NITRIC OXIDE AND PROSTAGLANDINS IN HUMAN PARTURITION

William James Benedict Dennes

A thesis submitted for the degree of

Doctor of Philosophy of the University of London

Imperial College School of Medicine

Queen Charlotte's and Chelsea Hospital

London W6 0XG

2001

To my Parents,

to whom I owe so much

ABSTRACT

This study investigates the roles of some of the key factors involved in the maintenance of human pregnancy and in the onset of labour. It investigates nitric oxide and prostaglandin synthesis in gestational tissue and both the regulation and inter-relationship of nitric oxide synthase (NOS) and cyclo-oxygenase (COX) expresssion within these tissues.

NOS and COX expression was investigated by reverse transcriptase polymerase chain reaction, Western analysis and *in situ* hybridisation in human fetal membranes and myometrium. An amnion-derived epithelial cell line (WISH cells) was used to study the inter-relationship of NOS and COX expression. The possible physiological role of NO in influencing myometrial contractility was investigated *in vitro*, in an organ bath system.

NOS mRNA is expressed in gestational tissue, but expression remains unchanged throughout gestation and with labour onset, suggesting in human pregnancy at least, that endogenous NO is not responsible for the onset of parturition. Whilst exogenous NO relaxes myometrial contractility *in vitro*, NOS mRNA does not appear to be expressed in the myocyte, suggesting that it is unlikely that NO has a role in maintaining myometrial quiescence through pregnancy. The intrinsic role of prostaglandins in parturition has been confirmed by data showing that in both fetal membranes and myometrium COX-2 mRNA and protein is up-regulated at term. iNOS and COX-2 mRNA induction by IL-1ß in a WISH cell model may involve NF-kB, however the inter-relationship between NOS and COX appears to be largely tissue-dependent.

TABLE OF CONTENTS

Abstract 3

Table of Contents 4

List of Figures 15

List of Tables 21

Acknowledgements 22

Abbreviations 23

1. INTRODUCTION 28

1.1 The Biochemistry of Parturition 28

1.1.1 Introduction 28

1.1.2 The phases of parturition 28

Phase 0: Pro-pregnancy factors 30

Progesterone 30

Nitric Oxide. 30

Phase 1: Pro-labour factors 31

Oxytocin 31

Corticotrophin releasing hormone (CRH) 31

Gap junction proteins 32

Ion Channels 33

Activin 33

Endothelin 34

Phase 2: Labour: an inflammatory reaction 34

Cytokines 34

The Cervix 35

Prostaglandins 35
Phase 3: Post labour 36

1.2 Relevant anatomy 37
1.2.1 Early placentation 37
1.2.2 Placenta 37
1.2.3 Fetal membranes 38
Amnion 39
Chorion 40
1.2.4 Myometrium 40
Myometrial anatomy 40
Myometrial contractions 41
Uterine smooth muscle 41
Myometrial contractility 41
Myometrial Signalling 43

1.3 The Biochemistry of Prostaglandins 44
1.3.1 Introduction 44
Historical perspective 44
Metabolic pathway 45
Structure and Nomenclature 46
1.3.2 Prostaglandin receptors 46
1.3.3 Arachidonic acid synthesis 47
1.3.4 Cyclo-oxygenase 49
COX-1 gene 50
COX-2 gene 51
COX transgenic mouse studies 52
COX expression in fetal membranes 53
COX expression in myometrium 54
1.3.5 Prostaglandin dehydrogenase 54

1.4 The Biochemistry of Nitric oxide 56
1.4.1 Introduction 56
1.4.2 Nitric oxide synthesis 56
1.4.3 Neuronal NOS (nNOS) 58

1.4.4 Inducible NOS (iNOS) 58
1.4.5 Endothelial NOS (e/cNOS) 59
1.4.6 Actions of nitric oxide 59
1.4.7 Nitric oxide donors 59
1.4.8 Nitric oxide synthase inhibitors 60
1.4.9 Nitric oxide synthase regulation 60
1.4.10 Nitric oxide synthase in pregnancy 61
1.4.11 Clinical use of nitric oxide donors 63

1.5 The aetiology and impact of preterm labour 66
1.5.1 Aetiology of preterm labour 66
Social and biological risk factors 66
Past obstetric history 67
Multiple pregnancy 67
Prelabour premature rupture of membranes 68
Infection 68
Iatrogenic preterm labour 69
Fetal factors 69
1.5.2 The impact of preterm labour 69
Incidence 69
Neonatal and Paediatric implications 69
Financial cost 70

1.6 The management of preterm labour 71
1.6.1 Supportive Management: 71
1.6.2 Tocolysis: 71
Analysis of effectiveness of management 71
Historical perspective: 72
Modern Tocolytics: 72
Betasympathomimetics: 73
Ritodrine: 73
Terbutaline: 75
Salbutamol: 75
Magnesium Sulphate: 75
Prostaglandin synthetase inhibitors: 76

Non-selective Prostaglandin synthetase inhibitors: 76
Sulindac: 78
Selective COX-2 inhibitors: 78
Oxytocin receptor antagonists: 78
Calcium channel blockers: 79
Nitric oxide donors: 79
Methylxanthines: 79

2. MATERIALS AND METHODS 81

2.1 Materials 81
2.1.1 Tissue and Cells 81
Fetal membranes 81
Myometrium 81
Cells 82
Tissue preparation 82
2.1.2 Reagents 82
2.1.3 Enzymes 82
2.1.4 Blotting and autoradiography 83
2.1.5 Radiolabelling and spun column chromatography 83
2.1.6 Gel electrophoresis 83
2.1.7 Bacterial strains 83
2.1.8 Antibodies 83
2.1.9 DNA samples 83
Molecular size markers and cloning vectors 83
Oligonucleotides 83
2.1.10 Solutions 85
Reverse transcriptase polymerase chain reaction 85
PCR buffer (10x KCl) 85
PCR buffer (10x NH_4^+) 85
Southern hybridisation 85
Southern pre-hybridisation solution 85
Southern hybridisation solution 85
Southern denaturing solution 85

Southern neutralising solution 85
10 x Denhardt's solution 85
SSC 85
T E buffer 85
E buffer (10 X, 1 L) 85
Oligo-labelling buffer 86
in situ hybridisation 86
Proteinase K buffer 86
Hybridisation buffer 86
Western blotting 86
PBS 0.1% TWEEN 86
T-Wash 86
7.45x Running gel 86
10x Stacking gel 86
10 x Running buffer 87
Electrophoresis transfer buffer 87
Gel shift assay 87
Nuclear protein extraction buffer A 87
Nuclear protein extraction buffer C 87
Nuclear protein extraction buffer D 87
5 x Gel shift binding buffer 87
RNA extraction 87
Solution D 87
Radio-immuno assay 87
RIA buffer 87
Charcoal / dextran buffer 87
PCR Sub-Cloning 88
LB medium 88
LB plates with ampicillin/IPTG/X-Gal 88
SOC medium 88
Preparation of plasmid DNA 88
Solution 1 88
Solution 2 88
Solution 3 88

2.1.11 Computing 88

2.2 Methods 89

2.2.1 RNA extraction 89

Preparation of solutions and glassware for RNA isolation 89

Isolation of RNA 89

2.2.2 Reverse transcriptase polymerase chain reaction (RT-PCR) 90

Introduction 90

PCR primer design 93

PCR conditions 94

Reverse transcription 94

Polymerase chain reaction 94

PCR cycle profiles 95

2.2.3 Agarose gel electrophoresis 95

2.2.4 Southern blotting 95

2.2.5 Dot blotting 96

2.2.6 Preparation of PCR products 96

2.2.7 Sub-Cloning of PCR products into plasmid vectors 97

pGEM-T ligation reaction 98

pGEM-T transformation reaction 98

2.2.8 Small scale isolation of plasmid DNA (Mini-prep procedure) 99

2.2.9 Restriction endonuclease digestion of DNA 99

2.2.10 DNA sequence verification 100

2.2.11 Radioactive labelling of double stranded DNA probes 100

Pre-hybridisation and hybridisation 101

Washing filters 101

2.2.12 Western Analysis 102

Preparation of samples for polyacrylamide gel electrophoresis 102

SDS-PAGE protein separation 102

Western blotting 102

Positive controls for Western analysis 103

COX-1 positive control 103

COX-2 positive control 103

iNOS positive control 104

e/cNOS positive control 104
2.2.13 *In situ* hybridisation 105
Introduction 105
in situ hybridisation techniques 106
Probe construction 106
Specimen preparation 108
Preparation of slides and coverslips 109
Fixation, embedding and sectioning of tissues 109
Pre-treatment of sections 109
RNA riboprobe transcription 109
Hybridisation 110
Removal of excess probe 110
Autoradiography 110
Developing 110
Analysis 111
2.2.14 Cell culture 111
Cell culture medium 111
Trypsinisation of cells 111
Cell viability 112
2.2.15 PGE_2 radio-immuno assay 112
2.2.16 Fetal membrane prostaglandin assays 113
2.2.17 NO Chemiluminescence assay 113
2.2.18 Gel Shift assay 114
Nuclear protein extraction 115
Oligonucleotide labelling 115
Gel shift assay reaction 115
2.2.19 Myometrial contractility studies 116
Sample preparation 117
Contractility experiments 117
Data analysis 117
2.2.20 Statistical analysis 118
Data interpretation 118
Data presentation 118

3. EXPERIMENTAL STRATEGY 120

4. RESULTS 122

4.1 Reverse transcription polymerase chain reaction 122
4.1.1 PCR cycle profiles 122
4.1.2 RT-PCR primers 123
iNOS 123
e/cNOS 123
nNOS 124
COX-2 126
COX-1 126
GAPDH 127
Calponin 127

4.2 NOS expression in pregnancy 128
4.2.1 NOS mRNA expression 128
Fetal membranes 128
NOS expression in relation to labour onset 129
NOS expression in relation to gestational age 135
Myometrium 139
NOS expression in relation to labour onset 141
NOS expression in relation to gestational age 144
4.2.2 *In situ* hybridisation 147
Introduction 147
NOS localisation in fetal membranes by in situ hybridisation 147
NOS localisation in myometrium by in situ hybridisation 151
4.2.3 NOS expression in myometrial cell culture 155
Introduction 155
4.2.4 Western analysis of NOS expression 158
Introduction 158
Optimisation of Western analysis 158
Western analysis of NOS protein in fetal membranes 158
Western analysis of NOS protein in myometrium 160

4.3 COX expression in pregnancy 162
4.3.1 COX mRNA expression 162
Fetal membranes 162
COX expression in relation to gestational age 162
Myometrium 166
COX mRNA expression in relation to labour onset 166
COX mRNA expression in relation to gestational age 168
4.3.2 Western analysis of COX expression 171
Western analysis of COX protein in fetal membranes 171
Western analysis of COX protein in myometrium 175
COX protein expression in myometrium in relation to labour onset 175
COX protein expression in myometrium in relation to gestational age 176
4.3.3 COX enzyme activity in fetal membranes 180

4.4 NOS regulation 184
4.4.1 Introduction 184
WISH cell culture 185
IL-1ß induction of NOS in WISH cells 185
iNOS mRNA 185
NOS enzyme activity 186
IL-1ß induction of COX in WISH cells 187
COX-2 mRNA 187
COX enzyme activity 188
IL-1ß electrophoretic mobility shift assays 189

4.5 Inter-relationship between NOS and COX 191
4.5.1 Introduction 191
4.5.2 The effect of COX and PGE_2 on NOS activity in WISH cells 193
4.5.3 The effect of NOS and NO on COX activity in WISH cells 194
Effects of inhibition of endogenous NOS on WISH cells PGE_2 synthesis 195
Effects of exogenous NO on WISH cell PGE_2 synthesis 196
4.5.4 The effect of NOS and NO on COX activity in the uterus 198
The effect of exogenous NO on COX activity in HOG-1 cells 200
The effect of exogenous NO on COX activity in HMSMC cells 201

4.6 Role of NO and in the control of myometrial contractility 203
4.6.1 Introduction 203
4.6.2 Effects of exogenous NO on myometrial contractility 203
Myometrial contractility 203
Effect of exogenous NO on COX-2 mRNA in myometrial strips 205
4.6.3 Effects of fetal membranes and inhibition of endogenous NO on myometrial contractility 207
4.6.4 COX-2 mRNA stability in myometrial strips 210
4.6.5 Effects of stretch on COX-2 mRNA in myometrial strips 210

5. DISCUSSION 212

5.1 General 212

5.2 NOS expression in pregnancy 213
5.2.1 Fetal membranes 213
5.2.2 Myometrium 216
5.2.3 Myometrial Cell culture 222

5.3 COX expression in pregnancy 224
5.3.1 Fetal membranes 224
5.3.2 Myometrium 226
5.3.3 Myometrial cell culture 228

5.4 NOS regulation 229

5.5 Inter-relationship between NOS and COX 231

5.6 The role of NO in myometrial contractility 235

5.7 Summary 237

6. REFERENCES 239

7. APPENDIX 266

7.1 Publications 266

7.1.1 Nitric oxide synthase mRNA expression in human fetal membranes: a possible role in parturition 266
7.1.2 Myometrial nitric oxide synthase messenger ribonucleic acid expression does not change throughout gestation or with the onset of labor 270
7.1.3 Expression of nitric oxide synthase isoforms in pregnant human myometrium 277
7.1.4 Expression of cyclo-oxygenase types-1 and -2 in human fetal membranes throughout pregnancy 289
7.1.5 Expression of cyclo-oxygenase types-1 and -2 in human myometrium throughout pregnancy 296
7.1.6 Region and labour-dependent synthesis of prostaglandin E2 by human fetal membranes 302
7.1.7 The Roles of the Cyclo-oxygenases Types One and Two in Prostaglandin Synthesis in Human Fetal Membranes at Term 305

7.2 Gene Sequences 312
7.2.1 Arrangement of the iNOS gene 312
7.2.2 Arrangement of the e/cNOS gene 313
7.2.3 Arrangement of the nNOS gene 314
7.2.4 Arrangement of the COX-1 gene 315
7.2.5 Arrangement of the COX-2 gene 316

LIST OF FIGURES

Fig. 1.01:	Parturitional phases	29
Fig. 1.02:	Human uterus, fetal membranes and placenta at 22 weeks	38
Fig. 1.03:	Diagrammatic representation of fetal membranes and attached maternal decidua	39
Fig. 1.04:	Schematic representation of arachidonic acid metabolism	45
Fig. 1.05:	Basic prostanoic acid structure	46
Fig. 1.06:	Structure of the COX-2 dimer, shown in ribbon representation	49
Fig. 1.07:	COX-1 and COX-2 gene structure	51
Fig. 1.08:	Molecular synthesis and actions of nitric oxide	57
Fig. 2.01:	Schematic representation of the polymerase chain reaction	92
Fig. 2.02:	pGEM-T Easy vector circle map	98
Fig. 2.03:	Principles of *in situ* hybridisation	105
Fig. 2.04:	pGEM-T vector map, with ligated e/cNOS PCR product	106
Fig. 2.05:	Sac I restriction endonuclease digestion of pGEM-T vector ligated with e/cNOS PCR product.	107
Fig. 2.06:	Fetal membrane-uterine muscle dual chamber system	116
Fig. 2.07:	Representatative box plot	119
Fig. 4.01:	iNOS cycle profile in pooled term chorion decidua	123
Fig. 4.02:	e/cNOS temperature profile	124
Fig. 4.03:	nNOS PCR in brain and myometrium at calculated annealing temperatures	125
Fig. 4.04:	nNOS PCR in brain and myometrium at elevated annealing temperatures	125
Fig. 4.05:	nNOS PCR temperature profile in brain and myometrium, using PCR primers 1943F and 2449R	126

Fig. 4.06: NOS mRNA expression in fetal membranes by RT-PCR 128

Fig. 4.07: iNOS mRNA expression in relation to labour, in term amnion 130

Fig. 4.08: iNOS mRNA expression in relation to labour, in term chorion decidua 131

Fig. 4.09: e/cNOS mRNA expression in relation to labour, in term amnion 132

Fig. 4.10: e/cNOS mRNA expression in relation to labour, in term chorion decidua 133

Fig. 4.11: GAPDH mRNA expression in fetal membranes in relation to labour 134

Fig. 4.12: iNOS expression in individual amnion samples related to gestational age 135

Fig. 4.13: iNOS expression in individual chorion decidua samples related to gestational age 136

Fig. 4.14: e/cNOS expression in individual amnion samples related to gestational age 137

Fig. 4.15: e/cNOS expression in individual chorion decidua samples related to gestational age 138

Fig. 4.16: GAPDH expression in fetal membranes throughout gestation 139

Fig. 4.17: NOS mRNA expression in myometrium, by RT-PCR 140

Fig. 4.18: iNOS mRNA expression in relation to labour, in term myometrium 141

Fig. 4.19: e/cNOS mRNA expression in relation to labour, in term myometrium 142

Fig. 4.20: nNOS mRNA expression in relation to labour, in term myometrium 143

Fig. 4.21: Calponin mRNA expression in relation to labour, in term myometrium 143

Fig. 4.22: iNOS expression in individual myometrial samples related to gestational age 144

Fig. 4.23: e/cNOS expression in individual myometrial samples related to gestational age 145

Fig. 4.24: nNOS expression in individual myometrial samples related to gestational age 146

Fig. 4.25: Calponin mRNA expression in relation to gestational age, in term myometrium 147

Fig. 4.26: iNOS expression localised in fetal membranes by *in situ* hybridisation (antisense riboprobe) 148

Fig. 4.27: iNOS expression localised in fetal membranes by *in situ* hybridisation (sense riboprobe) 149

Fig. 4.28: e/cNOS expression localised in fetal membranes by *in situ* hybridisation (antisense riboprobe) 150

Fig. 4.29: e/cNOS expression localised in fetal membranes by *in situ* hybridisation (sense riboprobe). 151

Fig. 4.30: iNOS expression localised in term myometrium, by *in situ* hybridisation (antisense riboprobe) 152

Fig. 4.31: e/cNOS expression localised in term myometrium, by *in situ* hybridisation (antisense riboprobe) 154

Fig. 4.32: NOS mRNA expression demonstrated by RT-PCR in myometrial tissue and myometrial smooth muscle cell culture 156

Fig. 4.33: iNOS, COX-2 and GAPDH expression in control and cytokine-stimulated myometrial cells at 24 and 48 hrs 157

Fig. 4.34: iNOS protein expression in term fetal membranes, by Western analysis 159

Fig. 4.35: e/cNOS protein expression in term fetal membranes by Western analysis 159

Fig. 4.36: e/cNOS protein expression in fetal membranes throughout gestation, by Western analysis 160

Fig. 4.37: e/cNOS protein expression in term myometrium in relationship to labour onset, by Western analysis 160

Fig. 4.38: COX-1 mRNA expression in amnion samples related to gestational age 162

Fig. 4.39: COX-1 mRNA expression in chorion decidua samples related to gestational age 163

Fig. 4.40: COX-2 mRNA expression in amnion samples related to gestational age 164

Fig. 4.41: COX-2 mRNA expression in chorion decidua samples related to gestational age. 165

Fig. 4.42: COX-1 mRNA expression in relation to labour, in term myometrium 166

Fig. 4.43: COX-2 mRNA expression in relation to labour, in term myometrium 167

Fig. 4.44: COX-1 expression in individual myometrial samples related to gestational age 168

Fig. 4.45: COX-2 expression in individual myometrial samples related to gestational age 169

Fig. 4.46: COX-1 mRNA expression in myometrium, throughout gestation and at term 170

Fig. 4.47: COX-2 mRNA expression in myometrium, throughout gestation and at term 171

Fig. 4.48: COX-1 protein expression in amnion, in relation to both gestational age and labour 172

Fig. 4.49: COX-1 protein expression in chorion decidua, in relation to both gestational age and labour 172

Fig. 4.50: COX-2 protein expression in amnion, in relation to both gestational age and labour 173

Fig. 4.51: COX-2 protein expression in chorion decidua, in relation to both gestational age and labour 174

Fig. 4.52: COX-1 protein expression in relation to labour, in term myometrium 175

Fig. 4.53: COX-2 protein expression in relation to labour, in term myometrium 176

Fig. 4.54: COX-1 protein expression in individual myometrial samples related to gestational age 177

Fig. 4.55: COX-2 protein expression in individual myometrial samples related to gestational age 177

Fig. 4.56: COX-1 protein expression in myometrium throughout gestation and at term 178

Fig. 4.57: COX-2 protein expression in myometrium throughout gestation and at term 179

Fig. 4.58: COX activity in fetal membrane discs throughout gestation 181

Fig. 4.59: COX activity in fetal membrane discs at term 182

Fig. 4.60: COX activity in fetal membrane discs throughout gestation and at term 183

Fig. 4.61: iNOS and GAPDH mRNA expression in IL-1 ß stimulated WISH cells 186

Fig. 4.62: Nitrite production by IL-1ß stimulated WISH cells 187

Fig. 4.63: COX-2 and GAPDH mRNA expression in IL-1 ß stimulated WISH cells 188

Fig. 4.64: PGE_2 production by IL-1ß stimulated WISH cells 189

Fig. 4.65: NF-κB EMSA. NF-κB gel retardation in IL-1ß stimulated WISH cells 190

Fig. 4.66: Amnion derived epithelial WISH cells in monolayer culture 192

Fig. 4.67: Indomethacin dose response inhibition of IL-1ß-stimulated PGE_2 synthesis in WISH cells at 24 hrs 193

Fig. 4.68: Indomethacin dose response inhibition of IL-1ß-stimulated NO synthesis in WISH cells at 24 hrs 194

Fig. 4.69: L-NAME dose response inhibition of IL-1ß stimulated PGE_2 synthesis in WISH cells at 24 hrs 195

Fig. 4.70: D-NAME dose response inhibition of IL-1ß stimulated PGE_2 synthesis in WISH cells at 24 hrs 196

Fig. 4.71: SNP dose response of PGE_2 synthesis in WISH cells - release over 24 hr 197

Fig. 4.72: SNP dose response of PGE_2 synthesis in WISH cells - activity with arachidonic acid 197

Fig. 4.73: MTT determination of SNP treated WISH cell viability 198

Fig. 4.74: HOG-1 epithelial cancer cells in monolayer culture. 199

Fig. 4.75: Human myometrial smooth muscle cells (HMSMC) in monolayer culture 200

Fig. 4.76: SNP dose response of PGE_2 synthesis in HOG-1 cells - release over 24 hr 200

Fig. 4.77: SNP dose response of PGE_2 synthesis in HOG-1 cells - activity with arachidonic acid 201

Fig. 4.78: SNP dose response of PGE_2 synthesis in HMSMC - activity with arachidonic acid 202

Fig. 4.79: Representative experimental record demonstrating the response of spontaneously contracting myometrial strips to increasing concentrations of sodium nitroprusside (SNP) 204

Fig. 4.80: SNP dose response of myometrial contractility in spontaneously contracting myometrial strips *in vitro* 205

Fig. 4.81: COX-2 mRNA expression in myometrial strips 206

Fig. 4.82: Representative experimental record demonstrating a cross-over response of myometrial strips to exposure to fetal membranes in the presence of L-NAME and D-NAME 208

Fig. 4.83: Effect of L-NAME and D-NAME on myometrial contractility, in spontaneously contracting myometrial strips, in the presence of fetal membranes 209

Fig. 4.84: Calponin mRNA stability in myometrial strips 210

Fig. 4.85: COX-2 mRNA expression in myometrial strips, in relation to applied stretch 211

LIST OF TABLES

Table 1.01: Prostaglandin receptors and their effects on myometrial contractility 47

Table 2.01: PCR primers and their uses 84

Table 4.01: nNOS PCR primers, product sizes and annealing temperatures 124

Table 4.02: COX assays: Gestational age and labour status of fetal membrane samples 181

ACKNOWLEDGEMENTS

The work presented in this thesis is the result of research carried out in the department of Paediatrics, Obstetrics and Gynaecology, at Queen Charlotte's and Chelsea Hospital, a division of Imperial College School of Medicine (formerly the Royal Postgraduate Medical School, Institute of Obstetrics and Gynaecology). The research was funded by grants from Wellbeing and the Dunhill trust.

I am deeply indebted to Dr. Donna Slater for all her help, guidance and tuition with many of the molecular techniques presented here. This research project has been a time of much enjoyment, stimulation and fulfilment and I gratefully acknowledge her significant contribution. Victoria Allport established the radio-immunoassay in our department and has provided much help and assistance with this and many other techniques. Dr. Robert Sawdy developed the myometrial contractility studies and has provided great help and enthusiasm in establishing these experiments and in analysing the large amounts of data generated.

Dr. Robert Newton at The National Heart and Lung Institute (Royal Brompton Hospital) has provided help and advice with many of the molecular techniques presented here and most specifically in helping to establish electrophoretic mobility shift assays in our department.

Richard Scrivener has generously helped to prepare slide presentations and illustrative materials at short notice. Dr. Tim Overton has provided much help with the formatting of this thesis and with the idiosyncrasies of Microsoft Word.

Members of the Myometrial Group (London) have provided spontaneous, relevant and willing assistance with collaborative research strategy. I am very grateful to Juan Campa and Professor Lucilla Poston at St. Thomas' Hospital for their help in providing tissue samples for analysis, and to Dr. Simon Bartlett at King's College, for providing myometrial cell cultures and technical advice in preparing nitric oxide synthase controls for Western analysis.

Finally, I would like to thank my supervisor, Professor Phil Bennett, for providing me with the opportunity to carry out this research. He has provided timely, helpful and often inspired advice throughout, and I am deeply indebted to him.

ABBREVIATIONS

AA	Arachidonic acid
Ac	Acetate
ACTH	Adrenocorticotrophic hormone
ADP	Adenosine diphosphate
APS	Ammonium persulphate
ATCC	American type culture collection
ATP	Adenosine triphosphate
BH_4	Tetrahydrobiopterin
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cf.	Compare (Latin: *Confer*)
CHAPS	3 [(3-cholamidopropyl) dimethylammonio] - 1 - propanesulphonate hydrate
COX	Cyclo-oxygenase
$cPLA_2$	Cytosolic phospholipase A_2
cps	Counts per second
CRE	cAMP response element
CRH	Corticotrophic releasing hormone
CRH-BP	Corticotrophic releasing hormone binding protein
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate

DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
dH_20	Distilled water
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
ECL	Enhanced chemiluminescence (Western analysis)
EDTA	Ethylene-diamine tetracetic acid
EDRF	Endothelin derived relaxation factor (Nitric oxide)
EMSA	Electrophoretic mobility shift assay
FAD	Flavin adenine dinucleotide
FDA	Food and Drink Administration (US)
FMN	Flavin mononucleotide
g	Acceleration due to gravity, 10m/s/s
GAPDH	Glyceraldehyde phosphate dehydrogenase
GDP	Guanosine diphospate
GMP	Guanosine monophospate
GTP	Guanosine triphospate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HETES	Hydroxyeicosatetraenoic acids

HMSMC	Human myometrial smooth muscle cell
HOG-1	Hammersmith obstetrics and gynaecology cervical epithelial cell line
HUVEC	Human umbilical vein endothelial cell
IgG H-RP	Immunoglobulin G horseradish peroxidase (Western analysis)
IL-1β	Interleukin-1β
kb	Kilobase
kDa	Kilo dalton
LPS	*E. coli* lipopolysaccharide
mRNA	Messenger ribonucleic acid
MIT	Mean integral tension
M-MLV	Moloney murine leukaemia virus
MOPS	Morpholinopropanesulfonic acid
MW	Molecular weight
MTT	3- [4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue
NADPH	Nicotinamide adenine dinucleotide phosphate dehydrogenase
NF-kB	Nuclear factor kappa beta
NF Il-6	Nuclear factor interleukin 6
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
OCT 1	Octamer transcription factor-1
OD	Optical density
$P <$	Probability less than
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PG	Prostaglandin
PGE_2	Prostaglandin E2
PKA	cyclic AMP-dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethlylsulfonyl fluoride
PSS	Physiological saline solution
RIA	Radio-immuno assay
RNA	Ribonucleic acid
RNAase	Ribonuclease
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
$sPLA_2$	Secretory phospholipase A_2
SR	Sarcoplasmic reticulum
SSC	Sodium chloride / Sodium citrate buffer
TEMED	N, N, N^1, N^1 tetramethylethylenediamine
TESPA	3-aminopropyltriethoxysilane

TGF	Tissue-like growth factor
TPA	O-tetradecanoylphorbol 13-acetate
TWEEN	Polyoxyethylenesorbitan monolaurate
TXA_2	Thromboxane A2
UV	Ultra-violet
WHO	World health organisation
WISH	Human amnion derived epithelial cells
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

All units are standard SI units.

1. INTRODUCTION

1.1 The Biochemistry of Parturition

1.1.1 Introduction

The control of myometrial contractility during pregnancy, and the changes associated with the onset of labour represents remarkable physiological adaptation. During pregnancy the uterus must remain in an unresponsive, quiescent state, allowing the developing fetus to grow. With the onset of labour, the myometrium undergoes significant changes to enable the development of powerful contractions and cervical dilatation, resulting in effective delivery of the fetus, and uterine involution.

These events are, for the most part controlled so that the onset of labour, and delivery of the mature fetus occurs at term, however in a minority of cases the regulatory mechanisms controlling labour onset fail, resulting in preterm labour. Whilst only affecting approximately 5% of all pregnancies, the impact of preterm labour on neonatal outcome is significant.

The development of strategies for both the prediction and prevention of preterm labour requires a better understanding of the mechanisms responsible for the onset of labour at term. The mechanisms which control the length of human pregnancy and signal the onset of labour have been the subject of much research.

1.1.2 The phases of parturition

During pregnancy the uterus expands to accommodate the growing fetus and placenta, without increasing contractility, whilst the cervix remains firm and closed. Throughout pregnancy 'pro-pregnancy' factors operate to inhibit myometrial contractility and to allow myometrial hypertrophy until near to term, 'pro-labour' factors begin to operate to mediate re-modelling of the cervix, to allow it to efface and dilate, and the uterus is stimulated to begin co-ordinated contractions. Labour has been considered as the result of the activation of a 'cassette of contraction-associated proteins' which act to convert the myometrium from a state of quiescence to a state of contractility (Lye, 1994). These contraction-associated proteins might include gap junction proteins, oxytocin and prostaglandin receptors, enzymes for the synthesis of prostaglandins or cytokines, and also components of cell

signalling mechanisms, which effect the way in which the uterus responds to receptor activation. It is likely that the factors which control the activation of the 'cassette of contraction-associated proteins' also activate factors in the fetal membranes that lead to the production of prostaglandins and cytokines associated with labour, and factors within the cervix which lead to cervical remodelling and ripening.

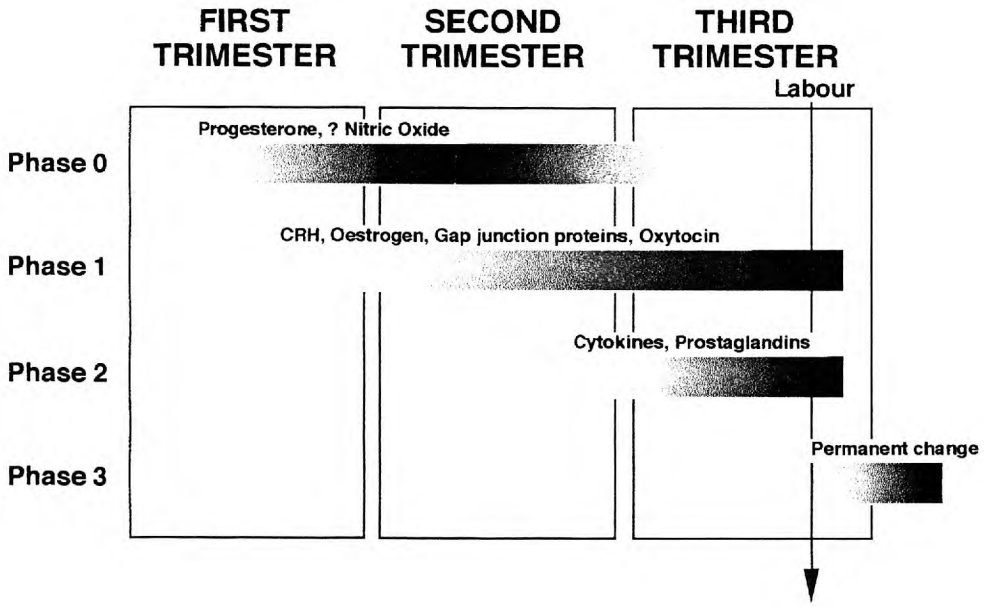


Fig. 1.01: Parturitional phases

Pregnancy may be considered in four parturitional phases (Fig. 1.01). The first phase (Phase 0), during the first and second trimesters, is dominated by 'pro-pregnancy factors' and is the period of myometrial growth and quiescence. The second phase (Phase 1), during the second, and mid third trimester, is also a phase of myometrial quiescence, but during which preparation for labour is made by up-regulation of myometrial, cervical and fetal membrane proteins which will be needed for labour. The third phase (Phase 2) is the phase of labour itself and has the character of an inflammatory reaction. During this third phase of pregnancy, the 'brake' on myometrial contractility caused by 'pro-pregnancy' factors is released and the spontaneous contractility of the uterus is augmented by oxytocic compounds such as prostaglandins and possibly oxytocin itself. The fourth parturitional phase (Phase 3) represents the state of the intrauterine tissues after the process of labour.

Phase 0: Pro-pregnancy factors

Progesterone

Progesterone, as its name suggests is the principle pro-pregnancy factor. In the human, maintenance of pregnancy in the first trimester is through release of progesterone by the corpus luteum, whose regression is inhibited by HCG. By about 7 weeks, progesterone production is taken over by the placenta, with subsequent luteal regression. Progesterone has a negative regulatory effect upon many of the 'contraction-associated proteins', associated with the formation of myometrial gap junctions (connexins), and the modulation of cervical ripening (IL-8). Progesterone also decreases uterine sensitivity to oxytocin; an effect that has been shown to be mediated through a non-genomic effect of progesterone on the oxytocin receptor (Grazzini et al., 1998). Progesterone is therefore probably important in mediating enlargement of the uterus without increasing contractility.

In many species progesterone is a requisite for the maintenance of uterine quiescence during pregnancy, and progesterone withdrawal immediately precedes the onset of labour. In non-primates labour is preceded by an increase in the oestrogen : progesterone ratio. In the human however there is no obvious systemic withdrawal of progesterone prior to labour. Pharmacological inhibition of the action of progesterone, using mifipristone (RU486) is well known clinically to induce abortion in the first trimester. In rhesus monkeys at term, mifipristone has been shown to induce myometrial contractions, although it does not induce the onset of labour which progresses to delivery (Haluska et al., 1987). Pharmacological inhibition of progesterone also up-regulates cervical IL-8 expression (Critchley et al., 1996), leading to cervical ripening. It is possible that in the human there is no actual or functional withdrawal of progesterone prior to labour, rather its 'pro-pregnancy' action is simply overwhelmed by 'pro-labour' factors.

The relative contribution of progesterone to myometrial quiescence remains uncertain, however there is evidence from studies of patients with impaired progesterone synthesis (placental aromatase deficiency)(Shozu et al., 1991) that minimal amounts of progesterone are compatible with both viable pregnancy, and spontaneous labour at term.

Nitric Oxide.

Nitric oxide (NO) has been proposed as a putative mediator of uterine smooth muscle relaxation, and myometrial quiescence (Buhimschi et al., 1995), and to have a pro-pregnancy function. Its regulation and control of myometrial quiescence and labour onset will be considered in detail later.

Phase 1: Pro-labour factors

Oxytocin

Oxytocin is a potent and specific mediator of uterine contractility. It has traditionally been considered the primary factor responsible for the onset of labour, and has been widely used clinically to augment labour. Oxytocin increases myometrial contractility by both increasing intracellular calcium concentrations $[Ca^{2+}]_i$, and myosin light chain phosphorylation. Whilst exogenous oxytocin induces uterine contractions, the role of endogenous oxytocin in the onset of labour is more equivocal. Oxytocin is released from the neurohypophysis in a pulsatile manner. Whilst there is no evidence that systemic concentrations of oxytocin increase, either with the onset or the progress of labour (Thornton et al., 1992), there is an apparent increase in the pulse frequency of its release (Hirst et al., 1991). In addition both the sensitivity (Fuchs et al., 1982) and density of myometrial oxytocin receptors increases towards term (Fuchs et al., 1984).

There is also evidence that oxytocin is generated from intrauterine tissues. Oxytocin mRNA has been identified in amnion, chorion and decidua (Chibbar et al., 1993), however the presence of oxytocinase in the placenta is likely to limit its physiological effects.

In addition to oxytocin's intrinsic uterotonic effects there is evidence that it stimulates prostaglandin release in amnion, chorion and decidua, and that this effect is significantly greater in tissues collected after the onset of labour (Larcher et al., 1995)(Fuchs et al., 1981). These data therefore suggest that oxytocin might amplify a prostaglandin-driven uterotonic cascade.

Corticotrophin releasing hormone (CRH)

Both McLean et al. (McLean et al., 1995) and Challis et al. (Jones et al., 1989) have suggested that the timing of labour is mediated through placental release of corticotrophin releasing hormone (CRH), whose concentration in maternal plasma begins to rise about 90 days prior to the onset of labour. Circulating CRH is principally in an inactive form, bound to a binding protein, CRH-BP, derived from the maternal liver. CRH-BP is itself negatively regulated by CRH, so that as placental release of CRH increases, maternal liver release of CRH-BP decreases. In the average pregnancy at about 37 weeks, CRH concentrations began to exceed those of CRH-BP, with effectively a large increase in the concentration of biological active CRH. An important difference between pituitary CRH

and placental CRH is that whilst pituitary CRH is under the negative feedback control of cortisol, placental release of CRH is increased by cortisol.

Both myometrium (Grammatopoulos et al., 1995) and fetal membranes (E. Linton personal communication) express CRH receptors, linked to G-protein adenyl cyclase second messenger systems (Chen et al., 1993)(Liaw et al., 1996). In addition to an excess of CRH relative to its binding protein, the affinity of the CRH receptors in the myometrium increases significantly late in gestation (Hillhouse et al., 1993) such that the receptor could theoretically cleave CRH from its binding protein. Free CRH up-regulates expression of the central prostaglandin synthetic enzyme cyclo-oxygenase type-2 (COX-2) (see below), and stimulates prostaglandin synthesis in both fetal membranes (Jones and Challis, 1990) and myometrium (Petraglia et al., 1995). CRH has also been shown to directly stimulate myometrial contractility (Challis et al., 1995). Whilst this seems contradictory to its action in increasing cellular cAMP, which would normally inhibit myometrial contractions (through inhibition of the activity of myosin light chain kinase), Grammatopoulos et al. (Grammatopoulos et al., 1996) have shown that myometrial CRH receptor coupling changes at term, to reduce cAMP generation by CRH. In addition CRH has been shown to augment oxytocin-induced myometrial contractility (Quartero et al., 1992).

Maternal CRH levels have also been shown to be a predictive marker of length of gestation (Wadhawa et al., 1998). Maternal CRH levels measured in circulating plasma in the third trimester significantly and negatively predicted gestational length. In addition, in deliveries preceded by spontaneous onset of labour CRH levels significantly and independently predicted both earlier onset of labour and preterm labour. These findings further support the premise that CRH is implicated in labour onset.

Gap junction proteins

Gap junctions facilitate the transmission of electrical stimuli and the exchange of second-messenger molecules between smooth muscle cells. The junctional resistance (a measurement of resistance between cells, itself an indicator of gap junction density) is approximately 100 Ω cm in non-pregnant myometrium compared with 50 Ω cm in pregnant myometrium at term. This generally reflects a high degree of cell-cell coupling when compared with other tissues (400 Ω cm in urinary bladder).

Connexin-43 (Cx-43) is the major gap junction protein in myometrium. An increase in gap junction proteins with the onset of labour has been shown in all species studied. Chow et

al. (Chow and Lye, 1994) have found that Cx-43 mRNA in human myometrium increases with the onset of labour. In rats and sheep there is a similar rise in Cx-43 mRNA in association with labour, that appears to be directly correlated with the oestrogen : progesterone ratio. There is evidence that stretch may also increase gap junction formation, possibly by a synergistic mechanism with oestrogen (Wathes and Porter, 1982). Garfield et al. have shown that in addition to a reduction in gap junction density, cell-cell communication may be decreased by elevated $[Ca^{2+}]_i$ (suggesting a possible negative feedback effect), lowered pH and increased levels of cAMP (Garfield, 1990).

Ion Channels

During pregnancy the resting membrane potential (RMP) of myometrium is relatively hyperpolarized, reducing the capability to initiate action potentials. With the onset of labour the RMP is relatively depolarized, enabling small changes in membrane potential to reach the critical threshold to induce an action potential (Crankshaw and Gaspar, 1992). This increase in excitability is probably a function of a change in expression of ion channels. The regulation of calcium channel synthesis in myometrium during pregnancy appears equivocal. There is some evidence from studies involving ovariectomized rats, that oestrogen therapy increased dihydropyridine binding and ^{45}Ca influx in the myometrium, which would be consistent with data suggesting an increase in intracellular calcium in endometrium following treatment with oestrogen (Batra, 1985). However Inoue et al. (Inoue and Sperelakis, 1991) found no change in calcium current densities, in the presence of increasing oestradiol levels, in rat myometrial cells, with increasing gestational age.

Activin

Activin is a 22KDa, dimeric protein, part of the TGF ß super family of proteins. It is closely related to Inhibin, incorporating two inhibin ß subunits (ßA and ßB). Inhibin is produced by the syncytiotrophoblast (Rabinovici et al., 1992) and mRNA for both subunits has been localised in amnion, chorion and decidua (Petraglia et al., 1990)(Petraglia et al., 1993)(de Kretser et al., 1994)(Petraglia et al., 1994b). Activin is widely bound to follistatin, its binding protein. The bioavailability of activin is therefore regulated by follistatin levels. The physiological role of activin remains unknown, although its close structural relationship with inhibin has suggested a possible antagonistic effect with this protein. Activin receptors have been shown in gestational tissue (Petraglia et al., 1994a), and receptor levels appear to change with gestation (Peng et al., 1993). In *vitro* there is evidence that activin increases both progesterone synthesis (Petraglia et al., 1989)

and aromatase activity (Song et al., 1996), suggesting activin may be able to modulate local oestrogen : progesterone ratio. In addition activin has been shown to stimulate prostaglandin synthesis in amnion cell culture (de Kretser et al., 1994) and stimulates oxytocin production by trophoblast cells (Florio et al., 1996). The role of activin in the onset of labour however remains equivocal, in the absence of any evidence suggesting that elevated activin levels induce labour.

Endothelin

The endothelins (ETs) are a group of powerful vasoconstrictor peptides, ET-1, -2 and -3. They interact with distinct G-protein-linked receptors (ET_A and ET_B). ET_A preferentially binds ET-1, whilst ET_B binds all peptides with equal affinity. Binding of the ligand to the ET_A receptor increases intracellular calcium concentrations, via stimulation of dihydropyridone-sensitive calcium channels. The ET_B receptor increases intracellular calcium via phospholipase C and production of inositol triphosphate. Myometrium contains both ET_A and ET_B receptors (Maggi et al., 1994) and pregnant myometrium is more sensitive to endothelin than non-pregnant tissue (Schiff et al., 1993). There is some evidence to suggest that ET-1 may potentiate the uterotonic actions of oxytocin in the pregnant uterus at term (Peri et al., 1992). Endothelin is produced by the amnion and is present in amniotic fluid. However levels of amniotic ET are not elevated in preterm labour, in the absence of intra-amniotic infection (Bracero et al., 1995).

Phase 2: Labour: an inflammatory reaction

Cytokines

There is a strong association between both term and preterm labour and increased production of the inflammatory cytokine interleukin-1ß within the uterus (Romero et al., 1990). IL-1ß concentrations have been shown to be elevated in amniotic fluid in term labour and just prior to labour in patients with chorio-amnionitis (Romero et al., 1992).

Whilst cytokine levels are markedly elevated in the presence of intra-uterine infection, such as chorio-amnionitis, there is evidence that cytokines may be involved in the physiological control of parturition. There is certainly evidence that cytokines mediate cervical ripening (see below), and IL-6 gene expression has been demonstrated to be elevated in both fetal membranes and myometrium obtained in active labour at term without demonstrable intra-

amniotic infection (Dudley et al., 1993). Expression of IL-1ß mRNA is also higher in fetal membranes after labour than before (Ammala et al., 1997) and IL-1ß expression also increases throughout the third trimester (Sullivan and Brown unpublished).

The Cervix

The cervix is composed mainly of a connective tissue of collagen and elastin embedded in a proteoglycan matrix with very little smooth muscle. The proteoglycan matrix of the cervix, consists of heteropolysaccharide side-chains (glycosaminoglycans), covalently linked to a central polypeptide core. At term, near to labour, the collagen of the cervix changes, undergoing collagenolysis. The fibrils become dissociated from their tightly organised bundles and are more widely scattered in an increased amount of ground substance, and there is loosening of the collagen bundles in the cervical stroma. There are also changes in the concentrations of glycosaminoglycans in the cervical matrix, at the time of parturition. These biochemical changes in the cervix are reflected as changes in its physical properties (Calder and Greer, 1992).

Much of the change in the cervix at term is due to increased collagenase activity. Fibroblasts present in the cervix, are capable of synthesising collagenase, but there is also an accumulation of neutrophils which release collagenase into the cervix, around the time of parturition. Cervical ripening therefore resembles an inflammatory reaction. It is currently thought that neutrophils are attracted into the cervix at term by the combination of increased prostaglandin synthesis and the 'neutrophil attractant peptide' interleukin-8 (IL-8), whose expression may be mediated by NF-kB (Calder, 1994)(El Maradny et al., 1996)(Kelly et al., 1992)(El Maradny et al., 1995)(Osmers et al., 1995a)(Osmers et al., 1995b).

Prostaglandins

Whilst many factors are thought to contribute to the onset of human parturition, their relative contributions remain elusive. What has become widely accepted however is the pivotal role that prostaglandins, and the control of prostaglandins synthesis plays in labour onset. There is clear evidence for the role of prostaglandins in the onset of parturition (Bennett and Elder, 1988), and this will be considered in detail below.

Phase 3: Post labour

Following labour and delivery the myometrium appears to undergo irreversible change, such that in subsequent pregnancy the speed and length of labour is significantly different. This is apparent clinically, where in multiparous patients pregnancy length tends to be shorter, and labour more rapid (Kieler et al., 1995). Contractions are also more effective and the uterus is more sensitive to oxytocin. As a result, multiparous labour is less likely to be associated with operative intervention than nulliparous labour. These observations probably reflect not only anatomical changes, but also changes in the sensitivity of gestational-associated tissues to pro-labour factors.

1.2 Relevant anatomy

1.2.1 Early placentation

The embryonic trophoblast forms the outer syncytial and inner cellular layers of the developing embryo. At day 11, before implantation is complete, lacunae form in the syncytiotrophoblast. These lacunae develop a brush border, and go on to fuse with maternal capillaries to form a blood-filled space - the future intervillous space. Primary villous stems form at day 13, with a lining of syncytiotrophoblast and an inner core of cytotrophphoblast. The cytotrophoblast invades both the syncytiotrophoblast and maternal decidua to form the primary villous stem. Trophoblast cells invading the myometrium form giant cells. Cells invading the lumen of spiral arteries result in degeneration of the tunica muscularis by fibrinoid necrosis, and a loss of resistance in placental circulation. A second wave of trophoblast invasion takes place during the early second trimester.

From day 21 the primary villous stems become vascularised and develop syncytial sprouts. They subsequently develop a cytotrophoblast, and then a mesenchymal core, and in so doing form the Primary villi. The primary stems undergo further division, forming Secondary stem villi just below the chorial plate. These give rise to Tertiary stem villi after a short lateral course. Tertiary stem villi develop in the intervillous space and turn back on themselves near the maternal surface. Tertiary stem villi go on to form the placental villi. At 12 weeks the placenta has achieved its definitive form and thickness. Subsequent development is circumferential. Placental growth is initially more rapid than fetal growth, however by 17 weeks both are of equal weight, until the fetus then overtakes.

Following implantation, and with the development of the embryo the decidua forms three distinct regions. The decidua basalis forms at the deepest embryonic pole, and ultimately forms the basis of the placental attachment. The decidua parietalis forms over the rest of the uterus except the implantation site. The decidua capsularis forms over the rest of the chorion and fuses with the decidua parietalis in the fifth month.

1.2.2 Placenta

The mature placenta is composed of some 200 lobules. Each lobule arises from a primary stem villus. The tertiary villi are arranged in circles around a hollow core. There is no anatomical relationship between the lobules and the morphologically visible cotyledons.

The umbilical arteries divide into primary, secondary and tertiary branches, corresponding to the stem villi. The tertiary branches break up into a capillary system in the terminal villi. Maternal spiral arteries in the myometrium terminate in the intervillous space and direct a stream of blood towards the chorionic plate. Spiral arteries undergo trophoblast invasion leading to dilatation. The normal term placenta shows perivillous fibrin deposition, and there is frequently evidence of infarction, intervillous thrombosis and calcification, all of which have no obvious pathological significance.

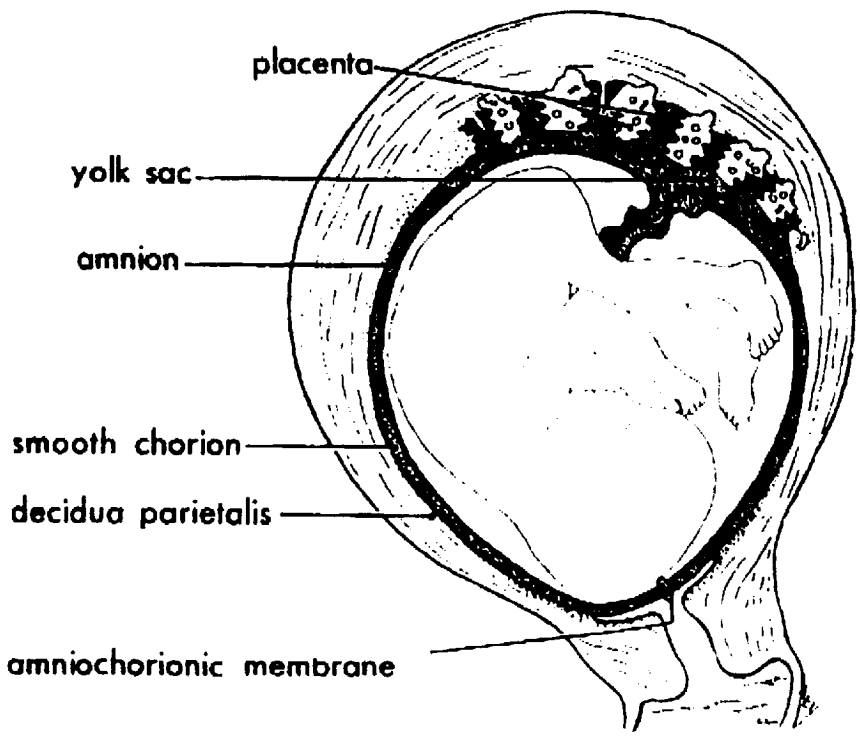


Fig. 1.02: Human uterus, fetal membranes and placenta at 22 weeks. Sagittal section of the gravid uterus, showing the relationship of the fetus, fetal membranes, placenta and uterus. From (Moore and Persaud, 1993) with permission.

1.2.3 Fetal membranes

The fetal membranes comprise the amnion and chorion decidua layers. When collected after delivery it is often difficult to separate or even distinguish the chorion from maternal decidua, these layers are therefore collected and stored as a single entity.

Amnion

The amnion forms the layer in closest proximity to the developing fetus, and in contact with the amniotic fluid. The amnion is smooth in appearance, physically strong, and ranges in thickness from between 20 - 500 μM. The amnion layer is composed of a number of histologically distinct layers (see Fig. 1.03).

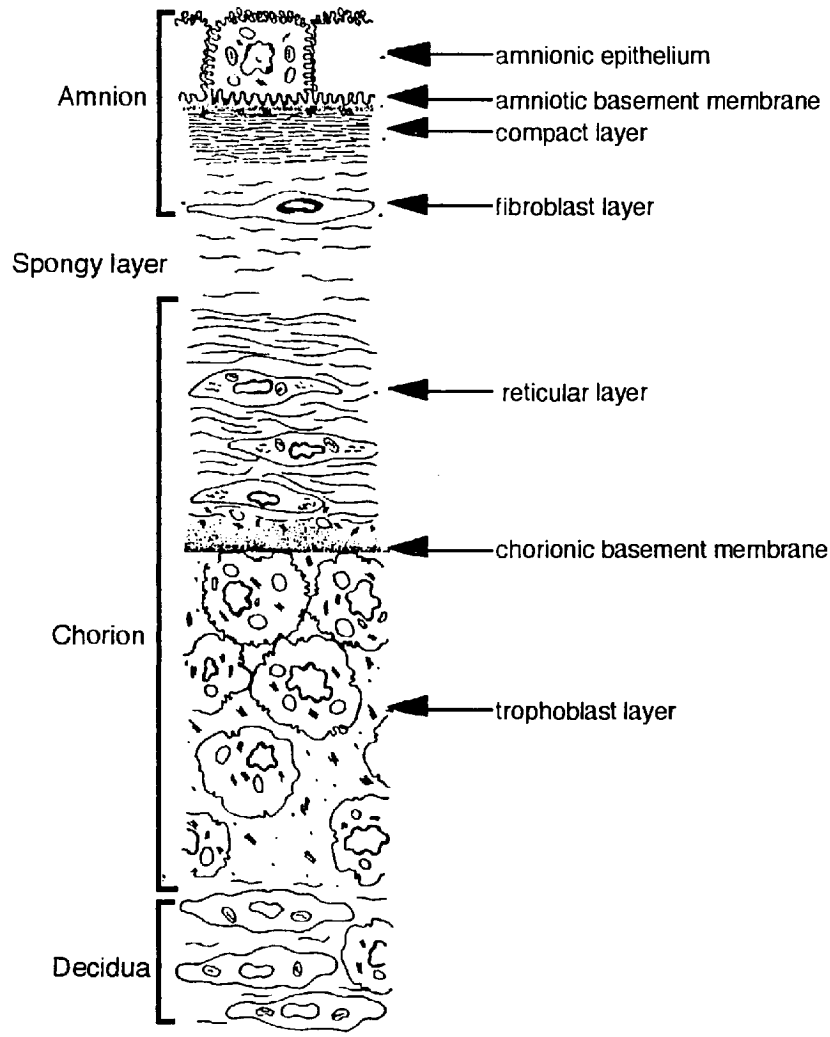


Fig. 1.03: Diagrammatic representation of fetal membranes and attached maternal decidua. From (Malak and Bell, 1994) with permission.

The amnionic epithelium is a single layer of non-ciliated cuboidal epithelial cells. These range from columnar over the placenta, to flattened cells over the reflected amnion. The epithelial cells are closely adherent to the amniotic basement membrane. The amnionic

basement membrane is formed by a well defined, narrow band of reticular tissue, formed over both the reflected and placental amnion. The compact layer forms the strongest component of the amnion. It is a dense acellular layer with a complex network of reticular fibres. The fibroblast layer is the thickest layer of the amnion, and is composed of a loose fibroblast network embedded in a mass of reticulin. The spongy layer is composed of extra-embryonic coelomic reticulum bathed in mucin. The structure of the spongy layer allows a certain degree of movement of the amnion over the chorion.

Chorion

The chorion lines the uterine cavity, and separates the maternal decidua and myometrium from the amnion. Its appearance is opaque, and it varies from between 20 - 200 μM in thickness. The chorion is composed of four distinct layers. The thin cellular layer is composed of an interlacing fibroblast network. The cellular layer is more apparent early on in gestation, and is not easily distinguished at term. The reticular layer provides the major component of the chorion, and is composed of a network of reticular fibres with embedded fibroblasts and Hofbauer cells. The pseudo-basement membrane is made up of dense connective tissue. It is firmly adherent to the reticular layer. The trophoblast varies from two to ten trophoblast cells in depth, the deepest of which are in contact with the maternal decidua.

1.2.4 Myometrium

Myometrial anatomy

The uterus develops embryologically at 8 weeks, by fusion of the müllerian ducts. The non-pregnant uterus is 7.5 cm long, and consists of a body (or corpus), a constricted region, which also includes the internal os(isthmus) and a narrow terminal region (cervix). In a nulliparous patient the uterus weighs 50g. In a multiparous patient it weighs 70g. During pregnancy the uterus increases in weight by up to 1 kg.

During the first trimester of pregnancy neither the cervix nor the isthmus change in length. Subsequently the isthmus elongates and becomes incorporated into the general uterine cavity to form the lower uterine segment. The cervix is 52 mm long until the 34th week, when the upper part becomes incorporated into the thin lower segment, so that by term the lower uterine segment is composed of 70% isthmus and 30% cervix. The uterus only

increases in weight during the first and second trimesters. Further enlargement is a result of expansion and thinning of the uterine walls, rather than continued growth.

Myometrial contractions

Uterine smooth muscle

The active contractile units of the myometrium are the myometrial smooth muscle cells. Smooth muscle cells are arranged in bundles, in a matrix of connective tissue composed of collagen. The collagen fibres facilitate transmission of the contractile forces. Uterine smooth muscle cells contain actin myofilaments (thin filaments) and myosin myofilaments (thick filaments) arranged randomly throughout the cytoplasm.

Myosin is a hexamer consisting of two identical heavy chains (200 kDa) and four light chains (two 20 kDa and two 15 - 17 kDa). Myosin provides both structural stability and can act as an enzyme, via ATP ase located on the myosin head, to convert ATP into the mechanical energy of muscle contraction. During gestation there is myometrial cell hypertrophy, and the content of both actin and myosin increases approximately fourfold (Word et al., 1993).

Myometrial contractility

The basis of smooth muscle contraction in the myometrium is a result of the interaction of actin with myosin. Contraction is achieved by the formation of an actomyosin complex, between myosin heads and actin cross-bridges, and pivoting of the myosin light chain arms on this complex generates a potential displacement of 100Å. The interaction of actin and myosin chains is fundamentally regulated by intracellular free calcium concentration - $[Ca^{2+}]_i$. It is generally accepted that free Ca^{2+} combines with calmodulin (an actin binding protein) to activate myosin light chain kinase (MLCK), which phosphorylates a serine residue (Ser 19) in the 20 kDa myosin light chain. Phosphorylated myosin then interacts with actin, resulting in smooth muscle contraction. When myosin is subsequently dephosphorylated by a type 1 phosphatase, relaxation occurs.

Ca^{2+}/calmodulin mediates smooth muscle contraction via MLCK, by converting the enzyme from an inactivated state by exposing two amino acid residues in the catalytic core of the enzyme (Word et al., 1993). However Ca^{2+}/calmodulin is also capable of achieving smooth muscle relaxation via a negative feedback loop on MLCK. Negative feedback occurs as Ca^{2+}/calmodulin not only activates MLCK but is also capable of activating

Ca^{2+}/calmodulin kinase II (CaM kinase II). CaM kinase II phosphorylates a specific serine residue in MLCK, reducing its activity. Since the $[Ca^{2+}]_i$ required for MLCK phosphorylation is higher than that required for the myosin light chain, as $[Ca^{2+}]_i$ rises myosin light chain are initially phosphorylated, resulting in smooth muscle contraction. As $[Ca^{2+}]_i$ rises further, MLCK is phosphorylated resulting in reduced MLCK activity and subsequent relaxation. MLCK may also be regulated by cAMP-dependent protein kinases. It has been proposed that protein kinase A (PKA) phosphorylates MLCK, decreasing its Ca^{2+} affinity resulting in relaxation (Nishikawa et al., 1984)(Yamagishi et al., 1994). This explains the inhibitory effect of tocolytics, such as the ß - sympathomimetics and NO donors, which increase myometrial cAMP levels.

The phasic pattern of uterine contractility is not solely a function of MLCK activity but also fluxes in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ may be altered either via the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels or through the release of Ca^{2+} from intracytoplasmic Ca^{2+}, stored within the sarcoplasmic reticulum (SR). The SR may act to either induce smooth muscle contraction by releasing Ca^{2+} into the cytoplasm, or relaxation by sequesting Ca^{2+} into the SR. The SR Ca^{2+} store is regulated by a Ca^{2+} / ATPase pump and an intraluminal Ca^{2+} - binding protein, Calreticulin (Nash et al., 1994). Currently two receptors have been identified on the SR; the ryanodine receptor involved in 'calcium-induced calcium release', and the inositol 1, 4, 5, - triphosphate ($InsP_3$) receptor. $InsP_3$ binds to the $InsP_3$ receptor in the SR and promotes the release of Ca^{2+} into the cytoplasm. Currently three $InsP_3$ receptors have been identified (I, II and III) (Newton et al., 1994) (Wojcikiewicz, 1995). The $InsP_3$ receptor expressed in human myometrium is highly specific for the physiologic Ins (1, 4, 5) P_3 isomer (Wojcikiewicz, 1995). The ryanodine receptor is structurally similar to the $InsP_3$ receptor. The ryanodine receptor is sensitive to an increase in $[Ca^{2+}]_i$, which induces the release of Ca^{2+} from the sarcoplasmic reticulum.

Non-painful 'Braxton-Hicks' contractions become demonstrable from 24 weeks. Whilst they do not result in cervical dilatation they are thought to attenuate the lower uterine segment. Uterine contractions sufficient to raise the intrauterine pressure to 25 mmHg are required to dilate the pregnant cervix. The mean uterine pressure during labour is 1100 kPa/ 15 min, and this generates pressures of between 60 - 70 mmHg at the peak of contractions. During labour the upper uterine segment actively contracts and shortens, while the lower segment passively dilates and lengthens.

Myometrial Signalling

Whilst myometrial contractility can be induced by action potentials (electro-mechanical coupling), the myometrium is also influenced by factors that mediate their effects via receptor interaction. Myometrial contractility may be influenced by hormones, classic transmitters or lipid mediators, all acting through second messengers. Receptors bind to their respective ligands, and initiate a signal for activation (or inhibition) to an effector within the cell, which is translated into a cellular response. The link between the receptor and the effector is frequently a regulatory GTP-binding protein (G-protein). These proteins have trans-membrane domains, and therefore communicate between the cell surface receptors and the second messenger. Binding of an agonist to the receptor results in a conformational change allowing the intracellular region of the receptor to interact with the G-protein. G-proteins are heterotrimers (α–, β–, and γ– subunits) and are classified by their α subunit. Conformational change in the G-protein induces the exchange of GDP for GTP on the G-protein α subunit. The activated α subunit dissociates from the β γ subunits, then both the α-GTP and β γ subunits interact with second messengers. $G\alpha_s$ acts via adenylate cyclase to increase cytoplasmic cAMP, activating PKA and MLCK phosphorylation, promoting myometrial relaxation. $G\alpha_i$ has both an inhibitory effect on adenylate cyclase, reducing cAMP, and a stimulatory effect on phospholipase C, both effects therefore promoting myometrial contraction. $G\alpha_q$ stimulates phospholipase C, resulting in the hydrolysis of phosphatidylinositol 4, 5,-biphosphate (PIP_2), and the generation of two second messengers, inositol 1, 4, 5, - triphosphate ($InsP_3$), and 1,2-diacylglycerol (DAG). $InsP_3$ mobilises Ca^{2+} from the SR. DAG activates PKC, resulting in protein phosphorylation, and also facilitates liberation of arachidonic acid. $InsP_3$ and DAG therefore both promote myometrial contraction. Peptides (oxytocin, vasopressin and endothelin), catecholamines (noradrenaline) and inflammatory mediators (platelet activating factor, bradykinin) are all believed to mediate myometrial contractility via the PLC/ $InsP_3$/DAG pathway. Prostanoids appear to both activate and inhibit this pathway through different receptors.

cGMP is thought to mediate myometrial smooth muscle relaxation by decreasing $[Ca^{2+}]_i$ and myosin light chain phosphorylation. This is thought to be the basis of the mode of action of nitric oxide donors (such as glyceryl trinitrate) that activate guanylate cyclase, increasing intracellular cGMP.

1.3 The Biochemistry of Prostaglandins

1.3.1 Introduction

Prostaglandins are a group of potent biological mediators, principally derived from arachidonic acid, with diverse physiological and pharmacological activities. As hormones, they are capable of exerting both autocrine and paracrine effects, via specific cell membrane receptors.

Prostaglandins are involved in a wide range of normal physiological and pathological conditions including; vasoconstriction and vasodilation, cellular transformation and differentiation, inflammation, asthma, arthritis, renal function, regulation of immune responses, malignant disease and pregnancy. In the human, the principle sources of uterine prostaglandins are the fetal membranes and myometrium.

There is clear evidence that prostaglandins are involved in the mechanisms responsible for the onset of parturition. Prostaglandins induce myometrial contractility (Bennett et al., 1987a), result in changes in extracellular matrix metabolism associated with cervical ripening (Greer, 1992) and participate in fetal membrane activation (Romero et al., 1994). These observations have been exploited clinically for many years, and prostaglandins are routinely used to induce early or late termination of pregnancy, and induce labour onset at term. The use of prostaglandin synthase inhibitors in the management of preterm labour offers huge potential for the future.

Historical perspective

Prostaglandins were originally discovered as a major constituent of seminal fluid which exhibited vaso-active properties (Von Euler, 1934). This vaso-active component was originally thought to be a single substance secreted by the prostate gland and was therefore termed "prostaglandin" by Von Euler. In fact, seminal prostaglandins are a product of the seminal vesicles. Although an association has been demonstrated between reduced prostaglandin levels and reduced male fertility (Hawkins and Labrum, 1961), beyond this little is known about the role of prostaglandins in seminal plasma.

Prostaglandin F was the first prostaglandin to be isolated in 1960, from sheep prostate glands (Bergstrom, 1966). Arachidonic acid was subsequently identified as a precursor of prostaglandin E_2 (Van Dorp et al., 1964). Further studies showed that both thromboxane

and prostacyclin were also derived from the same fatty acid precursors (Hamberg et al., 1975)(Moncada et al., 1976).

Metabolic pathway

Prostaglandins are formed from the precursor arachidonic acid which is itself a substrate for at least three enzyme groups (see Fig. 1.04). The cyclo-oxygenase, or prostaglandin endoperoxide synthase (COX) pathway produces prostaglandins. The lipoxygenase enzyme pathways produces a series of hydroxyeicosatetraenioc acids (HETEs), leukotrienes and lipoxins. Arachidonic acid metabolism via epoxygenase pathways produces epoxyeicosatetraenioc acids.

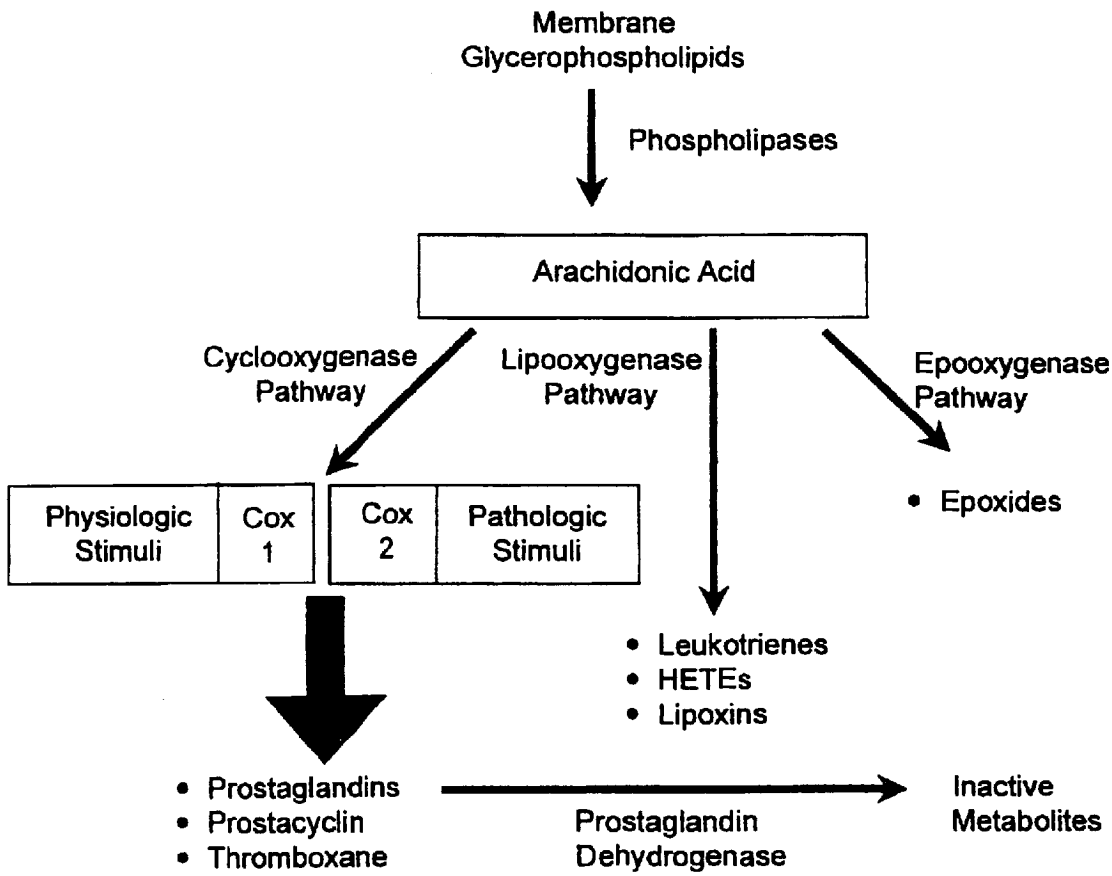


Fig. 1.04: Schematic representation of arachidonic acid metabolism

Prior to labour endogenous arachidonic acid metabolism in amnion is principally via the lipoxygenase enzyme pathways. With labour, there is an increase in arachidonic acid

metabolism and a change in the ratio of COX to lipoxygenase metabolism to favour synthesis of prostaglandin E_2 (Bennett et al., 1993). The roles of the lipoxygenase metabolites of arachidonic acid within the uterus are unknown, although 5-HETE may play a role in pre-labour (Braxton-Hicks) contractions (Bennett et al., 1987a)(Walsh, 1989).

Structure and Nomenclature

Prostaglandins are oxygenated polyunsaturated 20-carbon fatty acids, based on a twenty carbon parent compound, prostanoic acid.

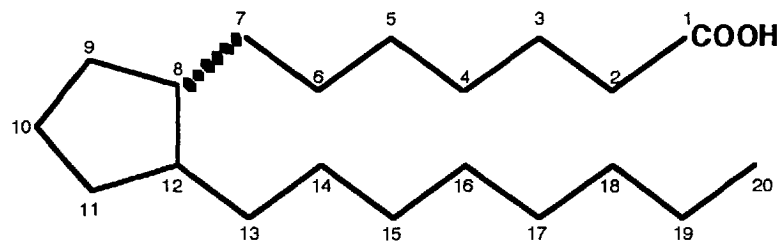


Fig. 1.05: Basic prostanoic acid structure

Nomenclature is based according to the nature and position of the various substituents on the cyclopentane ring, the number of double bonds found within the ring and on the alkyl side chains (Smith et al., 1990). Using this system the carbon skeleton is numbered sequentially from the carboxyl terminal, the letters A to J, divide the prostaglandins according to the substituents in the cyclopentane ring and numerals are used as a subscript to indicate the number of double bonds of the alkyl side chains. All prostaglandins have an α-hydroxyl group at carbon 15. Prostaglandins are divided into three series; those of the 1-series have a carbon 13-14 trans double bond, those of the 2 and 3-series have an additional double bond at carbon 5-6 and carbon 17-18 respectively. The spatial arrangement of the various substituents are described by the suffix α or β. Substituents lying below the plane of the cyclopentane ring are given the suffix α, and those lying above the plane of the ring are described by the suffix β.

1.3.2 Prostaglandin receptors

Each individual prostanoid binds to its own receptor. The effect of receptor binding, and their respective secondary messengers in human myometrium is shown in Table 1.01.

Currently four EP receptor subtypes have been identified. EP_1 and EP_3 mediate myometrial contractility by increasing $[Ca^{2+}]_i$ by activating PLC and Ca^{2+} entry through membrane channels and inhibition of adenyl cyclase. EP_2 and EP_4 inhibit myometrial contractility by stimulating adenyl cyclase. The presence of the EP_4 receptor in human myometrium remains equivocal. The EP_3 receptor exists in a number of different isoforms, due to alternative splicing of the mRNA. In human myometrium six isoforms have been identified (EP_{3a-f}) (Schmid et al., 1995).

Table 1.01: Prostaglandin receptors and their effects on myometrial contractility

Prostanoid	Receptor	G protein	Effector system	Effect
PGD_2	DP	G_s	AC↑	Relaxation
PGE_2	EP_1	G_i, G_q	Ca^{2+} channel↑	Contraction
			PLC↑	
	EP_2	G_s	AC↑	Relaxation
	$EP_{3a,b,c}$		AC↓, PLC↑	Contraction
	$EP_{3d,e,f}$		AC↓	Contraction
	EP_4	G_s	AC↑	Relaxation
PGF_2	FP	G_q	PLC↑	Contraction
PGI_2	IP	G_s	AC↑	Relaxation
TxA_2	TP	G_q	PLC↑	Contraction

Abbreviations: AC: adenyl cyclase; PLC: phospholipase C; ↑: stimulation; ↓: inhibition.

PGE_2 has the ability to both stimulate and inhibit myometrial contractility. The stimulatory effects appear to be mediated through a high affinity EP_1 subtype receptor, coupled to G_i, that does not activate PLC. In addition a low affinity receptor (EP_3) activates PLC via G_q (Asboth et al., 1996). The inhibitory effects (relaxation) of PGE_2 is probably mediated by either EP_2 or possible EP_4 coupled to G_s resulting in the stimulation of adenyl cyclase.

1.3.3 Arachidonic acid synthesis

Arachidonic acid is the major precursor of prostaglandins synthesis in mammalian cells. Arachidonic acid is synthesised directly from the essential fatty acid, linoleate, and is then stored in an esterified form in membrane phospholipid pools (phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol) within cells. The level of free

arachidonic acid reflects a balance between its release by specific hydrolases, and its incorporation by acyl transferases. Arachidonic acid is not incorporated during *de novo* synthesis of membrane phospholipids, rather it is incorporated later by the action of acyl transferases. Activity of the acyl transferases may be important in the regulation of prostaglandin synthesis.

Arachidonic acid is released from the phospholipid pool either by the direct action of specific acyl hydrolases: phospholipase A_2 and phospholipase A_1, or indirectly by the action of a phosphodiesterase, phospholipase C. Phospholipase A_2 releases arachidonic acid in a single step, catalysing the hydrolysis of the 2-acyl ester of phospholipid substrates. Whilst the phospholipase A_1 hydrolyses the 1-acyl ester resulting in the release of a 2-acyl lysophospholipid, which requires further enzyme action of a lysophopspholipase for the release of arachidonic acid.

Two forms of cellular phospholipase A_2 have been identified in intrauterine tissues, $sPLA_2$, a small molecular weight enzyme with homology to secreted PLA_2, and a larger cytosolic enzyme, $cPLA_2$. Placental $sPLA_2$ greatly exceeds expression in fetal membranes compared to $cPLA_2$, and there is no change in $sPLA_2$ expression during pregnancy or with the onset of labour (Bennett et al., 1994). Skannal et al. (Skannal et al., 1997) found that $cPLA_2$ activity in fetal membranes increases during pregnancy and is highest in anticipation of labour, becoming depleted after labour. This suggests that it is $cPLA_2$ and not $sPLA_2$ which mediates increases substrate arachidonic acid availability in fetal membranes with the onset of labour.

Until recently it was phospholipase activity which was thought to be the limiting factor in prostaglandin production in the fetal membranes. However any stimulus to prostaglandin production must also increase the activity of the enzyme cyclo-oxygenase (COX), since it has a short half life and undergoes destruction after a limited number of reactions (Marshall et al., 1987). Evidence that amnion produces predominately lipoxygenase metabolites of arachidonic acid before labour, and the change in the ratio of cyclo-oxygenase ; lipoxygenase metabolism with labour suggests that the activities of the two enzymes, COX and phospholipase, must be independently controlled (Bennett et al., 1987b)(Bennett et al., 1993).

1.3.4 Cyclo-oxygenase

Cyclo-oxygenase (COX) is the rate determining step of prostaglandin synthesis. Cyclo-oxygenase is a bifunctional, membrane-associated, haemprotein, that catalyses the conversion of free arachidonic acid to the unstable prostaglandin endoperoxide prostaglandin H_2 (PGH_2). PGH_2 is a short lived endoperoxide intermediate, rapidly metabolised by the various prostaglandin synthetic enzymes to the classical prostaglandins, thromboxanes and prostacyclins.

COX has two active sites catalysing two sequential reactions. First, fatty acid bis-oxygenase (cyclo-oxygenase) activity converts arachidonic acid to prostaglandin G_2 (PGG_2) and secondly, peroxidase activity catalyses the reduction of PGG_2 to PGH_2 (Hamberg and Samuelsson, 1967) (Hemler and Lands, 1980).

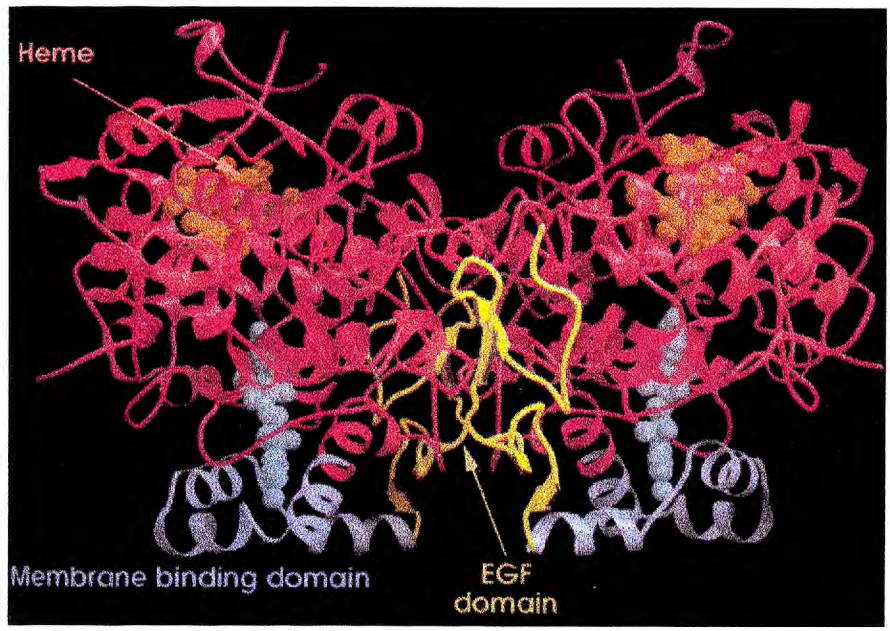


Fig. 1.06: Structure of the COX-2 dimer, shown in ribbon representation. The N-terminal (EGF domain) is shown in yellow, the membrane binding domain in blue, the catalytic C-terminal in magenta and the haem co-factor, required for peroxidase activity, in orange. From (Bazan et al., 1996) with permission.

Each of the cyclo-oxygenase homo dimers have three distinct domains. The N-terminal is referred to as the EGF domain, because of its similarity to other epidermal growth factor-like protein structures. The membrane binding domain contains four helices that both binds

the protein to the lipid membrane and provides access for substrates and inhibitors to the active cyclo-oxygenase site, from within the membrane. The third, and largest domain, is the catalytic C-terminal. The C-terminal domain includes both the active cyclo-oxygenase site, which is also the binding site for NSAID's and and the peroxidase active site containing the haem co-factor.

The cyclo-oxygenase activity of the COX enzyme provides a pharmacological target for many of the non-steroidal anti-inflammatory drugs, including aspirin, indomethacin and ibuprofen (Flower, 1974)(Vane and Botting, 1987). Non-steroidal anti-inflammatory drugs act either by competing with the arachidonic acid substrate for the active binding site on cyclo-oxygenase, or in the case of aspirin, by acetylating the enzyme in a time-dependent irreversible manner. Aspirin acetylates the hydroxyl side group of the Ser-530 amino acid residue. Acetylation of this side chain does not inhibit the catalytic site, but the presence of a bulky acetyl group interferes with the arachidonic acid binding site (DeWitt et al., 1990).

Currently two COX genes, expressing two distinct enzymes have been identified: COX-1 and COX-2. The possibility of further isoforms has not been excluded.

COX-1 gene

COX-1 cDNA was originally cloned in 1986 (Hla et al., 1986). Whilst the COX-1 and COX-2 genes map to different chromosomes (Ping et al., 1993), they encode proteins that share about 60% homology (Kujubu et al., 1991). The COX-1 gene is 25kb in length with 11 exons and 10 introns (Yokoyama and Tanabe, 1989).

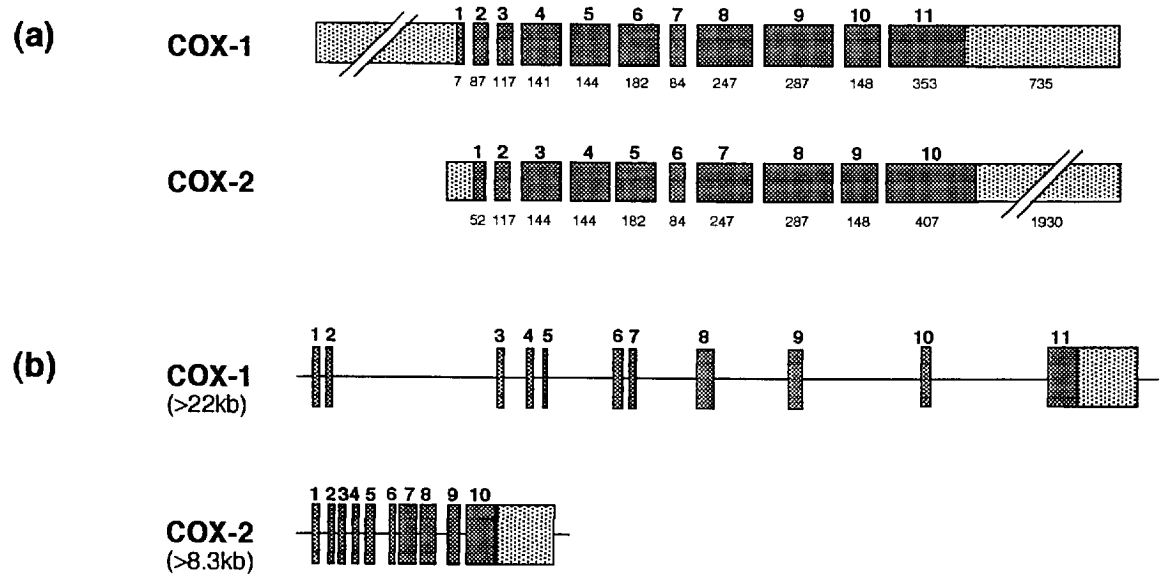


Fig. 1.07: COX-1 and COX-2 gene structure. **(a)** COX-1 and COX-2 mRNA structure, showing exon arrangement, and relative homolgy between genes. **(b)** COX-1 and COX-2 genomic DNA structure, showing exon and intron arrangement and the relative difference in sizes between the two genes. Darkly shaded boxes denote exons, with exon number indicated above, and size (in base pairs) below.

COX-1 has been considered a constitutively expressed gene, responsible for maintaining homeostatic mechanisms and background synthesis of prostaglandins in the gastric mucosa and renal tissue.

COX-2 gene

In contrast to COX-1, COX-2 is a 'primary response gene' (Herschman, 1991). Transcription of these genes is increased in response to various extracellular ligands, such as growth factors and hormones. However, *de novo* protein synthesis is not required. Transcription is induced when pre-existing latent transcription factors are activated, suggesting that it is capable of rapid activation and transcription and further evidence for this is provided by the small size of the gene (8.3 kb), and the number of introns. Transcriptional activation is a major mechanism by which COX-2 message is induced. The five prime (5') regulatory region of the COX-2 gene is known to contain putative binding sites for several different transcriptional factors, including NF-kB, NF-IL-6 and a cAMP-response element (CRE) (Tazawa et al., 1994). In contrast to the COX-1 protein, COX-2 has an 18 amino acid insertion near its carboxyl terminal, and this structural difference has

been exploited to generate antisera that can distinguish the COX-1 and COX-2 proteins (Kujubu et al., 1993).

COX transgenic mouse studies

Transgenic mice with knock-outs for COX-1, COX-2 and the prostaglandin EP_4 and FP receptors have been reported (Langenbach et al., 1995)(Morham et al., 1995)(Nguyen et al., 1997)(Sugimoto et al., 1997). Surprisingly, COX-1 knock-out mice lack the gastro-intestinal lesions that would be expected, given the importance of COX-1 mediated prostaglandin synthesis in the stomach. This may be because pharmacological inhibition of COX leaves intact the peroxidase activity. The only serious deficiency found in homozygous COX-1 knock-out mice was their failure to produce viable offspring when homozygous mice were crossbred. Heterozygotes of either sex, when mated with homozygotes, produced litters of normal size and survival, indicating that COX-1 expression in only 50% of pups, or their placentas, is sufficient for the health of the entire litter. Breeding studies with COX-1 knock-out mice ruled out the need for COX-1 during ovulation or spermatogenesis, but suggested an essential requirement for COX-1 during mouse parturition.

The first report of a COX-2 knock-out mouse (Morham et al., 1995) showed that homozygous COX-2 knock-out mice begin to die at around eight weeks of age, with few surviving for as long as sixteen weeks. All tissues examined in these mice were normal except the kidney. Nephropathy was seen by six weeks in these animals, which was gradually progressive until the animal succumbed. Examination of COX-2 deleted mice embryos showed that kidney maturation had ceased prematurely, after only a small percentage of nephrons had developed, with the vast majority of glomeruli and tubules remaining small and immature. During postnatal life the small number of functional nephrons begin to atrophy, with the development of glomerulosclerosis, interstitial inflammation and fibrosis, ultimately leading to kidney failure. This clearly shows an important role for COX-2 in the embryological development of the kidney.

Nguyen et al., (Nguyen et al., 1997) showed abnormal function of the ductus arteriosus in mice with deleted cyclic AMP-linked prostaglandin E receptor (EP_4). EP_4 receptor was found to be expressed in the smooth muscle of the mouse ductus arteriosus and homozygote deleted mice died in the postnatal period from failure of ductal closure. They suggested that the dependence of the ductus upon vaso-dilatory circulating prostaglandin

E_2 during fetal life depends upon the presence of the EP_4 receptor. In the absence of EP_4 receptor expression the ductus does not become dependant and so remains patent after prostaglandin E_2 withdrawal at birth.

Sugimoto et al., (Sugimoto et al., 1997) found that mice deleted from the prostaglandin F receptor (FP) had normal oestrous cycles, normal ovulation and normal implantation. Pregnant homozygote FP knock-out mice, however, failed to initiate labour because of failure of regression of the corpus luteum. In mice the onset of labour is stimulated by regression of the corpus luteum and withdrawal of ovarian progesterone, which allows upregulation of the uterine oxytocin receptor. Ovariectomy in FP knock-out homozygote mice released the progesterone inhibition allowing the onset of labour.

Whilst the respective roles of COX-1 and COX-2 in the menstrual cycle, ovulation and implantation may be similar in mice and humans, there appear to be fundamental differences both in the general mechanism of the onset of parturition and in the roles of COX-1 and -2 in parturition between the primate and the rodent. The corpus luteum regresses in the first trimester of human pregnancy and progesterone synthesis is taken over by the placenta and chorion. There is no large scale withdrawal of progesterone in the human prior to parturition, although there may be local withdrawal within the fetal membranes or changes in progesterone receptor function which leads to a functional progesterone withdrawal. Although in the mouse, COX-1-mediated prostaglandins appear to be important for normal parturition, in the human it is expression of COX-2 rather than COX-1 which increases within the uterus at term and it is therefore doubtful that COX-1 plays any significant role in the onset of human labour.

COX expression in fetal membranes

In situ hybridisation has localised COX-1 and COX-2 mRNA expression in fetal membranes (Slater et al., 1995). Expression of COX-2 is mainly within the amnion epithelial layer and also in the cells of the amnionic mesoderm, reticular layer of the chorion and the decidua. COX-2 is not expressed in the trophoblast layer of the chorion. COX-1 expression is more diffuse throughout the fetal membranes, in the amnionic epithelium, amnionic mesoderm and also in the chorionic mesoderm and decidua. RT-PCR has shown that in amnion at term, COX-2 mRNA has over 2 orders of magnitude greater abundance than COX-1 mRNA (Slater et al., 1994). Hirst et al. (Hirst et al., 1995) made similar findings using ribonuclease protection assays. COX-1 expression does not change

throughout pregnancy or labour. The expression of COX-2 in the fetal membranes increases throughout the third trimester and then doubles in association with labour (Hirst et al., 1995) (Slater et al., 1994). Enzyme kinetics studies suggest that the increase in COX activity with the onset of labour is due entirely to an increase in the synthesis of the COX enzyme (Smieja et al., 1993).

The increases in COX-2 expression in the fetal membranes closely mirror the concentration of free CRH in the maternal circulation, and CRH increase both COX-2 expression and prostaglandin synthesis in fetal membranes in tissue culture (Alvi et al., 1999). It has also been suggested that platelet activating factor (PAF), produced by the fetal lungs (Hoffman et al., 1990) (Moya et al., 1993) increases prostaglandin synthesis in amnion near to term, and that prostaglandins may then cross to the decidua, cervix and myometrium, to initiate labour. IL-1ß also increases prostaglandin synthesis in the fetal membranes (Romero et al., 1989) and in the myometrium (Bartlett et al., 1999) by a combination of increased phospholipase activity, secondary to phosphorylation of PLA_2 (Skannal et al., 1997), and up-regulation of COX-2 expression. It is possible that increased COX-2 expression during pregnancy is mediated through CRH, whilst the secondary increase occurring near to the onset of labour, is mediated by other factors such as IL-1ß, and in amnion, PAF.

Increasing cAMP concentrations leads to up-regulation of COX-2 in some cell types (Han et al., 1996). Both the PAF receptor and the IL-1 receptor are expressed in fetal membranes, decidua and myometrium (Allport et al., unpublished). PAF and IL-1ß act to up-regulate expression through the transcription factor NF-kB. The promoter regions of IL-1ß and IL-8 share a common NF-kB binding site, with the COX-2 promoter. Activation of NF-kB may therefore precipitate a positive feed back system of increased inflammatory and neutrophil attractant cytokines, leading to both a biochemical and cellular inflammatory reaction within the uterus.

COX expression in myometrium

In the myometrium there is expression of COX-1 and COX-2 mRNA at similar levels, but only COX-2 expression increases near to term, and with the onset of labour (Slater et al., 1999).

1.3.5 Prostaglandin dehydrogenase

Prostaglandins generated by the intrauterine tissues is rapidly inactivated by 15- hydroxy prostaglandin dehydrogenase (PGDH). Currently only a single gene has been identified, although other isoforms may be present as a result of alternative splicing of RNA (Slater et

al., unpublished). PGDH is principally located in the chorion, therefore limiting the paracrine effect of prostaglandins generated in the fetal membranes of amniotic fluid. Whether these prostaglandins exert an effect on myometrial contractility remains largely unproved. It has been proposed that PGDH present in the chorion serves to maintain the uterus in a quiescent state during pregnancy (Germain et al., 1994). There is some evidence to suggest that preterm labour may be associated with reduced chorionic PGDH (Challis, 1996). Furthermore Challis et al., has suggested that cortisol may play a role in downregulating chorionic PGDH, resulting in increased bioavailability of prostaglandins. This evidence would also correlate with the finding that cortisol induces COX-2 expression via CRH (Jones and Challis, 1990). Thus changes in expression of both PGDH (downregulated) and COX-2 (upregulated) may be responsible for the increased availability and bioeffect of prostaglandins associated with the onset of labour.

1.4 The Biochemistry of Nitric oxide

1.4.1 Introduction

Since nitric oxide (NO) was first identified and its properties described, there has been significant interest in its putative role in pregnancy and the onset of labour. Its physiological function as a potent mediator of smooth muscle relaxation implicates a role in myometrial quiescence in pregnancy, and changes in NO levels might be one of many factors responsible for the onset of labour.

1.4.2 Nitric oxide synthesis

NO is a highly reactive free radical with diverse biological activities. It was first identified in vascular endothelial cells (Furchgott and Zawadzki, 1980) where it was termed endothelial derived relaxation factor (EDRF) now synonymous with NO.

NO is synthesised by oxidative de-amination of the semi-essential amino acid L-arginine by isoforms of the transcriptionally regulated enzyme nitric oxide synthase (Palmer et al., 1988) (See Fig.1.08).

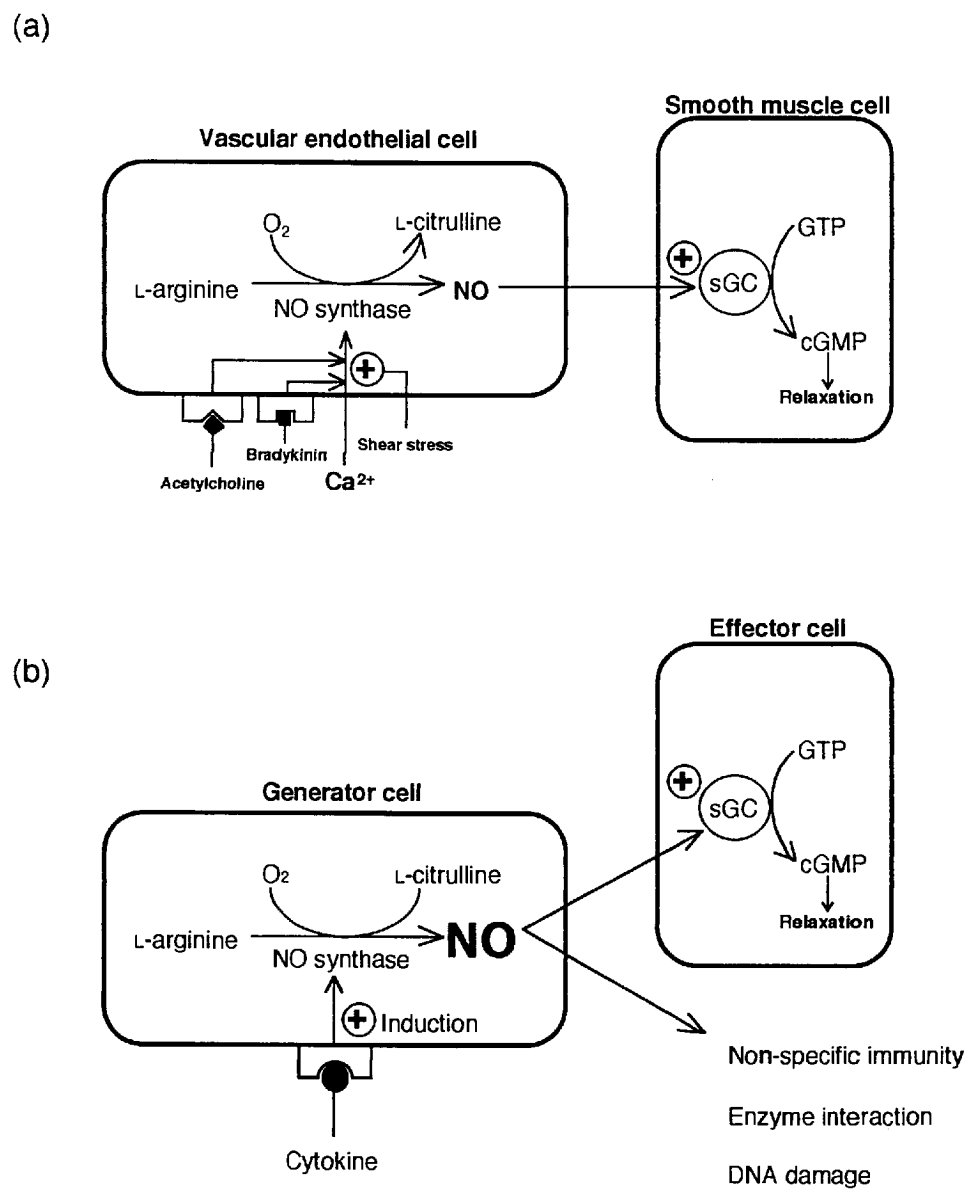


Fig. 1.08: Molecular synthesis and actions of nitric oxide. (**a**): Physiological role in vascular endothelial cells. Shear stress or receptor activation of vascular endothelium cells, result in calcium influx. Calcium-dependent NO synthase is stimulated to generate NO from L-arginine. NO diffuses to smooth muscle cells and stimulates soluble guanylate cyclase (sGC), resulting in enhanced synthesis of cyclic GMP (cGMP) from guanosine triphosphate (GTP), and smooth muscle relaxation. (**b**): Pathophysiological role in generator cells. Cytokine interaction with their receptors results in induction of calcium-independent NO synthase, with the resultant generation of NO. The high levels of NO generated from this system not only interact with soluble guanylate cyclase, but also exert additional cytotoxic effects.

The synthesis of NO requires L-arginine and NADPH and results in the formation of L-citrulline. Molecular oxygen is also required as a substrate for this reaction, which constitutes an L-arginine $N^{\omega}C^{G}$ dioxygenation, involving two separate, distinct steps. N^{ω}-hydroxyarginine is formed from a single O_2 molecule and NADPH in the presence of

tetrahydrobiopterin (BH_4). The second step is the oxidation of N^{ω}-hydroxyarginine to form NO and citrulline. The detailed mechanism of this reaction remains to be determined, although the presence of flavin coenzymes appears to be obligatory.

NOS exists in at least three isoforms, Type I: a constitutive, calcium-dependent neuronal form (nNOS), Type II: an inducible, calcium-independent form (iNOS), and Type III: a constitutive, calcium-dependent endothelial form (e/cNOS). NOS isoforms have common features with Cytochrome *P*-450 reductase (Bredt et al., 1991) with both oxidative and reductive domains.

1.4.3 Neuronal NOS (nNOS)

nNOS was the first NOS isoenzyme to be purified and cloned (Bredt et al., 1991) (NOS isoforms are variously titled Types I, II and III on the basis of the order in which they were originally identified and cloned, hence the title Type I for nNOS). nNOS mRNA is expressed at high levels in brain, in the central nervous system, some peripheral neurones and in skeletal muscle (Nakane et al., 1993). The nNOS gene maps to chromosome 12. It is thought that due to its widespread distribution this isoform constitutes the largest proportion of constitutive NOS activity in man. nNOS mRNA is approximately 10.5 kb in size expressing a 160.5 kDa protein. The nNOS protein shares 57% amino acid homology with e/cNOS, and 54% homology with iNOS. As for the other cloned isoenzymes there are consensus sequences in the protein for NADPH, FMN and FAD. Consensus sequences are also present for calmodulin and PKA and PKC.

1.4.4 Inducible NOS (iNOS)

The inducible isoform, whilst originally isolated from macrophages, is probably ubiquitous, such that all cell types are capable of expressing iNOS after appropriate cytokine induction. NO generated from iNOS differs significantly in concentration from that generated by the calcium-dependent isoforms (μM cf. pM concentrations). It is these high levels of NO generated in macrophages and other inflammatory cell types that is thought to mediate the cytotoxic effects of iNOS stimulation. DNA appears particularly sensitive to the destructive effects of NO. iNOS mRNA is only 4.7 kb in size (Chartrain et al., 1994), expressing a 133 kDa protein. The iNOS gene appears to map on either side of the centromere of chromosome 17. This may represent alternative forms of iNOS or rather intron-less pseudogenes. iNOS expression is highly transcription-dependent and iNOS is

rapidly induced by various cytokines (see below). The iNOS protein shares consensus binding sites for NADPH, FMN, FAD, PKA and somewhat surprisingly calmodulin. This paradox is resolved by the finding that iNOS binds calmodulin extremely tightly in an apparently calcium-independent manner (Cho et al., 1992). Calmodulin thus forms a subunit of iNOS.

1.4.5 Endothelial NOS (e/cNOS)

The endothelial NOS isoenzyme is functionally similar to nNOS and distinct from iNOS. e/cNOS is present in vascular endothelial cells and is probably responsible for NOS activity in the endothelium of all blood vessels. e/cNOS mRNA is 4.4 kb in size (Nadaud et al., 1994) generating a 161.5 kDa protein. The e/cNOS gene maps to chromosome 7. e/cNOS shares 51% amino acid homology with iNOS. e/cNOS is partially membrane bound and this is thought to be due to a consensus *N* myristoylation site on the N-terminus (Pollock et al., 1992).

1.4.6 Actions of nitric oxide

NO is a potent smooth muscle relaxant and platelet anti-aggregator. In the central nervous system it is thought to be involved in the formation of memory, and in the periphery, acts as a neurotransmitter in what were previously termed non-adrenergic non-cholinergic nerves, regulating various gastrointestinal, respiratory and genito-urinary tract systems. NO is also generated in activated macrophages and mediates non-specific immunity by its anti-microbial and anti-tumour activities. It is also thought to be responsible for regulating lymphocyte function. NO exerts its effect on smooth muscle by stimulation of the guanylate cyclase pathway. NO directly interacts with the haem moiety of this enzyme to increase levels of cyclic guanosine monophosphate and effect smooth muscle relaxation by altering intracellular calcium concentrations. NO has a half life of between 5 - 15 s, is widely diffusable and has a range of approximately 150 - 300 μm (Lancaster, 1996). It is rapidly oxidised to form nitrates and nitrites, or combines with superoxide anions to form peroxynitrites.

1.4.7 Nitric oxide donors

Diethylenetriamine-nitric oxide (DETA-NO) spontaneously releases nitric oxide, and therefore provides an appropriate source for the study of the effects of NO *in vitro*.

Similarly sodium nitroprusside (SNP) releases NO spontaneously (Noack and Feelisch, 1991). More complex molecules such as glyceryl trinitrate (GTN), isorbide mono- and isosorbide di- nitrate (ISMN and ISDN) require enzymatic metabolism to generate NO, and this must be considered when analysing NO kinetics.

1.4.8 Nitric oxide synthase inhibitors

The most widely used inhibitor of NOS is N^{ω}-monomethyl-L-arginine (L-NMMA). L-NAME is its methyl ester. L-NMMA is a competitive inhibitor of NOS, competing with L-arg . This inhibition is enantiomer specific, enabling the use of the respective stereoisomer as a control. L-NAME non-selectively inhibits all NOS isoforms. More recently compounds have been identified that are highly selective inhibitors of the NOS isoforms, allowing investigation of the relative contributions of the different isoforms to NO synthesis. L-N^6-(1-Iminoethyl)lysine (L-NIL) has been synthesised, and shown to be both a potent and highly selective inhibitor of iNOS (Moore et al., 1994).

An alternative inhibitor of NOS is diphenyleneiodonium. Whilst structurally unrelated to L-arg, diphenyleneiodonium inhibits NOS by competing with NADPH, one of its substrates. Diphenyleneiodonium inhibition can therefore be overcome at high NADPH concentrations, and inhibition is both progressive and irreversible.

Calmodulin antagonists such as trifluoroperazine and chlorpromazine have been shown to inhibit the constitutive, calcium dependent NOS isoforms, and paradoxically the calcium-independent isoform in rats (Evans et al., 1992).

2,4-diamino-6-hydroxypyrimidine (DAHP) inhibits the substrate BH_4, by inhibiting its synthesis. NO itself appears to exert a negative feedback on the activity of NOS. It is likely that this inhibition results from interaction of NO with the haem moiety of NOS.

1.4.9 Nitric oxide synthase regulation

iNOS is transcriptionally regulated, and expression can be induced by a number of different cytokines (Ding et al., 1988). Analysis of the 5' promoter region of the iNOS gene shows putative share putative response elements for NF-kB and NF-IL-6 (Nunokawa et al., 1994), which are shared with the COX-2 gene. Many effectors of NO production lead to simultaneous release of mediators from the COX pathway, suggesting a mechanism through which the NOS and COX pathways are capable of amplifying a physiological or

pathological response. Furthermore, interaction is possible between NO and COX at the enzyme level, due to the presence of a haem-iron moiety within the COX active site. The inter-relationship between NOS and COX is both complex and tissue dependent. Salvemini et al. (Salvemini et al., 1993) have shown in mouse macrophage cells that NO enhances COX activity through a cGMP-independent mechanism, and where both NOS and COX systems are present there is an NO-mediated increase in the production of pro-inflammatory prostaglandins that may result in an exacerbated inflammatory response. In contrast Swierkosz et al. (Swierkosz et al., 1994) have found *in vitro* that whilst both NOS and COX are co-induced by bacterial endotoxin, low levels of NO increase, whereas high levels inhibit both the expression and activity of COX-2. *In vivo* Dong et al. (Dong and Yallampalli, 1996) have found that in rat uteri at term there is a fall in nitrite production with an increase in PGE_2 during labour. In addition diethylenetriamine/NO, an NO donor, induced a dose-dependent increase in PGE_2, whilst L-NAME, a NOS inhibitor, decreased PGE_2 induction, suggesting that both exogenous and endogenous NO increases PGE_2 production.

Tetsuka et al. (Tetsuka et al., 1994) investigated the effect of PGE_2 on induction of NOS by IL-1ß in rat mesangial cells. They found that indomethacin, a non-selective COX inhibitor, enhanced both IL-1ß-induced iNOS mRNA levels and nitrite production. The addition of exogenous PGE_2 reversed the effects of indomethacin in a dose dependent manner. The inhibitory effect of indomethacin appeared to be independent of activation of adenylate cyclase. They suggest that PGE_2 might down-regulate iNOS transcription via the EP_1 receptor.

Calcium-dependent NOS expression may also be subject to regulation at the level of gene transcription. e/cNOS and nNOS are subject to oestrogen-induced transcriptional up-regulation, although the physiological significance of this finding remains unknown (Weiner et al., 1994b).

1.4.10 Nitric oxide synthase in pregnancy

Yallampalli et al. (Yallampalli et al., 1994b) have shown that in rats an L-arginine-nitric oxide-cyclic guanosine monophosphate system exists in the uterus. They found that the inhibitory effects of L-arginine on myometrial contractility were lower in samples taken during delivery or postpartum, suggesting that this system may contribute to the maintenance of uterine quiescence during pregnancy, but not during delivery. In the rabbit,

Sladek et al. (Sladek et al., 1993) found that NOS activity in the uterus fell on the last day of pregnancy. Similarly in the rat, Natuzzi et al. (Natuzzi et al., 1993) found the presence of different NOS isoforms within the uterus, with a fall in NOS activity with the onset of parturition. This suggests that in these animals, NO might play a role in maintaining myometrial quiescence during pregnancy until the onset of labour.

There is increasing evidence that the changes in NOS expression and activity seen in pregnancy are mediated by the sex steroid hormones. Weiner et al. (Weiner et al., 1994b) found that calcium-dependent NOS activity (nNOS and e/cNOS), measured indirectly by citrulline production, increased in various tissues in pregnancy, and following treatment with oestradiol. These changes in nitric oxide synthase activity occur early in pregnancy (Weiner et al., 1994a). Yallampalli et al. (Yallampalli et al., 1994a) examined the changes in rat uterine NOS activity. Nitric oxide concentrations, measured as a function of both cGMP and nitrite production, were relatively low in the non-pregnant group, but increased in pregnancy before falling with the onset of labour and post-partum. In contrast to Weiner, Yallampalli found that in uterine tissue nitric oxide levels fell in oestradiol treated animals, suggesting that the changes in NO levels seen in labour might be a reflection of increased oestrogen levels.

Buhimschi et al. (Buhimschi et al., 1995) have shown that a nitric oxide-cyclic guanosine monophosphate relaxation pathway exists in the human uterus and may be responsible for maintaining uterine quiescence during pregnancy. Whilst it is thought that the human uterus responds to NO by relaxation (Kumar et al., 1965), Jones et al. (Jones and Poston, 1997) have shown *in vitro* that the addition of L-arginine to spontaneously contracting human myometrial strips did not lead to any changes in contractility. They also showed that the addition of an inhibitor of nitric oxide synthase had no effect on myometrial contraction. They concluded that endogenous nitric oxide does not play an important role in the control of human, non-labouring uterine smooth muscle contractility. Norman et al., (Norman et al., 1997) have shown the relaxant effects of nitric oxide donors in a similar system. They found that spontaneous myometrial contractions were abolished in a concentration-dependent manner by both glyceryl trinitrate and sodium nitroprusside with a maximum inhibition, of between 33 and 47% and 31 and 47% respectively, at highest concentrations.

Localisation of NOS expression and activity in the uterus remains contentious. Localisation of NOS activity is difficult, due to notorious problems with NOS monoclonal antibodies in

immuno-localisation, and there are contradictory studies involving NOS expression within the uterus. Telfer et al. (Telfer et al., 1995) identified NOS in non-pregnant endometrium and pregnant myometrium, by both *in situ* hybridisation and immunocytochemistry. They demonstrated e/cNOS mRNA in endometrial glandular epithelium, endometrial stroma, myometrium and myometrial blood vessels. Thomson et al. (Thomson et al., 1997b) from the same group, reported immunoreactivity for all three NOS isoforms, but were unable to detect any changes in expression associated with parturition. In addition Howe et al. (Howe et al., 1996) whilst unable to identify iNOS expression in the uterus by immunohistochemistry, found no change in e/cNOS or nNOS expression by Western analysis before or after the onset of labour. In contrast Bansal et al., (Bansal et al., 1997) have shown, using immunostaining and Western analysis, that iNOS is expressed in human myometrial cells, and that expression falls significantly at term, and falls further (but not significantly) with the onset of labour. They concluded from these data that NO generated from iNOS might be relevant in the maintenance of myometrial quiescence during pregnancy, but a decline in NO generation was unlikely to promote the onset of parturition. They proposed that NO might function to prevent the co-ordinated uterine contractions required for labour, rather than to completely suppress all uterine activity.

Essentially the confounding factors are that type III (e/cNOS) expression or activity in uterine tissue might reflect the presence of vascular tissue, rather than true localisation within the myometrium. Similarly type II (iNOS) expression or activity might reflect the presence of activated macrophages.

1.4.11 Clinical use of nitric oxide donors

The use of nitric oxide donors in pregnancy is not new. In 1882 Fancourt Barnes, an obstetric physician had noted the profound hypotensive effects of amyl nitrate on vascular tone, and felt that amyl nitrate might exert a similar effect on the smooth muscle of the uterus. He administered amyl nitrate by inhalation, and was able to deliver the retained placenta of a patient with an hour-glass contraction of the uterus (Barnes, 1882). There is good evidence that nitric oxide donors have an inhibitory effect on pregnant human uterine contractility. Kumar et al. (Kumar et al., 1965) found, using intra-uterine pressure transducers, that NO donors were capable of decreasing the force of contraction of oxytocin-induced myometrial contractions. Peng et al. (Peng et al., 1989) subsequently exploited this observation clinically, with the use of intravenous nitroglycerin in the

management of retained placentae. More recently, Segal et al. (Segal et al., 1998) have shown in rats that the placenta appears to enhance uterine relaxation by nitroglycerin, possibly by enhancing the enzymatic release of NO from GTN.

Thomson et al. (Thomson et al., 1997a) have shown that NO donors (isosorbide mononitrate and glceryl trinitrate) when administered topically actually ripen the cervix, and with an improved side effect profile may confer benefits over the use of topical prostaglandins in the induction of labour (Thomson et al., 1998). Whether this evidence suggests that NO donors have a differential effect on distinct areas of the uterus, or than when administered systemically (transdermal) they fail to achieve high enough concentrations locally in the cervix to achieve cervical ripening, remains to be seen. Although to date there have been no reports of adverse effects on the cervix, in terms of cervical ripening associated with systemic NO donors. The differential effects of nitric oxide donors on myometrial activity and cervical ripening might be employed to beneficial effect in external cephalic version. Success at version might be improved through uterine relaxation, and subsequent labour onset (due to cervical ripening) might be desirable to prevent reversion (Groom et al., 2000).

El-Sayed et al. (El-Sayed et al., 1999) investigated both the safety of intravenous nitroglycerin and its tocolytic potential, compared to magnesium sulphate. Tocolytic failures (persistent contractions with cervical change or rupture of previously intact membranes) were more common with nitroglycerin compared with magnesium sulphate. In addition, haemodynamic alterations (persistent hypotension) complicated 25% of the nitroglycerin treatment group. Intravenous nitroglycerin has been used with apparent success for perioperative tocolysis in fetal surgery. Its use is associated with maternal systemic hypotension, increasing the risks of diminished utero-placental blood flow, and there have been case reports of pulmonary oedema associated with intravenous nitroglcerine following fetal surgery (DiFederico et al., 1996). Lung damage may have resulted from the combination of NO with exogenous oxygen, to form peroxynitrite, a known inhibitor of alveolar epithelial cell function. Intravenous nitroglycerin however appears to be the current drug of choice, at least in San Francisco, for perioperative tocolysis for fetal surgery.

Whilst NO donors appear to achieve uterine smooth muscle relaxation *in vivo*, their role in the management of preterm labour is less clear. Lees et al. (Lees et al., 1994) published a widely quoted observational study involving the use of glyceryl trinitrate (GTN) in the

arrest of preterm labour and the prolongation of pregnancy. The trial involved the use of 10 mg GTN patches applied topically to the abdominal skin every 24 hrs. Whilst the authors conceded that the diagnosis of preterm labour is difficult to make, as many women admitted with uterine contractions do not proceed to established labour and delivery, they quoted a mean prolongation of pregnancy of thirty-four days, and that all episodes of preterm labour responded to treatment. They went on to carry out a multi-centre, prospective, randomised control study comparing GTN with placebo, and GTN with intravenous ritodrine in the management of preterm labour (Lees et al., 1999). They found no overall difference between glyceryl trinitrate and ritodrine in the acute tocolysis of preterm labour. NO donors are well known to cause peripheral vasodilatation, resulting in side effects of hypotension and headache. The incidence of side effects in this trial was low and it is therefore possible then that the dose of GTN used was too low to mediate uterine smooth muscle relaxation.

The potential for nitroglycerin to prolong pregnancy in active preterm labour has also been investigated and compared to placebo (Smith et al., 1999). This small, randomised, double-blind study compared transdermal nitroglycerin patches with placebo, and found a smaller number of patients delivered by 48 hrs in the treatment group compared with placebo (6/17 compared with 10/16). Minor side effects of headache and flushing were recorded in the treatment group.

1.5 The aetiology and impact of preterm labour

Preterm delivery is defined as any delivery occurring before 37 weeks duration (WHO). The lower limit of definition of preterm delivery becomes the upper limit of definition of spontaneous abortion. As neonatal standards of resuscitation continue to improve, the gestational age associated with viability will continue to fall. Viability is clearly dependent on many factors, of which birth weight and standard of neonatal facilities are probably the most significant. In the UK viability is generally considered as a gestation beyond 25 weeks.

Preterm delivery affects up to 10% of all pregnancies, but represent approximately 60% of neonatal morbidity and mortality. Despite the widespread use of tocolytics there has been no documented decrease in preterm labour in the last two to three decades. The impact of preterm delivery on neonatal outcome remains significant.

1.5.1 Aetiology of preterm labour

The aetiology of preterm labour is multifactorial, and individual factors contributing to this syndrome may not be independent of each other. There is however compelling evidence for the involvement of various factors.

Social and biological risk factors

Low maternal age (≤ 20 yrs) is associated with preterm labour. Women in their first pregnancy also have a higher incidence of preterm labour.

Race and Ethnicity have a complex association with preterm labour, as assignment of race and ethnic group have more important implications for socio-economic group, nutrition and levels of antenatal care. These confounding factors are highlighted in a study analysing the incidence of preterm labour in military personnel. In a military population where the same standard of medical care was provided to all, the difference in the incidence of preterm labour between black and white patients was diminished (Cabral et al., 1990).

Cigarette smoking is causally associated with low birth weight when confounding factors have been excluded. The association with preterm labour however is more obscure. Since smoking is associated with prelabour premature rupture of membranes (PPROM) and placenta praevia, both precursors of preterm labour, it would be anticipated that there would be a direct association between smoking and preterm labour. However intervention

studies (stopping smoking) have failed to maka an impact on the rates of preterm labour (Lumley, 1995).

There is growing evidence that either maternal or fetal stress may be associated with preterm labour. Major life events have an association with prematurity (Newton and Hunt, 1984), as does the fetal stress of intrauterine growth retardation. A premature rise in CRH concentrations in maternal plasma has been associated with preterm labour (Sandman et al., 1997). Since placental CRH is up-regulated by corticosteroids, chronic up-regulation of maternal or fetal corticosteroid concentrations would lead to an early rise in CRH, signalling the early onset of labour.

Past obstetric history

The single most significant predictor of preterm labour is a previous preterm delivery. The risk of preterm delivery increases with the number of previous preterm deliveries: x 2.2 for one, x 3.7 for two and x 4.9 for more. The risk is reduced if a full term delivery intervenes (Bakketeig and Hoffman, 1981). The mechanisms behind these increased risks are unknown although it might be tempting to suggest that these patients might form a subset whose pro-labour factors outweigh their pro-pregnancy factors. The possibility of inherited factors cannot be excluded. There is no direct association between previous abortion and preterm labour.

Multiple pregnancy

Preterm labour complicates 44% of all multiple pregnancies. The concept that the length of pregnancy is controlled by a placental product, CRH, might explain the onset of preterm labour in the case of multiple pregnancy. It would be expected that a larger placental mass would generate higher levels of CRH. Down-regulation of CRH-BP would then occur prematurely, leading to an early rise in free active CRH in the maternal circulation. If parturitional phase three is signalled simply by a certain threshold of CRH, or of IL-1ß stimulated by CRH, then labour onset would occur earlier. Although progesterone protects the uterus against the effects of stretch it is probable that in severe polyhydramnios the degree of stretch is such that up-regulation of contraction-associated proteins nevertheless occurs early. It is likely that in addition to gap junction proteins, other contraction-associated proteins will be found to be stretch sensitive.

Prelabour premature rupture of membranes

Prelabour premature rupture of membranes (PPROM) is defined as rupture of the fetal membranes before labour onset. PPROM complicates approximately 30% of all cases of preterm labour, and has been estimated to account for up to 10% of all perinatal deaths (Kaltreider and Kohl, 1980). The aetiology of PPROM is largely unknown, although at term it is likely to reflect changes in the biomechanical and physiological properties of the fetal membranes (Naeye, 1982)(Evaldson et al., 1980). Many factors contribute to PPROM including, an incompetent cervix, multiple pregnancy, uterine anomalies, polyhydramnios, tobacco abuse, sexual intercourse, vaginal bleeding and infection.

Infection

In most cases it is thought intrauterine infection ascends from the vagina, although it may also be transplacental, or introduced during invasive procedures. It is probable that many cases of early preterm labour, thought to be associated with cervical incompetence, occur because vaginal pathogens enter the lower pole of the uterus through a partially open cervix, where they set up an inflammatory reaction

It seems logical that bacterial infection would initiate preterm labour by causing an inflammatory reaction within the uterus, and effectively switching on parturitional phase three at an earlier stage. Indeed this may reflect some form of survival behaviour, that prevents the fetus from prolonged exposure to intrauterine pathogens, so that delivery might confer a survival benefit, in spite of the obvious risks associated with prematurity.

The mechanisms by which bacteria initiate infections are probably multifactorial. Bacteria do not directly synthesise prostaglandins (Bennett and Elder, 1992), but they do release phospholipases which may liberate arachidonic acid from intracellular lipid pools, therefore increasing synthesis of prostaglandins. Lamont et al (Lamont et al., 1985) have demonstrated that bacteria release a substance which increases prostaglandin synthesis in amnion cells. This effect is probably mediated by phospholipase release (Bennett et al., 1990).

Another important mechanism by which bacteria might activate inflammatory mediators within the uterus is by the action of endotoxin. Endotoxin has been shown to stimulate prostaglandin production by monocytes. Romero et al (Romero et al., 1987) found that early spontaneous labour in prelabour premature rupture of membrane was more likely when endotoxin was detected. Endotoxin stimulates synthesis of inflammatory cytokines

including IL-1ß and IL-6, tumour necrosis factor and prostaglandins (Romero et al., 1988) in amnion cells and in intact fetal membranes (Rajasingam et al., 1998).

Iatrogenic preterm labour

Preterm labour might be considered iatrogenic following fetal intervention (amniocentesis or chorionic villus sampling) or fetal surgery, or delivery may be deliberately precipitated in the presence of maternal or fetal compromise. Elective delivery represents between 18 - 28% of all preterm births (Savitz et al., 1991).

Fetal factors

The principal fetal factor influencing preterm labour is male sex (Hall and Carr, 1982). The influence of male sex on prematurity is compounded by a worse prognostic outcome for male infants from the respiratory complications of prematurity. The reasons for this sex difference are unknown.

1.5.2 The impact of preterm labour

Incidence

Preterm labour occurs in 5 - 10% of all pregnancies. The incidence of preterm birth varies world-wide, from 5.8% in Western Australia and New Zealand to 10.6% in North America. The basis of these geographical differences is unknown.

Neonatal and Paediatric implications

In the neonatal period preterm infants are forty times more likely to die than term deliveries. Neonatal outcome however cannot be judged on mortality figures alone, as the surviving preterm infant is at increased risk from major complications including cerebral palsy, blindness, deafness, respiratory illness and complications of neonatal intensive care (1990) (McCormick, 1985). There is evidence to suggest the long term outcome for preterm infants may also be compromised. Lipper et al. (Lipper et al., 1990) found that of children whose birthweight was ≤ 800 g, 72% had neurological or developmental measurements in the 'suspect' or 'abnormal' range at seven years, requiring rehabilitation or early intervention services. More recently Hutton et al. (Hutton et al., 1997) carried out a longitudinal study to determine the differential effects of preterm birth and 'small for gestational age' (SGA) on both cognitive and motor ability. Assessing children born at ≤ 32 weeks gestation, at 9 years of age, they found that IQ was positively correlated with

birthweight ratio (the ratio of the observed birthweight, relative to expected birthweight, for a given gestational age). IQ was not associated with either birthweight or gestational age. Motor ability was positively correlated with birthweight, birthweight ratio and gestational age. They concluded that the effects of SGA and preterm birth differ. SGA was associated with impaired cognitive and motor ability whilst preterm birth was associated with impaired motor ability.

Financial cost

The obvious and immediate costs of preterm labour involve neonatal intensive care. The estimated cost for very low birth weight babies ranges from between £4,000 per survivor for babies weighing 1,000 to 1,500 g in the UK, to £100,000 per survivor for babies weighing less than 750 g (1986 costs) (Sandhu et al., 1986) (Boyle et al., 1983). When the costs of parents' loss of earnings, travel expenses, social and support services are considered the costs rapidly escalate. It was estimated that in 1990 the total annual cost to the NHS of neonatal intensive care for preterm infants was between £42 - £70 M (Griffin, 1993). The long term costs of preterm delivery are considerable, and it has been estimated that for all infants delivered below 2,500g birthweight in the UK, the total cost of special education would be £1.5 M per annum (Newns et al., 1984).

1.6 The management of preterm labour

1.6.1 Supportive Management:

Non-specific supportive management has been advocated in preterm labour. Its effectiveness has not been clearly defined due to the placebo effect associated with any treatment regimen in preterm labour. A combination of intravenous hydration and sedation is commonly advocated.

Arginine vasopressin (AVP) is a nine amino acid peptide, sharing close homology with oxytocin. AVP is released by the posterior pituitary in response to fluid depletion. There is evidence from both animal (Chan et al., 1996) and human (Maggi et al., 1990) studies that arginine vasopressin receptors (V1A) are present in the myometrium, and that AVP is capable of stimulating uterine activity by binding to both oxytocin and V1A receptors. A theoretical basis therefore exists for pre-hydration therapy of patients in threatened preterm labour, to reduce AVP levels, and hence potential uterine activity. Clinical studies however have shown that there is no advantage in prehydration (Freda and DeVore, 1996), and that the use of intravenous hydration in the management of preterm contractions is of no benefit (Guinn et al., 1997). Whilst pretherapy rehydration may prevent ketoacidosis, and possibly improve uteroplacental blood flow, intravenous hydration should be administered cautiously, to avoid the potential risks of fluid overload and pulmonary oedema associated with subsequent tocolytic therapy.

Sedation with narcotics or barbiturates does not result in myometrial relaxation, but may achieve a transitory decrease in uterine activity (Petrie et al., 1976). Their use should be cautioned by their profound effect on neonatal respiratory and central nervous system depression.

1.6.2 Tocolysis:

Analysis of effectiveness of management

The potential problem in analysing the effectiveness of tocolytics in the management of preterm labour is the establishment of the diagnosis. Preterm labour may be defined as uterine contractions before 37 weeks gestation, leading to cervical change. Clinically the differentiation between false and true labour may make diagnosis difficult. Even if the diagnosis is made, up to 60% of patients with painful uterine contractions and cervical

change, if left untreated, will resolve spontaneously and will not deliver until term (King et al., 1988). Tocolytics are not without significant side effects, and their clinical use must therefore be balanced by their perceived benefit. The criteria used to diagnose preterm labour have been proposed (Creasy and Herron, 1981) in an attempt to allow comparison between different clinical trials.

The decision as to when to treat preterm labour is largely dependent on gestational age, clinical scenario, and the level of neonatal care available. It is generally accepted that tocolytic intervention between 24 - 26 weeks is acceptable, although some might advocate their use between 20 - 24 weeks. The upper limit of therapy is more equivocal. There is some evidence to suggest that there is some maternal-fetal benefit if tocolytics are used up to 32 weeks, but at gestational ages of 34 weeks or more this benefit is lost (Macones et al., 1996).

Historical perspective:

The first agent proposed for use in preterm labour was isoxsuprine, a non-selective betamimetic (Bishop and Wouterxz, 1961). Its use was limited by its non-selective beta-adrenergic side effects. Intravenous alcohol has been proposed, however its clinical use was limited by poor tolerance, maternal and fetal toxicity and its questionable efficacy.

Modern Tocolytics:

Currently the most widely used tocolytics are the betasympathomimetics. Ritodrine is probably the mostly widely used betasympathomimetic in the United States. The effectiveness of betasympathomimetics at inhibiting uterine contractions and preterm labour is limited. Ritodrine is only effective at inhibiting preterm delivery for 24 - 48 hours (King et al., 1988). Short term delay in delivery improves neonatal outcome as a result of the administration of corticosteroids and in-utero transfer to a centre with neonatal intensive care facilities.

Magnesium sulphate has been used in the United States for many years in the management of pre-eclampsia. As a result of its widespread use, its actions as a potential tocolytic were recognised (Hall et al., 1959).

Prostaglandin synthetase inhibitors such as indomethacin, inhibit prostaglandin synthesis. Non-specific prostaglandin synthetase inhibitors are associated with significant materno-fetal side effects. The advent of selective COX inhibitors has provided a very real

breakthrough in tocolytic options, with the potential for preventing uterine contractions, in the absence of significant side effects.

More recently alternative therapeutic strategies have been explored including oxytocin receptor antagonists, calcium channel blockers, nitric oxide donors, and methylxanthines.

Betasympathomimetics:

Betasympathomimetics exert their action on the uterus by beta-adrenergic receptor stimulation. Intra-membranous beta-adrenergic receptors activate adenylate cyclase, resulting in an increase in intracellular cAMP (Scheid et al., 1979). cAMP reduces intracellular calcium and decreases the sensitivity of actin-myosin complexes to the uterotonic effects of calcium and prostaglandins (Carsten and Miller, 1987).

Ritodrine:

Ritodrine is the most widely used tocolytic in the United States. It was first introduced in 1972. Initial studies compared its use against ethanol and placebo (Merkatz et al., 1980). This study formed the basis of a Phase III FDA trial and found that ritodrine was significantly better than either ethanol or placebo in its ability to prolong pregnancy. Ritodrine was associated with a two to three week prolongation in pregnancy. Whilst the FDA was aware of potential complications of pulmonary oedema and fluid overload, in this trial, the incidence of these complications was no higher than in the other treatment groups (Barden et al., 1980). As a result the FDA awarded ritodrine a licence for use in pregnancy in 1980, and ritodrine remains the only tocolytic currently licensed for use by the FDA. Following these original studies it become apparent that the incidence of maternal cardio-vascular complications, and pulmonary oedema in particular, were being under-reported. With the release of an FDA alert bulletin, increasing the level of awareness of potential maternal complications, the true incidence of complications was established. Currently the incidence of betasympathomimetic pulmonary oedema is estimated between 0.01 and 9% (Leveno and Cunningham, 1992) (Benedetti, 1992).

Betamimetics cause a generalised vasodilatation, with resultant hypotension and compensatory increased cardiac output. Maternal heart rate, stroke volume and systolic blood pressure all increase. Cardiac output increases by up to 60% with betasympathomimetic therapy (Schwarz and Retzke, 1983). Antidiuretic effects of tocolytic therapy potentiate the risks of fluid overload and pulmonary oedema. Although fatal pulmonary oedema associated with ritodrine infusion is almost certainly multi-factorial, the

balance of evidence suggests that fluid overload is the most important single factor. There is some evidence that the incidence of cardiovascular complications is increased in higher order pregnancies (Gabriel et al., 1994). Metabolic complications include hyperglycaemia, primarily due to beta-adrenergic stimulation of glucagon release by the pancreas (Spellacy et al., 1978), and reactive hypoglycaemia is sometimes seen in fasting patients following cessation of treatment (Caldwell et al., 1987). Hypokalemia may also be associated with betasympathomimetic treatment, contributing to the risk of pulmonary oedema, and is attributable to tocolytic-induced intracellular shift of potassium by insulin (Cano et al., 1985).

Fetal side effects of betasympathomimetics are widely recognised. Fetal tachycardia is a direct result of myocardial beta-adrenergic stimulation, resulting in increased fetal cardiac output (Sharif et al., 1990). Ritodrine stimulates placental prostacyclin production (Jouppila et al., 1985), resulting in increased uteroplacental blood flow (Ylikorkala et al., 1983). Long term follow-up of betamimetics used in pregnancy, have shown no evidence of any significant growth disorders, or neurodevelopmental or social developmental alterations.

Notwithstanding its complications, ritodrine's efficacy as a tocolytic is by no means conclusive. Recent studies have failed to confirm its effectiveness at prolonging pregnancy. The Canadian Preterm labour Investigation Group (1992) demonstrated a 24 - 48 hour delay in delivery, with intravenous and oral ritodrine therapy. There was no significant difference in birth weight, perinatal mortality or neonatal morbidity between treatment and control groups. Meta-analysis of existing studies suggest that ritodrine is only successful at short term prolongation of pregnancy (King et al., 1988), and it is unlikely that the FDA would award ritodrine a licence for its use in pregnancy today.

Long term administration of betasympathomimetics is associated with tolerance to smooth muscle relaxation. This is probably mediated through adrenoreceptor down-regulation, and provides the basis for the clinical observation that the ability of these tocolytic agents to arrest preterm labour is transitory. The possibility of reducing tolerance, by pulsatile subcutaneous administration of betasympathomimetics has been explored (Casper and Lye, 1986), however evidence for its efficacy is currently limited, and only available from small, un-controlled studies (Lam et al., 1988).

Terbutaline:

Terbutaline is a selective betasympathomimetic, with similar efficacy and side effect profiles as ritodrine (Caritis et al., 1984). Comparative studies comparing tocolytic effects of intravenous terbutaline against intravenous magnesium sulphate, and subsequent oral maintenance therapy, failed to show any difference in delivery rates at either 48 hours (Beall et al., 1985), or after one week of therapy (Chau et al., 1992). Terbutaline is usually administered intravenously at a dose of 10μg/min, until either contractions stop or unacceptable side effects develop and increased to a maximal dose of 25μg/min. Similar efficacy and incidence of side effects has been observed when terbutaline is administered either subcutaneously or intravenously (Haspedis, 1988).

Salbutamol:

Salbutamol is another selective betasympathomimetic, whose efficacy, side effects and administration are similar to the other betamimetic agents. Comparative studies with terbutaline (Ryden, 1977) and ethanol (Sims et al., 1978) confirm no significant difference in side effects or efficacy. Anecdotally salbutamol is sometimes used when alternative betasympathomimetics have failed to abolish uterine contractions, although its efficacy in this context has not been proven. Salbutamol is administered intravenously at 10μg/min, gradually increased to a maximum of 45 μg/min, or until contractions stop or side effects intervene.

Magnesium Sulphate:

Magnesium sulphate has been used by physicians in the United States in the management of pre-eclampsia for many years, a result of which its additional ability to effect uterine relaxation is well known (Hall et al., 1959) (Hutchinson et al., 1965). Although the actual mechanism of uterine smooth muscle relaxation by magnesium sulphate remains largely unknown, it is likely that magnesium competes with calcium for entry into the cell via-voltage operated calcium gates (Guiet et al., 1985). Placebo controlled trials of magnesium sulphate are limited. Randomised comparative trials with ethanol (Steer and Petrie, 1977), ritodrine (Guiet et al., 1985) and terbutaline (Cotton et al., 1984) have shown no significant difference in delay in delivery at 48 hours. The incidence of maternal side effects was significantly lower with magnesium sulphate therapy compared with intravenous betasympathomimetics (2% cf. 38%) (Beall et al., 1985). As a result of the comparable efficacy of magnesium sulphate and betasympathomimetics, in the United States, magnesium sulphate represents first line tocolytic therapy. In Europe, where

magnesium sulphate use is less widespread, its application tends to be confined to cases where first line betasympathomimetic treatment has failed.

Side effects of magnesium sulphate therapy are limited if serum levels are maintained in the therapeutic range (5 - 6 mEq/l). Maternal tachycardia is not associated with magnesium therapy and cardiac output remains unchanged (Hollander et al., 1987) Serum calcium concentrations have been shown to fall with magnesium therapy (Cruikshank et al., 1981), and decreased bone density has been reported with long term therapy (Smith et al., 1992). Non-specific side effects such as nausea, vomiting, constipation and generalised muscle weakness are associated with higher levels of magnesium and long term therapy.

Significant complications of magnesium therapy are associated with magnesium toxicity. Levels above 8 mEq/l may result in loss of deep tendon reflexes, at levels between 12 - 15 mEq/l respiratory depression occurs and at levels in excess of 15 mEq/l cardiac arrest may occur. These complications are thought to be due to competition between magnesium and calcium at voltage-dependent calcium channels in muscle cells. Magnesium sulphate is usually administered intravenously, with a loading dose of 4 g (40 ml of 10% $MgSO_4$), infused over twenty minutes, followed by a maintenance dose of 2 g per hour. Serum magnesium levels should be measured one hour after the start of the maintenance dose, and six hourly thereafter. Renal function should be measured, and deep tendon reflexes elicited regularly. Fetal side effects are limited. There is no evidence of changes in uteroplacental blood flow (Keeley et al., 1993). Neonatal hypermagnesemia has been reported with prolonged therapy and following delivery during infusion (Lipsitz, 1971).

Prostaglandin synthetase inhibitors:

Non-selective Prostaglandin synthetase inhibitors:

Prostaglandins play a pivotal role in the onset of labour. The use of prostaglandin synthetase inhibitors should theoretically provide a useful therapeutic option in the management of preterm labour. Both placebo controlled (Zuckerman et al., 1984) and comparative studies with betasympathomimetics (Morales et al., 1989) and magnesium sulphate (Morales and Madhav, 1993) have shown that prostaglandin synthetase inhibitors are effective at delaying delivery, although none of the studies have analysed neonatal morbidity. Unfortunately the widespread maternal and fetal side effects have limited their application. More recently the use of selective prostaglandin synthetase inhibitors has

offered the potential of abolishing uterine contractions, with the absence of marked side effects.

Prostaglandins ripen the cervix, modulate calcium release from the sarcoplasmic reticulum and directly activate myometrial calcium channels. As already discussed prostaglandins are generated from arachidonic acid by the two cyclo-oxygenase isoforms, COX-1 and COX-2. Non-selective prostaglandin synthetase inhibitors inhibit both COX-1 and COX-2. The 'beneficial' effects of decreased prostaglandin synthesis are the result of COX-2 inhibition, whilst the side effects seen with prostaglandin synthetase inhibitors are largely a result of COX-1 inhibition. Gastro-intestinal irritation is commonly reported. Platelet dysfunction, whilst theoretically reversible, may result in prolonged bleeding times. Renal dysfunction and an altered immune response have also been reported. Whilst these complications may be undesirable, it is the fetal side effects that are more profound, and that have limited the application of non-selective prostaglandin synthetase inhibitors.

Oligohydramnios results from decreased fetal urine output. It complicates between 10 - 30% of cases treated with indomethacin. In the presence of normal renal blood flow, it is thought that oligohydramnios is a result of a prostaglandin-mediated alteration in tubular function (Mari et al., 1990). Urine output may fall by up to 50%. Oligohydramnios appears to be dose-dependent and resolves on stopping treatment. Isolated cases of fetal renal failure have been reported with indomethacin therapy (Buderus et al., 1993). Prostaglandin E_1 is responsible for maintaining dilatation of the fetal ductus ateriosus. Inhibition of prostaglandin E_1 by indomethacin results in ductal constriction. Constriction of the ductus arteriosus complicates between 20 - 50% of fetuses exposed to indomethacin therapy. Ductal constriction can be visualised ultrasonically within 2 hours of treatment and resolves after 24 hours. Ductal effects of indomethacin appear to be gestationally related and most significant after 32 weeks (Huhta et al., 1987). Chronic constriction of the ductus arteriosus has been associated with fetal pulmonary hypertrophy, pulmonary hypertension and hydrops. Persistent fetal pulmonary hypertension may also result in neonatal pulmonary hypertension (Levin et al., 1978). Indomethacin is usually administered orally, with a 50 mg loading dose followed by 25 mg every 6 hours. Amniotic fluid indices and ductal blood flows should be measured ultrasonically at least twice weekly during treatment with prostaglandin synthetase inhibitors.

Sulindac:

Adverse fetal side effects of non-specific prostaglandin synthetase inhibitors has prompted the use of agents with limited placental transfer. Sulindac (Clinoril) is a pro-drug closely resembling indomethacin. It is oxidised to a sulfinyl group, and subsequently reduced in the liver to the active sulphide form of the drug. Whilst fetal blood levels of the pro-drug are similar to those in the maternal circulation, levels of the active sulphide are lower in the fetus, due to decreased reduction of sulindac to the active sulphide in the fetal liver (Kramer et al., 1995). Evidence available from comparative studies suggest that sulindac is as effective as indomethacin at controlling preterm contractions, without associated changes in amniotic fluid indices (AFI) and ductal flows (Carlan et al., 1992) although this effect is not seen in placebo controlled studies (Carlan et al., 1995). Theoretically sulindac has some benefits over indomethacin, with an improved side effect profile, it does however inhibit both the constitutive and inducible COX isoforms.

Selective COX-2 inhibitors:

Indomethacin has roughly equal efficacy at inhibiting both COX-1 and COX-2. The advent of specific inhibitors of COX-2 has provided a very real development in potentially useful tocolytic agents. The use of selective COX-2 inhibitors is still being developed. Nimesulide is a selective COX-2 inhibitor, with approximately 100 fold greater inhibition of COX-2 compared with COX-1. There is early evidence from case reports that nimesulide is successful at inhibiting preterm contractions, with limited effects on AFI or ductal flow (Sawdy et al., 1997). As further highly COX-2 specific compounds are developed, the incidence of COX-1 related side effects will diminish, and with their background of proved efficacy it is likely that this group of drugs will supersede betasympathomimetics as first line therapy for preterm labour.

Oxytocin receptor antagonists:

Whilst no changes have been found in the concentrations of oxytocin, with either the onset, or the progress of labour (Thornton et al., 1992) the density of myometrial oxytocin receptors does increase towards term (Fuchs et al., 1984). Oxytocin antagonists, such as atosiban, competitively bind with cell surface oxytocin receptors, inhibiting the uterotonic effects of oxytocin in a dose-dependent manner (Akerlund et al., 1987). Atosiban has been shown to decrease uterine contractions in threatened preterm labour (Goodwin et al., 1994). Atosiban is usually administered intravenously. Atosiban crosses the placenta, although fetal levels are only 10% of maternal (Akerlund et al., 1987), and this results in

minimal fetal side effects. Recent studies (Moutquin et al., 2000) comparing atosiban with ritodrine have shown that atosiban was comparable in clinical effectiveness to conventional ritodrine therapy and was better tolerated, with no evidence of significant maternal or fetal adverse events. Neonatal morbidity, which was similar with both ritodrine and atosiban appeared to be related to the gestational age of the infant rather than to the exposure to either tocolytic agent.

Calcium channel blockers:

Calcium channel blockers such as nifedipine and nicardipine have a theoretical role in the management of preterm labour. They appear to block voltage-dependent calcium channels in myometrial cells (Braunwald, 1982). Calcium channel blockers appear to be potent mediators of uterine relaxation, and although clinical trials are limited, there is some evidence that calcium channel blockers are able to delay delivery in excess of 48 hrs (Read and Wellby, 1986).

Whilst their action may be complicated by maternal hypotension and reflex tachycardia, the incidence of cardiovascular side effects is lower than that associated with the betasympathomimetics (Read and Wellby, 1986). The effects of calcium channel blockers on placental blood flow appear equivocal. Loss of placental autoregulation has been reported with nifedipine treatment (Maigaard et al., 1984). Nifedipine is usually administered either sublingually or orally, at a dose of 20 mg, 6 hourly.

Nitric oxide donors:

The effects of nitric oxide on myometrial smooth muscle and the potential use of NO donors as tocolytics in the management of preterm labour has been previously discussed (section 1.4.11).

Methylxanthines:

Methylxanthines such as aminophylline are capable of decreasing uterine activity by inhibiting phosphodiesterase degradation of cAMP. Clinical studies are limited but suggests some prolongation of pregnancy with aminophylline (Liu and Blackwell, 1978). degradation of cAMP is more marked in association with ß adrenergic stimulation. There is therefore a theoretical indication for combining methylxanthines with betasympathomimetics, although this combination therapy has not been evaluated clinically.

Evidence to date suggests less maternal side effects associated with aminophylline compared with ritodrine. Currently its use as a tocolytic is limited to experimental protocols.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Tissue and Cells

Fetal membranes

Fetal membranes and placentae were collected from deliveries at Queen Charlotte's and Chelsea Hospital and The Hammersmith Hospital. Tissue was collected immediately after delivery, and washed in PBS to remove amniotic fluid and blood. The fetal membranes were divided from the placental tissue, and carefully separated into amnion and chorio decidual layers. Membranes were collected from term deliveries, before and after labour onset, and preterm deliveries throughout the second and third trimester. The availability of preterm tissues was limited. The placenta and fetal membranes are routinely discarded after delivery in these hospitals, and therefore informed consent was not required from patients for these tissues. First trimester tissue was collected at time of surgical termination of pregnancy. Tissue obtained by suction curettage was subsequently examined, and the fetal membranes and trophoblast tissue identified. Informed, written consent in line with hospital policy was obtained from all patients undergoing surgical termination of pregnancy.

Myometrium

Myometrial tissue was collected from patients undergoing caesarean section at St. Thomas' Hospital, Queen Charlotte's and Chelsea Hospital and The Hammersmith Hospital. Myometrial tissue was collected from the upper margin of uterine incision at time of lower segment caesarean section. Myometrial biopsies avoided decidual and vascular tissue. Three groups of patients were analysed, term labour (n = 5), term non-labour (n = 11) and preterm non-labour (n = 13). The term patients' gestational age range was 38 - 40 weeks, in the preterm non-labour group the gestational age range was 26 - 37 weeks. In the term labour group, all patients had regular painful contractions with associated cervical dilatation. The indications for caesarean section in the term labour group were associated with fetal distress. There was no evidence of uterine dysfunction. The non-labour group had no evidence of uterine contractions or cervical change. The indications for caesarean section in this group were breech presentation, hypertensive disease and previous

caesarean section. This study was approved by the hospital's ethics committee and all patients gave written, informed consent.

Cells

Human amnion derived epithelial cells (WISH) and U937 cells were obtained from ATCC. Human umbilical epithelial cells (HUVEC) and Human myometrial cells were a kind gift from Dr. Simon Bartlett (King's College, London). Hammersmith Obstetrics and Gynaecology 1; cervical epithelial cell line (HOG-1) cells were a kind gift from Dr. John White (Hammersmith Hospital, London).

Tissue preparation

Tissue for RNA, or protein extraction was immediately snap frozen in liquid nitrogen, and subsequently stored at -80°C. Tissue for *in situ* hybridisation was fixed in freshly prepared 4% paraformaldehyde in PBS, for 24 - 48 hours at 4°C. Fetal membranes for prostaglandin assays were placed in DMEM and assayed immediately. Myometrial tissue for contractility studies was placed in cold physiological salt solution (PSS), stored at 4°C, and assessed within 12 hr.

2.1.2 Reagents

All reagents, with the exception of those subsequently listed were analar grade, supplied by BDH Ltd. Trizma base, NaCl, BSA, ethidium bromide, dextran sulphate, orange G, N-laurylsarcosine, bromophenol blue, xylene cyanol, toluidine blue, tetracycline hydrochloride, ampicillin, X-gal, TESPA, Mayers hematoxylin, eosin, triethanolamine, 2-mercaptoethanol, acetic anhydride, spermidine, dithiothreitol, phorbol ester, interleukin-1ß, corticotrophin releasing hormone, diethylpyrocarbonate and dextran sulphate (Sigma Chemicals Co). Phenol (Rathbone Chemicals Ltd). Agar, tryptone, yeast extract (Difco Laboratories). Histoclear (National Diagnostics). RNAzol (Biogenesis). DMEM, antibiotics, antimycotics, fetal bovine serum (Gibco-BRL). UTP-S (Amersham International). Ultrapure dNTPs, random hexanucleotide primers (Pharmacia LKB).

2.1.3 Enzymes

Restriction endonucleases (Gibco-BRL, New England Biolabs, IBI). M-MLV reverse transcriptase, T4 polynucleotide kinase, Klenow, T7 and T3 RNA polymerase, placental RNAse inhibitor (Gibco-BRL). Terminal deoxynucleotidyl transferase (Promega). T4

DNA ligase, RNAase A, proteinase K, (Boehringer Mannheim). *Taq* DNA polymerase (Bioline, Gibco-BRL).

2.1.4 Blotting and autoradiography

Hybond N (Amersham International), 3 MM chromatography paper (Whatman Ltd), X ray Film (Kodak X-OMAT AR, Fuji RX or Konica AX), Instant photographs (Polaroid Ltd., type 667 film) Cassettes and intensifying screens (Genetic Research Instrumentation).

2.1.5 Radiolabelling and spun column chromatography

[α^{32}P] dCTP (3000 Ci/mmol), [γ^{32}P] dATP (3000 Ci/mmol), [α^{33}P] dATP (800 Ci/mmol), [α^{35}S] dATP (3000Ci/mmol) (Amersham International), sephadex G50 and G25 (Pharmacia), biopolymer wool (Interpret).

2.1.6 Gel electrophoresis

Agarose and low melting point agarose (BRL).

2.1.7 Bacterial strains

JM109 competent cells were used for DNA plasmid transformations (Promega).

2.1.8 Antibodies

Antibodies for Western analysis and EMSA for COX-1 (#SC-1752), COX-2 (#SC-1745), iNOS (#SC-649), e/cNOS (#SC-1654), NFkBp65 (#SC-372-G), Actin (#SC-1616) (Santa Cruz).

2.1.9 DNA samples

Molecular size markers and cloning vectors

1kb ladder, 100bp ladder (Gibco-BRL). pGEM-T Easy cloning vector system (Promega).

Oligonucleotides

Oligonucleotides used for PCR and in situ hybridisation are displayed in Table 2.01. iNOS sense and antisense primers were a kind gift from Dr. Robert Newton (National Heart and Lung Institute, Royal Brompton Hospital, London). All other oligonucleotides were synthesised commercially, and obtained deprotected and desalted from Genosys

Laboratories, Cambridge. Consensus and non-consensus oligonucleotides for gel shift assays were obtained from Promega.

Table 2.01: PCR primers and their uses

Name	Use	Sequence 5'-3'
iNOS sense	RT-PCR / *in situ*	GAGCTTCTACCTCAAGCTATC
iNOS antisense	RT-PCR / *in situ*	CCTGATGTTGCCATTGTTGGT
iNOS (antisense 871)	*in situ*	CCCGGGCAGTGGAACAGCTG
iNOS (antisense 901)	*in situ*	GCACGTGTCTGCAGATGTGT
iNOS (antisense 680-661)	*in situ*	CCGTAATATTGGTTGACAAA
iNOS (antisense 760-741)	*in situ*	AGGTTCCTGTTGTTTCTATC
iNOS (antisense 850-831)	*in situ*	GGTTGGACCACTGGATCCTC
e/cNOS sense	RT-PCR / *in situ*	GCACAGGAAATGTTCACCTAC
e/cNOS antisense	RT-PCR / *in situ*	CACGATGGTGACTTTGGCTAG
e/cNOS (antisense 1180)	*in situ*	GTATCCAGGTCCATGCAGAC
e/cNOS (antisense 1240)	*in situ*	AGCACGGCCACGTTGATTTC
e/cNOS (antisense 780-761)	*in situ*	GCCCGCGTAGCGCACCAGCT
e/cNOS (antisense 881-861)	*in situ*	TCGAAGCGACCGTTTCCTGG
e/cNOS (antisense 1110-1091)	*in situ*	GCCGATCTCAGTGCTCATGT
nNOS antisense 2449R	RT-PCR	GGGACAGGCGCTGAACTCCA
nNOS sense 1943F	RT-PCR	TGTGTGGGCAGGATCCAGTG
nNOS antisense 1962R	RT-PCR	CACTGGATCCTGCCCACACA
nNOS sense 1394F	RT-PCR	GGAATCCAGGTGGACAGAGA
COX-2 sense	RT-PCR	TTCAAATGAGATTGTGGGAAAATTGCT
COX-2 antisense	RT-PCR	AGATCATCTCTGCCTGAGTATCTT
GAPDH sense	RT-PCR	CCACCCATGGCAAATTCCATGGCA
GAPDH antisense	RT-PCR	TCTAGACGGCAGGTCAGGTCCACC
Calponin sense	RT-PCR	GATGGCATCATTCTTTGCGA
Calponin antisense	RT-PCR	TTGTAGTAGTTGTGTGCGTG

2.1.10 Solutions

Reverse transcriptase polymerase chain reaction

PCR buffer (10x KCl)

500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 0.1% (w/v) gelatin.

PCR buffer (10x NH_4^+)

160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20.

Southern hybridisation

Southern pre-hybridisation solution

6x SSC, 10x Denhardt's solution, 5mM EDTA, 100μg/ml microwaved salmon sperm DNA, 0.5% SDS and 0.02% w/v sodium pyrophosphate.

Southern hybridisation solution

4x SSC (6x SSC for oligonucleotides), 0.1% SDS, 5 x Denhardt's solution and 100μg/ml microwaved salmon sperm DNA.

Southern denaturing solution

1.5 M NaCl, 0.5 M NaOH.

Southern neutralising solution

1.5 M NaCl, 0.5 M Tris-HCl pH 7.4.

10 x Denhardt's solution

0.2% Ficoll, 0.2% bovine serum albumin and 0.2% polyvinylpyrrolidone.

SSC

0.15 M NaCl, 0.015 M sodium citrate pH 7.0 (usually made as 10x SSC containing 1.5 M NaCl, 0.15 M sodium citrate pH 7.0).

T E buffer

10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0.

E buffer (10 X, 1 L)

48.7 g Tris base, 16.4 g NaOAc, 7.4 g EDTA, pH with glacial acetic acid to pH 7.0.

Oligo-labelling buffer

Solution O: 1.25 M Tris-HCl, 0.125 M MgCl2 pH 8.0 (store at 4°C). Solution A: 1ml Solution O + 18 μl 2-mercaptoethanol + 5 μl each of 0.1M dATP, dTTP, and dGTP, dissolved in TE and pH adjusted to 7.0. Solution B: 2M HEPES pH to 6.6 with NaOH (store at -20°C). Solution C: Hexadeoxyribonucleotides evenly suspended in TE at 90 OD units/ml (store at -20°C). Oligolabelling buffer is A : B : C in the ratio 1 : 25 : 1.5 (store at -20°C).

in situ hybridisation

Proteinase K buffer

50 mM KCL, 10 mM Tris-HCL (pH 8.7), 0.25 mM $MgCl_2$, 0.05% TWEEN, 0.1 mg/ml Proteinase K stored at -20°C.

Hybridisation buffer

60% deionised formamide, 0.3 M NaCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA, 10 mM $NAPO_4$ (pH 6.8), 10% dextran sulphate, 1x Denhardt's, 0.5 μg/ml yeast RNA, 0.5 μg/ml poly A RNA, 50 mM DTT.

Western blotting

PBS 0.1% TWEEN

Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g $NaHPO_4$ and 0.24 g $KHPO_4$ in 1 l distilled water and equilibrate to pH 7.4. 10 ml TWEEN.

T-Wash

0.5% Triton-X 100, 5 mM NaF, 10 mM EDTA. 10 mM PMSF, 4 μg/ml pepstatin and 0.5 μg/ml leupeptin added fresh.

7.45x Running gel

7.5 ml 4 x Tris-HCl + SDS (pH 8.8), 7.5 ml Bis acrylamide 30%, 15 ml distilled water. 200 μl APS, 20 μl TEMED.

10x Stacking gel

2.5 ml 4 x Tris-HCl + SDS (pH 6.8), 1.0 ml Bis acrylamide 30%, 6.4 ml distilled water. 50 μl APS, 10 μl TEMED.

10 x Running buffer

30.2 g Tris-HCL, 144.2 g glycine and 10 g SDS in 1 l distilled water.

Electrophoresis transfer buffer

25 mM Tris-HCL, 192 mM glycine, 20% (v/v) methanol, pH 8.3.

Gel shift assay

Nuclear protein extraction buffer A

10 mM HEPES (pH 7.9 at 4°C), 1.4 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 0.5 mM DTT.

Nuclear protein extraction buffer C

20 mM HEPES (pH 7.9 at 4°C), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF.

Nuclear protein extraction buffer D

20 mM HEPES (pH 7.9 at 4°C), 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF.

DTT and PMSF added fresh to all nuclear protein extraction buffers.

5 x Gel shift binding buffer

20% (v/v) glycerol, 5 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 250 mM NaCl, 50 mM Tris-HCL (pH 7.5).

RNA extraction

Solution D

4 M guanidinium isothiocyanate, 25 mM Na citrate, 0.5% sarcosyl solution, 0.1 M beta mercaptoethanol.

Radio-immuno assay

RIA buffer

50% 0.5 M Tris-HCL (pH 7.4), 50% Tris-HCL and 0.1% gelatin.

Charcoal/ dextran buffer

50 ml 0.5 M Tris-HCL (pH 7.4), 0.2 g dextran sulphate, 1.0 g activated charcoal.

PCR Sub-Cloning

LB medium

10 g Bacto-Tryptone, 5 g Bacto-Yeast extract, 5 g NaCl. (pH 7.0).

LB plates with ampicillin/IPTG/X-Gal

Add 15 g of agar to 1 l of LB medium. Allow to cool to 50°C before adding ampicillin to a final concentration of 100 μg/ml. Supplement with 0.5 mM IPTG and 80 μg/ml X-Gal. Pour 35 ml of medium into 85 mm petri dishes.

SOC medium

2.0 g Bacto-Tryptone, 0.5 g Bacto-Yeast extract, 1 ml 1M NaCl, 0.25 ml 1M KCl, 1 ml 2M Mg^{2+} stock (1M $MgCl_2$. $6H_20$/1m $MgSO_4$. 7 H_20), 1ml 2M glucose.(pH 7.0).

Preparation of plasmid DNA

Solution 1

50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA.

Solution 2

0.2 N NaOH (freshly diluted from a 10 N stock), 1% SDS.

Solution 3

60 ml 5 M Potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_20.

2.1.11 Computing

General data storage and restriction enzyme sequence searches were performed using 'DNA Strider' on Apple Macintosh Computers. DNA homology searches of the EMBL and Genbank DNA data bases were carried out using an Apple Macintosh Computer as a terminal to the HGMP computer at Hinxton, Cambridgeshire. Word processing was performed using Microsoft Word 6.0.1 on an Apple Macintosh Performa 5400 Power PC, scanned images were processed with Adobe Photoshop 4.0, figures and tables were drawn with MacDraw Pro and Microsoft Excel 5.0. Data was analysed statistically with Statview 4.5 and Data Desk 6.0. The reference database was compiled using EndNote Plus 2.1.

2.2 Methods

2.2.1 RNA extraction

Preparation of solutions and glassware for RNA isolation

The principle difficulty in isolating undegraded RNA from tissue is the ubiquitous presence of RNAases, both within the tissues from which the RNA is to be extracted, and in the laboratory environment. RNA degradation is minimised by the use of extraction procedures which simultaneously disrupt cells or tissues and inactivate endogenous nucleases, by the use of RNAase free glass and plasticware, and by careful preparation of RNAase free solutions.

Sterile, disposable plasticware is generally RNAase free. Glass and metalware can be rendered RNAase free by baking overnight at 200°C. Non-sterile plasticware was treated with a solution of 0.1% diethylpyrocarbonate (DEPC), overnight in a well ventilated environment, followed by autoclaving to remove the DEPC. Solutions, except those containing Tris, were also DEPC treated and autoclaved. Gloves were changed frequently to minimise contamination by skin borne RNAases.

Isolation of RNA

A routine guanidinium isothiocyanate technique was employed for isolating RNA from tissue and cells (Chirgwin et al., 1979). This technique generates highly quality total RNA, suitable for RT-PCR analysis.

Small tissue samples (approximately 500 mg in weight) were homogenised from frozen in 700 μl of freshly prepared solution D. The homogenates were kept on ice and 90 μl of 2M NaAc (pH 4.0) was added to each sample. The samples were briefly vortexed, and 600 μl of water saturated phenol was added. The samples were vortexed and 150 μl of chloroform/isoamylalcohol (49:1) was added. The samples were then left at 4°C for 15 min, and subsequently centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant was removed and 200 μl of solution D and 20 μl of Na Ac was added. The samples were vortexed and 500 μl of 2M NaAc (pH 4.0) and 120 μl of chloroform/isoamylalcohol (49:1) was added. The samples were centrifuged for 10 min at 13,000 rpm at 4°C. 400 μl of the upper aqueous phase was removed, and an equal volume of propan-2-ol was added. The samples were then left overnight at - 80°C, and subsequently centrifuged, from frozen, for 30 min at 13,000 rpm at 4°C. The supernatant was removed and discarded, and

the RNA pellets washed in 75% ethanol. The samples were centrifuged for 10 min at 13,000 rpm at 4°C, and the RNA pellets allowed to air dry. The RNA extracted was re-suspended in 20 μl of 1 mM EDTA. The RNA yield was determined by spectrophotometry. 4 μl of re-suspended RNA was added to 196 μl of RNAse free distilled water. Absorbance was measured at 260 nM.

RNA yield (μg/ml) = 260 nM absorbance x 42 x 50

This method of RNA extraction can be adapted to isolate RNA from cell culture. Culture medium was removed from the cell culture plates, 700 μl of solution D was added per well, and the cells scraped. Since there was less cellular debris in cell culture, the second phenol/chloroform extraction technique was not required.

2.2.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

Introduction

Polymerase chain reaction (PCR) is a technique for the amplification of specific DNA sequences, by making multiple copies of the initial 'template' molecule. PCR relies on the ability of a DNA polymerase to make a complimentary copy of a single stranded DNA molecule in the 5' to 3' direction, but only when primed from a stretch of double stranded DNA at the 5' end. Amplification of DNA using PCR requires the nucleic acid sequence of the region at either end of the fragment to be known. Usually the sequence in between will also be known, but in diagnostic applications it is often the polymorphism which is found between the two primer sites which is to be examined. The PCR requires primers which are specific for the region of DNA to be amplified. Two primers are needed, one on each complementary strand, at each 5' end of the region to be amplified. The reaction mix also contains all four of the single nucleotides (dATP, dGTP, dCTP and dTTP) and DNA polymerase.

The DNA is first heated to 92°C, which breaks the bonds between the two complementary strands, forming single stranded DNA. The reaction is then cooled to a temperature at which the specific primers will anneal to the DNA at each 5' end. This temperature varies between primers, but ideally primer pairs are designed to have similar annealing temperatures. Primers concentration in the reaction is in great excess over the template DNA, so template/primer hybridisation is favoured over re-annealing of the two template strands. The temperature is then changed to 72°C, the optimal temperature for DNA

polymerase. The DNA polymerase synthesises a new strand of DNA complementary to the template, beginning at the primer site (Fig. 2.01).

The number of molecules of the specific region of DNA has now doubled. Repeating the temperature cycle denatures the newly formed double stranded DNA, allows the primers to anneal to the single strand templates and again doubles the amount of specific DNA present.

If the temperature cycle is repeated 20 or 30 times the specific region of DNA, originally present in nanogram quantities, can be amplified to microgram quantities, sufficient to be seen on an ethidium bromide stained agarose gel. PCR is automated using commercially available cycling heat blocks. PCR is extremely sensitive, allowing amplification of minute amounts of DNA such as that found in single cells. The extreme sensitivity of PCR makes it essential to avoid contamination of reactions with template DNA from other sources.

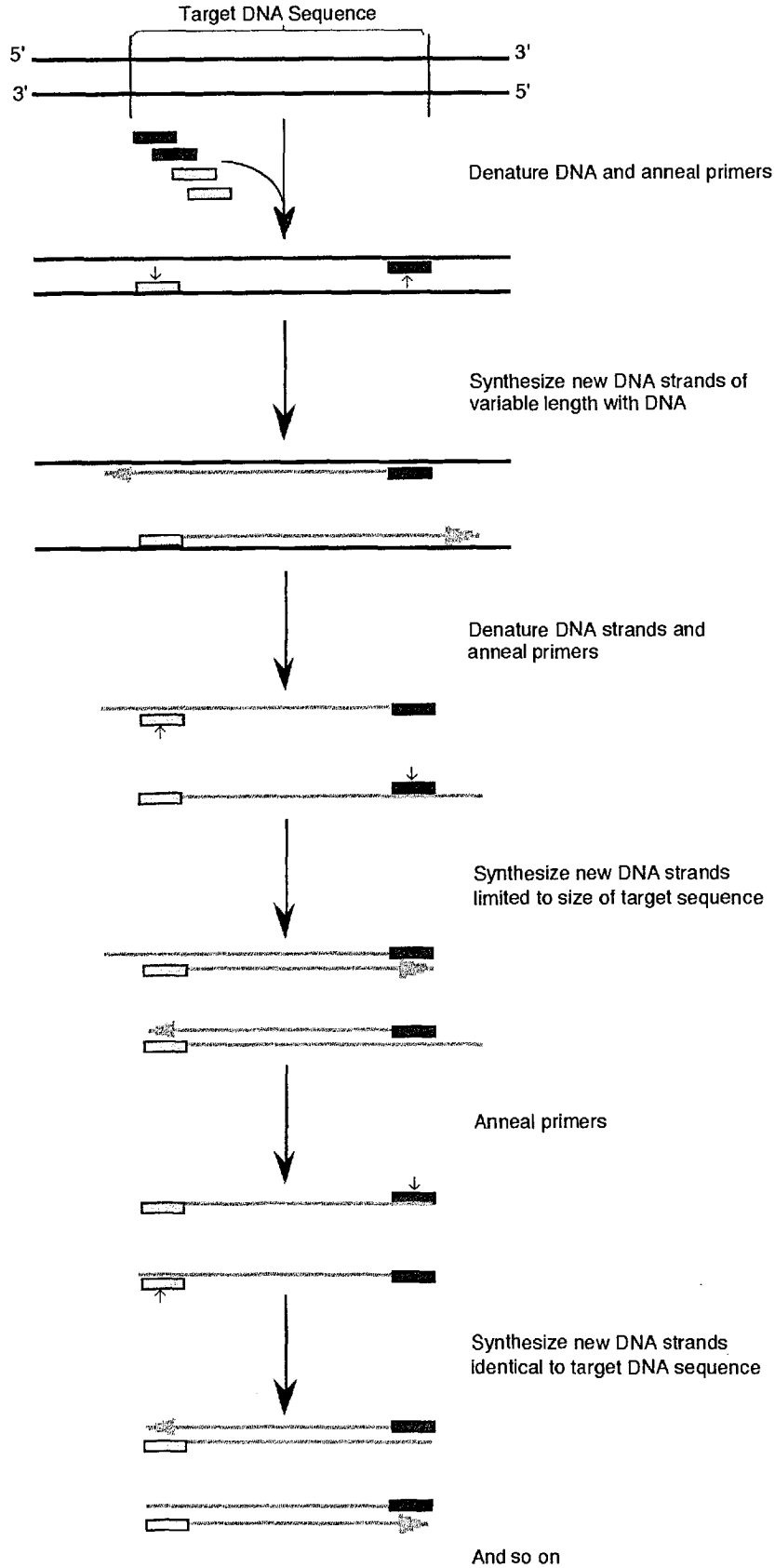


Fig. 2.01: Schematic representation of the polymerase chain reaction (PCR). Further cycles of denaturing, annealing and synthesis yield an exponential increase in the amount of PCR product.

PCR primer design

The design of PCR primers is fundamental to successful DNA amplification. Gene sequences were obtained from various databases such as the European Bio Informatics (EBI) or The Human Genome Mapping Project (HGMP) in Cambridge. The position of intron / exon splice sites was obtained from the literature, so that primers could be designed that spanned at least one intron. cDNA is intronless, having been generated by reverse transcription from mRNA. Genomic DNA however contains introns, so that PCR amplification of contaminating genomic DNA will generate a larger amplification product, identifying genomic contamination. All primers were therefore designed to span intron / exon splice sites.

Primer pairs should ideally share the same annealing temperature. Annealing temperature (Tm) can be calculated from the GC content of each primer, using the following formula:

$$Tm\,(^{\circ}C) = 4\,(G + C) + 2\,(A + T) - 5$$

It follows therefore that the relative GC content (and to a lesser extent, AT content) should be similar for both primers. In addition GC rich areas of the gene are avoided, as this will necessarily increase the Tm resulting in sub-optimal amplification. If the annealing temperature is set too low for the primers, primer specificity is compromised and non-specific amplification is likely to occur. Conversely if the temperature is set too high, primer binding will be compromised, resulting in reduced PCR sensitivity.

Whilst Tm can be calculated, it realistically only allows an estimation of the optimum annealing temperature, and in practice temperature profiles are carried out using temperatures, both above and below the calculated Tm, to establish the optimal Tm for each primer set (see below).

The optimum primer length, in terms of number of bases, is between 20 - 30. If the primer sequence is too short, the primers are more likely to be homologous with different areas of the genome, leading to non-specific amplification. If however primers are too long then primer annealing tends to be compromised.

Once primer sequences have been optimised then the potential PCR amplification products can be determined from the original gene sequence. This allows consideration of product size, and using appropriate computer software a restriction endonuclease map can be constructed, detailing relevant restriction site. This information is useful in subsequent sub-cloning experiments, for the orientation of PCR inserts in plasmid vectors. Finally a

comparison of sequence homomlogy for the PCR products from different enzyme isoforms can be determined using software available at the HGMP (bestfit). In this way primers can be designed that generate products, which show minimal homology, thus ensuring isoform-specific hybridisation and localisation, for subsequent *in situ* hybridisation studies.

PCR conditions

The initial denaturation step enables the double stranded DNA to denature into single strands, suitable for primer annealing. If this step is too short then the single strands tend to re-anneal to each other. If the step is too long then Taq polymerase will start to denature, decreasing enzyme efficiency. In general, an initial denaturation step of 4 min was used, followed by a 30 s step in each cycle.

Taq polymerase adds nucleotides to the complimentary strand during the extension step, at a rate of approximately 100 bases per second. The extension step can therefore be short, except in the case of very long PCR products (long range PCR). The extension step was routinely 30 s.

Magnesium is a co-factor in the PCR reaction for Taq polymerase DNA synthesis. The magnesium concentration also affects strand dissociation and primer annealing. The concentration of magnesium cannot be determined empirically, therefore concentrations must be determined for each primer set, by altering the concentrations, in magnesium profile experiments.

Reverse transcription

RNA samples (1 μg) were denatured at 70°C for 5 min and cooled to 37°C. Reverse transcription was carried out using random hexanucleotide primers (0.2 μg), x1 reverse transcriptase buffer, 10 mM dithiothreitol, 1 mM each dNTP, 40 units M-MLV reverse transcriptase, and 1 unit RNAse inhibitor was added to a final volume of 20 μl and incubated at 37°C for 60 min. Reverse transcription was terminated by heating to 90°C for 5 minutes and 80 μl of distilled water was added to each sample. cDNA generated from reverse transcription was stored at - 80°C.

Polymerase chain reaction

PCR was performed on 5 μl of the RT product (cDNA) with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 125 ng of each primer, and 1unit of Biotaq polymerase, to a total volume of 25μl.

Evaporation was prevented by adding a number of drops of mineral oil to each reaction mix. Reaction cycles were an initial denaturation step of 4 minutes at 94°C, followed by denaturation at 94°C for 30 seconds, an annealing step of 30 seconds, and extension at 72°C for 30 seconds. The temperature of the annealing step is dependent on the primers used. The final step was a further extension at 72°C for 5 minutes. All PCRs were performed on Hybaid Omnigene Thermocycling machines.

PCR cycle profiles

The optimal number of cycles of PCR amplification was determined for each gene and each separate reverse transcription reaction by PCR cycle profiles. Cycle profiles were carried out on pooled samples to determine the exponential phase of amplification, where final product concentration is proportional to starting cDNA concentration, and in this way semi-quantitation is achieved. PCR reactions were set up, and the range of cycle numbers were chosen from preliminary data, for each gene and tissue being investigated.

2.2.3 Agarose gel electrophoresis

PCR products were separated by agarose gel electrophoresis. DNA migrates through agarose gel at a rate dependent on its molecular size. The resolution of the gel depends upon the concentration of agarose. Gels with a high agarose concentration will separate small DNA molecules whereas low concentration gels will more efficiently separate large molecular weight DNA molecules. 1 % agarose gels were routinely used to separate generated PCR products. Ethidium bromide was added to both running buffer and agarose gels at a concentration of 0.5 μg/ml, to allow visualisation of DNA in longwave UV light. The oil layer was removed from each PCR reaction and orange-G loading buffer (1/10 final volume) was added to each sample. 10 μl of each sample was then loaded per well. Gels were visualised in UV light and photographed.

2.2.4 Southern blotting

Southern blotting was used to transfer size-fractionated PCR products from the agarose gel to a charged nylon membrane allowing hybridisation to a labelled probe (Southern, 1975). PCR products were separated by electrophoresis in an agarose gel. The gel was then soaked in denaturing solution for 40 min with gentle shaking. The denaturing solution was removed and the gel soaked in neutralising solution for a further 40 min.

In a large tray of 20x SSC, Whatman 3 mm filter paper was placed over a support to form a wick into the SSC solution. The gel was put onto the support and clingfilm placed over the gel to cover the entire tray. A central hole was cut in the clingfilm to delineate the area to be blotted, and a piece of nylon membrane (Hybond-N, Amersham) (cut to fit the gel) was soaked in 3x SSC and placed on top of the gel over the hole in the clingfilm. The clingfilm prevented any contact between the 20x SSC and the nylon membrane except through the gel where the DNA transferred. Air bubbles between the gel and the membrane were removed and a three sheets of Whatman filter paper were placed on top of the membrane followed by a stack of paper towels. Finally, a weight on a piece of strong glass or plastic was placed on top to keep all of the layers compressed. Transfer took place overnight. The lanes of the gel were carefully marked on to the membrane when the blot was dismantled. Confirmation that the DNA had transferred was made by examination of the gel under UV light. The membrane was allowed to air dry before irreversibly cross-linking the DNA to the membrane by exposure to UV light for 30 s.

2.2.5 Dot blotting

Dot blotting allows DNA to be transferred to a membrane directly. These types of blots are useful both for analysis of the presence or absence of a cross-hybridising sequence, and to semi-quantitate the PCR reaction, by subsequent radio-labelled hybridisation and radioactive counting.

Dot blots were made by aliquoting 5 μl of the RT-PCR product (before the addition of orange-G) onto membranes (Hybond) clearly marked with pencil. After air drying the DNA was denatured by placing the filter for 3 min onto a sheet of Whatman filter paper soaked in denaturing solution. The filter was then removed and placed onto a sheet of Whatman filter paper soaked in neutralising solution for 3 min, and finally placed onto a sheet of Whatman filter paper in 3 x SSC. The DNA was fixed to the membrane by cross-linking to the membrane by exposure to UV light for 30 s.

2.2.6 Preparation of PCR products

The products of PCR amplification were required for a number of different purposes. PCR products were used to construct radiolabelled probes, for subsequent hybridisation reactions to determine the relative amounts of DNA generated by PCR. In addition

products were ligated into plasmid vectors for riboprobe construction and *in situ* hybridisation. PCR products were also sequence verified.

Large scale (50 μl) PCR reactions were performed, and the entire product was loaded onto a low melting point (LMP) agarose gel. Products were separated as normal. The gel was examined under UV light, and the DNA excised as a band from the agarose gel. UV exposure time was limited, to prevent DNA degradation. The excised DNA was separated from the agarose using a commercially available Geneclean kit (Bio 101 inc.). The excised DNA (and agarose) was incubated with three volumes of sodium iodide stock solution contained in the kit, for 5 min at 45 - 55°C to dissolve the agarose. Glass milk was then added to the mixture (10 μl for every 100 mg of gel) and incubated for 15 min, at room temperature. The glass milk/DNA complex was pelleted by centrifugation at 13,000 rpm for a few seconds, and the supernatant discarded. The pellet was re-suspended in 30 - 50 μl glass milk preparation and washed three times by centrifugation at 13,000 rpm for 60 s at a time. After the final wash, the supernatant was discarded and the pellet spun once more to remove all of the supernatant. The pellet was re-suspended in 15 μl of distilled water and the DNA eluted from the glassmilk beads. The mixture was centrifuged once more and the supernatant carefully aspirated into a clean Eppendorf. This contained clean PCR product, whose presence could be confirmed by agarose gel electrophoresis.

2.2.7 Sub-Cloning of PCR products into plasmid vectors

PCR products were cloned into the pGEM-T Easy vector (Promega) to enable the generation of cDNA probes, RNA probes and for sequence verification. The pGEM-T Easy vector, when linearized add a 3' terminal thymidine to both ends of the potential insert site. Since the terminal event of polymerase extension, in the PCR amplification reaction, is to add an adenine base, this vector is particularly suitable for the ligation of PCR inserts. The pGEM-T Easy vector also contains T7 and SP6 RNA polymerase promoters flanking the insert site facilitating RNA transcription.

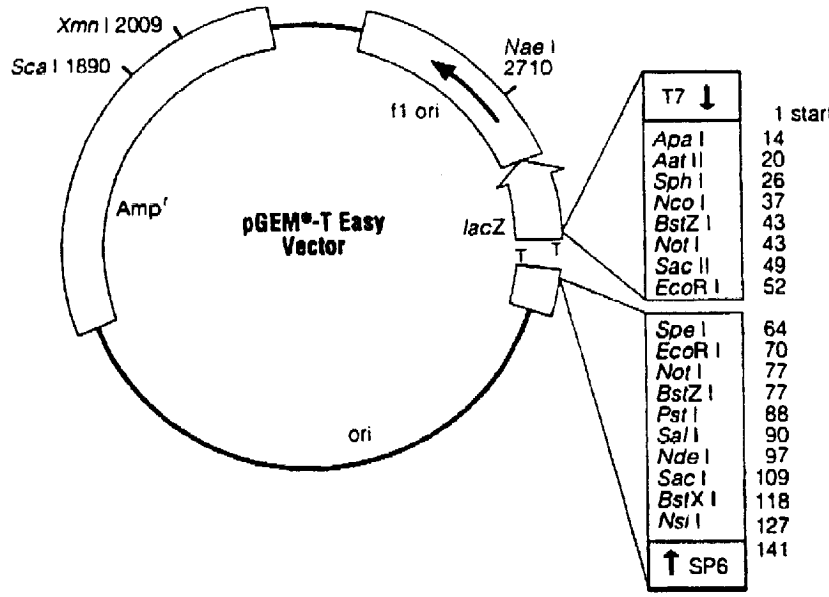


Fig. 2.02: pGEM-T Easy vector circle map.

The insert site is located within the α-peptide coding region of the enzyme β-galactosidase. Recombinant colonies can be identified using X-Gal selection, a chromogenic substrate of β -galactosidase which turns colonies white when the β-galactosidase gene is disrupted. If DNA has not been incorporated into the vector then it will produce functional β–galactosidase, which results in blue colouration of the colony when grown on X-Gal containing media. Recombinant clones containing inserts have a disrupted β-galactosidase, which is not functional and gives rise to white colonies.

pGEM-T ligation reaction

Ligation reactions were set up in Eppendorfs with known low DNA binding affinity. Ligation reactions contained: 1 μl T4 DNA ligase 10 x Buffer, 1 μl pGEM-T Easy Vector (50 ng), 5 μl PCR product, 1 μl T4 DNA ligase (3 units/μl) and 2 μl of dH_20. The reaction was mixed by pipetting and allowed to incubate overnight at 4°C.

pGEM-T transformation reaction

The ligated PCR product and vector were transformed into commercially available JM 109 High Efficiency Competent cells (Promega). Competent cells are those which will accept plasmid vectors into their cytoplasm as a result of a disrupted cellular membrane. Suitable host cells can be made competent for transformation of plasmid and subclones by treatment with calcium chloride. In this case the JM 109 cells had already been rendered competent.

Frozen JM 109 cells were gently thawed from frozen. 50 μl of cells were transferred to each ligation reaction. The cells were gently mixed, and placed on ice for 20 min. The cells were heat shocked for 45 - 50 s at exactly 42°C. The tubes were returned to ice for 2 min. 950 μl of SOC medium was added to each tube, and then incubated for at least 1.5 hrs, at 37°C, with shaking. 100μl of each transformation reaction was plated out onto LB plates with ampicillin/IPTG/X-Gal and allowed to grow up overnight. Plates were examined for Blue/white colonies. White colonies would be expected to contain inserts on the basis of β-galactosidase disruption. A number of white colonies were selected and isolated for Mini- and Maxi- prep DNA extraction.

2.2.8 Small scale isolation of plasmid DNA (Mini-prep procedure)

Selected colonies were picked from plates, and transferred into LB medium with ampicillin, added to a final concentration of 100 μg/ml. Cultures were grown overnight with shaking at 37°C. Bacterial cells were harvested by centrifugation at 2000 rpm. Cell pellets were re-suspended in 200 μl Solution-1. 400 μl of Solution-2 was added, and the tubes mixed by inversion, before incubation on ice for 2 - 3 min. 300 μl of Solution 3 was added, and the samples were shaken vigorously, and then stood on ice for 5 min allowing separation of plasmid DNA from bacterial chromosomal DNA. The bacterial DNA and protein was spun out of solution at 12,000 rpm at 4°C. 700 μl of plasmid solution was removed, and extracted with an equal volume of 50:50 phenol/chloroform. After a further chloroform extraction, an equal volume of isopropanol was added to precipitate the plasmid DNA. Each sample was re-suspended in 50 μl TE. The samples prepared in this manner contained a high proportion of RNA. Digestions were therefore carried out in the presence of 1 μl RNAase A (20 mg/ml stock).

2.2.9 Restriction endonuclease digestion of DNA

The presence and orientation of an insert in a plasmid vector can be determined by restriction endonuclease digestion. Restriction endonucleases cut DNA at specific sequences in double stranded DNA. Most enzymes recognise either four or six base pair sequences, although enzymes that cut infrequently in the genome may have larger recognition sequences. Most enzymes work in a pH range of 7.2 - 7.6, with magnesium as a co-factor. The predominant difference between enzymes is the dependence on ionic strength, varying from 0 - 150 mmol NaCl. Most manufacturers supply standard buffers

for each of their enzymes and a typical restriction reaction will contain, in addition to the DNA substrate, Tris buffer, magnesium ions, NaCl, 2-mercaptoethanol and bovine serum albumin. If a 'double-digest' is to be performed using two restriction enzymes simultaneously a buffer will be found in which both enzymes retain an acceptable degree of activity. Most enzymes are maximally active at 37°C but some, particularly those isolated from thermophiles, for example *Taq I*, are more efficient at higher temperatures.

2.2.10 DNA sequence verification

DNA obtained from plasmid mini-prep and subsequent restriction enzyme digest was separated by low melting point agarose gel electrophoresis, and extracted using a Geneclean technique. 100 - 200 ng/μl DNA samples were sent to The Department of Virology, Wolfson Molecular Biology Unit, Hammersmith Hospital, for automated sequence verification using an ABI 373A DNA sequencer. Sequences were then compared with the known cDNA sequences using an HGMP alignment program.

2.2.11 Radioactive labelling of double stranded DNA probes

DNA probes were labelled using a random priming method (Feinberg and Vogelstein, 1983). DNA was denatured to generate single stranded molecules, and then quench cooled. Random hexanucleotides were annealed to the DNA where they bound to complementary sequences. Klenow, primed by the short stretches of double stranded DNA where hexanucleotides had annealed, generated a new nucleotide sequence in a 5' to 3' direction. One of the nucleotides in the reaction mixture was radiolabelled, producing a radiolabelled product.

The digested PCR products which had been separated on a low melting point gel, were excised and placed in a small Sterilin container. The weight of the excised agarose (and DNA) was determined and 300 μl of distilled water was added for each 100 mg of gel. The gel was then melted at 65°C for 5 min and 34 μl of the mixture placed in a large Eppendorf. This was heated to 95°C for 5 min to denature the DNA. 10 μl of oligolabelling buffer together with 5 μl of the radioactive nucleotide (50 μCi $\{\alpha^{32}P\}$ dCTP) and 1 μl Klenow (5 u/μl) were added to give a final volume of 50 μl. The mixture was incubated at 37°C for a minimum of two hours.

The labelled probe was separated from un incorporated nucleotides by centrifugation through sephadex G-50 columns. A plug of polymer wool was placed in the bottom of a disposable 1 ml plastic syringe and a slurry of G-50 in 3x SSC pipetted to fill the syringe. The column was then spun at 1600 - 1800 rpm for 4 min. The labelling reaction was made up to 200 μl with 3x SSC and then carefully added to the top of the syringe. The column was spun again at 18,000 rpm for 5 min and the eluant collected in a fresh Eppendorf. The eluant contained the labelled probe, whilst the unincorporated nucleotides remain in the G-50 sephadex. The probe was boiled for 5 min and quenched on ice, to denature the DNA, before being used for hybridisation.

Pre-hybridisation and hybridisation

Pre-hybridisation is designed to block non-specific binding, and thus reduce background radioactivity on the filter. The cross-linked filters were placed in sealed glass jars, ensuring no air bubbles were trapped between the filters and the glass. 20 ml of Southern pre-hybridisation solution was added to the jars, which were placed in a hybridisation oven at 65°C for two to three hours. The jars were rotated within the oven to ensure good contact between the filters and the pre-hybridisation mixture. After pre-hybridisation the solution was replaced with 20 ml of Southern hybridisation solution with 200 μl of probe added. Hybridisation was allowed to occur overnight at 65°C.

Washing filters

The filters were washed to remove un-bound probe. The filters were removed from the hybridisation jars and placed in a plastic container with 1 l of 3x SSC, with 0.1% SDS, at room temperature. The container was gently shaken for 30 min, followed by a second wash in 3x SSC, 0.1% SDS at 65 °C for 30 min. With each subsequent wash the concentration of the SSC was decreased from 3x to 2x to 1x to 0.5x and finally to 0.1x. The filter was monitored, with a hand held Geiger counter and washing stopped when counts fell below 5 - 10 cps. The filters were air dried and then mounted on intensifying screens and autoradiographed at -70°C with slow pre-flashed X-ray film. Samples for radioactive counting were carefully cut out of the membrane, behind a protective perspex screen, and placed individually in counting cells. Radioactivity was measured in a Rak B scintillation counter, as total counts in 60 s.

2.2.12 Western Analysis

Western analysis was carried out using techniques developed from Laemmli et al. (Laemmli, 1970).

Preparation of samples for polyacrylamide gel electrophoresis

Protein extracts were prepared from tissue samples by homogenisation in 10 vol T- Wash for 30 seconds. The supernatant was separated from tissue debris by centrifugation at 1,000 X g, for 10 min, at 4°C. Protein concentrations were determined by using a commercially available Protein assay kit (Bio-Rad Laboratories) with reference to bovine serum albumin standards in T-Wash. Electrophoresis was carried out on 20 μg aliquots of protein samples, in 2 x loading buffer (4% SDS, 20% Glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125M Tris-HCl, pH 6.8). Samples were boiled for 5 min and then placed on ice prior to loading.

SDS-PAGE protein separation

Electrophoresis of protein samples was carried out using a 7.45% polyacrylamide running gel and 10% polyacrylamide stacking gel. Solutions were prepared, and both the APS and TEMED were added immediately prior to pouring as polymerisation occurs rapidly after their addition. Having poured the running gel, the gel was overlaid with a thin layer of water, whilst polymerisation took place. The water was subsequently removed, and replaced with stacking gel and the spacer comb placed between the glass plates. The gels were left for 1 - 2 hours before use. The comb was removed and the wells were flushed out, to remove any un-polymerised acrylamide. Protein samples were loaded and electrophoresis was carried out for 60 min at 200V (Bio-Rad Mini PROTEAN II electrophoresis cell).

Western blotting

Following electrophoresis, proteins were transferred to nitrocellulose using a Biorad transblot apparatus.

After electrophoresis the gel was equilibrated in electrophoresis transfer buffer for 20 - 30 minutes. While the gel was equilibrating the transfer membrane was soaked in transfer buffer. The transblot gel holder was set up with a fibre pad, two layers of Whatman paper, the gel, transfer membrane, two further layers of Whatman paper, and a further fibre pad. All components of the transblot system were pre-soaked in transfer buffer. Air bubbles between any of the layers were excluded. The transblot gel holder was placed in the buffer

tank so that the transfer membrane was orientated on the anode side of the gel. Transfer was carried out for 60 min at 100 V. The membrane was then removed and either used immediately for antibody detection, or stored dry at 4°C. Protein transfer was confirmed by staining with Ponceau's Solution.

Membranes were blocked in 5% Marvel-0.1% TWEEN-PBS, for 1hr at room temperature. Affinity-purified polyclonal antibodies, directed against the peptide immunogen, were used at dilutions of between 1: 500 - 2000, and incubated overnight at 4°C. Membranes were washed in 0.1% TWEEN-PBS, and then incubated with the appropriate IgG-HRP secondary antibody at a dilution of 1: 2000, for 1hr at room temperature. Detection was carried out using a commercially available ECL detection kit (Amersham Life Science), in accordance with conditions supplied by the manufacturer. Protein band size was determined using Rainbow coloured protein molecular weight markers (Amersham Life Science). Antibody specificity was confirmed using positive controls. The intensity of each specific immunoreactive band was quantified using densitometric scanning.

Positive controls for Western analysis

Positive controls were used in Western analysis to confirm antibody specificity

COX-1 positive control

Human platelets were used as a positive control for COX-1 protein. Time-expired human platelets intended for clinical use were obtained from Queen Charlotte's and Chelsea Hospital Haematology department. 10 ml aliquots were taken and centrifuged for 10 min at 4°C. The supernatant was removed and 1 ml of T-Wash was added to the depleted platelets. The samples were homogenised and subsequently centrifuged for 10 min at 4°C. The supernatant was removed and assayed for protein content. Samples were stored at -20°C. The platelet samples were not lymphocyte depleted, the extracted protein therefore contained and expressed some COX-2 protein.

COX-2 positive control

LPS stimulated human peripheral lymphocytes were used as a positive control for COX-2 protein. 14 ml of venous blood was obtained and diluted 1:1 with 1 x PBS. This was then suspended in histopaque 1077 (Sigma) and centrifuged for 30 min, at 2,000 rpm, at 24°C. The resultant gradient separated the sample, enabling the leucocytes present in the buffy coat, to be removed. The cells were re-suspended in 1 x PBS and centrifuged for 10 min,

at 2,000 rpm, at 24°C. The supernatant was removed and the cells were re-suspended in RPMI medium with 100 ng/ml LPS added. The cells were cultured for 24 hrs at 37°C, 5% CO_2. The medium was removed and the cells scraped and re-suspended in T-Wash. The samples were assayed for protein content.

iNOS positive control

ADP sepharose extracted IL-1ß stimulated A549 cells were used as a positive control for iNOS protein. NOS enzymes exhibit a high affinity for ADP, conferred by the NADPH-cytochrome P450 reductase-like domain common to all NOS isoforms. This technique preferentially extracts NOS proteins, and therefore increases the relative concentration of NOS protein in the extracted sample. Confluent A549 cells were cultured in DMEM with 10 ng/ml IL-1ß for 24 hrs at 37°C, 5% CO_2. The medium was removed and the cells washed with 1 x PBS. 500 μl of Lysis buffer (50 mM HEPES (pH 7.5), 2mM EDTA, 0.2% CHAPS, 1mM DTT,1 mM PMSF, 1 μg/ml Pepstatin, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin) was added to the cells on ice, and the cells were scraped and transferred to an Eppendorff and placed on dry ice for 10 min. Cells were thawed and then re-frozen on dry ice. The samples were then centrifuged for 5 min, at 13,000 rpm, at 4°C. The supernatant was transferred to a fresh tube and 20 μl of fully expanded ADP-Sepharose beads (Pharmacia) were added. The samples were mixed continuously for 45 min at 4°C. The samples were pulse spun to collect the beads and the supernatant was removed. The beads were washed with 250 μl of Lysis buffer, centrifuged and the supernatant removed. The beads were washed a further three times with Lysis buffer containing 0.5 ml NaCl, followed by two further washes with Lysis buffer. The beads were then re-suspended with 200 μl of Sample loading buffer. Samples were then boiled for 3 min and stored at -20°C. These samples were not assayed for protein content. 20 μl of sample was routinely loaded. ADP-Sepharose beads in the samples did not pass into the gel, or impede electrophoresis.

e/cNOS positive control

ADP sepharose extracted human umbilical vein endothelial cells (HUVEC) were used as a positive control for e/cNOS protein. HUVEC cells were grown in culture without cytokine stimulation. Protein extraction and sample preparation was identical to that for A549 cells.

2.2.13 *In situ* hybridisation

Introduction

In situ hybridisation is a technique by which radiolabelled probes are hybridised to tissue sections, revealing the cellular localisation of the complementary mRNA within the tissue. Whist RT-PCR analysis of homogenised tissue or cells provides information on gene expression it is unable to localise expression within the tissues examined. *In situ* hybridisation is a technique using either short oligonucleotide probes (DNA), or complementary riboprobes (RNA), to localise cellular RNA expression. Pilot data suggested low levels of expression of the genes of interest, within the tissues under investigation. The use of both riboprobes and radioactive expression improves sensitivity, and was therefore adopted in this study.

In situ hybridisation was originally described in 1969, by groups in both the United States (John et al., 1969) and the United Kingdom (Gall and Pardue, 1969). The principles of the technique involves pre-treatment of cellular preparations to reveal target nucleic acids, hybridisation of a (previously constructed) nucleic acid probe, with a complementary base sequence to the target. Finally the label attached to the probe is detected, allowing visualisation of the target (see Fig. 2.03).

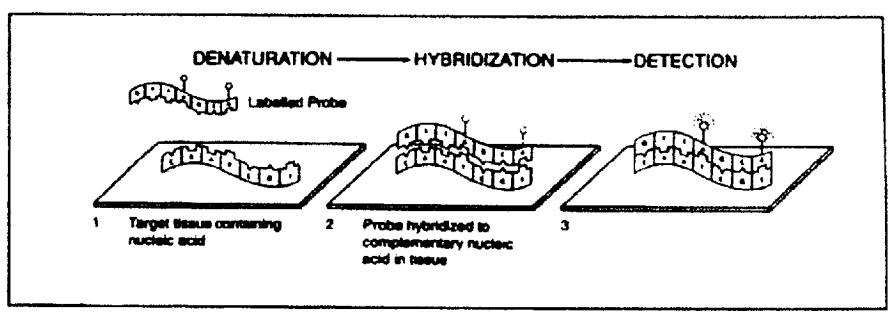


Fig. 2.03: Principles of *in situ* hybridisation

Whilst the principles involved are simple, in practice the technique requires significant preparation, optimisation, adequate controls and ultimately patience.

in situ hybridisation techniques

Probe construction

In principle *in situ* probes are either oligonucleotide probes or ribonucleotide probes (riboprobes). Oligonucleotides, as their name suggests are short DNA probes, homologous with the gene of interest. They are routinely end-labelled with a radioactive isotope for detection purposes. Oligonucleotides hybridise to the gene of interest by DNA-RNA binding. In contrast riboprobes are short RNA sequences, usually constructed by transcribing from a DNA template, incorporating radioactive nucleotides (UTP for example). Hybridisation is achieved by RNA-RNA binding to the consensus gene sequence. Riboprobes, whilst potentially more difficult to construct than oligonucleotides, bind with greater affinity to the target sequence and therefore result in greater sensitivity. Pilot data suggested that levels of expression of NOS isoforms in gestational tissue was low, and that oligonucleotide probes would not achieve the required level of sensitivity. The use of ribonucleotide probes was therefore adopted in this study.

Riboprobes were transcribed from sub-cloned PCR products in pGEM-T vector.

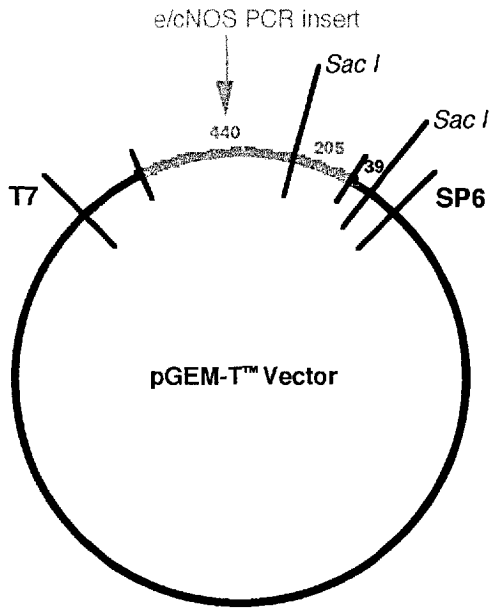


Fig. 2.04: pGEM-T vector map, with ligated e/cNOS PCR product. T7 and SP6 indicate RNA transcription start points. *Sac I* indicates the restriction endonuclease points, in both the e/cNOS PCR product and pGEM-T vector. Shaded area represents the e/cNOS PCR product (645 bp).

The orientation of the sub-cloned PCR product in the vector and the transcription initiation site, determines whether a sense (control) or antisense riboprobe is constructed. This is obviously fundamental to the success of the technique. PCR product orientation was determined by restriction endonuclease digestion, an example of which is shown in Fig. 2.04.

Restriction digestion with *Sac I* will cut the vector as shown, 39 bases from the insert site (see pGEM-T vector map Fig. 2.04). In addition a *Sac I* site in the PCR product will divide the e/cNOS product into a 440 and 205 bp product. When the PCR product is ligated into the vector (see section 2.2.7) the product may ligate either in a 5' - 3' direction, or in a 3' - 5' direction. If the product is orientated in the vector as shown (3' - 5'), digestion with *Sac I* will yield both a 243 bp product (205 + 39) and a 3 kb product (the remainder of the linearized vector). Conversely, if the product is orientated the opposite way (in a 5' - 3' direction), a 479 bp product (440 + 39) and a 3 kb will result.

Fig. 2.05 shows that restriction digestion of the pGEM-T vector for this particular ligation reaction generates a 244 bp product, confirming that the PCR product is orientated in a 3' - 5' direction.

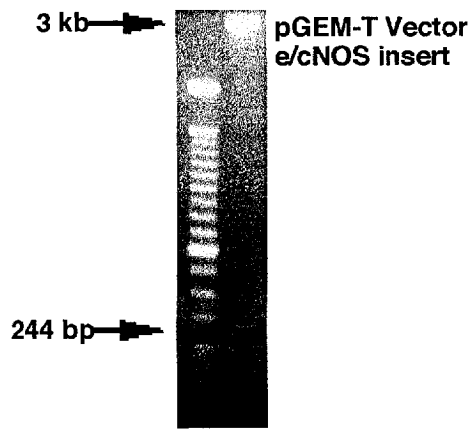


Fig. 2.05 Sac I restriction endonuclease digestion of pGEM-T vector ligated with e/cNOS PCR product. The restriction digest was carried out at 37 °C for 2 hr. Digest products were electrophoresed on a 1% ethidium-bromide stained agarose gel. Product size determines insert orientation in pGEM-T vector (see text).

Riboprobes are generated by transcribing directly from the ligated PCR product. Transcription initiated from the SP6 promoter site will generate, in the above example, an antisense riboprobe, whilst T7 will generate a sense (control) riboprobe.

Specimen preparation

RNA is highly susceptible to degradation by nuclease activity. *In situ* hybridisation using ribonucleotide probes is dependent on the presence of high quality RNA within the tissue samples. Nucleases are ubiquitous, and widely distributed on skin, clothing and work surfaces, and for this reason stringent precautions are used to limit RNA degradation. The tissues for *in situ* hybridisation in this study were prepared by embedding in paraffin blocks and sectioning, on a microtome into 7 μM sections, in the same way that sections are routinely prepared for haematoxylin and eosin staining for histological assessment. Somewhat paradoxically specimens fixed in paraffin blocks appear to be unaffected by 'normal' contamination levels of nucleases, and practically, stringent precautions to avoid RNA degradation are only necessary once the tissue has been sectioned.

The use of cross-linking fixatives, such as formalin, prior to paraffin embedding will tend to mask nucleic acid sequences. Proteolytic digestion with proteinase K is therefore employed to reverse this process. The degree of digestion required to present nucleic acids prior to hybridisation is clearly tissue dependent, and must be determined empirically for each tissue type.

The aim of hybridisation is to ensure maximal reaction of the probe with the target sequence whilst minimising the degree of non-specific interaction. Formamide and sodium chloride in the hybridisation solution controls the specificity of hybrid formation. Anionic macromolecules, such as dextran sulphate, reduce non-specific binding and the presence of EDTA limits nuclease activity by chelating magnesium, a co-factor for nuclease activity.

Controls are generated by the use of sense probes. Antisense riboprobes contain the consensus nucleotide sequence and thus hybridise to the target sequence. The sense probes contain the target sequence. They should not therefore hybridise to the target sequence, but in other respects, such as length they are identical to the antisense probes.

Whilst commercially available colour kits are available their sensitivity is limited, and radioactivity and photographic detection was therefore employed to further increase sensitivity, at the expense of exposure times.

Preparation of slides and coverslips

Racked slides were washed thoroughly overnight in 10% Decon, at 60°C. They were then rinsed in running hot tap water for at least 4 hours and left to stand in distilled water for at least an hour. The racks were wrapped in foil and baked overnight at 200°C. Slides were pre-treated with TESPA to improve tissue adherence. Slides were placed in acetone for 2 min, then in acetone containing 1% TESPA for 5 min. Slides were then rinsed in 2 washes of DEPC treated water, and allowed to air dry. Coverslips were prepared in a similar way.

Fixation, embedding and sectioning of tissues

Tissues samples were prepared and paraffin embedded in The Histopathology department, Hammersmith Hospital. Tissue sections were cut to a thickness of 7μm and dried onto TESPA treated slides before being stored at 4°C until use. Slides were used within one month of cutting. Precautions were taken at all stages to minimise RNA degradation.

Pre-treatment of sections

Sections were de-waxed twice, in histoclear, for 10 min each. The histoclear was removed with 99% ethanol, twice for two minutes. The sections were rehydrated by passing the slides sequentially through 95%, 85%, 70%, 50% and 30% ethanols for 1 min each, and then rinsed with saline and PBS. Sections were post-fixed in 4% formaldehyde in PBS, rinsed twice in PBS and then placed in Proteinase K buffer for 15 min at 37°C. The slides were rinsed in PBS, re-fixed for 5 min and immersed in freshly prepared 0.1 M triethanolamine with 0.25% acetic anhydride for 10 min at room temperature. Triethanolamine coats the slide and prevents non-specific binding of RNA. Slides were then rinsed in 2x SSC and dehydrated in graded ethanols, air dried and used on the same day for *in situ* hybridisation.

RNA riboprobe transcription

RNA riboprobes were transcribed from sub-cloned PCR products in pGEM-T easy vector. Antisense and sense (control) probes were transcribed. [^{35}S]dUTP labelled RNA probes for each gene, were generated using a commercially available MAXIscript in vitro transcription kit (Ambion).

Hybridisation

Radiolabelled probes were diluted to a concentration of $1x10^6$ cpm per 50 μl hybridisation buffer and heated to 80°C for two min and quenched on ice. 10 μl of radiolabelled probe was then placed over the tissue sections and overlaid with a coverslip. Slides were incubated in a humid chamber (50% formamide 4x SSC) for two hours and then placed in an oven, overnight, at 42°C.

Removal of excess probe

Excess probe was removed by removing the coverslips by immersing the slides in 2x SSC for 5 min. Slides were washed in 50% formamide, 2x SSC at 45°C. The solution was changed three times at 15 min intervals, followed by two 30 min. rinses of 1x SSC at room temperature. All slides were dehydrated by passing through a series of 50%, 70%, and 100% ethanol, and then air dried.

Autoradiography

Slides were developed under darkroom conditions. K5 emulsion (Ilford) was placed in a 50ml glass beaker heated, to 42°C, and diluted 1:1 with water. The emulsion was left for 10 min, to allow the emulsion to melt. The emulsion was transferred to the dipping chamber, and bubbles removed from the meniscus by dipping blank slides. Slides were dipped into the chamber for 5 s, and drained on the side of the dipping chamber. The backs of the slides were wiped, and the slides were then left to dry, in a light tight rack, for approximately two hours. Slides were then left to develop in a dry, light tight box at 4°C for 5 days to 4 weeks. Activated silica gel was enclosed with the slides to minimise humidity.

Developing

Slides were developed every week to determine optimal exposure times. Prior to development, slides were allowed to equilibrate at room temperature. Under darkroom conditions slides were removed from the box and placed in glass racks. The slides were then developed for 4 min in Phenisol, (diluted one in five with deionised water) with gentle agitation. The development was stopped by dipping the slides in Stop solution (0.5% acetic acid) for 30 s. The slides were then dipped in 30% sodium thiosulphate. Sodium thiosulphate strips away the emulsion leaving behind the silver grains. The slides were then rinsed for 30 minutes in running tap water, and counter stained with 0.02% Toluidine blue for 1 min. The sections were rehydrated by passing through an ethanol series of 50%,

70%, 100% for 30 seconds each, and then dipped in Histoclear for 10 min. The slides were mounted in a small amount of neutral mounting fluid. Coverslips were added and slides were left to dry overnight.

Analysis

Slides were examined with a Zeiss Axioskop microscope under both light and dark fields, and analysed using IPLab Spectrum Smart Capture (Signal Analytics).

2.2.14 Cell culture

Cell culture medium

All cells were cultured in DMEM, at an initial concentration of at 5 x 10^5/ml, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μl/ml) and cultured at 37°C in 5% carbon dioxide until confluent (2 - 3 days). HOG-1 cells were also supplemented with 10 nM oestradiol (diluted in medium from a 1 mM stock solution). 24 hours prior to the experimental period, the medium was changed to serum free DMEM. Once the cells had been 'serum free' for 24 hours the medium was removed and replaced with DMEM (no FBS) and the cells subjected to varying experimental conditions. The medium was removed at different time points for subsequent analysis. Medium was stored at -70°C and assayed for both PGE_2 (by RIA) and nitrites (by chemiluminescence).

An estimate of COX enzyme activity was determined by culturing the cells in the presence of excess arachidonic acid, the substrate for PG synthesis. PG synthesis is then a function of COX activity, rather than the mobilisation of endogenous arachidonic acid by phospholipase. Cells were incubated in fresh DMEM with 30 μM arachidonic acid for a further 10 min and the medium removed for PGE_2 assay.

The treated cells were stored at -70°C or processed immediately. Total cellular protein, nuclear protein or total RNA was isolated for Western immunoblotting, EMSA or RT-PCR analysis respectively.

Trypsinisation of cells

Cells were trypsinised when confluent, to generate further cell culture plates. The medium was removed and the cells were washed with 1 x PBS, and incubated with 0.05% trypsin / 0.02% EDTA until the cells began to detach from the plate. Trypsinisation was halted by the addition of DMEM containing 10% FBS - an inhibitor of trypsin. Cell suspensions

were removed and centrifuged at 1200 rpm for 5 min. The old medium was taken off and the cells were re-suspended in fresh medium and re-plated. Maintenance cultures were passaged every 4 to 6 days, while sub-confluent, and re-plated at 2 x 10^6 cells per 175 cm^2 flask. Cells used in culture experiments were between passages 2 and 6.

Cell viability

Cell viability following different experimental conditions was confirmed using MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue). MTT is a water soluble tetrazolium salt, which yields a yellow solution when prepared in medium. Dissolved MTT may be converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes. The cleavage and conversion of the soluble yellow dye to the insoluble purple formazan has been used to develop an assay of cell viability.

MTT stock solution (5 mg/ml in DMEM) was diluted 1:10 in fresh culture medium. The cells were incubated for a further 4 hrs following the experimental period. At the end of this incubation period the medium was removed and the converted dye solubilized by the addition of 0.1 N HCL in absolute propan-2-ol. Absorbance of the converted dye was measured at a wavelength of 570 nm.

2.2.15 PGE_2 radio-immuno assay

Cell culture supernatants and media from tissue culture experiments were collected, and stored at -70 °C until the RIA was carried out. The assay incubation consisted of 200 μl standard (5 - 1000 pg PGE_2) or unknown, 100 μl (^{3}H) PGE_2 (30 nCi) and 100 μl anti - PGE_2 antibody (raised in rabbits, to a PGE_2 - bovine serum albumin conjugate) in RIA buffer. Control A contained RIA buffer 1 and (^{3}H) PGE_2 only. Control B contained RIA buffer, (^{3}H) PGE_2, Charcoal / dextran buffer. All samples were covered with Saran wrap and incubated overnight, at 4°C. 200 μl of Charcoal / dextran buffer was added to all tubes (except Control A), to remove unbound antibody, and incubated on ice for 10 min, then spun at 3,000 rpm for 35 min at 4°C. The supernatant was decanted into fresh tubes, and 4 ml of scintillant (Picofluor 40, Packard) was added. Samples were then counted in a Wallac Scintillation counter. Individual PGE_2 sample values were derived from plots of the PGE_2 standards. Appropriate culture medium blanks were included in the assays.

2.2.16 Fetal membrane prostaglandin assays

The potential of fetal membranes to generate prostaglandin E_2 from endogenous and exogenous arachidonic acid, was investigated using a fetal membrane disc system.

Fetal membranes collected fresh following delivery, were separated from the placental tissue, and either separated into amnion and chorion decidua or left intact. Membranes were placed on a cork board and 'cored out', to form membrane discs, 1 cm in diameter, these were then placed in six well plates with 1 ml of DMEM. The membrane discs were allowed to equilibrate at 37°C, during a 60 min rest period. The medium was changed and the discs incubated for 15 min. The medium was removed and immediately snap frozen. The medium was replaced with DMEM containing 30 μM arachidonic acid (from a stock of 30 mM in ethanol). The discs were incubated for a further 15 min, and the medium was removed and immediately snap frozen. The media obtained from these experiments was subsequently analysed for PGE_2 production by RIA. The membrane discs were snap frozen for subsequent protein estimation.

2.2.17 NO Chemiluminescence assay

NO is extremely unstable in biological models and rapidly autoxidates in the liquid phase, to nitrite (NO_2^-). Studies of autoxidation in liquid phase, in the absence of haemproteins has shown that the product is exclusively NO_2^- (Ignarro et al., 1993). In biological systems however, the presence of oxidizing agents, such as haemproteins, favour the production of nitrates (NO_3^-). The comparative production of both nitrite and nitrate in cell culture has been previously studied. In the absence of haemproteins such as haemoglobin, where both nitrites, and nitrites and nitrates have been measured the predominant result of NO oxidation has been confirmed as nitrites (L. Buttery, Personal communication). Media samples obtained from cell culture experiments were therefore only analysed for NO_2^- levels.

Nitrite accumulation in the medium of cultured cells can be determined colourimetrically by a diazotisation reaction, using standard Greiss reagent (Green et al., 1982). Greiss reagent is reported to be sensitive to nitrite levels as low as 0.45 μM. In practice this reagent was unable to detect the levels of nitrite generated in this cell culture system. An alternative strategy of nitrite determination, involving NO chemiluminescence was therefore adopted.

The NO-chemiluminescence assay is based on the fact that when NO reacts with ozone (O_3) to form nitrite, some of the nitrite is in an excited state and dissipates energy (hv) on returning to ground state:

$$NO + O_3 \rightarrow NO_2^* + O_2 \rightarrow NO_2 + hv$$

The energy released as a result of this reaction can either be used in reactions between NO_2 and other gas molecules, or released as a photon. Light emission is therefore linearly related to NO content.

NO-chemiluminescence assays were carried out using a Sievens - 270S NO chemiluminescent analyser. The basic structure consists of a reaction chamber, a light detector to collect emitted light and convert to an electrical signal and a recorder. NO levels were determined in the gas phase. The reaction chamber contained 1M KI to reduce NO_2 to NO, and was driven by (NO free) air. Generated NO reacted with ozone in the chemiluminescence reaction chamber and light emission was measured. Initial calibration was achieved with 100 μl of known samples (1.0, 0.5 and 0.1 μM NO_2). Generated light emission was recorded using MacLab software, and determined as the integral of the resultant NO signal. Individual samples were then measured in an identical manner, in duplicate, and NO levels derived from a calculated standard curve.

2.2.18 Gel Shift assay

The gel shift or mobility shift assay is a rapid technique for detecting DNA binding proteins. It is widely used in the study of sequence-specific DNA binding proteins, such as transcription factors. Gel shift assay relies on the principle that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than either unbound DNA or double stranded oligonucleotides. The gel shift assay is performed by incubating a purified protein (nuclear or total protein) with a ^{32}P end-labelled oligonucleotide containing the putative protein binding site. The reaction is then separated on a non-denaturing polyacrylamide gel. Specificity of the DNA binding protein for the putative binding site is established by competition experiments using synthetic oligonucleotides corresponding to the binding sites of the same (consensus) and other (non-consensus) DNA binding proteins. Confirmation of correct protein-oligonucleotide complexes can be confirmed on the basis of supershift assays, involving the use of antibodies raised against the respective oligonucleotide. Protein-oligonucleotide-antibody

complexes are further retarded when separated on a non-denaturing polyacrylamide gel (hence the term super shift assay).

Nuclear protein extraction

Nuclear proteins were extracted from confluent cell culture using a technique adapted from Dignan et al. (Dignan et al., 1983).

Confluent cells in 24 well plates were scraped in 1 ml of culture medium. The cell suspension was removed, and centrifuged for 5 min at 13,000 rpm, at 4°C. The supernatant was removed and the cell pellets were disaggregated in 200 μl of Nuclear protein extraction buffer A. Cell suspensions were vortexed and centrifuged for 5 min at 13,000 rpm, at 4°C. The supernatant was removed and discarded. 15 μl of Nuclear protein extraction buffer C was added, the cells were agitated and rested for 1 hr on ice. Samples were subsequently centrifuged for 5 min at 13,000 rpm, at 4°C. The supernatant was removed and dialysed with 75 μl of Nuclear protein extraction buffer D. Samples were then assayed for protein content.

Oligonucleotide labelling

Consensus oligonucleotides were end-labelled with [γ- ^{32}P]ATP. Phosphorylation was carried out in a reaction containing: 2 μl consensus oligonucleotide (1.75 pM/μl), 1 μl T4 polynucleotide kinase 10 x buffer, 1 μl [γ- ^{32}P]ATP, 5 μl dH_20, 1 μl T4 polynucleotide kinase (5 - 10u/μl). The reaction was incubated at 37°C for 30 min. 200μl of TE buffer was subsequently added to the reaction, which was then centrifuged through a G25 column. Labelling was confirmed by radioactive monitoring.

Gel shift assay reaction

The gel shift assay reaction was composed of: 5 μg of protein (made up to a total volume of 15 μl with nuclear protein extraction buffer D) and 4 μl of 5 x Gel shift binding buffer. The reaction was incubated for 20 min on ice, and then, 1.5 μl of labelled oligonucleotide was added. Parallel controls were set up using consensus and non-consensus oligonucleotides. The reactions were incubated for a further 40 min, and 1.5 μl of bromophenol blue was then added, and the samples loaded on a 6% polyacrylamide gel. Electrophoresis was carried out for 2 hr at 220 V. Progress was monitored by the position of the bromophenol blue band. The gel was subsequently removed, placed on Whatman

paper and covered with cling film. The gel was dried using a standard vacuum gel drier, for 4 hrs. Autoradiography was subsequently carried out overnight.

2.2.19 Myometrial contractility studies

Myometrial contractility studies were carried out using a system devised in our department by Dr. Robert Sawdy and Dr. David Talbot. The system was an adaptation of one used by Collins et al. (Collins et al., 1993) to investigate the effects of fetal membranes on myometrial contractility. This system involved the use of a fetal membrane-uterine muscle dual chamber (Fig. 2.06).

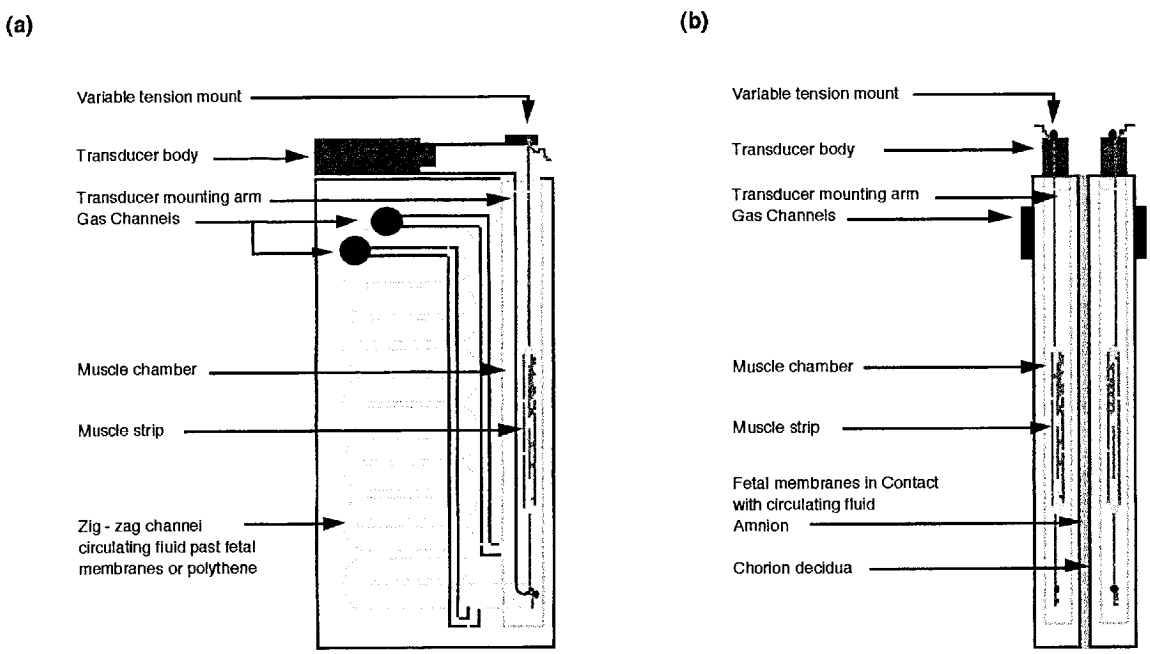


Fig. 2.06: Fetal membrane-uterine muscle dual chamber system. **(a)** Dual chamber system showing a muscle strip suspended in medium and connected to a transducer to monitor myometrial contractility *in vitro*. Perfusate circulates as a result of gas supplied via gas channels. Circulating perfusate comes into contact with either fetal membranes or polythene (blank), 'sandwiched' between the two chambers, by passing through channels in the plexiglass. **(b)** Dual chamber system shown in cross-section showing positioning of the suspended myometrial strips, and fetal membranes between the two chambers.

Myometrial strips were suspended, in tandem, in 15 ml chambers, in PSS at 37 °C. Gas (95% O_2, 5% CO_2) supplied via gas channels perfused the strips directly, and also circulated the perfusate through a zigzag set of channels within the plexiglass. Circulation through this system results in perfusate passing directly over fetal membranes (or

polythene) 'sandwiched' between the two chambers. This system therefore allows investigation of the influence of fetal membranes on myometrial contractility, in relation to fetal membrane orientation and in the presence of any administered agents. In experiments where the effects of administered agents on myometrial contractility were being studied in isolation, polythene was sandwiched between the chambers. Isometric transducers were constructed, and a unique computer program was devised to analyse uterine contractility.

Sample preparation

Specimens for contractility studies were received fresh in PSS. Myometrial strips were dissected from the biopsy samples, taking care to avoid both decidual and vascular tissue. A maximum of eight myometrial strips (10 x 2 x 2 mm) could be examined per experiment.

Contractility experiments

The strips were mounted vertically in 15 ml cassettes and maintained in PSS (37°C, pH 7.4) when gassed with 95% O_2, 5% CO_2 and stretched to a resting tension of 3 g. For experiments involving fetal membranes, these were separated from the placenta (from the same patient) and then placed between the experimental chambers. The orientation of the fetal membranes with respect to the amnion and chorion decidua was recorded. Control strips contained polythene between experimental chambers. The strips were allowed to equilibrate for 40 min prior to the experimental period, during which time spontaneous contractions had usually initiated. Contractions were recorded within a control period of 60 min and subsequently for a 60 min experimental period, during which time agonists were added to the circulating perfusate of contracting strips (with the exception of control strips), in increasing concentrations. Where myometrial contractility was abolished, tissue viability was established by eliciting oxytocin-induced contractions.

Tissue for both RNA and protein extraction were obtained by snap freezing in liquid nitrogen, tissue obtained at the time of strip dissection (t = 0, no stretch), tissue kept at 37°C in the organ bath without being suspended (t = 3 hrs, no stretch), and tissue from myometrial strips that had been suspended, and subjected to different experimental conditions.

Data analysis

Myometrial contractions were recorded using a photoelectric cell (transducer) connected directly to a variable tension mount and the suspended myometrial strip. DC current

generated by the transducer was transferred via a digital - analogue converter and analysed on an IBM personal computer, using specialist software developed in our department. A number of different parameters were measured including maximum tension, time to the onset of spontaneous contractions and contraction interval. An indication of mean myometrial contractility was determined by mean integral tension (MIT), defined as the sum of the integral tensions during the experimental period divided by the duration of the period.

2.2.20 Statistical analysis

Data interpretation

Data generated from individual experiments were analysed in Statview 4.5. Data was analysed for normal distribution, by means of Shapiro - Francia W' test for normal data. Means and standard errors were determined.

Data conforming to a normal distribution were then compared by one way analysis of variance (ANOVA). Differences between groups were assessed using Fisher's exact test. Data not conforming to a normal distribution were analysed using a non-parametric comparative analysis (Mann - U - Whitney test). Data generated from individual samples throughout pregnancy were analysed with respect to gestational age by simple regression analysis. Statistical significance was assumed if $p < 0.05$.

Data presentation

Data generated from RT-PCR analysis of gene expression compared by groups throughout gestation and at term was exhibited by Box plots. Western data was similarly shown. Box plots showed mean values, twenty-fifth centiles, and sample range. Extreme outlying samples were also shown (Fig. 2.07).

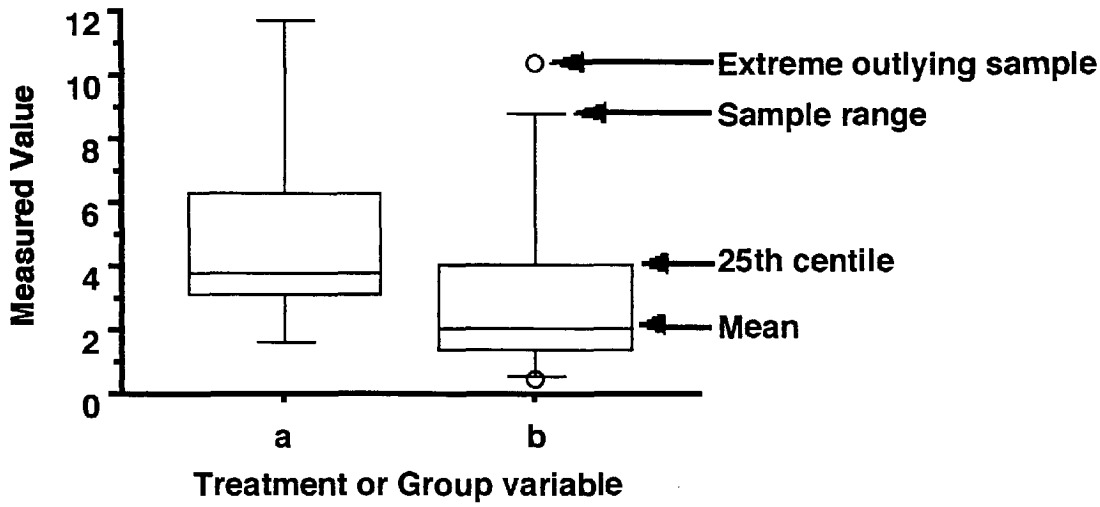


Fig. 2.07: Representative box plot. Box plot shows comparison of data between two treatment groups, showing sample mean, 25th centiles, sample range and extreme outlying samples.

RT-PCR and Western data from individual samples throughout gestation were shown as simple regression graphs. Cell culture, enzyme activity assays and myometrial contractility data were shown by cell bar charts, showing mean values with error bars showing two times the standard error of the mean ($\pm$ 2 SEM).

3. EXPERIMENTAL STRATEGY

The aim of this study was to examine, at a molecular level, expression of genes involved in both the maintenance of pregnancy and the onset of parturition. The expression of both NOS and COX isoforms was examined in fetal membranes and myometrial tissue, collected throughout gestation and at term, before and after labour. mRNA expression was analysed by RT-PCR (sections 4.2.1 and 4.3.1). Western blotting was used to investigate NOS and COX protein expression (section 4.2.4 and 4.3.2). Localisation of NOS expression was analysed in both fetal membranes and myometrium by *in situ* hybridisation (section 4.2.2).

In addition to Western analysis, COX protein (enzyme) activity was determined. COX enzyme activity was determined by measuring the synthesis of PGE_2 in fetal membrane disks, incubated in medium containing arachidonic acid. Enzyme activity in relation to both gestational age and labour onset was determined in whole (intact) membrane disks. The relative contribution of both amnion and chorion decidua, to total PGE_2 synthesis, was determined in separated fetal discs (section 4.3.3).

The regulation of both NOS and COX was investigated in an *in vitro* cell culture model, using human amnion-derived (WISH) cells (section 4.4.1). NOS and COX mRNA levels were determined by RT-PCR, in relation to cytokine induction with IL-1ß. iNOS and COX-2 share putative protein binding sites for the transcriptional factor NF-kB in their 5' promoter regions. The role of NF-kB in IL-1ß-induction of WISH cells was investigated by gel shift assay (EMSA).

The inter-relationship of NOS and COX was investigated *in vitro*, in WISH cells (section 4.5). Experiments were performed to investigate the influence of both endogenous and exogenous NO on COX enzyme activity (measured by PGE_2 synthesis). The influence of endogenous PGE_2 on NOS activity (measured indirectly by nitrite synthesis) was determined in cytokine (IL-1ß) stimulated WISH cells.

During the course of this project, evidence was published proposing that exogenous NO might ripen the cervix, facilitating cervical dilatation. This suggests that NO might have a differential effect on uterine tissue in the cervix, compared to the body or fundus. Using an *in vitro* cell culture model with cells from the cervix (HOG-1) and the lower uterine

segment (HMSMC) the influence of exogenous NO on PGE_2 synthesis was investigated (section 4.5.4).

The physiological role of NO in gestational tissue was investigated in *vitro* myometrial contractility studies (section 4.6). The effects of exogenous NO on myometrial contractility was investigated in isolated myometrial strips. The potential role of endogenous NO in maintaining myometrial quiescence during pregnancy was investigated using NOS inhibitors. COX mRNA levels were also measured, and analysed relative to both myometrial contractility and the influence of stretch.

The hypothesis being tested by this thesis was that NOS mRNA and protein was expressed in gestational tissue and that NO generated in these tissues might contribute to myometrial quiescence in pregnancy. In addition the hypothesis that NOS mRNA and protein expression falls prior to the onset of labour in human pregnancy was also examined. Since COX-2 expression has been shown to increase in association with labour, and both iNOS and COX-2 expression are subject to transcriptional regulation, it was postulated that in these tissues, different regulatory mechanisms might be involved in the transcriptional control of COX-2 and iNOS mRNA.

4. RESULTS

4.1 Reverse transcription polymerase chain reaction

4.1.1 PCR cycle profiles

PCR amplification increases the concentration of DNA generated exponentially until one or other of the substrates is exhausted. Substrate exhaustion is usually a result of nucleotide depletion. At this point PCR amplification will continue, but the reaction kinetics will be limited, resulting in loss of exponential amplification. Semi-quantitative PCR allows determination of starting template (cDNA), and hence mRNA, by determining the concentration of cDNA generated by the PCR amplification. PCR product is only proportional to starting template when amplification takes place in the exponential phase of the reaction. The exponential phase is determined using cycle profiles. It follows then that the exponential phase of the PCR reaction has to be determined for each primer set. In addition since reverse transcriptase efficiency may differ between reactions, cycle profiles must be carried out for each primer set, for each tissue and with each reverse transcriptase reaction.

Individual samples from an RT reaction were pooled, and identical PCR reactions set up on a thermal cycler. Eppendorfs were then removed after a pre-determined number of cycles and placed on ice. The products were dot-blotted and hybridised, the relative radioactivity is counted and the DNA concentration determined (Fig. 4.01). It is then possible to determine the optimal cycle number where amplification is exponential, and DNA product is proportional to starting template. This is the basis of semi-quantitative RT-PCR.

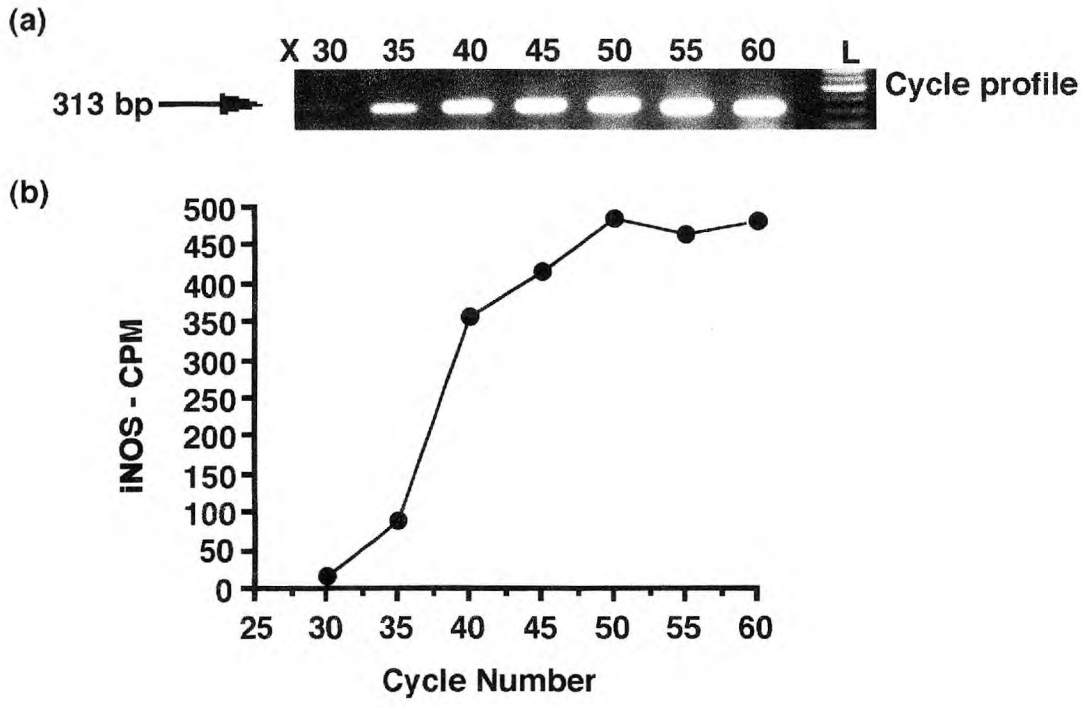


Fig. 4.01: iNOS cycle profile in pooled term chorion decidua. (**a**): Representative cycle profile of iNOS expression in term chorion Decidua. Cycle number was increased from 30 to 60 cycles to determine the exponential phase of amplification. L: 100 base pair DNA ladder. (**b**): Radioactivity, measured as counts per minute of hybridised dot blots, against cycle number. The cycle profile shows increasing PCR product with increasing cycle number

4.1.2 RT-PCR primers

iNOS

The sense primer maps to nucleotides 636 - 657, the antisense primer to 948 - 927 (Chartrain et al., 1994). The primers span three intron / exon splice sites, (see Appendix 7.2.1) and generate a 313 bp product. The optimal annealing temperature (62 °C) and magnesium concentration (1.5 mM) have been determined previously. The iNOS PCR product was verified by automated sequencing.

e/cNOS

The e/cNOS primers were designed from the published gene sequence (Nadaud et al., 1994). The sense primer maps to 629 - 649, the antisense maps to 1274 - 1253 (see Appendix 7.2.2). The primers span four intron / exon splice sites and generate a 645 bp product. The calculated Tm for this primer pair was 58 °C. A temperature profile however

showed that at 55°C, PCR product for e/cNOS was visible in chorion, but not at 58°C or 60°C, suggesting that the optimal Tm for this primer pair was 55 °C (Fig. 4.02).

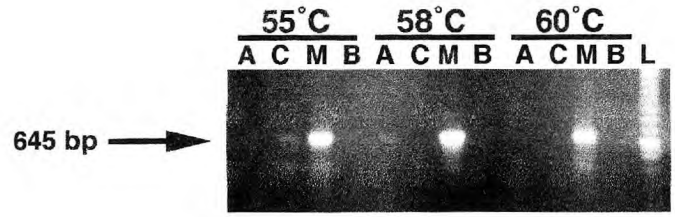


Fig. 4.02: e/cNOS temperature profile: e/cNOS expression in various gestational tissue at 45 cycles indicating an optimal annealing temperature of 55°C. Tissues are, A: amnion, C: chorion decidua, M: myometrium. B: Blank lane, no DNA template, L: 100 base pair DNA marker.

PCR amplification was consistent at a magnesium concentration of 1.5 mM. The e/cNOS PCR product was verified by automated sequencing.

nNOS

Primers were designed from the published nNOS sequence (Hall et al., 1994) (see Appendix 7.2.3). Two sets of primers were constructed to amplify different regions, with a view to identifying primers that both consistently and cleanly amplified template DNA. Primers were labelled nNOS sense 1394F (1394 - 1413), nNOS sense 1943F (1943 - 1962), nNOS antisense 1962 (1962 - 1943) and nNOS antisense 2449 (2449 - 2430) (Table 4.01). The influence of primer design and PCR conditions on the efficiency of amplification can be demonstrated by considering the techniques involved in optimising nNOS PCR amplification.

The predicted product size and calculated Tm for these primers pairs were as follows:

Table 4.01: nNOS PCR primers, product sizes and annealing temperatures

Sense Primer	Antisense primer	Product size / bp	Annealing Temp / °C
nNOS sense 1394F	nNOS antisense 1962	568	58
nNOS sense 1943F	nNOS antisense 2449	506	60
nNOS sense 1394F	nNOS antisense 2449	1056	59

PCR at these annealing temperatures produced non-specific amplification for all primer pairs (Fig. 4.03).

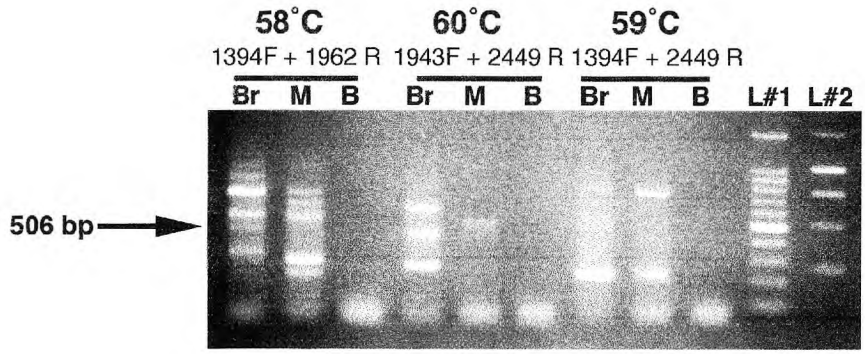


Fig. 4.03: nNOS PCR in brain and myometrium at calculated annealing temperatures. RT-PCR at 40 cycles. Br: fetal brain tissue, M: myometrium. B; blank lane, no starting DNA, L#1: 100 base pair DNA marker, L#2: 1 Kilo base pair DNA marker.

Increasing the annealing temperature increases annealing specificity. PCR amplification with the nNOS sense 1943F and nNOS antisense 2449R primers yielded a consistent product, of the correct size (506 bp) (Fig. 4.04):

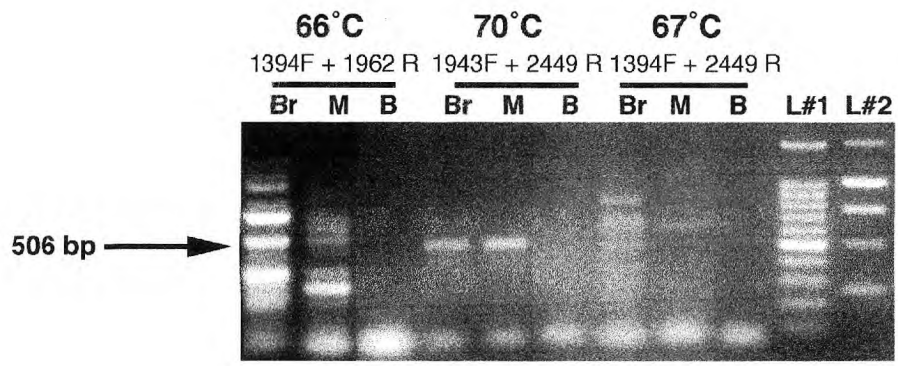


Fig. 4.04: nNOS PCR in brain and myometrium at elevated annealing temperatures. RT-PCR at 40 cycles. Br: fetal brain tissue, M: myometrium. B; blank lane, no starting DNA, L#1: 100 base pair DNA marker, L#2: 1 Kilo base pair DNA marker.

Having determined the optimal primer pairing, closer analysis of annealing temperature can further increase amplification efficiency. A subsequent temperature profile using 1943F

and 2449R, suggested that the optimal annealing temperature for this primer set was 68°C (Fig. 4.05):

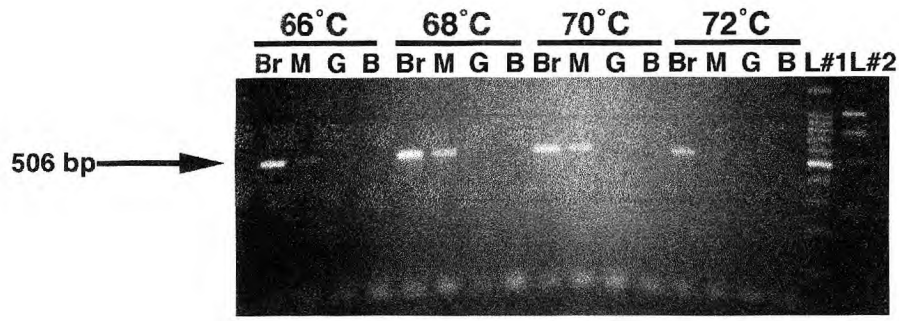


Fig. 4.05: nNOS PCR temperature profile in brain and myometrium, using PCR primers 1943F and 2449R. RT-PCR at 40 cycles. Br: fetal brain tissue, M: myometrium. B; blank lane, no starting DNA, L#1: 100 base pair DNA marker, L#2: 1 Kilo base pair DNA marker. The apparent discrepancy in PCR product size is due to minor differences in the rate of DNA electrophoresis throughout the agarose gel, confirmed by differences in rates of primer migration between lanes.

A PCR product is no longer generated from genomic DNA, due to the optimised conditions and resultant increased specificity. The genomic product would include introns, and would therefore generate an extremely large product, which is not favoured by these stringent conditions. PCR amplification was consistent at a magnesium concentration of 1.5 mM. The nNOS PCR product was verified by automated sequencing.

COX-2

The primers used for analysis of COX-2 expression have been previously optimised in our department (Slater et al., 1995) (see Appendix 7.2.5). The primers generated a PCR product of 305 bp which was subsequently sequence verified and accorded with the published sequence (Hla and Neilson, 1992). PCR conditions for these primers were an annealing temperature of 58°C, and a magnesium concentration of 1.5 mM.

COX-1

The COX-1 primers have been extensively used in our department and their characteristics identified (see Appendix 7.2.4). The COX-1 primers generate a 304 bp product, that corresponds with the published sequence (Chirgwin et al., 1979). PCR conditions for

these primers were an annealing temperature of 58°C, and a magnesium concentration of 1.5 mM.

GAPDH

Gylceraldehyde-3-phospate dehydrogenase is considered a 'housekeeping' gene expressed in all cells. GAPDH PCR is used as an internal control in semi-quantitative RT-PCR to control for mRNA concentrations. The GAPDH PCR primers used in these studies have been widely studied. The GAPDH primers generate a 598 bp product that corresponds to the published sequence (Tokunaga et al., 1987). PCR conditions for these primers were an annealing temperature of 58°C, and a magnesium concentration of 1.5 mM.

Calponin

Calponin is a cytoskeletal protein, specific for smooth muscle, whose expression is dependent on the differential state of smooth muscle cells (Gimona et al., 1990). Calponin was therefore used as an internal control in the myometrial studies, to reflect smooth muscle cell content and function.

The calponin primers used generated a 719 bp product, that corresponded to the published sequence for human basic calponin (Maguchi et al., 1995). The annealing temperature for these primers was 53°C, and the magnesium concentration 1.5 mM.

4.2 NOS expression in pregnancy

4.2.1 NOS mRNA expression

Fetal membranes

NOS expression in fetal membranes was initially investigated by analysing iNOS, e/cNOS and GAPDH expression in pooled samples of amnion, chorion decidua and placenta. HUVEC were used as a positive control for e/cNOS expression (Fig. 4.06):

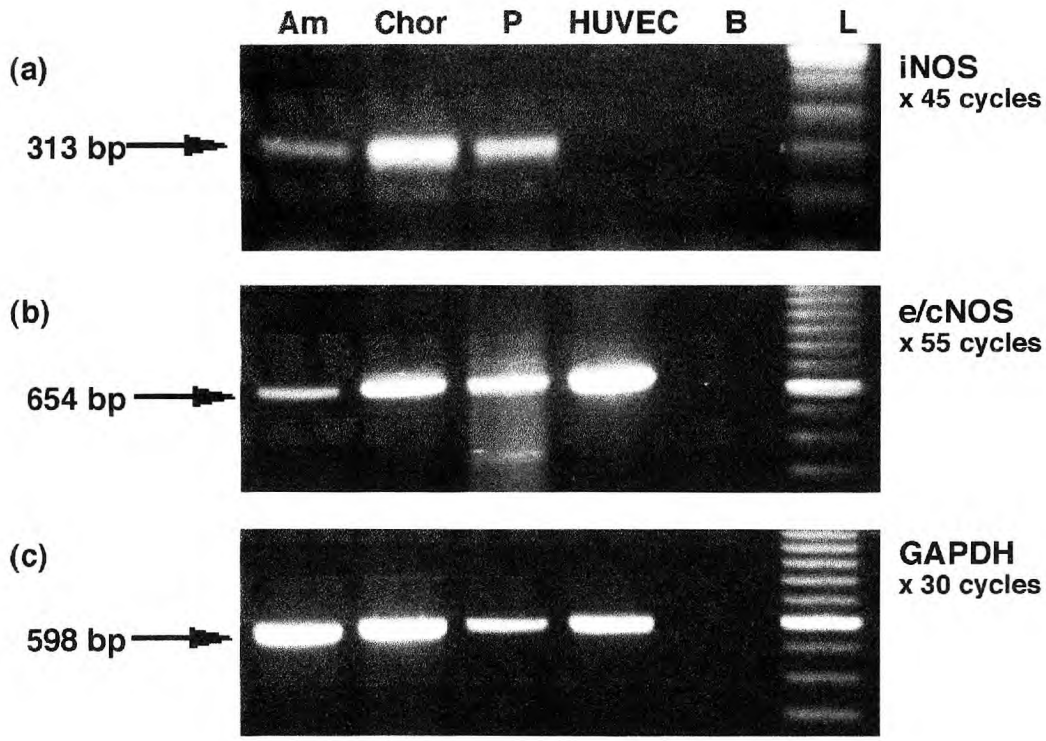


Fig. 4.06: NOS mRNA expression in fetal membranes by RT-PCR. (**a**) iNOS expression, (**b**) e/cNOS expression and (**c**) GAPDH. Samples are Am: amnion, Chor: chorion decidua, P: placenta, HUVEC: human umbilical vein endothelial cells, B: blank lane - no starting template DNA and L: 100 bp DNA ladder. HUVEC are a positive control for e/cNOS. GAPDH shows the presence of starting template DNA.

The number of cycles chosen for this study was derived from pilot data. The number of cycles required to produce a PCR product visible on an agarose gel is relatively high, for both iNOS and e/cNOS, implying low levels of mRNA expression. In terms of relative levels of NOS RNA in fetal membranes, data from cycle profiles suggest an approximate 100 fold higher concentration of e/cNOS in chorion decidua compared to amnion and

higher levels of iNOS expression in all tissues examined, with an approximate 30 fold higher concentration of iNOS mRNA in chorion decidua compared to amnion. HUVEC are a positive control for e/cNOS expression. iNOS expression would not be expected in this tissue. The presence of a PCR product in all samples with GAPDH confirms starting template in all samples.

The presence of nNOS mRNA in fetal membranes was not examined as nNOS PCR primers had not been designed or constructed at this point in the study. Furthermore, since nNOS is a constitutively expressed NOS isoform it was anticipated that mRNA expression would not be induced by labour or changes in gestational age.

Southern hybridisation, to previously generated and sequenced products, verified correct PCR products.

NOS expression in relation to labour onset

Semi-quantitative RT-PCR was used to analyse the effect, if any, of the onset of labour on levels of expression of iNOS and e/cNOS in amnion and chorion decidua. Tissue samples were collected either before labour onset (following elective caesarean section), n = 12, or following labour (following vaginal delivery), n = 12. RNA was extracted using a routine guanidinium isothiocyanate technique (as described in section 2.2.1). RNA was reverse transcribed and cycle profiles carried out on pooled amnion and chorion decidua samples for each gene. PCR amplification was then carried out on the individual samples using the appropriately derived cycle number.

The optimal cycle number determined for term amnion and chorion decidua, for these reverse transcriptase reactions, was 45 for iNOS and 35 for e/cNOS. The optimal cycle number determined for pooled amnion throughout gestation, was 42 for iNOS and 52 for e/cNOS. Finally the optimal cycle number determined for pooled chorion decidua throughout gestation, was 39 for iNOS and 47 for e/cNOS. Fig. 4.07 shows iNOS mRNA expression in term amnion in relation to labour:

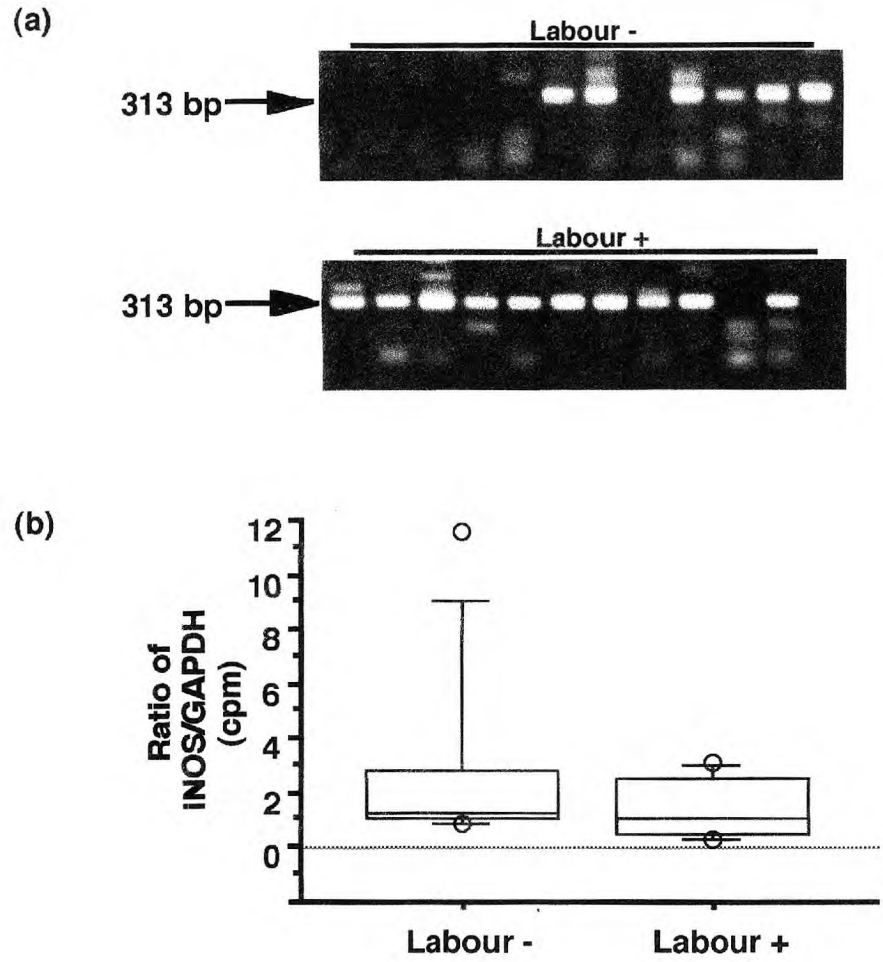


Fig. 4.07: iNOS mRNA expression in relation to labour, in term amnion. (**a**) RT-PCR with iNOS primers at 45 cycles on DNA isolated from term non-labour (L-, n = 12) and term labour (L+, n = 12) amnion samples. Correct product size is 313 base pairs. (**b**) Box plots of the ratio of iNOS expression (as cpm) to GAPDH. Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in iNOS expression before or after labour onset (p = 0.36).

iNOS mRNA expression was demonstrated in amnion samples at term using 45 PCR amplification cycles. Relative levels of expression between the pre labour (Labour -) and post labour (Labour+) groups were compared. Results were normalised with reference to GAPDH expression to standardise starting DNA template concentrations. There was no statistically significant difference in iNOS mRNA expression in term amnion before or after labour onset ($p = 0.36$).

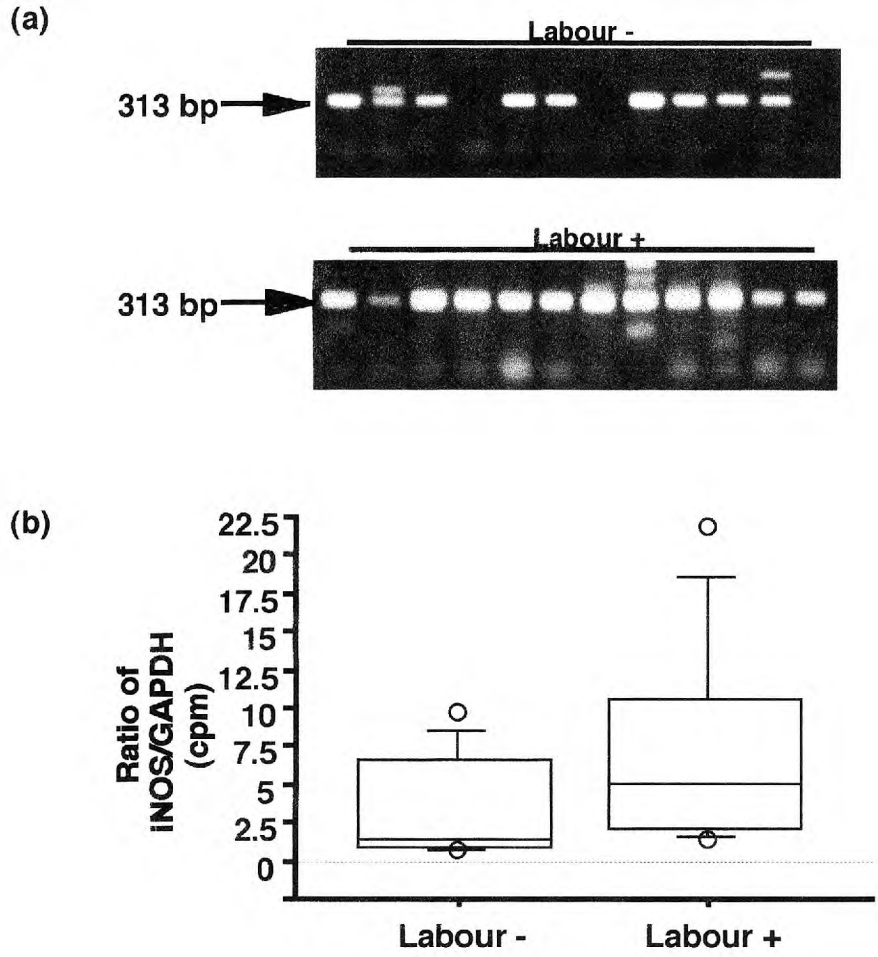


Fig. 4.08: iNOS mRNA expression in relation to labour, in term chorion decidua. (**a**) RT-PCR with iNOS primers at 45 cycles on DNA isolated from term non-labour (L-, n = 12) and term labour (L+, n = 12) chorion decidua samples. Correct product size is 313 base pairs. (**b**) Box plots of the ratio of iNOS expression (as cpm) to GAPDH. Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in iNOS expression before or after labour onset ($p = 0.06$).

iNOS mRNA expression in term chorion decidua relative to labour onset was similarly studied. There was no statistically significant difference in iNOS expression in chorion decidua before or after labour onset ($p = 0.06$).

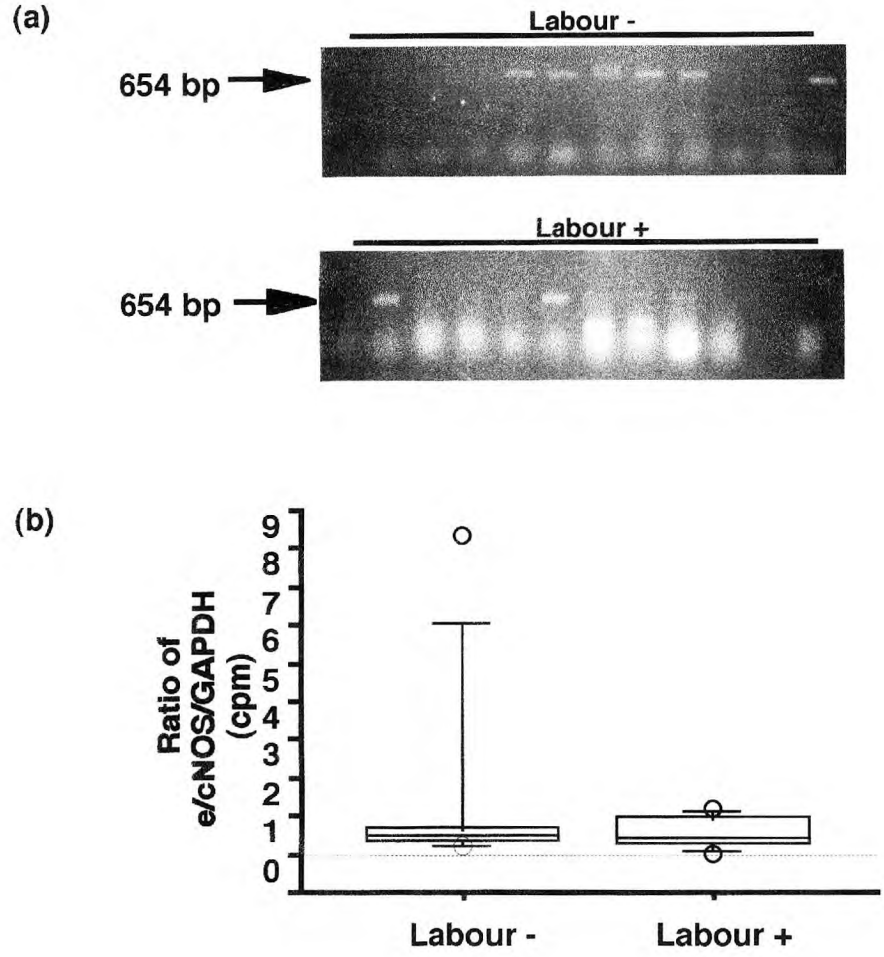


Fig. 4.09: e/cNOS mRNA expression in relation to labour, in term amnion. (**a**) RT-PCR with e/cNOS primers at 35 cycles on DNA isolated from term non-labour (L-, n = 12) and term labour (L+, n = 12) amnion samples. Correct product size is 654 base pairs. (**b**) Box plots of the ratio of e/cNOS expression (as cpm) to GAPDH. Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in e/cNOS expression before or after labour onset ($p = 0.82$).

e/cNOS mRNA expression was demonstrated in amnion samples at term using 35 PCR amplification cycles. Relative levels of expression between the pre labour (Labour-) and post labour (Labour+) groups were compared. Results were normalised with reference to GAPDH expression to standardise RNA concentrations. There was no statistically significant difference in e/cNOS mRNA expression in term amnion before or after labour onset ($p = 0.82$).

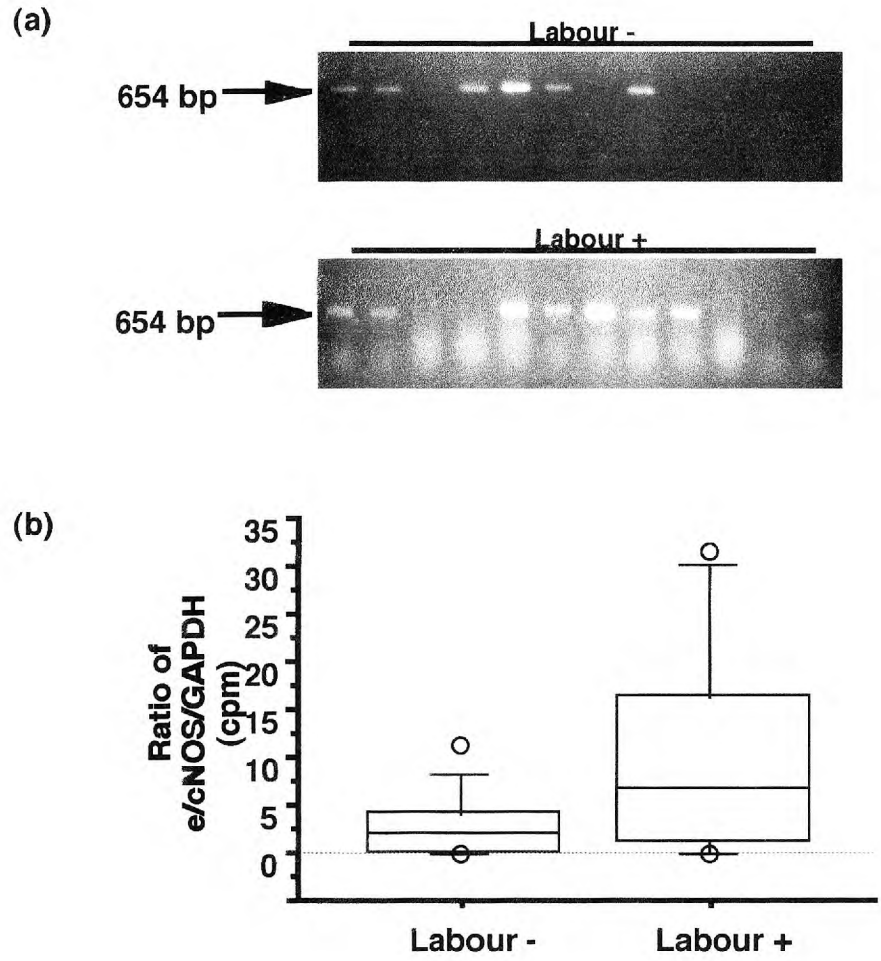


Fig. 4.10: e/cNOS mRNA expression in relation to labour, in term chorion decidua. **(a)** RT-PCR with e/cNOS primers at 35 cycles on DNA isolated from term non-labour (L-, n = 12) and term labour (L+, n = 12) chorion decidua samples. Correct product size is 654 base pairs. **(b)** Box plots of the ratio of e/cNOS expression (as cpm) to GAPDH. Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in e/cNOS expression before or after labour onset ($p = 0.08$).

e/cNOS mRNA expression in term chorion decidua relative to labour onset was similarly studied. There was no statistically significant difference in e/cNOS expression in chorion decidua before or after labour onset ($p = 0.08$).

Data from cycle profile experiments has shown that the optimal cycle number (where amplification is logarithmic) for iNOS is 45 and for e/cNOS is 35. Whilst potential differences in PCR efficiency between different primers make it difficult to compare absolute levels of expression these data suggests that in these tissues e/cNOS mRNA is expressed at a higher level than iNOS.

NOS expression is expressed as a ratio to GAPDH, to control for RNA concentration. GAPDH expression in fetal membranes before and after labour onset is shown in Fig. 4.11.

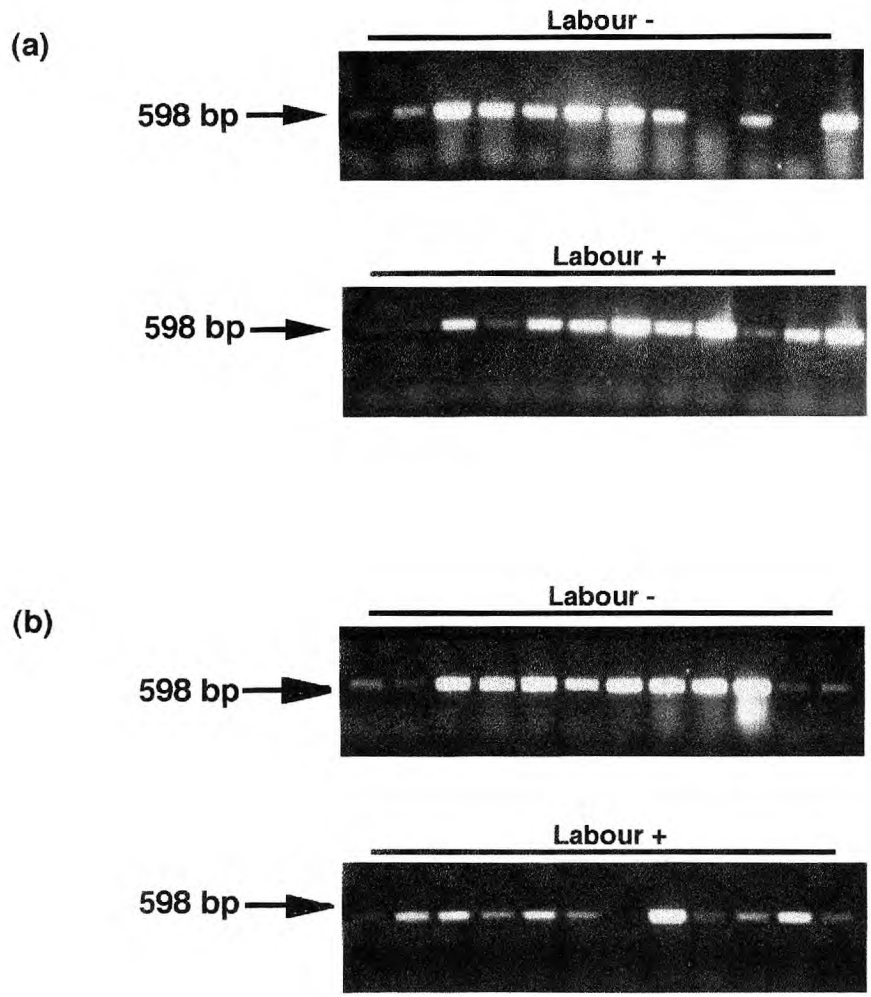


Fig. 4.11: GAPDH mRNA expression in fetal membranes in relation to labour. The panels show GAPDH expression by RT-PCR in **(a)** amnion and **(b)** chorion decidua for individual samples.

GAPDH mRNA expression was demonstrated in both amnion and chorion decidua samples at term using 32 PCR amplification cycles. There was no significant difference in the levels of GAPDH expression before or after labour (data not shown), suggesting that GAPDH can reliably be employed as an internal control.

NOS expression in relation to gestational age

The relationship of NOS expression to gestational age was considered by analysing NOS expression in fetal membranes samples collected throughout gestation. Preterm deliveries between 25 and 37 weeks (n = 14) were associated with intra-uterine growth retardation and hypertensive disorders. Samples were not collected from patients with prolonged premature ruptured membranes. There was no clinical evidence of chorioamnionitis. First and early second trimester tissue (n = 14) was collected from patients undergoing termination of pregnancy. iNOS expression in amnion samples throughout gestation is shown in Fig. 4.12.

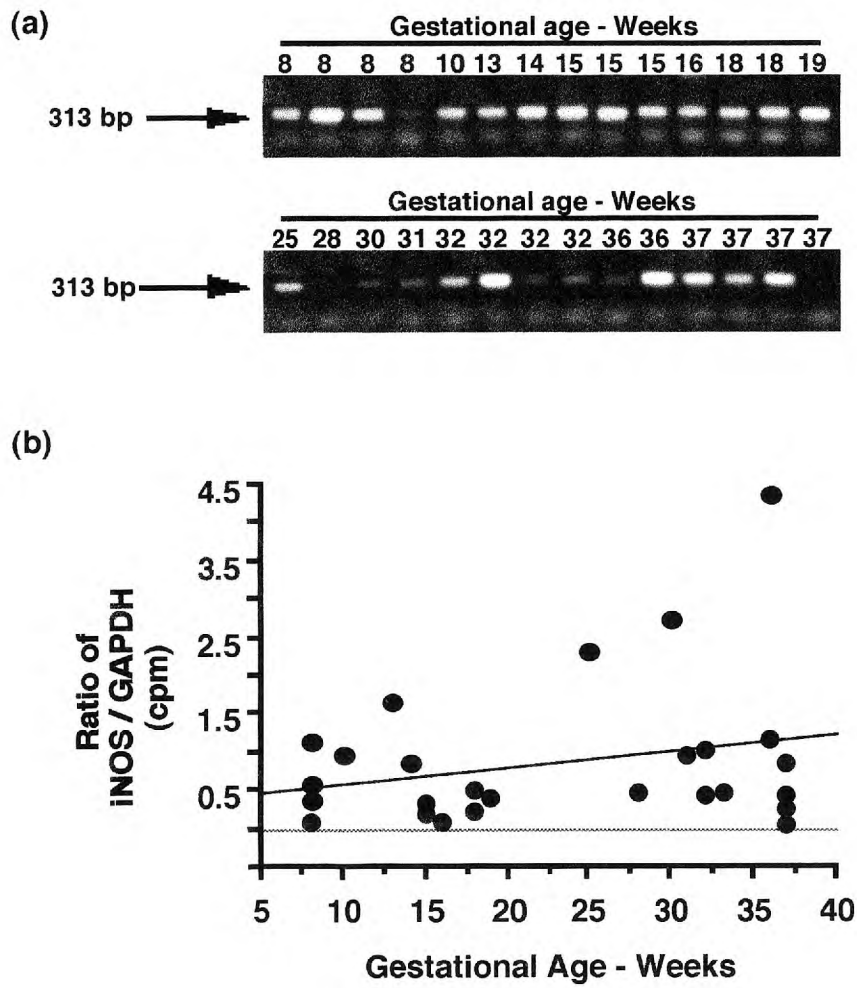


Fig. 4.12: iNOS expression in individual amnion samples related to gestational age. The top panel (**a**) shows iNOS mRNA expression by RT-PCR at 42 cycles, for individual amnion samples. The bottom panel (**b**) shows regression of iNOS expression on gestational age (as cpm), as a ratio to GAPDH expression. Simple regression analysis showed no significant correlation between iNOS expression in amnion and gestational age ($R = 0.25$, $p = 0.23$).

iNOS mRNA expression is demonstrated by RT-PCR at 42 cycles in amnion samples collected throughout gestation. There was no statistically significant association between levels of iNOS expression and gestational age ($R = 0.25$, $p = 0.23$).

iNOS mRNA expression in chorion decidua, in paired samples, throughout gestation is shown in Fig. 4.13:

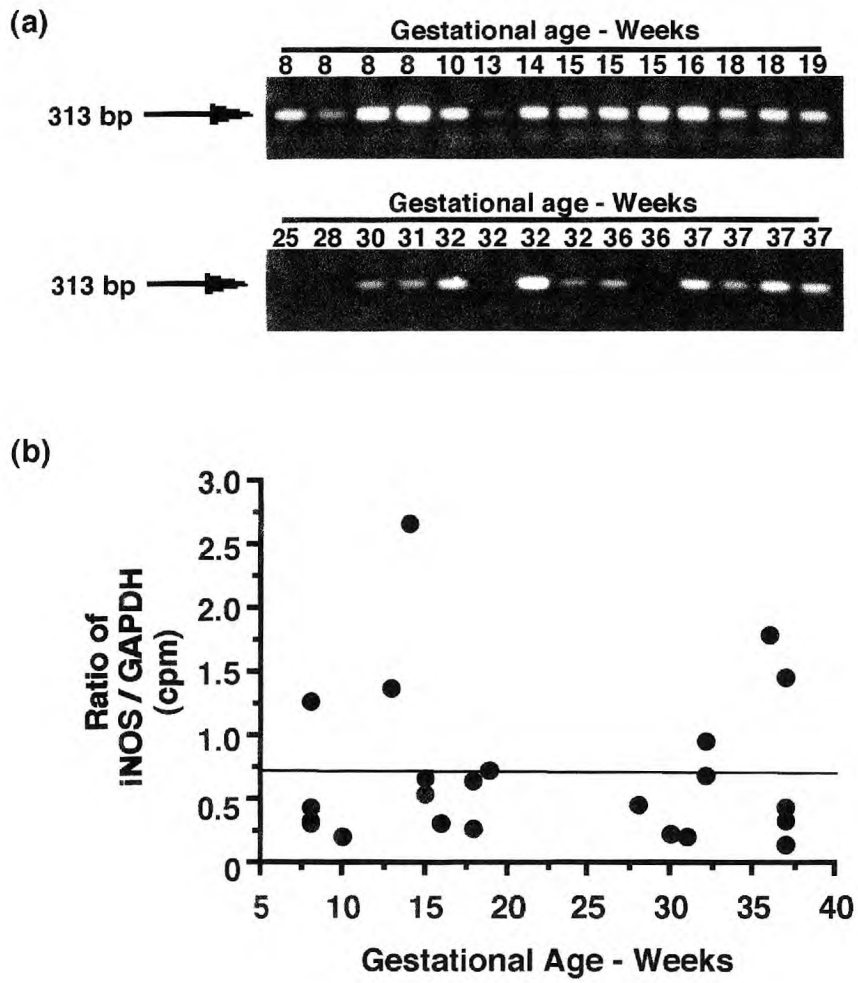


Fig. 4.13: iNOS expression in individual chorion decidua samples related to gestational age. The top panel (**a**) shows iNOS mRNA expression by RT-PCR at 39 cycles, for individual chorion decidua samples. The bottom panel (**b**) show regression of iNOS expression on gestational age (as cpm), as a ratio to GAPDH expression. Simple regression analysis showed no significant correlation between iNOS expression in amnion and gestational age ($R = 0.02$, $p = 0.94$).

iNOS mRNA expression is demonstrated by RT-PCR at 39 cycles in chorion decidua throughout gestation. There was no statistically significant association between levels of iNOS expression and gestational age (R = 0.02, p = 0.94). Data from cycle profiles from tissue throughout gestation suggest higher levels of iNOS mRNA expression in chorion decidua compared with amnion.

Similarly e/cNOS expression in amnion is shown in Fig. 4.14:

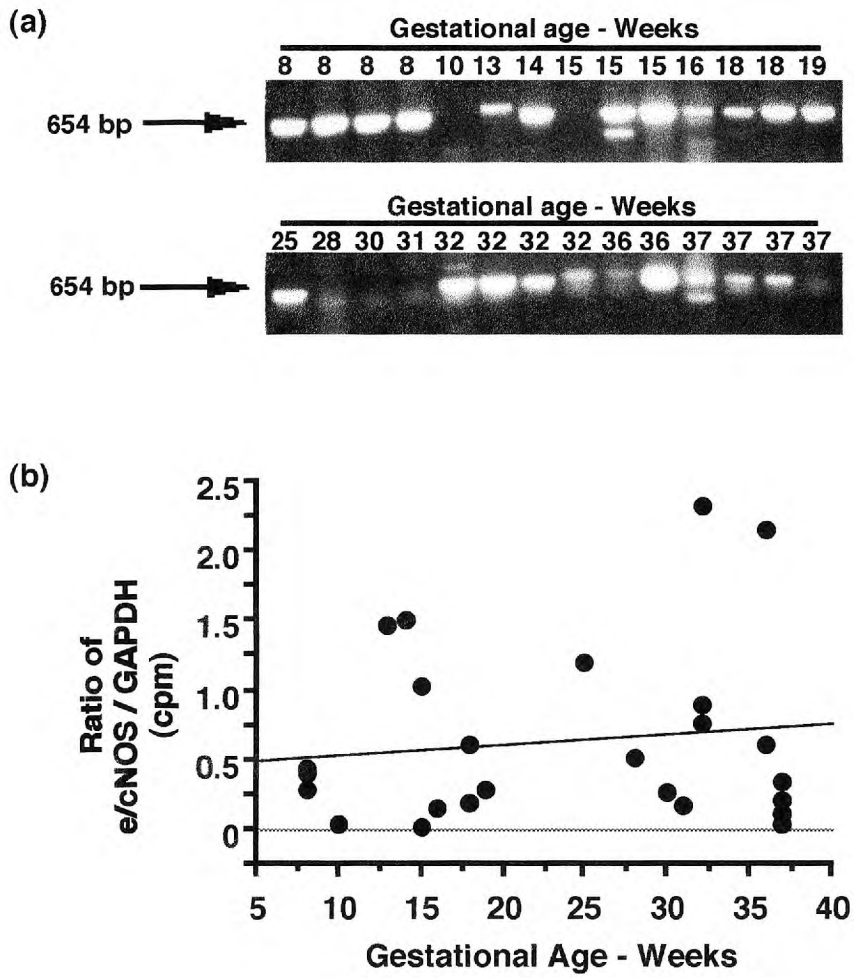


Fig. 4.14: e/cNOS expression in individual amnion samples related to gestational age. The top panel (**a**) shows e/cNOS mRNA expression by RT-PCR at 52 cycles, for individual amnion samples. The bottom panel (**b**) show regression of e/cNOS expression on gestational age (as cpm), as a ratio to GAPDH expression. Simple regression analysis showed no significant correlation between iNOS expression in amnion and gestational age (R = 0.13, p = 0.53).

e/cNOS mRNA expression is demonstrated by RT-PCR at 52 cycles in amnion samples collected throughout gestation. There was no statistically significant association between levels of e/cNOS expression and gestational age ($R = 0.13$, $p = 0.53$).

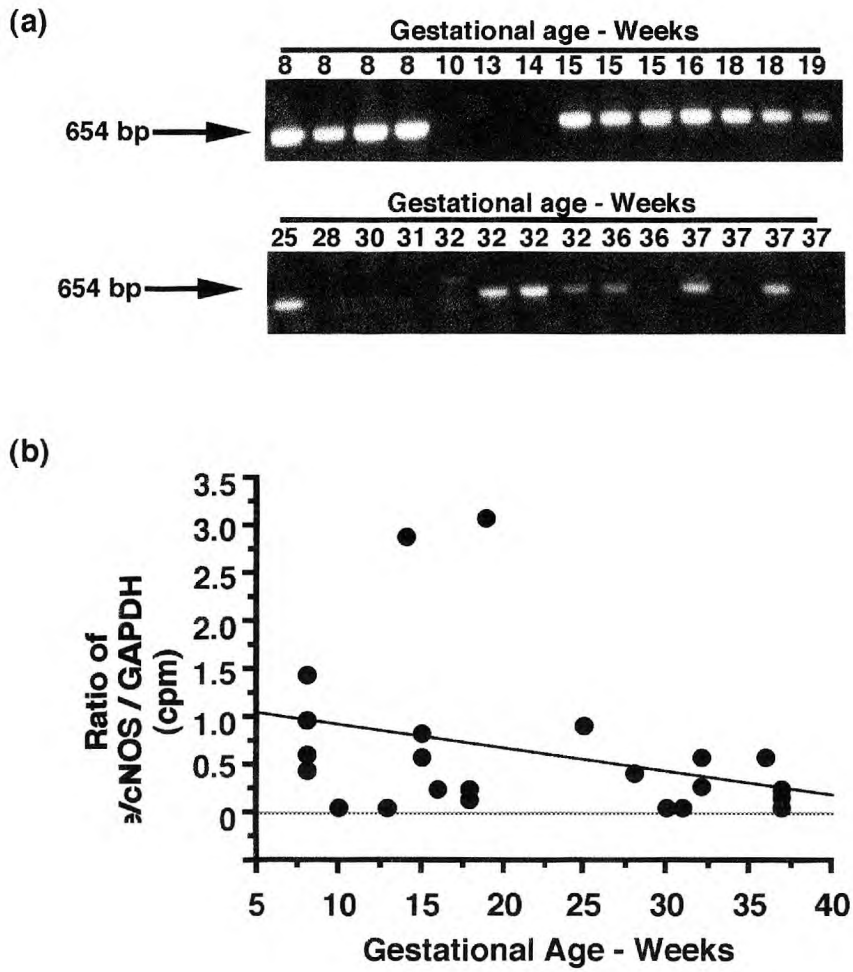


Fig. 4.15: e/cNOS expression in individual chorion decidua samples related to gestational age. The top panel (**a**) shows e/cNOS mRNA expression by RT-PCR at 47 cycles, for individual chorion decidua samples. The bottom panel (**b**) show regression of e/cNOS expression on gestational age (as cpm), as a ratio to GAPDH expression. Simple regression analysis showed no significant correlation between iNOS expression in amnion and gestational age ($R = 0.13$, $p = 0.53$).

e/cNOS mRNA expression is demonstrated by RT-PCR at 47 cycles in chorion decidua throughout gestation. There was no statistically significant association between levels of iNOS expression and gestational age ($R = 0.13$, $p = 0.53$). Data from cycle profiles from

tissue throughout gestation suggest higher levels of e/cNOS mRNA expression in chorion decidua compared with amnion.

NOS expression is expressed as a ratio to GAPDH, to control for RNA concentration. GAPDH expression in fetal membranes throughout gestation is shown in Fig. 4.16.

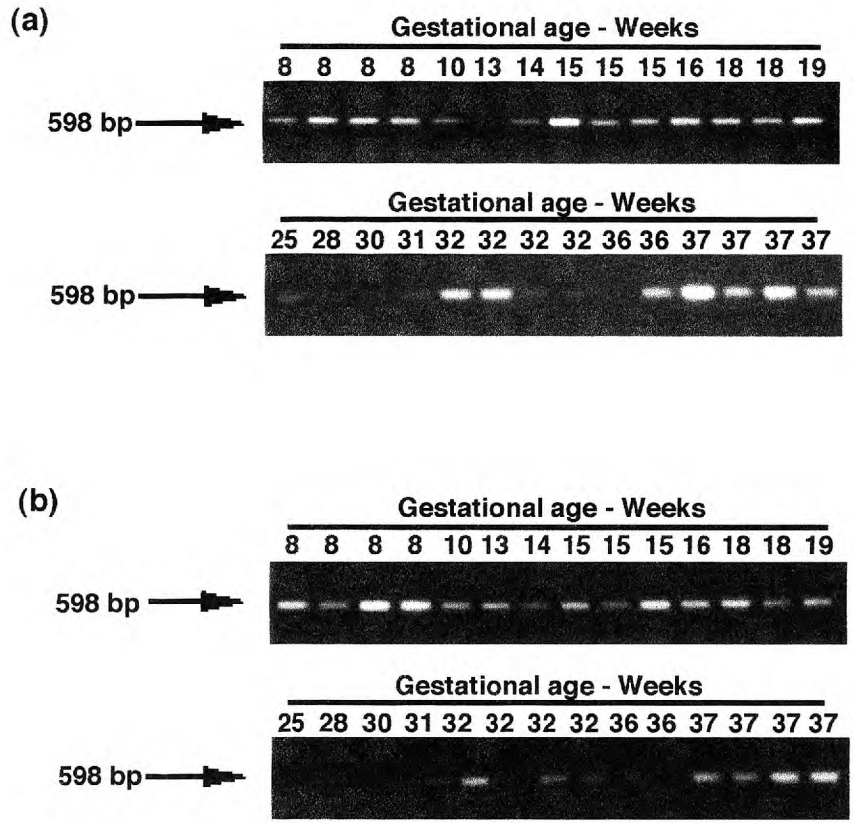


Fig. 4.16: GAPDH mRNA expression in fetal membranes throughout gestation. The panels show GAPDH mRNA expression by RT-PCR at 22 cycles in both (**a**) amnion and (**b**) chorion decidua, for individual samples.

Myometrium

NOS expression in myometrial tissue was initially examined using cycle profiles on pooled samples. Cycle profiles were used to determine optimal cycle numbers for each NOS isoform and to indirectly compare levels of expression between isoforms. Samples were collected from subjects either after labour onset at term, (term labour, n = 5), before labour at term, (term non-labour, n = 11), and before preterm labour (preterm non-labour, n = 13). The term patient's gestational age range was 38 - 40 weeks, in the preterm non-labour

group the gestational age range was 26 - 37 weeks. In the term labour group, all patients had regular painful contractions with associated cervical dilatation. The indications for caesarean section in the term labour group were not associated with uterine dysfunction. The non-labour group had no evidence of uterine contractions or cervical change.

The expression of nNOS in skeletal muscle has been reported (Nakane et al., 1993). The possibility that nNOS might also be expressed in uterine smooth muscle was investigated by analysing not only iNOS and e/cNOS expression, but also the neuronal isoform in these tissues.

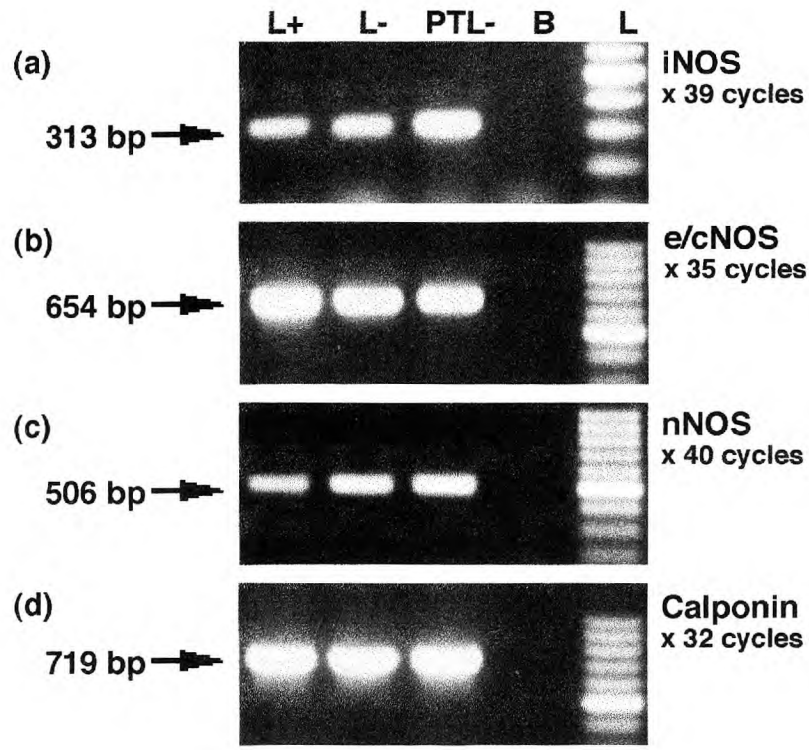


Fig. 4.17: NOS mRNA expression in myometrium, by RT-PCR. Panels show NOS mRNA expression by RT-PCR, for pooled samples for (**a**) iNOS, (**b**) e/cNOS, (**c**) nNOS and (**d**) calponin. Pooled samples are L+: term labour, L-: term non-labour, PTL-: preterm non-labour, B: blank lane - no starting template DNA and L: 100 bp DNA marker. Calponin shows the presence of starting template DNA.

NOS mRNA was demonstrated by RT-PCR in myometrial tissue collected at term, before and after labour and in pooled samples throughout gestation. Data from cycle profile numbers would suggest that levels of e/cNOS mRNA are generally higher in pooled myometrial samples than both iNOS and nNOS. Calponin mRNA expression

demonstrated by RT-PCR confirms starting template DNA. The relative levels of the NOS mRNA isoforms was investigated in individual myometrial samples, both in relation to labour onset (at term) and throughout gestation.

NOS expression in relation to labour onset

The relationship of NOS expression with labour onset in myometrium was investigated by analysing mRNA expression in individual samples, by RT-PCR. The optimal cycle number for each gene was derived from cycle profiles.

The optimal cycle number determined for pooled myometrium for this reverse transcriptase reaction, was 39 for iNOS, 35 for e/cNOS, 40 for nNOS and 32 for calponin.

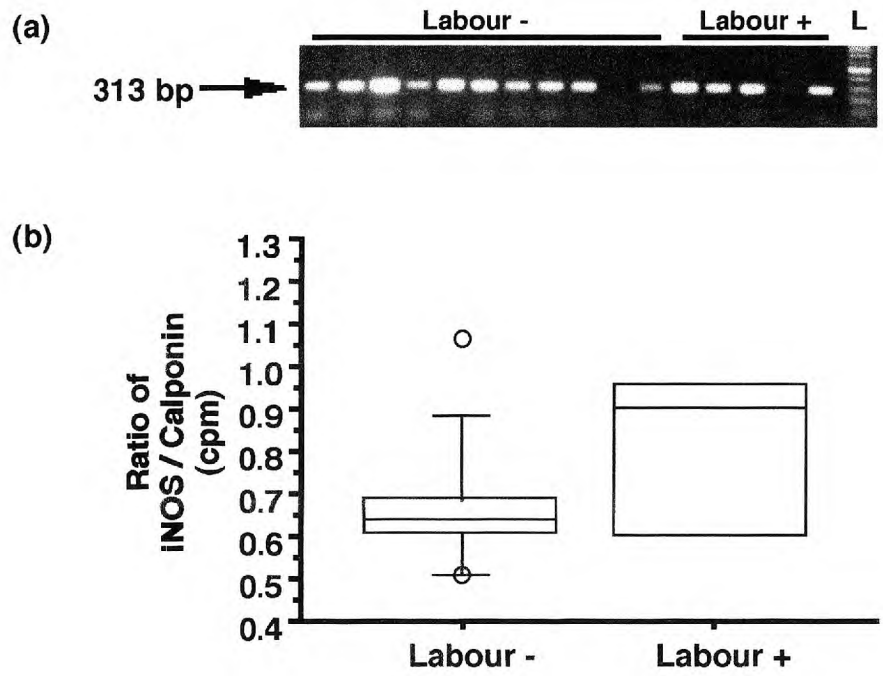


Fig. 4.18: iNOS mRNA expression in relation to labour, in term myometrium. (**a**) RT-PCR with iNOS primers at 39 cycles on DNA isolated from term non-labour (L-, n = 11) and term labour (L+, n = 5) myometrial samples. L: 100 bp DNA marker. Correct product size is 313 base pairs. (**b**) Box plots of the ratio of iNOS expression (as cpm) to calponin. Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in iNOS expression before or after labour onset ($p = 0.61$).

iNOS mRNA expression is demonstrated by RT-PCR at 39 cycles in myometrial samples collected at term. There was no statistically significant difference in levels of iNOS expression before or after the onset of labour ($p = 0.61$).

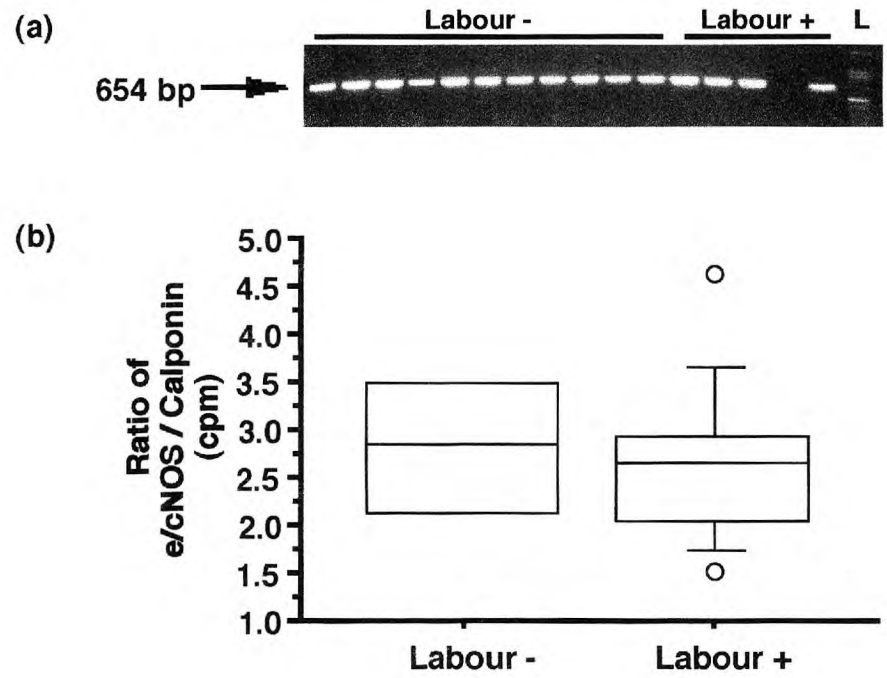


Fig. 4.19: e/cNOS mRNA expression in relation to labour, in term myometrium. (**a**) RT-PCR with e/cNOS primers at 35 cycles on DNA isolated from term non-labour (L-, n = 11) and term labour (L+, n = 5) myometrial samples. L: 100 bp DNA marker. Correct product size is 654 base pairs. (**b**) Box plots of the ratio of e/cNOS expression (as cpm) to calponin. Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in iNOS expression before or after labour onset ($p = 0.58$).

e/cNOS mRNA expression is demonstrated by RT-PCR at 35 cycles in myometrial samples collected at term. There was no statistically significant difference in levels of e/cNOS expression before or after the onset of labour ($p = 0.58$).

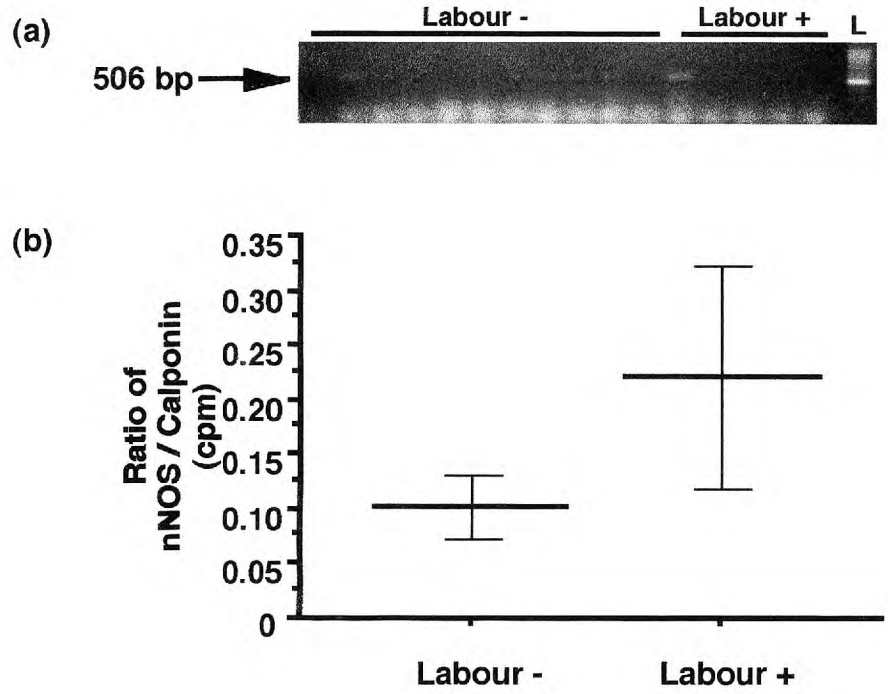


Fig. 4.20: nNOS mRNA expression in relation to labour, in term myometrium. (**a**) RT-PCR with nNOS primers at 40 cycles on DNA isolated from term non-labour (L-, n = 11) and term labour (L+, n = 5) myometrial samples. L: 100 bp DNA marker. Correct product size is 506 base pairs. (**b**) Plots of the ratio of e/cNOS expression (as cpm) to calponin. Data shows means $\pm$ x 1 SE. There was no statistically significant difference in nNOS expression before or after labour onset ($p = 0.46$).

nNOS mRNA expression is demonstrated by RT-PCR at 40 cycles in myometrial samples collected at term. There was no statistically significant difference in levels of nNOS expression before or after the onset of labour ($p = 0.46$).

NOS expression in myometrium is expressed as a ratio to calponin rather than GAPDH (as in the fetal membranes) with the intention of normalising for smooth muscle cell content. Calponin expression in term myometrium is shown in Fig. 4.21.

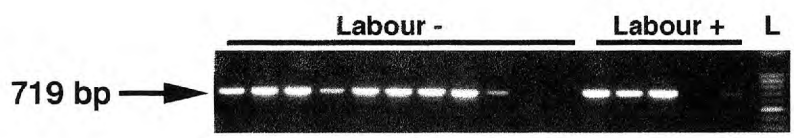


Fig. 4.21: Calponin mRNA expression in relation to labour, in term myometrium. RT-PCR with calponin primers at 32 cycles on DNA isolated from term non-labour (L-, n = 11) and term labour (L+, n = 5) myometrial samples. L: 100 bp DNA marker. Correct product size is 719 base pairs.

Calponin mRNA expression was demonstrated in term myometrium using 32 PCR amplification cycles. There was no significant difference in the levels of calponin expression before or after labour (data not shown).

NOS expression in relation to gestational age

The relationship of NOS expression to gestational age was considered by analysing NOS expression in myometrial samples collected throughout gestation. The preterm non-labour group of patients had a gestational age range from 26 - 37 weeks. The indications for delivery in this group were associated with intra-uterine growth retardation and hypertensive disorders. There was no clinical evidence of preterm labour in this group. iNOS, e/cNOS and nNOS expression is shown in Fig. 4.22.

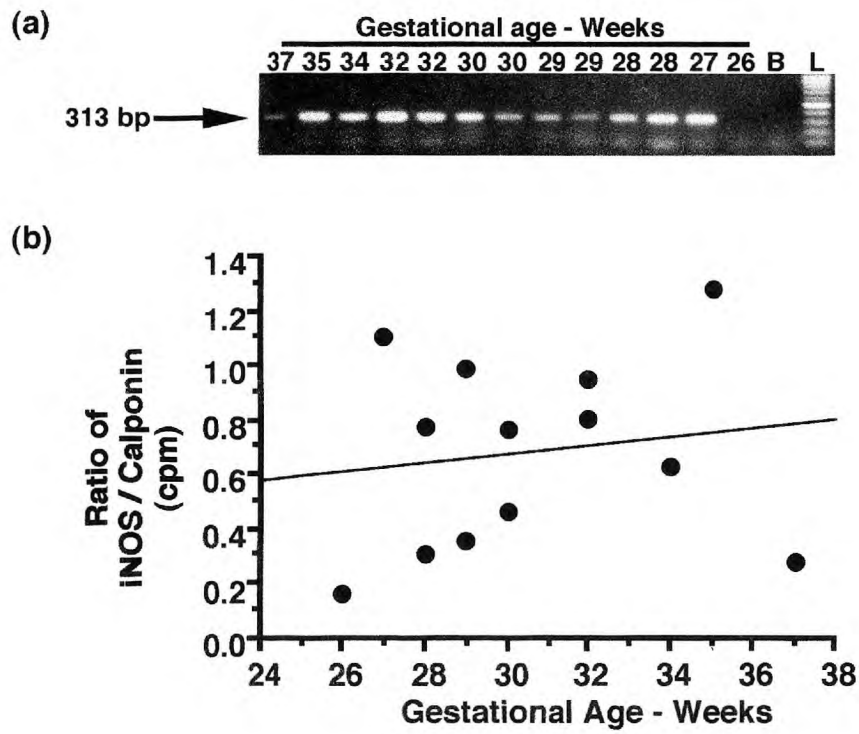


Fig. 4.22: iNOS expression in individual myometrial samples related to gestational age. The top panel (**a**) shows iNOS mRNA expression by RT-PCR at 39 cycles, for individual myometrial samples. B: blank lane - no starting template DNA and L: 100 bp DNA marker. Correct product size is 313 base pairs. The bottom panel (**b**) show regression of iNOS expression on gestational age (as cpm), as a ratio to calponin expression. Simple regression analysis showed no significant correlation between iNOS expression in myometrium and gestational age ($R = 0.15$, $p = 0.62$).

iNOS mRNA expression is demonstrated by RT-PCR in myometrial samples throughout gestation. There was no significant correlation between iNOS expression in myometrium and gestational age ($R = 0.15$, $p = 0.62$).

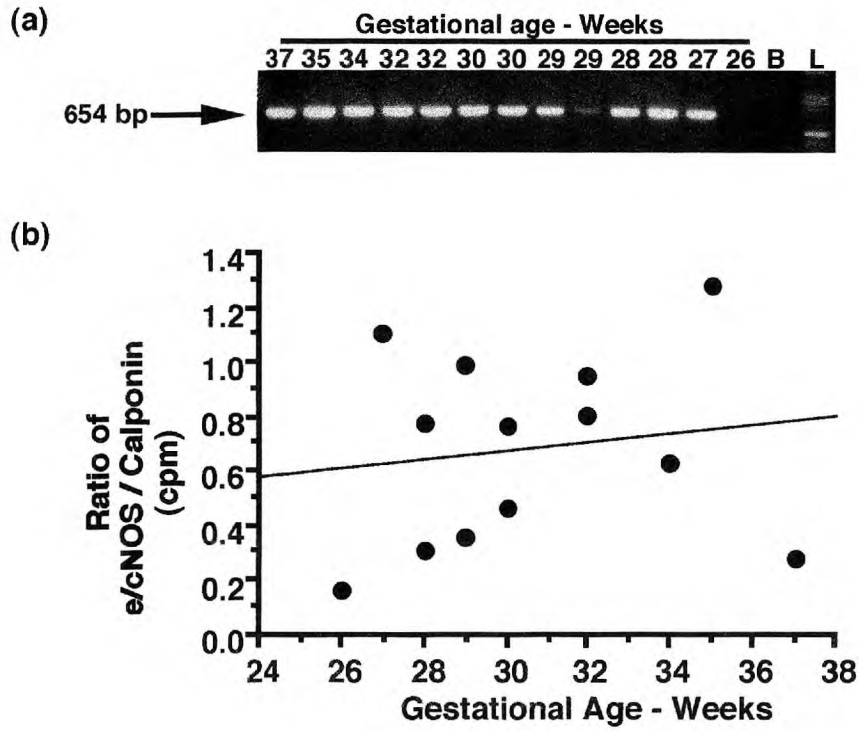


Fig. 4.23: e/cNOS expression in individual myometrial samples related to gestational age. The top panel **(a)** shows e/cNOS mRNA expression by RT-PCR at 35 cycles, for individual myometrial samples. B: blank lane - no starting template DNA and L: 100 bp DNA marker. Correct product size is 654 base pairs. The bottom panel **(b)** show regression of e/cNOS expression on gestational age (as cpm), as a ratio to calponin expression. Simple regression analysis showed no significant correlation between e/cNOS expression in myometrium and gestational age ($R = 0.48$, $p = 0.09$).

e/cNOS mRNA expression is demonstrated by RT-PCR in myometrial samples throughout gestation. There was no significant correlation between e/cNOS expression in myometrium and gestational age ($R = 0.48$, $p = 0.09$).

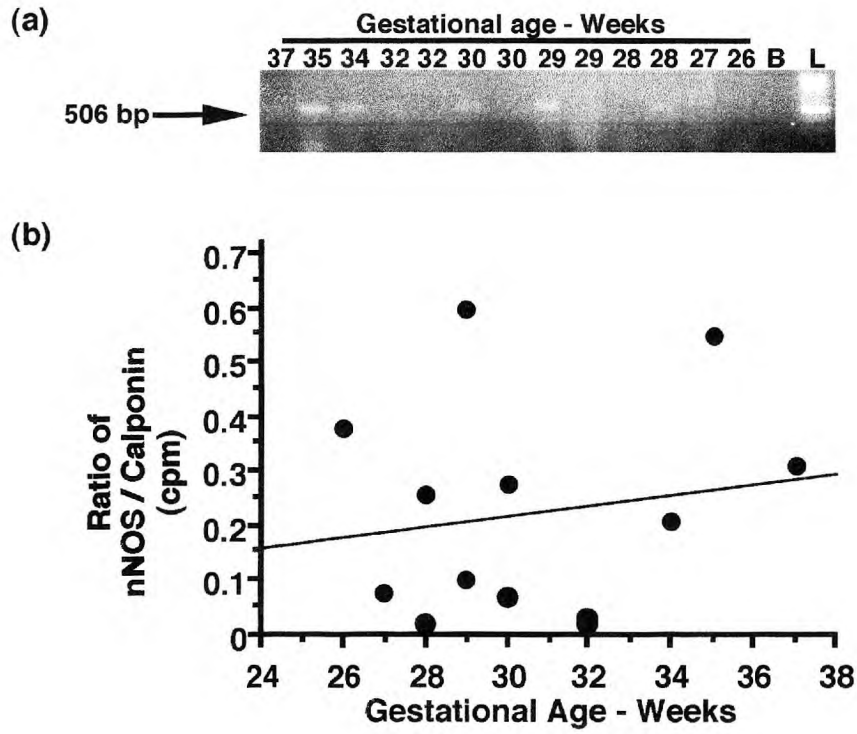


Fig. 4.24: nNOS expression in individual myometrial samples related to gestational age. The top panel (**a**) shows nNOS mRNA expression by RT-PCR at 40 cycles, for individual myometrial samples. B: blank lane - no starting template DNA and L: 100 bp DNA marker. Correct product size is 506 base pairs. The bottom panel (**b**) show regression of nNOS expression on gestational age (as cpm), as a ratio to calponin expression. Simple regression analysis showed no significant correlation between nNOS expression in myometrium and gestational age ($R = 0.17$, $p = 0.59$).

nNOS mRNA expression is demonstrated by RT-PCR in myometrial samples throughout gestation. There was no significant correlation between nNOS expression in myometrium and gestational age ($R = 0.17$, $p = 0.59$).

Calponin expression in myometrium throughout gestation is shown in Fig. 4.25.

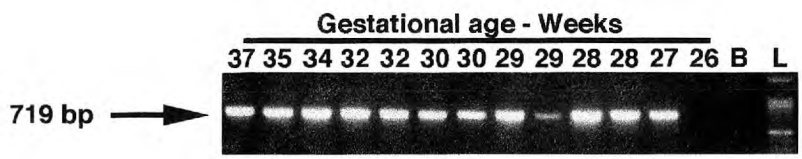


Fig. 4.25: Calponin mRNA expression in relation to gestational age, in myometrium. RT-PCR with calponin primers at 32 cycles on DNA isolated from individual myometrial samples throughout gestation. L: 100 bp DNA marker. Correct product size is 719 base pairs.

Calponin mRNA expression was demonstrated in term myometrium using 32 PCR amplification cycles. There was no significant difference in the levels of calponin expression before or after labour (data not shown).

4.2.2 *In situ* hybridisation

Introduction

In situ hybridisation is a technique that identifies cellular localisation of gene expression. In contrast RT-PCR relies on homogenisation and RNA extraction from tissue as a whole, and while this provides information on gene expression within individual tissue samples, it provides no further information on cellular localisation. Thus whilst NOS expression has been identified in fetal membranes and myometrium by RT-PCR, the exact location of expression of these genes has not been identified.

In fetal membranes there is some speculation that iNOS expression might be related to cells from an inflammatory cell type (CD 14 macrophages for example) present in the amnionic mesenchyme (Eis et al., 1997). Similarly in myometrium iNOS expression might be related to monocyte / macrophage infiltration, and e/cNOS expression to vascular endothelial cells, in small vessels contained in the individual samples. The purpose of these in situ hybridisation studies was then to resolve these questions.

NOS localisation in fetal membranes by in situ hybridisation

NOS expression is localised in fetal membranes by in situ hybridistion. Fig. 4.26 shows iNOS expression in fetal membranes collected after labour at term (n = 1).

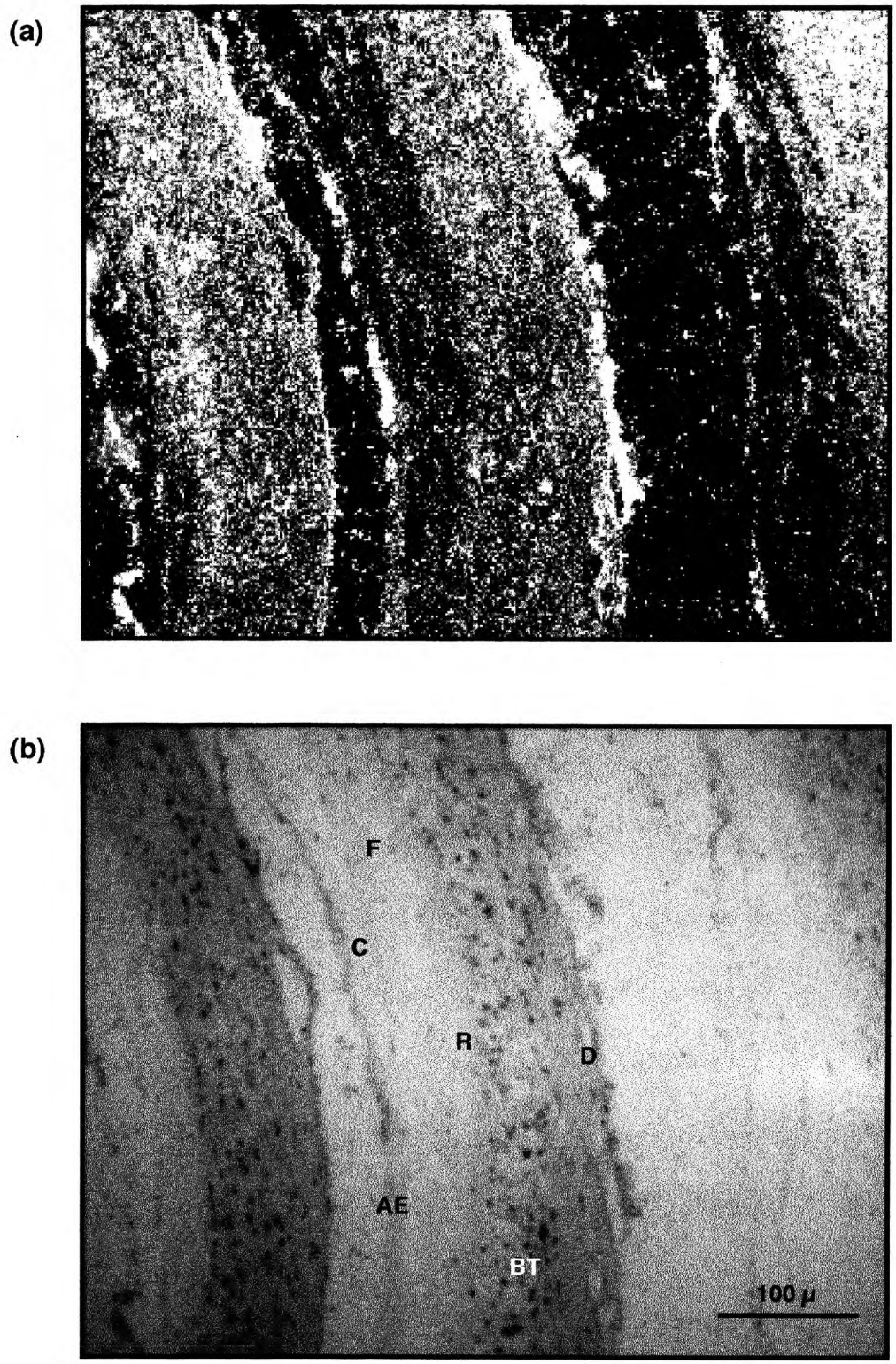


Fig. 4.26: iNOS expression localised in fetal membranes by *in situ* hybridisation (antisense riboprobe). **(a)** iNOS expression in fetal membranes, antisense riboprobe: dark field microscopy, x 10 magnification. **(b)** iNOS expression in fetal membranes: light field microscopy, x10 magnification. AE: amniotic epithelium, C: acellular compact layer, R: reticular layer of the chorion, F: fibroblast layer, BT: basal cytotrophoblasts, D: decidua, 100 μ: 100 μM size bar.

This study confirms RT-PCR data, that iNOS is expressed in amnion and chorion decidua. iNOS expression appears to be greater in the amniotic epithelial layer, and the chorionic cytotroblast and decidua. This concords with iNOS cycle profiles that suggested that there was higher levels of iNOS expression in chorion decidua (x 39) compared with amnion (x 42). iNOS expression does not appear to be solely related to macrophage / monocyte distribution. *In situ* hybridisation with the sense (control) ribonucleotide showed no hybridisation (Fig. 4.27).

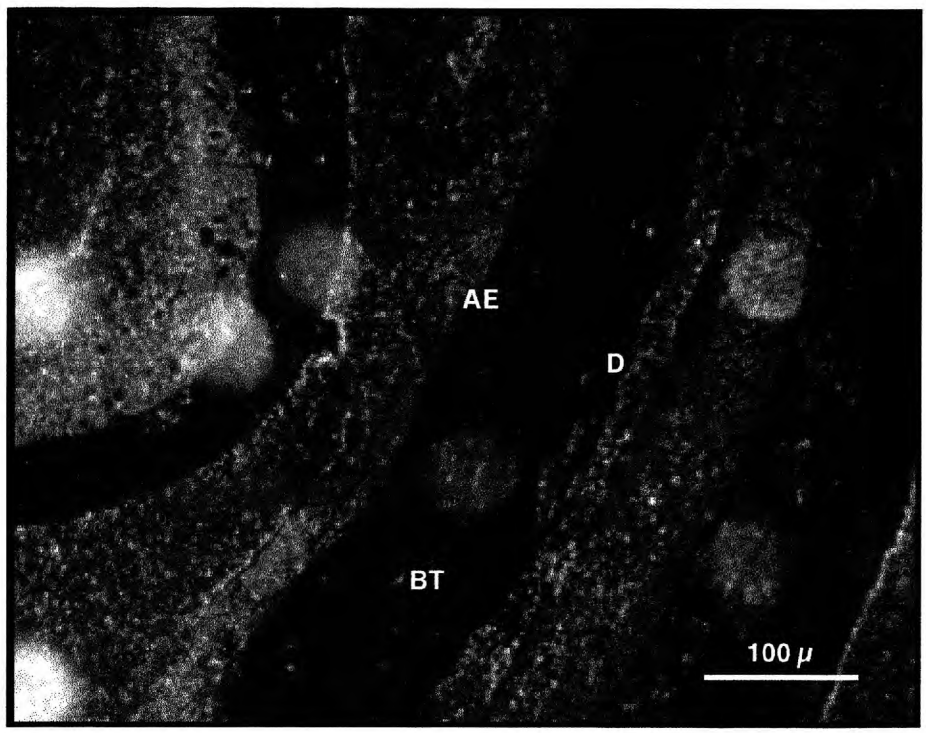


Fig. 4.27: iNOS expression localised in fetal membranes by *in situ* hybridisation (sense riboprobe). iNOS expression in fetal membranes, sense riboprobe: dark field microscopy, x10 magnification. AE: amniotic epithelium, BT: basal cytotrophoblasts, D: decidua. 100 μ: 100 μM size bar.

e/cNOS localisation in fetal membranes may be similarly demonstrated.

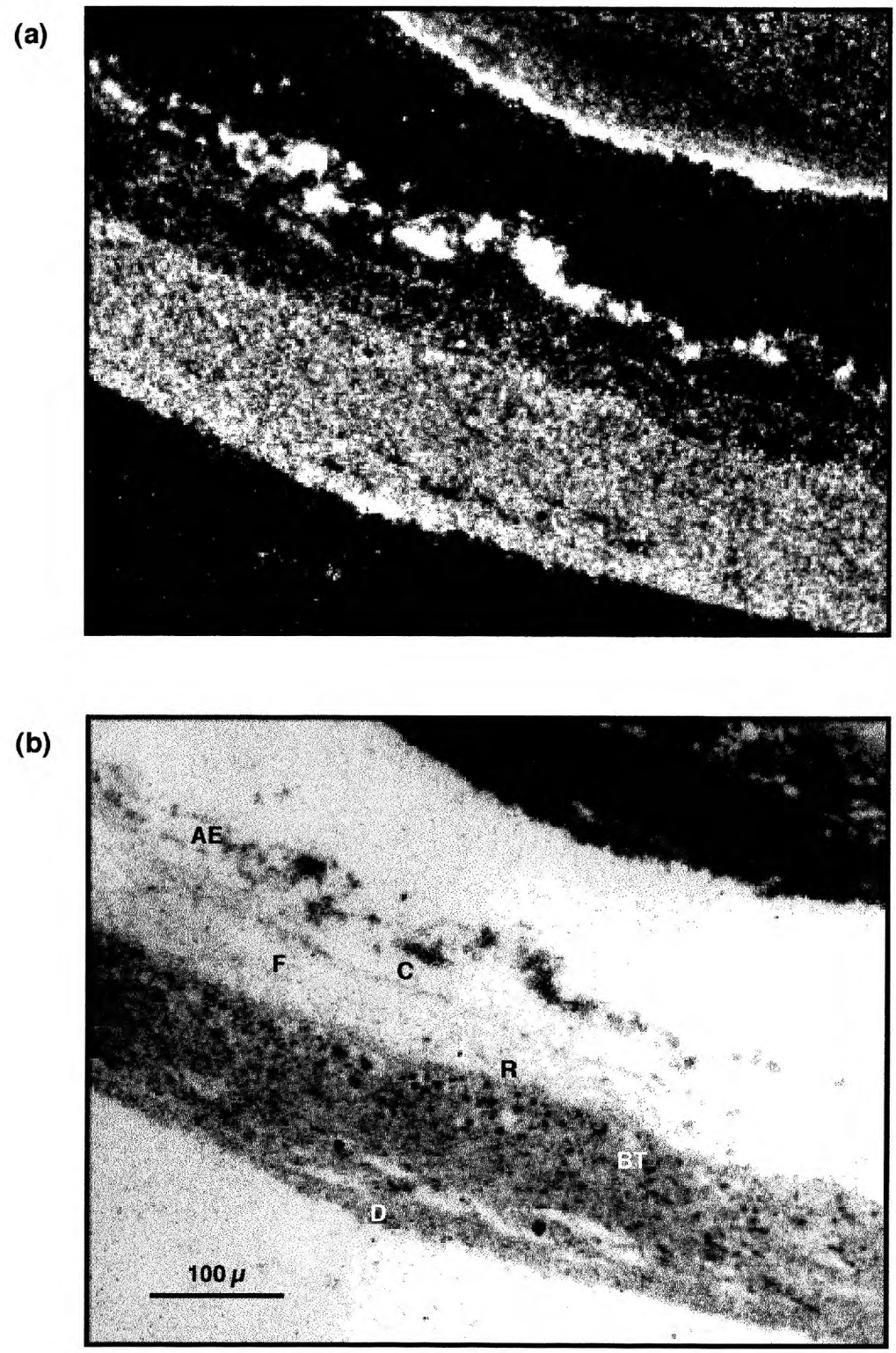


Fig. 4.28: e/cNOS expression localised in fetal membranes by *in situ* hybridisation (antisense riboprobe). **(a)** e/cNOS expression in fetal membranes, antisense riboprobe: dark field microscopy, x10 magnification. **(b)** e/cNOS expression in fetal membranes: light field microscopy, x10 magnification. AE: amniotic epithelium, C: acellular compact layer, R: reticular layer of the chorion, F: fibroblast layer, BT: basal cytotrophoblasts, D: decidua. 100 μ: 100 μM size bar.

e/cNOS is expressed throughout the amnion and chorion decidua. Expression is localised in the amnion epithelial layer, the reticular layer of the chorion and in the decidua. In a similar manner to iNOS expression is preferentially localised in the chorion decidual layers confirming RT-PCR data derived from cycle numbers, suggesting higher levels of expression in the chorion decidua (x 47, compared with x 52 in the amnion).

In situ hybridisation with the sense (control) ribonucleotide showed no hybridisation (Fig. 4.29).

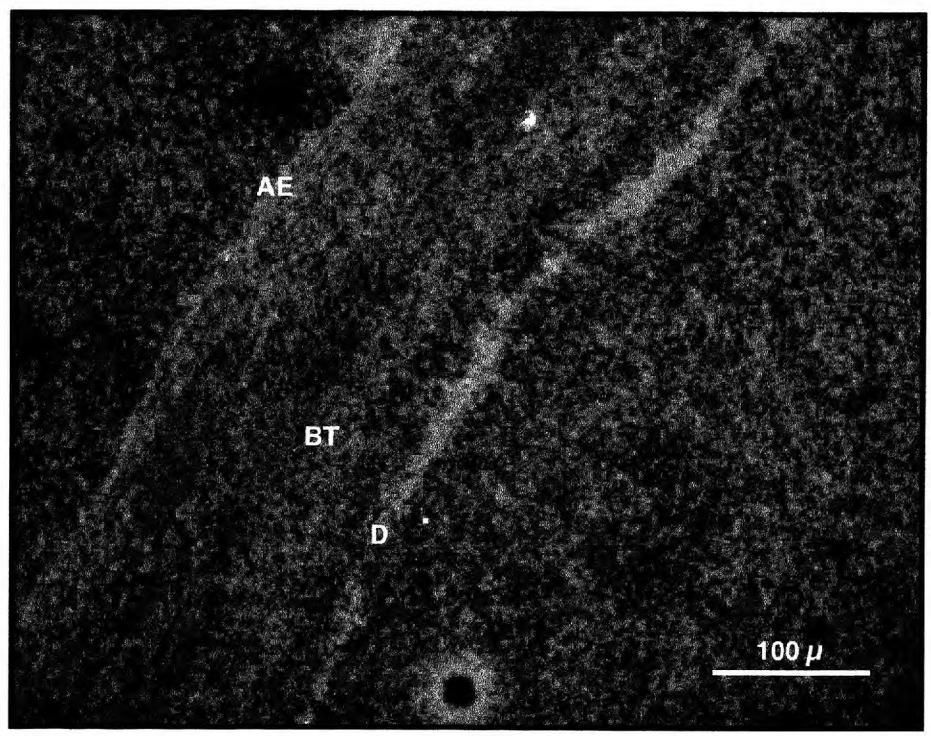


Fig. 4.29: e/cNOS expression localised in fetal membranes by *in situ* hybridisation (sense riboprobe). e/cNOS expression in fetal membranes,: dark field microscopy, x10 magnification. AE: amniotic epithelium, BT: basal cytotrophoblasts, D: decidua. 100 μ: 100 μM size bar.

NOS localisation in myometrium by in situ hybridisation

NOS localisation was examined in a myometrial biopsy obtained following labour onset (n = 1). iNOS expression is shown in Fig. 4.30.

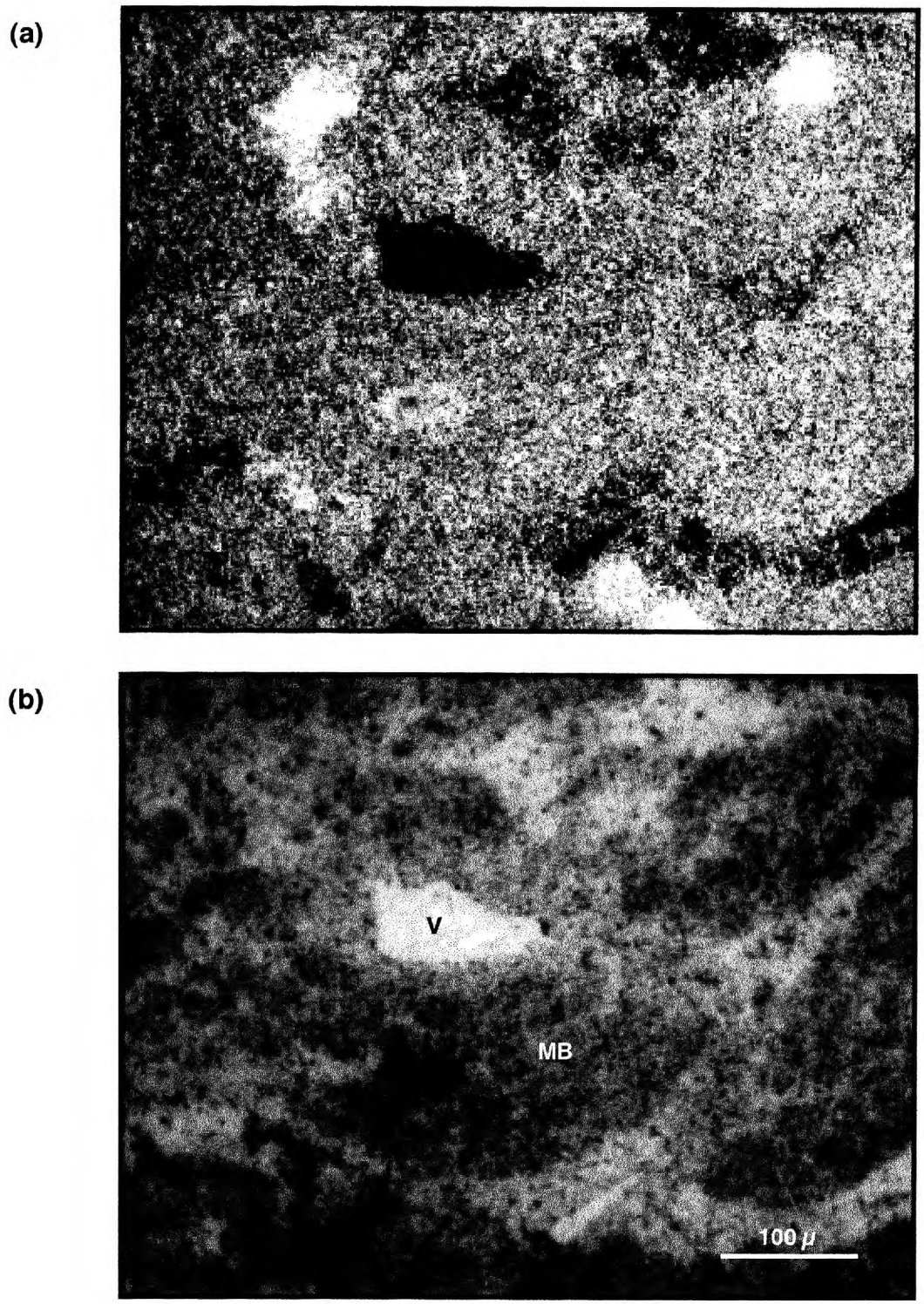


Fig. 4.30: iNOS expression localised in term myometrium, by *in situ* hybridisation (antisense riboprobe). **(a)** iNOS expression in myometrium, antisense riboprobe: dark field microscopy, x10 magnification. **(b)** iNOS expression in myometrium: light field microscopy, x10 magnification. MB: myometrial smooth muscle bundle, V: venule. 100 μ: 100 μM size bar.

The hybridisation pattern seen with the antisense riboprobe suggests widespread non-specific hybridisation. This could be due to a number of different factors. The riboprobe might be hybridising non-specifically, the RNA present in the tissue may have been subject to nuclease activity or ultimately iNOS expression in iNOS might in fact be diffuse. The riboprobes used in this study were identical to those employed in the fetal membrane *in situ* experiments, where hybridisation was both specific and consistent. Evidence for the probable explanation for these results is provided by the light field. The tissue morphology and definition appears markedly disrupted, probably as a result of tissue preparation. This may also have been associated with nuclease activity. At any rate the results are at best sub-optimal, and realistically uninterpretable. The question as to whether iNOS is expressed in myometrial smooth muscle cells is investigated further in section 4.2.3.

e/cNOS expression in myometrium is shown in Fig.4.31:

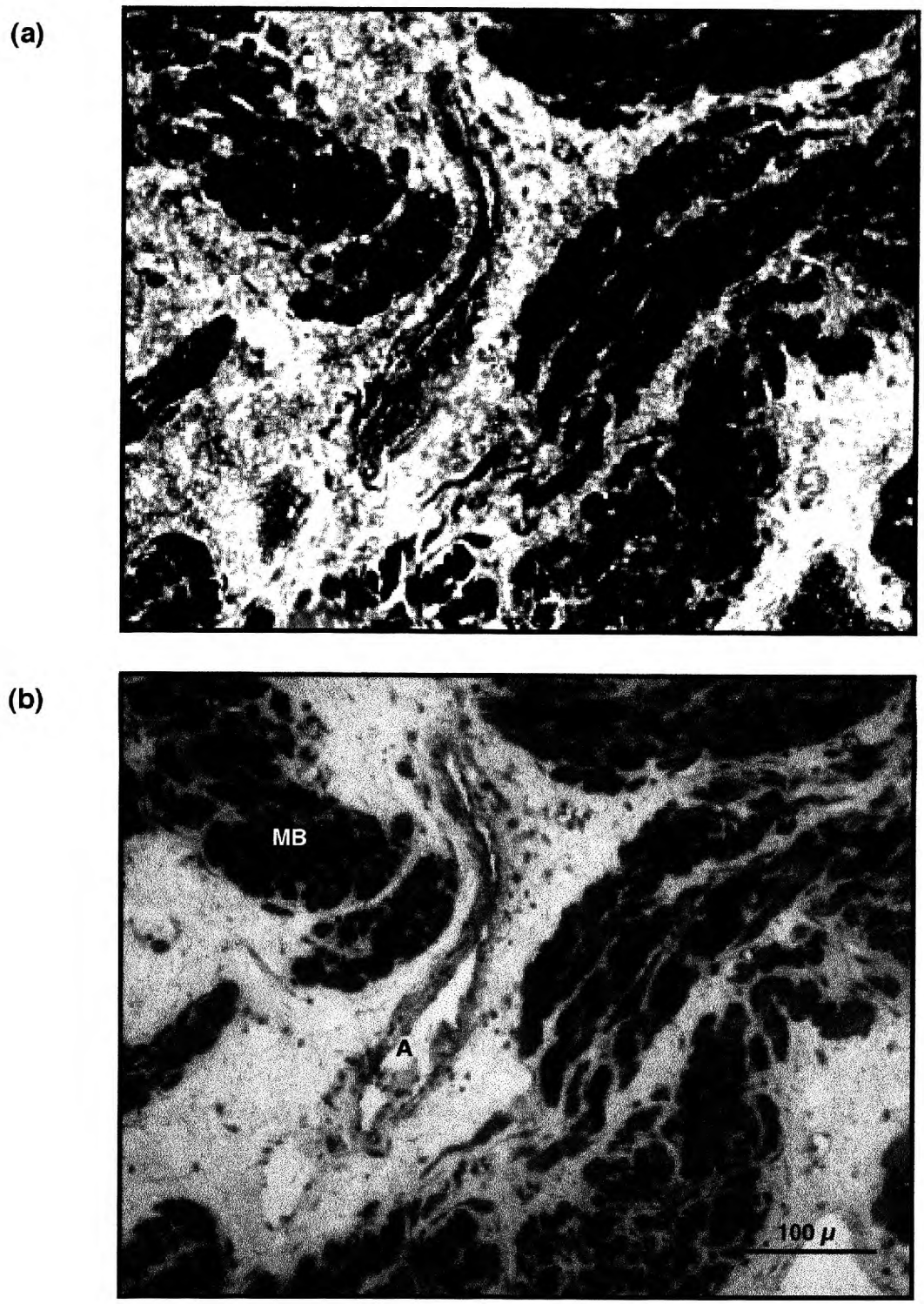


Fig. 4.31: e/cNOS expression localised in term myometrium, by *in situ* hybridisation (antisense riboprobe). (**a**) e/cNOS expression in myometrium, antisense riboprobe: dark field microscopy, x10 magnification. (**b**) e/cNOS expression in myometrium: light field microscopy, x10 magnification. MB: myometrial smooth muscle bundle, A: arteriole. 100 μ: 100 μM size bar.

e/cNOS expression is localised to myometrial connective tissue and vascular endothelial tissue. e/cNOS expression in myometrial smooth muscle cells was not identified. e/cNOS expression in vascular tissue is expected, and it was perhaps fortuitous that the section studied contained vascular tissue to confirm appropriate hybridisation.

4.2.3 NOS expression in myometrial cell culture

Introduction

The expression of NOS isoforms has been investigated in myometrium by both RT-PCR and *in situ* hybridisation. Whilst iNOS is expressed in myometrial tissue by RT-PCR, its cellular localisation was not clearly identified by *in situ* hybridisation, and an alternative experimental strategy was therefore employed to resolve this question. Myometrial cells derived from myometrial explants provide information on NOS expression in myometrial cells provide they result from pure culture. Results obtained from cell culture provide some information on both the localisation of expression and capacity for these cells to express NOS. However they provide little evidence on the physiological role, if any, of NOS in myometrium as the cells are necessarily manipulated to establish in culture, and this may influence both their morphology and phenotype.

Myometrial cells established in culture were a kind gift from Dr. Simon Bartlett (King's College, London). Cells were cultured to confluence, and all cells used in these studies were between 3 - 5 passages. Cells were either cultured non-stimulated, in the presence of 10% FBS, or stimulated, with 10% FBS and a cytokine stimulus. Cells were obtained frozen, and RNA was extracted and reverse transcribed using routine techniques in our laboratory (see section 2.2.1).

NOS expression in myometrial cell culture was compared with NOS expression in pooled myometrial tissue samples (Fig. 4.32).

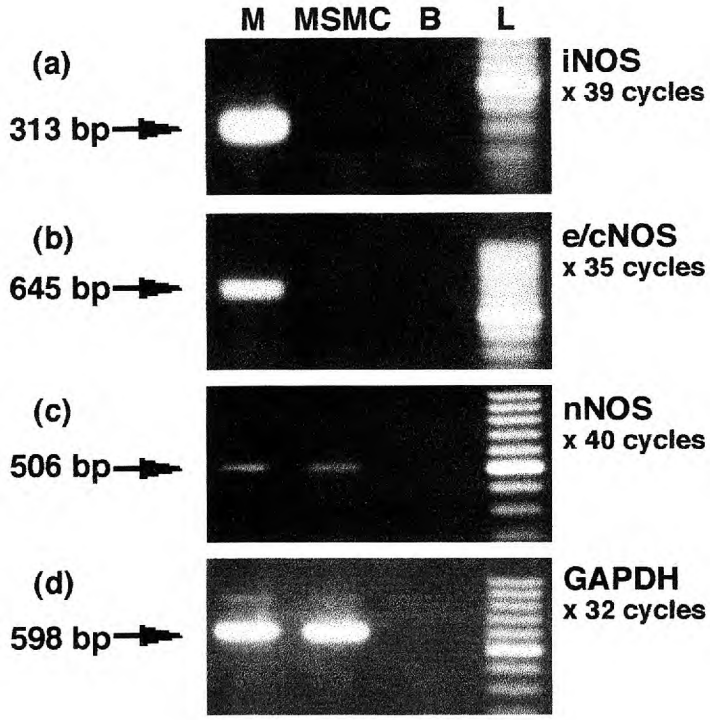


Fig. 4.32: NOS mRNA expression demonstrated by RT-PCR in myometrial tissue and non-stimulated myometrial smooth muscle cell culture. **(a)** iNOS expression, **(b)** e/cNOS expression, **(c)** nNOS expression and **(d)** GAPDH expression. M - pooled myometrial tissue, MSMC - myometrial smooth muscle cell culture, B: blank lane - no starting template DNA and L: 100 bp DNA marker.

iNOS expression is confirmed in myometrial tissue, but not found in unstimulated, myometrial cell culture. This suggests that iNOS expression in myometrial tissue observed by RT-PCR might reflect expression in cell types other than myometrial cells. Similarly e/cNOS expression is demonstrated in myometrial tissue, but not cell culture, suggesting that expression seen in myometrial tissue might reflect expression in vascular tissue. Conversely nNOS appears to be expressed in both myometrial tissue and cell culture, suggesting that nNOS is expressed in myometrium.

GAPDH expression is shown in Fig. 4.32 (**d**) confirming starting template in both PCR reactions.

The ability of myometrial cells to express iNOS (and COX-2) following cytokine induction was further investigated by RT-PCR (Fig.4.33). Cells were stimulated for 24 hrs with a combination of 100 μg / ml LPS and 10ng / ml each of IL-1ß, IFN-γ and TNF-α.

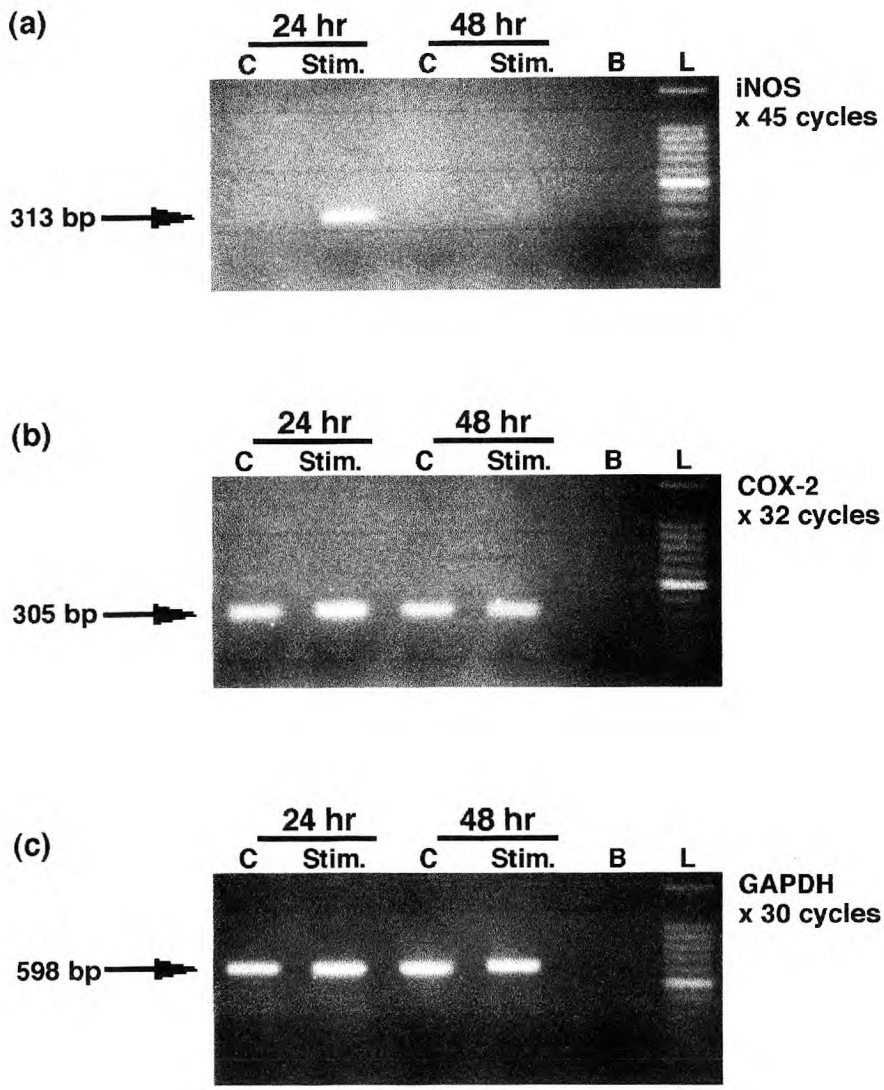


Fig. 4.33: iNOS, COX-2 and GAPDH mRNA expression in control and cytokine-stimulated myometrial cells, at 24 and 48 hrs. (**a**) iNOS expression, (**b**) COX-2 expression and (**c**) GAPDH expression.

Fig. 4.33 (**a**) shows that iNOS message is up-regulated by cytokine induction at 24 hrs. No PCR product is seen at 48 hrs suggesting that iNOS induction is short lived, or that iNOS mRNA is not stable over this time period. In contrast Fig. 4.33 (**b**) shows that COX-2 message is present in all samples, and does not appear to be significantly further upregulated by cytokine induction. COX-2 might be already be maximally induced by culture conditions. GAPDH Fig. 4.33 (**c**) confirms starting DNA template.

To summarise these cell culture experiments, iNOS is not expressed in cell culture, in the absence of cytokine induction, however cytokine stimulation upregulates iNOS message at

24 hrs. e/cNOS is not expressed in myometrial cell culture. nNOS is expressed in myometrial cell culture in the absence of cytokine stimulation. GAPDH is expressed in myometrial cell culture.

4.2.4 Western analysis of NOS expression

Introduction

Western analysis is a technique for analysing protein expression in tissue. In principle it involves extracting total protein from tissue samples, and separating proteins on the basis of their size, on non-denaturing polyacrylamide by gel electrophoresis. Proteins are subsequently transferred to a nylon membrane for membrane blocking and binding with primary antibodies directed against the protein of interest. Secondary antibodies bind to the primary antibody and allow for detection by chemiluminescence (see sect 2.2.12 for details). The fundamental basis of Western analysis is determination of protein levels in separate tissue samples. Data from these experiments provides direct information on the relative abundance of protein within these samples. Whilst providing evidence on protein levels it does not share the sensitivity of analysis of mRNA by RT-PCR, and is heavily dependent on antibody specificity. Thus it use in analysis of proteins expressed at low levels is limited.

Optimisation of Western analysis

The sensitivity of Western analysis to detect the appropriate proteins is largely dependent on the specificity of the primary antibodies. Primary antibodies were obtained commercially. It is impossible to determine their specificity in tissue with any certainty. The only controls available with which to determine their specificity are the peptide against which they were originally raised. It is anticipated that antibodies will bind to these peptides, and therefore their sensitivity to NOS proteins can only be inferred.

The relative concentrations of both primary and secondary antibodies was determined empirically for each tissue, from pilot data.

Western analysis of NOS protein in fetal membranes

Expression of iNOS protein in term fetal membrane samples is shown in Fig. 4.34.



Fig. 4.34: iNOS protein expression in term fetal membranes, by Western analysis. Tissue Sample is a representative sample, collected at term before the onset of labour. Lanes are: Am: amnion, Chor: chorion decidua, P: placenta and Control: ADP-sepharose extracted cytokine induced A549 cells. Exposure time refers to ECL exposure time (see section 2.2.12 for methods).

Protein bands can be seen for amnion, chorion decidua and placenta at the correct size for iNOS protein (130 kDa). However the presence of multiple protein bands makes interpretation of relative levels of protein difficult. Cytokine induced A549 cells should be a good source of iNOS protein, and therefore a satisfactory positive control, but even in this lane antibody-protein binding is not very great. The ECL exposure time was a balance between protein detection and background development. As a result of this lack of specificity the relationship of iNOS protein to gestational age and labour onset was not examined.

e/cNOS protein expression in fetal membranes is similarly highly antibody dependent. Expression of e/cNOS protein in term fetal membrane samples is shown in Fig. 4.35:

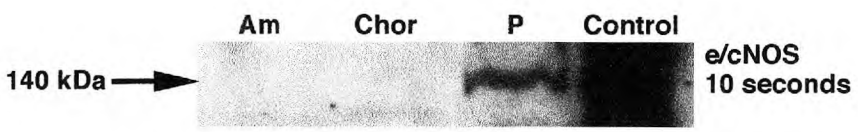


Fig. 4.35: e/cNOS protein expression in term fetal membranes by Western analysis. Tissue Sample is a representative sample, collected at term before the onset of labour. Lanes are: Am: amnion, Chor: chorion decidua, P: placenta and Control: ADP sepharose-extracted HUVEC. Exposure time refers to ECL exposure time (see methods).

e/cNOS protein is detected in placental samples but not amnion or chorion decidua. The bands seen correlate with the putative size of e/cNOS protein (140 kDa). The control sample was ADP sepharose extracted HUVEC (see section 2.2.12 for details of this technique). ADP-sepharose extraction exploits the affinity of NADPH binding of NOS

proteins, to increase the relative concentration of NOS proteins in the control sample. The presence of other protein bands suggests some non-specific binding.

The relationship of e/cNOS expression to gestational age is shown if Fig. 4.36:

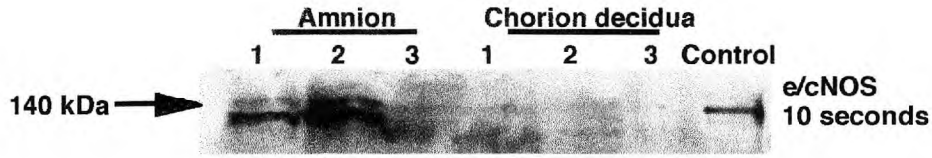


Fig. 4.36: e/cNOS protein expression in fetal membranes throughout gestation, by Western analysis. Samples are: Amnion, 1: 14 weeks gestation (labour-), 2: Term (labour-) and 3: Term (labour+), and Chorion, 1: 14 weeks gestation (labour-), 2: Term (labour-) and 3: Term (labour+), and Control: ADP sepharose extracted HUVEC. Exposure time refers to ECL exposure time (see methods).

In this study e/cNOS expression is shown in amnion and chorion decidua at 14 weeks and at term. e/cNOS protein expression appears greater in amnion than chorion decidua. The presence of multiple protein bands makes interpretation of gestational and labour onset changes difficult.

Western analysis of NOS protein in myometrium

e/cNOS expression in myometrium is shown in Fig. 4.37:

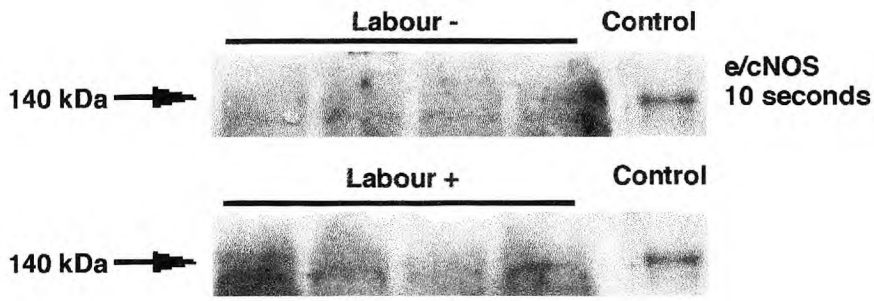


Fig. 4.37: e/cNOS protein expression in term myometrium in relationship to labour onset, by Western analysis. Lanes are: Labour - : Term non-labour samples (four individual representative samples), Labour + : Term labour samples (four individual representative samples) and Control: ADP sepharose extracted HUVEC. Exposure time refers to ECL exposure time (see methods).

e/cNOS protein expression can be identified, and the size of band correlates both with the positive control (ADP sepharose extracted HUVEC), and the putative size of the e/cNOS protein. However the presence of multiple non-specific bands, and the very faint antibody - protein binding makes analysis of relative levels of protein, in relation to labour onset, difficult.

The expression of iNOS protein in myometrium was not investigated.

4.3 COX expression in pregnancy

4.3.1 COX mRNA expression

Fetal membranes

COX expression in relation to gestational age

COX expression was investigated in fetal membrane samples collected throughout pregnancy. The number of cycles chosen for this study were derived from pilot data. COX-1 and COX-2 mRNA expression was investigated in both amnion and chorion decidua by RT-PCR. COX-1 expression in amnion samples collected throughout pregnancy is shown in Fig. 4.38:

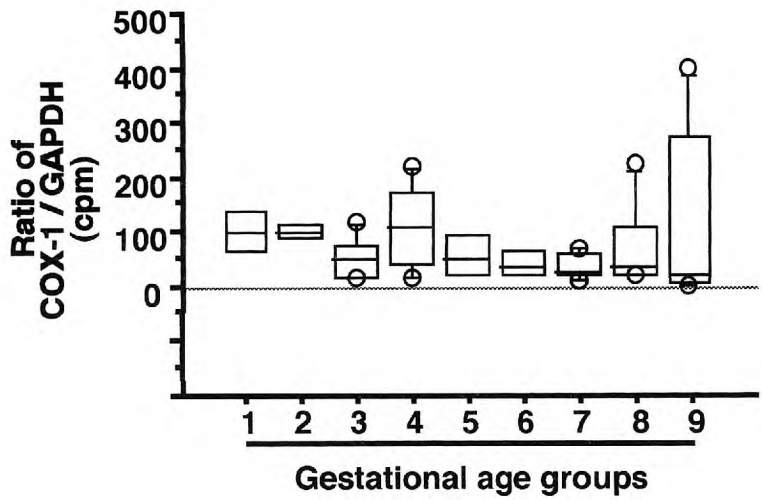


Fig. 4.38: COX-1 mRNA expression in amnion samples related to gestational age. Gestational age groups are: 1: 6 - 8 weeks (n = 4), 2: 9 - 10 weeks (n = 4), 3: 11 - 12 weeks (n = 6), 4: 13 - 15 weeks (n = 8), 5: 16 - 19 weeks (n = 4), 6: 20 - 28 weeks (n = 4), 7: 29 - 34 weeks (n = 6), 8: 35 - 37 weeks (n = 6), 9: 38 - 40 weeks (prior to labour) (n = 6). Box plots show means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample.

COX-1 mRNA expression was identified in amnion samples collected throughout gestation. When comparing COX-1 expression between different gestational age groups there was no statistically significant difference in expression in relation to gestational age.

COX-1 mRNA expression in chorion decidua is similarly shown in samples collected throughout gestation (Fig. 4.39).

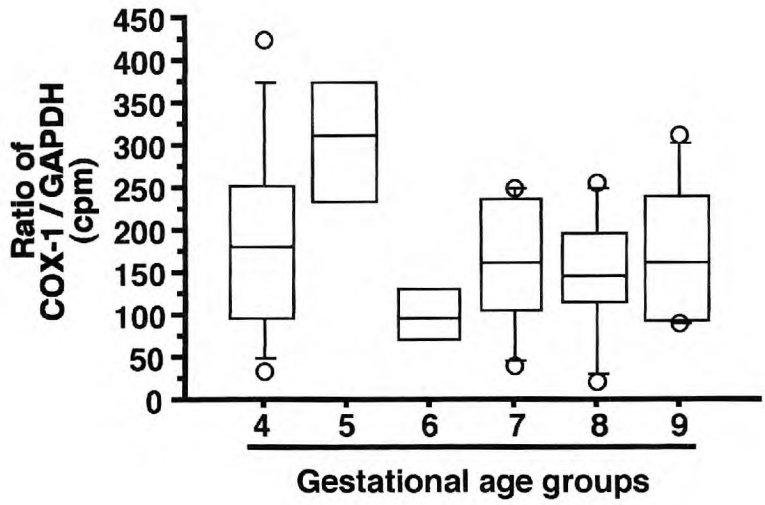


Fig. 4.39: COX-1 mRNA expression in chorion decidua samples related to gestational age. Gestational age groups are: 4: 13 - 15 weeks (n = 8), 5: 16 - 19 weeks (n = 4), 6: 20 - 28 weeks (n = 4), 7: 29 - 34 weeks (n = 6), 8: 35 - 37 weeks (n = 6), 9: 38 - 40 weeks (prior to labour) (n = 6). Box plots show means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample.

COX-1 mRNA expression was demonstrated in all samples collected throughout gestation. Due to limits in tissue collection, samples between 6 to 13 weeks gestation (Groups 1 - 3) were not available for testing. Whilst there is a statistically significant rise in COX-1 mRNA expression in group 5 (compared with groups 6, 7, 8 or 9) this does not appear to represent an association with gestational age changes, as there were no statistically significant differences in COX-1 mRNA expression between any of the other groups tested.

COX-2 mRNA expression in amnion throughout gestation is shown in Fig. 4.40:

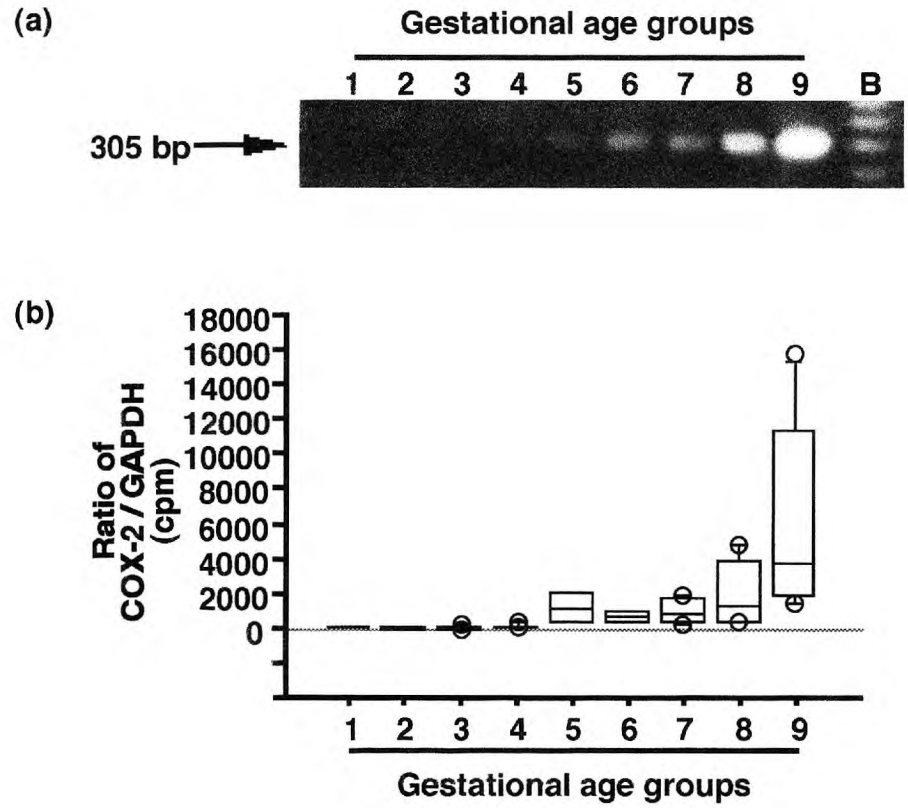


Fig. 4.40: COX-2 mRNA expression in amnion samples related to gestational age. The top panel (**a**) shows COX-2 mRNA expression by RT-PCR at 31 cycles, for representative amnion samples, throughout gestation. B - Blank lane, no DNA template. The bottom panel (**b**) show COX-2 mRNA expression as a ratio to GAPDH expression. Gestational age groups are: 1: 6 - 8 weeks (n = 4), 2: 9 - 10 weeks (n = 4), 3: 11 - 12 weeks (n = 6), 4: 13 - 15 weeks (n = 8), 5: 16 - 19 weeks (n = 4), 6: 20 - 28 weeks (n = 4), 7: 29 - 34 weeks (n = 6), 8: 35 - 37 weeks (n = 6), 9: 38 - 40 weeks (prior to labour) (n = 6). Box plots show means $\pm$ 25th centiles, and sample range. ○ denotes an extreme outlying sample.

COX-2 mRNA expression in amnion was undetectable (by RT-PCR) until the second trimester, when levels increased towards term. COX-2 mRNA expression at 38 - 40 weeks (Group 9) was significantly increased, compared to all other groups ($p \leq 0.01$). There was a statistically significant rise in expression between 35 -37 weeks and 38 - 40 weeks (prior to labour) ($p < 0.01$).

COX-2 mRNA expression in chorion decidua is similarly shown in Fig. 4.41:

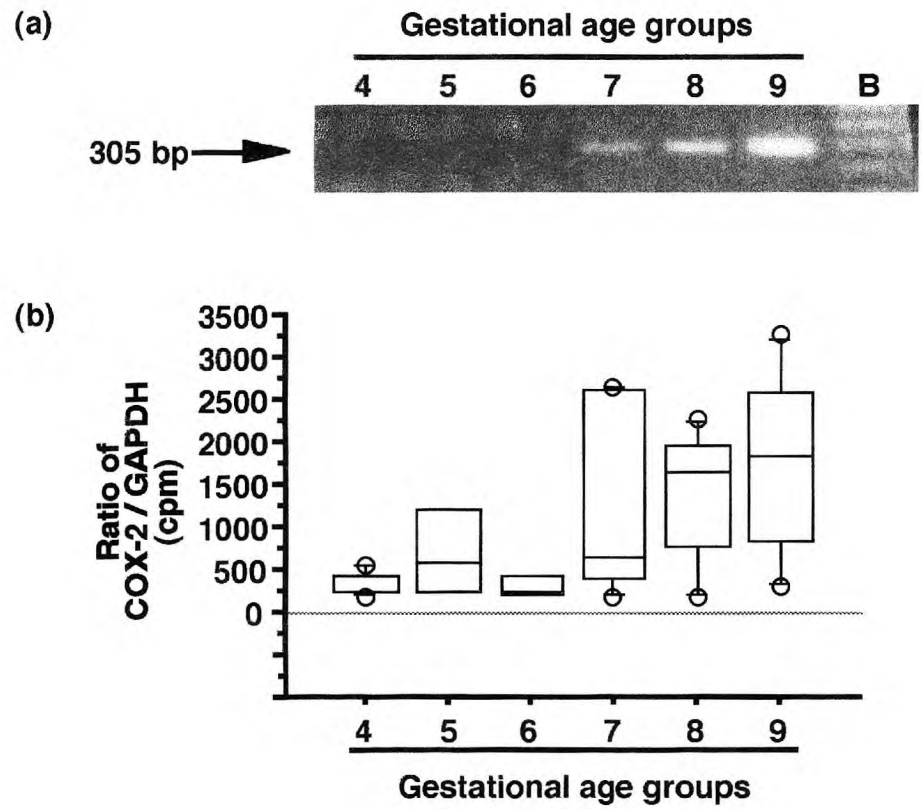


Fig. 4.41: COX-2 mRNA expression in chorion decidua samples related to gestational age. The top panel (**a**) shows COX-2 mRNA expression by RT-PCR at 33 cycles, for representative chorion decidua samples, throughout gestation. B - Blank lane, no DNA template. The bottom panel (**b**) show COX-2 mRNA expression as a ratio to GAPDH expression. Gestational age groups are: 4: 13 - 15 weeks (n = 8), 5: 16 - 19 weeks (n = 4), 6: 20 - 28 weeks (n = 4), 7: 29 - 34 weeks (n = 6), 8: 35 - 37 weeks (n = 6), 9: 38 - 40 weeks (prior to labour) (n = 6). Box plots show means $\pm$ 25th centiles, and sample range. ○ denotes an extreme outlying sample.

COX-2 mRNA expression in chorion decidua was low until the third trimester, when levels increased towards term. COX-2 mRNA expression at 35 - 37 weeks was significantly increased, compared to 13 - 15 weeks ($p = 0.02$) and 20 - 28 weeks ($p < 0.01$). COX-2 expression at 38 - 40 weeks was significantly increased compared to all gestational age groups below 29 weeks ($p \leq 0.04$) (Groups 4, 5 and 6).

Compared to COX-1 mRNA, COX-2 mRNA was expressed at higher levels in both amnion and chorion decidua, throughout pregnancy as indicated by optimal cycle number (COX-1 amnion and chorion decidua: 35, COX-2 amnion: 31 and COX-2 chorion decidua 33).

Myometrium

COX expression in myometrial tissue was examined using the same samples as those used to study NOS expression in myometrium. Samples were collected from subjects after labour onset at term, (term labour, n = 5), before labour at term, (term non-labour, n = 11), and before preterm labour (preterm non-labour, n = 13). The term patients' gestational age range was 38 - 40 weeks, in the preterm non-labour group the gestational age range was 26 - 37 weeks. In the term labour group, all patients had regular painful contractions with associated cervical dilatation. The indications for caesarean section in the term labour group were not associated with uterine dysfunction. The non-labour group had no evidence of uterine contractions or cervical change. Expression of both the COX-1 and COX-2 genes were investigated.

COX mRNA expression in relation to labour onset

The relationship of COX expression with labour onset in myometrium was investigated by analysing mRNA expression in individual samples, by RT-PCR.

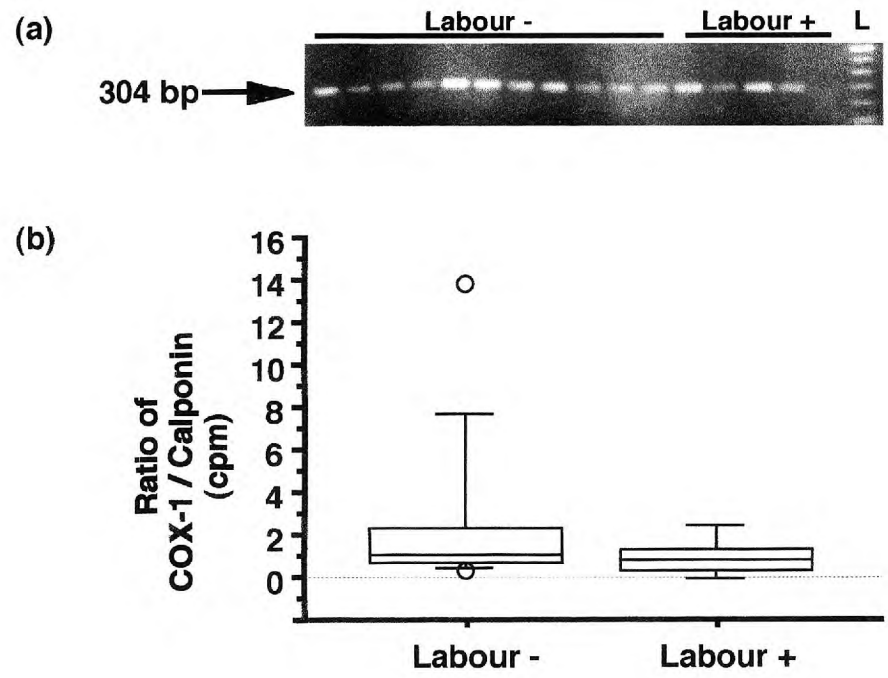


Fig. 4.42: COX-1 mRNA expression in relation to labour, in term myometrium. (**a**) RT-PCR with COX-1 primers at 39 cycles on DNA isolated from term non-labour (L-, n = 11) and term labour (L+, n = 5) myometrial samples. L: 100 bp DNA marker. Correct product size is 304 base pairs. (**b**) Box plots of the ratio of COX-1 expression (as cpm) to calponin. Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in iNOS expression before or after labour onset ($p = 0.39$).

The optimal cycle number for each gene was derived from cycle profiles. The optimal cycle number determined for pooled myometrium for this reverse transcriptase reaction, was 31 for both COX-1 and COX-2, and 32 for calponin. COX-1 expression in myometrium before and after labour is shown in Fig. 4.42.

There was no significant difference in levels of COX-1 mRNA determined by RT-PCR, expressed as a ratio to calponin expression, before or after the onset of labour (p = 0.39). COX-2 expression in myometrium before and after labour is shown in Fig. 4.43:

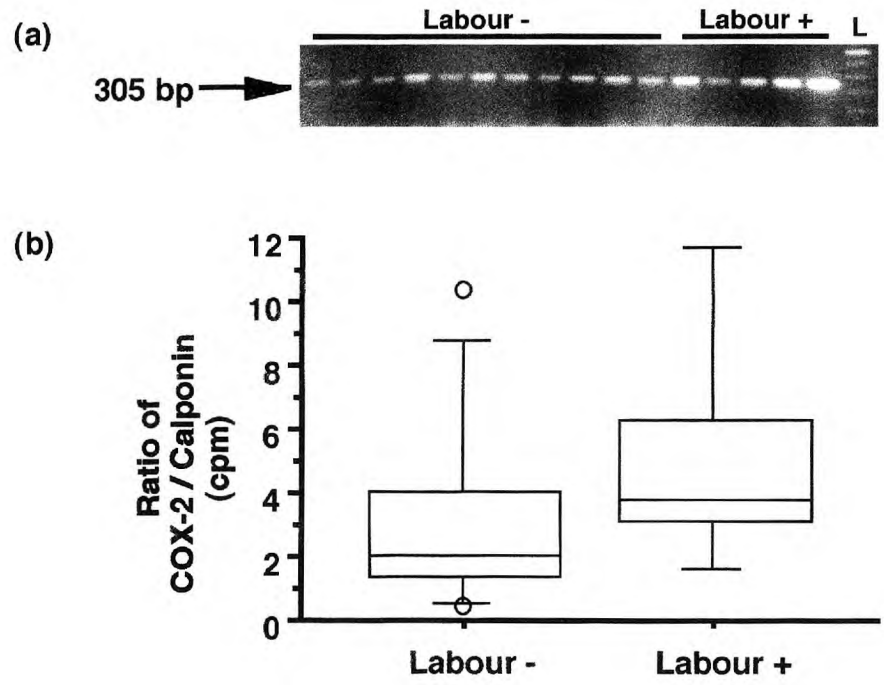


Fig. 4.43: COX-2 mRNA expression in relation to labour, in term myometrium. (**a**) RT-PCR with COX-2 primers at 39 cycles on DNA isolated from term non-labour (L-, n = 11) and term labour (L+, n = 5) myometrial samples. L: 100 bp DNA marker. Correct product size is 305 base pairs. (**b**) Box plots of the ratio of COX-1 expression (as cpm) to calponin. Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in iNOS expression before or after labour onset (p = 0.34).

When levels of COX-2 expression in myometrium are quoted as a ratio to calponin, and compared before and after the onset of labour at term there is no statistically significant difference in levels of expression (p = 0.34).

COX expression in myometrium is expressed as a ratio to calponin rather than GAPDH (as in the fetal membranes) with the intention of normalising for smooth muscle cell content. Calponin expression in term myometrium is shown in Fig. 4.21 (see section on NOS).

COX mRNA expression in relation to gestational age

The relationship of COX expression to gestational age was considered by analysing expression in myometrial samples collected throughout gestation. The preterm non-labour group of patients had a gestational age range from 26 - 37 weeks (n = 13). The indications for delivery in this group were associated with intra-uterine growth retardation and hypertensive disorders. There was no clinical evidence of preterm labour in this group.

COX-1 expression in myometrium throughout gestation relative to calponin expression is shown in Fig. 4.44:

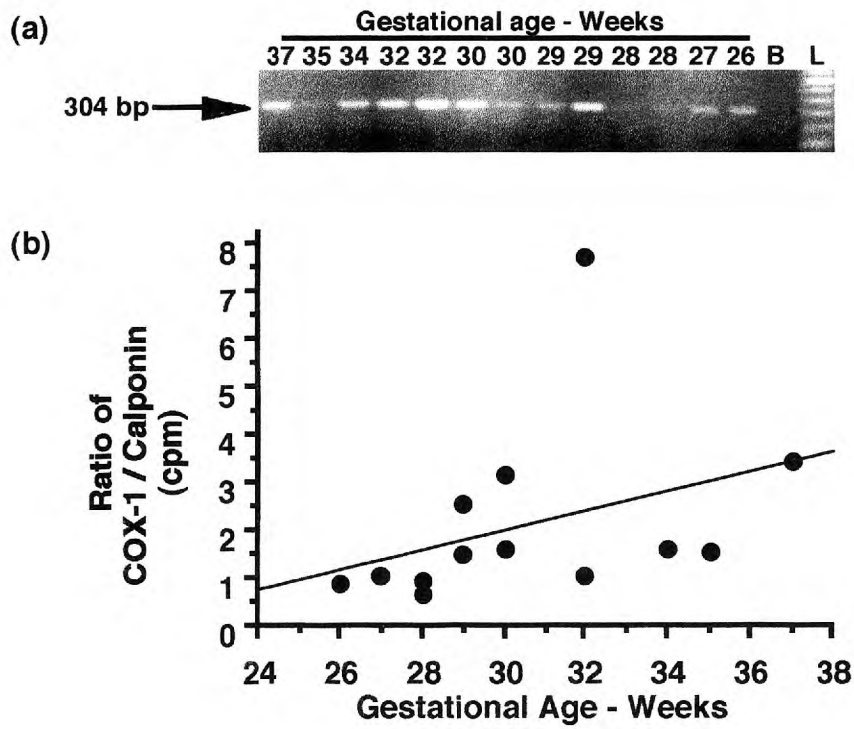


Fig. 4.44: COX-1 expression in individual myometrial samples related to gestational age. The top panel (**a**) shows COX-1 mRNA expression by RT-PCR at 39 cycles, for individual myometrial samples. B: blank lane - no starting template DNA and L: 100 bp DNA marker. Correct product size is 304 base pairs. The bottom panel (**b**) show regression of COX-1 expression on gestational age (as cpm), as a ratio to calponin expression. Simple regression analysis showed no significant correlation between COX-1 expression in myometrium and gestational age ($R = 0.36$, $p = 0.23$).

Linear regression analysis of COX expression (as a ratio to calponin) on gestational age, showed no significant association between expression COX-1 and gestational age (COX-1 expression versus gestational age, $R = 0.36$, $p = 0.23$.

COX-2 expression in myometrium throughout gestation, relative to calponin expression is shown in Fig. 4.45:

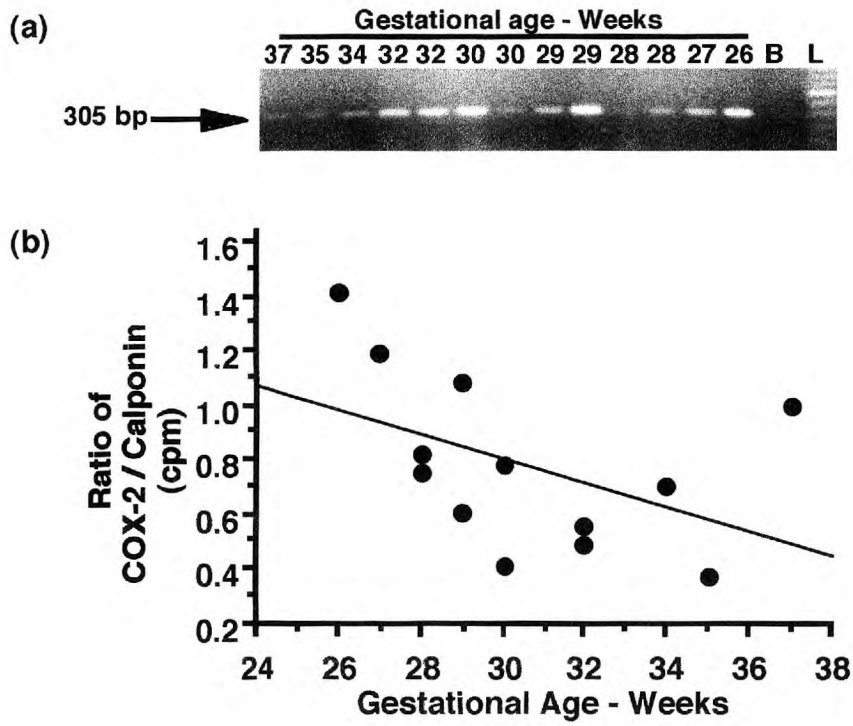


Fig. 4.45: COX-2 expression in individual myometrial samples related to gestational age. The top panel **(a)** shows COX-2 mRNA expression by RT-PCR at 39 cycles, for individual myometrial samples. B: blank lane - no starting template DNA and L: 100 bp DNA marker. Correct product size is 305 base pairs. The bottom panel **(b)** show regression of COX-2 expression on gestational age (as cpm), as a ratio to calponin expression. Simple regression analysis showed no significant correlation between COX-expression in myometrium and gestational age ($R = 0.47$, $p = 0.10$).

Linear regression analysis of COX expression (as a ratio to calponin) on gestational age, showed no significant association between expression of COX-2 and gestational age ($R = 0.47$, $p = 0.10$).

Calponin expression in myometrium throughout gestation is shown in Fig. 4.25 (see NOS section).

However if COX expression throughout gestation is compared to both expression at term and in association with labour a different trend is observed. The samples for further analysis, were divided into four separate groups on the basis of their gestational age and labour status. Groups were preterm samples, between 26 - 32 weeks gestation (n = 8), an intermediate group between 33 - 37 weeks (n = 5), term non-labour samples (n = 11) and term labour samples (n = 5).

COX-1 expression throughout gestation and at term, on the basis of these groupings is shown in Fig. 4.46:

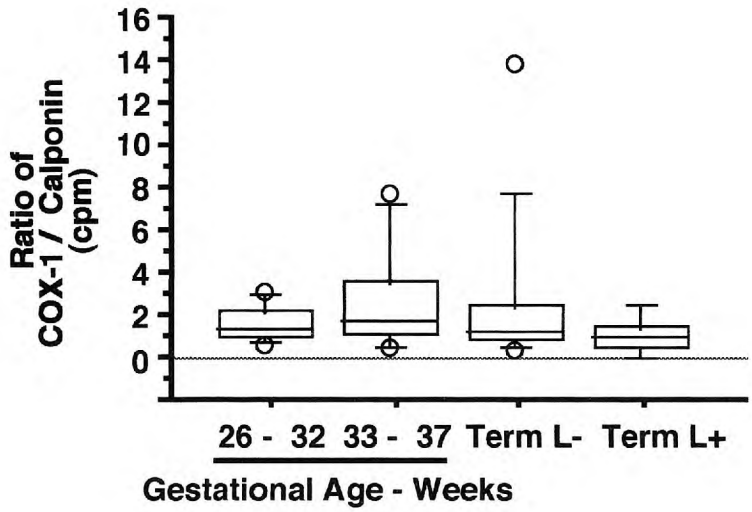


Fig. 4.46: COX-1 mRNA expression in myometrium, throughout gestation and at term. Groups are preterm samples, between 26 - 32 weeks gestation (n = 8), preterm samples between 33 - 37 weeks (n = 5), term non-labour samples, Term L-, (n = 11) and term labour samples Term L+, (n = 5). Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample.

Comparison of expression between these groups showed no significant difference for COX-1 expression (26 - 32 weeks gestation versus 33 - 37 weeks gestation ($p = 0.46$), 26 - 32 weeks gestation versus term non-labour ($p = 0.42$), 26 - 32 weeks gestation versus term labour ($p = 0.73$), 33 - 37 weeks gestation versus term non-labour ($p = 0.97$), 33 - 37 weeks gestation versus term labour ($p = 0.33$), and term non-labour versus term labour ($p = 0.38$).

COX-2 expression throughout gestation and at term, on the basis of these groupings is shown in Fig. 4.47:

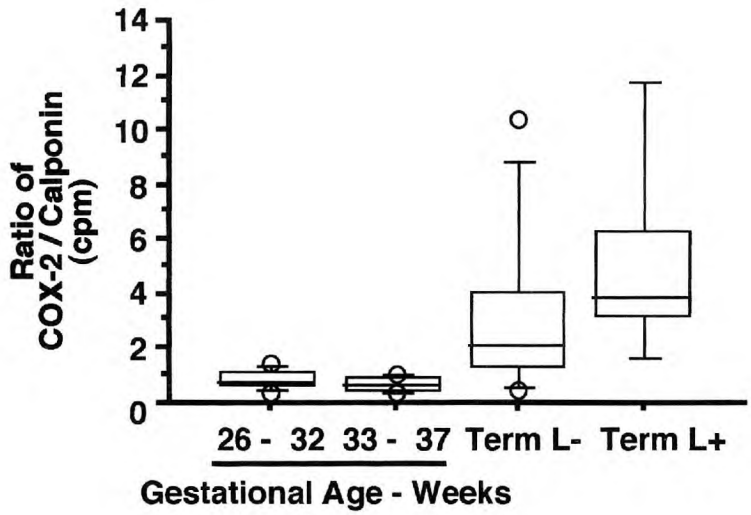


Fig. 4.47: COX-2 mRNA expression in myometrium, throughout gestation and at term. Groups are preterm samples, between 26 - 32 weeks gestation (n = 8), preterm samples between 33 - 37 weeks (n = 5), term non-labour samples, Term L-, (n = 11) and term labour samples Term L+, (n = 5). Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample.

Comparison of COX-2 expression between both of the preterm groups and the two term groups showed a highly statistically significant increase in COX-2 mRNA expression (26 - 32 weeks gestation versus 33 - 37 weeks gestation ($p = 0.88$), 26 - 32 weeks gestation versus term non-labour ($p = 0.05$), 26 - 32 weeks gestation versus term labour ($p < 0.01$), 33 - 37 weeks gestation versus term non-labour ($p = 0.05$), 33 - 37 weeks gestation versus term labour ($p < 0.01$), and term non-labour versus term labour ($p = 0.34$). These data demonstrate that COX-2 mRNA expression increases significantly at term, prior to labour onset, and increases further (though not significantly) with the onset of labour.

4.3.2 Western analysis of COX expression

Western analysis of COX protein in fetal membranes

COX protein expression in fetal membranes was investigated by Western analysis. Representative fetal membrane samples were collected from patients in the first trimester (10 - 14 weeks), at term prior to labour (38 - 40 weeks) and at term, after labour (38 - 40 weeks) (n = 4 for each group). Western analysis showed COX-1 protein at 70 Kda. Representative COX-1 protein expression in amnion is shown in Fig. 4.48:

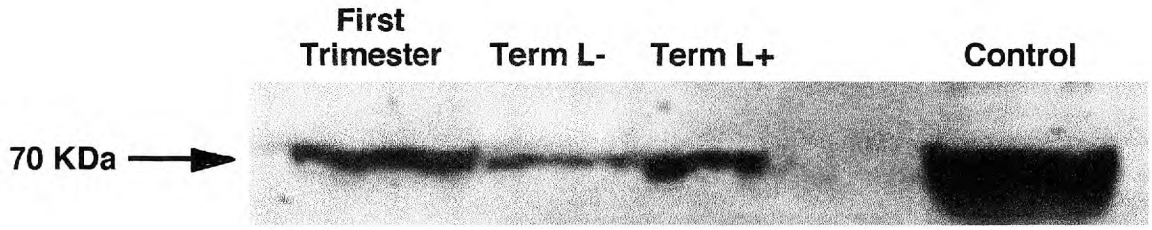


Fig. 4.48: COX-1 protein expression in amnion, in relation to both gestational age and labour. Western analysis of COX-1 protein. Representative samples of first trimester, term non-labour (Term L-) and term labour (Term L+) amnion samples. Control: COX-1 positive control (human platelets). Correct protein size is 70 KDa. There was no apparent difference in COX-1 protein expression in amnion samples in relation to either gestational age or labour.

COX-1 protein was demonstrated in amnion samples collected throughout pregnancy. Confirmation of correct protein size was made using standard protein size markers. Antibody specificity was confirmed by the presence of identical bands in the positive control. Comparison of relative COX-1 protein expression was made between samples collected in the first trimester and before and after labour onset. Protein expression was determined by measuring mean densitometry of each protein band. There was no apparent change in COX-1 protein expression in amnion between the first trimester and term, or before and after the onset of labour at term.

COX-1 protein expression in chorion decidua is shown in representative samples in Fig. 4.49:

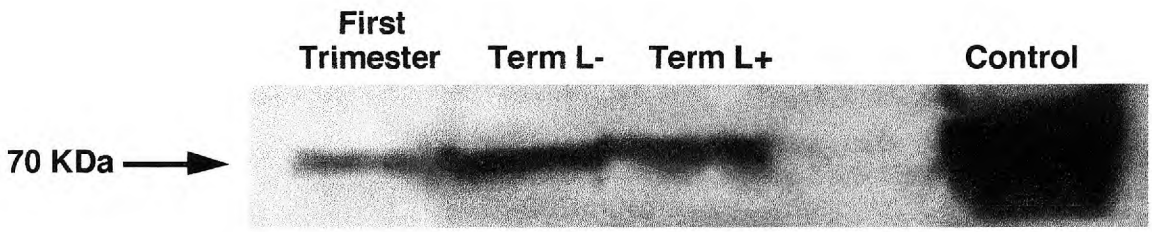


Fig. 4.49: COX-1 protein expression in chorion decidua, in relation to both gestational age and labour. Western analysis of COX-1 protein. Representative samples of first trimester, term non-labour (Term L-) and term labour (Term L+) chorion decidua samples. Control: COX-1 positive control (human platelets). Correct protein size is 70 KDa. There was no apparent difference in COX-1 protein expression in chorion decidua samples in relation to either gestational age or labour.

COX-1 protein was demonstrated in chorion decidua samples collected throughout pregnancy. There was no apparent change in COX-1 protein expression in chorion decidua between the first trimester and term, or before and after the onset of labour at term.

COX-2 protein expression in amnion is shown in representative samples in Fig. 4.50:

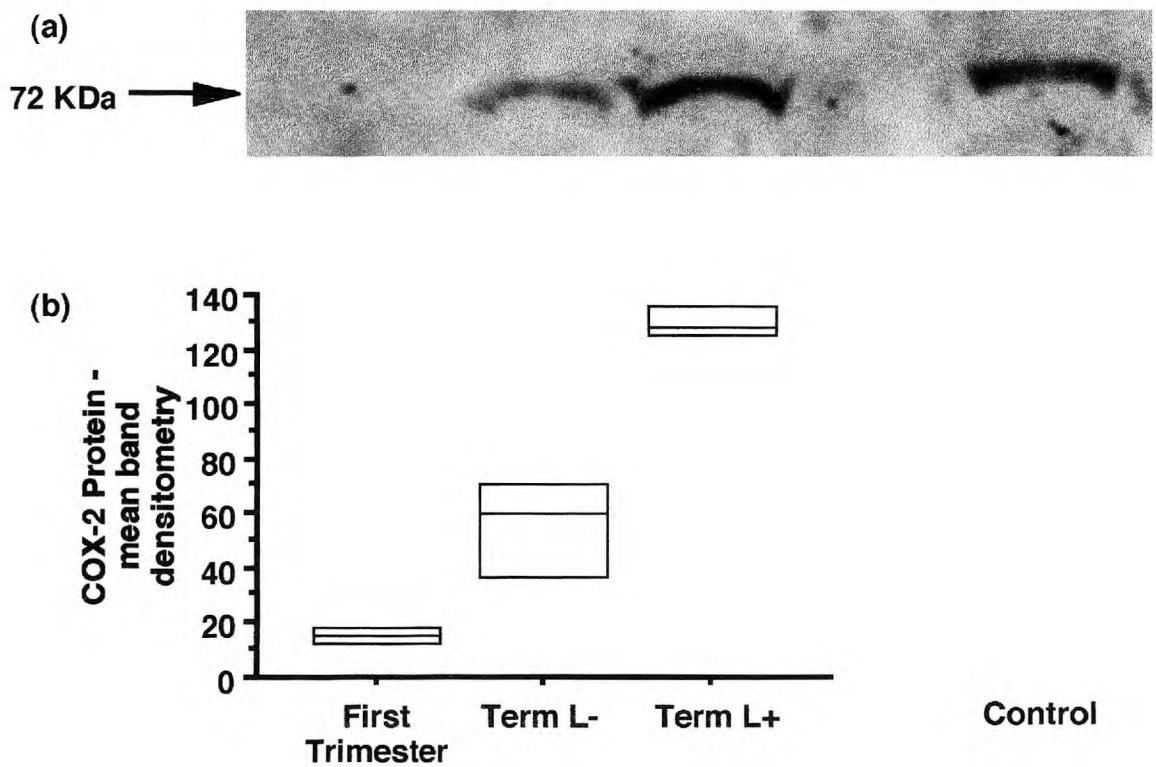


Fig. 4.50: COX-2 protein expression in amnion, in relation to both gestational age and labour. **(a)** Western analysis of COX-2 protein. Representative samples of first trimester, term non-labour (Term L-, n = 4) and term labour (Term L+, n = 4) amnion samples. Control: COX-2 positive control (LPS-stimulated human peripheral lymphocytes). Correct protein size is 72 KDa. **(b)** Box plot of COX- 2 protein (as a function of Western protein band densitometry), n = 4 for each group. Data shows means ± 25th centiles. There was a statistically significant increase in COX-2 protein expression in amnion samples when compared between first trimester and Term L- ($p = 0.05$) and Term L+ ($p < 0.01$), and with the onset of labour (Term L- versus Term L+) ($p < 0.01$).

COX-2 protein was demonstrated in amnion samples collected throughout pregnancy. COX-2 protein levels in the first trimester were almost undetectable. Levels of COX-2 protein increased significantly between samples collected in the first trimester and at term, before the onset of labour (Term L-) ($p = 0.05$). There was also a statistically significant

increase in COX-2 protein levels between first trimester samples and at term, after labour onset (Term L+) ($p < 0.01$). Levels of COX-2 protein expression further increased at term, and with the onset of labour (Term L- versus Term L+) ($p < 0.01$).

COX-2 protein expression in chorion decidua is shown in representative samples in Fig. 4.51:

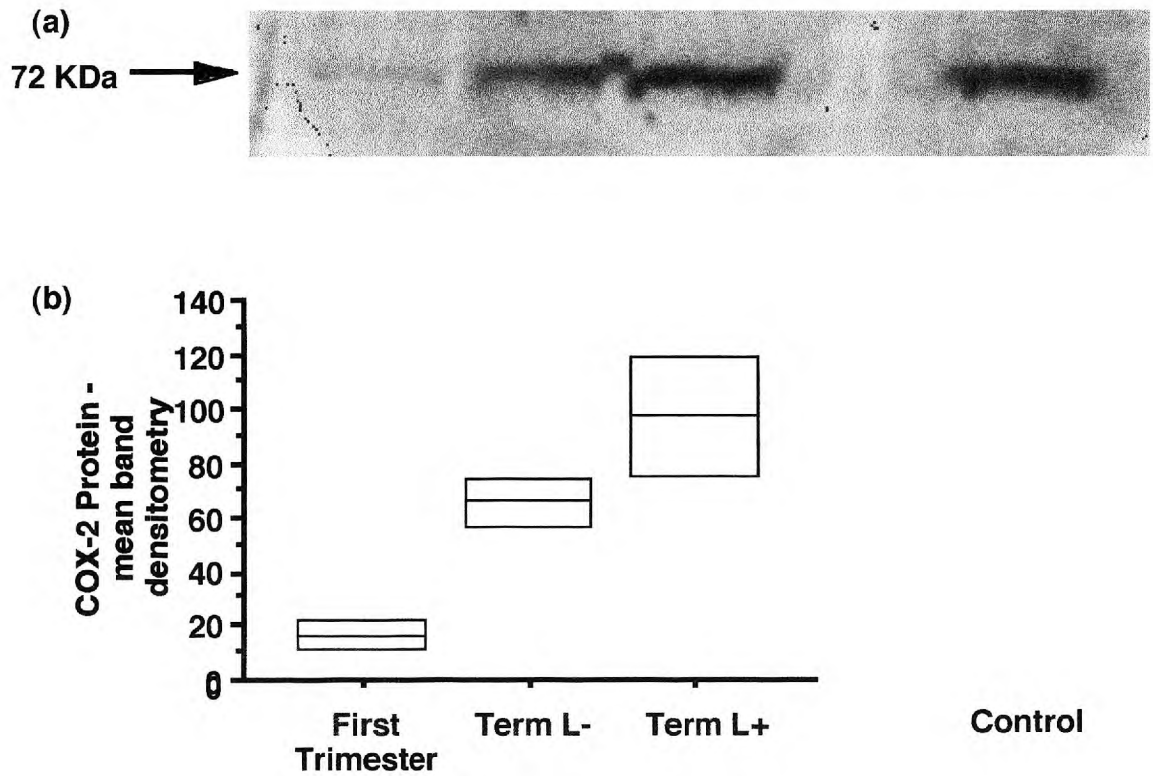


Fig. 4.51: COX-2 protein expression in chorion decidua, in relation to both gestational age and labour. (**a**) Western analysis of COX-2 protein. Representative samples of first trimester, term non-labour (Term L-, n = 4) and term labour (Term L+, n = 4) chorion decidua samples. Control: COX-2 positive control (LPS-stimulated human peripheral lymphocytes). Correct protein size is 72 KDa. (**b**) Box plots of COX- 2 protein (as a function of Western protein band densitometry), n = 4 for each group. Data shows means ± 25th centiles. There was a statistically significant increase in COX-2 protein expression in chorion decidua samples when compared between first trimester and Term L- ($p < 0.01$) and Term L+ ($p < 0.01$), and with the onset of labour (Term L- versus Term L+) ($p = 0.03$).

COX-2 protein was demonstrated in chorion decidua samples collected throughout pregnancy. In chorion decidua COX-2 protein concentration increased significantly between the first trimester and term (Term L-) ($p < 0.01$). There was also a statistically significant increase in COX-2 protein levels between first trimester samples and at term,

after labour onset (Term L+) ($p < 0.01$).There was a significant difference in COX-2 protein expression between chorion decidua collected before and after labour (Term L- versus Term L+) ($p < 0.03$).

Western analysis of COX protein in myometrium

COX protein expression in myometrium in relation to labour onset

COX protein expression was investigated in myometrium by Western analysis. Protein samples were obtained from identical myometrial samples from which RNA was extracted, for RT-PCR analysis. In this way a direct comparison could be made between RNA and protein levels in the same myometrial samples.

COX-1 protein expression in myometrial tissue before and after the onset of labour is shown in Fig. 4.52:

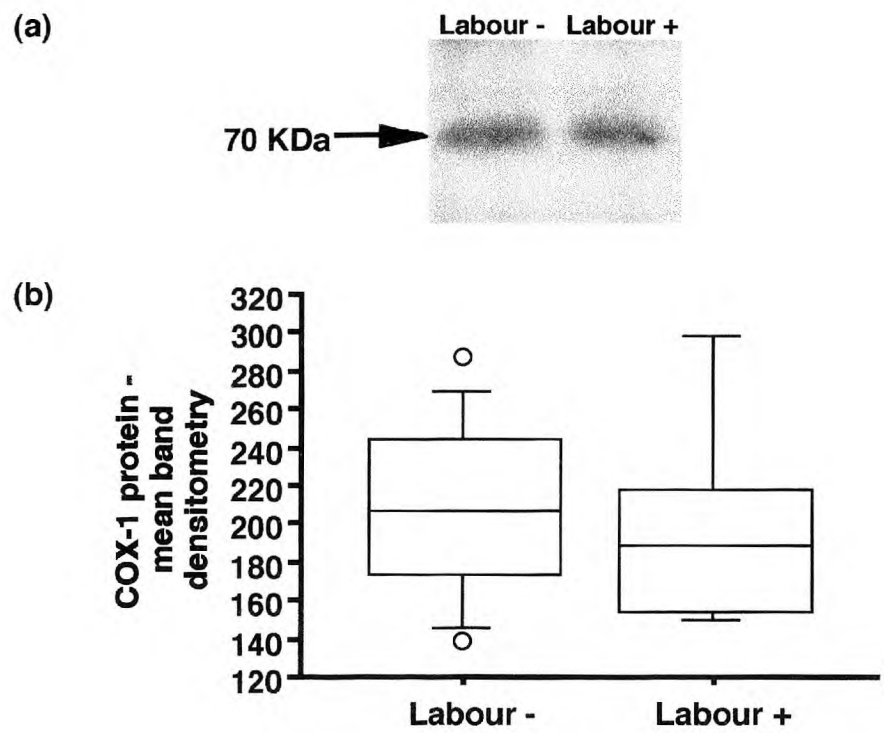


Fig. 4.52: COX-1 protein expression in relation to labour, in term myometrium. (**a**) Western analysis of COX-1 protein. Representative samples of term non-labour (L-, n = 11) and term labour (L+, n = 5) myometrial samples. Correct protein size is 70 KDa. (**b**) Box plots of the ratio of COX-1 protein (as a function of Western protein band densitometry). Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in COX-1 protein expression before or after labour onset ($p = 0.72$).

There was no significant difference in levels of COX-1 protein expression before and after the onset of labour ($p = 0.72$).

COX-2 protein expression in term myometrium before and after labour is shown in Fig. 4.53:

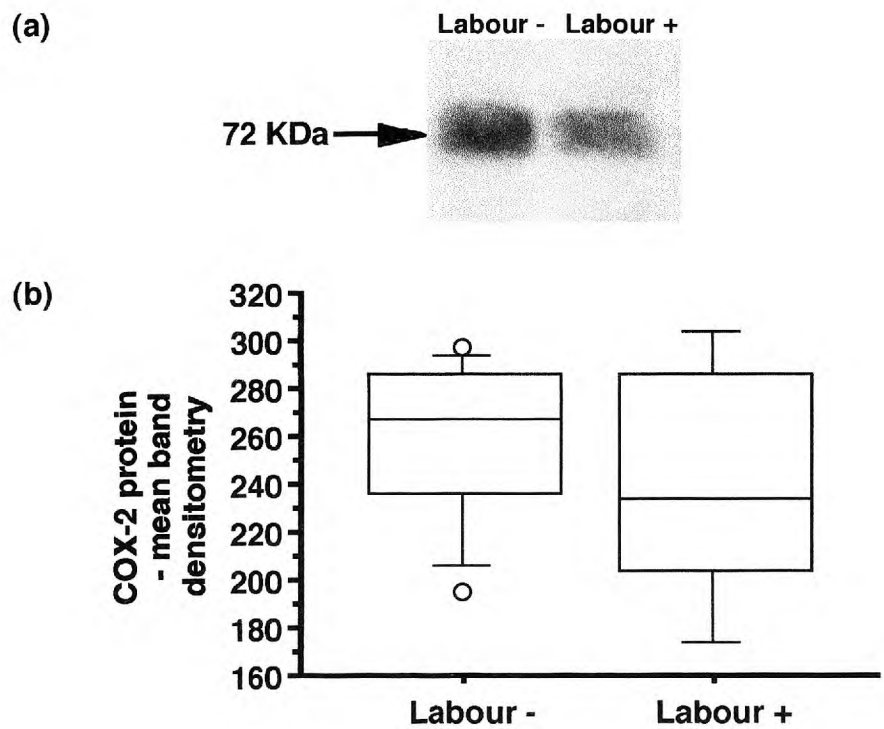


Fig. 4.53: COX-2 protein expression in relation to labour, in term myometrium. (**a**) Western analysis of COX-2 protein. Representative samples of term non-labour (L-, n = 11) and term labour (L+, n = 5) myometrial samples. Correct protein size is 72 KDa. (**b**) Box plots of the ratio of COX-2 protein (as a function of Western protein band densitometry). Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample. There was no statistically significant difference in COX-2 protein expression before or after labour onset ($p = 0.44$).

COX-2 protein expression in myometrium, when compared before and after the onset of labour at term, shows no significant change in expression ($p = 0.44$).

COX protein expression in myometrium in relation to gestational age

COX-1 protein expression, analysed by Western analysis, in myometrial tissue throughout gestation is shown in Fig. 4.54:

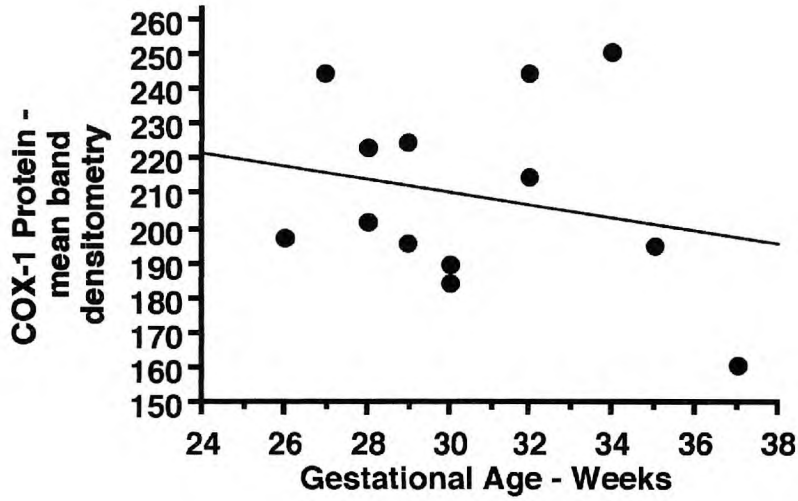


Fig. 4.54: COX-1 protein expression in individual myometrial samples related to gestational age. The top panel **(a)** shows COX-2 protein expression by Western analysis, for individual myometrial samples. Correct product size is 70 KDa. The bottom panel **(b)** show regression of COX-1 protein on gestational age (as a function of Western protein band densitometry). Simple regression analysis showed no significant correlation between COX-1 protein in myometrium and gestational age ($R = 0.22$, $p = 0.46$).

Gestational age had no impact on levels of expression of COX-1 protein in myometrium ($R = 0.22$, $p = 0.46$). COX-2 protein expression in myometrial tissue throughout gestation, demonstrated by Western analysis is shown in Fig. 4.55:

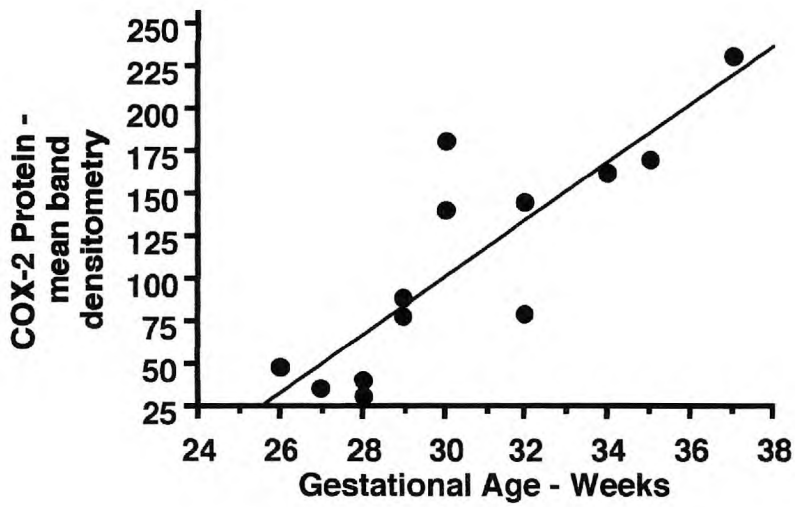


Fig. 4.55: COX-2 protein expression in individual myometrial samples related to gestational age. The top panel **(a)** shows COX-2 protein expression by Western analysis, for individual myometrial samples. Correct product size is 72 KDa. The bottom panel **(b)** show regression of COX-2 protein on gestational age (as a function of Western protein band densitometry). Simple regression analysis showed significant correlation between COX-2 protein in myometrium and gestational age ($R = 0.85$, $p < 0.01$).

Fig. 4.55 shows that COX-2 protein, in myometrial tissue, increases significantly with gestational age. Simple regression analysis of COX-2 protein in myometrium, on gestational age is highly statistically significant ($R = 0.85$, $p < 0.01$).

If the protein data is analysed in a similar manner to the results generated from RT-PCR, then comparison can be made between different gestational and parturitional groups. COX-1 protein expression, throughout gestation and in relation to labour onset is shown by groups, in Fig. 4.56:

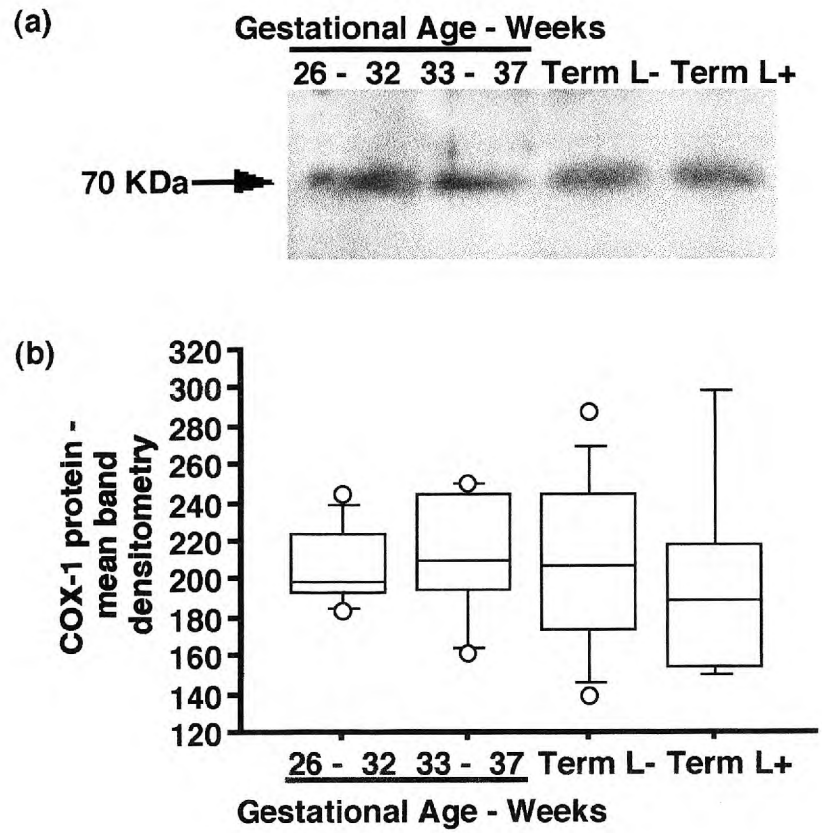


Fig. 4.56: COX-1 protein expression in myometrium throughout gestation and at term. (**a**) Western analysis of COX-1 protein. Representative samples of 26 - 32 weeks gestation (n = 8), preterm samples between 33 - 37 weeks (n = 5), term non-labour samples, Term L-, (n = 11) and term labour samples Term L+, (n = 5). Correct protein size is 70 KDa. (**b**) Box plots of COX-1 protein (as a function of Western protein band densitometry) divided by groups. Data shows means ± 25th centiles, and sample range. ○ denotes an extreme outlying sample.

Analysis of COX-1 protein by groups showed no significant change in levels of protein expression throughout gestation, and in association with labour (26 - 32 weeks gestation versus 33 - 37 weeks gestation ($p = 0.81$), 26 - 32 weeks gestation versus term non-labour ($p = 0.99$), 26 - 32 weeks gestation versus term labour ($p = 0.67$), 33 - 37 weeks gestation versus term non-labour ($p = 0.80$), 33 - 37 weeks gestation versus term labour ($p = 0.56$), and term non-labour versus term labour ($p = 0.72$)). In contrast a significant increase in COX-2 protein expression is shown, both with increasing gestational age and labour onset (Fig. 4.57).

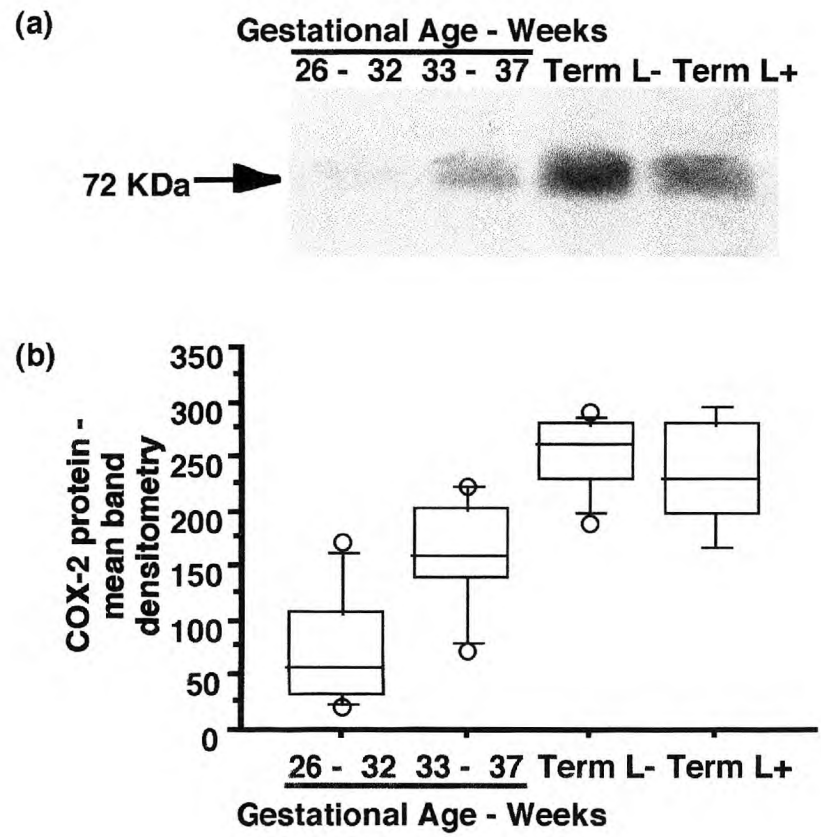


Fig. 4.57: COX-2 protein expression in myometrium throughout gestation and at term. (a) Western analysis of COX-2 protein. Representative samples of 26 - 32 weeks gestation (n = 8), preterm samples between 33 - 37 weeks (n = 5), term non-labour samples, Term L-, (n = 11) and term labour samples Term L+, (n = 5). Correct protein size is 72 KDa. (b) Box plots of COX-2 protein (as a function of Western protein band densitometry) divided by groups. Data shows means + 25th centiles, and sample range. ○ denotes an extreme outlying sample.

COX-2 protein expression increases significantly at 33 - 37 weeks, with respect to early in gestation (26 - 32 weeks) ($p < 0.01$), and increases further and significantly with respect to

early in gestation (26 - 32 weeks) both at term non-labour ($p < 0.01$) and term labour ($p < 0.01$). In addition protein levels show a further, significant increase with respect to mid-pregnancy levels (33 - 37 weeks) both at term non-labour ($p < 0.01$) and term labour ($p < 0.01$). There was no significant difference in levels of COX-2 protein expression before or after the onset of labour at term ($p = 0.44$).

4.3.3 COX enzyme activity in fetal membranes

Smieja et al. (Smieja et al., 1993), have shown using enzyme kinetics studies, that in amnion the increase in COX activity with the onset of labour is due entirely to an increase in the synthesis of the COX enzyme. The relative contribution of amnion-derived COX activity to COX activity in fetal membranes as a whole, was determined by analysing COX activity in the separate components of the fetal membranes, both throughout gestation and in relation to labour onset.

COX activity was determined using a fetal membrane disk system (see section 2.2.16). Fetal membranes were collected throughout gestation and at term, and either separated into amnion and chorion decidua or left intact. Membrane discs were cut and placed in medium. Prostaglandin synthesis was determined by measuring PGE_2 by RIA in the medium, after a incubation period of 15 min. This was designated the background PGE_2 synthesis and felt to represent basal prostaglandin synthesis. Discs were subsequently incubated in medium containing arachidonic acid, the substrate for the COX enzyme, and the medium removed after 15 min, and assayed for PGE_2. The relative COX activity was considered as PGE_2 generated during incubation in the presence of arachidonic acid, minus background PGE_2 synthesis. Whilst discs were of the same diameter, a difference in the thickness between the amnion and chorion decidua resulted in a higher cellular content in the chorion decidua discs. COX activity was therefore normalised for protein concentration, by calculating PGE_2 synthesis per mg of total protein.

Relative COX activity was determined in membranes collected throughout gestation (n = 8) (Table 4.02), and at term before (Labour - : n = 6) and after (Labour + : n = 6) the onset of labour. Each membrane provided four discs

Table 4.02: COX assays: Gestational age and labour status of fetal membrane samples

Trimester	Gestation - Weeks	Labour Status
First	8	L-
	8	L-
	9	L-
	10	L-
	10	L-
Second	14	L-
	21	L-
Third	29	L-

Membranes were either separated into component amnion and chorion decidua or left intact. Four discs for each group were obtained from each fetal membrane sample, and incubated in twelve well plates. The purpose of analysing COX activity in intact (i.e. non-separated) fetal membranes was to study the effect, if any, of prostaglandin dehyrogenase on PGE_2 synthesis as a whole in fetal membranes. COX activity in amnion, chorion decidua and intact fetal membranes throughout gestation is shown in Fig. 4.58:

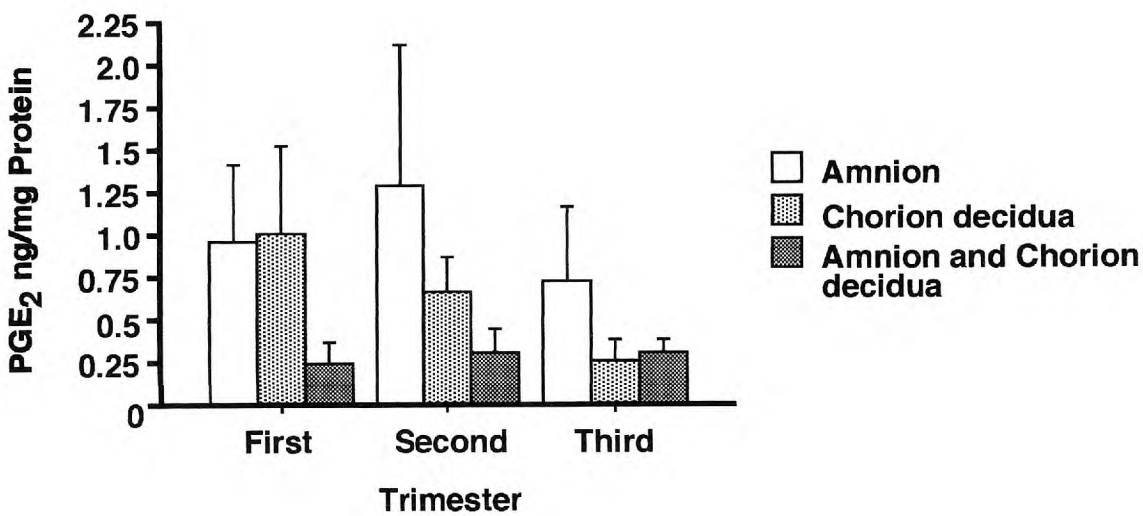


Fig. 4.58: COX activity in fetal membrane discs throughout gestation. PGE_2 was measured by RIA. Groups were: First Trimester (n = 5, 8 - 10 weeks), Second Trimester (n = 2, 14 - 21 weeks) and Third Trimester (n = 1, 29 weeks). Cell bar chart shows mean values $\pm$ 2 SEM.

COX activity in amnion remains unchanged throughout gestation. COX activity in chorion decidua falls with increasing gestational age, and this becomes statistically significant between the first and third trimesters (p = 0.01). COX activity in intact fetal membranes did not change with gestational age.

COX activity in relationship to labour was studied in term amnion, chorion decidua and intact membrane discs (Fig. 4.59).

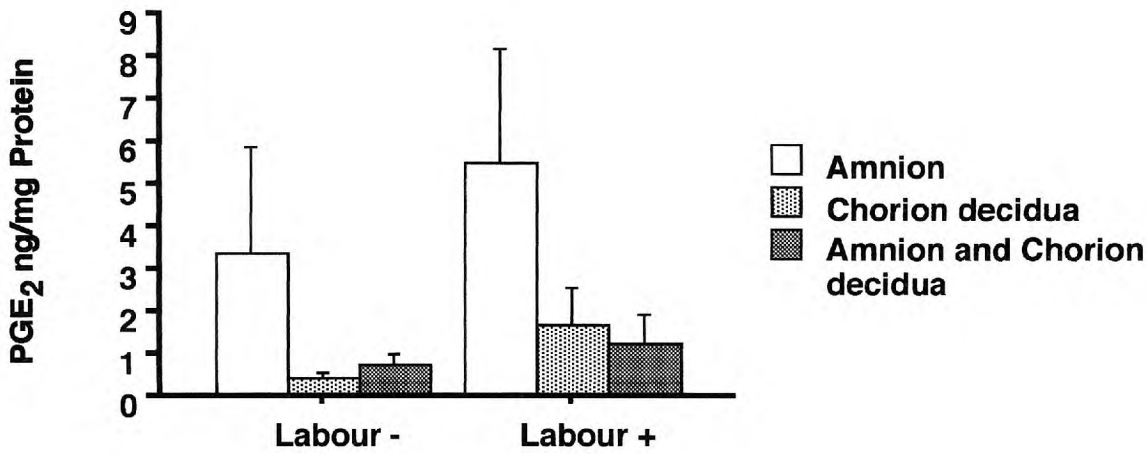


Fig. 4.59: COX activity in fetal membrane discs at term. PGE_2 was measured by RIA. Groups were: Labour - (n = 6, Term non-labour) and Labour + (n = 6, Term labour). Cell bar chart shows mean values ± 2 SEM.

COX activity in amnion increases with labour onset, but not significantly (p = 0.27). COX activity in chorion decidua increases significantly with labour onset (p = 0.01). COX activity in intact fetal membrane discs remains unchanged with labour onset (p = 0.13).

Combining the data from membranes collected both throughout gestation and at term, the relative contribution of the amnion to prostaglandin synthesis in labour can be clearly seen (Fig. 4.60).

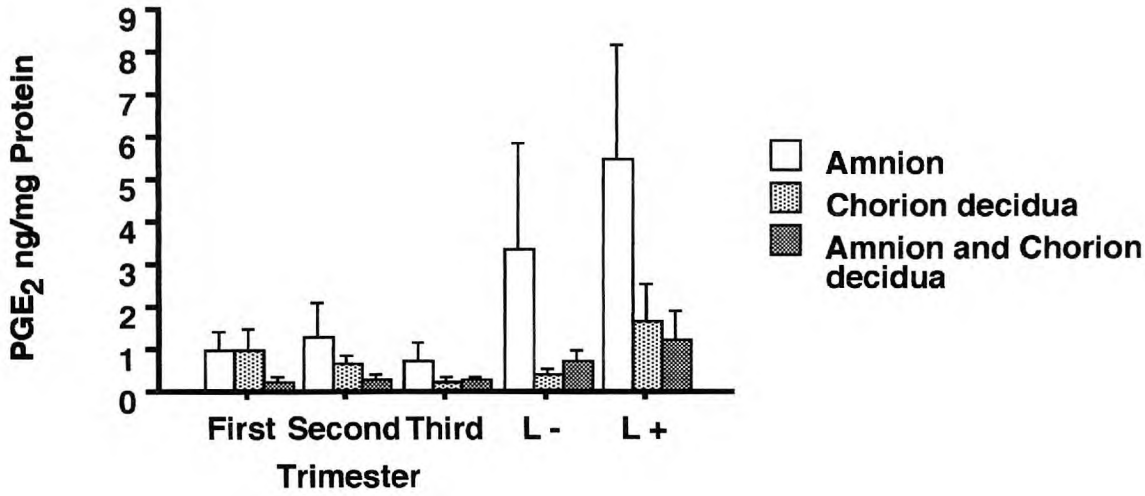


Fig. 4.60: COX activity in fetal membrane discs throughout gestation and at term. Cell bar chart shows mean values $\pm$ 2 SEM.

4.4 NOS regulation

4.4.1 Introduction

NOS has both constitutive (e/cNOS and nNOS) and inducible isoforms (iNOS). Whilst there is some evidence that the constitutive, calcium-dependent isoforms may be subject to transcriptional regulation by steroid hormones (Weiner et al., 1994b), expression of the inducible isoform is known to be subject to transcriptional regulation by cytokines (Ding et al., 1988). In these respects the NOS isoforms show close similarity with the COX isoforms. NOS and COX have both constitutive and inducible isoforms. The constitutive isoforms are widely distributed in many different cell types, and are largely responsible for the maintenance of normal physiological function. The inducible isoforms are considered early response genes and their expression may be widely induced. Analysis of the 5' promoter region of the iNOS gene shows putative response elements, shared with the COX-2 gene, for NF-kB and NF-IL-6 (Nunokawa et al., 1994).

iNOS expression in both fetal membranes and myometrium does not change significantly throughout gestation, or with the onset of labour. In contrast COX-2 expression in fetal membranes increases significantly with the onset of labour, and expression in myometrium increases throughout gestation, and further increases with the onset of labour. In models of inflammation cytokines induce both iNOS and COX-2 transcription, suggesting a mechanism for amplifying an inflammatory response. In contrast, in these gestational tissues, COX-2 is upregulated in the absence of any change in iNOS expression. The possibility therefore exists that in gestational tissues, and the uterus as a whole, cytokine induction of COX-2 may differ from that of iNOS. Potentially, then, a different regulatory mechanism may exist in these tissues.

The regulation of iNOS (and COX-2) mRNA and protein activity by cytokines was investigated in a gestational cell line. The choice of cell type was dictated by choosing an appropriate cell type that is capable of expressing both iNOS and COX-2, is subject to cytokine induction, and ideally has been immortalised.

WISH cells have been extensively investigated in our department, and provide a reliable model for studying gene regulation *in* vitro. The WISH cell line is an immortalised cell line derived from human amnion epithelial cells.

IL-1ß is an inflammatory cytokine whose mRNA and protein levels are significantly increased in decidua in association with labour (Ammala et al., 1997). IL-1ß is capable of activating NF-κB, which then serves as an inducible transcriptional regulatory element in both the COX-2 and iNOS 5' promoter. IL-1ß is therefore a cytokine that is both upregulated with labour onset, and also has putative binding sites in both the COX-2 and iNOS promoter. IL-1ß was therefore used to investigate induction of NOS and COX mRNA and enzyme activity, in WISH cells, in an *in vitro* model.

WISH cell culture

WISH cells were cultured in 12 well plates and grown to confluence (see section 2.2.14 for culture conditions). All WISH cells in these experiments were used at less than 10 passages. Cells were maintained in serum free medium for 24 hrs prior to the experimental period. The optimal concentration of IL-1ß for maximal stimulation of WISH cells was determined as 1ng/ml.

An IL-1ß time course was used for both iNOS and COX-2 to determine whether IL-1ß induction of these genes occurred in a time-dependent manner. Time points were set up in duplicate, and IL-1ß added to the medium at time zero, and removed at 15, 30, 60 and 120 min intervals. Non-stimulated controls at all time points, and a time zero control were set up. Medium was removed and snap frozen for subsequent PGE_2 assay by RIA, and nitrite assay by chemiluminescence. Cold solution D was added to each well, and RNA extracted using a routine guanidinium isothiocyanate technique (see section 2.2.1). RNA was subsequently transcribed and PCR amplification carried out using cycle numbers determined from cycle profiles.

IL-1ß induction of NOS in WISH cells

iNOS mRNA

Time dependent IL-1 ß induction of iNOS mRNA is shown by RT-PCR in Fig. 4.61:

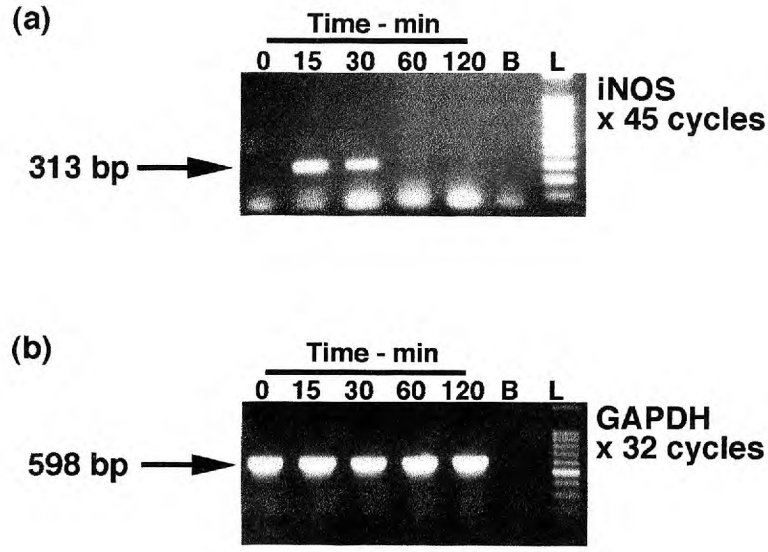


Fig. 4.61: iNOS and GAPDH mRNA expression in IL-1 ß stimulated WISH cells. The top panel (**a**) shows iNOS mRNA expression by RT-PCR at 45 cycles, in WISH cells stimulated with IL-1 ß. Cells were cultured in medium with IL-1 ß, at a final concentration of 1 ng/ml. Cells were collected and snap frozen at the time points indicated. The bottom panel (**b**) shows GAPDH mRNA expression by RT-PCR at 32 cycles, in the same samples, as a parallel control. B: blank lane - no starting template DNA and L: 100 bp DNA marker. Correct product size for iNOS is 313 base pairs and for GAPDH is 598 base pairs.

iNOS mRNA is induced by 1ng/ml IL-1ß at 15 min. There was no background expression of iNOS, demonstrated by the absence of expression at t = 0. iNOS induction persisted to 30 min, after which time PCR product was not visible. These data suggest that iNOS expression in WISH cells is rapidly induced by IL-1ß, but that iNOS mRNA is not stable, and is equally rapidly inactivated. GAPDH confirms starting template for all samples.

NOS enzyme activity

NOS activity was determined indirectly in the IL-1ß stimulated WISH cells by measuring nitrite concentrations in the medium. Nitrite levels after IL-1ß stimulation are shown in Fig. 4.62:

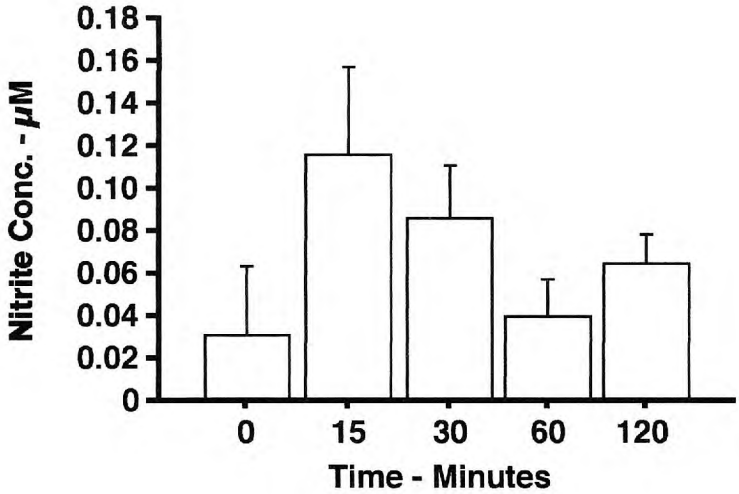


Fig. 4.62: Nitrite production by IL-1ß stimulated WISH cells. Cell bar chart shows Nitrite levels in WISH cell culture between 0 and 120 min. Cells were cultured in medium with IL-1 ß (1 ng/ml). Media was collected and snap frozen at the time points indicated. NO production was subsequently determined by measuring nitrite concentration by chemiluminescence. Cell bar chart shows mean values $\pm$ 2 SEM.

Fig. 4.62 demonstrates that WISH cells generate NO, in response to stimulation with IL-1ß. Data from these experiments have shown that NO production is significantly increased in response to IL-1ß (compared with control, t = 0) by 15 minutes ($p \leq 0.01$), and that NO levels remain significantly elevated at 30 min ($p \leq 0.01$) before gradually declining over time. These data correlate directly with RNA data (Fig 4.61) showing up-regulation of iNOS mRNA at 15 and 30 min, in response to stimulation with 1ng/ml IL-1ß, suggesting that the observed changes in NO levels in IL-1ß stimulated WISH cells is a direct result of up-regulation of iNOS mRNA.

IL-1ß induction of COX in WISH cells

COX-2 mRNA

Time dependent IL-1 ß induction ofCOX-2mRNA is shown by RT-PCR in Fig. 4.63:

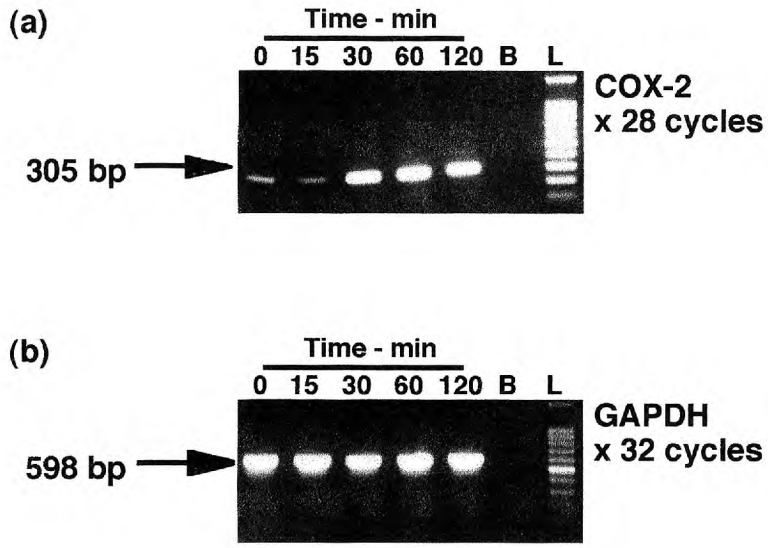


Fig. 4.63: COX-2 and GAPDH mRNA expression in IL-1 ß stimulated WISH cells. The top panel (**a**) shows COX-2 mRNA expression by RT-PCR at 28 cycles, in WISH cells stimulated with IL-1 ß. Cells were cultured in medium with IL-1 ß, at a final concentration of 1 ng/ml. Cells were collected and snap frozen at the time points indicated. The bottom panel (**b**) shows GAPDH mRNA expression by RT-PCR at 32 cycles, in the same samples, as a parallel control. B: blank lane - no starting template DNA and L: 100 bp DNA marker. Correct product size for COX-2 is 305 base pairs and for GAPDH is 598 base pairs.

COX-2 mRNA is significantly induced by IL-1ß by 30 min. COX-2 message persists to 120 min, suggesting that COX-2 message may be more stable than iNOS. COX-2 mRNA expression can be seen at t = 0, suggesting that in WISH cells, COX-2 may be expressed continuously, at basal levels.

COX enzyme activity

COX enzyme activity was determined indirectly, by measuring PGE_2 content in the medium removed from the cells. PGE_2 levels in IL-1ß stimulated WISH cells is shown as a time course between 4 hr and 24 hr in Fig. 4.64:

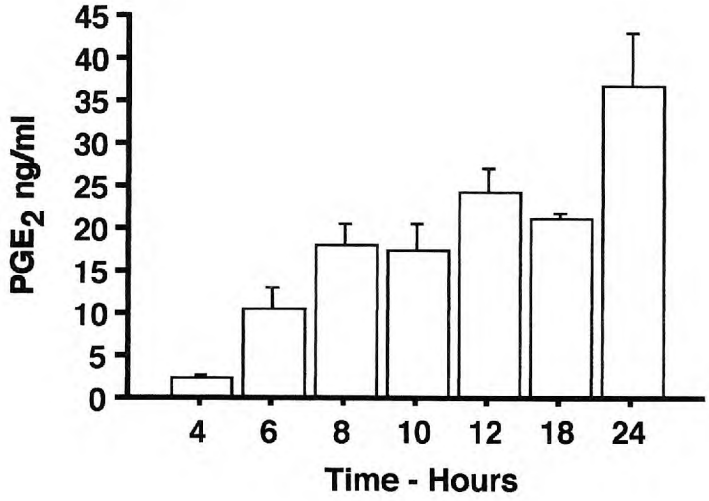


Fig. 4.64: PGE_2 production by IL-1ß stimulated WISH cells. Cell bar chart shows PGE_2 in WISH cell culture between 4 and 24 hrs. Cells were cultured in medium with IL-1 ß (1 ng/ml). Media was collected and snap frozen at the time points indicated. PGE_2 production was subsequently determined by RIA. Cell bar chart shows mean values ± 2 SEM.

Monolayer WISH cells in culture generate PGE_2 in response to cytokine stimulation with 1 ng/ml IL-1ß. Data from these experiments suggest that PGE_2 is detectable by RIA after 4 hrs stimulation. PGE_2 continues to be generated up to 24 hrs of stimulation.

IL-1ß electrophoretic mobility shift assays

The mechanism involved in IL-1ß induction of NOS and COX is thought to be mediated through the inducible transcription regulatory element, NF-κB. NF-κB is predominantly associated with I-κB, a cytoplasmic inhibitory protein. Release of NF-κB from I-κB allows rapid transfer of NF-κB into the nucleus. Genes regulated by NF-κB can therefore be transcriptionally activated within minutes following induction. NSAID's, such as ibuprofen influence cytokine induction by preventing dissociation of NF-κB from I-κB, thus effectively inhibiting gene transcription, and providing the mechanistic basis for the clinical effects of NSAID's (Scheuren et al., 1996).

To investigate whether IL-1ß induction of NOS and COX is, as anticipated, a result of NF-κB activation and nuclear transfer, electrophoretic mobility shift assays (gel shift assays) were performed on nuclear protein extracts from IL-1ß induced WISH cells.

An IL-1ß time course was set up as before to analyse the transfer and presence of NF-κB in the nucleus in a time-dependent manner. Time points were set up in quadruplicate, and

IL-1ß (1 ng/ml) added to the medium at time zero, and removed at 30, 60, 120, and 360 min intervals. Non-stimulated controls at 360 min, and a time zero control were set up. Medium was removed and snap frozen for subsequent PGE_2 assay by RIA, and nitrite assay by chemiluminescence. Nuclear protein extracts and gel shift assays were carried out as indicated in section 2.2.18. NF-κB consensus and OCT 1 non-consensus oligonucleotides were used. The result of electrophoretic shift assay using IL-1ß stimulated WISH nuclear proteins is shown in Fig. 4.65:

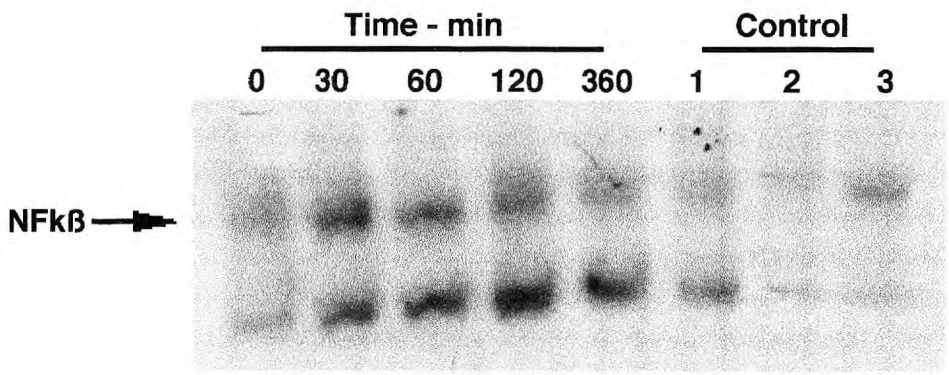


Fig. 4.65: NF-κB EMSA. NF-κB gel retardation in IL-1ß stimulated WISH cells. WISH cells were cultured in medium with IL-1 ß (1 ng/ml). Cells were collected and snap frozen at the time points indicated. Nuclear proteins were subsequently extracted. Samples were incubated with radio-labelled NF-κB consensus sequences. The presence of NF-κB sequences in the samples results in complex formation and gel shift retardation. Controls are 1: Non-stimulated WISH cells (360 min), 2: Unlabelled NF-κB consensus sequence and 3: Unlabelled non-consensus sequence (OCT1).

Fig . 4.65 shows NF-κB gel retardation in IL-1ß stimulated WISH cells. NF-κB nuclear levels can be seen to rise from background, at 30 min, this persists at 60 and 120 min, and then shows a small fall. Interestingly, at time zero NF-κB can be identified in the nuclear component. This might represent a response to cellular handling and incubation. Control 1 is non stimulated at 360 min, and shows levels similar to background at time zero. Control 2 involves competitive binding with an unlabelled consensus oligonucleotide (negative control). Control 3 involves competition with unlabelled non-consensus (OCT1) oligonucleotide (positive control). This experiment suggests that IL-1ß may act via NF-κB in WISH cell induction.

4.5 Inter-relationship between NOS and COX

4.5.1 Introduction

The observation that in gestational-associated tissue there are changes in both Cyclo-oxygenase (Type 2) mRNA and protein expression, in association with labour onset and gestational age, in the absence of any statistically significant change in nitric oxide synthase (iNOS) mRNA, protein and enzyme activity is unexpected. The regulation of both COX-2 and iNOS expression is considered to be transcriptional, and both iNOS and COX-2 share putative protein binding sites in their 5' promoter regions. In addition EMSA studies confirm that cytokine stimulation of WISH cells with IL-1ß, appears to induce transcription via activation of NF-kB. It is therefore paradoxical that in gestational tissue, which is known to be subject to cytokine induction, particularly in association with labour, there is COX-2 upregulation in the absence of iNOS upregulation. Co-induction of both these genes is a consistent feature of acute inflammation. This raises the possibility that in gestational tissue both the control of expression and interaction of NOS and COX may be distinct from other tissues and inflammatory models.

The potential for interactions between NOS and COX exist not only at the transcriptional level but also at the protein level. The COX enzymes are potential targets for NO due to the presence of an iron-haem centre at their active site, and the majority of the effects mediated by NO are a consequence of its interaction with iron, or iron containing moieties. The binding of NO to the haem - Fe^{2+} prosthetic group of soluble guanylate cyclase, is one example of this type of interaction.

The inter-relationship of NOS and COX was studied in an *in vitro* cell culture model. The benefits of a cell culture model is that culture conditions can be carefully maintained, and reproducible results can be obtained with immortalised cells in confluent culture. The choice of cell type was fundamental. Ideally the cell type should be derived from gestational tissue, or at least be representative of a gestational cell type. It should be capable of responding to cytokine induction, and should express both COX-2 and iNOS mRNA and protein, and exhibit their respective enzyme activity. A number of different cell types were originally investigated to establish the optimal cell line for future experiments.

WISH cells were chosen for *in vitro* cell culture. WISH cells represent immortalised amnion epithelial cells, and as such provide a model for a wholly gestational-related cell type. Co-induction of COX-2 and iNOS has been previously demonstrated in WISH cells.



Fig 4.66: Amnion derived epithelial WISH cells in monolayer culture. x 30 magnification.

Amnion has been established *in vivo* as an important source of intra-uterine prostaglandins in pregnancy (Slater et al., 1995), and this is largely a result of activity of the Type 2 COX enzyme (Slater et al., 1994). Similarly iNOS mRNA expression has been shown by RT-PCR in snap-frozen human amnion samples (Dennes et al., 1997). Use of an amnion cell culture model might therefore generate information on the inter-relationship of COX and NOS *in vitro*, which might be extrapolated to provide some insight into the interactions of these two enzymes *in* vivo.

Cell culture experiments were carried out in standard 24 well plates, with each experimental condition carried out in quadruplicate. WISH cells were allowed to establish confluence and were then maintained in serum-free conditions for 24 hr prior to each experimental period. Cells were cultured for 24 hr under experimental conditions, when the culture media was then removed and analysed

4.5.2 The effect of COX and PGE_2 on NOS activity in WISH cells

The inter-relationship of NOS and COX was initially investigated in WISH cells by examining the effect of PGE_2 on NOS activity (determined by nitrite production) in the presence of a non-selective COX inhibitor. WISH cells were stimulated with IL-1ß at a concentration of 1 ng/ml, in the presence of an increasing concentration of indomethacin (a non-selective NSAID) from 0.1 μM to 100 μM. Controls were set up with no IL-1ß stimulation and no indomethacin treatment (negative control), and IL-1ß stimulation with no indomethacin treatment (positive control). The effects of IL-1ß on PGE_2 production by WISH cells in the presence of indomethacin is shown in Fig. 4.67:

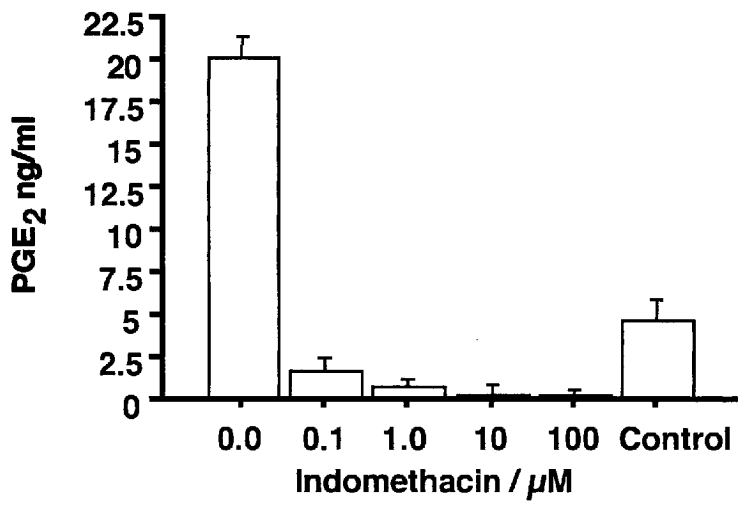


Fig. 4.67: Indomethacin dose response inhibition of IL-1ß-stimulated PGE_2 synthesis in WISH cells at 24 hrs. Control group represents the negative control (background PGE_2 synthesis): no IL-1ß stimulation and no indomethacin treatment. Cell bar chart shows mean values ± 2 SEM.

As anticipated IL-1ß stimulates PGE_2 synthesis in WISH cells. PGE_2 synthesis is inhibited in this model by indomethacin, in a dose response manner (relative to no indomethacin inhibition: $p < 0.01$ for all indomethacin concentrations). PGE_2 synthesis is inhibited significantly relative to basal synthesis (control) ($p < 0.01$) for all indomethacin concentrations). MTT colour conversion showed no statistically significant difference in conversion between different treatment groups, confirming cell viability.

The effect of reducing PGE_2 synthesis on NOS was investigated by measuring nitrite concentrations, by chemiluminescence, in the medium removed from these cells. Nitrite production by WISH cells in the presence of indomethacin is shown in Fig. 4.68:

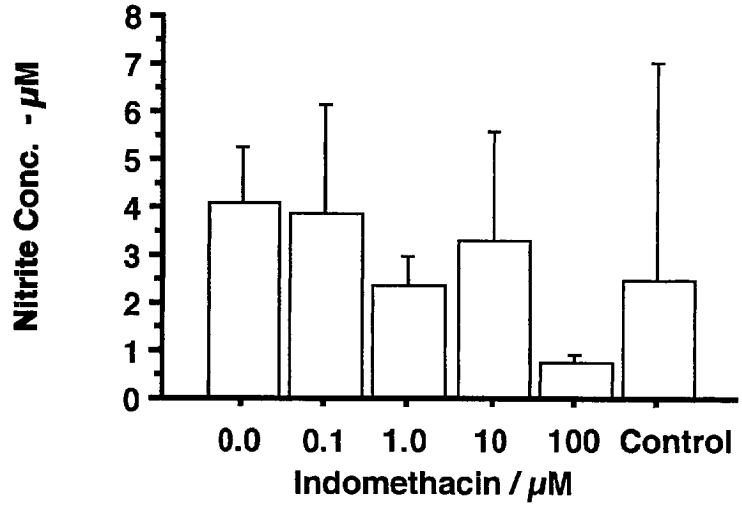


Fig. 4.68: Indomethacin dose response inhibition of IL-1ß-stimulated NO synthesis in WISH cells at 24 hrs. Control group represents the negative control (background NO synthesis): no IL-1ß stimulation and no indomethacin treatment. Cell bar chart shows mean values ± 2 SEM.

Fig. 4.68 shows that in WISH cells stimulated with 1 ng/ml IL-1ß, inhibition of endogenous PGE_2 synthesis by indomethacin appears to have no statistically significant effect on NO production, compared to control (no Il-1ß stimulation, no indomethacin). Furthermore at indomethacin concentrations at which COX-2 activity in WISH cells is almost entirely inhibited (≥ 0.1 μM indomethacin) (Fig. 4.67), there is no significant effect on NOS activity, suggesting that inhibition of endogenous PGE_2 synthesis has no effect on NOS activity. NOS activity does appear to be reduced (although not significantly) at an indomethacin concentration of 100 μM, and this might reflect an indirect effect, via intracellular calcium concentrations.

4.5.3 The effect of NOS and NO on COX activity in WISH cells

The effects of NOS and NO on PGE_2 synthesis in WISH cells can be examined using a number of different approaches. The effects of inhibition of endogenously generated NO

can be investigated by inhibiting endogenous NO synthesis using a non-selective NOS inhibitor, such as L-NAME. The possible non-specific effects of the addition of L-NAME can be controlled for by running identical experiments using D-NAME, the pharmacologically inactive enatiomer. In the absence of cytokine stimulation, inhibition of endogenous NOS activity is likely to represent largely constitutive NOS activity (e/c or nNOS).

An alternative strategy is to examine the effects of exogenous NO on PGE_2 synthesis. Exogenous NO can be generated using NO donors, such as sodium nitroprusside (SNP).

Effects of inhibition of endogenous NOS on WISH cells PGE_2 synthesis

Endogenous NOS was inhibited with L-NAME. IL-1ß stimulated WISH cells were treated with L-NAME (and D-NAME), and PGE_2 was determined in the medium by RIA. WISH cells were stimulated with IL-1ß (1ng/ml) in the presence of L-NAME (and D-NAME) in concentrations between 10-1000 μM. PGE_2 synthesis was determined by RIA. The influence of L-NAME on IL-1ß stimulated PGE_2 synthesis is shown in Fig. 4.69:

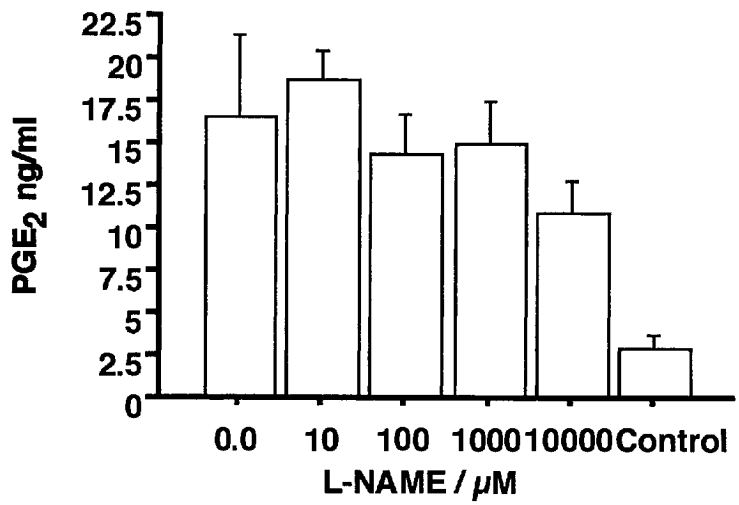


Fig. 4.69: L-NAME dose response inhibition of IL-1ß stimulated PGE_2 synthesis in WISH cells at 24 hrs. Control group represents the negative control (background PGE_2 synthesis) no IL-1ß stimulation or L-NAME treatment. Cell bar chart shows mean values $\pm$ 2 SEM.

Inhibition of PGE_2 synthesis (relative to no L-NAME) becomes statistically significant at 10000 μM concentration ($p < 0.01$). Inhibition however is by no means complete and levels do not fall to control (basal, non-stimulated) levels. These data suggest that

inhibition of NOS, with associated falls in NO synthesis might reduce PGE_2 synthesis. To confirm that this effect is specific for L-NAME, the influence of D-NAME at 10000 μM on IL-1ß stimulated PGE_2 synthesis is shown in Fig. 4.70:

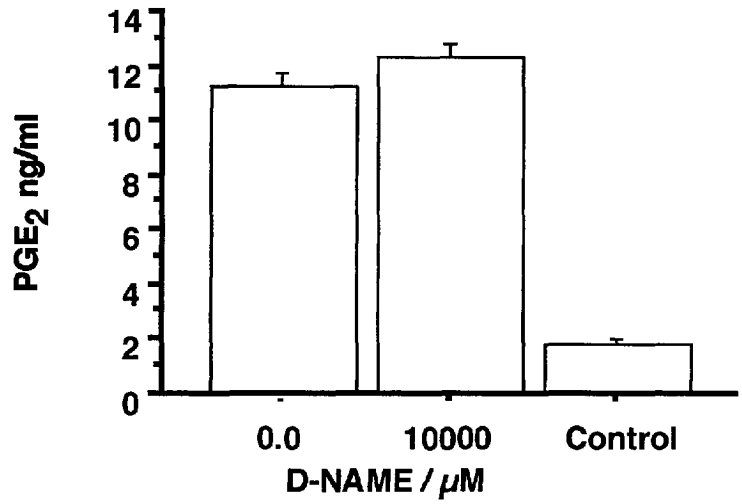


Fig. 4.70: D-NAME dose response inhibition of IL-1ß stimulated PGE_2 synthesis in WISH cells at 24 hrs. Control group represents the negative control (background PGE_2 synthesis) no IL-1ß stimulation or D-NAME treatment. Cell bar chart shows mean values $\pm$ 2 SEM.

D-NAME at a concentration where L-NAME has maximal inhibition (10000 μM) has no significant effect on PGE_2 synthesis in IL-1ß stimulated WISH cells (p = 0.47). This suggests that the inhibition of PGE_2 synthesis seen with L-NAME is specific for this agent, and is therefore likely to represent the result of NOS inhibition. MTT conversion data confirms cell viability, at all concentrations of L-NAME.

Effects of exogenous NO on WISH cell PGE_2 synthesis

The effects of exogenous NO on PGE_2 synthesis in WISH cells was determined using SNP, an NO donor. SNP was added to the WISH cell culture medium in a dose response manner, the cells were cultured for 24 hr, the medium removed and assayed for PGE_2 synthesis. An estimation of COX activity was determined by adding medium containing arachidonic acid (final concentration - 30 μM) in the presence of SNP, for a 10 min incubation period. Concentrations of SNP used in these experiments were determined both empirically, from pilot data, and from published series involving other cell types

(Salvemini et al., 1993). The effect of increasing concentration of SNP on WISH cell PGE_2 synthesis over 24 hr is shown in Fig. 4.71:

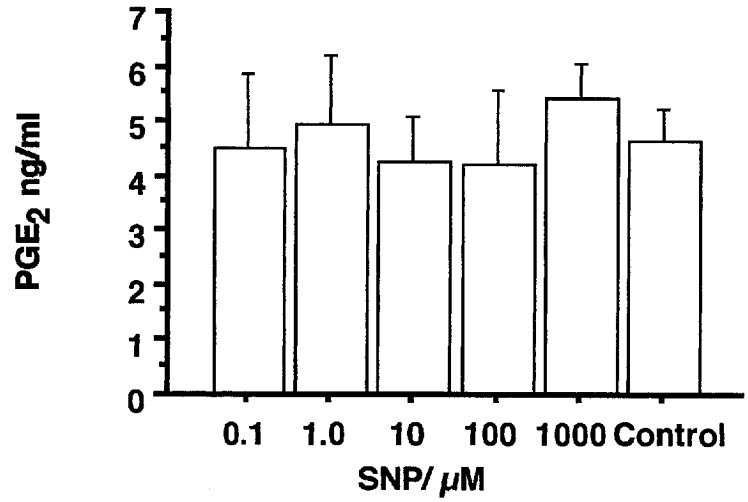


Fig. 4.71: SNP dose response of PGE_2 synthesis in WISH cells - release over 24 hr. Control group represents the negative control (background PGE_2 synthesis), no SNP treatment. Cell bar chart shows mean values $\pm$ 2 SEM.

There was no significant effect of SNP, at any concentration, from 1.0 - 1000 μM on PGE_2 synthesis over 24 hr in WISH cells. PGE_2 synthesis in the presence of arachidonic acid (as an indicator of COX activity) with SNP is shown in Fig. 4.72:

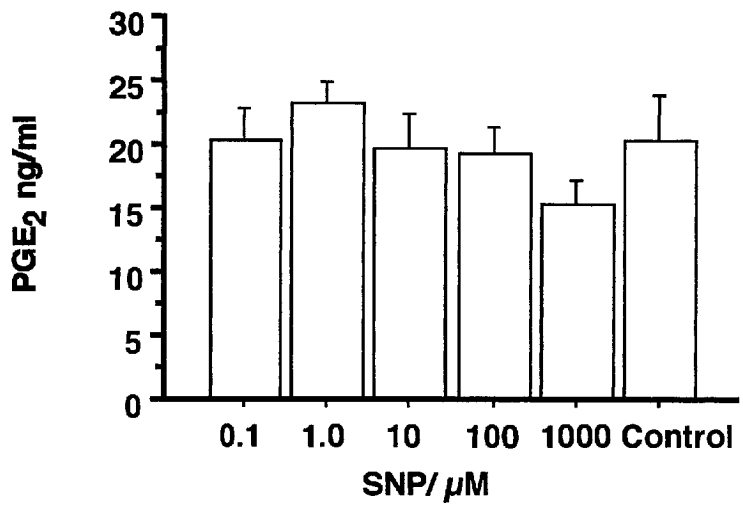


Fig. 4.72: SNP dose response of PGE_2 synthesis in WISH cells - activity with arachidonic acid. Control group represents the negative control (background PGE_2 synthesis), no SNP treatment. Cell bar chart shows mean values $\pm$ 2 SEM.

PGE_2 synthesis in WISH cells cultured in the presence of arachidonic acid remains unchanged at concentrations of between 1.0 - 100 μM SNP. At a concentration of 1000 μM SNP there is a statistically significant fall in PGE_2 synthesis (p = 0.01). However MTT conversion data (Fig. 4.73) shows that at this SNP concentration, MTT conversion relative to control (no SNP), is also diminished ($p < 0.01$), suggesting decreased cell viability. The observed fall in PGE_2 synthesis seen with the highest concentration of SNP might therefore reflect decreased cell number and viability as a result of the toxic effects of high dose SNP.

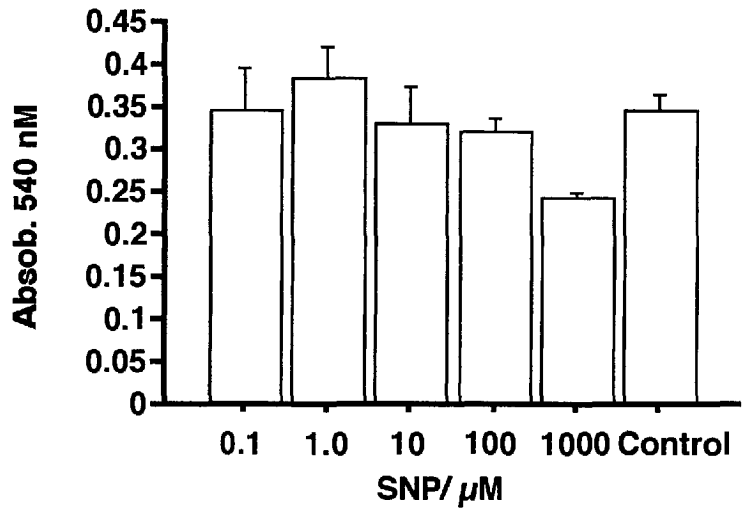


Fig. 4.73: MTT determination of SNP treated WISH cell viability. Cell bar chart shows mean absorbance at 540 nM ± 2 SEM. Viable cells convert yellow MTT dye to purple, with absorbance at 540 nM. Decreased absorbance therefore implies decreased cell viability.

4.5.4 The effect of NOS and NO on COX activity in the uterus

Clinical studies suggest that NO donors may exert a differential effect on distinct areas of the uterus. Whilst there is some evidence from both animal (Yallampalli et al., 1994b) and human (Buhimschi et al., 1995) studies that NO might relax the uterus, more recently there is evidence that NO donors might ripen the uterine cervix (Thomson et al., 1997a). The molecular basis for this apparent difference was investigated using an *in vitro* cell culture model. The differential effect of an NO donor on PGE_2 synthesis of cells from different

areas of the uterus was investigated by establishing cell culture with HOG-1 and human myometrial smooth muscle cells (HMSMC).

HOG-1 cells are an immortalised, cervical epithelial cancer cell line established originally at The Hammersmith Hospital. As such they would provide information on the cervical response to NO donors.

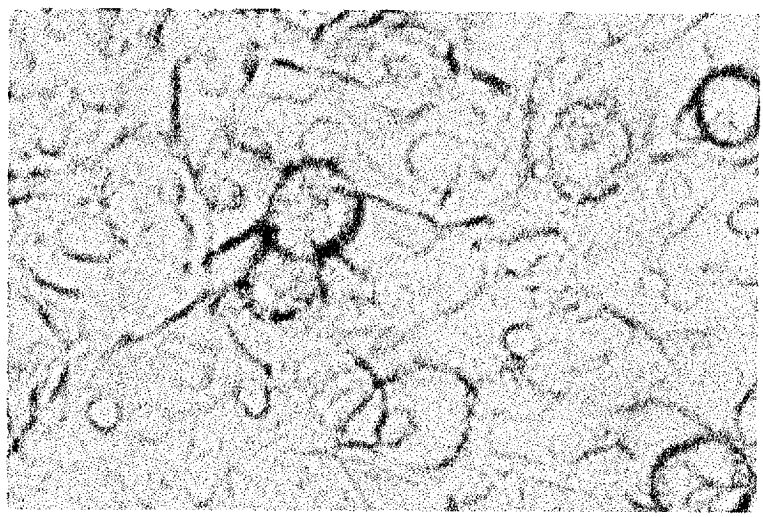


Fig. 4.74: HOG-1 epithelial cancer cells in monolayer culture. x 20 magnification.

HMSMC are a primary cell line established in culture from myometrial explants. Myometrial explants are obtained at caesarean section from the lower uterine segment. Rarely, and usually only at caesarean-hysterectomy, tissue may be obtained from the fundus. Tissue obtained at caesarean-hysterectomy may however be uninformative in studies analysing physiological events related to labour, due to the often prolonged temporal relationship between delivery and biopsy. Additional maternal pathological events may further compromise the value of the sample. In contrast to WISH and HOG-1 cells HMSMC are a primary culture. Whilst their phenotype should be closer to the original cell type, the cells remain viable for experimental purposes for a shorter period.

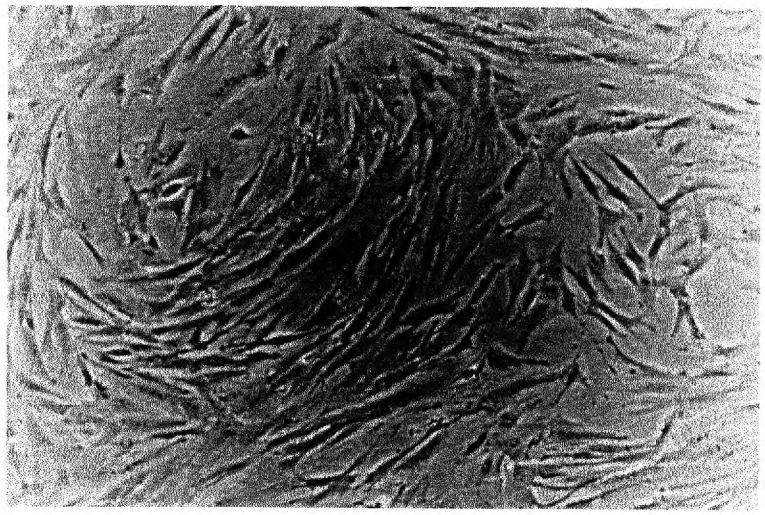


Fig. 4.75: Human myometrial smooth muscle cells (HMSMC) in monolayer culture. x 20 magnification.

The effect of exogenous NO on COX activity in HOG-1 cells

The effect of exogenous NO on COX activity in HOG-1 cells was investigated using an SNP dose response experiment. The initial concentration of SNP used in these experiments was between 0.1 and 5.0 mM. PGE_2 synthesis in HOG-1 cells over 24 hr treated with an increasing concentration of SNP is shown in Fig. 4.76:

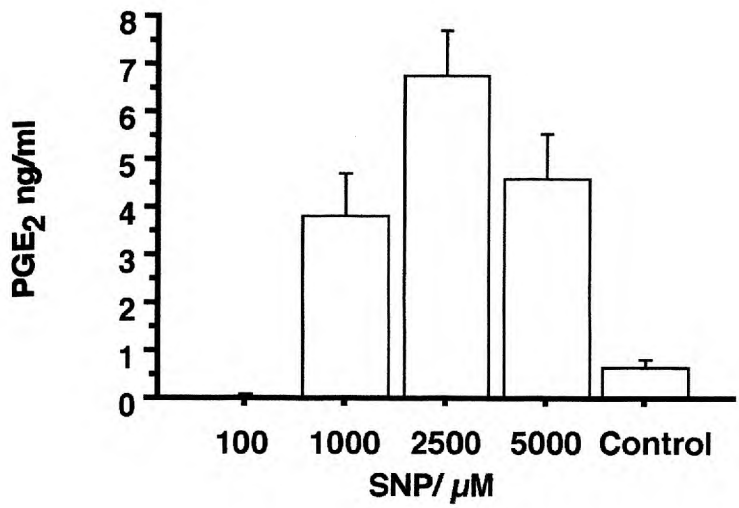


Fig. 4.76: SNP dose response of PGE_2 synthesis in HOG-1 cells - release over 24 hr. Control group represents no SNP. Cell bar chart shows mean values ± 2 SEM.

SNP appears to induce PGE_2 synthesis in HOG-1 cells at these concentrations. At all concentrations above 100 μM SNP there is a statistically significant induction of PGE_2 synthesis ($p < 0.01$ relative to control). There appeared to be cell damage at SNP concentrations above 1000 μM, and determined PGE_2 synthesis may therefore represent the result of cell damage. This possibility is further supported by evidence from COX activity experiments. An estimation of COX activity was determined by adding medium containing arachidonic acid (final concentration - 30 μM) in the presence of SNP, for a 10 min incubation period. The effects of SNP on PGE_2 synthesis in the presence of arachidonic acid are shown in Fig. 4.77:

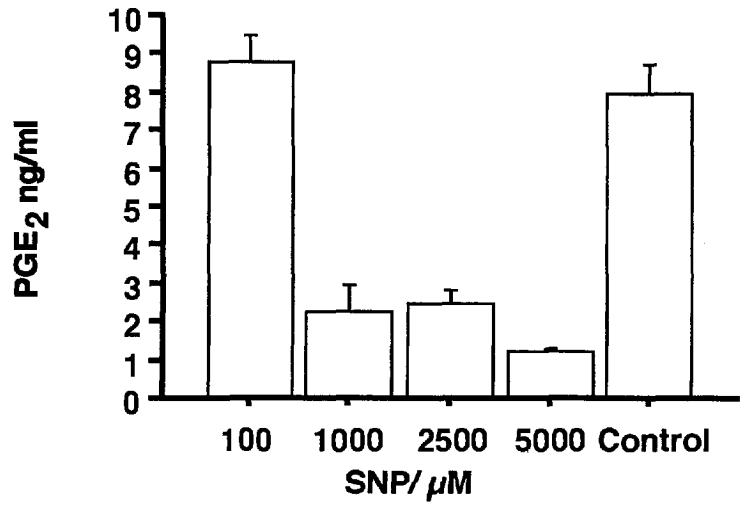


Fig. 4.77: SNP dose response of PGE_2 synthesis in HOG-1 cells - activity with arachidonic acid. Control group represents no SNP. Cell bar chart shows mean values $\pm$ 2 SEM.

In the presence of arachidonic acid, PGE_2 synthesis is reduced in a highly significant manner with SNP concentrations above 100 μM ($p < 0.01$, for concentrations of 1000, 2500 and 5000 μM SNP respectively, relative to control). The fact that COX enzyme activity is so significantly affected at SNP concentrations above 100 μM provides further evidence to support the theory that cell damage result at these concentrations of SNP.

The effect of exogenous NO on COX activity in HMSMC cells

The effect of exogenous NO on COX activity in HMSMC cells was investigated using an SNP dose response experiment. The concentration of SNP used in these experiments was

identical to that used in the HOG-1 experiments (0.1 - 5.0 mM). PGE_2 synthesis in HMSMC cells, over 24 hr, treated with an increasing concentration of SNP was undetectable by RIA. This suggests that if PGE_2 was synthesised by HMSMC in this experiment, levels fell below the sensitivity of the RIA, suggesting pM concentrations. The effects of SNP on PGE_2 synthesis in HMSMC in the presence of arachidonic acid are shown in Fig. 4.78:

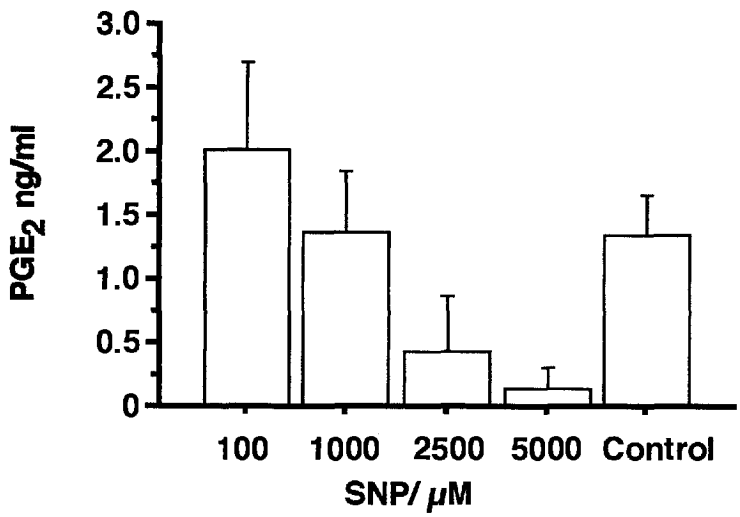


Fig. 4.78: SNP dose response of PGE_2 synthesis in HMSMC - activity with arachidonic acid. Control group represents no SNP. Cell bar chart shows mean values ± 2 SEM.

Whilst PGE_2 is generated from these cells in response to SNP in the presence of arachidonic acid, the levels are low compared to HOG-1 cells (approximately 20%). In addition PGE_2 synthesis is statistically significantly reduced at SNP concentrations of 2500 and 5000 μM ($p < 0.01$, relative to control). It is likely that this inhibition of PGE_2 synthesis represents the result of cell damage, in a similar manner to that observed in HOG-1 cells.

4.6 Role of NO and in the control of myometrial contractility

4.6.1 Introduction

The effects on myometrial contractility of modulating endogenous and exogenous nitric oxide were examined *in vitro* using a fetal membrane-uterine muscle dual chamber system, as described in section 2.2.19. The effects of exogenous NO on myometrial contractility in this system was examined using an NO donor (SNP).

Pilot data from our group, investigating the effects of fetal membranes on myometrial contractility, suggested that fetal membranes when apposed to myometrial strips, in this system, increase both latent time to onset of spontaneous contractions, and reduce MIT (R.J. Sawdy, Personal communication). The orientation of the fetal membranes to the myometrial strip did not influence these effects. These data suggested that the fetal membranes were inhibiting myometrial contractility through some, as yet unknown, mechanism. The possibility that these effects might be mediated by endogenous NO generated in the fetal membranes was investigated in this system in the presence of fetal membranes using an NOS inhibitor (L-NAME) and its inactive enantiomer (D-NAME).

4.6.2 Effects of exogenous NO on myometrial contractility

The influence of exogenous NO on myometrial contractility was investigated in the dual chamber system in the presence of SNP, an NO donor. The concentration of SNP used in this study was derived from published data (Norman et al., 1997).

Myometrial contractility

The effect of increasing concentration of SNP was determined on myometrial contractility by measuring MIT. A representative dose response of SNP on spontaneous rhythmic myometrial contractions is shown in Fig. 4.79:

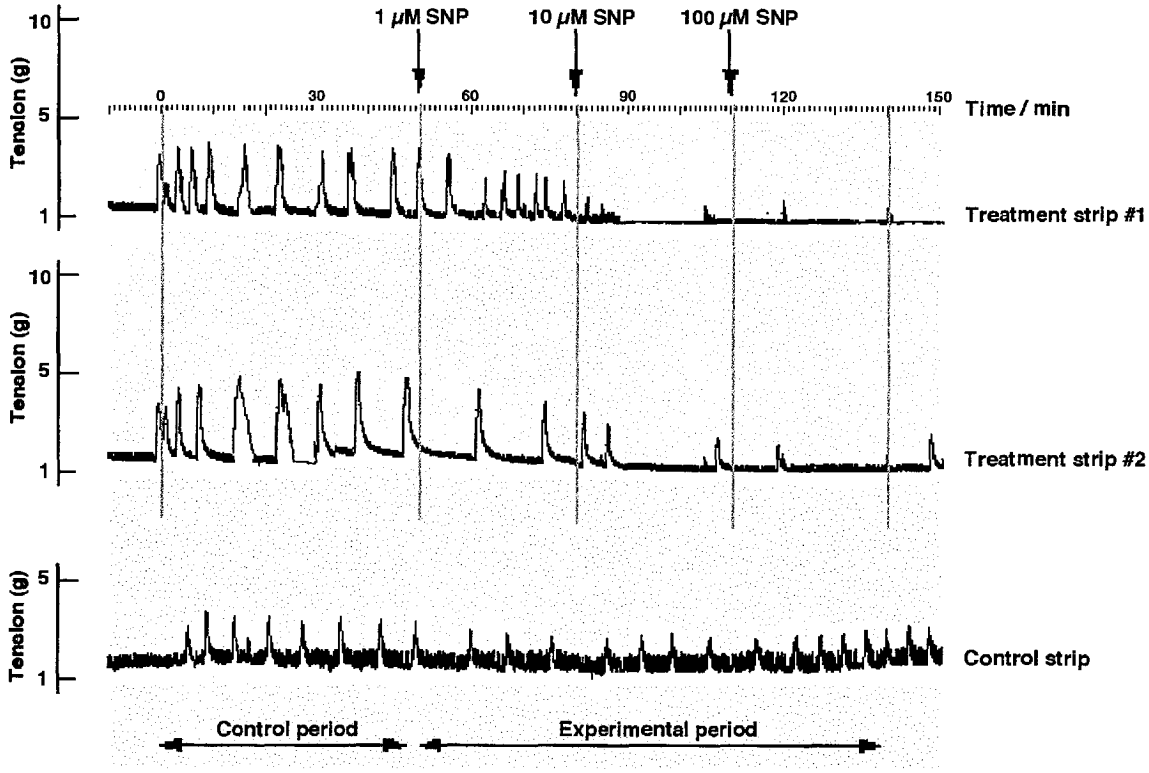


Fig. 4.79: Representative experimental record demonstrating the response of spontaneously contracting myometrial strips to increasing concentrations of sodium nitroprusside (SNP). The two upper tracings show representative myometrial strips. After a control period of 50 min, during which time spontaneous contractions are maintained, SNP was addedto circulating PSS, in increasing concentrations from 1 - 100 μM. Myometrial contractility was confirmed in these strips at the end of the treatment period by eliciting a contractile response to oxytocin (not shown). The control strip shows that myometrial contractility is maintained throughout the experimental period in the absence of pharmacological agents.

Fig. 4.79 shows that the nitric oxide donor SNP inhibits myometrial contractility in spontaneously contracting myometrial strips *in vitro* .

Analysis of the effects of SNP on myometrial contractility were carried out by analysing mean integral tension (MIT), defined as the sum of the integral tensions during the experimental period, divided by the duration of the period. The response of the myometrium to the addition of SNP was expressed as a ratio to the mean integral tension during the baseline period (Control). Combined data from SNP dose response experiments (n = 3) are shown in Fig. 4.80:

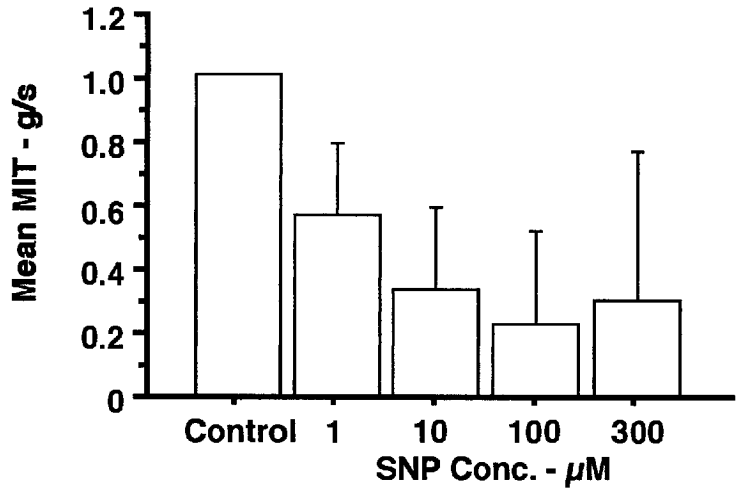


Fig. 4.80: SNP dose response of myometrial contractility in spontaneously contracting myometrial strips *in vitro*. Figure shows myometrial contractility (MIT), relative to the initial control period (no SNP). SNP was added to circulating PSS in increasing concentrations and MIT determined over 30 min treatment periods. SNP concentrations were 1, 10 100 and 300 μM. Cell bar chart shows mean values $\pm$ 2 SEM. MIT was significantly inhibited by SNP at all concentrations. (Control vs. 1, 10, 100 and. 300 μM SNP: $P < 0.01$).

Fig. 4.80 demonstrates that myometrial contractility is significantly inhibited in myometrial strips *in vitro* at concentrations of 1 μM and above. In addition SNP appears to have an almost immediate effect at modulating myometrial contractility and this would be consistent with the accepted mechanism of spontaneous NO release by SNP.

Effect of exogenous NO on COX-2 mRNA in myometrial strips

Following the experimental period myometrial strips were removed from the organ chamber, and snap frozen. RNA was then extracted from individual myometrial strips, as described in section 2.2.1. RT-PCR was then carried out to analyse COX-2 expression within these tissues. Cycle profiles for both COX-2 and calponin were carried out, on pooled myometrial samples, to determine the appropriate cycle number for this reverse transcriptase reaction. Optimal cycle number for COX-2 was 32, and for calponin was 30.

Myometrial strips were divided into those in which contractions were never established (no contractions), those in which contractions were established, but which were both treated and responded to increasing concentrations of SNP (SNP treated), and control strips in which spontaneous contractions were both established and maintained throughout the experimental period, and were untreated (control). COX-2 mRNA expression in myometrial strips demonstrated by RT-PCR is shown in Fig. 4.81:

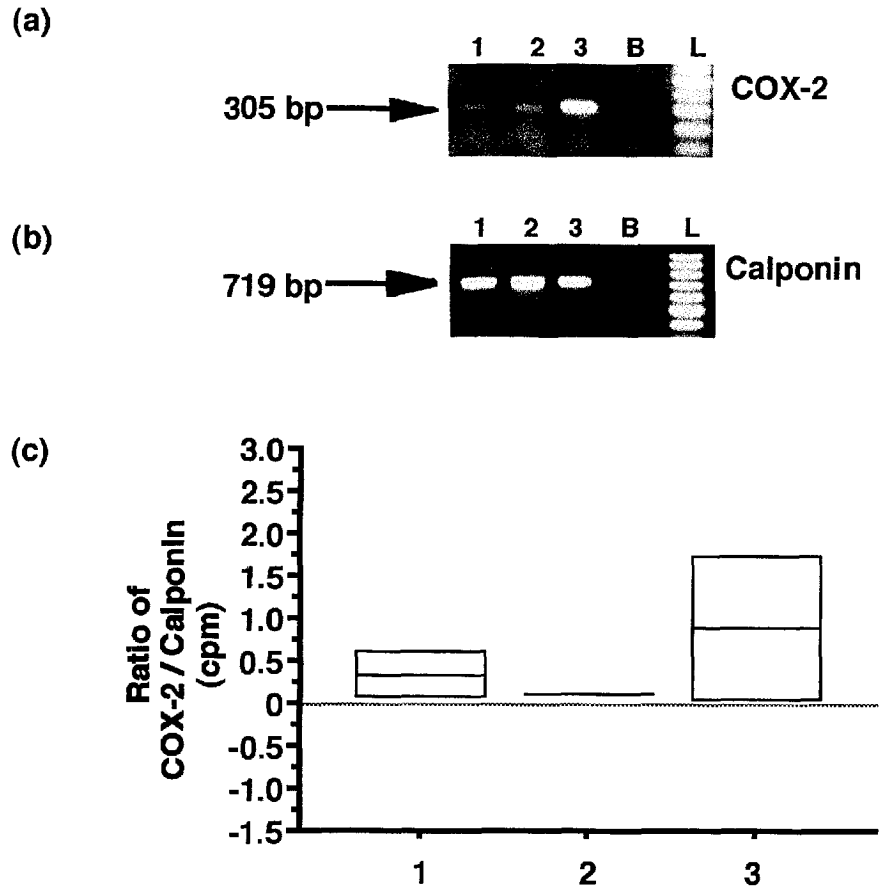


Fig. 4.81: COX-2 mRNA expression in myometrial strips. (**a**) RT-PCR with COX-2 primers at 32 cycles on DNA isolated from isolated myometrial strips. Correct product size is 305 base pairs. (**b**) RT-PCR with calponin primers at 30 cycles on DNA isolated from isolated myometrial strips. Correct product size is 719 base pairs. Samples are 1: no contractions (n = 2), 2: spontaneous contractions and SNP treated (increasing SNP concentration - 1 - 100 μM) (n = 2) and 3: Control, spontaneous contractions, no treatment (n = 2). B: blank lane - no starting template DNA and L: 100 bp DNA marker. (**c**) Box plots of COX-2 expression, shown as a ratio to calponin (as cpm). Data shows mean $\pm$ 25th centiles, and sample range.

The influence of exogenous NO on myometrial COX-2 expression in an *in vitro* contractility model is shown in Fig. 4.80. Relative to the control strip, SNP reduces COX-2 expression, but not significantly (p = 0.36). In addition, this experiment demonstrates that in myometrial strips that do not establish spontaneous myometrial contractions, the level of COX-2 mRNA, demonstrated by RT-PCR, is lower than that of control strips, although this difference does not achieve statistical significance (p = 0.51).

4.6.3 Effects of fetal membranes and inhibition of endogenous NO on myometrial contractility

The effects of inhibiting endogenous NO on myometrial contractility have been previously investigated using non-specific NOS inhibitors on myometrial strips in an organ bath system (Jones and Poston, 1997). No significant change in myometrial contractility was observed either by inhibiting endogenous NO synthesis with L-NAME, or with the addition of the NOS substrate, L-Arginine.

The influence of fetal membranes and the potential role of fetal membrane-generated NO on myometrial contractility was investigated in the same organ bath system. The system was set up with fetal membranes 'sandwiched' between the two chambers. Fetal membranes were pre-incubated in PSS containing either 1 mM L-NAME or D-NAME at 37°C for 2 hrs. Chambers were then assembled with myometrial strips and either polythene (blank) or fetal membranes in contact with the circulating PSS. Fetal membrane orientation was noted.

After an initial equilibrium period of 60 min contractility was determined for a 60 min experimental period. Myometrial strips were then transferred to their corresponding chambers in a cross-over manner. Strips that had been in contact with amnion or chorion decidua were transferred to chambers in contact with polythene (blank), and vice versa. The circulating PSS was not changed. The nature of the cross-over experimental design provides an internal control, therefore no further controls were required and experiments were set up with a total of eight myometrial strips transferred between fetal membranes and blank. A representative fetal membrane cross-over experiment, with L-NAME and D-NAME is shown in Fig. 4.82.

Fig. 4.82 shows that myometrial strips set up in this system in blank chambers (the two upper and lower strips) initiate spontaneous myometrial contractions, within the initial experimental period. In comparison myometrial strips set up in the presence of fetal membranes fail to initiate spontaneous myometrial contractions, even following cross-over to blank chambers. The presence of L-NAME or D-NAME does not appear to influence this response.

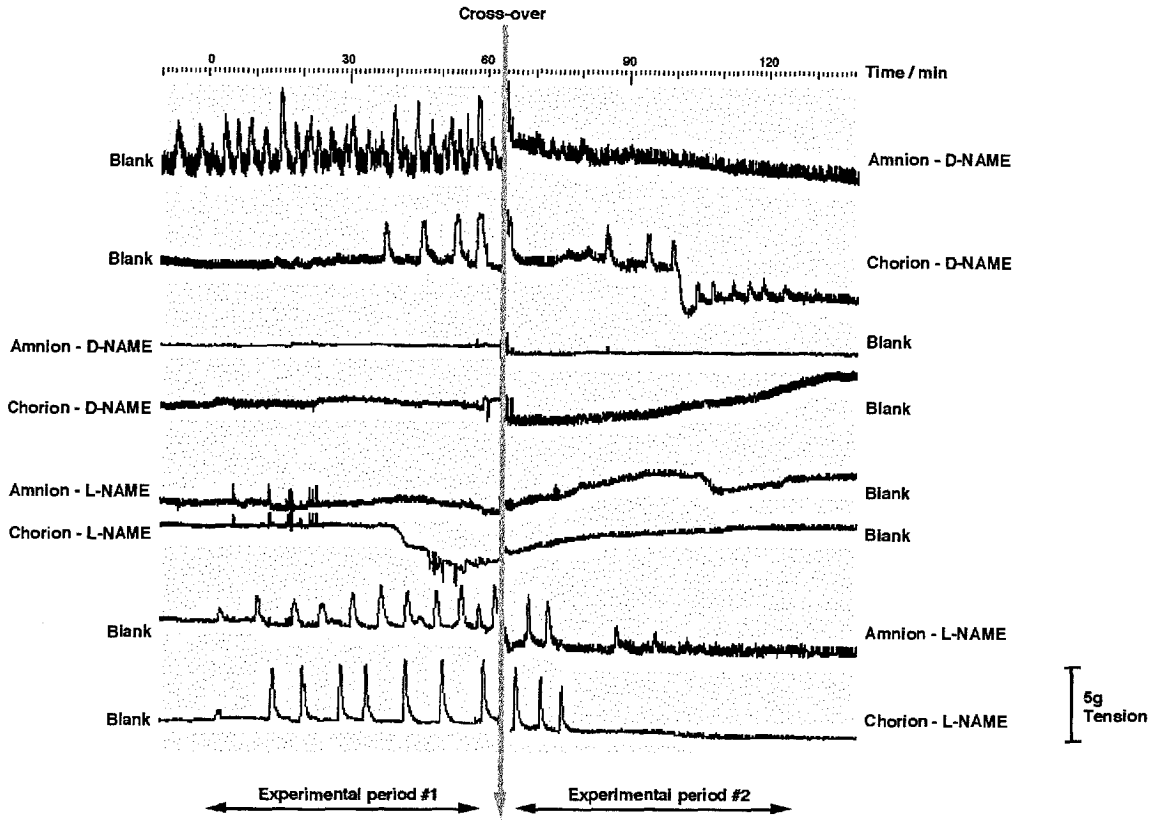


Fig. 4.82: Representative experimental record demonstrating a cross-over response of myometrial strips to exposure to fetal membranes in the presence of L-NAME and D-NAME. The two upper tracings show myometrial strips initially set up in blank chambers crossed-over to chambers with amnion and chorion decidua respectively, in the presence of 1 mM D-NAME. Similarly subsequent strips were established in chambers with amnion and chorion decidua in the presence of 1 mM D-NAME and crossed-over to blank chambers. Similar strips were set up in exactly the same manner with 1 mM L-NAME. Myometrial contractility was confirmed in all strips at the end of the experiment by eliciting a contractile response to oxytocin (not shown).

Strips set up in blank chambers, where spontaneous contractions have been established, when crossed-over to chambers with fetal membranes consistently show a decrease in contractility that is evident within 10 min of cross-over. This effect appears to be more significant when strips are transferred from blank chambers to chambers with amnion, rather than chorion decidua. There appears to be no difference in this observed effect when strips are transferred to chambers in the presence of either L-NAME or D-NAME. The fact that all strips elicited a contractile response to oxytocin, confirmed both tissue viability and potential for myometrial contractions.

The effect of the presence of fetal membranes on myometrial contractility (as a function of MIT), in the presence of both L-NAME and D-NAME is shown in Fig. 4.83:

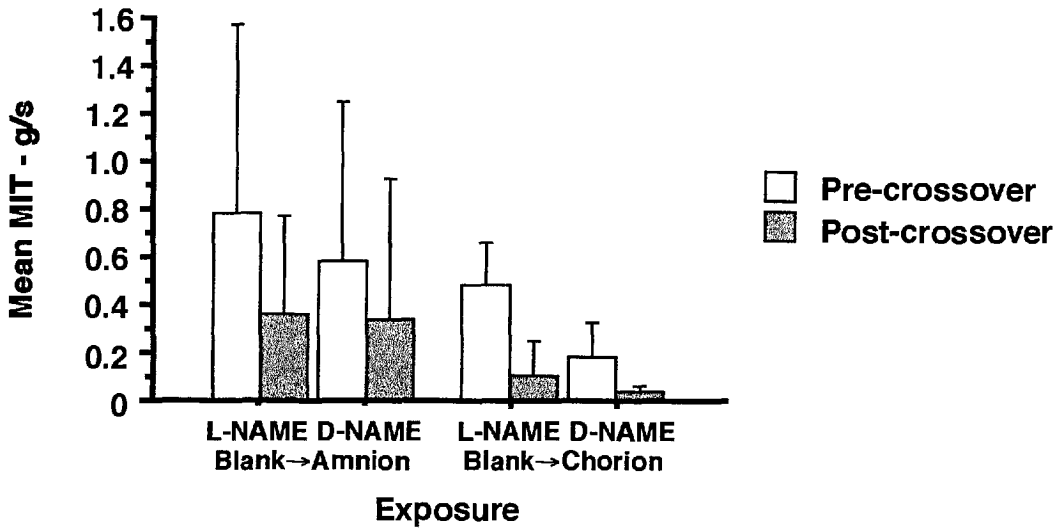


Fig. 4.83: Effect of L-NAME and D-NAME on myometrial contractility, in spontaneously contracting myometrial strips, in the presence of fetal membranes. Figure shows myometrial contractility (MIT) in strips initially exposed to blank (Pre-crossover, no membranes), transferred to chambers with either amnion or chorion decidua (Post-crossover) in the presence of either 1 mM L-NAME or 1 mM D-NAME. Cell bar chart shows mean values ± 2 SEM.

Strips exposed to fetal membranes (either amnion or chorion decidua) in the first exposure did not initiate spontaneous myometrial contractions, and were not subject to investigation in this analysis. Mean MIT was reduced (although not significantly) on crossing over myometrial strips to chambers with both amnion and chorion decidua. This reduction in myometrial contractility was observed in the presence of both L-NAME, a NOS inhibitor, and D-NAME its inactive enantiomer.

This study has confirmed data from earlier studies carried out in our department showing that the presence of fetal membranes increases both latent time to onset of spontaneous contractions, and reduces myometrial contractility. L-NAME and D-NAME have been shown in other studies (Jones and Poston, 1997), using a similar system, to have no direct effect on myometrial contractility. It is therefore unlikely that this observation is a result of a toxic effect of L-NAME or D-NAME. Furthermore since NOS inhibition has no effect on this observed response, it is unlikely that this effect is a result of NO synthesis within the fetal membranes, but rather that some factor present in fetal membranes, and amnion in particular, may be negatively modulating myometrial contractility.

4.6.4 COX-2 mRNA stability in myometrial strips

The stability of RNA in tissue employed in prolonged contractility studies was unknown. It was assumed whilst tissue continued to maintain spontaneous contractions, in physiological PSS at 37°C, RNA should remain stable, and therefore suitable for extraction using routine techniques. The stability of RNA was determined by comparing levels of RNA expression of tissue that had been immediately snap frozen, following collection compared with myometrial strips maintained in PSS for 3 hrs. Calponin expression in myometrial strips immediately snap frozen (t = 0), and maintained in PSS for 3 hrs (t = 3 hr) is demonstrated by RT-PCR in Fig. 4.84:

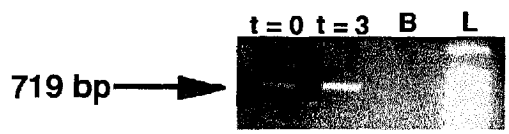


Fig. 4.84: Calponin mRNA stability in myometrial strips. RT-PCR with calponin primers at 32 cycles on DNA isolated from isolated myometrial strips. Correct product size is 719 base pairs. Samples are: t = 0, immediately snap frozen, t = 3 hr, maintained in PSS for 3 hrs. B: blank lane - no starting template DNA and L: 100 bp DNA marker.

Fig. 4.84 shows that RNA remains stable and is suitable for analysis by RT-PCR, in tissue maintained for up to 3 hr in physiological PSS at 37°C.

4.6.5 Effects of stretch on COX-2 mRNA in myometrial strips

The influence of applying stretch to myometrial strips was investigated in the same system. Myometrial strips were routinely vertically mounted, in an organ bath, and 3g of resting tension applied. Expression of COX-2, determined by RT-PCR, was compared in strips that were mounted with stretch applied, against strips maintained in PSS, but not stretched. The experimental period was similar for both groups (t = 3 hr). COX-2 mRNA expression in stretched and unstretched myometrial strips is shown in Fig. 4.85:

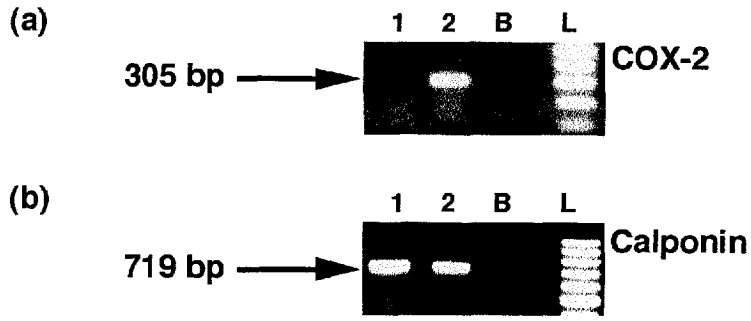


Fig. 4.85: COX-2 mRNA expression in myometrial strips, in relation to applied stretch. **(a)** RT-PCR with COX-2 primers at 32 cycles on DNA isolated from isolated myometrial strips. Correct product size is 305 base pairs. **(b)** RT-PCR with calponin primers at 30 cycles on DNA isolated from isolated myometrial strips. Correct product size is 719 base pairs. Myometrial strips are 1: No stretch, or 2: 3 g of applied resting tension (stretch). B: blank lane - no starting template DNA and L: 100 bp DNA marker.

COX-2 mRNA expression in myometrial strips, in relation to applied stretch is shown in Fig. 4.85. COX-2 mRNA expression, identified by RT-PCR, in isolated myometrial strips increased in relation to applied stretch. Whilst quantitative data comparing COX-2 mRNA in stretched and unstretched strips is not available, similar, consistent results were obtained on repeating this experiment.

These data might suggest that physiological stress may be a factor involved in up-regulation of COX-2 mRNA expression in myometrium, supporting a possible hypothesis that stretch may be one of the factors involved in the initiation of labour onset.

5. DISCUSSION

5.1 General

This study has analysed some of the molecular factors involved in both the maintenance of pregnancy, and the onset of parturition. Preterm labour remains a major therapeutic challenge. The motivation for investigating the molecular basis of labour onset was to develop a more advanced understanding of some of the factors involved in parturition, with a view to identifying potential targets for therapeutic intervention. Recent clinical studies have proposed a number of different therapeutic approaches. These are often based on empirical, clinical observations rather than on basic science. One of the areas that this study was specifically designed to address, was the molecular basis for the potential use of NO donors in clinical practice.

Principally this study has investigated the expression of nitric oxide synthase, and the putative roles of nitric oxide in gestational tissue. The relationship of COX expression to gestational age and labour onset has also been investigated. The inter-relationship between these two genes in both gestational tissue and in an *in vitro* cell model system has been investigated, with respect to both their mechanisms of induction and putative interactions. Finally, the influence of nitric oxide and nitric oxide donors on spontaneous myometrial contractility was investigated in an *in vitro*, fetal membrane-uterine muscle dual chamber system.

5.2 NOS expression in pregnancy

5.2.1 Fetal membranes

Nitric oxide synthase mRNA expression was demonstrated by RT-PCR in fetal membranes (Dennes et al., 1997). The neuronal isoform (nNOS) was not investigated in fetal membranes as this isoform represents a constitutive NOS isoenzyme, and whilst its expression has been identified in skeletal muscle, it was thought unlikely to contribute to NO generation in fetal membranes. In terms of relative levels of NOS RNA in fetal membranes, data from cycle profiles suggest a higher concentration of e/cNOS in chorion decidua compared to amnion and higher levels of iNOS expression in all tissues examined, with a higher concentration of iNOS mRNA in chorion decidua compared to amnion. Analysis of NOS expression in amnion and chorion decidua showed no significant correlation with either labour onset, or gestational age.

These findings concur with other studies suggesting that fetal membranes are capable of generating nitric oxide. Di-Iulio et al. (Di-Iulio et al., 1995) indirectly studied NOS activity in fetal membranes and placenta, by measuring the conversion of radio-labelled L-arginine to L-citruline. They identified NOS activity in amnion and placental tissue, but were unable to identify NOS activity in the chorion decidua. Furthermore there was no significant difference in NOS activity in either amnion or placenta, before or after labour onset. Evidence presented in this thesis has unequivocally demonstrated NOS mRNA in the chorion decidua, both at term and throughout gestation. The presence of NOS mRNA implies the potential for that tissue to generate NO. Whilst NOS mRNA might be subject to post-transcriptional regulation, it seems more likely that the observed differences seen between these two studies, in terms of the potential for chorion decidua to generate NO, is a function of different experimental approaches. Di Iulio et al. suggest that the absence of NOS activity seen in the chorion decidua might be the result of the presence of arginase in this tissue, limiting the availability of NOS substrate.

This study was unable to identify any changes in levels of expression of any of the NOS isoforms in association with gestational age. This is in contrast to Ramsay et al. (Ramsay et al., 1996), who studied NOS activity (again measured indirectly by the conversion of radio-labelled L-arginine to L-citruline) in villous trophoblast and myometrium. They found that that levels of NOS activity in villous trophoblast were relatively high during the first trimester with a significant fall in activity in the third trimester. Whilst these

observations might represent post-transcriptional regulation, they do not appear to be associated with changes in levels of NOS mRNA.

More recent evidence appears to support the data obtained on NOS RNA expression in fetal membranes. Thomson et al. (Thomson et al., 1997b) studied NOS activity, determined by conversion of radio-labelled L-arginine to L-citrulline, in homogenates of frozen fetal membranes (with attached decidua) and placenta. No difference was found in either the expression or enzyme activity of NOS in fetal membranes or placenta, before and during labour at term. These results suggest that, in contrast to animal studies, a decrease in NOS enzyme activity is not involved in the onset of parturition at term in the human.

The role of nitric oxide generated by fetal membranes remains speculative. The possibility that nitric oxide, generated in the fetal membranes, might contribute to myometrial quiescence in pregnancy has been proposed. Whilst NO diffuses rapidly, isotropically, in all directions from its source, its finite half-life of between 5 - 15 s limits its bioavailable range to between 150 - 300 μM (Lancaster, 1996). It is therefore unlikely that nitric oxide generated in the fetal membranes would be capable of diffusing across the feto-maternal interface to reduce myometrial tone. It is therefore more likely that nitric oxide exerts an autocrine, rather than a paracrine role in fetal membranes. Ticconi et al. (Ticconi et al., 1996) investigated the release of PGE_2 in response to exogenous NO in an *in vitro* model, using fetal membrane explants. They found that incubation of fetal disks with SNP increased PGE_2 production, suggesting that NO might stimulate PGE_2 release in these tissues.

An alternative role for NO in the placenta has been proposed. Sun et al. (Sun et al., 1997) investigated regulation of 11-beta-hydroxysteroid dehyrogenase (11-beta-HSD) activity in *in vitro* cultured human placental trophoblast and chorionic cell preparations. 11-beta-HSD is involved in the interconversion of cortisol and cortisone, and is thought to possibly control the passage of maternal glucocoticoids across the placenta. Two types of 11-beta-HSD have been identified: Type 1 has both oxidase and reductase activities, interconverting cortisol and cortisone. Type 2 has only oxidase activity, converting cortisol to cortisone. 11-beta-HSD activity in cultured human placental trophoblast is predominantly type 2, and is inhibited in a dose dependent manner by SNP. Type 2 mRNA expression was similarly inhibited. Partial inhibition with 8-bromo cGMP suggested that this effect was mediated at least partially through the cGMP pathway. In this respect NO appears to be mediating an autocrine effect within the placenta.

e/cNOS, the calcium-dependent, constitutively expressed NOS isoform is principally involved in homeostatic mechanisms, such as the maintenance of vascular tone. The observation in this study, that e/cNOS mRNA is not induced by labour or changes in gestational age, is therefore consistent with these concepts. This study has not compared levels between non-pregnant and pregnant subjects, which might have shown evidence of oestrogen-induced transcriptional up-regulation (Weiner et al., 1994b). In contrast iNOS is transcriptionally regulated by a wide number of cytokines and growth factors (de Vera et al., 1996). Labour is associated with an increase in cytokine production, throughout different feto-maternal compartments (Olah et al., 1996), and it is therefore more likely that labour would be associated in fetal membranes, with both an increase in iNOS expression and NO levels. It is possible that falls in NOS mRNA expression in association with labour onset, as seen in animal studies, might be obscured by iNOS mRNA induction, as a result of increased cytokine levels associated with labour. However the onset of labour in animals is also associated with an increase in cytokine production.

In situ hybridisation studies localised iNOS and e/cNOS expression in fetal membranes. Localisation of NOS expression in fetal membranes, in other studies, has been limited to immuno-localisation. Thomson et al. (Thomson et al., 1997b) identified expression of all three NOS isoforms in fetal membranes by immunocytochemistry. They found e/cNOS protein expression in the amniotic epithelium and the trophoblast, but absent in the chorionic connective tissue. nNOS protein was present in amnion, chorion and trophoblast. iNOS protein expressed was antibody dependent. Using iNOS affinity purified antibodies from Santa Cruz, as used for Western analysis in the study here, iNOS protein was weakly localised to the amniotic epithelial layer, and more strongly localised to the chorionic connective tissue. *In situ* hybridisation localised e/cNOS mRNA expression in the amnion epithelial layer, the reticular layer of the chorion and in the decidua. iNOS mRNA expression appeared to be greatest in the amniotic epithelial layer, the chorionic cytotroblast and decidua. The discrepancy between the results obtained from immunolocalisation studies and *in situ* hybridisation might reflect differing levels of sensitivity, rather than real differences in patterns of expression. *In situ* hybridisation is a highly sensitive technique, and the required prolonged exposure times (up to 30 days) imply very low levels of mRNA expression, which would suggest similarly low levels of NOS protein.

Eis et al (Eis et al., 1997) similarly investigated NOS protein expression in fetal membranes. They demonstrated co-localisation of iNOS with CD14 positive decidual macrophages and fibroblasts. They suggested that iNOS expression in fetal membranes is a function of macrophage and fibroblast infiltration, rather than actual expression in fetal membranes. The *in situ* studies presented here show iNOS expression within the amnionic mesenchyme, although co-localisation with CD14 was not determined.

Western analysis demonstrated both e/cNOS and iNOS protein expression in fetal membranes. e/cNOS protein was demonstrated in term placental trophoblast but not in amnion or chorion decidua at term. However e/cNOS protein was expressed in both amnion and chorion decidua at 14 weeks, but not at term suggesting a fall in e/cNOS protein at term. The presence of a clearly defined protein band with the e/cNOS positive control (ADP-sepharose extracted HUVEC) implied specific and appropriate protein-antibody binding. However, due to the presence of non-specific bands, and excess background, this relationship could not be analysed quantitatively.

iNOS protein was present in amnion, chorion decidua and placenta at term. iNOS Westerns were carried out using a commercially available antibody (Santa Cruz - #SC-649). Conflicting patterns of iNOS protein expression have been reported with different commercially available antibodies (Thomson et al., 1997b). In the study presented here iNOS expression, as determined by Western analysis, confirms the results obtained by Thomson et al., however the presence of multiple non-specific protein bands prevented analysis of iNOS protein expression in relation to labour onset and gestational age.

5.2.2 Myometrium

This study has demonstrated iNOS, e/cNOS and nNOS mRNA expression by RT-PCR, in second and third trimester myometrial tissue. Whilst no direct comparison can be made as to the absolute amount of RNA present, due to potential differences in PCR efficiency between the two genes, data from cycle profiles suggest a higher concentration of e/cNOS (x35) than iNOS (x39). There was no association between either the onset of labour or gestational age and iNOS, e/cNOS or nNOS mRNA concentrations (Dennes et al., 1999). Western analysis demonstrated e/cNOS protein in myometrial tissue. iNOS protein was not identified in myometrial tissue.

RNA data presented here supports evidence from previous studies analysing e/cNOS expression by both *in situ* hybridisation and immunocytochemistry, in non-pregnant uterine tissue (Telfer et al., 1995). Similarly iNOS-like immunoreactivity was been detected in human uterine tissue obtained in the first trimester of pregnancy (Telfer et al., 1997a). Bansal et al. (Bansal et al., 1997) investigated NOS expression in the uterus, both in non-pregnant samples, and pregnant samples in relation to labour and delivery. Using qualitative immuno-localisation and Western analysis they found iNOS protein expression present in samples throughout gestation, but absent in non-pregnant myometrium. Expression of iNOS was highest in myometrium of preterm, non-labour samples, and iNOS expression was observed to fall by 75% in association with preterm and term labour. However examination of the data presented in their study showed strong staining of bands with a molecular weight below that of iNOS, even in the positive control. Although this discrepancy was not discussed in their paper, this might possibly represent the result of prolonged boiling, resulting in hydrolytic cleavage of NOS proteins. Whilst Bansal's findings might represent post-translational modification, to date, post-translational iNOS modification has only been reported in murine macrophages (Pan et al., 1996), and has not been seen in humans.

Expression of all three NOS isoforms has recently been studied by immunocytochemistry and Western analysis, in both myometrium and myometrial cell culture (Bartlett et al., 1999). e/cNOS and iNOS proteins were not detected in human myometrial myocytes. Expression of e/cNOS was restricted to vascular endothelial cells, within the myometrium, and was not detected in myometrial cells cultured from term, non-labouring myometrium. nNOS protein was detected in myometrial samples throughout gestation and at term, although protein levels were low, and showed no association with gestational age, or labour onset.

Finally, and most recently, Norman et al. (Norman et al., 1999) studied NOS protein expression in non-pregnant and pregnant myometrium, by immunohistochemistry and Western blotting. e/cNOS and nNOS protein expression was compared in non-pregnant, preterm and term myometrial samples. This study found that e/cNOS expression was localised to myometrial smooth muscle cells and vascular endothelium. nNOS was localised to both myometrial smooth muscle cells and vascular endothelium. Western analysis demonstrated significantly higher levels of expression of both e/cNOS and nNOS

in preterm compared with non-pregnant samples. Levels of expression of both isoforms fell at term.

There therefore appears to be little consensus, in the published literature, regarding NOS protein expression in myometrium. Certainly NOS mRNA levels are low for all isoforms, suggesting low levels of NOS protein which might confound NOS protein studies. In addition the antibodies used in the study by Norman et al. for e/cNOS (Affiniti) and nNOS (Serotec) differed from those used by both Bansal and Bartlett et al. (e/cNOS, iNOS polyclonal antibodies - Santa Cruz, e/cNOS, iNOS monoclonal antibodies - Transduction). It is possible that these apparently conflicting results are the consequence of differences in antibody-protein specificity. Whilst human myometrium has the potential to generate endogenous NO, its capacity to generate NO remains largely unknown.

The possible role of endogenously generated NO remains obscure. Buhimschi et al. (Buhimschi et al., 1995) have identified an NO-cGMP relaxation pathway in the uterus, and proposed a role in maintaining myometrial quiescence. However, the role of this system in regulating myometrial contractility remains controversial. Yallampalli et al. (Yallampalli et al., 1994b) demonstrated in rats that SNP inhibited spontaneous contractions, in myometrial strips obtained from 18-day pregnant rats. Although no cGMP measurements were made in this study, it was concluded that this effect was mediated via cGMP. However there was an apparent latent period of between 15 - 20 min, between application of SNP (5 mM) and myometrial response, which ultimately resulted in complete cessation of myometrial contractions. Cyclic GMP levels are elevated by SNP in smooth muscle preparations in under a minute (Janis and Diamond, 1979), it therefore appears unlikely that the observed response to SNP was mediated via cGMP, rather this response may represent a non-specific toxic effect of high levels of SNP.

Hennan et al. (Hennan and Diamond, 1998) found in both pregnant and ovariectomized rats that SNP (5 mM) had no effect on spontaneous contractions in myometrial samples. SNP however elevated cGMP and cyclic GMP-dependent protein kinase (PKG) in the same samples, suggesting that the absence of any observed effects on myometrial contractility was not due to a failure of SNP-elevated cGMP to activate PKG. They concluded from this study that cGMP does not play an important role in the control of myometrial contractility in the rat uterus.

Kuenzi et al. (Kuenzli et al., 1998) studied the effects of NO donors and endogenous NO on myometrial contractility in monkeys. They found that CysNO (S-nitroso-L-cysteine) an NO donor both reduced spontaneous contractility, and elevated cGMP levels *in vitro*. However CysNO reduced myometrial contractility in the presence of guanylate cyclase inhibitors, suggesting that this effect was not mediated through an increase in cGMP. Furthermore analogues of cGMP had no significant effect on myometrial contractility. N^{ω}-nitro-l-arginine (L-NOARG) a competitive NOS inhibitor significantly increased spontaneous myometrial contractility. D-NOARG the inactive enatiomer had no effect. They conclude that in monkeys endogenous NO and CysNO reduce myometrial contractility in a cGMP-independent manner. The failure of cGMP analogues to influence myometrial contractility argues against a role for cGMP in the regulation of monkey uterine contractility. However increases in contractility seen with NOS inhibitors does suggest a role for NO in the regulation of contractile activity in the uterus.

The activity of nitric oxide may be modulated by biological mediators present in the uterus. Superoxide dismutase is responsible for scavenging superoxide anions, and as such prolongs the biological effect of nitric oxide. Changes in levels of superoxide dismutase might then influence nitric oxide activity. Similarly xanthine oxidase, the enzyme responsible for superoxide synthesis may reduce nitric oxide synthesis, by generating superoxide anions, which rapidly combine with nitric oxide, to form peroxynitrite. Telfer et al. (Telfer et al., 1997b) identified the presence of both super oxide dismutase and xanthine oxidase by immunocytochemistry in the uterus, placenta and fetal membranes. Whilst they were unable to identify any changes in activity or expression of either enzyme in association with labour, they suggest that the presence of these enzymes in the feto-placental system might be important both in the regulation of nitric oxide activity in pregnancy and in the maintenance of uterine quiescence.

In human myometrial strips the relaxant effects of SNP are well known (Norman et al., 1997), and similar results are reported here. A corresponding effect with 8-bromo cGMP implicates a role for cGMP in NO-mediated uterine relaxation in human myometrial tissue. There is convincing data that endogenous NO does not play a significant role in the control of human myometrial contractility during pregnancy. Jones et al. (Jones and Poston, 1997) found that neither L-arginine or L-NAME influenced spontaneous myometrial contractility.

In summary then, in human pregnancy NO donors appear to reduce myometrial contractility. Whilst cGMP has been traditionally regarded as the secondary messenger for

NO in smooth muscle, NO may be acting via some other mechanism, to reduce myometrial contractility. The data on NOS protein expression in myometrium is conflicting. Myometrial tissue possesses the potential to generate NO, however endogenous NO does not appear to play an important role in the control of human myometrial contractility during pregnancy. It therefore seems unlikely that NO contributes to myometrial quiescence. The absence of changes in levels of NOS mRNA and NOS protein, prior to labour suggest, that the initiation of labour is not a result of NO withdrawal.

Myometrial biopsies obtained in these studies in pregnancy, were all taken from the lower uterine segment (uterine incision) at caesarean section. The lower uterine segment develops more rapidly late in pregnancy and undergoes significant changes at term and close to labour, when it becomes thinner and the relative smooth muscle content falls. In labour, it stretches significantly to allow passage of the fetus, but probably plays a small role in generating uterine contractions and in increasing uterine pressure to expel the fetus. A number of physiological differences have been observed between the upper and lower uterine segments. Fuchs et al. (Fuchs et al., 1984) identified an oxytocin receptor concentration gradient between the cervix, lower segment and fundus. They found that oxytocin receptor concentrations in the fundus and corpus were similar, and significantly higher than in the lower part of the uterine segment. They concluded that these observed changes were consistent with a functional role of endogenous oxytocin in the uterus during pregnancy and labour. More recently (Sparey et al., 1999) the spatial expression of a number of different contraction-associated proteins in the uterus has been investigated. Whilst connexin-43 protein appears to be expressed at much greater levels in the upper uterine segment compared to the lower, myometrial COX-1 and COX-2 proteins were expressed at greater levels in the lower segment compared to the upper. Therefore whilst current ethical considerations have limited analysis in this thesis to samples from the lower uterine segment it may be reasonable to extrapolate these results to include the uterus as a whole. Furthermore, Luckas et al. (Luckas and Wray, 2000) analysed the contractile characteristics of myometrium taken from upper and lower uterine segments in an organ bath system. They found no significant differences in the contractile rate and force production produced by myometrium from the upper and lower segments, and concluded that there was no apparent functional regionality in terms of contractility, within the uterus. The regional functionality of NOS expression in the human uterus has not been clearly determined although there is evidence from animal studies, that there is differential

regulation of NO in the uterus and cervix during pregnancy and labour (Buhimschi et al., 1996), and it is therefore possible that there may also be differential NOS expression throughout the human uterus.

Partial thickness biopsies were obtained in this study to specifically exclude decidual tissue, however iNOS expression may still be a function of macrophage or lymphocyte expression, rather than true myocyte expression. Similarly e/cNOS expression may be a result of expression in vascular endothelial cells within the myometrial biopsy.

Clinical studies have provided evidence that in addition to NO regulation, the role of endogenous NO might alter between distinct areas of the uterus. Thomson et al. (Thomson et al., 1997a) investigated the use of nitric oxide donors (glyceryltrinitrate and isosorbide mononitrate) to ripen the cervix prior to surgical termination of pregnancy. When administered vaginally in the first trimester, they found that topical NO donors could induce effective cervical ripening. These findings were confirmed in a subsequent prospective randomised controlled trial (Thomson et al., 1998), where topical NO donors were found to ripen the cervix, with less associated systemic side effects than prostaglandin analogues. Therefore whilst the potential exists that NO donors might relax uterine smooth muscle contractility, this may be associated with cervical ripening. This observation would limit the tocolytic potential for NO donors, as cervical ripening is clearly undesirable in the presence of threatened preterm labour. Chwalisz et al. (Chwalisz and Garfield, 1998) have considered this apparent dual effect of NO donors on the myometrium. They propose a model for cervical ripening, where NO, induced as a result of cytokine induction, is the final metabolic mediator, acting at the end of the ripening cascade. The actual effects of NO on the cervix are not fully understood, although it is likely that NO is involved in an increase in vascular permeability, changes in glycosaminoglycan synthesis, and apoptosis. They suggest that the pro-inflammatory effects of NO might be amplified, by induction of COX-2 by NO, to induce prostaglandin synthesis. They suggest that the differential effects of NO donors administered both topically and systemically maybe a result of drug bioavailability, and observe that there have been no reports (to date) of adverse effects on the cervix (cervical ripening) with transdermal NO donors. However whilst this paradox remains unexplained the clinical use of NO donors in the management of preterm labour cannot be supported.

5.2.3 Myometrial Cell culture

The somewhat ambiguous results obtained with *in situ* hybridisation for iNOS mRNA, and conflicting published immuno-localisation data prompted a different experimental strategy. Since myometrial biopsies contain a relatively heterogeneous cell population, despite attempts to macroscopically avoid vascular and connective tissue, the possibility of developing a cell culture model was explored. Primary myometrial cell cultures were obtained which had been established in culture from myometrial explants. Confirmation of cell phenotype was by inspection of morphology, and staining with a mono-clonal anti-smooth muscle α–actin antibody. Myometrial smooth muscle cell culture therefore provided a homologous myometrial cell culture, to which sensitive RT-PCR could be applied, and in which gene expression could then be confidently localised to the myocyte.

Analysis of both stimulated and non-stimulated myometrial cell cultures provided evidence for the presence and induction of NOS mRNA. In non-stimulated culture neither iNOS or e/cNOS mRNA could be identified by RT-PCR. This suggests that in non-stimulated myocytes iNOS and e/cNOS are not expressed. This would further support the hypothesis that iNOS and e/cNOS expression seen in (homogenised) myometrial tissue by RT-PCR is a reflection of the presence of both inflammatory cell types (monocytes and macrophages) and vascular endothelium.

Stimulated myometrial cell culture cells did however demonstrate that myocytes are capable of expressing iNOS mRNA, following appropriate cytokine induction. This observation that iNOS can be induced provides little information on the possible physiological role of iNOS as most cell types appear to be able to express iNOS if subject to cytokine induction. iNOS mRNA was stable following 24 hr stimulation with IL-1ß, but absent at 48 hr showing that iNOS mRNA maybe relatively unstable.

An alternative explanation for the absence of iNOS mRNA at 48 hrs might be negative modulation of NF-kB by IkB. Data presented in this thesis suggests that IL-1ß stimulates both COX-2 and iNOS mRNA transcription via NF-kB. NF-kB when present in the cell cytoplasm is bound to IkB, an inhibitory molecule, forming an inactive complex. Cytokine induction (IL-1ß) results in dissociation of the cytoplasmic complexes and translocation of free NF-kB into the nucleus. Two major forms of IkB have been identified, IkB-α and IkB-β. IkB-α regulates NF-kB through a feedback loop. Induction of NF-kB results in phosphorylation and rapid loss of IkB-α protein through proteolysis. However induced nuclear NF-kB mRNA results in subsequent up-regulation of IkB-α mRNA levels, due to

the presence of NF-kB sites in the IkB-α promoter. Newly synthesised IkB-α mRNA is translated, and accumulated IkB-α protein shuts down the NF-kB response, ensuring that responsive genes are only activated transiently. More recently there has been evidence to suggest that cytokine induction with an alternative class of inducers, such as LPS, results in both IkB-α and IkB-β activation (Thompson et al., 1995). NF-kB is unable to induce transcription of IkB-β mRNA, unlike IkB-α, suggesting that NF-kB released from IkB-β is not down-regulated. Activation of NF-kB with 'persistent' activators (LPS) results in release of NF-kB from IkB-α and then from IkB-β complexes. This biphasic response would lead to an initial transient phase, mediated through IkB-α and a persistent phase mediated through IkB-β. In these studies iNOS mRNA is present at 24 hrs and not 48 hrs, suggesting that NF-kB activation by IL-1ß is via IkB-α.

e/cNOS was not detected in stimulated myometrial cell culture confirming the constitutive nature of this gene.

nNOS mRNA appeared to be expressed constitutively in myometrial cell culture, confirming its presence in myocytes, The role of nNOS in the myometrium is speculative. A neurotransmitter role has been proposed for nNOS (Sheng et al., 1992), suggesting a possible non-adrenergic, non-cholinergic innervation of the uterus. However if this was the case it might be anticipated that nNOS expression would be confined to neuronal tissue and not the myocyte itself. Furthermore in this study there was no association of nNOS expression (in myometrial samples) with either labour onset or gestational age, and this combined with the constitutive nature of this gene tend to suggest that it is unlikely to be involved in the initiation of labour.

5.3 COX expression in pregnancy

5.3.1 Fetal membranes

COX mRNA expression in fetal membranes at term, and changes in association with labour onset have been demonstrated previously (Slater et al., 1995). COX-2 and COX-1 mRNA was expressed in amnion. COX-2 mRNA in amnion increased in association with labour, suggesting that it is the inducible COX-2 isoform that mediates an increase in prostaglandin synthesis with labour. In this study the expression of both COX-1 and COX-2 mRNA was investigated in amnion and chorion decidua samples collected throughout gestation, with the purpose of identifying any possible change in levels of expression throughout pregnancy and at term, prior to labour onset.

Tissue was routinely available from first trimester termination of pregnancy or third trimester preterm and term delivery. However due to clinical limitations only a small number of samples were available between sixteen and twenty five weeks. Western analysis was therefore confined to samples collected from the first trimester and at term (before and after labour).

COX-1 mRNA expression in both amnion and chorion decidua remained largely unchanged throughout gestation. There was similarly no apparent change in COX-1 protein concentrations between the first trimester, pre-labour (term) and post-labour (term). Since COX-1 is considered a constitutively expressed gene these observations are consistent with our current hypothesis that the increase in prostaglandin seen at term, with labour onset is the result of an increase in COX-2 mRNA expression (Hirst et al., 1995).

COX-2 mRNA expression in amnion increases significantly at term compared with the first trimester. Western analysis of COX-2 protein, in both amnion and chorion decidua, showed increases in protein concentration between the first and third trimester and before and after labour, paralleling observations at the mRNA level.

Western analysis suggests similar levels of COX-1 and COX-2 protein in fetal membranes, although absolute levels were not determined due to potential differences in the affinity of the two COX antibodies for their epitopes. However in terms of relative levels of COX mRNA expression, cycle numbers derived from pooled amnion and chorion samples suggest higher levels of COX-2 mRNA (31 cycles in amnion, 33 cycles in chorion) compared with COX-1 mRNA (35 cycles in both amnion and chorion). This apparent

discrepancy between COX mRNA and protein might be explained in terms of COX enzyme kinetics. COX has been considered 'a suicide enzyme', undergoing destruction during catalysis (Marshall et al., 1987). Increased prostaglandin synthesis via COX-2 would therefore mean increased turnover of the enzyme itself. Increased COX-2 protein turnover would necessarily require increased COX-2 mRNA concentrations. However due to simultaneous COX-2 destruction, protein levels at any one time would be increased to a lesser extent than mRNA levels. Conversely if the enzyme is not active, as is the case for COX-1 in fetal membranes, COX-1 protein levels may be similar to COX-2 whilst mRNA concentrations would be low.

The relative contribution of COX-1 and COX-2 to prostaglandin synthesis in fetal membranes has been investigated using selective COX inhibitors (Sawdy et al., 2000). COX-1 and COX-2 protein was demonstrated by Western analysis in amnion and chorion decidua at term. Prostaglandin synthesis in intact fetal membrane discs were inhibited by either a COX-1 (SC-560) or COX-2 (SC-236) selective inhibitor. The COX-2 selective inhibitor significantly reduced prostaglandin synthesis both in its COX-2 specific and higher, non-selective concentration ranges. The COX-1 selective inhibitor had no significant effect on prostaglandin at COX-1 selective concentrations, but did significantly reduce prostaglandin synthesis at higher non-selective (COX-1 and COX-2) concentrations. Therefore whilst fetal membranes appear to express both COX-1 and COX-2 mRNA and both COX-1 and COX-2 protein is present, it is the COX-2 isoform that contributes to prostaglandin synthesis. This observation has prompted increased interest in the use of COX-2 selective inhibitors in the management of preterm labour (Sawdy et al., 1997).

COX enzyme activity was determined in this study, in fetal membranes, by measuring PGE_2 synthesis in intact fetal membrane disks incubated in medium containing arachidonic acid. Whilst this does not represent a direct measurement of COX activity, by providing arachidonic acid, the synthesis of prostaglandins is independent of arachidonic acid release by phospholipase. Since COX enzyme activity is the other rate limiting step of prostaglandin synthesis, this experimental design provides information on COX activity within the fetal membrane samples. The relative contributions however, of COX-1 and COX-2 were not determined. The chorion is a major source of prostaglandin dehydrogenase (PGDH), which catalyses the conversion of prostaglandins to inactive metabolites (Cheung and Challis, 1989). The concentration of PGE_2 in the culture medium

of fetal membrane explants is determined by the balance between prostaglandin release and prostaglandin metabolism. PGE_2 synthesis in the chorion decidua detected in this system is therefore likely to not only represent the result of COX activity but also PGDH inactivation.

Prostaglandin synthesis in separated fetal membranes disks was greater in the amnion compared with the chorion decidua and placenta, probably as a result of PGDH activity. In this study prostaglandin synthesis in the amnion did not change significantly with gestational age, however prostaglandin synthesis increased (but not significantly) at term and further increased with labour onset. These data support evidence from enzyme kinetic studies, suggesting that in amnion the increase in COX activity with the onset of labour is due to an increase in synthesis of COX enzyme (Smieja et al., 1993). Further evidence to support increased COX activity and prostaglandin synthesis in the amnion at term is provided by RNA data (section 4.3.1). Analysis of COX mRNA by Northern analysis confirmed that the increase in prostaglandin synthesis seen in fetal membranes with the onset of labour is a result of an increase in COX mRNA expression (Bennett et al., 1992). The increase in prostaglandin synthesis in amnion in association with labour was subsequently shown to be a result of up-regulation of the type 2 COX isoform (Slater et al., 1995).

The impact of both region and labour on prostaglandin synthesis in human fetal membranes has been examined using a similar *in vitro* system (Sawdy et al., 1999). Regional fetal membrane prostaglandin synthesis was examined to investigate any possible association between rupture site and PG synthesis. Fetal membranes were collected, and disks obtained from the cervical region (close to the site of spontaneous rupture); the periplacental region; and a region midway between the two, following spontaneous labour and delivery, or at elective caesarean section prior to labour. In the presence of exogenous arachidonic acid prostaglandin synthesis increased with labour onset, although no regional difference in prostaglandin synthesis was identified.

5.3.2 Myometrium

In this study both COX protein and mRNA expression have been analysed in human myometrium in relation to labour onset and throughout gestation. COX-1 and COX-2 mRNA expression was identified in all myometrial samples. There was no correlation between COX-1 mRNA expression and gestational age or labour status. A similar pattern

was shown with COX-1 protein. In contrast COX-2 mRNA increased significantly at term, and further increased with labour onset. COX-2 protein expression increased by 33 - 37 weeks gestation, relative to the preterm samples, and showed a further significant increase at term. The fact that COX-2 mRNA expression shows significant up-regulation in myometrium late in pregnancy and in association with labour suggests that COX-2 might represent one of the 'cassette of contraction-associated proteins'.

Western analysis showed increases in COX-2 protein concentrations between preterm and term tissues, paralleling that seen at the mRNA level. The differences in expression at the protein level were not as great as those seen at the mRNA level and appeared to occur at earlier gestational ages. These observations parallel those seen in fetal membranes, where similar enzymes kinetics would explain the apparent discrepancy between COX mRNA and protein levels. During prostaglandin synthesis, COX is destroyed after a limited number of reactions, resulting in an effectively short half life. Any stimulus to prostaglandin production must therefore also increase synthesis of new COX protein, to replace that destroyed during catalysis. It is possible that the increase in COX-2 protein between 33 and 37 weeks, without an associated increase in mRNA, represents the accumulation of protein in anticipation of an increase in prostaglandin synthesis, associated with labour at term. Synthesis of oxytocic prostaglandins, during labour, would consume COX protein whose replacement would require either increased COX gene transcription, or increased mRNA stability. This hypothesis would be consistent with the observation that there is a greater increase in mRNA compared with protein at term.

In myometrium the principle prostaglandin synthesised is prostacyclin (Bamford et al., 1980). In this study COX-1 mRNA is expressed with a similar abundance to COX-2 in myometrium. In contrast, in the fetal membranes COX-2 expression greatly exceeds that of COX-1 (Slater et al., 1994). Expression of the 'constitutive' COX-1 enzyme in myometrium probably reflects constitutive prostacyclin synthesis. Prostacyclin is a vasodilator and may contribute to the necessary increased myometrial blood flow during pregnancy. Myometrial COX-2 expression increases with a pattern similar to that of the chorion decidua, suggesting a possible common mechanism of control. Zuo et al. (Zuo et al., 1994) used *in situ* hybridisation and immunocytochemistry to show that human pregnant myometrium expresses both COX-1 and COX-2. They found that COX-1 expression decreased with advancing gestational age, but did not change with labour, and that COX-2 expression increased with gestational age, but decreased with labour. Their

findings are not entirely consistent with the results presented here, however their studies were based upon qualitative scoring of histological sections, and did not allow for statistical analysis. However their data, both in addition to that presented here, and data from fetal membrane studies, showing increased COX-2 expression with the onset of labour (Slater et al., 1995) (Hirst et al., 1995) support the overall hypothesis that it is COX-2 and not COX-1, whose expression is increased in both the myometrium and the fetal membranes with the onset of labour, and which catalyses the formation of oxytocic prostaglandins.

5.3.3 Myometrial cell culture

COX-2 mRNA expression in myometrial tissue was confirmed by myometrial cell culture studies. COX-2 mRNA expression was demonstrated by RT-PCR in both cytokine stimulated, and non-stimulated cell cultures. In addition COX-2 message was found to be present following both 24 hr and 48 hr cytokine stimulation, showing that relative to iNOS mRNA, COX-2 mRNA appears either more stable or subject to different regulatory factors. COX-2 mRNA was present in non-stimulated cell culture, suggesting that some function of either the cell culture conditions or RNA extraction have led to COX-2 induction.

5.4 NOS regulation

Constitutive NOS expression has been shown to be subject to transcriptional regulation by oestrogen (Weiner et al., 1994b). However the regulation of e/cNOS and nNOS mRNA was not examined in this study as it was felt that the inducible (iNOS) isoform was more likely to be involved in an inflammatory process such as labour. Transcriptional regulation of iNOS is likely to be mediated via putative response element protein binding sites in its 5' promoter region. The iNOS gene has response element binding sites for γ–IFN (eight), NF-IL6 (one), and NF-kB (one) in its 1.1 kb, 5' promoter region (Nunokawa et al., 1994). γ–IFN is known to be one of the key factors which induce NOS in macrophages and vascular smooth muscle cells (Charles et al., 1993). Eight γ–IFN response elements are reported to be necessary for γ–IFN induction (Yang et al., 1990). NF-IL6 and NF-kB binding sites are required for cytokine induction by TNF-α, endotoxin and IL-1ß (Chu et al., 1998).

An *in vitro* WISH cell culture model was used to analyse IL-1ß cytokine induction of iNOS mRNA. iNOS message was up-regulated within 15 min, at an Il-1ß concentration of 1 ng/ml. Message remained present at 30 min, but was absent by 60 min, exhibiting the rapid inactivation and short half-life of iNOS mRNA in cell culture.

The relationship of NF-κB to IL-1ß induction in WISH cells was explored using NF-κB electrophoretic mobility shift assays (Molloy, 2000). Activation of the inducible transcription regulatory element, NF-κB is regarded as the mechanism of IL-1ß induction. NF-κB is predominantly associated with I-κB, a cytoplasmic inhibitory protein. Release of NF-κB from I-κB allows rapid transfer of NF-κB into the nucleus. Genes regulated by NF-κB can therefore be transcriptionally activated within minutes following induction. NF-κB electrophoretic mobility shift assays of IL-1ß stimulated WISH cells showed that NF-κB is increased rapidly in the nucleus, within 30 min of induction. NF-κB remains elevated until levels start to fall at 120 min. In this particular study (Fig. 4.65) nuclear NF-κB levels were elevated at both time zero and in the non-stimulated sample, suggesting that some function of either the culture conditions or nuclear protein extraction technique had resulted in activation of NF-κB, and transfer into the nucleus. If NF-κB activation is exclusively responsible for iNOS induction in WISH cells, nuclear NF-κB levels would rise prior to the observed iNOS mRNA induction seen by 15 min. iNOS induction by IL-1ß in WISH cells appears to be mediated by NF-κB, and results in rapid transcription of iNOS mRNA.

iNOS and COX-2 are both subject to induction by IL-1ß, and this appears to be mediated by NF-κB. This results in rapid induction of both genes, making them both 'primary response genes' (Herschman, 1991). In both fetal membranes and myometrium this study has shown that COX-2 mRNA is up-regulated with the onset of labour, and in the case of myometrium, COX-2 mRNA is also up-regulated at term. In contrast, and in fact in identical tissue samples, no association has been found in either fetal membranes or myometrium between iNOS mRNA and both the onset of labour and gestational age. Labour is regarded as an acute inflammatory reaction associated with an increase in inflammatory cytokines throughout different materno-fetal compartments (Olah et al., 1996). COX-2 up-regulation in association with labour might therefore be expected in view of its capacity for cytokine induction. In contrast iNOS mRNA up-regulation was not observed. Since both genes share common protein binding sites for NF-κB in their 5' promoter regions it may be that the regulation of iNOS transcription in gestational tissue is not mediated via NF-κB. Recently there has been evidence to suggest that iNOS transcription may not be solely dependent on 5' promoter activity (Nunokawa et al., 1997). Nunokawa et al. designed luciferase reporter constructs containing the iNOS 5'-flanking region and 3'-untranslated region (UTR). They found that the construct containing both 5'-flanking region and 3'-UTR, plus its downstream region cancelled the constitutive activity of the reporter gene, and was more responsive to the cytokine stimulation. It is possible that the 3'-untranslated region (UTR) might modulate both iNOS mRNA transcription and mRNA stability in these gestational tissues (Shaw and Kamen, 1986).

5.5 Inter-relationship between NOS and COX

The possible inter-relationship of NOS and COX was investigated in this study *in vitro* using a WISH cell culture model. It was established that WISH cells, following appropriate cytokine induction would express both iNOS and COX mRNA and generate PGE_2 and NO. The interactions of NOS and COX may occur at a number of different levels. In their transcriptional control, in protein translation or in terms of their enzyme activity.

In this study PGE_2 synthesis was induced in WISH cells following cytokine induction with IL-1ß (1 ng/ml). IL-1ß stimulation is associated with an increase in COX-2 mRNA. This occurs early after stimulation and COX-2 mRNA levels can be seen to rise within 30 min of IL-1ß stimulation. Analysis of COX activity in WISH cells, determined indirectly by the generation of endogenous PGE_2 has shown that an increase in PGE_2 levels can be detected by RIA at 4 hrs. These data suggest not only that increased PGE_2 synthesis is a result of increased COX-2 activity, following COX-2 mRNA transcription, but also confirm the temporal relationship between COX-2 mRNA up-regulation and COX-2 protein synthesis. Indomethacin, as expected inhibited PGE_2 synthesis in cytokine stimulated WISH cells in a dose response manner. However since indomethacin is a non-specific inhibitor of COX, the relative contribution of COX-1 and COX-2 to PGE_2 synthesis in this system was not deduced.

IL-1ß stimulation of WISH cells is also associated with up-regulation of iNOS mRNA. iNOS mRNA levels increased rapidly after IL-1ß stimulation, with significant levels detectable by RT-PCR at 15 and 30 min. iNOS mRNA levels subsequently rapidly fall and become undetectable by 60 min, suggesting iNOS mRNA is unstable in WISH cell culture. NOS activity in WISH cells was determined by measuring nitrite levels. Nitrite levels rise rapidly (and significantly) by 15 min, and subsequently return to control levels by 60 min. NOS activity therefore correlates closely with iNOS mRNA data, suggesting that the change in NOS activity is a reflection of iNOS transcription and subsequent NOS protein translation. The influence of PGE_2 on NOS activity was investigated by inhibiting endogenous prostaglandin synthesis with indomethacin. Indomethacin appeared to have no significant effect on nitrite production by IL-1ß stimulated WISH cells, at concentrations where COX-2 activity was almost completely inhibited. These data support evidence from other authors (Salvemini and Masferrer, 1996), who have found that in LPS-stimulated murine macrophages (RAW 264.7) indomethacin (0.01 - 10 μM) inhibited PGE_2

synthesis, but not NO production. Furthermore, MTT data suggests that WISH cells remained viable at all indomethacin concentrations, and that the reduced NO levels (with increasing indomethacin concentrations) were therefore unlikely to represent cell damage. The observed fall in nitrite levels at an indomethacin concentration of 100 μM, whilst not statistically significant, might represent an indirect effect of indomethacin on calcium-dependent NOS activity. Sawdy et al. (Sawdy et al., 1998) have shown that indomethacin modulates myometrial contractility not only by inhibiting COX activity (COX-1 and COX-2) but also by inhibition of voltage-gated Ca^{2+} channel currents, resulting in decreased intra-cellular calcium levels. The observed fall in nitrite levels, at indomethacin concentrations in excess of 100 μM might reflect decreased calcium-dependent NOS activity (e/cNOS and nNOS). However it is likely that the majority of NO generated in response to IL-1ß in WISH cells would result from induction of the calcium-independent NOS isoform (iNOS) rather than the constitutively expressed, calcium-dependent, isoforms.

The influence of endogenous NO on COX enzyme activity was studied in cytokine-stimulated WISH cells in the presence of L-NAME, a non-specific NOS inhibitor. L-NAME, at a concentration of 1 mM significantly inhibited PGE_2 synthesis. Treatment with D-NAME, the inactive enantiomer showed no effect on PGE_2 synthesis, confirming that the observed results with L-NAME were not a result of non-specific activity. It therefore appears that in this system endogenous NO generated as a result of cytokine induction increases PGE_2 synthesis. These data would support evidence from published studies. Salvemini et al. (Salvemini et al., 1993) found in the mouse macrophage cell line when stimulated with LPS (1 μg/ml for 18 hr) that L-NAME (1 - 300 μM) inhibited PGE_2 and (nitrite synthesis) in a concentration-dependent manner. In addition they found that in cells pre-stimulated with LPS (1 μg/ml for 18 hr) exogenous NO (generated from SNP or GTN - 50 - 200 μM) in the presence of arachidonic acid enhanced the production of PGE_2. In addition Franchi et al. (Franchi et al., 1994) studying eicosanoid synthesis in oestrogen-treated rat uteri, found that prostaglandin synthesis was markedly stimulated with SNP (100 μM) and inhibited by L-NMMA. In the study presented in this thesis, exogenous NO generated from SNP had no effect on either PGE_2 release or COX activity (in the presence of arachidonic acid) in unstimulated WISH cells, at any concentration below the toxic range. This suggests that NO might only increase PGE_2 synthesis following cytokine induction. Cytokine induction induces COX-2 mRNA, and it is possible that following

appropriate induction NO acts directly on newly synthesised COX-2 protein, to increase PGE_2 synthesis. However in contrast to these findings, Dong et al. (Dong and Yallampalli, 1996) found that NO donors (DETA-NO) increased PGE_2 synthesis in uterine tissue obtained from (day 18) pregnant rats in a concentration-dependent manner, in the absence of obvious cytokine induction. Day 18 represents a preterm pregnancy in rat gestation, so that cytokine events associated with term gestation and labour onset, are likely to have been confounding factors in this study. However the authors concede that levels of PGE_2 detected at day 18, in the presence of elevated NOS activity, might reflect lower levels of COX protein rather than modulation of COX activity. Furthermore Salvemini et al. (Salvemini et al., 1995) found that in rats, *in vivo*, exogenous NO (generated from either SNP or GTN) was associated with an increase in prostaglandin synthesis, but COX-2 mRNA or protein is not found in the same organs obtained from normal animals (Seibert and Masferrer, 1994). The authors therefore concluded that this increase in prostaglandin synthesis was a result of activation of COX-1, rather than COX-2.

Conflicting results were published by Swierkosz et al. (Swierkosz et al., 1995) who studied the co-induction of NOS and COX *in vitro* in murine J774.2 macrophages. They found that in LPS (1 ng/ml) stimulated macrophages SNP (0.1 - 1000 μM) inhibited both prostaglandin synthesis and COX-2 activity. Furthermore L-NMMA (1 μM - 10 mM) increased prostaglandin synthesis in LPS treated macrophages in a concentration-dependent manner. They conclude that high levels of (exogenous) NO (generated by SNP) inhibit COX activity, whilst low levels of (endogenous) NO (inhibited by L-NMMA) increase COX activity. They acknowledge that this data conflicts with other published studies, and suggest that the influence of NO on prostanoid synthesis may be dependent on both the cell type investigated, the rate of prostanoid synthesis, the state of cell activation and the levels of COX-2 and iNOS proteins present. They suggest that mechanistically the interaction of NO with COX is more likely to result in inhibition of COX due to the ability of NO to reduce the ferric-active form of COX to the ferrous-inactive form (Kanner et al., 1992), or to nitrosylate tyrosine groups within the COX enzyme (Shimokawa et al., 1990).

The presence of a haem-iron moiety in the COX active site, and evidence that NO interacts with iron-containing enzymes (Moncada et al., 1991) provides a mechanistic basis for the interaction NO and COX enzyme. Salvemini et al. (Salvemini et al., 1993) propose that the

interaction between NO and COX might represent a regulatory step at which interactions between pro- and anti-inflammatory mediators occur. NO, by modulating COX protein activity and PGE_2 synthesis may represent a mechanism by which an initial inflammatory response might be amplified or attenuated. Salvemini's study however involved the investigation of PGE_2 synthesis in response to both exogenous and endogenous NO in cytokine stimulated mouse macrophage cell line (RAW 264.7). Since these cells represent an inflammatory cell type their response to cytokine stimulation might differ from that in WISH cells, an epithelial-derived amnion cell type. Thus whilst NO might induce PGE_2 synthesis in an inflammatory process, the response of gestational tissue to a similar stimulus appears to have no effect on PGE_2 synthesis.

5.6 The role of NO in myometrial contractility

The possibility that NO is responsible for maintaining myometrial quiescence during pregnancy, and that changes in NO in association with labour might modulate the changes in myometrial contractility seen with the onset of labour was investigated *in vitro* using a fetal membrane-uterine muscle dual chamber system designed and developed in our department.

Pilot data from our group, investigating the effects of fetal membranes on myometrial contractility, suggested that fetal membranes when apposed to myometrial strips, in this system, both increase latent time to onset of spontaneous contractions and reduce Mean integral tension (MIT) (Robert Sawdy, personal communication). The orientation of the fetal membranes to the myometrial strip did not influence these effects. These data suggested that the fetal membranes were inhibiting myometrial contractility through some mechanism.

Collins et al. (Collins et al., 1993) using a dual chamber-fetal membrane-uterine muscle model, investigated the effects of the presence of fetal membranes on myometrial contractility *in vitro*. Whilst this study was limited by the fact that they analysed the effects of human fetal membranes on oestrogenised rat myometrial strips, they found that fetal membranes caused a 40% decrease in uterine contractions. They concluded that fetal membranes inhibit uterine contractility, and suggested that this might contribute to myometrial quiescence in pregnancy. Nitric oxide is a potent mediator of smooth muscle relaxation. It is therefore possible that the observed reduction in myometrial contractility seen in these experiments in the presence of fetal membranes might be mediated by NO generated in the fetal membranes. Whether NO generated in the fetal membranes would be capable of diffusing across the feto-maternal interface to reduce myometrial tone is unknown. However the finite half-life of NO of between 5 - 15s limits its bioavailable range to between 150 - 300 μM (Lancaster, 1996). To test this hypothesis the effects of exogenous NO on myometrial contractility in this system were investigated using an NO donor (SNP). The possibility that these membrane effects might be mediated by endogenous NO generated in the fetal membranes was investigated in this system in the

presence of fetal membranes using an NOS inhibitor (L-NAME) and its inactive enantiomer (D-NAME).

SNP (1-100 μM) inhibited spontaneous myometrial contractility, in a concentration-dependent manner, and this confirms previous studies (Norman et al., 1997). These data confirm that nitric oxide is capable of reducing myometrial contractility *in vitro*, probably via an effect on cGMP levels. The presence of fetal membranes in the dual chamber system inhibited spontaneous myometrial contractility. The addition of both L-NAME and D-NAME in crossover studies had no effect on myometrial contractility (MIT), compared to control strips. Inhibition of endogenous NO synthesis in either fetal membranes or myometrium therefore had no demonstrable effect on myometrial contractility. Therefore whilst the presence of fetal membranes, in close apposition to spontaneously contracting myometrial strips reduces myometrial contractility and even prevents the establishment of spontaneous contractions, inhibition of NOS does not appear to affect this response, suggesting that nitric oxide is not responsible for the observed changes in myometrial contractility seen in the presence of fetal membranes. Similarly the orientation of the fetal membranes to the circulating perfusate (either amnion or chorion decidua) had no impact on any observed effects.

5.7 Summary

In summary this thesis has investigated at a molecular level the expression and regulation of nitric oxide and prostaglandins in human fetal membranes and myometrial tissue.

NOS mRNA expression has been demonstrated in fetal membranes and myometrium by RT-PCR, however there was no difference in levels of expression throughout gestation or with the onset of labour. *In situ* hybridisation localised iNOS and e/cNOS expression in fetal membranes. In myometrium e/cNOS mRNA expression was localised to vascular tissue. Cell culture studies demonstrated that neither e/cNOS or iNOS mRNA is expressed in myometrial smooth muscle cell culture. Western analysis confirmed the presence of NOS protein in fetal membranes. Whilst myometrial contractility studies have shown that exogenous nitric oxide inhibits myometrial contractility *in vitro*, NOS mRNA data from this thesis suggests that in contrast to animal studies endogenous nitric oxide synthesis does not change throughout gestation or with labour onset, suggesting that in humans, nitric oxide is not responsible for the physiological onset of labour.

COX mRNA expression was demonstrated in fetal membranes and myometrium. COX-2 mRNA and protein concentrations in fetal membranes and myometrium increased throughout gestation, and in myometrium increased further with labour onset. COX-1 mRNA and protein showed no significant change in relation to gestation or labour onset. These data confirm the role of COX-2 in prostaglandin synthesis in gestational tissue, and provide a sound basis for the further development of selective COX-2 inhibitors for use in the management of preterm labour.

COX-2 and iNOS regulation was investigated in Il-1ß-stimulated WISH cells, and appears to be mediated via the transcription factor NF-kB. iNOS mRNA is only transiently elevated after Il-1ß stimulation, suggesting possible regulation via IkB-α. Conversely COX-2 mRNA remains elevated at 48 hrs suggesting alternative regulatory factors.

The inter-relationship of NOS and COX was investigated in cell culture. Inhibition of endogenous nitric oxide synthesis reduced prostaglandin synthesis in Il-1ß stimulated WISH cells.

Myometrial contractility studies have shown that exogenous nitric oxide reduces myometrial contractility. Whilst the presence of co-incubation with fetal membranes reduces myometrial contractility, this effect is not mediated via nitric oxide. The viability of

mRNA in these experiments have been confirmed by the presence of mRNA suitable of RT-PCTR analysis after three hours of experimental conditions. The application of applied tension to individual myometrial strips appears to induce COX-2 mRNA, raising the possibility that physiological stretch late in pregnancy may be one of the factors responsible for the observed up-regulation of COX-2 mRNA, and subsequent labour onset.

6. REFERENCES

(1990) European Community collaborative study of outcome of pregnancy between 22 and 28 weeks' gestation. Working Group on the Very Low Birthweight Infant. *Lancet.* **336:** 782-784.

(1992) Treatment of preterm labor with the beta-adrenergic agonist ritodrine. The Canadian Preterm Labor Investigators Group. *N Engl J Med.* **327:** 308-312.

Akerlund, M., Stromberg, P., et al. (1987) Inhibition of uterine contractions of premature labour with an oxytocin analogue. Results from a pilot study. *Br J Obstet Gynaecol.* **94:** 1040-1044.

Alvi, S. A., Rajasingam, D., et al. (1999) The production of interleukin-1beta from human fetal membranes is not obligatory for increased prostaglandin output. *Immunology.* **97:** 249-256.

Ammala, M., Nyman, T., Salmi, A. and Rutanen, E. M. (1997) The interleukin-1 system in gestational tissues at term: effect of labour. *Placenta.* **18:** 717-723.

Asboth, G., Phaneuf, S., et al. (1996) Prostaglandin E2 activates phospholipase C and elevates intracellular calcium in cultured myometrial cells: involvement of EP1 and EP3 receptor subtypes. *Endocrinology.* **137:** 2572-2579.

Bakketeig, L. S. and Hoffman, H. J. (1981) In *Preterm labour*(Eds, Elder, M. G. and Hendricks, C. H.) Butterworth's International Medical Reviews. London.

Bamford, D. S., Jogee, M. and Williams, K. I. (1980) Prostacyclin formation by the pregnant human myometrium. *Br J Obstet Gynaecol.* **87:** 215-218.

Bansal, R. K., Goldsmith, P. C., et al. (1997) A decline in myometrial nitric oxide synthase expression is associated with labor and delivery. *J Clin Invest.* **99:** 2502-2508.

Barden, T. P., Peter, J. B. and Merkatz, I. R. (1980) Ritodrine hydrochloride: a betamimetic agent for use in preterm labor. I. pharmacology, clinical history, administration, side effects, and safety. *Obstet Gynecol.* **56:** 1-6.

Barnes, F. (1882) Hour-Glass contraction of the uterus treated with Nitrite of Amyl. *Br Med J.* **1::** 377.

Bartlett, S. R., Bennett, P. R., et al. (1999) Expression of nitric oxide synthase isoforms in pregnant human myometrium. *J Physiol (Lond).* **521 (Part 3):** 705-716.

Batra, S. (1985) Characterization of [3H]-nitrendipine binding to uterine smooth muscle plasma membrane and its relevance to inhibition of calcium entry. *Br J Pharmacol.* **85:** 767-774.

Bazan, N., Botting, J. and Vane, J. (Eds.) (1996) *New Targets in Inflammation: Inhibitors of COX-2 or Adhesion Molecules.* Kluwer Academic. London.

Beall, M. H., Edgar, B. W., Paul, R. H. and Smith, W. T. (1985) A comparison of ritodrine, terbutaline, and magnesium sulfate for the suppression of preterm labor. *Am J Obstet Gynecol.* **153:** 854-859.

Benedetti, T. J. (1992) Treatment of preterm labor with the beta-adrenergic agonist ritodrine. *N Engl J Med.* **327:** 1758.

Bennett, P., Slater, D., Berger, L. and Moor, G. (1994) The expression of phospholipase A2 and lipocortins (annexins) I, II and V in human fetal membranes and placenta in association with labour. *Prostaglandins.* **48:** 81-90.

Bennett, P. R. and Elder, M. G. (1988) Extreme prematurity: the aetiology of preterm delivery. *Br Med Bull.* **44:** 850-860.

Bennett, P. R. and Elder, M. G. (1992) The mechanisms of preterm labor: common genital tract pathogens do not metabolize arachidonic acid to prostaglandins or to other eicosanoids. *Am J Obstet Gynecol.* **166:** 1541-1545.

Bennett, P. R., Elder, M. G. and Myatt, L. (1987a) The effects of lipoxygenase metabolites of arachidonic acid on human myometrial contractility. *Prostaglandins.* **33:** 837-844.

Bennett, P. R., Elder, M. G. and Myatt, L. (1990) Secretion of phospholipases by bacterial pathogens may initiate preterm labor. *Am J Obstet Gynecol.* **163:** 241-242.

Bennett, P. R., Henderson, D. J. and Moore, G. E. (1992) Changes in expression of the cyclooxygenase gene in human fetal membranes and placenta with labor. *Am J Obstet Gynecol.* **167:** 212-216.

Bennett, P. R., Rose, M. P., Myatt, L. and Elder, M. G. (1987b) Preterm labor: stimulation of arachidonic acid metabolism in human amnion cells by bacterial products. *Am J Obstet Gynecol.* **156:** 649-655.

Bennett, P. R., Slater, D., et al. (1993) Changes in amniotic arachidonic acid metabolism associated with increased cyclo-oxygenase gene expression. *Br J Obstet Gynaecol.* **100:** 1037-1042.

Bergstrom, S. (1966) The prostaglandins. *Recent Prog Horm Res.* **22:** 153-175.

Bishop, E. H. and Wouterxz, T. B. (1961) Isoxsuprine, a myometrial relaxant. *Obstet Gynecol.* **17:** 442-446.

Boyle, M. H., Torrance, G. W., Sinclair, J. C. and Horwood, S. P. (1983) Economic evaluation of neonatal intensive care of very-low-birth-weight infants. *N Engl J Med.* **308:** 1330-1337.

Bracero, L. A., Reale, M. R., et al. (1995) Endothelin-1 levels in the maternal-fetal dyad. *Gynecol Obstet Invest.* **39:** 221-225.

Braunwald, E. (1982) Mechanisms of action of calcium channel blocking agents. *N Engl J Med.* **307:** 1618-1627.

Bredt, D. S., Hwang, P. M., et al. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature.* **351:** 714-718.

Buderus, S., Thomas, B., Fahnenstich, H. and Kowalewski, S. (1993) Renal failure in two preterm infants: toxic effect of prenatal maternal indomethacin treatment? *Br J Obstet Gynaecol.* **100:** 97-98.

Buhimschi, I., Ali, M., et al. (1996) Differential regulation of nitric oxide in the rat uterus and cervix during pregnancy and labour. *Hum Reprod.* **11:** 1755-1766.

Buhimschi, I., Yallampalli, C., Dong, Y. L. and Garfield, R. E. (1995) Involvement of a nitric oxide-cyclic guanosine monophosphate pathway in control of human uterine contractility during pregnancy. *Am J Obstet Gynecol.* **172:** 1577-1584.

Cabral, H., Fried, L. E., et al. (1990) Foreign-born and US-born black women: differences in health behaviors and birth outcomes. *Am J Public Health.* **80:** 70-72.

Calder, A. A. (1994) Prostaglandins and biological control of cervical function. *Aust N Z J Obstet Gynaecol.* **34:** 347-351.

Calder, A. A. and Greer, I. A. (1992) Prostaglandins and the cervix. *Baillieres Clin Obstet Gynaecol.* **6:** 771-786.

Caldwell, G., Scougall, I., Boddy, K. and Toft, A. D. (1987) Fasting hyperinsulinemic hypoglycemia after ritodrine therapy for premature labor. *Obstet Gynecol.* **70:** 478-480.

Cano, A., Tovar, I., Parrilla, J. J. and Abad, L. (1985) Metabolic disturbances during intravenous use of ritodrine: increased insulin levels and hypokalemia. *Obstet Gynecol.* **65:** 356-360.

Caritis, S. N., Toig, G., Heddinger, L. A. and Ashmead, G. (1984) A double-blind study comparing ritodrine and terbutaline in the treatment of preterm labor. *Am J Obstet Gynecol.* **150:** 7-14.

Carlan, S. J., O'Brien, W. F., et al. (1995) Outpatient oral sulindac to prevent recurrence of preterm labor. *Obstet Gynecol.*.

Carlan, S. J., O'Brien, W. F., O'Leary, T. D. and Mastrogiannis, D. (1992) Randomized comparative trial of indomethacin and sulindac for the treatment of refractory preterm labor. *Obstet Gynecol.* **79:** 223-228.

Carsten, M. E. and Miller, J. D. (1987) A new look at uterine muscle contraction. *Am J Obstet Gynecol.* **157:** 1303-1315.

Casper, R. F. and Lye, S. J. (1986) Myometrial desensitization to continuous but not to intermittent beta-adrenergic agonist infusion in the sheep. *Am J Obstet Gynecol.* **154:** 301-305.

Challis, J. (1996) In *The Third International conference on the uterus* NYU School of Medicine and The First Institute of Obstetrics and Gynaecology , University of Bologna, Italy.

Challis, J. R., Matthews, S. G., Van, M. C. and Ramirez, M. M. (1995) Current topic: the placental corticotrophin-releasing hormone-adrenocorticotrophin axis. *Placenta.* **16:** 481-502.

Chan, W. Y., Wo, N. C. and Manning, M. (1996) The role of oxytocin receptors and vasopressin V1a receptors in uterine contractions in rats: implications for tocolytic therapy with oxytocin antagonists. *Am J Obstet Gynecol.* **175:** 1331-1335.

Charles, I. G., Palmer, R. M. J., et al. (1993) *Proc Natl Acad Sci U S A.* **90:** 11419-11423.

Chartrain, N. A., Geller, D. A., et al. (1994) Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. *J Biol Chem.* **269:** 6765-6772.

Chau, A. C., Gabert, H. A. and Miller, J. J. (1992) A prospective comparison of terbutaline and magnesium for tocolysis. *Obstet Gynecol.* **80:** 847-851.

Chen, R., Lewis, K. A., Perrin, M. H. and Vale, W. W. (1993) Expression cloning of a human corticotropin-releasing-factor receptor. *Proc Natl Acad Sci U S A.* **90:** 8967-8971.

Cheung, P. Y. and Challis, J. R. (1989) Prostaglandin E2 metabolism in the human fetal membranes. *Am J Obstet Gynecol.* **161:** 1580-1585.

Chibbar, R., Miller, F. D. and Mitchell, B. F. (1993) Synthesis of oxytocin in amnion, chorion, and decidua may influence the timing of human parturition. *J Clin Invest.* **91:** 185-192.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* **18:** 5294-5299.

Cho, H. J., Xie, Q. W., et al. (1992) Calmodulin is a subunit of nitric oxide synthase from macrophages. *J Exp Med.* **176:** 599-604.

Chow, L. and Lye, S. J. (1994) Expression of the gap junction protein connexin-43 is increased in the human myometrium toward term and with the onset of labor. *Am J Obstet Gynecol.* **170:** 788-795.

Chu, S. C., Marks, K. J., et al. (1998) Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: characterization of differences between human and mouse iNOS promoters. *Biochem Biophys Res Commun.* **248:** 871-878.

Chwalisz, K. and Garfield, R. E. (1998) Nitric oxide as the final metabolic mediator of cervical ripening. *Hum Reprod.* **13:** 245-248.

Collins, P. L., Idriss, E. and Moore, J. J. (1993) Human fetal membranes inhibit spontaneous uterine contractions. *J Clin Endocrinol Metab.* **77:** 1479-1484.

Cotton, D. B., Strassner, H. T., et al. (1984) Comparison of magnesium sulfate, terbutaline and a placebo for inhibition of preterm labor. A randomized study. *J Reprod Med.* **29:** 92-97.

Crankshaw, D. J. and Gaspar, V. (1992) Effects of prostanoids on the rat's myometrium in vitro during pregnancy. *Biol Reprod.* **46:** 392-400.

Creasy, R. K. and Herron, M. A. (1981) Prevention of preterm birth. *Semin Perinatol.* **5:** 295-302.

Critchley, H. O., Kelly, R. W., et al. (1996) Sex steroid regulation of leukocyte traffic in human decidua. *Hum Reprod.* **11:** 2257-2262.

Cruikshank, D. P., Pitkin, R. M., Donnelly, E. and Reynolds, W. A. (1981) Urinary magnesium, calcium, and phosphate excretion during magnesium sulfate infusion. *Obstet Gynecol.* **58:** 430-434.

de Kretser, D. M., Foulds, L. M., Hancock, M. and Robertson, D. M. (1994) Partial characterization of inhibin, activin, and follistatin in the term human placenta. *J Clin Endocrinol Metab.* **79:** 502-507.

de Vera, M. E., Shapiro, R. A., et al. (1996) Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: initial analysis of the human NOS2 promoter. *Proc Natl Acad Sci U S A.* **93:** 1054-1059.

Dennes, W. J., Slater, D. M. and Bennett, P. R. (1997) Nitric oxide synthase mRNA expression in human fetal membranes: a possible role in parturition. *Biochem Biophys Res Commun.* **233:** 276-278.

Dennes, W. J. B., Slater, D. M., Poston, L. and Bennett, P. R. (1999) Myometrial nitric oxide synthase messenger ribonucleic acid expression does not change throughout gestation or with the onset of labor. *Am J Obstet Gynecol.* **180:** 387-392.

DeWitt, D. L., El-Harith, E. A., et al. (1990) The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J. Biol. Chem.* **265:** 5192-5198.

Di-Iulio, J. L., Gude, N. M., King, R. G. and Brennecke, S. P. (1995) Human placental and fetal membrane nitric oxide synthase activity before, during and after labour at term. *Reprod-Fertil-Dev.* **7:** 1505-1508.

DiFederico, E. M., Harrison, M. and Matthay, M. A. (1996) Pulmonary edema in a woman following fetal surgey. *Chest.* **109:** 1114-1117.

Dignan, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic acids research.* **11:** 1475-1489.

Ding, A. H., Nathan, C. F. and Stuehr, D. J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J Immunol.* **141:** 2407-2412.

Dong, Y. L. and Yallampalli, C. (1996) Interaction between nitric oxide and prostaglandin E2 pathways in pregnant rat uteri. *Am J Physiol.* **270:** E471-E476.

Dudley, D. J., Collmer, D., Mitchell, M. D. and Trautman, M. S. (1993) In *40th Annual Meeting of the Society for Gynecologic Investigation*Toronto, Canada.

Eis, A. L., Brockman, D. E. and Myatt, L. (1997) Immunolocalization of the inducible nitric oxide synthase isoform in human fetal membranes. *Am J Reprod Immunol.* **38:** 289-294.

El Maradny, E., Kanayama, N., et al. (1995) Effects of neutrophil chemotactic factors on cervical ripening. *Clin Exp Obstet Gynecol.* **22:** 76-85.

El Maradny, E., Kanayama, N., et al. (1996) Biochemical changes in the cervical tissue of rabbit induced by interleukin-8, interleukin-1beta, dehydroepiandrosterone sulphate and prostaglandin E2: a comparative study. *Hum Reprod.* **11:** 1099-1104.

El-Sayed, Y. Y., Riley, E. T., et al. (1999) Randomized comparison of intravenous nitroglycerin and magnesium sulfate for treatment of preterm labor. *Obstet Gynecol.* **93:** 79-83.

Evaldson, G., Lagrelius, A. and Winiarski, J. (1980) Premature rupture of the membranes. *Acta Obstet Gynecol Scand.* **59:** 385-393.

Evans, T., Carpenter, A. and Cohen, J. (1992) Purification of a distinctive form of endotoxin-induced nitric oxide synthase from rat liver. *Proc Natl Acad Sci U S A.* **89:** 5361-5365.

Feinberg, A. P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem.* **132:** 6-13.

Florio, P., Lombardo, M., et al. (1996) Activin A, corticotropin-releasing factor and prostaglandin F2 alpha increase immunoreactive oxytocin release from cultured human placental cells. *Placenta.* **17:** 307-311.

Flower, R. J. (1974) Drugs which inhibit prostaglandin biosynthesis. *Pharmacol Rev.* **26:** 33-67.

Franchi, A. M., Chaud, M., et al. (1994) Role of nitric oxide in eicosanoid synthesis and uterine motility in estrogen-treated rat uteri. *Proc Natl Acad Sci U S A.* **91:** 539-543.

Freda, M. C. and DeVore, N. (1996) Should intravenous hydration be the first line of defense with threatened preterm labor? A critical review of the literature. *J Perinatol.* **16:** 385-389.

Fuchs, A. R., Fuchs, F., Husslein, P. and Soloff, M. S. (1984) Oxytocin receptors in the human uterus during pregnancy and parturition. *Am J Obstet Gynecol.* **150:** 734-741.

Fuchs, A. R., Fuchs, F., et al. (1982) Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor. *Science.* **215:** 1396-1398.

Fuchs, A. R., Husslein, P. and Fuchs, F. (1981) Oxytocin and the initiation of human parturition. II. Stimulation of prostaglandin production in human decidua by oxytocin. *Am J Obstet Gynecol.* **141:** 694-697.

Furchgott, R. F. and Zawadzki, J. V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* **288:** 373-376.

Gabriel, R., Harika, G., et al. (1994) Prolonged intravenous ritodrine therapy: a comparison between multiple and singleton pregnancies. *Eur J Obstet Gynecol Reprod Biol.* **57:** 65-71.

Gall, J. G. and Pardue, M. L. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc Natl Acad Sci (USA).* **63:** 378-383.

Garfield, R. E. (1990) In *Uterine Contractility* (Ed, Garfield, R. E.) Serono Symposia. Norwell, MA pp. 21-40.

Germain, A. M., Smith, J., Casy, M. L. and McDonald, P. M. (1994) Human fetal membrane contribution to the prevention of parturition: uterotonin degradation. *J Clin Endocrinol metab.* **78:** 463.

Gimona, M., Herzog, M., Vandekerckhove, J. and Small, J. V. (1990) Smooth muscle specific expression of calponin. *Febs Lett.* **274:** 159-162.

Goodwin, T. M., Paul, R., et al. (1994) The effect of the oxytocin antagonist atosiban on preterm uterine activity in the human. *Am J Obstet Gynecol.* **170:** 474-478.

Grammatopoulos, D., Stirrat, G. M., Williams, S. A. and Hillhouse, E. W. (1996) The biological activity of the corticotropin-releasing hormone receptor-adenylate cyclase complex in human myometrium is reduced at the end of pregnancy. *J Clin Endocrinol Metab.* **81:** 745-751.

Grammatopoulos, D., Thompson, S. and Hillhouse, E. W. (1995) The human myometrium expresses multiple isoforms of the corticotropin-releasing hormone receptor. *J Clin Endocrinol Metab.* **80:** 2388-2393.

Grazzini, E., Guillon, G., Mouillac, B. and Zingg, H. H. (1998) Inhibition of oxytocin receptor function by direct binding of progesterone. *Nature.* **392:** 509-512.

Green, L. C., Wagner, D. A., et al. (1982) Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Anal Biochem.* **126:** 131-138.

Greer, I. (1992) *Cervical ripening.* Springer-Verlag. London.

Griffin, J. (1993) Office of Health Economics. London.

Groom, K. M., Bennett, P. R. and Shennan, A. H. (2000) Randomised, double-blind, placebo controlled pilot study assessing nitroglycerin as a tocolytic. *Br J Obstet Gynaecol.* **107:** 1182-1183.

Guiet, B. A., Bara, M. and Durlach, J. (1985) Comparative study of the effects of two tocolytic agents (magnesium sulfate and alcohol) on the ionic transfer through the isolated human amnion. *Eur J Obstet Gynecol Reprod Biol.* **20:** 297-304.

Guinn, D. A., Goepfert, A. R., et al. (1997) Management options in women with preterm uterine contractions: a randomized clinical trial. *Am J Obstet Gynecol.* **177:** 814-818.

Hall, A. V., Antoniou, H., et al. (1994) Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J Biol Chem.* **269:** 33082-33090.

Hall, D. G., McGaughery, H. S. J., Corey, E. L. and Thornton, N. (1959) The effects of magnesium therapy on the duration of labor. *Am J Obstet Gynecol.* **78:** 27-32.

Hall, M. H. and Carr, H. R. (1982) Impact of sex ratio on onset and management of labour. *Br Med J Clin Res Ed.* **285:** 401-403.

Haluska, G. J., Stanczyk, F. Z., Cook, M. J. and Novy, M. J. (1987) Temporal changes in uterine activity and prostaglandin response to RU486 in rhesus macaques in late gestation. *Am J Obstet Gynecol.* **157:** 1487-1495.

Hamberg, M. and Samuelsson, B. (1967) Oxygenation of unsaturated fatty acids by the vesicula gland of sheep. *J. Biol. Chem.* **242:** 5344-5354.

Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci U S A.* **72:** 2994-2998.

Han, S. W., Lei, Z. M. and Rao, C. V. (1996) Up-regulation of cyclooxygenase-2 gene expression by chorionic gonadotropin during the differentiation of human endometrial stromal cells into decidua. *Endocrinology.* **137:** 1791-1797.

Haspedis, L. (1988) Terbutaline in the treatment of preterm labor: a comparison of intravenous and subcutaneous administration. *J Am Osteopath Assoc.* **88:** 489-493.

Hawkins, D. F. and Labrum, A. H. (1961) Semen prostaglandin levels in men attending a fertility clinic. *Reprod Fert.* **2:** 1.

Hemler, M. E. and Lands, W. E. M. (1980) Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J. Biol. Chem.* **255:** 6253-6261.

Hennan, J. K. and Diamond, J. (1998) Evidence that spontaneous contractile activity in the rat myometrium is not inhibited by NO-mediated increases in tissue levels of cyclic GMP. *Br J Pharmacol.* **123:** 959-967.

Herschman, H. R. (1991) *Primary response genes induced by growth factors and tumour promoters.* Annual Reviews Inc. Palo Alto, California.

Hillhouse, E. W., Grammatopoulos, D., Milton, N. G. and Quartero, H. W. (1993) The identification of a human myometrial corticotropin-releasing hormone receptor that increases in affinity during pregnancy. *J Clin Endocrinol Metab.* **76:** 736-741.

Hirst, J. J., Haluska, G. J., et al. (1991) Comparison of plasma oxytocin and catecholamine concentrations with uterine activity in pregnant rhesus monkeys. *J Clin Endocrinol Metab.* **73:** 804-810.

Hirst, J. J., Teixeira, F. J., Zakar, T. and Olson, D. M. (1995) Prostaglandin endoperoxide-H synthase-1 and -2 messenger ribonucleic acid levels in human amnion with spontaneous labor onset. *J Clin Endocrinol Metab.* **80:** 517-523.

Hla, T., Farrell, M., Kumar, A. and Bailey, J. M. (1986) Isolation of the cDNA for human prostaglandin H synthase. *Prostaglandins.* **32:** 829-845.

Hla, T. and Neilson, K. (1992) Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci U S A.* **89:** 7384-7388.

Hoffman, D. R., Romero, R. and Johnston, J. M. (1990) Detection of platelet-activating factor in amniotic fluid of complicated pregnancies. *Am J Obstet Gynecol.* **162:** 525-528.

Hollander, D. I., Nagey, D. A. and Pupkin, M. J. (1987) Magnesium sulfate and ritodrine hydrochloride: a randomized comparison. *Am J Obstet Gynecol.* **156:** 631-637.

Howe, D. T., Sangha, R., et al. (1996) Identification and expression of nitric oxide synthase isoforms in human myometrium before and after the onset of labour. *J Soc Gynecol Invest.* **3:** 326A.

Huhta, J. C., Moise, K. J., et al. (1987) Detection and quantitation of constriction of the fetal ductus arteriosus by Doppler echocardiography. *Circulation.* **75:** 406-412.

Hutchinson, H. T., Nichols, M. M., Kuhn, C. R. and Vasicka, A. (1965) Effects of magnesium sulfate on uterine contractility, intrauterine fetus and infant. *Am J Obstet Gynecol.* **88:** 747-758.

Hutton, J. L., Pharoah, P. O., Cooke, R. W. and Stevenson, R. C. (1997) Differential effects of preterm birth and small gestational age on cognitive and motor development. *Arch Dis Child Fetal Neonatal Ed.* **76:** F75-81.

Ignarro, L. J., Fukuto, J. M., et al. (1993) Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L- arginine. *Proc Natl Acad Sci U S A.* **90:** 8103-8107.

Inoue, Y. and Sperelakis, N. (1991) Gestational change in Na+ and Ca2+ channel current densities in rat myometrial smooth muscle cells. *Am J Physiol.* **260:** C658-C663.

Janis, R. A. and Diamond, J. (1979) Relationship between cyclic nucleotide levels and drug-induced relaxation of smooth muscle. *J Pharmacol Exp Ther.* **211:** 480-484.

John, H. A., Birnstiel, M. L. and Jones, K. W. (1969) RNA-DNA hybrids at the cytological level. *Nature.* **223:** 582-587.

Jones, G. D. and Poston, L. (1997) The role of endogenous nitric oxide synthesis in contractility of term or preterm human myometrium. *Br J Obstet Gynaecol.* **104:** 241-245.

Jones, S. A., Brooks, A. N. and Challis, J. R. (1989) Steroids modulate corticotropin-releasing hormone production in human fetal membranes and placenta. *J Clin Endocrinol Metab.* **68:** 825-830.

Jones, S. A. and Challis, J. R. (1990) Effects of corticotropin-releasing hormone and adrenocorticotropin on prostaglandin output by human placenta and fetal membranes. *Gynecol Obstet Invest.* **29:** 165-168.

Jouppila, P., Kirkinen, P., Koivula, A. and Ylikorkala, O. (1985) Ritodrine infusion during late pregnancy: effects on fetal and placental blood flow, prostacyclin, and thromboxane. *Am J Obstet Gynecol.* **151:** 1028-1032.

Kaltreider, D. F. and Kohl, S. (1980) Epidemiology of preterm delivery. *Clin Obstet Gynecol.* **23:** 17-31.

Kanner, J., Harel, S. and Granit, R. (1992) Nitric oxide, an inhibitor of lipid oxidation by lipoxygenase, cyclooxygenase and hemoglobin. *Lipids*. **27:** 46-49.

Keeley, M. M., Wade, R. V., Laurent, S. L. and Hamann, V. D. (1993) Alterations in maternal-fetal Doppler flow velocity waveforms in preterm labor patients undergoing magnesium sulfate tocolysis. *Obstet Gynecol*. **81:** 191-194.

Kelly, R. W., Leask, R. and Calder, A. A. (1992) Choriodecidual production of interleukin-8 and mechanism of parturition. *Lancet*. **339:** 776-777.

Kieler, H., Axelsson, O., Nilsson, S. and Waldenstrom, U. (1995) The length of human pregnancy as calculated by ultrasonographic measurement of the fetal biparietal diameter. *Ultrasound Obstet Gynecol*. **6:** 353-357.

King, J. F., Grant, A., Keirse, M. J. and Chalmers, I. (1988) Beta-mimetics in preterm labour: an overview of the randomized controlled trials. *Br J Obstet Gynaecol*. **95:** 211-222.

Kramer, W. B., Saade, G., et al. (1995) Placental transfer of sulindac and its active sulfide metabolite in humans. *Am J Obstet Gynecol*. **172:** 886-890.

Kuenzli, K. A., Buxton, I. L. O. and Bradley, M. E. (1998) Nitric oxide regulation of monkey myometrial contractility. *Br J Pharmacol*. **124:** 63-68.

Kujubu, D. A., Fletcher, B. S., et al. (1991) TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J Biol Chem*. **266:** 12866-12872.

Kujubu, D. A., Reddy, S. T., Fletcher, B. S. and Herschman, H. R. (1993) Expression of the protein product of the prostaglandin synthase-2/TIS 10 gene in mitogen-stimulated Swiss 3t3 cells. *J Biol Chem*. **268:** 5425-5430.

Kumar, D., Zourlas, P. A. and Barnes, A. C. (1965) In Vivo effect of amyl nitrite on human pregnant uterine contractility. *Am J Obstet Gynecol*. **91:** 1066-1068.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227:** 680-685.

Lam, F., Gill, P., et al. (1988) Use of the subcutaneous terbutaline pump for long-term tocolysis. *Obstet Gynecol*. **72:** 810-813.

Lamont, R. F., Rose, M. and Elder, M. G. (1985) Effect of bacterial products on prostaglandin E production by amnion cells. *Lancet.* **2:** 1331-1333.

Lancaster, J. R. (1996) Diffusion of free nitric oxide. *Methods Enzymol.* **268:** 31-50.

Langenbach, R., Morham, S. G., et al. (1995) Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulcertation. **83:** 483-492.

Larcher, A., Neculcea, J., et al. (1995) Oxytocin receptor gene expression in the rat uterus during pregnancy and the estrous cycle and in response to gonadal steroid treatment. *Endocrinology.* **136:** 5350-5356.

Lees, C., Campbell, S., et al. (1994) Arrest of preterm labour and prolongation of gestation with glyceryl trinitrate, a nitric oxide donor. *Lancet.* **343:** 1325-1326.

Lees, C. C., Lojacono, A., et al. (1999) Glyceryl trinitrate and ritodrine in tocolysis: an international multicenter randomized study. GTN Preterm Labour Investigation Group. *Obstet Gynecol.* **94:** 403-408.

Leveno, K. J. and Cunningham, F. G. (1992) Beta-adrenergic agonists for preterm labor. *N Engl J Med.* **327:** 349-351.

Levin, D. L., Fixler, D. E., Morriss, F. C. and Tyson, J. (1978) Morphologic analysis of the pulmonary vascular bed in infants exposed in utero to prostaglandin synthetase inhibitors. *J Pediatr.* **92:** 478-483.

Liaw, C. W., Lovenberg, T. W., et al. (1996) Cloning and characterization of the human corticotropin-releasing factor-2 receptor complementary deoxyribonucleic acid. *Endocrinology.* **137:** 72-77.

Lipper, E. G., Ross, G. S., Auld, P. A. and Glassman, M. B. (1990) Survival and outcome of infants weighing less than 800 grams at birth [see comments]. *Am J Obstet Gynecol.* : 146-150.

Lipsitz, P. J. (1971) The clinical and biochemical effects of excess magnesium in the newborn. *Pediatrics.* **47:** 501-509.

Liu, D. T. and Blackwell, R. J. (1978) The value of a scoring system in predicting outcome of pre-term labour and comparing the efficacy of treatment with aminophylline and salbutamol. *Br J Obstet Gynaecol.* **85:** 418-424.

Luckas, M. J. and Wray, S. (2000) A comparison of the contractile properties of human myometrium obtained from the upper and lower uterine segments. *Br J Obstet Gynaecol.* **107:** 1309-1311.

Lumley, J. (1995) Strategies for reducing smoking in pregnancy. *Cochrane Pregnancy and Childbirth Database.* .

Lye, S. J. (1994) *The initiation and inhibition of labour - Toward a molecular understanding.* .

Macones, G. A., Bader, T. J. and Asch, D. A. (1996) Determining the optimal gestational age limit for tocolysis: A decision analysis. *Am J Obstet Gynecol.* **174:** 308.

Maggi, M., Del, C. P., et al. (1990) Human myometrium during pregnancy contains and responds to V1 vasopressin receptors as well as oxytocin receptors. *J Clin Endocrinol Metab.* **70:** 1142-1154.

Maggi, M., Vannelli, G. B., et al. (1994) Endothelin in the human uterus during pregnancy. *J Endocrinol.* **142:** 385-396.

Maguchi, M., Nishida, W., et al. (1995) Molecular cloning and gene mapping of human basic and acidic calponins. *Biochem Biophys Res Commun.* **217:** 238-244.

Maigaard, S., Forman, A. and Andersson, K. E. (1984) Effects of nifedipine on human placental arteries. *Gynecol Obstet Invest.* **18:** 217-224.

Malak, T. M. and Bell, S. C. (1994) Structural characteristics of term human fetal membranes (amniochorion and decidua): a novel zone of morphological alteration within the rupture site. *Br J Obstet Gynaecol.* **101:** 375-386.

Mari, G., Moise, K. J., et al. (1990) Doppler assessment of the renal blood flow velocity waveform during indomethacin therapy for preterm labor and polyhydramnios. *Obstet Gynecol.* **75:** 199-201.

Marshall, P. J., Kulmacz, R. J. and Lands, W. E. (1987) Constraints on prostaglandin biosynthesis in tissues. *J Biol Chem.* **262:** 3510-3517.

McCormick, M. C. (1985) The contribution of low birth weight to infant mortality and childhood morbidity. *N Engl J Med.* **312:** 82-90.

McLean, M., Bisits, A., et al. (1995) A placental clock controlling the length of human pregnancy. *Nat Med.* **1:** 460-463.

Merkatz, I. R., Peter, J. B. and Barden, T. P. (1980) Ritodrine hydrochloride: a betamimetic agent for use in preterm labor. II. Evidence of efficacy. *Obstet Gynecol.* **56:** 7-12.

Molloy, P. L. (2000) Electrophoretic mobility shift assays. *Methods Mol Biol.* **130:** 235-246.

Moncada, S., Gryglewski, R., Bunting, S. and Vane, J. R. (1976) An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature.* **263:** 663-665.

Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1991) *Pharmacol Rev.* **43:** 109-142.

Moore, K. L. and Persaud, T. V. N. (1993) *The Developing Human.* W.B. Saunders Company. Philadelphia.

Moore, W. M., Webber, R. K., et al. (1994) L-N6-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. *J Med Chem.* **37:** 3886-3888.

Morales, W. J. and Madhav, H. (1993) Efficacy and safety of indomethacin compared with magnesium sulfate in the management of preterm labor: a randomized study. *Am J Obstet Gynecol.* **169:** 97-102.

Morales, W. J., Smith, S. G., et al. (1989) Efficacy and safety of indomethacin versus ritodrine in the management of preterm labor: a randomized study. *Obstet Gynecol.* **74:** 567-572.

Morham, S. G., Langenbach, R., et al. (1995) Prostalgnadin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell.* **83:** 473-482.

Moutquin, J. M., Sherman, D., et al. (2000) Double-blind, randomized, controlled trial of atosiban and ritodrine in the treatment of preterm labor: a multicenter effectiveness and safety study. *Am J Obstet Gynecol.* **182:** 1191-1199.

Moya, F. R., Hoffman, D. R., Zhao, B. and Johnston, J. M. (1993) Platelet-activating factor in surfactant preparations. *Lancet.* **341:** 858-860.

Nadaud, S., Bonnardeaux, A., Lathrop, M. and Soubrier, F. (1994) Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. *Biochem Biophys Res Commun.* **198:** 1027-1033.

Naeye, R. L. (1982) Factors that predispose to premature rupture of the fetal membranes. *Obstet Gynecol.* **60:** 93-98.

Nakane, M., Schmidt, H. H., et al. (1993) Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *Febs Lett.* **316:** 175-180.

Nash, P. D., Opas, M. and Michalak, M. (1994) Calreticulin: not just another calcium-binding protein. *Mol Cell Biochem.* **135:** 71-78.

Natuzzi, E. S., Ursell, P. C., et al. (1993) Nitric oxide synthase activity in the pregnant uterus decreases at parturition. *Biochem Biophys Res Commun.* **194:** 1-8.

Newns, B., Drummond, M. F., Durbin, G. M. and Culley, P. (1984) Costs and outcomes in a regional neonatal intensive care unit. *Arch Dis Child.* **59:** 1064-1067.

Newton, C. L., Mignery, G. A. and Sudhof, T. C. (1994) Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate (InsP3) receptors with distinct affinities for InsP3. *J Biol Chem.* **269:** 28613-28619.

Newton, R. W. and Hunt, L. P. (1984) Psychosocial stress in pregnancy and its relation to low birth weight. *Br Med J Clin Res Ed.* **288:** 1191-1194.

Nguyen, M. T., Camenisch, T., et al. (1997) The prostaglandin receptor EP_4 triggers remodelling of the cardiovascular system at birth. *Nature.* **390:** 78-81.

Nishikawa, M., de, L. P., Lincoln, T. M. and Adelstein, R. S. (1984) Phosphorylation of mammalian myosin light chain kinases by the catalytic subunit of cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase. *J Biol Chem.* **259:** 8429-8436.

Noack, E. and Feelisch, M. (1991) Molecular mechanisms of nitrovasodilator bioactivation. *Basic Res Cardiol.* **2:** 37-50.

Norman, J. E., Thomson, A. J., et al. (1999) Myometrial constitutive nitric oxide synthase expression is increased during human pregnancy. *Mol Human Reprod.* **5:** 175-181.

Norman, J. E., Ward, L. M., et al. (1997) Effects of cGMP and the nitric oxide donors glyceryl trinitrate and sodium nitroprusside on contractions in vitro of isolated myometrial tissue from pregnant women. *J Reprod Fertility.* **110:** 249-254.

Nunokawa, Y., Ishida, N. and Tanaka, S. (1994) Promoter analysis of human inducible nitric oxide synthase gene associated with cardiovascular homeostasis. *Biochem Biophys Res Commun.* **200:** 802-807.

Nunokawa, Y., Oikawa, S. and Tanaka, S. (1997) Expression of human inducible nitric oxide synthase is regulated by both promoter and 3'-regions. *Biochem Biophys Res Commun.* **233:** 523-526.

Olah, K. S., Vince, G. S., et al. (1996) Interleukin-6, interferon-gamma, interleukin-8, and granulocyte-macrophage colony stimulating factor levels in human amniotic fluid at term. *J Reprod Immunol.* **32:** 89-98.

Osmers, R. G., Adelmann, G. B., et al. (1995a) Biochemical events in cervical ripening dilatation during pregnancy and parturition. *J Obstet Gynaecol.* **21:** 185-194.

Osmers, R. G., Blaser, J., Kuhn, W. and Tschesche, H. (1995b) Interleukin-8 synthesis and the onset of labor. *Obstet Gynecol.* **86:** 223-229.

Palmer, R. M., Ashton, D. S. and Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature.* **333:** 664-666.

Pan, J., Burgher, K. L., Szczepanik, A. M. and Ringheim, G. E. (1996) Tyrosine phosphorylation of inducible nitric oxide synthase: implications for potential post-translational regulation. *Biochem J.* **314:** 889-894.

Peng, A. T., Gorman, R. S., et al. (1989) Intravenous nitroglycerin for uterine relaxation in the postpartum patient with retained placenta. *Anesthesiology.* **71:** 172-173.

Peng, C., Huang, T. H., et al. (1993) Expression of the type II activin receptor gene in the human placenta. *Endocrinology.* **133:** 3046-3049.

Peri, A., Vannelli, G. B., et al. (1992) Endothelin in rabbit uterus during pregnancy. *Am J Physiol.* **263:** E158-E167.

Petraglia, F., Anceschi, M. M., et al. (1993) Inhibin and activin in human fetal membranes: evidence for a local effect on prostaglandin release. *J Clin Endocrinol Metab.* **77:** 542-548.

Petraglia, F., Benedetto, C., et al. (1995) Effect of corticotropin-releasing factor-binding protein on prostaglandin release from cultured maternal decidua and on contractile activity of human myometrium in vitro. *J Clin Endocrinol Metab.* **80:** 3073-3076.

Petraglia, F., Calza, L., et al. (1990) Presence and synthesis of inhibin subunits in human decidua. *J Clin Endocrinol Metab.* **71:** 487-492.

Petraglia, F., Gallinelli, A., et al. (1994a) Activin at parturition: changes of maternal serum levels and evidence for binding sites in placenta and fetal membranes. *Obstet Gynecol.* **84:** 278-282.

Petraglia, F., Gallinelli, A., et al. (1994b) Local production and action of follistatin in human placenta. *J Clin Endocrinol Metab.* **78:** 205-210.

Petraglia, F., Vaughan, J. and Vale, W. (1989) Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placental cells. *Proc Natl Acad Sci U S A.* **86:** 5114-5117.

Petrie, R. H., Wu, R., et al. (1976) The effect of drugs on uterine activity. *Obstet Gynecol.* **48:** 431-435.

Ping, X. W., Warden, C., et al. (1993) Chromosomal organization of the inducible and constitutive prostaglandin synthase / cyclooxygenase genes in mouse. *Genomics.* **15:** 458-460.

Pollock, J. S., Klinghofer, V., Forstermann, U. and Murad, F. (1992) Endothelial nitric oxide synthase is myristylated. *Febs Lett.* **309:** 402-404.

Quartero, H. W., Srivatsa, G. and Gillham, B. (1992) Role for cyclic adenosine monophosphate in the synergistic interaction between oxytocin and corticotrophin-releasing factor in isolated human gestational myometrium. *Clin Endocrinol Oxf.* **36:** 141-145.

Rabinovici, J., Goldsmith, P. C., Librach, C. L. and Jaffe, R. B. (1992) Localization and regulation of the activin-A dimer in human placental cells. *J Clin Endocrinol Metab.* **75:** 571-576.

Rajasingam, D., Bennett, P. R., et al. (1998) Stimulation of prostaglandin production from intact human fetal membranes by bacteria and bacterial products. *Placenta.* **19:** 301-306.

Ramsay, B., Sooranna, S. R. and Johnson, M. R. (1996) Nitric oxide synthase activities in human myometrium and villous trophoblast throughout pregnancy. *Obstet Gynecol.* **87:** 249-253.

Read, M. D. and Wellby, D. E. (1986) The use of a calcium antagonist (nifedipine) to suppress preterm labour. *Br J Obstet Gynaecol.* **93:** 933-937.

Romero, R., Durum, S., et al. (1989) Interleukin-1 stimulates prostaglandin biosynthesis by human amnion. *Prostaglandins.* **37:** 13-22.

Romero, R., Hobbins, J. C. and Mitchell, M. D. (1988) Endotoxin stimulates prostaglandin E2 production by human amnion. *Obstet Gynecol.* **71:** 227-228.

Romero, R., Kadar, N., Hobbins, J. C. and Duff, G. W. (1987) Infection and labor: the detection of endotoxin in amniotic fluid. *Am J Obstet Gynecol.* **157:** 815-819.

Romero, R., Mazor, M., et al. (1992) Interleukin-1 alpha and interleukin-1 beta in preterm and term human parturition. *Am J Reprod Immunol.* **27:** 117-123.

Romero, R., Mazor, M., et al. (1994) The preterm labor syndrome. *Ann N Y Acad Sci.* **734:** 414-429.

Romero, R., Parvizi, S. T., et al. (1990) Amniotic fluid interleukin-1 in spontaneous labor at term. *J Reprod Med.* **35:** 235-238.

Ryden, G. (1977) The effect of salbutamol and terbutaline in the management of premature labour. *Acta Obstet Gynecol Scand.* **56:** 293-296.

Salvemini, D. and Masferrer, J. L. (1996) Interactions of nitric oxide with cyclooxygenase: in vitro, ex vivo, and in vivo studies. *Methods Enzymol.* **269:** 12-25.

Salvemini, D., Misko, T. P., et al. (1993) Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci U S A*. **90:** 7240-7244.

Salvemini, D., Settle, S. L., et al. (1995) Regulation of prostaglandin production by nitric oxide; an in vivo analysis. *Br J Pharmacol.* **114:** 1171-1178.

Sandhu, B., Stevenson, R. C., Cooke, R. W. and Pharoah, P. O. (1986) Cost of neonatal intensive care for very-low-birthweight infants. *Lancet.* **1:** 600-603.

Sandman, C. A., Wadhwa, P. D., et al. (1997) Maternal stress, HPA activity, and fetal/infant outcome. *Ann N Y Acad Sci.* **814:** 266-275.

Savitz, D. A., Blackmore, C. A. and Thorp, J. M. (1991) Epidemiologic characteristics of preterm delivery: etiologic heterogeneity. *Am J Obstet Gynecol.* **164:** 467-471.

Sawdy, R., Knock, G. A., et al. (1998) Effect of nimesulide and indomethacin on contractility and the Ca2+ channel current in myometrial smooth muscle from pregnant women. *Br J Pharmacol.* **125:** 1212-1217.

Sawdy, R., Slater, D., et al. (1997) Use of a cyclo-oxygenase type-2-selective non-steroidal anti-inflammatory agent to prevent preterm delivery. *Lancet.* **350:** 265-266.

Sawdy, R. J., Dennes, W. J., et al. (1999) Region and labour-dependent synthesis of prostaglandin E2 by human fetal membranes. *Placenta.* **20:** 181-184.

Sawdy, R. J., Slater, D. M., et al. (2000) The roles of the cyclo-oxygenases types one and two in prostaglandin synthesis in human fetal membranes at term. *Placenta.* **21:** 54-57.

Scheid, C. R., Honeyman, T. W. and Fay, F. S. (1979) Mechanism of beta-adrenergic relaxation of smooth muscle. *Nature.* **277:** 32-36.

Scheuren, N., Pahl, A., Bang, H. and Brune, K. (1996) In *10 th International conference on prostaglandins and related compounds* Vienna, Austria.

Schiff, E., Ben, B. G., et al. (1993) Endothelin-1 receptors in the human myometrium: evidence for different binding properties in post-menopausal as compared to premenopausal and pregnant women. *Clin Endocrinol Oxf.* **38:** 321-324.

Schmid, A., Thierauch, K. H., Schleuning, W. D. and Dinter, H. (1995) Splice variants of the human EP_3 receptor for prostaglandin E_2. *Eur J Biochem.* **228:** 23-30.

Schwarz, R. and Retzke, U. (1983) Cardiovascular effects of terbutalin in pregnant women. *Acta Obstet Gynecol Scand.* **62:** 419-423.

Segal, S., Csavoy, A. N. and Datta, S. (1998) Placental tissue enhances uterine relaxation by nitroglycerin. *Anesth Analg.* **86:** 304-309.

Seibert, K. and Masferrer, J. L. (1994) Role of inducible cyclooxygenase (COX-2) in inflammation. *Receptor.* **4:** 17-23.

Sharif, D. S., Huhta, J. C., et al. (1990) Changes in fetal hemodynamics with terbutaline treatment and premature labor. *J Clin Ultrasound.* **18:** 85-89.

Shaw, G. and Kamen, R. (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell.* **46:** 659-667.

Sheng, H., Schmidt, H. H., et al. (1992) Characterization and localization of nitric oxide synthase in non-adrenergic non-cholinergic nerves from bovine retractor penis muscles. *Br J Pharmacol.* **106:** 768-773.

Shimokawa, T., Kulmacz, R. J., De Witt, D. L. and Smith, W. L. (1990) Tyrosine 385 of prosatglandin endoperoxide synthase is required for cyclooxygenase catalysis. *J Biol Chem.* **265:** 20073-20076.

Shozu, M., Akasofu, K., Harada, T. and Kubota, Y. (1991) A new cause of female pseudohermaphroditism: placental aromatase deficiency. *J Clin Endocrinol Metab.* **72:** 560-566.

Sims, C. D., Chamberlain, G. V., Boyd, I. E. and Lewis, P. J. (1978) A comparison of salbutamol and ethanol in the treatment of preterm labour. *Br J Obstet Gynaecol.* **85:** 761-766.

Skannal, D. G., Eis, A. L., et al. (1997) Immunohistochemical localization of phospholipase A2 isoforms in human myometrium during pregnancy and parturition. *Am J Obstet Gynecol.* **176:** 878-882.

Sladek, S. M., Regenstein, A. C., Lykins, D. and Roberts, J. M. (1993) Nitric oxide synthase activity in pregnant rabbit uterus decreases on the last day of pregnancy. *Am J Obstet Gynecol.* **169:** 1285-1291.

Slater, D., Berger, L., et al. (1994) The relative abundance of type 1 to type 2 cyclo-oxygenase mRNA in human amnion at term. *Biochem Biophys Res Commun.* **198:** 304-308.

Slater, D. M., Berger, L. C., et al. (1995) Expression of cyclooxygenase types 1 and 2 in human fetal membranes at term. *Am J Obstet Gynecol.* **172:** 77-82.

Slater, D. M., Dennes, W. J., et al. (1999) Expression of cyclo-oxygenase types-1 and -2 in human myometrium throughout pregnancy. *Mol Hum Reprod.* **5:** 880-884.

Smieja, Z., Zakar, T., Walton, J. C. and Olson, D. M. (1993) Prostaglandin endoperoxide synthase kinetics in human amnion before and after labor at term and following preterm labor. *Placenta.* **14:** 163-175.

Smith, G. N., Walker, M. C. and McGrath, M. J. (1999) Randomised, double-blind, placebo controlled pilot study assessing nitroglycerin as a tocolytic. *Br J Obstet Gynaecol.* **106:** 736-739.

Smith, L. J., Burns, P. A. and Schanler, R. J. (1992) Calcium homeostasis in pregnant women receiving long-term magnesium sulfate therapy for preterm labor. *Am J Obstet Gynecol.* **167:** 45-51.

Smith, W. L., Borgat, P., et al. (1990) Nomenclature. *Meth. Enzymol.* **187:** 1-9.

Song, Y., Keelan, J. and France, J. T. (1996) Activin-A stimulates, while transforming growth factor beta 1 inhibits, chorionic gonadotrophin production and aromatase activity in cultured human placental trophoblasts. *Placenta.* **17:** 603-610.

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol.* **98:** 503-517.

Sparey, C., Robson, S. C., et al. (1999) The differential expression of myometrial connexin-43, cyclooxygenase-1 and -2, and Gs alpha proteins in the upper and lower segments of the human uterus during pregnancy and labor. *J Clin Endocrinol Metab.* **84:** 1705-1710.

Spellacy, W. N., Cruz, A. C., Buhi, W. C. and Birk, S. A. (1978) The acute effects of ritodrine infusion on maternal metabolism: measurements of levels of glucose, insulin, glucagon, triglycerides, cholesterol, placental lactogen and chorionic gonadotropin. *Am J Obstet Gynecol.* **131:** 637-642.

Steer, C. M. and Petrie, R. H. (1977) A comparison of magnesium sulfate and alcohol for the prevention of premature labor. *Am J Obstet Gynecol.* **129:** 1-4.

Sugimoto, Y., Yamasaki, A., et al. (1997) Failure of parturition in mice lacking the prostaglandin F receptor. *Science.* **277:** 681-683.

Sun, K., Yang, K. and Challis, J. R. (1997) Differential regulation of 11 beta-hydroxysteroid dehydrogenase type 1 and 2 by nitric oxide in cultured human placental trophoblast and chorionic cell preparation. *Endocrinology.* **138:** 4912-4920.

Swierkosz, T. A., Mitchell, J. A., et al. (1994) Relationship between different isoforms of nitric oxide synthase and cyclooxygenase in various cell types. *Pol J Pharmacol.* **46:** 587-592.

Swierkosz, T. A., Mitchell, J. A., et al. (1995) Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids. *Br J Pharmacol.* **114:** 1335-1342.

Tazawa, R., Xu, X. M., Wu, K. K. and Wang, L. H. (1994) Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. *Biochem Biophys Res Commun.* **203:** 190-199.

Telfer, J. F., Irvine, G. A., et al. (1997a) Expression of endothelial and inducible nitric oxide synthase in non-pregnant and decidualized human endometrium. *Mol Hum Reprod.* **3:** 69-75.

Telfer, J. F., Lyall, F., Norman, J. E. and Cameron, I. T. (1995) Identification of nitric oxide synthase in human uterus. *Hum Reprod.* **10:** 19-23.

Telfer, J. F., Thomson, A. J., et al. (1997b) Expression of superoxide dismutase and xanthine oxidase in myometrium, fetal membranes and placenta during normal human pregnancy and parturition. *Hum Reprod.* **12:** 2306-2312.

Tetsuka, T., Daphna, I. D., et al. (1994) Cross-talk between cyclooxygenase and nitric oxide pathways: prostaglandin E2 negatively modulates induction of nitric oxide synthase by interleukin 1. *Proc Natl Acad Sci U S A*. **91:** 12168-12172.

Thompson, J. E., Phillips, R. J., et al. (1995) I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. *Cell.* **80:** 573-582.

Thomson, A. J., Lunan, C. B., et al. (1997a) Nitric oxide donors induce ripening of the human uterine cervix: a randomised controlled trial. *Br J Obstet Gynaecol.* **104:** 1054-1057.

Thomson, A. J., Lunan, C. B., et al. (1998) Randomised trial of nitric oxide donor versus prostaglandin for cervical ripening before first-trimester termination of pregnancy. *Lancet.* **352:** 1093-1096.

Thomson, A. J., Telfer, J. F., et al. (1997b) Nitric oxide synthase activity and localization do not change in uterus and placenta during human parturition. *Hum Reprod.* **12:** 2546-2552.

Thornton, S., Davison, J. M. and Baylis, P. H. (1992) Plasma oxytocin during the first and second stages of spontaneous human labour. *Acta Endocrinol Copenh.* **126:** 425-429.

Ticconi, C., Zicari, A., et al. (1996) Nitric oxide in human fetal membranes at term gestation: effect on prostaglandin E2 release. *Eur J Obstet Gynecol Reprod Biol.* **69:** 135-139.

Tokunaga, K., Nakamura, Y., et al. (1987) Enhanced expression of a glceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. *Cancer Res.* **47:** 5616-5619.

Van Dorp, D. A., Beerthuis, R. K., Nugteren, D. H. and Vonkeman, H. (1964) The biosynthesis of prostaglandins. *Biochim. Biophys. Acta.* **90:** 204-207.

Vane, J. and Botting, R. (1987) Inflammation and the mechanism of action of anti-inflammatory drugs. *Faseb J.* **1:** 89-96.

Von Euler, U. S. (1934) Uber die spezifische blutdrucksekende Substanz des menschlichen Prostata und Samenblasensekretes. *Klinische Wochenschrift.* **14:** 1182-1183.

Wadhawa, P. D., Porto, M., et al. (1998) Maternal corticotrophin-releasing hormone levels in the early third trimester predict length of gestation in human pregnancy. *Am J Obstet Gynecol.* **179:** 1079-1085.

Walsh, S. W. (1989) 5-Hydroxyeicosatetraenoic acid, leukotriene C4, and prostaglandin F2 alpha in amniotic fluid before and during term and preterm labor. *Am J Obstet Gynecol.* **161:** 1352-1360.

Wathes, D. C. and Porter, D. G. (1982) Effect of uterine distension and oestrogen treatment on gap junction formation in the myometrium of the rat. *J Reprod Fertil.* **65:** 497-505.

Weiner, C. P., Knowles, R. G. and Moncada, S. (1994a) Induction of nitric oxide synthases early in pregnancy. *Am J Obstet Gynecol.* **171:** 838-843.

Weiner, C. P., Lizasoain, I., et al. (1994b) Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc Natl Acad Sci U S A.* **91:** 5212-5216.

Wojcikiewicz, R. J. (1995) Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J Biol Chem.* **270:** 11678-11683.

Word, R. A., Stull, J. T., Casey, M. L. and Kamm, K. E. (1993) Contractile elements and myosin light chain phosphorylation in myometrial tissue from nonpregnant and pregnant women. *J Clin Invest.* **92:** 29-37.

Yallampalli, C., Byam, S. M., Nelson, S. O. and Garfield, R. E. (1994a) Steroid hormones modulate the production of nitric oxide and cGMP in the rat uterus. *Endocrinology.* **134:** 1971-1974.

Yallampalli, C., Izumi, H., Byam, S. M. and Garfield, R. E. (1994b) An L-arginine-nitric oxide-cyclic guanosine monophosphate system exists in the uterus and inhibits contractility during pregnancy. *Am J Obstet Gynecol.* **170(1 Pt 1)::** 175-185.

Yamagishi, T., Yanagisawa, T., Satoh, K. and Taira, N. (1994) Relaxant mechanisms of cyclic AMP-increasing agents in porcine coronary artery. *Eur J Pharmacol.* **251:** 253-262.

Yang, Z., Sugawara, M., et al. (1990) Interferon gamma response region in the promoter of the human DPA gene. *Proc Natl Acad Sci U S A.* **87:** 9226-9230.

Ylikorkala, O., Jouppila, P., Kirkinen, P. and Viinikka, L. (1983) Maternal prostacyclin, thromboxane, and placental blood flow. *Am J Obstet Gynecol.* **145:** 730-732.

Yokoyama, C. and Tanabe, T. (1989) Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. *Biochem Biophys Res Commun.* **165:** 888-894.

Zuckerman, H., Shalev, E., Gilad, G. and Katzuni, E. (1984) Further study of the inhibition of premature labor by indomethacin. Part II double-blind study. *J Perinat Med.* **12:** 25-29.

Zuo, J., Lei, Z. M., et al. (1994) Differential cyclooxygenase-1 and -2 gene expression in human myometria from preterm and term deliveries. *J Clin Endocrinol Metab.* **79:** 894-899.

7. Appendix

7.1 Publications

7.1.1

Nitric oxide synthase mRNA expression in human fetal membranes: a possible role in parturition

William J. B. Dennes, Donna M. Slater and Phillip R. Bennett

Biochemical and Biophysical Research Communications (1997), **233**, 276-278.

Author's Contribution:

Experimental design and methodology, RT-PCR analysis and writing and submission of the manuscript.

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 233, 276–278 (1997)
ARTICLE NO. RC976439

Nitric Oxide Synthase mRNA Expression in Human Fetal Membranes: A Possible Role in Parturition

William J. B. Dennes,[1] Donna M. Slater, and Phillip R. Bennett
Institute of Obstetrics and Gynaecology, Royal Postgraduate Medical School, Queen Charlotte's and Chelsea Hospital, London W6 0XG, United Kingdom

Received March 6, 1997

Nitric oxide (NO) is a potent endogenous smooth-muscle relaxant. It is synthesised from l-arginine by isoforms of nitric oxide synthase (NOS). Whilst it is clear that the uterus responds to NO by relaxation, NOS expression has not been investigated in fetal membranes or myometrium in human pregnancy. This study has shown, using semi-quantitative RT-PCR, expression of cNOS mRNA in human amnion, chorion-decidua, and placenta. iNOS mRNA expression was demonstrated in human amnion, chorion-decidua, and placenta. It is possible that NO synthesised in fetal membranes may act either directly to inhibit myometrial contractility or indirectly to interact with other labour-associated genes, such as cyclo-oxygenase, to coordinate the onset of labour. © 1997 Academic Press

The mechanisms that initiate the onset of labour in humans are poorly understood. It is our hypothesis that parturition occurs through the up-regulation of a group of pro-labour genes, such as oxytocin receptors and gap junction proteins. We have previously shown (1) that labour is associated with increased expression of cyclo-oxygenase (COX), the central enzyme in prostaglandin synthesis. With labour there is increased expression of the inducible, type-2 isoform, with no change in expression of the constituitive, type-1 isoform.

NO is a highly reactive inorganic free radical with widespread biological effects. It inhibits platelet aggregation, is a potent smooth muscle relaxant, and can act as a cellular messenger. Its effects on smooth muscle are mediated via stimulation of the cyclic guanylate cyclase pathway. Both animal and human studies (2,3) have shown that there is a NO-cGMP relaxation pathway in the uterus with decreased responsiveness to NO at term, suggesting that NO may decrease uterine contractility, maintaining the uterus in a state of quiescence during pregnancy.

NO is synthesised by oxidative de-amination of the semi-essential amino acid L-arginine by isoforms of the transcriptionally regulated enzyme nitric oxide synthase (NOS,EC1.14.23) (4). NOS exists in at least three isoforms: a constituitive, calcium-dependent endothelial form (cNOS), a constituitive, calcium-dependent neuronal form (nNOS) and an inducible, calcium-independent form (iNOS). NOS activity in myometrium has been demonstrated in animal studies, and been shown to fall both on the last day of pregnancy (5) and with the onset of labour (6). Telfer et al has identified cNOS in non-pregnant human endometrium and myometrium.

Whilst it is clear that NO relaxes myometrial smooth muscle, the production of NO by either fetal membranes or myometrium itself has not been directly studied in human pregnancy. This study investigates the expression of cNOS and iNOS in term fetal membranes.

MATERIALS AND METHODS

Human term fetal membranes and placenta were collected (n=12), washed with phosphate buffered saline, split into amnion, chorion-decidua, and placental trophoblast, and immediately snap frozen in liquid nitrogen. Human umbilical endothelial cells (HUVEC) a positive control for cNOS, were obtained from established culture. RNA was extracted using a standard guanidium isothiocyanate technique (7). RNA samples (1μg) were denatured at 70°c for 5 minutes and cooled to 37°c. Reverse transcription was carried out using random hexanucleotide primers (0.2μg), ×1 reverse transcriptase buffer, 10mM dithiothreitol, 1mM each dNTP, 40units M-MLV reverse transcriptase (Gibco BRL) and 1unit RNAse inhibitor (Pharmacia Biotech) were added to a final volume of 20μl and incubated at 37°c for 60 minutes. Reverse transcription was terminated by heating to 90°c for 5 minutes.

PCR amplification from reverse transcribed cDNA was carried out using specifically designed PCR primers for cNOS and iNOS: cNOS sense: 5′-GCACAGGAAATGTTCACCTAC-3′, cNOS antisense: 5′-CACGATGGTGACTTTGGCTAG-3′ (8), iNOS sense: 5′-GAGCTTCTACCTCAAGCTATC-3′, iNOS antisense: 5′-CCTGATGTTGCCATTGTTGGT-3′ (9). PCR amplification for Glyceraldehyde-3-mono-

[1] To whom correspondence should be addressed. Fax: ++ 44 181 383 1838. E-mail: w.dennes@rpms.ac.uk.

0006-291X/97 $25.00
Copyright © 1997 by Academic Press
All rights of reproduction in any form reserved.

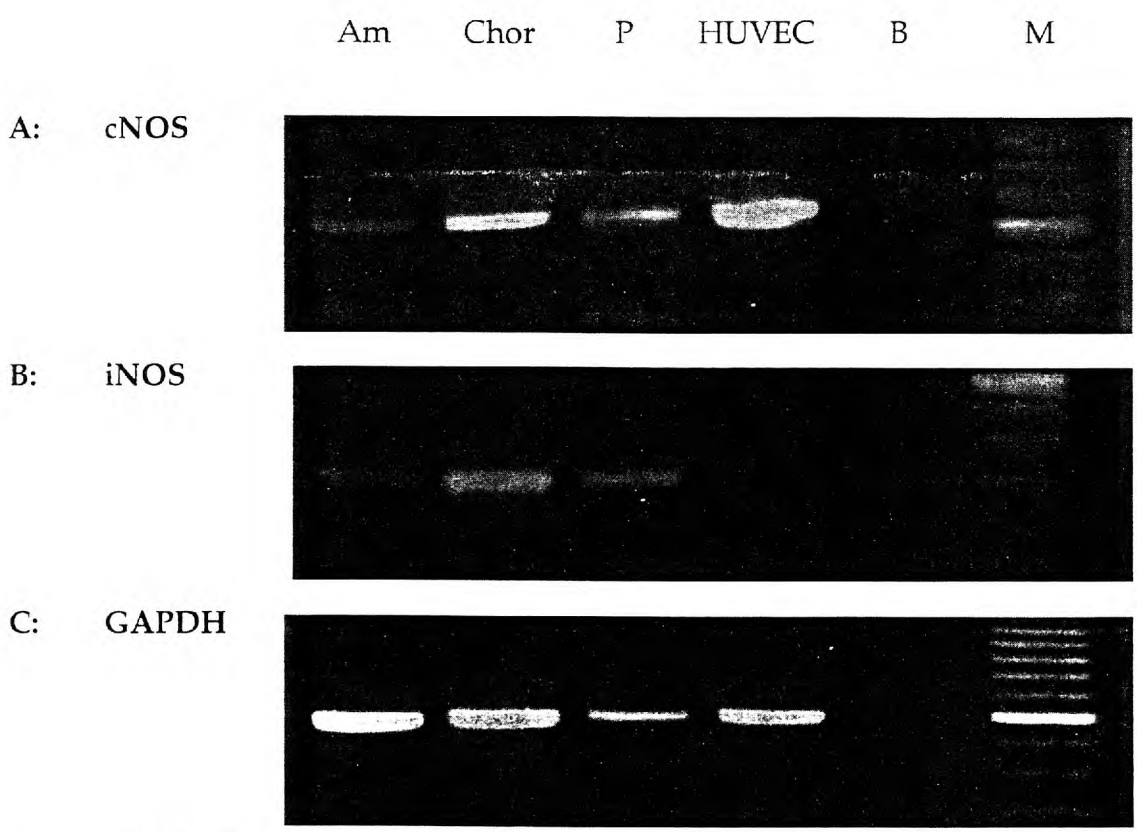


FIG. 1. Expression of NOS isoforms and GAPDH (as control) in term human amnion (Am), chorion-decidua (Chor), placenta (P), and human umbilical vein endothelial cell (HUVEC). B, Blank (PCR with no DNA template); M, 100bp DNA ladder.

phosphate dehydrogenase (GAPDH) was carried out on the same samples as a parallel control. GAPDH sense: 5'-CCACCCATGGCAAATTCCATGGCA-3', GAPDH antisense: 5'-TCAAGACGGCAGGTCAGGTCCACC-3' (10).

PCR was performed on a 1/40 volume of the RT reaction with 1.5mM magnesium chloride, 0.2mM dNTPs, 125ng of each primer, and 1unit of Biotaq polymerase (Biotaq) in a final volume of 25μl. Reaction cycles were an initial denaturation step of 4 minutes at 94°c, followed by denaturation at 94°c for 30 seconds, annealing at 55°c (cNOS) or 62°c (iNOS) for 30 seconds, and extension at 72°c for 30 seconds for the appropriate number of cycles, followed by a further 5 minute extension time at 72°c. 10μl aliquots of the PCR product were separated by horizontal gel electrophoresis, on a 1% agarose ethidium bromide stained gel. DNA was transferred to a nylon transfer membrane (MSI) for Southern blotting. Filters were hybridised with [^{32}P]dCTP labelled cDNA probes, for cNOS, iNOS and GAPDH and washed to high stringency. Autoradiography was carried out to confirm the identity of the PCR products. PCR products were initially sub-cloned, ligated into pGEM-T vector (Promega) and subsequently sequence verified.

For each target gene, cycle profiles were carried out on pooled samples to determine the exponential phase of amplification, where final product concentration is proportional to starting cDNA concentration. Cycles were carried out from 25 to 60, at 5 cycle intervals. Since the initial amount of RNA template in each case is identical (1μg), semi-quantitation is achieved.

RESULTS

cNOS expression was shown in amnion, chorion-decidua, placenta and HUVEC (positive control). The expected product size was 654bp. Figure 1A. shows cNOS PCR amplification at 55 cycles. Visually there was increased amplification product concentration from chorion-decidua. This was confirmed by cycle profiles which indicated an optimal cycle number for amnion as 52, and for chorion-decidua as 45 (data not shown).

iNOS expression was shown in amnion, chorion-decidua and placenta. Expected product size was 313bp. Figure 1B. shows iNOS amplification at 45 cycles. There was no iNOS expression in HUVEC cells. In a similar expression pattern to cNOS there appeared to be increased amplification product concentration from chorion-decidua and this was confirmed by cycle profiles, indicating optimal cycles for amnion as 45 and for chorion-decidua 40 (data not shown).

GAPDH PCR amplification (expected product size 598bp) confirmed the presence of cDNA starting template and amplification products (Figure1C).

PCR products were subsequently verified by Southern hybridization to previously generated and sequenced products.

DISCUSSION

This study has for the first time demonstrated the expression of cNOS and iNOS mRNA in human fetal

membranes at term. In addition cycle profiles indicate an approximate 100 fold higher amplification product concentration of cNOS mRNA in chorion-decidua compared to amnion, and higher levels of iNOS expression in all tissues examined with an approximate 30 fold higher concentration of iNOS mRNA in chorion-decidua compared to amnion.

The role of NO generated from NOS expression within fetal membranes is speculative. NO might exert a direct effect on myometrial contractility, although the short half-life of NO (4 seconds) and anatomical considerations might suggest this unlikely. We propose that NO might interact with other labour-associated genes exerting an indirect effect on myometrial activity and possibly the initiation of labour. Prostaglandins have been shown to play a pivotal role in the onset of parturition. Prostaglandins are generated from arachidonic acid by the actions of cyclo-oxygenase. At present two cyclo-oxygenase enzymes have been identified, a constituitive isoform COX-1 and an inducible isoform COX-2. We have shown previously (1) that fetal membranes, and amnion in particular, express COX-2 and are a significant source of PGE_2. Swierkosz et al. (11) have shown in endothelial cells that low levels of NO induce COX whilst high levels inhibit. In addition Tetsuka et al. (12) have proposed that in rat mesangial cells iNOS-generated NO activates cyclo-oxygenase, resulting in the production of PGE_2 which, via the EP_1 receptor, then down-regulates iNOS. It is possible that a similar pathway is active within these tissues and that changes in NOS expression and therefore NO levels may influence PGE_2 production and the onset of labour.

ACKNOWLEDGMENTS

Grateful acknowledgement is made to Dr. Robert Newton for kindly providing iNOS primers and sub-cloned probe. This work was supported by a Wellbeing grant.

REFERENCES

1. Slater, D. M., Berger, L. C., Newton, R., Moore, G. E., and Bennett, P. R. (1995) *Am. J. Obstet. Gynecol.* **172,** 77–82.
2. Yallampalli, C., Izumi, H., Byam, S. M., and Garfield, R. E. (1994) *Am. J. Obstet. Gynecol.* **170,** 175–85.
3. Buhimschi, I., Yallampalli, C., Dong, Y. L., and Garfield, R. E. (1995) *Am. J. Obstet. Gynecol.* **172,** 1577–84.
4. Palmer, R. M., Ashton, D. S., and Moncada, S. (1988) *Nature* **333,** 664–6.
5. Sladek, S. M., Regenstein, A. C., Lykins, D., and Roberts, J. M. (1993) *Am. J. Obstet. Gynecol.* **169,** 1285–91.
6. Natuzzi, E. S., Ursell, P. C., Harrison, M., Buscher, C., and Riemer, R. K. (1993) *Biochem. Biophys. Res. Commun.* **194,** 1–8.
7. Chirgwin, J. M., Przybyla, A. E., MacDonald. R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–9.
8. Nadaud, S., Bonnardeaux, A., Lathrop, M., and Soubrier, F. (1994) *Biochem. Biophys. Res. Commun.* **198,** 1027–33.
9. Chartrain, N. A., Geller, D. A., Koty, P. P., Sitrin, N. F., Nussler, A. K., Hoffman, E. P., Billiar, T. R., Hutchinson, N. I., and Mudgett, J. S. (1994) *J. Biol. Chem.* **269,** 6765–72.
10. Cheung, P. Y., and Challis, J. R. (1989) *Am. J. Obstet. Gynecol.* **161,** 1580–5.
11. Swierkosz, T. A., Mitchell, J. A., Tomlinson, A., Botting, R. M., Warner, T. D., and Vane, J. R. (1994) *Pol. J. Pharmacol.* **46,** 587–92.
12. Tetsuka, T., Daphna, I. D., Srivastava, S. K., Baier, L. D., DuMaine, J., and Morrison, A. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12168–72.
13. Di Iulio, J., Gude, N. M., King, R. G., and Brennecke, S. P. (1995) *Reprod. Fertil. Dev.* **7,** 1505–8.

7.1.2

Myometrial nitric oxide synthase messenger ribonucleic acid expression does not change throughout gestation or with the onset of labor

William J. B. Dennes, Donna M. Slater, Lucilla Poston and Phillip R. Bennett

American Journal of Obstetrics and Gynaecology (1999), **180**, 2, 387-392.

Author's Contribution:

Experimental design and methodology, RT-PCR analysis and writing and submission of the manuscript.

Myometrial nitric oxide synthase messenger ribonucleic acid expression does not change throughout gestation or with the onset of labor

William J.B. Dennes, MB, BS,[a] Donna M. Slater, PhD,[a] Lucilla Poston, PhD,[b] and Phillip R. Bennett, MD, PhD[a]

London, United Kingdom

OBJECTIVE: The purposes of this study were to examine expression of nitric oxide synthase isoforms in human myometrium and to determine any changes in expression with gestational age and with the onset of labor at term.

STUDY DESIGN: Myometrial samples were collected from patients undergoing cesarean delivery at term before and after the onset of labor (n = 17) and throughout gestation (n = 13). Expressions of inducible, calcium-independent nitric oxide synthase and constitutive, calcium-dependent endothelial nitric oxide synthase were determined by semiquantitative reverse transcriptase–polymerase chain reaction.

RESULTS: Messenger ribonucleic acid for inducible, calcium-independent nitric oxide synthase and constitutive, calcium-dependent endothelial nitric oxide synthase is expressed in human myometrium at term and throughout the second and third trimesters. Levels of messenger ribonucleic acid for both inducible, calcium-independent nitric oxide synthase and constitutive, calcium-dependent endothelial nitric oxide synthase do not change with either gestational age or the onset of labor.

CONCLUSION: Changes in myometrial nitric oxide synthase expression and thus of levels of endogenous nitric oxide are unlikely to be directly involved in myometrial quiescence or the onset of human parturition. (Am J Obstet Gynecol 1999;180:387-92.)

Key words: Endothelial nitric oxide synthase, inducible nitric oxide synthase, reverse transcriptase–polymerase chain reaction, pregnancy, uterus

Preterm labor accounts for ≤10% of births but >60% of neonatal morbidity. In the last 20 years there have been no significant advances in tocolytic therapy, and the improved survival of preterm infants is largely a reflection of improvements in neonatal resuscitation and intensive care. The mechanisms involved in the onset of labor at term remain ill defined, and as a result the factors responsible for the onset of preterm labor are similarly poorly understood. The progression of pregnancy to term relies on profound physiologic and anatomic changes within the uterus and on a state of prolonged myometrial quiescence. Quiescence is a function of decreased myometrial excitability, with resistance to activation and with associated incomplete cell-cell coupling, resulting in low basal smooth muscle tone and absence of contractions. At present no single factor has been shown to be responsible for these physiologic changes in pregnancy. Pregnancy may rather represent a balance between "propregnancy" factors, such as progesterone, that maintain myometrial quiescence and allow uterine expansion and "prolabor" factors, such as prostaglandins, that result in the onset of uterine contractions, cervical remodeling, and ultimately delivery of the fetus.

Nitric oxide has been proposed as a putative mediator of uterine smooth muscle relaxation and myometrial quiescence,[1] and it has been proposed to have a propregnancy function. Nitric oxide, a highly reactive free radical with diverse biologic activities, is synthesized by oxidative deamination of the semiessential amino acid L-arginine by isoforms of nitric oxide synthase (NOS).[2] NOS exists in at least 3 isoforms. Type I is a constitutive, calcium-dependent neuronal form (nNOS), type II is an inducible, calcium-independent form (iNOS), and type III is a constitutive, calcium-dependent endothelial form (e/cNOS). There is good evidence that nitric oxide donors have an inhibitory effect on pregnant human uterine contractility. Amyl nitrate, a nitric oxide donor, has been shown to decrease the magnitude of oxytocin-induced contractions

From the Division of Paediatrics, Obstetrics and Gynaecology, Imperial College School of Medicine, Queen Charlotte's and Chelsea Hospital,[a] and the Fetal Health Research Laboratory, Division of Obstetrics and Gynaecology, United Medical and Dental School, St Thomas' Hospital.[b]
Received for publication April 15, 1998; revised June 18, 1998; accepted August 17, 1998.
Reprint requests: William J.B. Dennes, MB, BS, Division of Obstetrics and Gynaecology, Imperial College School of Medicine, Queen Charlotte's and Chelsea Hospital, London, United Kingdom W6 0XG.
Copyright © 1999 by Mosby, Inc.
0002-9378/99 $8.00 + 0 6/1/93883

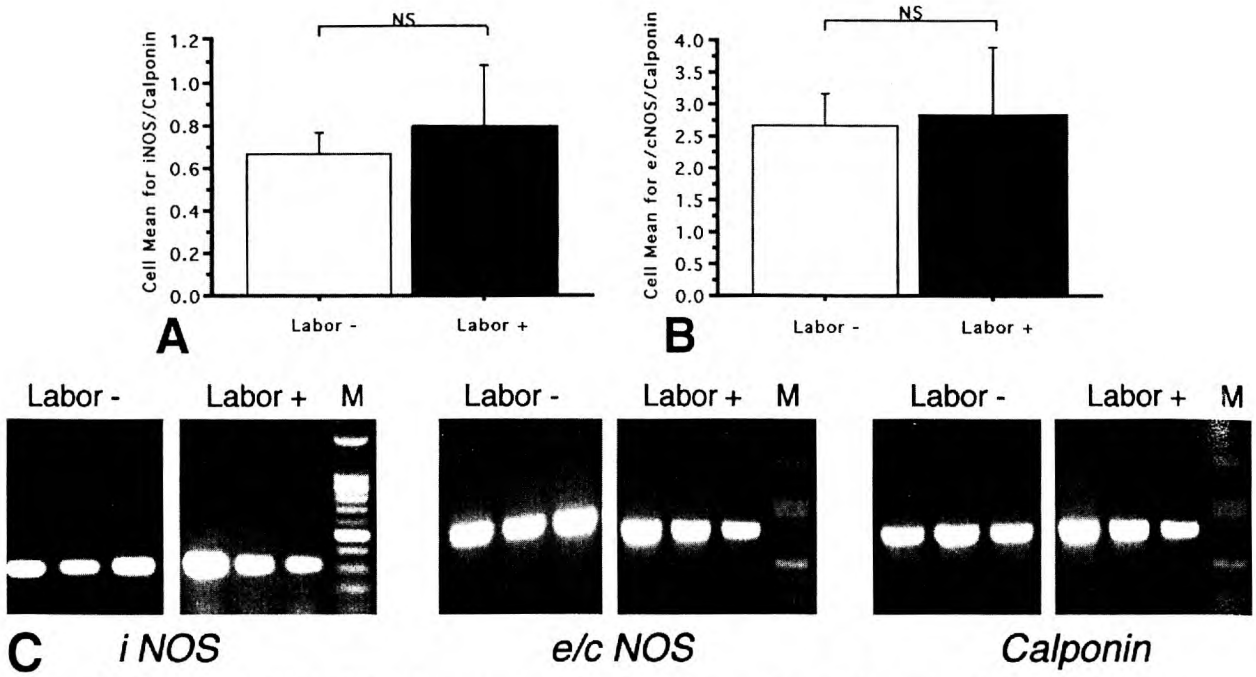


Fig 1. Expression of NOS mRNA in human myometrium by reverse transcriptase–polymerase chain reaction. NOS expression is shown as a function of mean counts per minute before (n = 11, *Labor –*) and after (n = 5, *Labor +*) the onset of labor for iNOS **(A)** and e/cNOS **(B)**. *NS*, Not significant. *Error bars* represent 2 SEM. **C,** Representative samples before *(Labor –)* and after *(Labor +)* the onset of labor. *M* represents a 100–base pair DNA marker. Dot blots were probed with radiolabeled complementary DNA probes.

but not of spontaneous contractions.[3] Intravenous nitroglycerin has been used to relax the uterus post partum for extraction of retained placenta,[4] and it has been suggested that glyceryl trinitrate may arrest preterm labor and prolong pregnancy.[5] Although exogenous nitric oxide appears to relax the uterus, the role of endogenous nitric oxide and the generation of nitric oxide within the human myometrium are less clear.

Nitric oxide exerts its effect on smooth muscle by stimulation of the guanylate cyclase pathway. Both animal and human studies[1, 6] have suggested that a nitric oxide–cyclic guanosine monophosphate relaxation pathway exists in the pregnant uterus, with decreased responsiveness to nitric oxide at term. In both rabbits[7] and rats[8] NOS activity within the uterus has been reported to fall on the last day of pregnancy and with the onset of labor. Human studies have demonstrated expression of e/cNOS in the nonpregnant uterus,[9] and both e/cNOS and iNOS have been reported in the uterus during early pregnancy.[10] Bansal et al[11] showed, by means of immunostaining and Western blot analysis, that iNOS is expressed in human myometrial cells and that expression falls significantly at term and falls further (but not significantly) with the onset of labor. In contrast to this evidence suggesting that endogenous nitric oxide may be directly associated with the onset of labor, Jones and Poston,[12] in their in vitro studies of human myometrial strips, were unable to demonstrate any effect on spontaneous myometrial contractions after the addition of either L-arginine, the substrate for NOS, or L-arginine methyl ester hydrochloride, a NOS inhibitor. They therefore concluded that nitric oxide was unlikely to be involved in the maintenance of uterine quiescence.

There is good evidence that NOS isoforms are subject to transcriptional regulation. Messenger ribonucleic acid (mRNA) for iNOS can be induced by both cytokine and bacterial factors.[13] The calcium-dependent isoforms, traditionally termed *constitutive,* may also be subject to transcriptional regulation. Levels of both protein and mRNA of e/cNOS and constitutive, calcium-dependent neuronal NOS are substantially increased in the guinea pig during pregnancy in both brain and skeletal muscle, probably as a result of estrogen induction.[14] Previous studies of changes in NOS expression in human myometrium have been confined to NOS protein levels and enzyme activity. We examined expression of NOS mRNA by semiquantitative reverse transcriptase–polymerase chain reaction, both throughout gestation and before and after the onset of labor at term, to establish whether changes in NOS mRNA transcription supported the hypothesis that endogenous nitric oxide production within the myometrium is directly involved in the physiologic control of human parturition.

Methods

Tissue collection. Myometrial tissue was collected from the upper margin of uterine incision at time of lower segment cesarean delivery. Myometrial biopsy samples avoided decidual and vascular tissue. Three groups of patients were analyzed: those in labor at term (n = 5), those at term not in labor (n = 11), and preterm patients not in labor (n = 13). The term patients' gestational age range was 38 to 40 weeks; in the preterm nonlabor group the gestational age range was 26-37 weeks. In the term labor group all patients had regular painful contractions with associated cervical dilatation. The indications for cesarean delivery in the term labor group were associated with fetal distress. There was no evidence of uterine dysfunction. The nonlabor groups had no evidence of uterine contractions or cervical change. The indications for cesarian delivery in this group were breech presentation, hypertensive disease, and previous cesarian delivery. This study was approved by the hospital's ethics committee, and all patients gave written, informed consent.

Semiquantitative reverse transcriptase–polymerase chain reaction. Myometrial tissue was immediately snap frozen in liquid nitrogen. Ribonucleic acid (RNA) was extracted by means of a routine guanidine isothiocyanate technique.[15] RNA samples (1 μg) were denatured at 70°C for 5 minutes and cooled to 37°C. Reverse transcription was carried out with random hexanucleotide primers (0.2 μg), ×1 reverse transcriptase buffer, 10 mmol/L dithiothreitol, 1 mmol/L each deoxyribonucleoside triphosphate (all obtained from Promega, Madison Wis), 40 units Moloney murine leukemia virus reverse transcriptase obtained from Gibco BRL (Life Technologies Ltd, Paisley, Scotland), and 1 unit ribonuclease inhibitor obtained from Pharmacia Biotech (Uppsala, Sweden) were added to a final volume of 20 μL and incubated at 37°C for 60 minutes. Reverse transcription was terminated by heating to 90°C for 5 minutes. Polymerase chain reaction amplification was carried out with polymerase chain reaction primers specifically designed for e/cNOS and iNOS. Polymerase chain reaction amplification for calponin, a smooth muscle cell marker, was carried out on the same samples as a parallel control. Primers used were as follows:

e/cNOS sense: 5′-GCACAGGAAATGTTCACCTAC-3′
e/cNOS antisense[16]: 5′-CACGATGGTGACTTTGGCTAG-3′
iNOS sense: 5′-GAGCTTCTACCTCAAGCTATC-3′
iNOS antisense[17]: 5′-CCTGATGTTGCCATTGTTGGT-3′
calponin sense: 5′-GATGGCATCATTCTTTGCGA-3′
calponin antisense[18]: 5′-TTGTAGTAGTTGTGTGCGTG-3′

Polymerase chain reaction was performed on 1/40 of the volume of the reverse transcription reaction with 1.5 mmol/L magnesium chloride, 0.2 mmol/L deoxyribonucleoside triphosphate, 125 ng of each primer, and 1 unit Biotaq polymerase obtained from Biotaq (Bioline, London, United Kingdom) in a final volume of 25 μL.

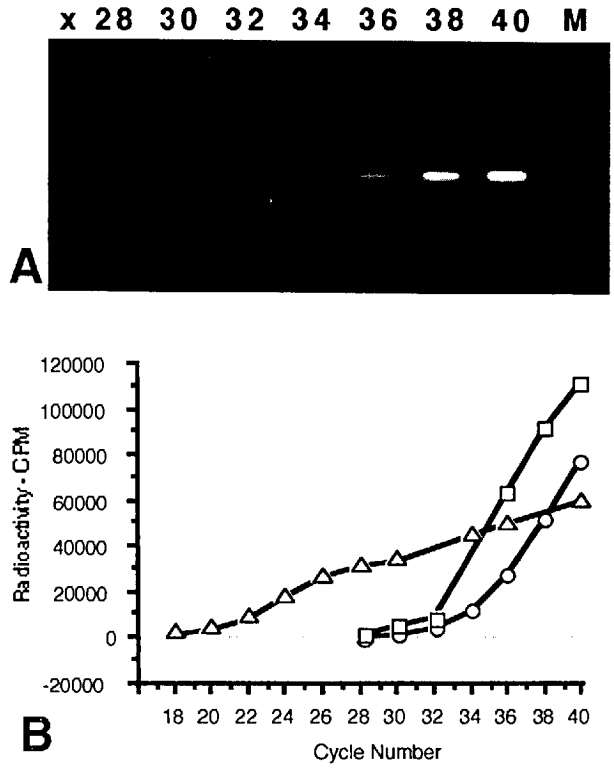


Fig 2. A, Representative myometrial iNOS reverse transcriptase–polymerase chain reaction cycle profile. Cycle number was increased from 28 to 40 cycles to determine the range of exponential amplification. *M* represents a 100–base pair DNA marker. **B,** Radioactivity measured as counts per minute of hybridized dot blots against cycle number for iNOS *(open circles)*, e/cNOS *(open squares)*, and calponin *(open triangles)*.

Reaction cycles were an initial denaturation step of 4 minutes at 94°C followed by denaturation at 94°C for 30 seconds; annealing at 55°C (e/cNOS), 62°C (iNOS) or 53°C (calponin) for 30 seconds; and extension at 72°C for 30 seconds for the appropriate number of cycles. This was followed by a further 5 minute extension time at 72°C. Aliquots (10 μL) of the polymerase chain reaction product were separated by horizontal gel electrophoresis on a 1% agarose, ethidium bromide stained gel. Deoxyribonucleic acid (DNA) was transferred to a nylon transfer membrane obtained from MSI (Micron Separations Inc, Westborough, Mass) for Southern blotting. A further 5-μL aliquot of the polymerase chain reaction product was dotted directly on nylon transfer membrane to create a dot blot. Filters were hybridized with complementary DNA probes for e/cNOS, iNOS and calponin labeled with phosphorus 32–tagged deoxycytidine triphosphate by means of standard techniques[19] and washed to high stringency. Autoradiography was carried out to confirm the identity of the polymerase chain reaction products. Polymerase chain reaction products were subcloned, ligated into pGEM-T vector obtained from Promega (Madison, Wis) and subsequently verified for sequence. The dot blot membranes

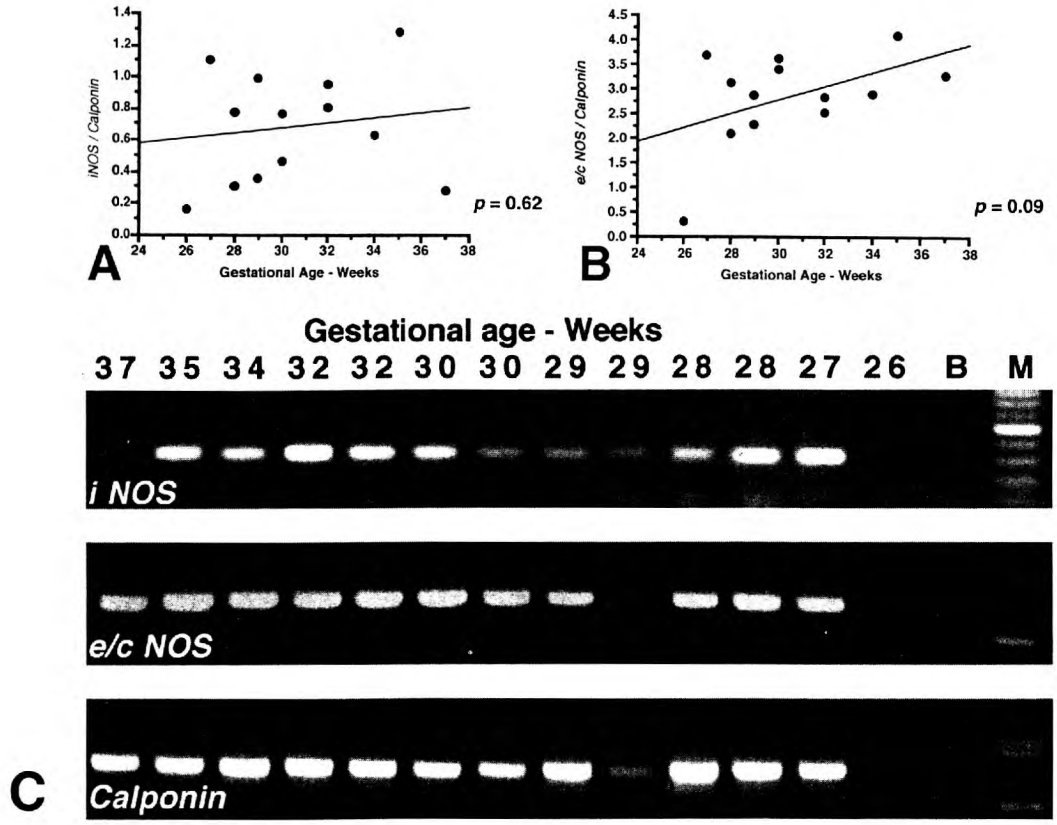


Fig 3. NOS mRNA expression in human myometrium between 26 and 37 weeks' gestation. Linear regression analysis of NOS expression on gestational age between 26 and 37 weeks' gestation (n = 13) is shown as a function of mean counts per minute for iNOS (**A**) and e/cNOS (**B**). **C,** iNOS, e/cNOS, and calponin expressions by reverse transcriptase–polymerase chain reaction. Dot blots were probed with radiolabeled complementary DNA probes. *M* represents a 100-base pair DNA marker. *B,* Blank lane—no starting template DNA.

were placed in a scintillation counter, and radioactivity was measured as a function of counts per minute for each individual polymerase chain reaction.

Cycle profiles were carried out on pooled samples for each target mRNA to determine the exponential phase of amplification, where final product concentration is proportional to starting complementary DNA concentration. Cycles were carried out at 2-cycle intervals from 28 to 40 for iNOS and e/cNOS and from 18 to 40 cycles for calponin. Expression of NOS was represented as a ratio to that of calponin to control for RNA concentration.

Statistical analysis. Data comparing changes in levels of expression with labor onset were analyzed nonparametrically with the Mann-Whitney *U* test (Statview; Abacus concepts Inc, Berkeley, Calif), and expressed as means with 2 SE. The relationship of NOS expression to gestational age was analyzed by linear regression analysis of NOS expression on gestational age. Statistical significance was assumed at $P \leq .05$.

Results

Nitric oxide synthase expression in myometrium. Expressions of iNOS and e/cNOS mRNA were demonstrated in human pregnant myometrium. Fig 1, *C,* shows representative NOS expression in both term labor and term nonlabor myometrial samples. Southern blotting and hybridization verified correct polymerase chain reaction product. The expected polymerase chain reaction product sizes with these primers were 313 base pairs for iNOS and 654 base pairs for e/cNOS.

Cycle profiles. A representative cycle profile is shown in Fig 2, *A.* Fig 2, *B,* shows radioactivity in counts per minute for hybridized dot blots of cycle profiles for iNOS, e/cNOS, and calponin. The optimal cycle numbers determined were 39 for iNOS, 35 for e/cNOS, and 35 for calponin.

Nitric oxide synthase expression and labor. Fig 1 shows NOS expression as a function of mean counts per minute for both iNOS (Fig 1, *A*) and e/cNOS (Fig 1, *B*) in term myometrium before (n = 11) and after (n = 5) the onset of labor. There were no significant differences in expression of either iNOS or e/cNOS in term myometrium before and after the onset of labor (iNOS labor versus nonlabor, $P = .61$; e/cNOS labor versus nonlabor, $P = .58$)

Nitric oxide synthase expression and gestation. Fig 3, *C,* shows iNOS and e/cNOS expressions between 26 and 37 weeks' gestation (n = 13). Linear regression analysis of

NOS expression by gestational age showed no significant association between expression of either iNOS (Fig 3, *A*) or e/cNOS (Fig 3, *B*) and gestational age (iNOS expression versus gestational age, $P = .62$; e/cNOS expression versus gestational age, $P = .09$).

Comment

This study shows that mRNA for iNOS and e/cNOS is expressed in human myometrial tissue in both the second and third trimesters. This finding supports evidence from previous studies of NOS expression in pregnancy.[10] In contrast to other studies,[11] however, we were unable to find any changes in expression with either the onset of labor or increasing gestation. Although Bansal et al's findings[11] could represent posttranslational modification, to date posttranslational iNOS modification has only been reported in murine macrophages[20] and has not been investigated in human pregnancy.

The calcium-dependent, constitutively expressed NOS isoform, e/cNOS, is principally involved in homeostatic mechanisms, such as the maintenance of vascular tone. The observation in this study that e/cNOS mRNA is not induced by labor or changes in gestational age is consistent with these concepts. We did not compare levels between nonpregnant and pregnant subjects, which might have shown evidence of estrogen-induced transcriptional upward regulation.[14] In contrast, iNOS is transcriptionally regulated by a wide number of cytokines and growth factors.[13] Labor is associated with an increase in cytokine production throughout different fetomaternal compartments,[21] and it is therefore more likely that labor would be associated with an increase in iNOS expression and nitric oxide levels, rather than previously observed falls.[11] We were unable to find up-regulation of iNOS mRNA in human myometrial tissue associated with labor. We previously showed that cyclo-oxygenase type 2, the principal enzyme involved in prostaglandin synthesis, is up-regulated in both human fetal membranes[22] and human myometrium[23] with advancing gestation and with labor. Cyclo-oxygenase type 2 and iNOS share putative response elements in their 5′ promoter regions for nuclear factor kappa B and interleukin 6.[24, 25] In labor, however, there is an increase in myometrial cyclo-oxygenase type 2 mRNA but not a concomitant rise in iNOS mRNA. Because iNOS mRNA is not up-regulated with labor but cyclo-oxygenase type 2 mRNA is, it is possible that iNOS transcription may not be regulated by the same factors as those that regulate cyclo-oxygenase type 2 transcription.

Previous studies suggest localization of e/cNOS immunoreactivity in vascular endothelial cells of the nonpregnant myometrium and of iNOS immunoreactivity in decidual stromal cells.[10] Immunostaining for iNOS has also been seen in diffusely dispersed myocytes in the pregnant uterus.[11] Myometrial biopsy samples in our study were taken from the upper uterine incision at cesarean delivery. The lower uterine segment develops more rapidly late in pregnancy and undergoes significant changes at term and close to labor, when it becomes thinner and the relative smooth muscle content falls. In labor it stretches significantly to allow passage of the fetus, but it probably plays only a small role in generating uterine contractions and in increasing uterine pressure to expel the fetus. It is therefore possible that there is differential NOS expression throughout the uterus, and there is evidence from animal studies that there is differential regulation of nitric oxide in the uterus and cervix during pregnancy and labor.[26] Partial-thickness biopsy samples were taken specifically to exclude decidual tissue; however, iNOS expression in our study may still be a function of macrophage or lymphocyte expression rather than of true myocyte expression. Similarly, e/cNOS expression may be a result of expression in vascular endothelial cells within the myometrial biopsy sample.

Levels of expression of both NOS isoforms in this study were low. Semiquantitative reverse transcriptase–polymerase chain reaction is a highly sensitive and reproducible technique for studying mRNA expression of genes expressed at low levels. The optimal cycle numbers determined in this study in myometrium were 39 for iNOS and 35 for e/cNOS. Although no direct comparison can be made regarding the absolute amount of RNA present, because of potential differences in polymerase chain reaction efficiency between the 2 genes, these high cycle numbers suggest extremely low levels of mRNA for both NOS isoforms and further question the physiologic role, if any, of endogenous nitric oxide.

REFERENCES

1. Buhimschi I, Yallampalli C, Dong YL, Garfield RE. Involvement of a nitric oxide-cyclic guanosine monophosphate pathway in control of human uterine contractility during pregnancy. Am J Obstet Gynecol 1995;172:1577-84.
2. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 1988;333:664-6.
3. Kumar D, Zourlas PA, Barnes AC. In vivo effect of amyl nitrite on human pregnant uterine contractility. Am J Obstet Gynecol 1965;91:1066-8.
4. Peng AT, Gorman RS, Shulman SM, DeMarchis E, Nyunt K, Blancato LS. Intravenous nitroglycerin for uterine relaxation in the postpartum patient with retained placenta. Anesthesiology 1989;71:172-3.
5. Lees C, Campbell S, Jauniaux E, Brown R, Ramsay B, Gibb D, et al. Arrest of preterm labour and prolongation of gestation with glyceryl trinitrate, a nitric oxide donor. Lancet 1994;343:1325-6.
6. Yallampalli C, Izumi H, Byam SM, Garfield RE. An L-arginine-nitric oxide-cyclic guanosine monophosphate system exists in the uterus and inhibits contractility during pregnancy. Am J Obstet Gynecol 1994;170:175-85.
7. Sladek SM, Regenstein AC, Lykins D, Roberts JM. Nitric oxide synthase activity in pregnant rabbit uterus decreases on the last day of pregnancy. Am J Obstet Gynecol 1993;169(5):1285-91.
8. Natuzzi ES, Ursell PC, Harrison M, Buscher C, Riemer RK. Nitric oxide synthase activity in the pregnant uterus decreases at parturition. Biochem Biophys Res Commun 1993;194:1-8.
9. Telfer JF, Lyall F, Norman JE, Cameron IT. Identification of nitric oxide synthase in human uterus. Hum Reprod 1995; 10(1):19-23.

10. Telfer JF, Irvine GA, Kohnen G, Campbell S, Cameron IT. Expression of endothelial and inducible nitric oxide synthase in non-pregnant and decidual human endometrium. J Mol Reprod 1997;3:69-75.
11. Bansal RK, Goldsmith PC, He Y, Zaloudek CJ, Ecker JL, Riemer RK. A decline in myometrial nitric oxide synthase expression is associated with labor and delivery. J Clin Invest 1997;99:2502-8.
12. Jones G, Poston L. The role of endogenous nitric oxide synthesis in contractility of term and preterm human myometrium. Br J Obstet Gynaecol 1997;104:241-5.
13. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. J Immunol 1988; 141:2407-12.
14. Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S. Induction of calcium-dependent nitric oxide synthases by sex hormones. Proc Natl Acad Sci U S A 1994;91: 5212-6.
15. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979;18:5294-9.
16. Nadaud S, Bonnardeaux A, Lathrop M, Soubrier F. Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. Biochem Biophys Res Commun 1994;198:1027-33.
17. Chartrain NA, Geller DA, Koty PP, Sitrin NF, Nussler AK, Hoffman EP, et al. Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. J Biol Chem 1994;269:6765-72.
18. Maguchi M, Nishida W, Kohara K, Kuwano A, Kondo I, Hiwada K. Molecular cloning and gene mapping of human basic and acidic calponins. Biochem Biophys Res Commun 1995;217:238-44.
19. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1989.
20. Pan J, Burgher KL, Szczepanik AM, Ringheim GE. Tyrosine phosphorylation of inducible nitric oxide synthase: implications for potential post-translational regulation. Biochem J 1996;314:889-94.
21. Olah KS, Vince GS, Neilson JP, Deniz G, Johnson PM. Interleukin-6, interferon-gamma, interleukin-8, and granulocyte-macrophage colony stimulating factor levels in human amniotic fluid at term. J Reprod Immunol 1996;32:89-98.
22. Slater DM, Berger LC, Newton R, Moore GE, Bennett PR. Expression of cyclooxygenase types 1 and 2 in human fetal membranes at term. Am J Obstet Gynecol 1995;172:77-82.
23. Slater DM, Dennes WJ, Jones JD, Poston L, Bennett PR. Expression of COX-1 and COX-2 in human myometrium: changes in relation to gestational age and labour onset [abstract]. J Soc Gynecol Invest 1997;4 Suppl 1:115A.
24. Nunokawa Y, Ishida N, Tanaka S. Promoter analysis of human inducible nitric oxide synthase gene associated with cardiovascular homeostasis. Biochem Biophys Res Commun 1994;200:802-7.
25. Tazawa R, Xu XM, Wu KK, Wang LH. Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. Biochem Biophys Res Commun 1994;203:190-9.
26. Buhimschi I, Ali M, Jain V, Chwalisz K, Garfield RE. Differential regulation of nitric oxide in the rat uterus and cervix during pregnancy and labour. Hum Reprod 1996;11:1755-66.

Make researching easier with the Ten-Year Cumulative Index (1978-1987) to the American Journal of Obstetrics and Gynecology

The Ten-Year Cumulative Index is a complete guide to more than 30,000 pages of original articles, case reports, letters, and editorials published in the American Journal of Obstetrics and Gynecology from 1978 through 1987 (volumes 130 to 157). Numbering more than 800 pages, the Cumulative Index includes both a subject and an author index. This hard-cover volume is $64.50 ($69 international). Prices include shipping. *Payment must accompany all orders and must be in US funds, drawn on a US bank.*

Contact Mosby, Inc, Subscription Services, 11830 Westline Industrial Dr, St Louis, MO 63146-3318, USA; telephone (314)453-4351. In the US call toll-free: (800)453-4351. Please allow 6 weeks for delivery.

7.1.3

Expression of nitric oxide synthase isoforms in pregnant human myometrium

Simon R. Bartlett, Phillip R. Bennett, Juan S. Campa, William J. B. Dennes, Donna M. Slater, Giovanni E. Mann, Lucilla Poston, Robin Poston

Journal of Physiology (1999), **521**, 3, 705 - 716.

Author's Contribution:

Contributed to experimental design and methodology, carried out RT-PCR analysis and contributed to Discussion.

Expression of nitric oxide synthase isoforms in pregnant human myometrium

Simon R. Bartlett*, Phillip R. Bennett†, Juan S. Campa‡, William J. B. Dennes†, Donna M. Slater†, Giovanni E. Mann*, Lucilla Poston*‡ and Robin Poston*

*Centre for Cardiovascular Biology and Medicine and ‡Fetal Health Research Group, GKT Schools of Biomedical Sciences and Medicine, King's College London, London SE1 1UL and †Division of Obstetrics and Gynaecology, Imperial College of Medicine, Queen Charlottes's and Chelsea Hospital, London W6 0XG, UK

(Received 26 July 1999; accepted after revision 11 October 1999)

1. Endogenous nitric oxide has been proposed to play a role in the control of myometrial contractility in pregnancy. In this study, the expression, localisation and regulation of nitric oxide synthase (NOS) isoforms have been examined in human pregnant myometrium and cultured human myometrial smooth muscle cells, by immunoblotting, immunohistochemistry and reverse transcription-polymerase chain reaction.

2. Immunoblotting of extracts from freshly isolated myometrial tissue, affinity-enriched for NOS proteins by precipitation with ADP–sepharose, revealed expression of endothelial NOS (eNOS or NOS3) in tissues from preterm, term non-labour and active labour at term. Inducible NOS (iNOS or NOS2) and neuronal NOS (nNOS or NOS1) proteins were not detected at any stage of pregnancy.

3. Immunohistochemical detection showed that expression of eNOS protein was restricted to the endothelium of the myometrial vasculature, with no staining detected in myometrial smooth muscle cells.

4. Messenger RNA for all three NOS isoforms was detected, although iNOS and nNOS mRNAs were detectable only with high cycle number, implying a low copy number.

5. NOS isoforms were not detectable in human myometrial smooth muscle cells cultured from term non-labour pregnancies. Cytokine stimulation of cultured myometrial cells did not induce iNOS expression or nitrite accumulation in the culture medium, although both iNOS protein and nitrite release were detected in the human pulmonary epithelial cell line A549.

6. Levels of eNOS protein and of NOS mRNA expression were not correlated with gestational stage, suggesting that endogenously produced NO is not likely to be a modulator of myometrial tone during human pregnancy.

The initiation of labour in pregnant women is poorly understood. Uterine muscle is responsive both to labour 'promoting' and labour 'inhibitory' stimuli, and quiescent factors have been suggested to predominate during gestation. The mechanisms maintaining myometrial quiescence are likely to be multifactorial. Exogenous nitric oxide (NO) promotes uterine relaxation and has prompted interest in the use of NO donors as tocolytic agents (Lees *et al.* 1994). Thus endogenous production of NO may be involved in the regulation of myometrial tone in pregnancy, and a decline in NO production at term could play an important role in the initiation of, or preparation for, parturition (Natuzzi *et al.* 1993; Sladek *et al.* 1993; Yallampalli *et al.* 1994; Bansal *et al.* 1997).

NO is a short-lived free radical gas involved in the regulation of vascular smooth muscle tone, inflammation, cell-mediated immunity and coagulation (Knowles & Moncada, 1994). The effects of NO on cell function are mediated through a variety of interactions, such as binding to haem-containing enzymes including soluble guanylyl cyclase (sGC) (Moncada *et al.* 1991). NO-induced activation of sGC stimulates synthesis of intracellular guanosine 3′,5′-cyclic monophosphate (cGMP), which mediates smooth muscle relaxation (Palmer *et al.* 1987). NO is synthesised from L-arginine via nitric oxide synthases (NOS), of which three distinct isoforms have been identified (Knowles & Moncada, 1994). The two constitutive isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), are calcium/calmodulin

dependent, whilst the activity of the calcium/calmodulin-insensitive inducible isoform (iNOS) is predominantly regulated at the transcriptional level in response to cytokines and bacterial lipopolysaccharide (Palmer *et al.* 1987; Nathan & Xie, 1994).

Endogenous production of NO by the pregnant myometrium may play a key role in the maintenance of uterine quiescence in a number of species. NO synthesis is increased in uterine tissues of pregnant rats and rabbits, and declines towards term (Sladek *et al.* 1993; Natuzzi *et al.* 1993; Riemer *et al.* 1997). Consistent with this, expression of iNOS has been demonstrated in rat myometrial smooth muscle and declines at term (Riemer *et al.* 1997). Cultured rat myometrial smooth muscle cells express eNOS basally and iNOS upon stimulation with cytokines (Gangula *et al.* 1997). Moreover, expression of calcium-dependent NOS isoforms in a number of tissues appears to be modulated in the guinea-pig by steroid hormones, which are elevated during pregnancy (Weiner *et al.* 1994).

Two groups have claimed to detect NOS proteins in human myometrial tissue during pregnancy. Using a range of antibodies, Thomson *et al.* (1997) reported immunohistochemical staining for all three NOS isoforms in term non-labouring and labouring tissue, with no differences observed between the two stages. However, the staining patterns obtained with different iNOS antibodies were quite different. In a subsequent study, the same group reported an elevated level of expression of eNOS and nNOS protein, detected on immunoblots, in preterm non-labouring samples compared with term and non-pregnant tissue (Norman *et al.* 1999). Bansal *et al.* (1997) examined the expression of iNOS by immunohistochemistry and immunoblotting and claimed that protein is expressed in preterm non-labouring human myometrium and declines markedly prior to term and in preterm labour. Taken together these studies support the hypothesis that endogenously produced NO maintains human myometrial quiescence early in pregnancy. However, their biochemical evidence is not consistent with the findings from *in vitro* studies of human myometrial function (Morrison *et al.* 1996; Jones & Poston, 1997), which demonstrated in muscle strips from preterm and term non-labouring women that the NOS inhibitor N^G-nitro-L-argininemethyl ester (L-NAME) had no effect on spontaneous contractility. This is in marked contrast to findings in rat myometrial strips, in which L-NAME increased tension in a concentration-dependent manner (Yallampalli *et al.* 1994), as would be predicted for a tissue in which NOS activity is regulating muscle tone.

In the light of these conflicting data, we undertook to investigate the expression of all three NOS isoforms in human myometrium. Included in this study were non-labouring tissue from preterm and term deliveries, tissue from women in labour at term and human myometrial smooth muscle cells in culture. Protein expression was analysed by immunoblotting and immunohistochemistry, using characterised antibodies. Messenger RNA was monitored by reverse transcription-polymerase chain reaction (RT-PCR). Preliminary accounts of this work have been presented in abstract form (Bartlett *et al.* 1998; Campa *et al.* 1998).

METHODS

Tissue collection

Myometrial tissue was obtained from biopsies at Caesarean section from non-labouring women at term ($n = 30$, gestation $38{\cdot}4 \pm 0{\cdot}7$ weeks) or preterm ($n = 16$, gestation $28{\cdot}9 \pm 2{\cdot}4$ weeks). The indications for elective section were breech delivery or previous Caesarean section. The non-labouring group had no evidence of uterine contractions or cervical change. Myometrial tissue from labouring women was collected at emergency Caesarean section ($n = 7$, gestation $38{\cdot}4 \pm 0{\cdot}8$ weeks). In this group all patients had regular painful contractions with associated cervical dilation greater than 3 cm, and Caesarean section was indicated by fetal distress. There was no evidence of uterine dysfunction. The study was approved by the local Ethics Committee of St Thomas' Hospital, UK, where all tissues were collected, and all patients gave written, informed consent. Biopsies were obtained from the midline of the upper edge of the lower uterine segment incision.

Drugs and chemicals

Normal sera, biotinylated secondary antibodies and avidin–biotin–peroxidase complex (ABC) were from Dako Ltd (High Wycombe, UK); ADP–sepharose, RNAse inhibitor, enhanced chemiluminescence reagents (ECL+plus) from Amersham Pharmacia Biotech; proteinase K, M-MLV reverse transcriptase, MCDB 131 medium, and Dulbecco's modified Eagle's medium (DMEM) from Gibco Life Technologies; Biotaq polymerase from Biotaq Bioline (London, UK); Nylon transfer membrane from Micron Separations Inc. (Westborough, MA, USA); lipopolysaccharide (LPS) from Difco (Surrey, UK); recombinant human interleukin-1 beta (IL-1β), interferon gamma (IFN-γ), and tumour necrosis factor alpha (TNF-α) from R & D systems (Abingdon, UK); recombinant human nNOS was kindly provided by Dr Valentina Riveros Moreno (King's College London). All other reagents were obtained from Sigma Chemical Co.

NOS antibodies. Rabbit polyclonal antibodies directed against the carboxyl termini of eNOS, iNOS and nNOS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies raised against eNOS and iNOS were purchased from Transduction Laboratories (Lexington, NJ, USA). Control normal rabbit immunoglobulin fractions and purified mouse immunoglobulin (IgG_1 and IgG_{2a}) were obtained from Dako.

Isolation and primary culture of human myometrial smooth muscle cells (HMSMCs)

Biopsies from term non-labouring pregnancies were collected in sterile culture medium. In order to obtain rapid outgrowth, cells were initially cultured in MCDB 131 culture medium. Explants (2 mm^3, visibly free of blood vessels) were dissected and placed onto the surface of 25 cm^2 culture flasks for 2 h (37 °C humidified 5% CO_2/95% air atmosphere), before being submerged in MCDB 131 medium containing 10% fetal calf serum (FCS), 10 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and supplemented with 2·5 μg ml^{-1} amphotericin B, 50 μg ml^{-1} gentamicin, 10 ng ml^{-1} human recombinant epidermal growth factor (EGF) and 1 μg ml^{-1} hydrocortisone. After 4 days the medium was replaced with MCDB 131 without added amphotericin B and gentamicin. Primary

human myometrial smooth muscle cell (HMSMC) cultures were established within 10 days. After passage 1, cells were cultured in MCDB 131 without EGF or hydrocortisone. Cells exhibited typical smooth muscle morphology by phase contrast microscopy, and cultures stained positively with a monoclonal anti-smooth muscle α-actin antibody (data not shown). Confluent HMSMCs between passages 2 and 5 were used in all experiments.

Cytokine stimulation of cultured myometrial smooth muscle cells

For experiments, cells were seeded into 9 cm diameter culture dishes. In initial experiments, we discovered that cells cultured in MCDB 131 medium showed a much weaker induction of cyclo-oxygenase-2 (COX-2) expression in response to cytokine stimulation than cells cultured in a less enriched medium such as DMEM (data not shown; see Bartlett *et al.* 1999). Therefore, before addition of agonists, confluent HMSMCs were serum deprived for 16–24 h in DMEM, supplemented with 0·5% FCS, 4 mM L-glutamine, 100 U ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. Cells were stimulated for 24 or 48 h with a combination of 100 $\mu g\ ml^{-1}$ LPS and 10 ng ml^{-1} each of IL-1β, IFN-γ and TNF-α. The human epithelial carcinoma cell line A549, which is known to express iNOS (Watkins *et al.* 1997), was cultured under the same conditions and treated similarly as a positive control. After cytokine incubation the medium was removed, and cells washed with ice-cold phosphate-buffered saline (PBS) and lysed immediately for RNA or protein extraction.

Affinity extraction of ADP-binding proteins from whole tissue and cultured myometrial cells

NOS enzymes are labile, and for this reason a wide range of protease inhibitors were included in the extraction procedure and samples were heated only for 3 min, and then maintained at 4 °C before gel electrophoresis. Initial immunblot analysis of whole-tissue homogenates and cell lysates showed no evidence of expression of any of the NOS isoforms. In order to detect low amounts of protein we exploited the high affinity of these enzymes for ADP which is conferred by the NADPH–cytochrome P450 reductase-like domain common to all NOS isoforms (Knowles & Moncada, 1994). This was achieved by extracting ADP-binding proteins from homogenates and cell lysates with ADP–sepharose beads (Thomsen *et al.* 1994). The composition of the homogenising/lysis buffer (buffer A) was as follows: 0·2% [(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS), 50 mM Hepes (pH 7·5), 2 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 $\mu g\ ml^{-1}$ pepstatin, 1 $\mu g\ ml^{-1}$ leupeptin, 1 $\mu g\ ml^{-1}$ aprotinin, 5 $\mu g\ ml^{-1}$ chymostatin, 100 $\mu g\ ml^{-1}$ antipain and 100 $\mu g\ ml^{-1}$ soybean trypsin inhibitor. Snap-frozen myometrial tissue samples (1 g wet weight) were homogenised with 2 × 20 s bursts of an Ultra-Turrax T50 homogeniser set at 12000 r.p.m. at 4 °C in 5 ml buffer A and centrifuged (15000 *g*, 30 min, 4 °C). Cultured myometrial cells were lysed in buffer A by three cycles of freezing and thawing (10 min dry ice–hand warming), followed by centrifugation (13000 *g*, 5 min, 4 °C). Supernatants of homogenates and cell lysates were then adsorbed onto ADP–sepharose beads (45 min, 4 °C), washed three times with buffer A containing 0·5 M NaCl, followed by three washes in buffer A, and eluted with electrophoresis sample buffer (62·5 mM Tris HCl pH 6·8, 2·3% w/v SDS, 5% v/v glycerol, 5% v/v 2-mercaptoethanol and 0·02% w/v Bromophenol Blue).

Immunoblotting

ADP eluates were heated at 95 °C for 3 min, and centrifuged (9000 *g* for 2 min). ADP–sepharose-extracted proteins equivalent to 400 mg wet weight tissue or 3×10^6 cultured cells were separated by SDS-PAGE on 7·5% gels (Laemmli, 1970). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore), blocked overnight at 4 °C in blocking solution (3% w/v BSA in PBS and 0·1% v/v Tween 20 (PBS-T)), and incubated with agitation at room temperature for 1 h with polyclonal anti-NOS antibodies (each diluted 1:1000 in blocking solution). Secondary antibody was a horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody (diluted 1:10000), and antibody-bound protein was visualised with ECL+plus. Monoclonal antibodies raised against eNOS and iNOS were used between 1:750 and 1:1000 dilution in blocking solution, with an HRP-conjugated rabbit anti-mouse antibody at 1:10000 as the secondary antibody. Positive controls for immunoblotting were human umbilical vein endothelial cell (HUVEC) lysates (not shown) and ADP–sepharose-extracted placental tissue (eNOS), lysates of cytokine-stimulated cells of the human epithelial carcinoma cell line A549 (iNOS), and recombinant human nNOS.

Determination of nitrite

Nitrite accumulation in the conditioned medium of cultured cells was determined colorimetrically by a diazotisation reaction using the standard Griess reagent (Green *et al.* 1982), as described previously (Bogle *et al.* 1992; Baydoun *et al.* 1993). Cell cultures from six uteri obtained at term in the absence of labour were used in these experiments, and determinations using cell cultures from individual uteri were performed in quadruplicate.

Immunohistochemistry

Serial cryostat sections (5 μm) were cut and immunohistochemistry performed using the avidin–biotin–peroxidase method. Non-specific binding was prevented by incubation with 10% normal serum of the same species as the biotin-conjugated secondary antibody. Sections were then incubated for 1 h at room temperature with primary antibodies diluted at either 1:100 (monoclonal) or 1:200 (polyclonal) in PBS containing 5% FCS. Negative controls were run in parallel, with a non-immune rabbit immunoglobulin fraction for the polyclonals, and purified mouse IgG_1 and IgG_{2a} for the monoclonal anti-eNOS and anti-iNOS antibodies, respectively. After washing in PBS, sections were incubated successively for 30 min each with biotinylated secondary antibody (rabbit anti-mouse 1:500, or swine anti-rabbit 1:400) and freshly prepared avidin–biotin–peroxidase complex. Peroxidase activity was developed with diaminobenzidine.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from snap-frozen myometrial tissue and cultured cells (Chirgwin *et al.* 1979), and samples (1 μg) were denatured (70 °C, 5 min) and cooled to 37 °C. Reverse transcription (RT) was carried out at 37 °C for 1 h in a final volume of 20 μl, with 0·2 μg random hexanucleotide primers, 1 × RT buffer, 10 mM DTT, 1 mM of each dNTP, 40 units M-MLV reverse transcriptase, and 1 unit RNAse inhibitor. The reaction was terminated by heating at 90 °C for 5 min. Polymerase chain reaction (PCR) amplification was performed with specifically designed PCR primers for eNOS, iNOS and nNOS. PCR amplification for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was carried out on the same samples as a parallel control. The primer sets used were as follows: eNOS, sense 5′-GCACAGGAAATGTTCACCTAC-3′; antisense 5′-CACGATGGTGACTTTGGCTAG-3′ (Nadaud *et al.* 1994): iNOS, sense 5′-GAGCTTCTACCTCAAGCTATC-3′; antisense 5′-CCTGATGTTGCCATTGTTGGT-3′ (Chartrain *et al.* 1994): nNOS, sense 5′-TGTGTGGGCAGGATCCAGTG-3′; antisense 5′-GGGACAGGCGCTGAACTCCA-3′ (Hall *et al.* 1994): GAPDH, sense 5′-CCACCCATGGCAAATTCCATGGCA-3′; antisense 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (Tokunaga *et al.* 1987).

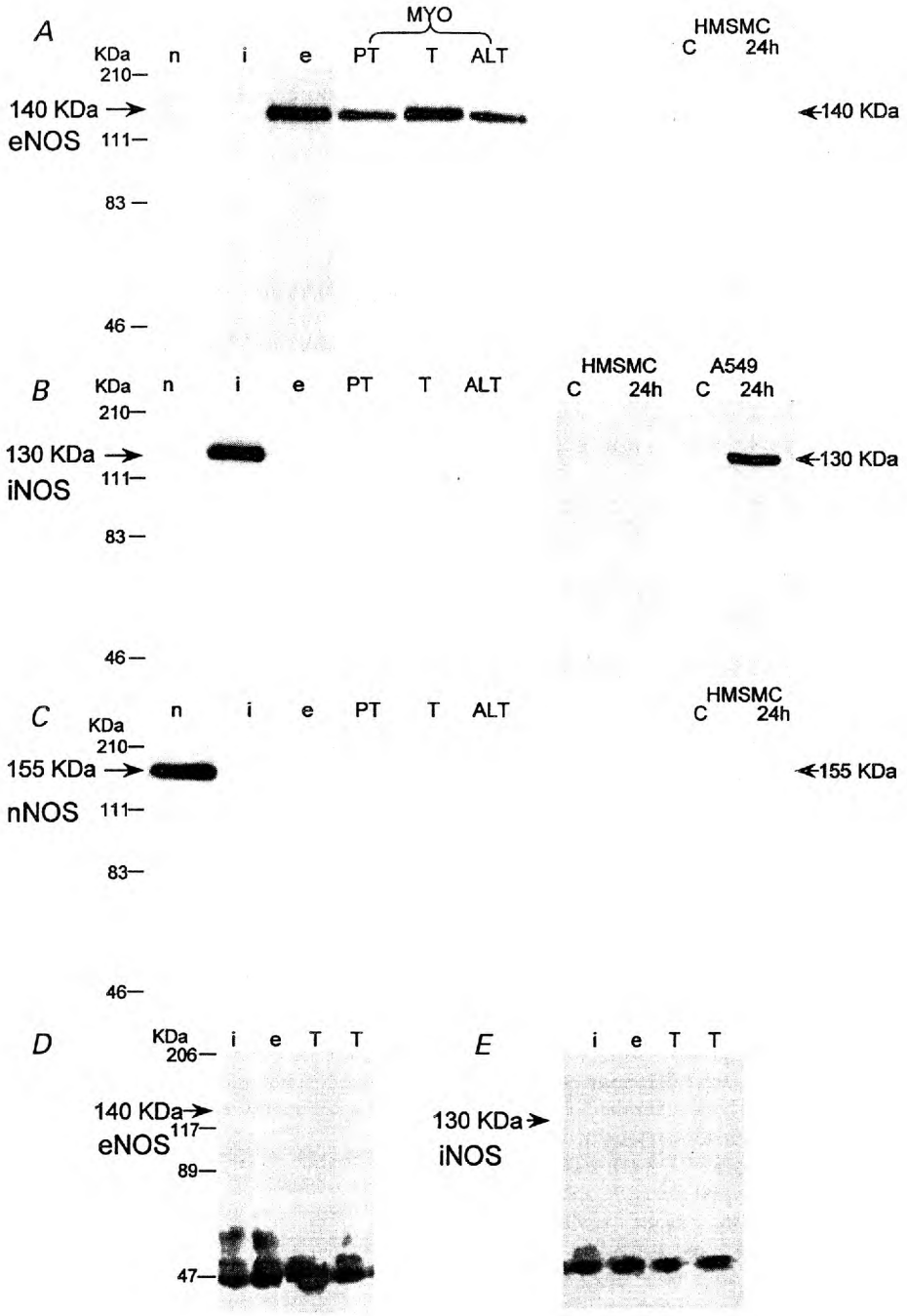


Figure 1. Immunodetection of NOS isoforms in ADP–sepharose extracts of human pregnant myometrial tissue (MYO) and human myometrial smooth muscle cells (HMSMCs) cultured from term non-labouring myometrium

Representative immunoblots for eNOS (*A*), iNOS (*B*) and nNOS (*C*) with rabbit polyclonal antibodies. *D* and *E* are representative blots for eNOS and iNOS obtained with mouse monoclonal antibodies. See Methods for details. Lanes n, i and e: positive controls for nNOS, iNOS and eNOS, respectively. MYO (ADP–sepharose extract of ~400 mg wet weight per lane): PT, preterm non-labour; T, term non-labour; ALT, active labour at term. HMSMC or, for comparison, A549 epithelial cells (ADP–sepharose extract of $\sim 3 \times 10^6$ cells per lane): C, control unstimulated cells; 24h, cells stimulated for 24 h with 100 μg ml^{-1} LPS and 10 ng ml^{-1} each of IL-1β, IFN-γ and TNF-α.

Table 1. NOS primary antibodies

Antibody	Immunogen	Dilution		Presentation	Source
		Immunoblot	Immuno-histochemistry		
eNOS polyclonal	a.a. 1183–1202 of human eNOS	1/1000	1/200	Affinity purified	Santa Cruz, sc-654
nNOS polyclonal	a.a. 1400–1419 of rat nNOS	1/1000	1/200	Affinity purified	Santa Cruz, sc-648
iNOS polyclonal	a.a. 1135–1153 of human iNOS	1/1000	1/200	Affinity purified	Santa Cruz, sc-649
eNOS monoclonal	a.a. 1030–1209 of human eNOS	1/750–1000	1/100	Purified IgG_1	Transduction, N30020
iNOS monoclonal	a.a. 961–1144 of human iNOS	1/750–1000	1/100	Purified IgG_{2a}	Transduction, N32020

a.a., amino acid.

A 1/40 volume of the RT reaction was used for PCR with 1·5 mM magnesium chloride, 0·2 mM dNTPs, 125 ng of each primer and 1 unit of Biotaq polymerase in a final volume of 25 μl. Reaction cycles were two denaturation steps (94 °C, 4 min, 94 °C, 30 s), followed by annealing for 30 s at 55 °C (eNOS), 62 °C (iNOS), 68 °C (nNOS) or 58 °C (GAPDH), and extension at 72 °C for 30 s. Initially 28–40 cycles were carried out, at 2 cycle intervals, in order to determine the cycle profile of linearity for each primer set. The number of cycles used for experiments were as indicated (see Results). Amplification was terminated with a further 5 min extension at 72 °C. Aliquots (10 μl) of the PCR product were separated by horizontal gel electrophoresis on a 1% agarose gel. Identity was confirmed by sequence-verification of the amplified products (Sambrook *et al.* 1989).

Statistics

All values of nitrite levels are given as means ± S.E.M. of measurements in six cultures. Statistical analyses were performed using Student's unpaired *t* test.

RESULTS

Detection of NOS isoforms by immunoblotting

Initial immunoblots carried out using whole-tissue homogenates failed to show staining for any of the NOS isoforms (data not shown). Partial enrichment of NOS proteins was achieved by extracting ADP-binding proteins from tissue homogenates and cell lysates with ADP–sepharose beads. With this procedure, all three NOS polyclonal antibodies used in this study (Table 1) were confirmed to be specific for their respective isoforms. Immunoblot analysis of known sources (positive controls) of human eNOS (human umbilical vein endothelial cells and human placental tissue), iNOS (cytokine/LPS-stimulated A549 cells), and of recombinant human nNOS protein showed single protein bands of the correct molecular masses, with no cross-reaction with other NOS isoforms (Fig. 1). When applied to the same samples, the anti-iNOS and anti-eNOS monoclonal antibodies (Table 1) did not stain bands at the appropriate molecular masses in any samples, or in positive controls. They did, however, stain bands at approximately 47 kDa in extracts of myometrial tissue and in all positive controls (Fig. 1*D* and *E*).

The myometrial tissue samples in Fig. 1*A–C* show representative examples of immunoblots obtained. ADP–sepharose extracts of approximately 400 mg myometrial tissue showed staining of an eNOS protein band (140 kDa; Fig. 1*A*), present in myometrial tissue from preterm (PT), term non-labour (T) and active labour at term (ALT). No protein bands for iNOS or nNOS were detected at any stage of pregnancy in any of the samples analysed (Fig. 1*B* and *C*).

None of the NOS isoforms were detected in ADP–sepharose extracts of non-stimulated cultured myometrial smooth muscle cells (Fig. 1*A–C*). There was also no evidence of iNOS protein after stimulation of HMSMCs for 24 h (Fig. 1*B*) and 48 h (data not shown) with a cytokine mix of 100 μg ml^{-1}

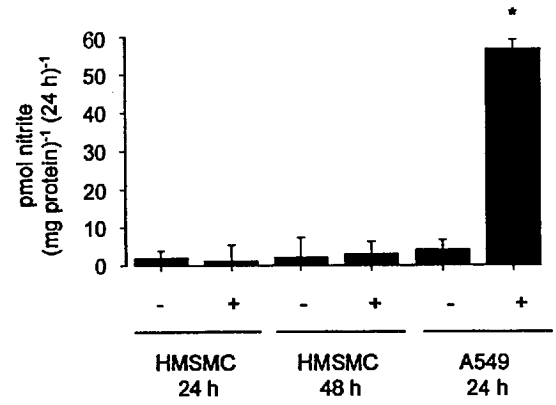


Figure 2. Effect of lipopolysaccharide and cytokines on nitrite accumulation in HMSMCs and A549 epithelial cells

HMSMCs and A549 cells were stimulated with 100 μg ml^{-1} LPS and 10 ng ml^{-1} each of IL-1β, IFN-γ and TNF-α. Nitrite accumulation in the culture medium was determined over 24 and 48 h (HMSMCs) and 24 h (A549). +, stimulated cells; −, control unstimulated cells. Determinations on cell cultures from individual uteri were performed in quadruplicate, and values are the means ± S.E.M. of cell cultures from 6 uteri obtained at term in the absence of labour. * $P < 0·001$ compared with unstimulated HMSMCs and A549 cells.

LPS and 10 ng ml^{-1} each of IL-1β, IFN-γ and TNF-α. A549 epithelial cells, cultured under the same conditions and stimulated concurrently, showed detectable protein expression after stimulation for 24 h, which was accompanied by an accumulation of nitrite (an index of NO synthesis) in the culture medium of these cells (Fig. 2), as described previously by Watkins *et al.* (1997). No accumulation of nitrite was detected in six HMSMC cultures tested. Similar results were obtained from HMSMC cultures that were established without supplementation with hydrocortisone and EGF (data not shown).

Localisation of NOS isoforms within human myometrium

Immunostaining of myometrial tissue sections with the polyclonal anti-eNOS antibody (Santa Cruz Biotechnology) revealed immunoreactivity localised to the endothelial cells of the small vessels within the myometrium, but not to myocytes (Fig. 3, top row). The same staining pattern was seen in preterm, term non-labour and term active labour samples. Immunoreactivity for iNOS and nNOS with the Santa Cruz polyclonal antibodies (Fig. 3, rows 2 and 3) was not above background levels obtained with non-immune serum in any of the sections (Fig. 3, row 4).

We also examined the immunoreactivity of the two monoclonal antibodies (Transduction Laboratories) used in previous studies (Thomson *et al.* 1997; Norman *et al.* 1999) against myometrial tissue sections. In contrast to the staining pattern seen with the polyclonal antibodies, the monoclonal anti-eNOS and iNOS antibodies (both of which failed to detect NOS proteins in immunoblots of positive controls) produced widespread immunoreactivity throughout sections of term non-labouring tissue (Fig. 4).

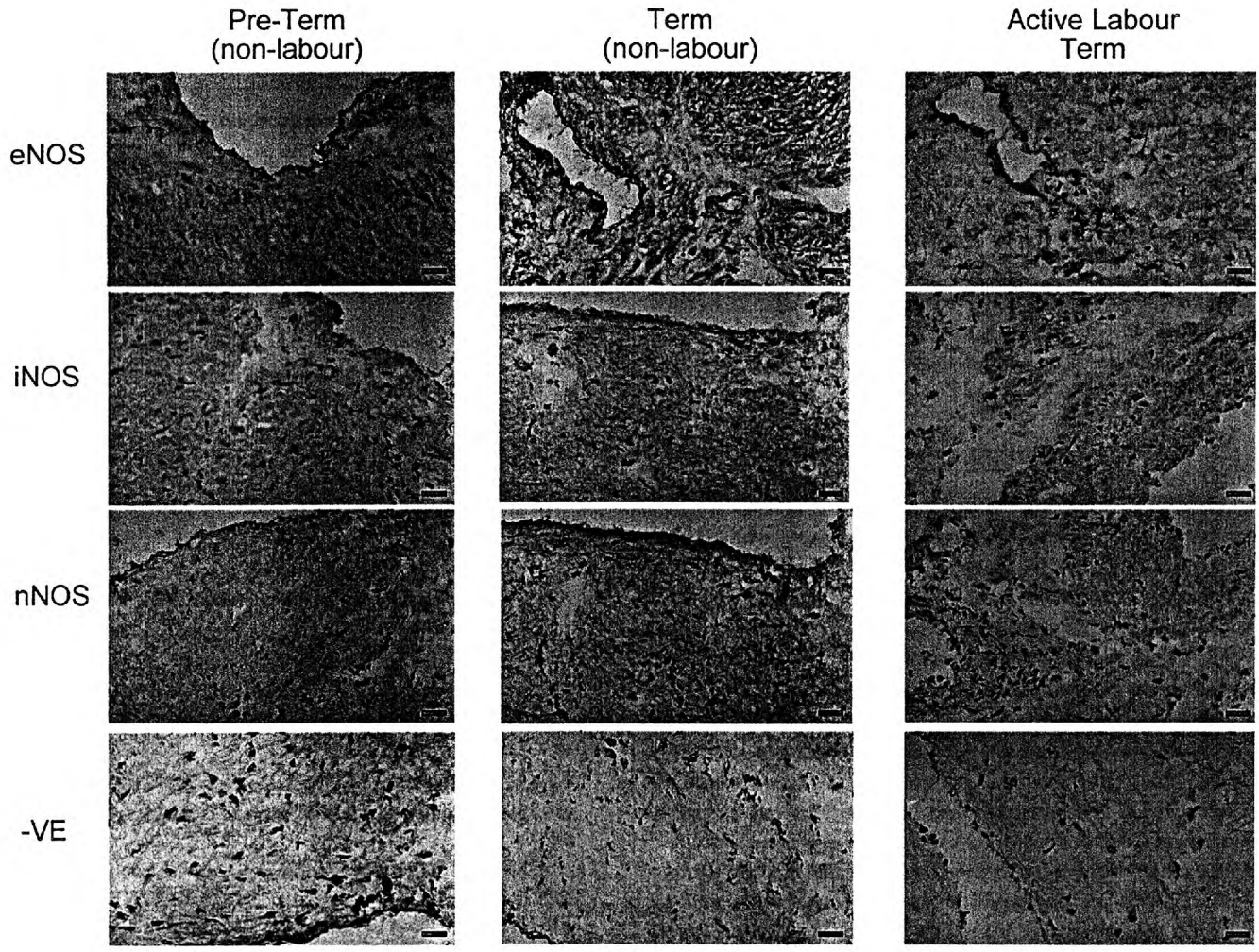


Figure 3. Immunohistochemical staining of sections of human pregnant myometrial tissue with polyclonal anti-NOS antibodies

Representative sections are shown from preterm and term non-labouring myometrium and from tissue of women in active labour at term. Cryostat sections 5 μm thick were incubated with polyclonal antibodies raised against eNOS, iNOS and nNOS. Negative controls were incubated with non-immune serum. Staining was by avidin–biotin–peroxidase.

Reverse transcription-polymerase chain reaction analysis of human myometrial NOS isoform expression

In a previous study, we demonstrated the expression of NOS mRNA in myometrial tissue throughout gestation (Dennes *et al.* 1999). In the present study we re-examined NOS mRNA expression by RT-PCR and confirmed that all three NOS isoforms were detected in myometrial tissue from preterm, term non-labour and term active labour (Fig. 5*A*). Detection of each isoform was achieved within the linear range of amplification cycles. However, iNOS and nNOS were only detectable after 40 cycles, suggesting a low copy number. In HMSMCs, eNOS, nNOS and iNOS mRNAs were not detected (Fig. 5*B*).

DISCUSSION

This study has examined in detail the expression, localisation and regulation of all three NOS isoforms in human pregnant myometrium. Previous reports that NO release from myometrial cells suppresses myometrial contraction during pregnancy have suggested expression of NOS in amounts that would easily be detectable by the techniques employed here. We have demonstrated with antibodies of proven specificity that eNOS, iNOS and nNOS proteins are not expressed at detectable levels in myocytes of human myometrium at any stage of pregnancy.

To date, investigation into NOS activity and expression in human pregnant myometrium has been inconclusive. Metabolism of radiolabelled L-arginine to L-citrulline has been observed in homogenates of human myometrium (Ramsay *et al.* 1996; Thomson *et al.* 1997). This may indicate the presence of NOS enzymes but the contribution of NOS from vascular tissue could not be excluded in these studies. Firm evidence requires the observation of increased contractility of myometrial muscle strips in response to inhibitors of NOS activity and the detection of NOS enzymes in myometrial tissue. Relaxation of murine and human myometrial strips in response to high concentrations of

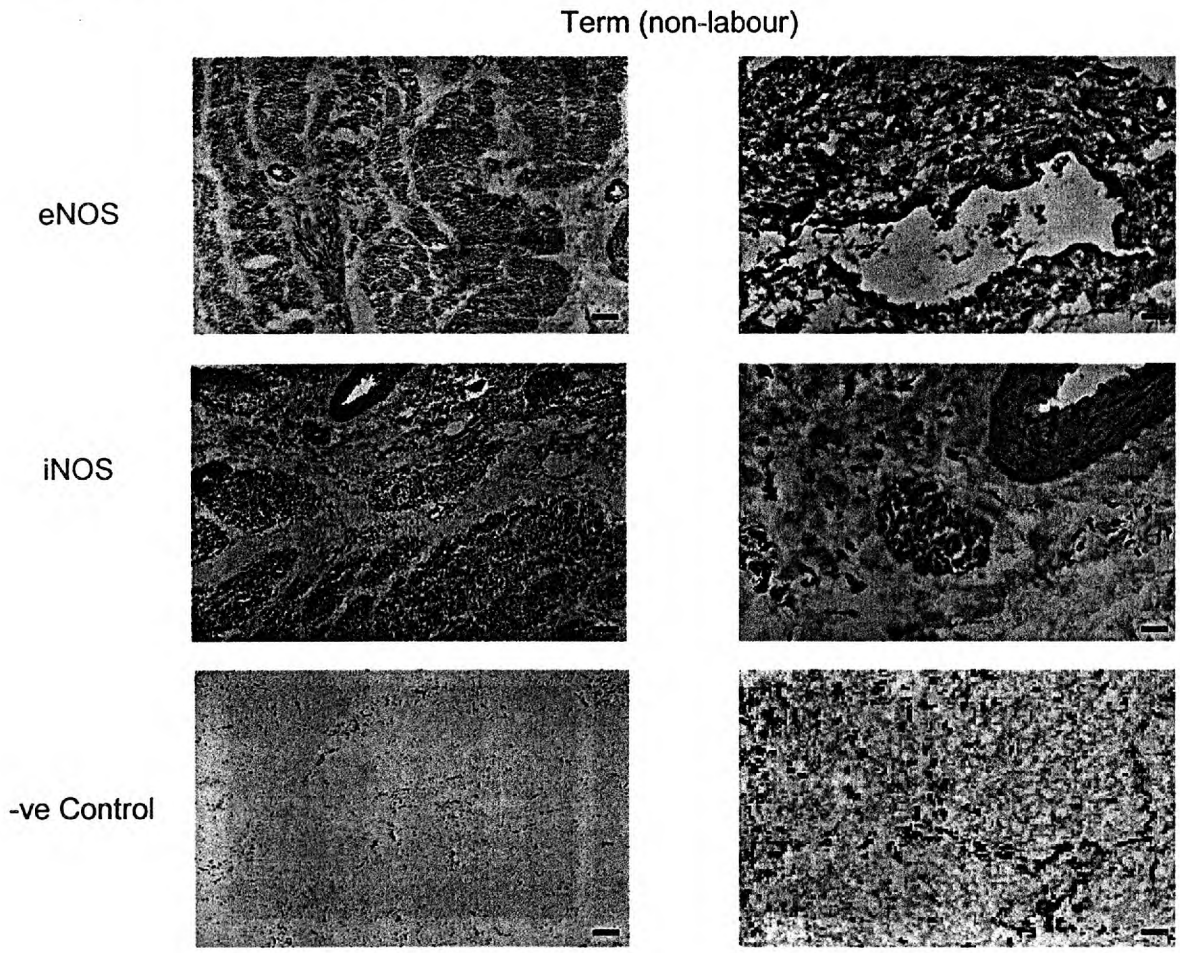


Figure 4. Immunohistochemical staining of sections of human pregnant myometrial tissue with monoclonal anti-NOS antibodies

Representative sections are shown of term non-labouring tissue. Cryostat sections 5 μm thick were incubated with monoclonal antibodies raised against eNOS and iNOS. Negative controls were incubated with non-immune mouse IgG. Staining was by avidin–biotin–peroxidase.

L-arginine has been observed (Izumi *et al.* 1993). However, at physiological plasma levels, L-arginine is not rate limiting for eNOS or iNOS activity, and it is possible that changes in tone were due to the cationic charge of L-arginine. Subsequent studies have not confirmed L-arginine-induced relaxation of preterm or term non-labouring human myometrial strips and, most significantly, neither have they observed increased contraction upon treatment with inhibitors of NOS activity, as would be predicted if the tissue was expressing functionally active NOS enzymes (Morrison *et al.* 1996; Jones & Poston, 1997). Claims of NO production, detected with an NO electrode, by human cultured pregnant myometrial smooth muscle cells stimulated with LPS, IL-1α and IL-1β, suggest that iNOS can be induced in this cell type (Nakaya *et al.* 1996; Yamamoto *et al.* 1997). However, the presence of NOS protein or mRNA, and inhibition of NOS activity by NOS inhibitors was not confirmed in those studies.

Two groups have examined the expression of NOS proteins in human myometrium during pregnancy. Thomson *et al.* (1997) reported immunoreactivity for all three isoforms,

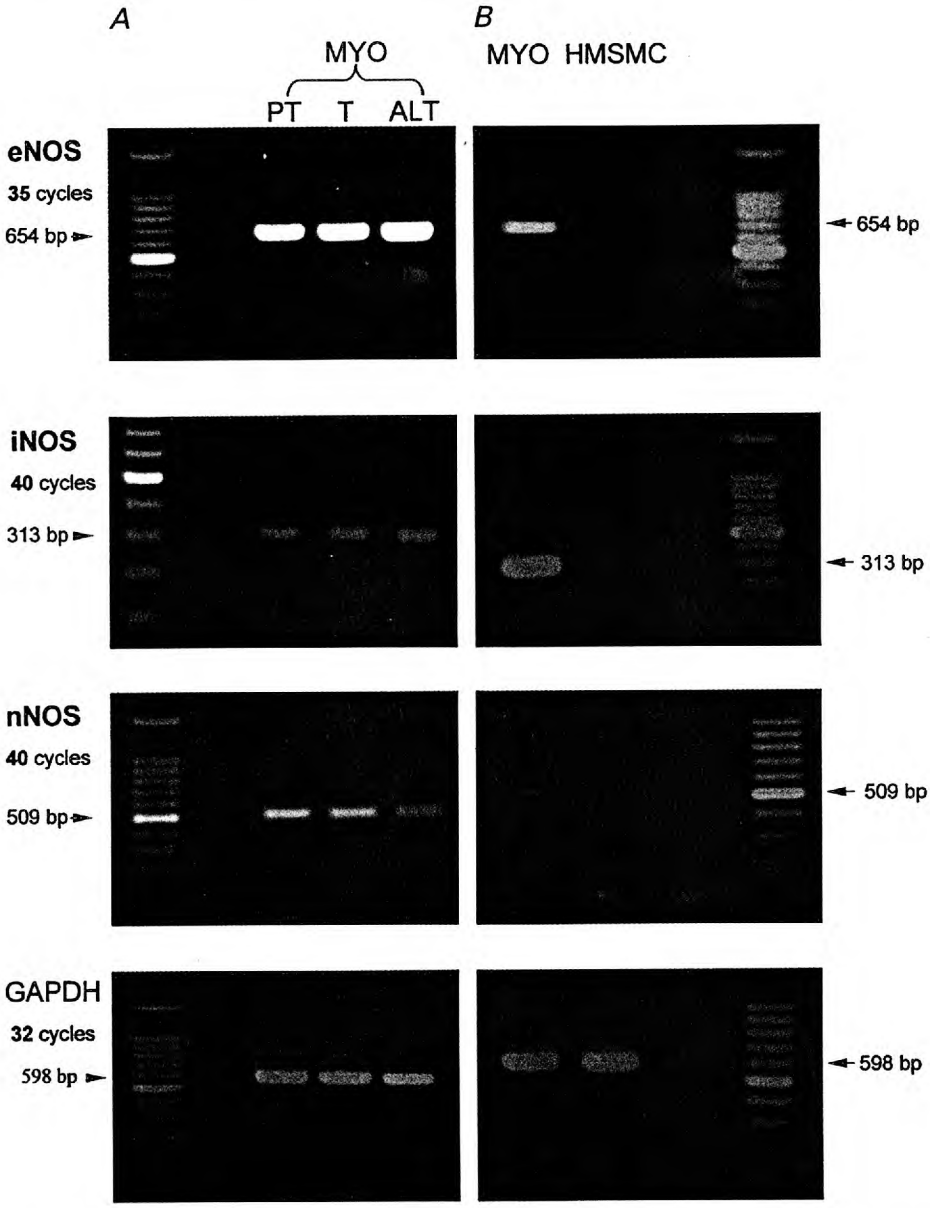


Figure 5. RT-PCR for NOS isoforms from human pregnant myometrial tissue (MYO) (*A*) and HMSMCs (*B*)

Sequences of eNOS, iNOS and nNOS were amplified from cDNAs derived from preterm non-labour (PT), term non-labour (T) and active labour at term (ALT) myometrial tissue, and from HMSMCs, at the cycle numbers indicated. In *B* the left-hand lane (MYO) represents pooled cDNAs from myometrial tissue as a positive control. Amplification of a sequence from the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was carried out as a parallel control.

with no detected changes associated with labour, but the staining presented was not consistent between different anti-iNOS antibodies used, and included staining with the same monoclonal antibody which we have shown in the present study to be non-specific. In a subsequent study (Norman *et al.* 1999), these authors reported increased expression of eNOS and nNOS protein by immunoblotting and immunohistochemistry in human preterm myometrium in comparison with term and non-pregnant tissue. The expression of iNOS was not reported. Bansal *et al.* (1997) presented immunohistochemical and immunoblotting evidence suggesting expression of iNOS in preterm, but not term, non-labouring myometrium. Taken together, these observations do not support a role for a decline in NO generation promoting parturition, but favour a model in which NO, generated by NOS isoforms within the uterine smooth muscle, promote uterine quiescence in the early stages of pregnancy. Nevertheless, such a conclusion is not consistent with functional studies (Morrison *et al.* 1996; Jones & Poston, 1997), which found no effect of NOS inhibitors on contractility of myometrial muscle strips at any stage of pregnancy. In view of the proposal for the use of NO donors in clinical practice, it is important that these apparent paradoxes are clarified.

We sought first to demonstrate the presence of NOS isoforms by immunoblotting. NOS enzymes are susceptible to proteolytic cleavage during extraction for electrophoresis (Lowe *et al.* 1996). This can reduce or eliminate the signal at the expected molecular mass on an immunoblot and also result in staining of lower molecular mass fragments, which would be difficult to distinguish from non-specific bands. Great care was therefore taken to ensure the preservation of full length NOS polypeptides during extraction and ADP–sepharose purification, and we were able unambiguously to detect all three NOS isoforms in positive controls with the polyclonal antibodies (Santa Cruz) on immunoblots, in the absence of any non-specific bands. We also attempted immunoblots with two monoclonal antibodies directed against eNOS and iNOS, respectively (Transduction Laboratories; see Table 1). When used to analyse the same samples, neither of these antibodies stained bands at the appropriate NOS molecular masses in positive controls or ADP–sepharose-extracted tissue, but did detect bands at approximately 47 kDa (Fig. 1*D* and *E*). We therefore conclude that the widespread staining seen with these antibodies on the myometrial sections (Fig. 4) is unreliable and likely to be non-specific.

The data on eNOS expression obtained with the polyclonal antibody, in immunoblots and tissue sections, suggested the presence of this isoform in the vascular endothelium (Figs 1 and 3). This was supported by the failure to detect eNOS mRNA or protein in extracts of cultured myometrial smooth muscle cells. Quantification of the eNOS signal between samples was not attempted, as variable sampling of vascular tissue would influence the levels of the protein detected. However, no gestationally related trends in protein expression were obvious. Based on these findings, we conclude that eNOS is unlikely to play any role in the regulation of myometrial tone during pregnancy. It could, however, be argued that diffusion of NO generated within the endothelium of the myometrial vasculature might mediate uterine relaxation, although the low levels of eNOS detected (after ADP–sepharose purification) argue against this. Moreover, if this were the case, NOS inhibitors should increase myometrial tone and contraction, which has not been found (Morrison *et al.* 1996; Jones & Poston, 1997).

We did not detect iNOS protein expression in tissues from any gestational stage, which is at odds with the previously reported detection, using the same antibody, of iNOS in preterm samples in the absence of labour (Bansal *et al.* 1997). In the authors' published blot the putative iNOS band at 130 kDa is indicated for the positive control cell lysate. However, the authors make no comment about the very much stronger staining of bands at lower molecular weights in all samples (including the positive control). It is thus difficult to reconcile the putative iNOS band in the preterm sample (which is at a different molecular weight from the band indicated in the positive control) with iNOS expression. It is also likely that the prolonged boiling in the homogenisation procedure that was used would have resulted in hydrolytic cleavage of any NOS proteins present. We therefore suggest that our study and that of Bansal *et al.* (1997) both fail to demonstrate the expression of iNOS in human myometrium throughout pregnancy.

Immunohistochemistry using the polyclonal antibody for iNOS confirmed the lack of iNOS expression in myometrium at all gestational stages. We were unable to detect iNOS staining in preterm sections as reported by Bansal *et al.* (1997), although this may reflect differences in the fixation procedure. RT-PCR did detect transcripts of iNOS after 40 cycles of amplification, which implies expression of iNOS mRNA at low copy number. A more extensive study of iNOS mRNA expression in human myometrial tissue has been presented elsewhere and confirms the absence of gestationally related changes as described here (Dennes *et al.* 1999). Expression of iNOS in inflammatory cell types within uterine tissues, including myometrium, has been reported during human pregnancy (Bansal *et al.* 1997; Eis *et al.* 1997; Myatt *et al.* 1997), and the PCR signal we identified is likely to have arisen from leukocytes and macrophages within the tissue. This is supported by the lack of expression of iNOS mRNA in cultured human myometrial smooth muscle cells. We also failed to detect the expression of nNOS protein in tissue homogenates and sections. The apparently low copy number of nNOS mRNA we detected may represent expression within the myocytes or in nerve cells supplying the myometrium.

Our data from the cultured cells were consistent with that from whole tissue. It is intriguing that near-micromolar concentrations of NO have apparently been detected after 24 h in the supernatant of cultured pregnant myometrial cells stimulated by cytokines (Nakaya *et al.* 1996; Yamamoto *et*

al. 1997). Although this is within the concentration range detectable by the Griess reaction employed in the current study, we could not detect nitrites released from cytokine-stimulated myometrial myocytes, nor could we detect iNOS protein or mRNA in these cells. We have also measured cGMP within HMSMCs, as a more sensitive index of NO production, and found no accumulation in response to cytokine stimulation (data not shown). Other cell types, derived from tissues capable of expressing iNOS, can maintain this capacity in culture (Nussler *et al.* 1995; Saura *et al.* 1996; Watkins *et al.* 1997). If this capacity were reproduced in HMSMCs, a positive finding would provide supporting evidence for a role of iNOS in the function of this tissue. The lack of response we found may be due to a loss of responsiveness under the culture conditions. However, A549 cells exposed to the same conditions express iNOS, and HMSMCs stimulated with the same cytokine mix or with IL-1β alone show a dramatic induction of COX-2 expression and activity (Bartlett *et al.* 1998, 1999). When taken together with the observations in fresh tissue, we propose that the lack of iNOS expression in cultured cells indicates that this cell type does not have the potential to generate NO in significant quantities via iNOS.

A potential limitation of our study is that tissue samples were obtained from the lower uterine segment which, it is suggested, may be more cervical in character, and less contractile than the fundus (Fuchs *et al.* 1984; Smith *et al.* 1998). However, all other published studies that have suggested that NOS is functionally expressed in the human myometrium in pregnancy have used tissue from the same anatomical region (Izumi *et al.* 1993; Bansal *et al.* 1997; Thomson *et al.* 1997; Norman *et al.* 1999). Moreover, no differences are seen in the sensitivity of human pregnant myometrium from the fundus *versus* lower segment to oxytocin or potassium chloride (Word *et al.* 1993), suggesting that at the cellular level, there may be little difference between these two regions.

Low sensitivities of rat and primate myometrium to relaxation by cGMP analogues and NO donors have recently been reported (Hennan & Diamond, 1998; Kuenzli *et al.* 1998; Word & Cornwell, 1998), and cGMP-dependent protein kinase expression is reduced in the myometrium from pregnant rats (Word & Cornwell, 1998). Similar observations in human pregnant myometrium support these findings (Jones & Poston, 1996; Norman *et al.* 1997) and are in keeping with the data we present here, suggesting that the human myometrium during pregnancy is not a source of NO production. Published evidence on the expression of NOS isoforms in human myometrium implicating endogenous NO in the regulation of tone early in pregnancy contradicts the data from functional studies. Under the rigorous experimental conditions described in this study, we have been unable to detect protein expression of NOS isoforms in human myometrial smooth muscle during pregnancy. The mRNA we detected for all three NOS isoforms may reflect expression by smooth muscle and/or contributions from other cell types. The presence of mRNA does not confirm translation into protein, but there may be translation into functional NOS proteins at levels below the sensitivity of immunoblotting and immunohistochemical techniques. Such a situation exists in human and rabbit gastrointestinal smooth muscle cells, where eNOS has been identified functionally, and expression of its mRNA detected by RT-PCR and Northern blot in the absence of detectable eNOS protein (Murthy & Makhlouf, 1994; Teng *et al.* 1998). Similarly, expression of nNOS has also been reported in gastrointestinal smooth muscle cells (Chadker *et al.* 1998). The levels of NO required to relax smooth muscle are not known, so it is not certain whether such low expression in myometrium would be sufficient to regulate tone. Therefore a role for endogenous NO production by the myometrium cannot be ruled out completely. However, human myometrial contractility is insensitive to L-NAME (Morrison *et al.* 1996; Jones & Poston, 1997), demonstrating an absence of NOS function, consistent with the lack of detectable NOS protein in the present study. Furthermore, no gestationally related changes in NOS mRNA expression were apparent. We therefore conclude that the weight of experimental evidence indicates that NO produced by the myometrium plays an insignificant role in the regulation of uterine smooth muscle tone during human pregnancy.

BANSAL, R. K., GOLDSMITH, P. C., HE, Y. P., ZALOUDEK, C. J., ECKER, J. L. & RIEMER, R. K. (1997). Decline in myometrial nitric oxide synthase expression is associated with labor and delivery. *Journal of Clinical Investigation* 99, 2502–2508.

BARTLETT, S., SAWDY, R., DENNES, W., BENNETT, P. & MANN, G. E. (1998). Cytokine-induced regulation of nitric oxide synthase and cyclooxygenase 2 in cultured human myometrial cells. *Journal of the Society for Gynecologic Investigation* 5, 183A (abstract).

BARTLETT, S. R., SAWDY, R. & MANN, G. E. (1999). Induction of cyclo-oxygenase-2 expression in human myometrial smooth muscle cells by interleukin-1: involvement of p38 mitogen-activated protein kinase. *Journal of Physiology* 520, 399–406.

BAYDOUN, A. R., BOGLE, R. G., PEARSON, J. D. & MANN, G. E. (1993). Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in murine macrophage J774 cells. *British Journal of Pharmacology* 110, 1401–1406.

BOGLE, R. G., BAYDOUN, A. R., PEARSON, J. D., MONCADA, S. & MANN, G. E. (1992). L-Arginine transport is increased in macrophages generating nitric oxide. *Biochemical Journal* 284, 15–18.

CAMPA, J. S., JONES, G. D., POSTON, R. & POSTON, L. (1998). Nitric oxide synthase (eNOS) is localized to the vascular endothelium in term (non-labouring) myometrium of pregnant women. *Journal of the Society for Gynecologic Investigation* 5, 182A (abstract).

CHAKDER, S., BANDYOPADHYAY, A. & RATTAN, S. (1997). Neuronal NOS gene expression in gastrointestinal myenteric neurons and smooth muscle cells. *American Journal of Physiology* 273, C1868–1875.

CHARTRAIN, N. A., GELLER, D. A., KOTY, P. P., SITRIN, N. F., NUSSLER, A. K., HOFFMAN, E. P., BILLIAR, T. R., HUTCHINSON, N. I. & MUDGETT, J. S. (1994). Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. *Journal of Biological Chemistry* 269, 6765–6772.

CHIRGWIN, J. M., PRZYBYLA, A. E., MACDONALD, R. J. & RUTTER, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.

DENNES, W. J., SLATER, D. M., POSTON, L. & BENNETT, P. R. (1999). Myometrial nitric oxide synthase messenger RNA expression does not change throughout gestation or with the onset of labor. *American Journal of Obstetrics and Gynecology* **180**, 387–392.

EIS, A. L. W., BROCKMAN, D. E. & MYATT, L. (1997). Immunolocalization of the inducible nitric oxide synthase isoform in human fetal membranes. *American Journal of Reproductive Immunology* **38**, 289–294.

FUCHS, A. R., FUCHS, F., HUSSLEIN, P. & SOLOFF, M. S. (1984). Oxytocin receptors in the human uterus during pregnancy and parturition. *American Journal of Obstetrics and Gynecology* **150**, 734–741.

GANGULA, P. R. R., DONG, Y. L. & YALLAMPALLI, C. (1997). Rat myometrial smooth muscle cells express endothelial nitric oxide synthase. *Human Reproduction* **12**, 561–568.

GREEN, L. C., WAGNER, D. A., GLOGOWSKI, J., SKIPPER, P. L., WISHNOK, J. S. & TANNENBAUM, S. R. (1982). Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Analytical Biochemistry* **126**, 131–138.

HALL, A. V., ANTONIOU, H., WANG, Y., CHEUNG, A. H., ARBUS, A. M., OLSON, S. L., LU, W. C., KAU, C. L. & MARSDEN, P. A. (1994). Structural organisation of the human neuronal nitric oxide synthase gene (NOS 1). *Journal of Biological Chemistry* **269**, 33082–33090.

HENNAN, J. K. & DIAMOND, J. (1998). Evidence that spontaneous contractile activity in the rat myometrium is not inhibited by NO-mediated increases in tissue levels of cyclic GMP. *British Journal of Pharmacology* **123**, 959–967.

IZUMI, H., YALLAMPALLI, C. & GARFIELD, R. E. (1993). Gestational changes in L-arginine-induced relaxation of pregnant rat and human myometrial smooth muscle. *American Journal of Obstetrics and Gynecology* **169**, 1327–1337.

JONES, G. & POSTON, L. (1996). The influence of modulators of nitric oxide synthesis on spontaneous human myometrial contractility *in vitro*. *Journal of Physiology* **491.P**, 167–168*P*.

JONES, G. D. & POSTON, L. (1997). The role of endogenous nitric oxide synthesis in contractility of term or preterm human myometrium. *British Journal of Obstetrics and Gynaecology* **104**, 241–245.

KNOWLES, R. G. & MONCADA, S. (1994). Nitric-oxide synthases in mammals. *Biochemical Journal* **298**, 249–258.

KUENZLI, K. A., BUXTON, I. L. O. & BRADLEY, M. E. (1998). Nitric oxide regulation of monkey myometrial contractility. *British Journal of Pharmacology* **124**, 63–68.

LAEMMLI, E. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

LEES, C., CAMPBELL, S., JAUNIAUX, E., BROWN, R., RAMSAY, B., GIBB, D., MONCADA, S. & MARTIN, J. F. (1994). Arrest of preterm labour and prolongation of gestation with glyceryl trinitrate, a nitric oxide donor. *Lancet* **343**, 1325–1326.

LOWE, P. N., SMITH, D., STAMMERS, D. K., RIVEROSMORENO, V., MONCADA, S., CHARLES, I. & BOYHAN, A. (1996). Identification of the domains of neuronal nitric-oxide synthase by limited proteolysis. *Biochemical Journal* **314**, 55–62.

MONCADA, S., PALMER, R. M. J. & HIGGS, E. A. (1991). Nitric-oxide – physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* **43**, 109–142.

MORRISON, J. J., PERERA, D., MARSHALL, I. & RODECK, C. H. (1996). Effects of GSNO, SNAP, SIN-1, GTN, L-ARG, L-NOARG, and L-NAME on contractility of isolated human pregnant myometrium. *Journal of the Society for Gynecologic Investigation* **3**, 333A (abstract).

MURTHY, K. S. & MAKHLOUF, G. M. (1994). VIP/PACAP-mediated activation of membrane-bound NO synthase in smooth muscle is mediated by pertussis toxin-sensitive G_{i1-2}. *Journal of Biological Chemistry* **269**, 15977–15980.

MYATT, L., EIS, A. L. W., BROCKMAN, D. E., KOSSENJANS, W., GREER, I. & LYALL, F. (1997). Inducible (type ii) nitric oxide synthase in human placental villous tissue of normotensive, pre-eclamptic and intrauterine growth-restricted pregnancies. *Placenta* **18**, 261–268.

NADAUD, S., BONNARDEAUX, A., LATHROP, M. & SOUBRIER, F. (1994). Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. *Biochemical and Biophysical Research Communications* **198**, 1027–1033.

NAKAYA, Y., YAMAMOTO, S., HAMADA, Y., KAMADA, M., AONO, T. & NIWA, M. (1996). Inducible nitric-oxide synthase in uterine smooth-muscle. *Life Sciences* **58**, PL249–255.

NATHAN, C. & XIE, Q. W. (1994). Regulation of biosynthesis of nitric-oxide. *Journal of Biological Chemistry* **269**, 13725–13728.

NATUZZI, E. S., URSELL, P. C., HARRISON, M., BUSCHER, C. & RIEMER, R. K. (1993). Nitric-oxide synthase activity in the pregnant uterus decreases at parturition. *Biochemical and Biophysical Research Communications* **194**, 1–8.

NORMAN, J. E., THOMSON, A. J., TELFER, J. F., YOUNG, A., GREER, I. A. & CAMERON, I. T. (1999). Myometrial constitutive nitric oxide synthase expression is increased during human pregnancy. *Molecular Human Reproduction* **5**, 175–181.

NORMAN, J. E., WARD, L. M., MARTIN, W., CAMERON, A. D., MCGRATH, J. C., GREER, I. A. & CAMERON, I. T. (1997). Effects of cGMP and the nitric oxide donors glyceryl trinitrate and sodium nitroprusside on contractions *in vitro* of isolated myometrial tissue from pregnant women. *Journal of Reproduction and Fertility* **110**, 249–254.

NUSSLER, A. K., DISILVIO, M., LIU, Z. Z., GELLER, D. A., FREESWICK, P., DORKO, K., BARTOLI, F. & BILLIAR, T. R. (1995). Further characterization and comparison of inducible nitric-oxide synthase in mouse, rat, and human hepatocytes. *Hepatology* **21**, 1552–1560.

PALMER, R. M. J., FERRIGE, A. G. & MONCADA, S. (1987). Nitric-oxide release accounts for the biological-activity of endothelium-derived relaxing factor. *Nature* **327**, 524–526.

RAMSAY, B., SOORANNA, S. R. & JOHNSON, M. R. (1996). Nitric-oxide synthase activities in human myometrium and villous trophoblast throughout pregnancy. *Obstetrics and Gynecology* **87**, 249–253.

RIEMER, R. K., BUSCHER, C., BANSAL, R. K., BLACK, S. M., HE, Y. P. & NATUZZI, E. S. (1997). Increased expression of nitric oxide synthase in the myometrium of the pregnant rat uterus. *American Journal of Physiology* **272**, E1008–1015.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Plainview, New York.

SAURA, M., PEREZSALA, D., CANADA, F. J. & LAMAS, S. (1996). Role of tetrahydrobiopterin availability in the regulation of nitric-oxide synthase expression in human mesangial cells. *Journal of Biological Chemistry* **271**, 14290–14295.

SLADEK, S. M., REGENSTEIN, A. C., LYKINS, D. & ROBERTS, J. M. (1993). Nitric oxide synthase activity in pregnant rabbit uterus decreases on the last day of pregnancy. *American Journal of Obstetrics and Gynecology* **169**, 1285–1291.

SMITH, G. C. S., BAGUMA-NIBASHEKA, M., WU, W. X. & NATHANIELSZ, P. W. (1998). The reduced contraction of baboon lower uterine segment (LUS) to prostaglandin (PG) E_2 is paralleled by increased EP_2 and decreased EP_3 receptor mRNA compared with the fundus. *Journal of the Society for Gynecologic Investigation* **5**, 64A (abstract).

7.1.4

Expression of cyclo-oxygenase types-1 and -2 in human fetal membranes throughout pregnancy

D. Slater, W. Dennes, R. Sawdy, V. Allport and P. Bennett

Journal of Molecular Endocrinology (1999), **22**, 125 - 130.

Author's Contribution:

Contributed to experimental design and methodology, carried out Fetal membrane COX activity and prostaglandin synthesis experiments and contributed to both the writing and submission of the manuscript.

Expression of cyclo-oxygenase types-1 and -2 in human fetal membranes throughout pregnancy

D Slater, W Dennes, R Sawdy, V Allport and **P Bennett**

Parturition Research Group, Imperial College School of Medicine, Institute of Obstetrics and Gynaecology, Queen Charlotte's and Chelsea Hospital, Goldhawk Road, London W6 0XG, UK

(Requests for offprints should be addressed to D Slater)

ABSTRACT

Human labour is associated with increased prostaglandin synthesis within the fetal membranes. We have studied the expression of the two isoforms of the central prostaglandin synthetic enzyme, cyclo-oxygenase (COX-1 and COX-2), in human fetal membranes throughout pregnancy, at mRNA, protein and activity levels.

COX-1 mRNA expression was low in human amnion and chorion–decidua and did not change with gestational age. COX-2 mRNA expression in fetal membranes increased with gestational age, with significant up-regulation prior to the onset of labour and in association with labour. Protein concentrations of COX-1 did not change, whilst concentrations of COX-2 increased from the first to the third trimester. COX activity increased with gestational age and in association with labour, although prostaglandin production in fetal membranes collected after labour was reduced, suggesting reduced substrate supply.

These data suggest that it is up-regulation of COX-2, rather than of COX-1, which mediates increased prostaglandin synthesis within the fetal membranes at term. Much of the increase in COX-2 expression precedes the onset of labour, suggesting that it is a cause, rather than a consequence, of labour.

Journal of Molecular Endocrinology (1999) **22**, 125–130

INTRODUCTION

There is considerable evidence to support a central role for prostaglandins in human parturition. Labour is associated with increased prostaglandin synthesis within the uterus (Keirse *et al.* 1977), particularly from the fetal membranes (Skinner & Challis 1985). Prostaglandins mediate cervical ripening, and directly stimulate uterine contractions (Crankshaw & Dyal 1994) and indirectly increase fundally dominant myometrial contractility by up-regulation of oxytocin receptors and synchronisation of contractions (Liggins 1989, Garfield *et al.* 1990).

Prostaglandins are formed from the precursor arachidonic acid, which itself is a substrate for at least three enzyme groups. The cyclo-oxygenase (COX) pathway produces prostaglandins. The lipoxygenase enzyme pathways produce a series of hydroxyeicosatetraenoic acids (HETEs). Arachidonic acid metabolism via epoxygenase pathways produces epoxyeicosatetraenoic acids. Prior to labour, endogenous arachidonic acid metabolism in amnion is principally via the lipoxygenase enzyme pathways. With labour, there is an increase in arachidonic acid metabolism and a change in the ratio of COX to lipoxygenase metabolism to favour synthesis of prostaglandin E_2 (Saeed & Mitchell 1982, Bennett *et al.* 1993). The roles of the lipoxygenase metabolites of arachidonic acid within the uterus are unknown, although 5-HETE may play a role in pre-labour (Braxton–Hicks) contractions (Bennett *et al.* 1987, Walsh 1989).

The switch from lipoxygenase to COX metabolism implies up-regulation of COX. Two COX genes have been described, the constitutively expressed COX-1 and the inducible COX-2 (Hla *et al.* 1986, Hla & Neilson 1992). *In situ* hybridisation has localised expression of COX-1 and COX-2 mRNA in the fetal membranes (Slater *et al.* 1995). Expression of COX-2 is mainly within the amnionic epithelial layer and also in cells of the amnionic mesoderm, and in the reticular layer of both chorion and decidua. COX-2 is not expressed in the trophoblast layer of the chorion. COX-1 expression is more diffuse, and is seen in the amnionic epithelium, amnionic mesoderm and also in the chorionic mesoderm and decidua. In amnion

Journal of Molecular Endocrinology (1999) 22, 125–130 Online version via http://www.endocrinology.org
0952–5041/99/022–125 © 1999 Society for Endocrinology *Printed in Great Britain*

at term, expression of the COX-2 mRNA is some 100-fold higher than that of COX-1 mRNA. The expression of COX-2 doubles in association with labour (Slater *et al.* 1994, 1995, Hirst *et al.* 1995). In chorion–decidua at term, COX-2 mRNA is 8-fold more abundant than COX-1 mRNA, and COX-2 expression increases 4-fold in association with labour (Slater *et al.* 1998). Enzyme kinetic studies suggest that the increase in COX activity with the onset of labour is due entirely to an increase in the synthesis of the COX enzyme (Smieja *et al.* 1993).

There is conflicting evidence as to whether the increased prostaglandin synthesis associated with labour is a cause or an effect of labour. MacDonald & Casey (1993) found that the increase in prostaglandin concentrations in the amniotic fluid occurred earlier and was greater in the fore-bag (in front of the fetal head) compared with the hind-bag, and that hind-bag prostaglandin concentrations in early labour were not increased above pre-labour concentrations. They inferred from these data that prostaglandin formation was a consequence of labour. However, studies where sequential amniocentesis was performed, on patients before and during labour, showed that increased prostaglandin concentrations preceded the onset of labour (Romero *et al.* 1996).

In this study we have examined COX-1 and COX-2 mRNA, protein and activity levels in human fetal membranes at different stages of pregnancy. We have tested the hypothesis that up-regulation of COX-2 within the human fetal membranes occurs before the onset of labour and is therefore a cause, and not a consequence, of labour.

MATERIALS AND METHODS

Tissue samples

For mRNA studies, fetal membranes were collected with consent and ethics committee approval between 6 and 22 weeks at surgical termination of pregnancy, between 28 and 37 weeks at elective caesarean section prior to the onset of labour, in cases of maternal cardiac, renal or hypertensive disease, and after 38 weeks at elective caesarean section prior to labour in cases of breech presentation or previous caesarean section.

Reverse transcription–polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described (Slater *et al.* 1995). Primers used were: COX-2 sense (5′-TTCAAATGAGATTTGGGAAAATTGCT), COX-2 antisense (5′-AGATCATCTCTGCCTGAGTATCTT), COX-1 sense (5′-TGCCCAGCTCCTGGCCCGCCGCTT), COX-1 antisense (5′-CCATGGCCCAAGGCCTTG), glyceraldehyde 3-phosphate dehydrogenase (GAP) DH sense (5′-CCACCCATGGCAAATTCCATGGCA), GAPDH antisense (5′-TCTAGACGGCAGGTCAGGTCCACC) (Hla *et al.* 1986, Hla & Neilson 1992). The reaction cycles were denaturing at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s.

To determine the exponential phase of amplification, where product formation is related to starting template concentration, a cycle profile was performed for each tissue. An average sample was created by mixing equal amounts of cDNA from all of the RT reactions from a single tissue type. PCR was performed using the 'average sample' template cDNA for between 20 and 40 cycles using primers specific for COX-1, COX-2 and GAPDH. PCR products were dotted onto Hybond N membranes (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) and hybridised overnight to the appropriately radiolabelled cDNA probe (Slater *et al.* 1994). Membranes were washed to high stringency conditions with 0·1 × sodium citrate buffer at 65 °C, and Cerenkov counted (Johnson 1969). In these experiments optimal cycle numbers were: 35 for COX-1 in amnion and chorion–decidua, 31 and 33 for COX-2 in amnion and chorion–decidua respectively, and 22 for GAPDH in all three tissues. These cycle numbers were used in subsequent experiments. To control for RNA concentration the expression of COX-1 and COX-2 was calculated as a ratio to that of GAPDH for each individual sample.

Western analysis

Protein extracts were prepared from fetal membrane samples by homogenisation in 10 vols T-Wash (50 mM Tris buffer, 10 mM EDTA, 1% Triton-100, with 10 mM phenylmethylsulfonyl fluoride, 4 μg/ml pepstatin and 0·5 μg/ml leupeptin) for 30 s. Proteins (20 μg) were separated on a 10% SDS-PAGE gel and transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked in 5% Marvel, 0·1% Tween in PBS and incubated with either COX-1 or COX-2 specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:500. Membranes were washed in 0·1% Tween in PBS and incubated with anti-goat IgG–horseradish peroxidase secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:2000. Antibody specificity was confirmed using positive controls. Human platelets were used as a

positive control for COX-1 and interleukin-1β-stimulated human lymphocytes for COX-2. Western autoradiographs were quantified by digital densitometry.

Fetal membrane COX activity and prostaglandin synthesis

Prostaglandin synthesis from exogenous substrate sources by fetal membranes was measured to assess COX activity. Membranes were washed in PBS, and 2 cm discs of separated amnion and chorion were cut and suspended in serum-free medium 199 for 1 h. Membrane discs were then incubated with fresh medium containing arachidonic acid (30 μM) for 15 min. Prostaglandin E_2 concentration in the medium was measured by RIA according to manufacturer's instructions (Sigma-Aldrich, Poole, Dorset, UK). To assess prostaglandin synthesis from endogenous substrate sources intact fetal membrane discs were suspended in 1·5 ml serum-free medium 199 with insulin (1 μM), transferrin (0·1 μM) and selenium (0·05 μM) (Sigma-Aldrich) for 2 h. Prostaglandin E_2 concentration was measured by enzyme immunoassay (Amersham Pharmacia Biotech).

Statistical analysis

Data were analysed in groups, respectively estimating the mean and standard error before comparison was made using one way analysis of variance (ANOVA). Differences between groups were assessed using Fisher's exact test. Statistical significance was assumed if $P<0·05$.

RESULTS

COX mRNA expression, as a ratio to the expression of the constitutively expressed GAPDH, was compared among ten gestational age groups. COX-1 mRNA expression in amnion and in chorion–decidua was low throughout pregnancy and did not change with gestational age or the onset of labour (Fig. 1). COX-2 mRNA expression in amnion and in chorion–decidua was low or undetectable until the second trimester, when it then increased towards term and doubled with labour (Fig. 2). ANOVA showed a significant effect of gestational age and labour on COX-2 expression both in amnion and in chorion. There was no correlation between COX-1 expression and gestational age.

Western analysis showed COX-2 protein at 72 kDa and COX-1 protein at 70 kDa. Protein

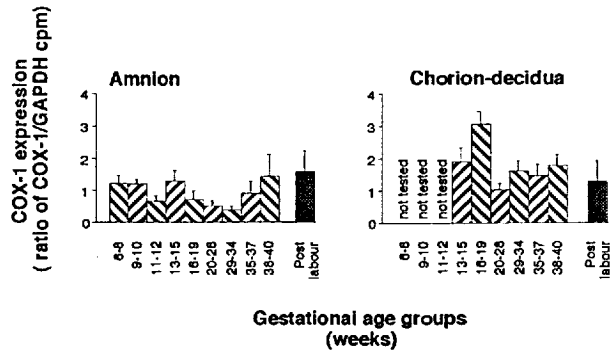


FIGURE 1. Expression of COX-1 mRNA, by RT-PCR, in amnion and chorion–decidua throughout gestation. Results are expressed as means and S.E. of the ratio of c.p.m. COX-1/GAPDH from gestational age groups; 6–8 weeks (n=4), 9–10 weeks (n=4), 11–12 weeks (n=6), 13–15 weeks (n=8), 16–19 weeks (n=4), 20–28 weeks (n=4), 29–34 weeks (n=6), 35–37 weeks (n=6), 38–40 weeks (prior to labour) (n=6), and 38–40 weeks (after labour) (n=6).

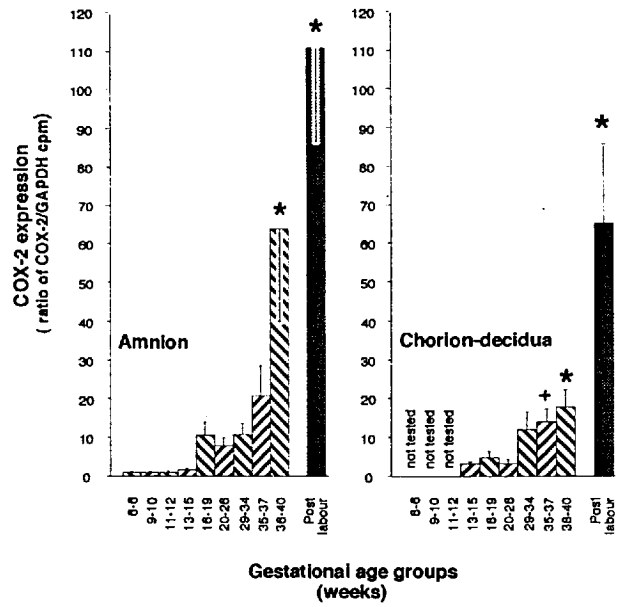


FIGURE 2. Expression of COX-2 mRNA, by RT-PCR, in amnion and chorion–decidua throughout gestation. Results are expressed as means and S.E. of the ratio of c.p.m. COX-2/GAPDH from gestational age groups; 6–8 weeks (n=4), 9–10 weeks (n=4), 11–12 weeks (n=6), 13–15 weeks (n=8), 16–19 weeks (n=4), 20–28 weeks (n=4), 29–34 weeks (n=6), 35–37 weeks (n=6), 38–40 weeks (prior to labour) (n=6), and 38–40 weeks (after labour) (n=6). In both amnion and chorion–decidua ANOVA showed a significant effect of gestational age upon COX-2 expression ($P<0·05$). *$P<0·05$ compared with all other groups; +$P<0·05$ compared with all groups below 28 weeks.

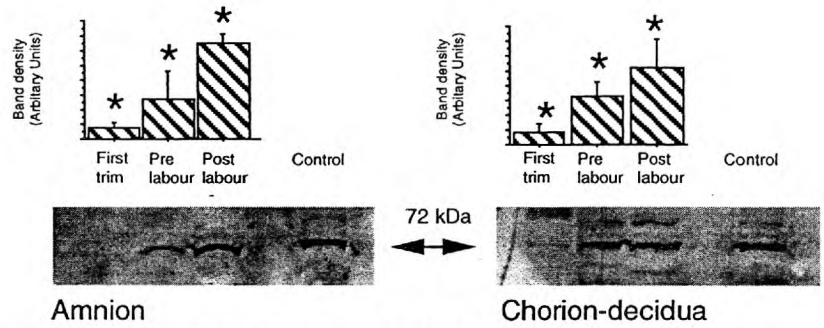


FIGURE 3. Western blot analysis of COX-2 protein in fetal membranes. Lower panels show original gels; upper panels show densitometric analysis of blots (means ± S.E., n=4). Expression of COX-2 protein was compared in fetal amnion and chorion–decidua from gestational age groups; 10–14 weeks (first trimester), 38–40 weeks (prior to labour), and 38–40 weeks (after labour). *P<0·05 compared with all other groups. P values as assessed by ANOVA and Fisher's exact test.

concentrations of COX-1 were similar at all gestational ages and did not change significantly with gestational age or with labour in either amnion or chorion–decidua (data not shown). In amnion, protein concentrations of COX-2 increased from almost undetectable levels in the first trimester to maximum levels in samples collected after labour (Fig. 3). In chorion–decidua, COX-2 protein concentration increased significantly between the first trimester and term. There was also a significant difference between chorion–decidua collected before and after labour.

COX activity was assessed in three gestational age groups, 10–14 weeks, 38–40 weeks prior to labour, and 38–40 weeks after labour. In amnion, COX activity increased between the first and third trimesters and was further increased in tissues collected following labour (Fig. 4). In chorion–decidua there was no change in activity between the first and third trimesters, but there was a significant increase in activity following the onset of labour. Production of prostaglandin E_2 from endogenous substrate sources in intact fetal membranes increased between the first and third trimesters, but there was a decrease post-labour when compared with pre-labour (Fig. 5).

DISCUSSION

During pregnancy the uterus expands to accommodate the growing fetus and placenta whilst remaining relatively quiescent, and the cervix remains firm and closed. At term these changes reverse to allow the uterus to contract, and the cervix to soften and dilate. Lye (1994) has suggested that labour is the result of the activation of a 'cassette of contraction associated proteins' which might include gap junction proteins, oxytocin and prostaglandin receptors. We have previously shown that, at the mRNA level, it is principally COX-2 which is expressed in both amnion and chorion–decidua, and that its expression increases with the onset of labour, whilst the expression of COX-1 in both tissues is unchanged (Slater *et al.* 1995, 1998). We

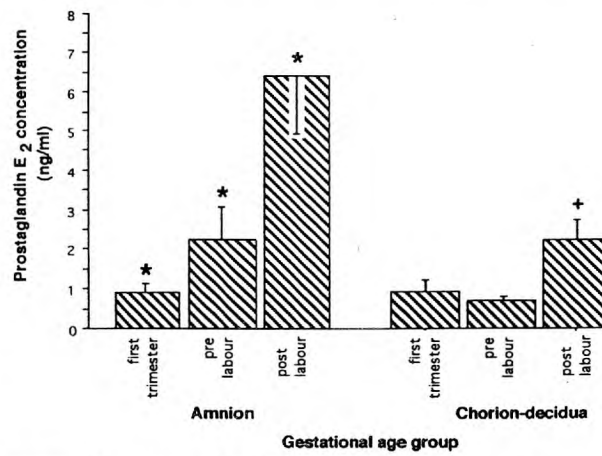


FIGURE 4. Assay of COX activity in amnion and chorion–decidua. Production of prostaglandin E_2 by fetal membranes from exogenous arachidonic acid was compared between 10–14 weeks (first trimester), 38–40 weeks (prior to labour), and 38-40 weeks (after labour) (n=5). Membranes were incubated with 30 μM arachidonic acid for 15 min and prostaglandin E_2 production was measured by RIA. Means ± S.E., *P<0·05 compared with all other groups; +P<0·05 compared with term pre-labour group. P values as assessed by ANOVA and Fisher's exact test.

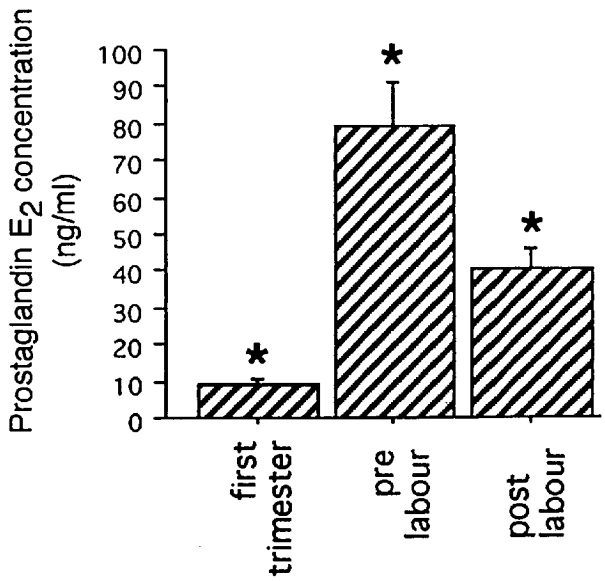


FIGURE 5. Production of prostaglandin E_2 by fetal membranes from endogenous arachidonic acid was compared between 10–14 weeks (first trimester), 38–40 weeks (prior to labour), and 38–40 weeks (after labour) (n=5). Membranes were incubated in medium for 2 h and the prostaglandin E_2 produced measured by ELISA. Means ± S.E., *P<0·05 compared with all other groups. P values as assessed by ANOVA and Fisher's exact test.

have confirmed our hypothesis that the central prostaglandin synthetic enzyme COX might also be a member of the 'cassette of contraction associated proteins', by our finding of significant up-regulation of COX-2 expression in late pregnancy prior to labour. In sheep, the principal source of prostaglandins in late gestation is the placental cotyledon rather than the fetal membranes (Mitchell & Flint 1977, Rice *et al.* 1988). Up-regulation of COX-2 expression in sheep placenta takes place gradually, as pregnancy advances, and COX-1 expression does not change (Wimsatt *et al.* 1993, Rice *et al.* 1995). The temporal pattern of COX expression is therefore similar in the two species, but in different tissues.

The major differences in COX-2 mRNA expression occur between the first and third trimesters, and before and after labour. Because of the difficulty of obtaining adequate numbers of tissue samples in the second and early third trimester, we confined COX activity studies and Western analysis to tissues obtained in the first trimester and at term, before and after labour. Western analysis showed increases in COX-2 protein concentrations between the first and third trimester and before and after labour, paralleling observations at the mRNA level. We found no change in COX-1 expression at either the mRNA or protein level. These data, together with previous studies showing increased COX-2 expression in fetal membranes with the onset of labour (Hirst *et al.* 1995, Slater *et al.* 1995, 1998), suggest that it is up-regulation of COX-2 in fetal membranes which mediates the increased prostaglandin synthesis associated with labour and delivery in the human.

Prostaglandin synthesis requires liberation of substrate arachidonic acid through the activity of a phospholipase. We have studied COX activity by measuring synthesis of prostaglandins from exogenous arachidonic acid because both endogenous arachidonic acid concentrations and phospholipase A_2 activity are known to fall in fetal membranes during labour (Curbelo *et al.* 1981, Skannal *et al.* 1997). This assay eliminates constraints on prostaglandin synthesis by endogenous substrate depletion or reduced phospholipase activity, but it does depend upon prostaglandin synthase E activity to produce prostaglandin E_2. We also measured synthesis of prostaglandin E_2 from intact fetal membranes without exogenous arachidonic acid, to examine their overall capacity for prostaglandin synthesis. Prostaglandin synthesis from endogenous sources increased between the first and third trimesters, in parallel with the increase in COX-2 mRNA and protein concentrations. The decrease in prostaglandin synthesis in fetal membranes collected after labour is consistent with lower endogenous arachidonic acid concentrations (Curbelo *et al.* 1981) and phospholipase A_2 activity (Skannal *et al.* 1997) following labour. The combination of increased COX-2 mRNA and protein expression, and maximum prostaglandin synthesis from endogenous substrate sources prior to labour, shows that increased prostaglandin synthesis within the fetal membranes precedes, and is therefore a cause, rather than a consequence, of labour.

ACKNOWLEDGEMENTS

This study was funded through grants made by Wellbeing and the Medical Research Council (UK).

REFERENCES

Bennett PR, Elder MG & Myatt L 1987 The effects of lipoxygenase metabolites of arachidonic acid on human myometrial contractility *in vitro*. *Prostaglandins* 33 837–844.

Bennett PR, Slater DM, Sullivan MHF, Elder MG & Moore GE 1993 Changes in arachidonic acid metabolism in amnion cells associated with increased cyclo-oxygenase gene

expression at parturition. *British Journal of Obstetrics and Gynaecology* **100** 1037–1042.
Crankshaw DJ & Dyal R 1994 Effects of some naturally occurring prostanoids and some cyclo-oxygenase inhibitors on the contractility of the human lower uterine segment *in vitro*. *Canadian Journal of Physiology and Pharmacology* **72** 870–874.
Curbelo V, Bejar R & Benirschke K 1981 Prostaglandin precursors in human placental membranes. *Obstetrics and Gynaecology* **57** 473–478.
Garfield RE, Tabb T & Thilander G 1990 Intercellular coupling and modulation of uterine contractility. In *Uterine Contractility*, pp 21–40. Ed RE Garfield. Norwell, MA, USA: Serono Symposia.
Hirst JJ, Teixeira FJ, Zakar T & Olson DM 1995 Prostaglandin endoperoxide-H synthase-2 expression increases in human gestational tissues with spontaneous labour onset. *Reproduction, Fertility and Development* **7** 633–637.
Hla T & Neilson K 1992 Human cyclo-oxygenase-2 cDNA. *Proceedings of the National Academy of Sciences of the USA* **89** 7384–7388.
Hla T, Farrell M, Kumar A & Bailey JM 1986 Isolation of the cDNA for human prostaglandin H synthase. *Prostaglandins* **32** 829–845.
Johnson MK 1969 Counting of Cerenkov radiation from 32P in nonaqueous media. *Analytical Biochemistry* **29** 348–350.
Keirse MJ, Mitchell MD & Turnbull AC 1977 Changes in prostaglandin F and 13,14-dihydro-15-keto-prostaglandin F concentrations in amniotic fluid at the onset of and during labour. *British Journal of Obstetrics and Gynaecology* **84** 743–746.
Liggins GC 1989 Initiation of labour. *Biology of the Neonate* **55** 366–375.
Lye S 1994 The initiation and inhibition of labour – toward a molecular understanding. In *Seminars in Reproductive Endocrinology*, vol 12, pp 284–294. Ed MD Mitchell. New York, USA: Thieme Medical Publishers, Inc.
MacDonald PC & Casey ML 1993 The accumulation of prostaglandins (PG) in amniotic fluid is an after effect of labour and not indicative of a role for PGE_2 or $PGF_{2\alpha}$ in the initiation of human parturition. *Journal of Clinical Endocrinology and Metabolism* **76** 1332–1339.
Mitchell MD & Flint A 1977 Prostaglandin concentrations in intrauterine tissues from late pregnant sheep before and after labour. *Prostaglandins* **14** 563–569.
Rice GE, Wong MH & Thorburn GD 1988 Gestational changes in prostaglandin synthase activity of cotyledonary microsomes. *Journal of Endocrinology* **118** 265–270.
Rice GE, Freed KA, Aitken MA & Jacobs RA 1995 Gestational and labour associated changes in relative abundance of prostaglandin G/H synthase-1 and -2 mRNA in ovine placenta. *Journal of Molecular Endocrinology* **14** 237–244.
Romero R, Munoz H, Gomez R, Parra M, Polanco M, Valverde V, Hasbun J, Garrido J, Ghezz F, Mazor M, Tolosa JE & Mitchell MD 1996 Increase in prostaglandin bioavailability precedes the onset of human parturition. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **54** 187–191.
Saeed SA & Mitchell MD 1982 Formation of arachidonic acid metabolites by human fetal membranes, uterine decidua vera and placenta. *Prostaglandins, Leukotrienes and Medicine* **8** 635–640.
Skannal DG, Brockman DE, Eis AL, Xue S, Siddiqi TA & Myatt L 1997 Changes in activity of cytosolic phospholipase A_2 in human amnion at parturition. *American Journal of Obstetrics and Gynecology* **177** 179–184.
Skinner KA & Challis JRG 1985 Changes in the synthesis and metabolism of prostaglandins by human fetal membranes and decidua at labor. *American Journal of Obstetrics and Gynecology* **151** 519–523.
Slater DM, Berger L, Newton R, Moore GE & Bennett PR 1994 The relative abundance of type 1 to type 2 cyclo-oxygenase mRNA in human amnion at term. *Biochemical and Biophysical Research Communications* **198** 304–308.
Slater DM, Berger L, Newton R, Moore GE & Bennett PR 1995 Changes in the expression of types 1 and 2 cyclo-oxygenase in human fetal membranes at term. *American Journal of Obstetrics and Gynecology* **172** 77–82.
Slater DM, Allport V & Bennett PR 1998 Changes in the expression of the type-2 but not the type-1 cyclo-oxygenase enzyme in chorion–decidua with the onset of labour. *British Journal of Obstetrics and Gynaecology* **105** 745–748.
Smieja Z, Zakar T, Walton JC & Olson DM 1993 Prostaglandin endoperoxide synthase kinetics in human amnion before and after labour at term and following preterm labour. *Placenta* **14** 163–175.
Walsh SW 1989 5-Hydroxyeicosatetraenoic acid, leukotriene C4 and prostaglandin $F_{2\alpha}$ in amniotic fluid before and during term and preterm labor. *American Journal of Obstetrics and Gynecology* **161** 1352–1360.
Wimsatt J, Nathanielsz PW & Sirois J 1993 Induction of prostaglandin endoperoxide synthase isoform-2 in ovine cotyledonary tissues during late gestation. *Endocrinology* **133** 1068–1073.

REVISED MANUSCRIPT RECEIVED 6 October 1998

7.1.5

Expression of cyclo-oxygenase types-1 and -2 in human myometrium throughout pregnancy

Donna M. Slater, William J. B. Dennes, Juan S. Campa, Lucilla Poston and Phillip R. Bennett

Molecular Human Reproduction (1999), **5**, 9, 880-884.

Author's Contribution:

Contributed to experimental design and methodology, carried out Western analyses of COX protein and mRNA expression in myometrium and contributed to both the writing and submission of the manuscript.

Molecular Human Reproduction vol.5 no.9 pp. 880–884, 1999

Expression of cyclo-oxygenase types-1 and -2 in human myometrium throughout pregnancy

Donna M.Slater[1,3], William J.B.Dennes[1], Juan S.Campa[2], Lucilla Poston[2] and Phillip R.Bennett[1]

[1]The London Myometrial Group Division of Paediatrics, Obstetrics and Gynaecology, Imperial College School of Medicine, Institute of Obstetrics and Gynaecology, Queen Charlottes and Chelsea Hospital, Goldhawk Road, London W6 OXG, and [2]Fetal Health Research Group, Department of Obstetrics and Gynaecology, Guy's, King's and St Thomas', School of Medicine, St Thomas' Hospital, London SE1 7EH, UK

[3]To whom correspondence should be addressed

Human labour is associated with increased prostaglandin synthesis within the uterus. The aim of this study was to examine the expression of the two isoforms of the central prostaglandin synthetic enzyme, cyclo-oxygenase (COX-1 and COX-2) in human myometrium throughout pregnancy and to test the hypothesis that COX in the myometrium may play a role in labour onset. Expression of *COX-1* and *COX-2* at the mRNA level was analysed using reverse transcriptase–polymerase chain reaction (RT–PCR) and at the protein level using Western blotting. No significant changes of *COX-1* RNA or protein expression were observed either with gestational age or labour. *COX-2* mRNA and protein expression increased at term with significant up-regulation occurring prior to the onset of labour ($P < 0.005$). These data would suggest that up-regulation of *COX-2*, rather than *COX-1*, mediates increased prostaglandin synthesis in human myometrium at term. The increased COX-2 expression observed preceded labour onset, suggesting that COX-2 has a role in labour onset, rather than its presence merely a consequence of labour.

Key words: cyclo-oxygenase/labour/myometrium/pregnancy/prostaglandins

Introduction

Preterm labour occurs in up to 10% of all births but is associated with 85% of perinatal deaths in otherwise normal babies. The aetiology of preterm labour remains largely unknown, since the mechanisms involved in the onset of normal labour at term are not fully understood. Throughout pregnancy the uterus undergoes profound physiological changes, expanding to accommodate the growing fetus, whilst remaining in a quiescent state. The myometrium is resistant to activation, which is associated with incomplete cell–cell coupling, resulting in low basal smooth muscle tone, and decreased uterine activity. No single factor has been demonstrated as responsible for these pregnancy-associated changes. It is likely that there is a balance of 'pro-pregnancy' factors, such as progesterone, maintaining uterine quiescence and 'pro-labour' factors such as gap junctions, calcium-sensitive potassium channels (Khan *et al.*, 1998), and connexins, that result in the onset of uterine contractions, cervical dilation and delivery of the fetus (for review see Garfield *et al.*, 1998).

Prostaglandins play a central role in human parturition. Labour is associated with increased prostaglandin synthesis within the uterus (Turnbull, 1977) prostaglandins are used for the induction of labour and prostaglandin synthesis inhibitors are the most effective tocolytic agents (Keirse, 1995). Prostaglandins act to mediate cervical ripening and to stimulate uterine contractions (Crankshaw and Dyal, 1994) and indirectly to increase fundally dominant myometrial contractility by up-regulation of oxytocin receptors and synchronization of contractions (Garfield *et al.*, 1987; Liggins, 1989). Prostaglandins are formed from the precursor arachidonic acid which itself is a substrate for at least three enzyme groups. The cyclo-oxygenase (COX) or prostaglandin endoperoxide synthase pathway produces prostaglandin endoperoxides, which are then synthesized to prostaglandins by specific synthase enzymes. The fetal membranes are a major source of prostaglandins within the human uterus and much attention has been focused upon the regulation of prostaglandin synthesis within the fetal membranes and its association with the onset of labour. Fetal membrane prostaglandin synthesis increases in association with labour (Skinner and Challis, 1985). In amnion, *COX-2* mRNA expression exceeds that of *COX-1* by a factor of 100 (Slater *et al.*, 1994) and the large increase in synthesis of prostaglandin E_2 is associated with increased expression of *COX-2* at term (Hirst *et al.*, 1995; Slater *et al.*, 1995). Increased expression of *COX-2* and prostaglandin production in fetal membranes precedes labour onset (Slater *et al.*, 1999). This would imply COX-2 in the fetal membranes has a role in labour onset as opposed to being merely a consequence of labour. The chorion is a major site of prostaglandin dehydrogenase (PGDH) (Cheung and Challis, 1989; Cheung *et al.*, 1990, 1992). It has been suggested that there may be a down-regulation of PGDH activity in association with the onset of labour that would facilitate prostaglandin E_2 access to the myometrium (Van Meir *et al.*, 1997).

Myometrium expresses receptors for the E and F series prostaglandins (Matsumoto *et al.*, 1997) linked to both inhibitory and excitatory mechanisms (Senior *et al.*, 1993). Prostaglandin synthesis inhibitors decrease uterine activity and reduce

© European Society of Human Reproduction and Embryology

contractility in in-vitro preparations of rat (Williams and Vane, 1975) and human (Johnson *et al.*, 1975) myometrium. The principal prostaglandin product of human pregnant myometrium is prostacyclin (Bamford *et al.*, 1980), which has no effect upon uterine contractility (Crankshaw and Dyal, 1994). Myometrium also synthesizes thromboxane and prostaglandins E_2 and $F_{2\alpha}$, each of which is potentially oxytocic (Crankshaw and Dyal, 1994).

The aim of this study was to test the hypothesis that COX in the myometrium plays a role in the onset and/or maintenance of human labour. We studied the expression of *COX-1* and *COX-2* mRNA and protein, by reverse transcription–polymerase chain reaction (RT–PCR) and Western blotting, in human pregnant myometrium in the second and third trimesters of pregnancy and before and after labour at term.

Materials and methods

Tissue collection

Myometrial tissue ~0.5×2 cm was taken from the upper margin of the lower segment incision at Caesarean section. The local Research Ethics Committees of St Thomas' Hospital and Hammersmith Hospital Trust, UK, approved the study. All patients gave written informed consent. Patients age ranged from 18–40 years. The data were analysed in four groups: at term in non-labouring (38–41 weeks, n = 11), and labouring women (38–40 weeks, n = 5), and preterm in non-labouring women. The preterm patients were divided into two groups, early preterm (26–32 weeks, n = 8) and late preterm (32–36 weeks, n = 6). These are arbitrary groupings based upon the clinical distinction between preterm (>32 weeks) and very preterm (<32 weeks). The indications for Caesarean section in the non-labour groups were breech presentation, hypertensive disease, and previous Caesarean delivery. No patients were on medication. The non-labouring groups had no evidence of uterine contractions or cervical change. There was no evidence of uterine dysfunction. Myometrial tissue from labouring women was collected during emergency Caesarean section. In this group all patients had regular painful contractions with associated cervical dilation (>3 cm), and Caesarean section was indicated by fetal distress. There was no prior use of prostaglandins or oxytocin and the progress of labour had previously been normal.

RT–PCR

Myometrial tissue was immediately snap frozen in liquid nitrogen. Total RNA was isolated using a standard guanidine isothyocyamate technique (Chirgwin *et al.*, 1979) and reverse transcribed into cDNA for use as the template for PCR. RNA samples (1 µg) were denatured at 70°C for 5 min. Reverse transcription was performed at 37°C for 60 min in a reaction volume of 20 µl containing random hexanucleotide primers, 0.2 µg (Pharmacia, UK), reverse transcriptase buffer, 0.1 mol/l dithiothreitol (DTT), 1 IU RNAse inhibitor, 1 mmol/l of each dNTP and 40 IU of mouse myeloma leukaemia virus reverse transcriptase (Promega, Southampton, UK). The reverse transcription reaction was stopped by heating at 90°C for 5 min. A 1/40 volume of the generated cDNA reaction was then used in subsequent amplification by PCR. PCR was performed in a 25 µl volume containing 1.5 mmol/l magnesium chloride, 0.2 mmol/l deoxynucleotide triphosphates (dNTPs), 125 ng of each sense and antisense primer, and 1 IU of Biotaq polymerase (Bioline UK). Following an initial denaturation step of 4 min at 94°C, the reaction cycles were denaturing at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s for the appropriate number of cycles. Primers used (Hla *et al.*, 1986; Hla and Neilson, 1992) were; **COX-2**; 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′ (sense), 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′ (antisense), **COX-1**; 5′-TGC CCA GCT CCT GGC CCG CCG CTT-3′ (sense), 5′-CCA TGG CCC AAG GCC TTG-3′ (antisense), glyceraldehyde-3-phosphate dehydrogenase (**G-APDH**); 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ (sense), 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ (antisense). Aliquots of the PCR reaction were separated by agarose gel electrophoresis, transferred onto nylon membranes, and subsequently hybridized to radiolabelled COX-1, COX-2 or GAPDH probes to confirm PCR product identity.

To determine the exponential phase of amplification, where product formation is related to starting template concentration, a PCR cycle profile was performed. An aliquot of cDNA, collected from each of the myometrial samples at 27–41 weeks of pregnancy, was pooled to create an 'average' sample. PCR was performed using between 20 and 40 cycles with primers specific for *COX-1*, *COX-2* and *GAPDH*. A 5 µl aliquot of each PCR was dot blotted onto nylon membrane (MSI), hybridized to specific radiolabelled probes, and washed under high stringency conditions. Dotted membrane filters were then placed in a scintillation counter and radioactivity determined as a function of counts per minute for each individual sample. The ratio of product concentration to template concentration was determined. The optimal cycle number was defined as the midpoint of cycle numbers giving a linear relationship between template and product concentrations. In these experiments, optimal cycle numbers were found to be 31 for each of COX-1 and COX-2 and 22 for GAPDH. These cycle numbers were therefore used in studies of COX expression. To control for RNA concentration, COX expression was calculated as a ratio to that of GAPDH.

Comparison of amplification efficiency

We have previously reported experiments showing that the efficiency of amplification of *COX-1* and *COX-2* is similar using these primers (Slater *et al.*, 1994, 1998). Briefly, *COX-1* and *COX-2* RT–PCR products were subcloned into the Bluescribe vector (Stratagene, La Jolla, CA, USA). The concentration of Bluescribe plasmid DNA containing either *COX-1* or *COX-2* PCR product inserts was determined spectrophotometrically, and compared visually by agarose gel electrophoresis. Stepwise (1 in 10) dilution series' were prepared for each plasmid to give equivalent concentrations of each target sequence and PCR was performed. Amplification efficiency was found to be similar for the two sequences.

Western analysis

Protein extracts were prepared from myometrial samples by homogenization in 10 vol T-Wash (50 mmol/l Tris buffer, 10 mmol/l EDTA, 1% Triton-100, with 10 mmol/l phenylmethylsulphonyl fluoride, 4 µg/ml pepstatin and 0.5 µg/ml leupeptin) for 30 s. The supernatant was separated from tissue debris by centrifugation at 1000 *g*, for 10 min, at 4°C. Protein concentrations were determined by Protein assay (Bio-Rad Laboratories, Richmond, CA, USA) and bovine serum albumin (BSA) reference standards. Electrophoresis was carried out on 20 µg aliquots of protein samples, in 2× loading buffer [4% sodium dodecyl sulphate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 mol/l Tris–HCl, pH 6.8]. Samples were boiled for 5 min, quenched on ice, and subsequently run on a 10% SDS–polyacrylamide gel.

Western blotting was carried out following electrophoretic transfer, in 25 mmol/l Tris, 192 mmol/l glycine and 20% v/v methanol, pH 8.3, on to Hybond ECL nitro-cellulose membrane (Amersham Life Science, Little Chalfont, UK). Membranes were blocked in 5%

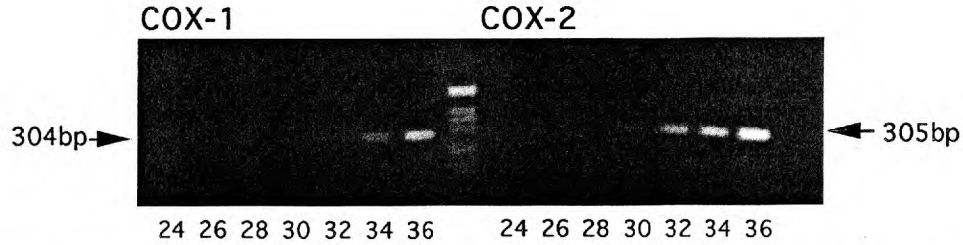


Figure 1. Reverse transcription–polymerase chain reaction (RT–PCR) cycle profile for *COX-1* and *COX-2* in human myometrium. Representative agarose gel stained with ethidium bromide. Cycle number was increased from 24–36 cycles to determine the exponential phase of amplification.

Marvel-0.1% Tween–phosphate-buffered saline (PBS), for 1 h at room temperature. Affinity-purified goat polyclonal antibodies, directed against the peptide immunogen, were used for COX-1 and COX-2 (COX-1 No. sc-1752, COX-2 No. sc-1745: Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:500, and incubated overnight at 4°C. Membranes were washed in 0.1% Tween–PBS and then incubated with anti-goat immunoglobulin G–horseradish peroxidase (IgG–HRP) secondary antibody at a dilution of 1:2000, for 1 h at room temperature. ECL Western blotting detection was carried out using standard protocols (Amersham Life Science). Protein band size was determined using Rainbow coloured protein molecular weight markers (Amersham Life Science). Antibody specificity was confirmed using positive controls. Human platelets were used as a positive control for COX-1 and interleukin-1β stimulated human lymphocytes for COX-2. Western autoradiographs were quantified by digital densitometry using the Image Master VDS gel documentation system and Image Master VDS Software (Pharmacia Biotech). Protein bands were digitized, ensuring that the range of pixel densities did not extend to either the minimum or maximum values. Mean pixel density for each band was assessed using a sample gate of the same size. To allow comparisons between blots prepared on different occasions a single control sample was included on each blot. The final pixel density was adjusted to ensure that this control sample carried the same value for each blot.

Statistical analysis

Data were analysed in groups, estimating the mean and SE respectively, before comparisons were made using analysis of variance (ANOVA). Differences between groups were assessed by post-hoc analysis using Fisher's exact test with the StatView 4.5 statistics software (Abacus Concepts Inc. Berkeley, CA, USA). $P < 0.05$ was considered to be statistically significant.

Results

COX-1 and *COX-2* were expressed at both mRNA and protein levels in all myometrial samples examined. No significant differences of *GAPDH* expression were observed in any of the samples examined. Western analysis showed COX-2 protein at a size of 72 kDa and COX-1 protein at 74 kDa. *COX-1* and *COX-2* expression data were analysed in four groups: early preterm (26–32 weeks, $n = 8$); late preterm (33–37 weeks, $n = 6$); term not in labour (38–41 weeks, $n = 11$); and term in labour (38–40 weeks, $n = 5$).

Cycle profiles ($n = 3$) were performed on pooled myometrial samples and the number of PCR cycles used to measure both COX-1 and COX-2 in this study was 31. Figure 1 depicts a representative cycle profile. It was observed that whilst the relative abundance of *COX-1* to *COX-2* mRNA in myometrium was of the same order of magnitude, in general it appeared that *COX-1* was expressed at levels slightly higher than those of *COX-2*.

A decrease of *COX-1* mRNA expression was observed in association with labour compared with all other groups; however, this decrease was not statistically significant (Figure 2a). No further changes in *COX-1* expression at either the mRNA or protein level were seen between any of the gestational age groups, or in association with labour (Figure 2a,b).

Expression of *COX-2* mRNA and protein was significantly ($P < 0.05$) higher in the term in-labour group compared with both early and late preterm groups. Expression of *COX-2* was also higher in the term non-labour group compared with either of the preterm groups, however, only increased protein values reached statistical significance ($P < 0.05$). No differences were observed for *COX-2* mRNA expression between either the early or late preterm groups. COX-2 protein expression was significantly higher in late preterm compared with early preterm ($P < 0.05$). No significant differences were observed in association with labour (Figure 3a,b). Linear regression analysis of COX expression by gestational age showed no significant association between expression of either COX-1 or COX-2 and gestational age (data not shown).

Discussion

During pregnancy the uterus expands to accommodate the growing fetus and placenta whilst remaining relatively quiescent, and the cervix remains firm and closed. At term these changes need to be reversed to allow the uterus to contract and the cervix to soften and dilate (for review see Garfield *et al.*, 1998). It has been suggested that labour is the result of the activation of a 'cassette of contraction associated proteins' which might include gap junction proteins, oxytocin and prostaglandin receptors (Lye, 1994). We have previously shown that, at the mRNA level, it is principally *COX-2* which is expressed in both amnion and chorion decidua and that its expression increases with the onset of labour, whilst the expression of *COX-1* in both tissues is unchanged (Slater *et al.*, 1995, 1998). We postulated that the central prostaglandin synthetic enzyme cyclo-oxygenase (COX) might also be a member of the 'cassette of contraction associated proteins' and this is supported by these findings of significant up-regulation of *COX-2* expression at the mRNA level in myometrium in late pregnancy and in association with labour.

Western analysis showed increases in COX-2 protein concen-

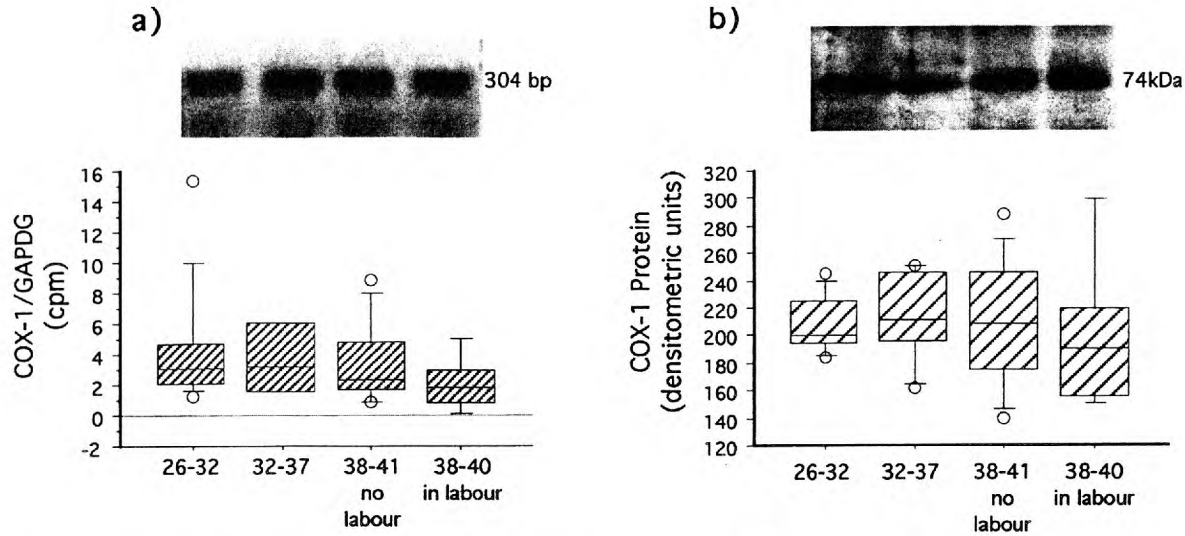


Figure 2. COX-1 expression by (**a**) reverse transcription–polymerase chain reaction (RT–PCR) and (**b**) Western blotting analysis. Upper panels show (**a**) representative autoradiograph following RT–PCR, transfer to nylon membrane and subsequent hybridization with a radiolabelled COX-1 probe, and (**b**) Western blot. Graphs depict results expressed as mean and SE of: (**a**) ratio of counts per minute (cpm) *COX-1/GAPDH* and (**b**) mean and SEM relative optical density of bands of COX-1 protein from early preterm (26–32 weeks, $n = 8$), late preterm (32–37 weeks, $n = 6$), term not in labour (38–41 weeks, $n = 11$) and term in-labour (38–40 weeks, $n = 5$) groups.

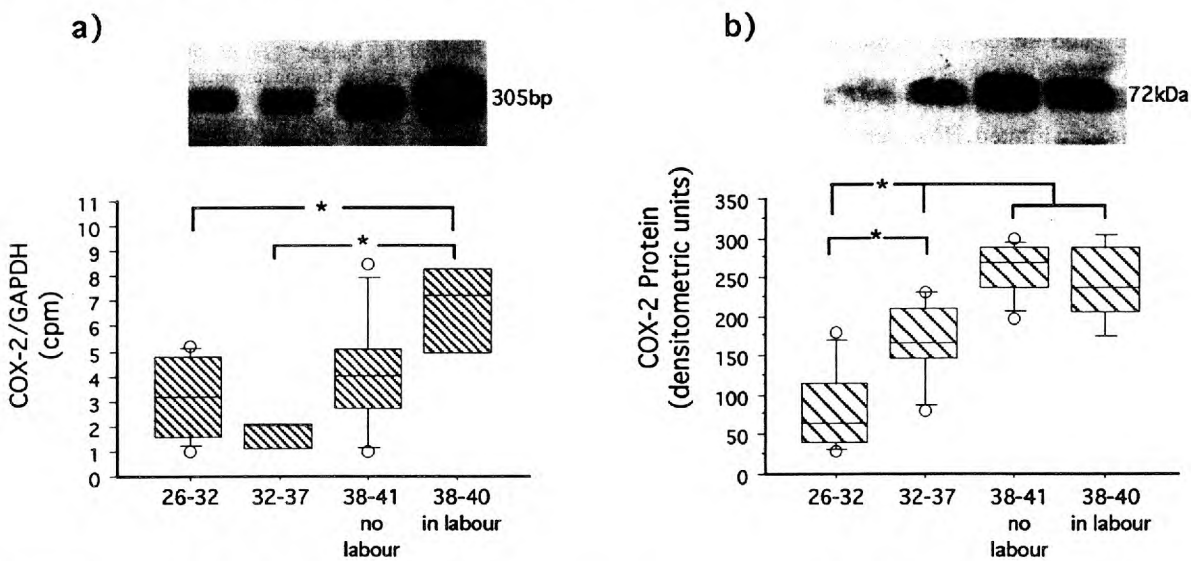


Figure 3. COX-2 expression by (**a**) reverse transcription–polymerase chain reaction (RT–PCR) and (**b**) Western blotting analysis. Upper panels show (**a**) representative autoradiograph following RT–PCR, transfer to nylon membrane and subsequent hybridization with a radiolabelled COX-2 probe and (**b**) Western blot. Graphs depict results expressed as mean and SE of (**a**) ratio of counts per minute (cpm) *COX-2/GAPDH* and (**b**) mean and SEM relative optical density of bands of COX-2 protein from early preterm (26–32 weeks, $n = 8$), late preterm (32–37 weeks, $n = 6$), term not in labour (38–41 weeks, $n = 11$) and term in-labour (38–40 weeks, $n = 5$) groups. *Significant ($P < 0.05$).

trations between preterm and term tissues, paralleling that seen at the mRNA level. The differences in expression at the protein level were not as great as those seen at the mRNA level and appeared to occur at earlier gestational ages. This may reflect the 'suicide' nature of the COX enzyme. When COX is not catalysing the synthesis of prostaglandins, for example in the circulating platelet, it is relatively stable. During synthesis of prostaglandins, however, COX undergoes destruction after a limited number of reactions (Marshall *et al.*, 1979) leading to an effectively short half-life. Any stimulus to prostaglandin production must therefore increase synthesis of new COX protein, to replace that destroyed during catalysis. It is possible that the increase in COX-2 protein which we have seen to occur at 33–37 weeks, without an associated increase in mRNA, represents the accumulation of protein in anticipation of an increase in prostaglandin synthesis associated with labour at term. Synthesis of oxytocic prostaglandins, during labour, would consume COX protein whose replacement would require either increased *COX* gene transcription or increased mRNA stability. This hypothesis would be consistent with our finding a greater increase in mRNA than protein concentrations at term prior to onset of labour.

In myometrium, prostacyclin is the principle prostaglandin synthesized (Bamford *et al.*, 1980). In this study we found that COX-1 is expressed in myometrium with a relative abundance similar to that of COX-2 whereas in the fetal membranes COX-2 expression greatly exceeds that of COX-1 (Slater *et al.*, 1994). Expression of the 'constitutive' COX-1 enzyme in myometrium probably reflects constitutive prostacyclin synthesis. Prostacyclin is a vasodilator and may contribute to the necessary increases in myometrial blood flow during pregnancy. Myometrial COX-2 expression increases with a pattern similar to that of the chorion–decidua suggesting common mechanisms of control (Slater *et al.*, 1998). In-situ hybridization and immunocytochemistry were used to show that human pregnant myometrium expresses both *COX-1* and *COX-2* (Zuo *et al.*, 1994). These authors found that *COX-1* expression decreased with advancing gestational age but did not change with labour and that *COX-2* expression increased with gestational age but decreased with labour. Their findings are not entirely consistent with our results. However, their conclusions were based upon qualitative scoring of histological sections which did not permit statistical analysis. Myometrial samples in this study were taken from the lower segment of the uterus. The lower uterine segment develops more rapidly late in pregnancy and undergoes significant changes at term. It would be of interest to know if the same changes also occurred in the fundal segment of the uterus. Access to these fundal samples is very limited. However, these data still suggest a gestational stage-related trend for increased prostaglandin synthetic capacity in human myometrium. Our current data, together with those from previous studies showing increased COX-2 expression in fetal membranes with the onset of labour (Hirst *et al.*, 1995; Slater *et al.*, 1995, 1999), support the overall hypothesis that it is *COX-2* and not *COX-1* whose expression is increased in both the myometrium and the fetal membranes with the onset of labour and which catalyses the formation of oxytocic prostaglandins.

Acknowledgements

Funding from Wellbeing and the Tommy's Campaign supported this work.

References

Bamford, D.S., Jogee, M. and Williams, K.I. (1980) Prostacyclin formation by the pregnant human myometrium. *Br. J. Obstet. Gynaecol.*, **87**, 215–218.

Cheung, P.Y. and Challis, J.R. (1989) Prostaglandin E_2 metabolism in the human fetal membranes. *Am. J. Obstet. Gynecol.*, **161**,1580–1585.

Cheung, P.Y., Walton, J.C., Tai, H.H. *et al.* (1990) Immunocytochemical distribution and localization of 15-hydroxyprostaglandin dehydrogenase in human fetal membranes, decidua, and placenta. *Am. J. Obstet. Gynecol.*, **163**, 1445–1449.

Cheung, P.Y., Walton, J.C., Tai, H.H. *et al.* (1992) Localization of 15-hydroxy prostaglandin dehydrogenase in human fetal membranes, decidua, and placenta during pregnancy. *Gynecol. Obstet. Invest.*, **33**, 142–146.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. *et al.* (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*, **18**, 5294–5299.

Crankshaw, D.J. and Dyal, R. (1994) Effects of some naturally occurring prostanoids and some cyclo-oxygenase inhibitors on the contractility of the human lower uterine segment *in vitro*. *Can. J. Physiol. Pharmacol.*, **72**, 870–874.

Garfield, R.E., Hayashi, R.H. and Harper, M.J. (1987) *In vitro* studies on the control of human myometrial gap junctions. *Int. J. Gynaecol. Obstet.*, **25**, 241–248.

Garfield, R.E., Saade, S., Buhimschi, C., *et al.* (1998) Control and assessment of the uterus and cervix during pregnancy and labour. *Hum. Reprod. Update*. **4**, 673–695.

Hirst, J.J., Teixeira, F.J., Zakar, T. *et al.* (1995) Prostaglandin endoperoxide-H synthase-1 and –2 messenger ribonucleic acid levels in human amnion with spontaneous labor onset. *J. Clin. Endocrinol. Metab.*, **80**, 517–523.

Hla, T., Farrell, M., Kumar, A. *et al.* (1986) Isolation of the cDNA for human prostaglandin H synthase. *Prostaglandins*, **32**, 829–45.

Hla, T. and Neilson, K. (1992) Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. USA*, **89**, 7384–7388.

Johnson, W.L., Harbert, G.M., and Martin, C.B. (1975) Pharmacologic control of uterine contractility. *In vitro* human and *in vivo* monkey studies. *Am. J. Obstet. Gynecol.*, **123**, 364–375.

Keirse, M. (1995) Indomethacin tocolysis in preterm labour. *Pregnancy and Childbirth; Cochran Database of Systematic Reviews*. Cochran Collaboration: Oxford. 4383.

Khan, R.N., Smith, S.K. and Ashford, M.L.J. (1998) Contribution of calcium-sensitive potassium channels to NS1619-induced relaxation in human pregnant myometrium. *Hum. Reprod.*, **13**, 208–213.

Liggins, G. (1989) Initiation of labour. *Biol. Neonate*, 55, 366–375.

Lye, S. (1994) The initiation and inhibition of labour – Toward a molecular understanding. *Sem. Reprod. Endocrinol.*, **12**, 284–294.

Marshall. P., Kulmacz, R. and Lands, W. (1979) Constraints on prostaglandin synthesis in tissues. *J. Biol. Chem.*, **262**, 3510–3517.

Matsumoto, T., Sagawa, N., Yoshida, M. *et al.* (1997) The prostaglandin E_2 and $F_{2\alpha}$ receptor genes are expressed in human myometrium and are down-regulated during pregnancy. *Biochem. Biophys. Res. Commun.*, **238**, 838–841.

Senior, J., Marshall, K., Sangha, R. *et al.* (1993) *In vitro* characterization of prostanoid receptors on human myometrium at term pregnancy. *Br. J. Pharmacol.*, **108**, 501–506.

Skinner, K.A. and Challis, J.R. (1985) Changes in the synthesis and metabolism of prostaglandins by human fetal membranes and decidua at labor. *Am. J. Obstet. Gynecol.*, **151**, 519–523.

Slater, D.M., Allport, V. and Bennett, P.R. (1988) Changes in the expression of the type-2 but not the type-1 cyclo-oxygenase enzyme in chorion-decidua with the onset of labour. *Br. J. Obstet. Gynaecol.*, **105**, 745–748.

Slater, D.M., Berger, L., Newton, R. *et al.* (1994) The relative abundance of type 1 to type 2 cyclo-oxygenase mRNA in human amnion at term. *Biochem. Biophys. Res. Commun.*, **198**, 304–308.

Slater, D.M., Berger, L., Newton, R. *et al.* (1995) Expression of cyclooxygenase types 1 and 2 in human fetal membranes at term. *Am. J. Obstet. Gynecol.*, **172**, 77–82.

Slater, D., Dennes, W., Sawdy, R. *et al.* (1999) Expression of cyclo-oxygenase types-1 and –2 in human fetal membranes throughout pregnancy. *J. Mol. Endocrinol.*, **22**, 125–130.

Turnbull, A. (1977) *The Fetus and Birth*. Elsevier, London, UK.

Van Meir, C.A., Ramirez, M.M., Matthews, S.G. *et al.* (1997) Chorionic prostaglandin catabolism is decreased in the lower uterine segment with term labour. *Placenta*, **18**, 109–114.

Williams, K.I. and Vane, J.R. (1975) Inhibition of uterine motility: the possible role of the prostaglandins and aspirin-like drugs. *Pharmacol. Ther.*, **1**, 89–113.

Zuo, J., Lei, Z.M., Rao, C.V. *et al.* (1994) Differential cyclooxygenase-1 and -2 gene expression in human myometria from preterm and term deliveries. *J. Clin. Endocrinol. Metab.*, **79**, 894–899.

Received on February 17, 1999; accepted on June 8, 1999

7.1.6

Region and labour-dependent synthesis of prostaglandin E2 by human fetal membranes

R. J. Sawdy, W. J. Dennes, V. Allport, D. M. Slater, M. G. Elder, M. H. Sullivan and P. R. Bennett.

Placenta(1999), **20**, 2-3.

Author's Contribution:

Contributed to experimental design and methodology, carried out COX assays in intact fetal membranes and contributed to the Discussion.

Placenta (1999), 20, 181–184

Region and Labour-dependent Synthesis of Prostaglandin E2 by Human Fetal Membranes

R. J. Sawdy, W. J. B. Dennes, V. Allport, D. M. Slater, M. G. Elder, M. H. F. Sullivan[a] and P. R. Bennett

Parturition Research Group, Imperial College School of Medicine, Institute of Obstetrics and Gynaecology, Queen Charlottes and Chelsea Hospital, Goldhawk Road, London W6 0XG, UK

Paper accepted 21 October 1998

To examine the effect of region and labour upon prostaglandin synthesis in human fetal membranes, intact membranes from three regions, the cervical region, the periplacental region and a region midway between the two, were collected following spontaneous labour and delivery or at elective caesarean section prior to labour. Discs of 2-cm diameter were cut from each of three regions and incubated for 1, 2, 4, 6, 12 or 24 h after which prostaglandin E2 concentration in the supernatant was measured. We found that there was an overall decrease in prostaglandin synthesis in tissues collected after labour, but that this effect could be reversed if exogenous arachidonic acid substrate was supplied. We found no differences in prostaglandin synthesis between tissues collected from each of the three regions. We conclude that prostaglandin synthesis from the fetal membranes during labour leads to depletion of arachidonic acid substrate and that regional changes in prostaglandin dehydrogenase activity do not appear to have a significant effect upon overall prostaglandin synthesis. © 1999 W. B. Saunders Company Ltd
Placenta (1999), 20, 181–184

INTRODUCTION

Prostaglandins play a central role in human parturition. They mediate cervical ripening and directly stimulate uterine contractions (Crankshaw and Dyal, 1994). Prostaglandins also indirectly increase fundally dominant myometrial contractility by upregulation of oxytocin receptors and synchronization of contractions (Liggins, 1989; Garfield, Tabb and Thilander, 1990). A major source of prostaglandin synthesis at the time of labour is the fetal membranes. Numerous studies have shown that both primary cell cultures (Olsen, Skinner and Challis, 1983; Skinner and Challis, 1985; Bennett et al., 1987, 1993) and intact fetal membranes (Brennand et al., 1995; Kredentser, Embree and McCoshen, 1995) show increased prostaglandin synthesis when collected after labour. The fetal membranes synthesize both PGE2 and PGF2a. PGE2 is the predominant product of amnion (Skinner and Challis, 1985). The decidua produces both PGE2 and PGF2α (Rajasingam et al., 1998).

The chorion is a major source of prostaglandin dehydrogenase (PGDH), which catalyses the conversion of prostaglandins to inactive metabolites (Cheung and Challis, 1989; Sangha et al., 1994). Van Meir et al. (1996) found specific regional changes in prostaglandin dehydrogenase activity in fetal membrane samples collected at term. In samples collected at elective caesarean section before labour PDGH immunoreactivity and enzyme activity were highest in the cervical region when compared to the peri-placental region or a region midway between the two. After labour PDGH immunoreactivity and enzyme activity was lowest in the cervical region. This suggests that a reduction in fetal membrane PGDH activity near to the cervix at the time of labour allows the release of active prostaglandins to mediate cervical ripening.

Region dependent changes in fetal membrane PDGH activity suggest that there may also be regional changes in prostaglandin synthesis. Intact fetal membrane tissue explants are frequently used as a model to study fetal membrane biochemistry without reference to the site of origin of the sample. A knowledge of whether there are regional differences in prostaglandin synthesis may be important both in understanding the control of prostaglandin synthesis in fetal membranes and in interpretation of experiments using the explant tissue culture model. We have therefore undertaken a series of experiments to examine the effect of membrane region and the clinical onset of labour upon prostaglandin E2 release by intact fetal membrane explants.

MATERIAL AND METHODS

Tissue samples

Fetal membranes were collected with informed consent and ethics committee approval at elective caesarean section prior to labour in cases of breech presentation or previous caesarean section ($n=5$) and following the spontaneous onset of labour at term and normal vaginal delivery ($n=5$).

[a]To whom correspondence should be addressed.

0143–4004/99/020181+04 $12.00/0

© 1999 W. B. Saunders Company Ltd

Fetal membranes were washed in PBS and 2-cm discs were cut from each of three regions; cervical ($n=12$), periplacental ($n=12$) and midway between the two ($n=12$). In the case of spontaneous vaginal delivery the cervical region was defined as that near to the rupture site. In the case of caesarean section the precise position of the membranes within the uterus was noted and the cervical region marked. The cut discs were placed in individual tissue culture plate wells, suspended in 1.5 ml of serum-free Medium 199 with insulin transferin and selenium (ITS) and incubated for 1, 2, 4, 6, 12 or 24 h. At the end of the incubation period the medium was stored at −20°C until prostaglandin E2 concentration was measured by enzyme-immunoassay (Amersham Life Science, Amersham Pharmacia Biotech, Amersham, Bucks, UK). To determine whether prostaglandin synthesis in fetal membranes was limited by substrate availability membrane discs from the mid-position ($n=5$ pre-labour, 5 post-labour) were incubated with arachidonic acid (30 μM) for 15 min. The medium was then aspirated and prostaglandin E2 concentration measured.

Statistical analysis

Data were analysed in groups, respectively estimating the mean and standard error before comparison was made using analysis of variance. Differences between groups were assessed using Fisher's PLSD test.

RESULTS

When prostaglandin E2 concentration was analysed independently of region there was a significant effect of labour upon prostaglandin concentration. Individual time point analysis showed that there was a general decrease in prostaglandin E2 in the medium between 2 and 24 h of culture, but this reached statistical significance only at 4–6 h of culture (Figure 1). There were no significant changes in the ouput of PGE2 at different time points in pre-labour tissues, although levels were highest after 2 h and declined slowly thereafter. In discs collected after labour, there again were no significant differences, although levels were lower from 4 to 6 h (Figure 1).

The effect of region (periplacental, mid-membrane or rupture site) was analysed separately for discs collected pre-labour and post-labour. Overall there was no effect of region upon prostaglandin concentration and post-hoc analysis of individual time points showed no significant differences (Figures 2 and 3).

Incubation of fetal membrane discs collected prior to labour or post-labour with exogenous arachidonic acid reversed the effect of labour upon prostaglandin E2 concentrations. In the presence of exogenous substrate prostaglandin E2, concentrations were significantly higher where discs were collected post-labour (Figure 4).

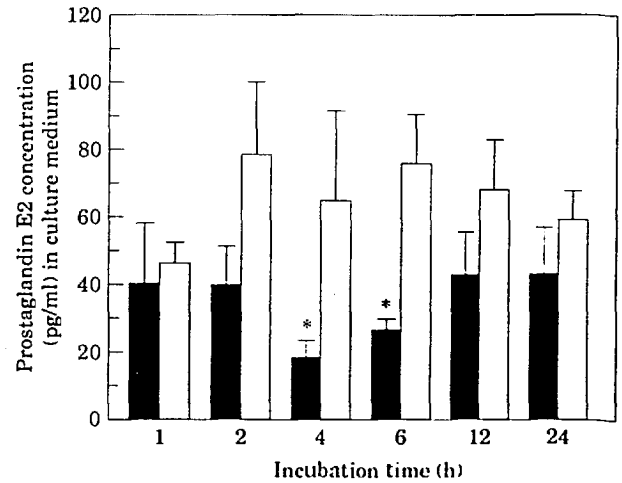


Figure 1. Overall PG synthesis before (□) and after (■) labour independent of in which region in the membranes samples were taken. Mean ± SEM, $n=5$. *Significantly different from pre-labour, $P<0.05$.

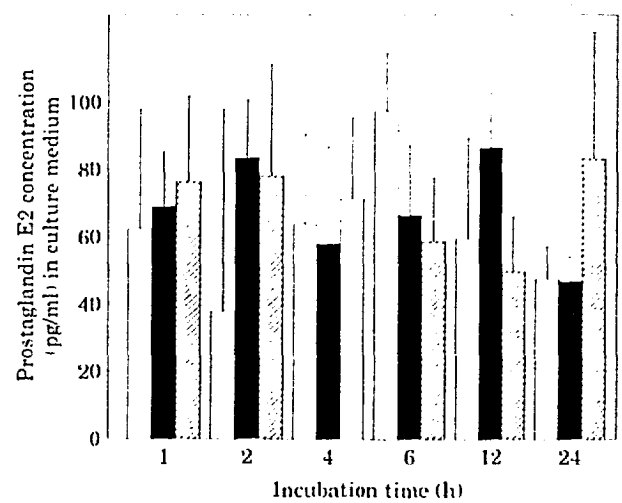


Figure 2. PG synthesis before labour in each of the three regions of the fetal membranes: □, mid; ■, placenta; ▨, rupture. Mean ± SEM, $n=5$ different patients.

DISCUSSION

The concentration of prostaglandin E2 in the culture medium of fetal membrane explants is determined by the balance between prostaglandin release and prostaglandin metabolism. Prostaglandin E2 synthesis is principally in the amnion, but also in the chorion-decidua (Skinner and Challis, 1985) whilst prostaglandin metabolism is in the chorion (Cheung and Challis, 1989). Synthesis of prostaglandin E2 requires an adequate supply of substrate arachidonic acid. Where there is no exogenous supply of arachidonic acid endogenous substrate is de-esterified from storage within membrane lipids by the

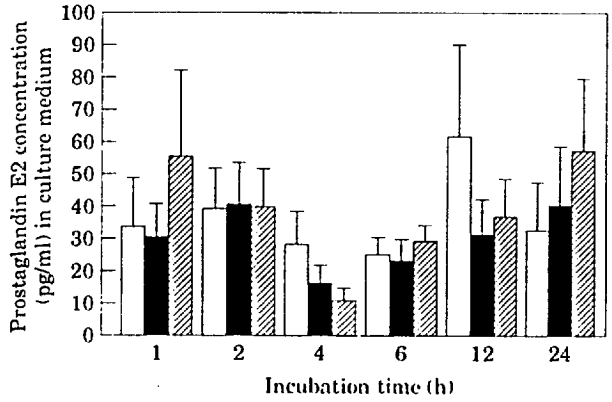


Figure 3. PG synthesis after labour in each of the three regions of the fetal membranes: □, mid; ■, placenta; ▨, rupture. Mean ± SEM, $n=5$ different patients.

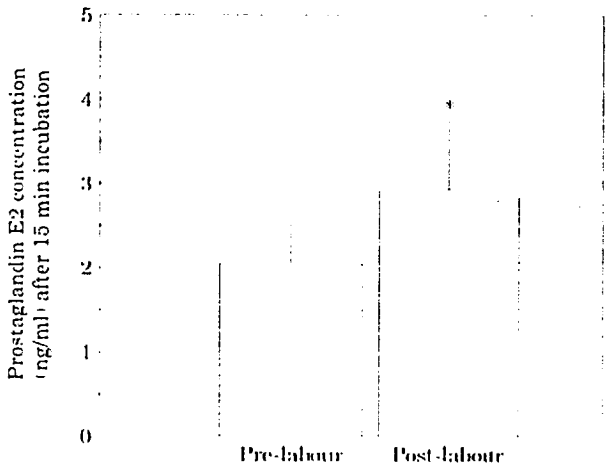


Figure 4. COX assay by production of prostaglandin E2 by intact fetal membranes from exogenous arachidonic acid. Mean + SEM, $n=5$ patients per group; *significantly different to pre labour, $P<0.05$.

action of phospholipase A2. De-esterified arachidonic acid is then metabolized to the intermediate prostaglandin endoperoxides by cyclo-oxygenase and then to prostaglandin E2 via a synthase. The predominant cyclo-oxygenase active in fetal membranes at term is the inducible, type-2 cyclo-oxygenase (Slater et al., 1994).

Studies of the production of prostaglandins by fetal membrane explants have used incubation times ranging between 1 and 24 h. Our data show that there is no significant effect of time upon prostaglandin concentration in the culture medium where discs are collected before labour. This suggests a balance between prostaglandin synthesis and metabolism to prevent an accumulation of prostaglandins with time. Cheung and Challis (1989) found that approximately 70–100 per cent of exogenously added prostaglandin E2 was metabolized by isolated chorion cells in 4 h. Rajasingam et al. (1998) have previously reported that there is little increase in prostaglandin E2 concentrations in the medium where intact fetal membranes are cultured for more than 2 h, but that there is an increase in prostaglandin metabolite concentrations. This suggests that, after the first 2 h of culture, prostaglandin synthesis is matched by prostaglandin dehydrogenase activity. Where fetal membrane discs were collected post-labour we found a limited decrease in prostaglandin E2 concentrations between 4 and 6 h. This may represent either a peak of prostaglandin dehydrogenase activity or a nidus of prostaglandin synthesis.

Unlike many previous studies which have shown an increase in prostaglandin synthesis in fetal membranes collected following labour we found a significant decrease. This is probably because most previous studies have incubated cells or tissue in medium containing serum, which represents a supply of exogenous arachidonic acid. Our experiments were performed in medium lacking both serum and arachidonic acid. Endogenous arachidonic acid concentrations are known to fall in fetal membranes during labour (Curbello et al., 1984) and phospholipase A2 activity also decreases (Skannal et al., 1997). It is likely that the reduction in prostaglandin E2 synthesis which we have seen following labour is because of depletion of substrate arachidonic acid. This is supported by our findings that, when supplied with exogenous substrate, the capacity of fetal membranes to synthesize prostaglandin is increased following labour.

In addition our unit policy has changed over the past 5 years such that elective caesarean section is most commonly performed close to 39 completed weeks of pregnancy, whereas previously caesarean section was more commonly performed close to 37 weeks. We have found that activation of the membranes, with an associated increase in prostaglandin synthesis, occurs some days prior to the onset of labour (Brown et al., 1998). It is possible that if prostaglandin synthesis between membranes collected at 37 weeks and post-labour is compared there will appear to be a labour-associated increase, but when comparison is made with membranes collected at 39 weeks the difference is eliminated or reversed.

The data obtained by van Meir et al. (1996) were on tissues which were all delivered by caesarean section, before or after the onset of labour. Our post-labour samples were delivered vaginally, which could also affect the data obtained. Passage through the birth canal could contribute to the data shown, but there may be an alternative explanation. The chorion has a capacity to metabolize PGE2 at concentrations which greatly exceed those found physiologically (Roseblade et al., 1990; Khan et al., 1991). This may partially explain how a major loss of prostaglandin-metabolizing enzymes associated with labour could have relatively little effect on the levels of PGE2 present—the remaining enzymes retain the ability to metabolize most of the primary prostaglandin.

We have shown that, although there are marked differences in fetal membrane prostaglandin synthesis in association with labour, there are no differences in prostaglandin synthesis between regions of the same membrane. Regional changes in prostaglandin dehydrogenase activity do not appear to have a significant effect upon overall prostaglandin synthesis.

ACKNOWLEDGEMENT

We thank Wellbeing, Regent's Park, London, UK, for funding this study.

REFERENCES

Bennett PR, Rose M, Myatt L & Elder MG (1987) Preterm labor; stimulation of arachidonic acid metabolism in human amnion cells by bacterial products. *Am J Obstet Gynecol*, 156, 649–655.

Bennett PR, Slater D, Elder MG & Moore GE (1993) Changes in arachidonic acid metabolism in amnion cells associated with increased cyclo-oxygenase gene expression at parturition. *Br J Obstet Gynaecol*, 100, 1037–1042.

Brennand JE, Leask R, Kelly RW, Greer IA, Calder AA (1995) Changes in prostaglandin synthesis and metabolism associated with labour, and the influence of dexamethasone, RU 486 and progesterone. *Eur J Endocrinol*, 133, 527–533.

Brown NL, Alvi S, Elder MG, Bennett PR & Sullivan MHF (1998) A spontaneous induction of fetal membrane prostaglandin production precedes clinical labour. *J Endocrinol*, 157, R1–R6.

Crankshaw-DJ & Dyal R (1994) Effects of some naturally occurring prostanoids and some cyclo-oxygenase inhibitors on the contractility of the human lower uterine segment in vitro. *Can J Physiol Pharmacol*, 728, 870–874.

Cheung PY & Challis JRG (1989) Prostaglandin E2 metabolism in the human fetal membranes. *Am J Obstet Gynecol*, 161, 1580–1585.

Curbelo V, Bejar R, Benirschke K & Gluck L (1981) Premature labor. I. Prostaglandin precursors in human placental membranes. *Obstet Gynecol*, 57, 473–478.

Garfield RE, Tabb T & Thilander G (1990) Intercellular coupling and modulation of uterine contractility. In *Uterine Contractility* (Ed.) Garfield RE, pp. 21–40. Norwell, MA, USA: Serono Symposia.

Khan II, Sullivan MHF, Roseblade CK, Helmig R, Uldbjerg N & Elder MG (1991) Quantitative production of PGE2 and PGE2 metabolites by human fetal membranes. *Br J Obstet Gynaecol*, 98, 712–715.

Kredentser JV, Embree JE & McCoshen JA (1995) Prostaglandin F2 alpha output by amnion-chorion-decidua: relationship with labor and prostaglandin E2 concentration at the amniotic surface. *Am J Obstet Gynecol*, 173, 199–204.

Liggins GC (1989) Initiation of labour. *Biol Neonate*, 55, 366–375.

Olson DM, Skinner K & Challis JRG (1983) Prostaglandin output in relation to parturition by cells dispersed from human intrauterine tissues. *J Clin Endocrinol Metab*, 57, 694–698.

Rajasingam D, Elder MG, Slater D, Bennett PR & Sullivan MHF (1995) Stimulation of prostaglandin in human fetal membranes by bacteria and bacterial products. *Placenta*, 19, 301–306.

Roseblade CK, Sullivan MHF, Khan H, Lumb MR & Elder MG (1990) Limited transfer of prostaglandin E2 across the fetal membranes before and after labour. *Acta Obstet Gynecol Scand*, 69, 399–403.

Sangha RK, Walton JC, Ensor CM, Tai HH & Challis JRG (1994) Immunohistochemical localization, mRNA abundance and activity of 15-hydroxyprostaglandin dehydrogenase in placenta and fetal membranes during term and preterm labour. *J Clin Endocrinol Metab*, 78, 982–989.

Skannal DG, Brockman DE, Eis AL, Xue S, Siddiqi TA & Myatt L (1997) Changes in activity of cytosolic phospholipase A2 in human amnion at parturition. *Am J Obstet Gynecol*, 177, 179–184.

Skinner KA & Challis JRG (1985) Changes in synthesis and metabolism of prostaglandins by human fetal membranes and decidua at labor. *Am J Obstet Gynecol*, 151, 159–162.

Slater DM, Berger L, Newton R, Moore GE & Bennett PR (1994) The relative abundance of type 1 to type 2 cyclo-oxygenase mRNA in human amnion at term. *Biochem Biophys Res Commun*, 198, 304–308.

van Meir CA, Sangha RK, Walton JC, Mathews SG, Keirse MJNC & Challis JRG (1996) Immunoreactive 15-hydroxyprostaglandin dehydrogenase (PGDH) is reduced in fetal membranes from patients at preterm delivery in the presence of infection. *Placenta*, 17, 291–297.

7.1.7

The Roles of the Cyclo-oxygenases Types One and Two in Prostaglandin Synthesis in Human Fetal Membranes at Term

R. J. Sawdy, D. M. Slater, W. J. B. Dennes, M. H. F. Sullivan and P. R. Bennett

Placenta (2000), **21**, 54-57.

Author's Contribution:

Contributed to experimental design and methodology, carried out Western analyses of COX protein in amnion and chorion-decidua and contributed to both the writing and submission of the manuscript.

Placenta (2000), 21, 54–57
Article No. plac.1999.0438

The Roles of the Cyclo-oxygenases Types One and Two in Prostaglandin Synthesis in Human Fetal Membranes at Term

R. J. Sawdy, D. M. Slater, W. J. B. Dennes, M. H. F. Sullivan and P. R. Bennett[a]

Parturition Research Group, Imperial College School of Medicine, Institute of Obstetrics and Gynaecology, Queen Charlottes and Chelsea Hospital, Goldhawk Road, London W6 0XG, UK

Paper accepted 23 June 1999

The aim of this study was to determine the relative contributions of cyclo-oxygenase (COX) types 1 and 2 to prostaglandin synthesis at term.

Methods: Fetal membranes were collected from 6 pregnancies after elective caesarean section at term, prior to labour. The presence of COX-1 and COX-2 protein was determined using Western analysis. The relative contributions of the two isoforms of COX to prostaglandin synthesis were determined by incubation of fetal membrane discs with either a COX-2 selective inhibitor, SC236, or a COX-1 selective inhibitor, SC560, and measurement of prostaglandin release during 24 h using enzyme-linked immuno-sorbent assay (ELISA).

Results: Both COX-1 and COX-2 protein were demonstrated in amnion and chorion-decidua. The COX-2 selective inhibitor, SC-236, significantly reduced prostaglandin synthesis, both in its COX-2 specific and higher, non-specific concentration ranges. The COX-1 selective inhibitor, SC-560, had no effect upon prostaglandin synthesis in its COX-1 specific concentration range, but did significantly reduce prostaglandin synthesis at higher, non-selective concentrations.

Conclusions: Fetal membranes contain both COX-1 and COX-2 at term, but only COX-2 contributes towards prostaglandin synthesis. COX-2 selective NSAI drugs will be as effective as non-selective agents in inhibition of fetal membrane prostaglandin synthesis and may represent a new strategy for tocolysis.
© 2000 Harcourt Publishers Ltd
Placenta (2000), 21, 54–57

INTRODUCTION

Human labour, both term and preterm, is associated with increased prostaglandin synthesis within the uterus (Turnbull, 1977). The principle sites of prostaglandin synthesis are the fetal membranes, in particular the amnion and the decidua (Skinner and Challis, 1985). It is currently believed that during most of pregnancy prostaglandin synthesis in the amnion is low, and that the decidua and myometrium are protected by the presence of prostaglandin dehydrogenase, principally within the chorion. At term, prostaglandin synthesis increases whilst prostaglandin dehydrogenase synthesis decreases. This process may begin near the cervix before extending throughout the uterus (Nakla et al., 1986; Cheung and Challis, 1989; Van Meir et al., 1996). Prostaglandins act both to mediate cervical ripening and to stimulate uterine contractions (Crankshaw and Dyal, 1994). Prostaglandins may also act indirectly to increase fundally dominant myometrial contractility by upregulation of oxytocin receptors and synchronization of contractions (Liggins, 1989; Garfield and Hertzberg, 1990). Prostaglandin synthesis in fetal membranes is maximally upregulated prior to the onset of labour (Brown et al., 1998; Sawdy et al., 1999). Non-steroidal-anti-inflammatory (NSAI) drugs such as indomethacin and sulindac, which act through inhibition of prostaglandin synthesis, have been shown to be highly effective in prevention of preterm delivery but their use is limited by adverse fetal and neonatal effects.

Prostaglandins are formed from the precursor arachidonic acid via the action of the cyclo-oxygenase (COX) enzyme. This enzyme, which is the target for non-steroidal anti-inflammatory drugs, is known to exist in two forms. COX-1 is constitutively expressed in tissues, such as gastric mucosa, which synthesize prostaglandins constantly. COX-2 is inducible and is expressed at sites of inflammation (Hla et al., 1986; Hla and Neilson, 1992). We have shown that, at the mRNA level, COX-2 expression in amnion and chorion-decidua exceeds that of COX-1 and that COX-2 but not COX-1 mRNA concentrations increase near to term and with the onset of labour (Slater et al., 1995; Slater et al., 1999). Since fetal synthesis of prostaglandins in, for example, the kidney or vascular system is constitutive, this is likely to be via COX-1. This suggests that NSAI drugs which are selective for the COX-2 enzyme may have a role in prevention of preterm delivery with fewer fetal side effects than are seen

[a] To whom correspondence should be addressed. Fax: +44 181 383 1838.

0143–4004/00/010054+04 $35.00/0

© 2000 Harcourt Publishers Ltd

with non-selective COX inhibitors such as indomethacin (Sawdy et al., 1997).

Cyclo-oxygenase is a 'suicide enzyme' which undergoes destruction during catalysis (Marshall, Kulmacz and Lands, 1979). Therefore, in general, an increase in prostaglandin synthesis implies an increase in synthesis of the COX enzyme. Where prostaglandins are not being actively synthesized however, COX may be stable. An example of this is in the circulating platelet which contains COX, but has no nucleus and cannot synthesize new COX. Platelet activation leads to synthesis of prostaglandins via stored COX, but once that COX has been inactivated the platelet is no longer functional. Although COX-2 mRNA concentrations in fetal membranes greatly exceed those of COX-1, it remains possible that there is stored COX-1 protein which contributes to the increased prostaglandin synthesis associated with labour.

We have previously found that the time of maximal prostaglandin synthesis in intact fetal membranes collected after labour is reduced when compared to prior to labour (Sawdy et al., 1999). Fetal membranes collected prior to labour show two alternate patterns of prostaglandin synthesis. Prostaglandin synthesis is either high, and cannot be further stimulated by IL1β, or is lower and can be increased by IL1β (Brown et al., 1998). We believe that this represents the difference between tissues collected from women close too, or some time away from, the natural onset of labour.

In this study we have used Western analysis to show the presence of COX-1 and COX-2 proteins within humna fetal membranes. The contribution that each of the two COX isoforms makes to prostaglandin synthesis at term has been studied using inhibitors which are selective for either COX-1 or COX-2. SC236 is a Searle compound whose IC 50 (concentration at which enzyme activity is reduced to 50 per cent) for COX-1 and COX-2 is approximately 10 μM and 10 nM SC560 is a Searle compound whose IC 50 for COX-1 and COX-2 is approximately 1 nM and 100 nM. Fetal membranes were selected to have high basal prostaglandin synthesis that could not be increased by IL1β, therefore representing the maximum degree of endogenous prostaglandin synthesis.

MATERIAL AND METHODS

Tissue samples

Fetal membranes were collected, with consent and ethics committee approval, at elective caesarean section, after 38 weeks, prior to labour in cases of breech presentation or previous caesarean section. The membranes were divided into amnion and chorion decidua for Western analysis of COX-1 and COX-2 protein expression. Alternatively membranes were retained intact to study prostaglandin synthesis in the presence of COX-1 and COX-2 selective inhibitors.

Western analysis

Protein extracts were prepared from fetal membrane samples by homogenization in 10 vol. T-Wash (50 mM Tris-Buffer, 10 mM EDTA, 1 per cent Triton X-100, with 10 mM PMSF, 4 μg/ml Pepstatin and 0.5 μg/ml Leupeptin) for 30 sec. Proteins were separated on a 10 per cent SDS-PAGE gel. Immunodetection was carried out following electrophoretic transfer on to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Life Science, Little Chalfont, UK). Membranes were blocked in 5 per cent Marvel–0.1 per cent TWEEN-PBS and incubated with either COX-1 or COX-2 specific antibodies (COX-1 No. sc-1752, COX-2 No. sc-1745: Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1 : 500. Membranes were washed in 0.1 per cent TWEEN-PBS and incubated with anti-goat IgG-HRP secondary antibody at a dilution of 1 : 2000. ECL Western blotting detection was carried out using standard protocols (Amersham). Antibody specificity was confirmed using positive controls. Human platelets were used as a positive control for COX-1 and interleukin 1β stimulated human lymphocytes for COX-2.

Fetal membrane prostaglandin synthesis studies

To assess the relative contribution of COX-1 and COX-2 to prostaglandin synthesis fetal membranes were washed in PBS and 2 cm discs were cut and placed in individual tissue culture plate wells. Membrane discs were suspended in 1.5 ml of serum free Medium 199 with insulin transferin and selenium (ITS, Sigma Aldrich, Dorset UK) and incubated for 24 h in the presence of the COX-1 selective inhibitor SC560 (0.01, 0.1, 1 or 10 μM) or the COX-2 selective inhibitor SC236 (0.001, 0.01, 0.1 or 10 μM). Control incubations were performed without inhibitor with or without interleukin 1β (1 ng/ml). At the end of the incubation period the medium was stored at −20°C until prostaglandin E2 concentration was measured by enzyme-immuno-assay (Amersham).

RESULTS

Western analysis showed the presence of both COX-1 and COX-2 protein in the relevant positive controls and in both amnion and chorion-decidua. The COX-1 protein content in amnion and chorion-decidua was similar as was the COX-2 protein content. There appeared to be similar concentrations of COX-1 and COX-2 proteins in each of the tissues (Figure 1).

To ensure that only fetal membranes with maximum potential prostaglandin synthesis were studied only those in which interleukin 1β did not stimulate any increase in basal prostaglandin synthesis were included in analysis. In those membranes studied, therefore, there were no differences in prostaglandin synthesis between control or control with IL1β. SC-236 significantly reduced prostaglandin synthesis at concentrations of 0.1 and 1 μM, which are within its COX-2 specific range, and at 10 μM at which concentration it becomes non-selective. SC-560 had no effect upon prostaglandin synthesis at concentrations of 0.001, 0.01 or 0.1 μM, which are within its COX-1

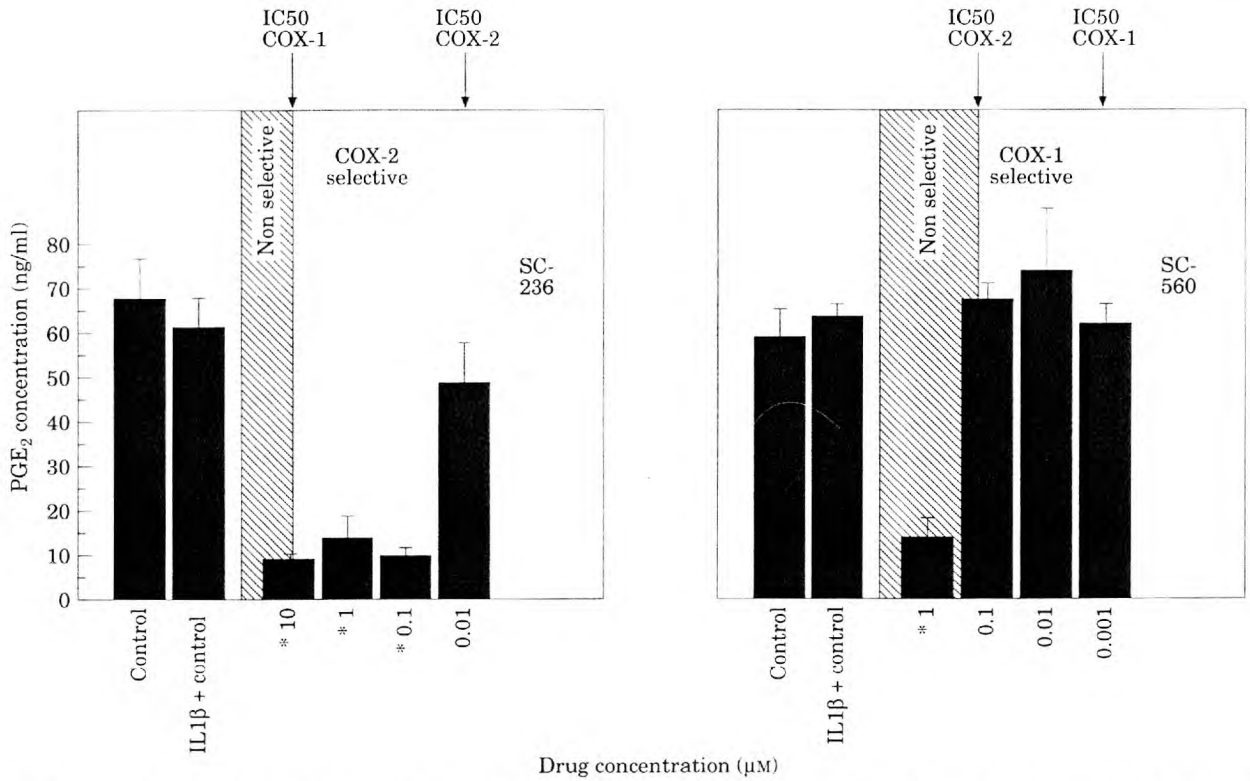


Figure 1. Production of prostaglandin E2 by intact fetal membranes in tissue culture. Membranes were selected on the basis of high prostaglandin production which could not be further stimulated by IL1β. Membranes cultured with either SC236 or SC560 were not simultaneously exposed to IL1β. IC50 indicates the concentration at which 50 per cent enzyme inhibition occurs ($n=6$ per group, Mean ± SE, * indicates significantly different to control ($P<0.05$ Student's t-test).

specific range, but did significantly reduce prostaglandin synthesis at 1 μM at which concentration it becomes non-selective. The combination of SC560, at any of its COX-1 specific concentrations, with SC236 at any of its COX-2 specific concentrations did not reduce prostaglandin synthesis any greater than SC236 at any COX-2 selective concentration alone (Figure 2).

DISCUSSION

The principle aim of this study was to determine what COX-1 contributes to the synthesis of prostaglandins at term. We have previously shown that, at term, amnion COX-2 expression at the mRNA level, exceeds that of COX-1 by 100 fold. In the chorion-decidua COX-2 mRNA expression is eight fold higher than that of COX-1 labour (Slater et al., 1995; Slater et al., 1999). In this study Western analysis showed that there was both COX-1 and COX-2 protein present within the fetal membranes. Although it is not possible to make accurate comparisons between the protein concentrations for COX-1 and COX-2, because of possible differences in the affinity of the two antibodies for their epitopes, the protein concentrations do not appear to reflect the mRNA concentrations. Since SC236 inhibited prostaglandin synthesis in fetal membranes at both COX-2 selective and non-selective concentrations, but SC560 was only inhibitory at concentrations

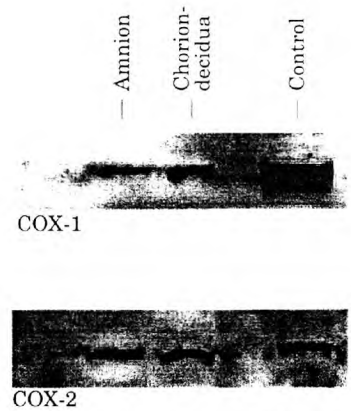


Figure 2. Expression of COX-1 and COX-2 protein in amnion and chorion-decidua shown by Western analysis. The control for COX-1 was human platelets. The control for COX-2 was interleukin 1β stimulated human lymphocytes.

above its COX-1 selective range, it appears that the COX-1 protein present within the fetal membranes plays virtually no role in prostaglandin synthesis at term.

This suggests that substrate arachidonic acid is not available for metabolism by COX-1. There must therefore be compartmentalization within the cells of the fetal membranes which ensures that arachidonic acid liberated by the action of phospholipase can only be converted to prostaglandin endoperoxides by COX-2. This has been found in other cell

types. For example Reddy and Herschman (1996) have shown that in fibroblasts and macrophages which contain both COX-1 and COX-2, only COX-2 is involved in mitogen stimulated prostaglandin synthesis from endogenous substrate sources, whereas exogenously supplied arachidonic acid can be metabolized by COX-1.

The apparent discrepancy between mRNA and protein concentrations for COX-1 and COX-2 can also be explained in terms of the known functions of the enzymes. COX is a suicide enzyme which undergoes destruction during catalysis (Marshall, Kulmacz and Lands, 1979). Increased prostaglandin synthesis via COX-2 would therefore mean increased turnover of the enzyme itself. This would be reflected by a large increase in mRNA concentrations, to drive protein synthesis, but, because the protein itself is also being actively destroyed, the COX-2 protein concentration measured at any one time would be increased to a much lesser extent. If the enzyme is not active, as is the case for COX-1 in the fetal membranes, the protein may be present at concentrations similar to that of COX-2 but mRNA concentrations would be very low.

We have previously suggested that non-steroidal anti-inflammatory drugs which are selective for COX-2 may have the tocolytic efficacy of indomethacin (Keirse, 1995) without the fetal side effects (Sawdy et al., 1997). This data shows that, although fetal membranes contain COX-1, it plays no significant role in prostaglandin synthesis. The fetal membranes are the principle site of prostaglandin synthesis within the uterus and therefore COX-2 selective NSAI drugs should be as effective as non-selective agents in inhibition of intrauterine prostaglandin synthesis and prevention of preterm contractions.

ACKNOWLEDGEMENTS

We thank Searle for supplying us with the COX-1 and COX-2 selective compounds SC236 and SC560 and Wellbeing for grant funding.

REFERENCES

Brown NL, Alvi SA, Elder MG, Bennett PR & Sullivan MH (1998) A spontaneous induction of fetal membrane prostaglandin production precedes clinical labour. *J Endocrinol*, 157, R1–R6.

Cheung PY & Challis JR (1989) Prostaglandin E2 metabolism in the human fetal membranes. *Am J Obstet Gynecol*, 161, 1580–1585.

Crankshaw DJ & Dyal R (1994) Effects of some naturally occurring prostanoids and some cyclooxygenase inhibitors on the contractility of the human lower uterine segment in vitro. *Can J Physiol Pharmacol*, 72, 870–874.

Garfield RE & Hertzberg EL (1990) Cell-to-cell coupling in the myometrium: Emil Bozler's prediction. *Prog Clin Biol Res*, 327, 673–681.

Hla T, Farrell M, Kumar A & Bailey JM (1986) Isolation of the cDNA for human prostaglandin H synthase. *Prostaglandins*, 32, 829–845.

Hla T & Neilson K (1992) Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci USA*, 89, 7384–7388.

Keirse M (1995) Indomethacin tocolysis in preterm labour. In *Pregnancy and Childbirth: Cochrane Database of Systematic Reviews*, p. 4383. Oxford: Cochrane Collaboration.

Liggins G (1989) Initiation of labour. *Biol Neonate*, 55, 366–375.

Marshall P, Kulmacz R & Lands W (1979) Constrants on prostaglandin synthesis in tissues. *J Biol Chem*, 262, 3510–3517.

Nakla S, Skinner K, Mitchell BF & Challis JR (1986) Changes in prostaglandin transfer across human fetal membranes obtained after spontaneous labor. *Am J Obstet Gynecol*, 155, 1337–1341.

Reddy ST & Herschman HR (1996) Transcellular prostaglandin production following mast cell activation is mediated by proximal secretory phospholipase A2 and distal prostaglandin synthase 1. *J Biol Chem*, 271, 186–191.

Sawdy RJ, Dennes WJB, Allport V, Slater DM, Elder MG, Sullivan MH & Bennett PR (1999) Region and labour dependent synthesis of prostaglandin E2 by human fetal membranes. *Placenta*, 20, 181–184.

Sawdy R, Slater DM, Fisk N, Edmonds DK & Bennett PR (1997) Use of a cyclo-oxygenase type-2-selective non-steroidal anti-inflammatory agent to prevent preterm delivery [letter]. *Lancet*, 350, 265–266.

Skinner KA & Challis JR (1985) Changes in the synthesis and metabolism of prostaglandins by human fetal membranes and decidua at labor. *Am J Obstet Gynecol*, 151, 519–523.

Slater DM, Dennes WJB, Sawdy R, Allport VL & Bennett PR (1999) Expression of cyclo-oxygenase types-1 and -2 in human fetal membranes throughout pregnancy. *J Mol Endocrinol*, 22, 125–130.

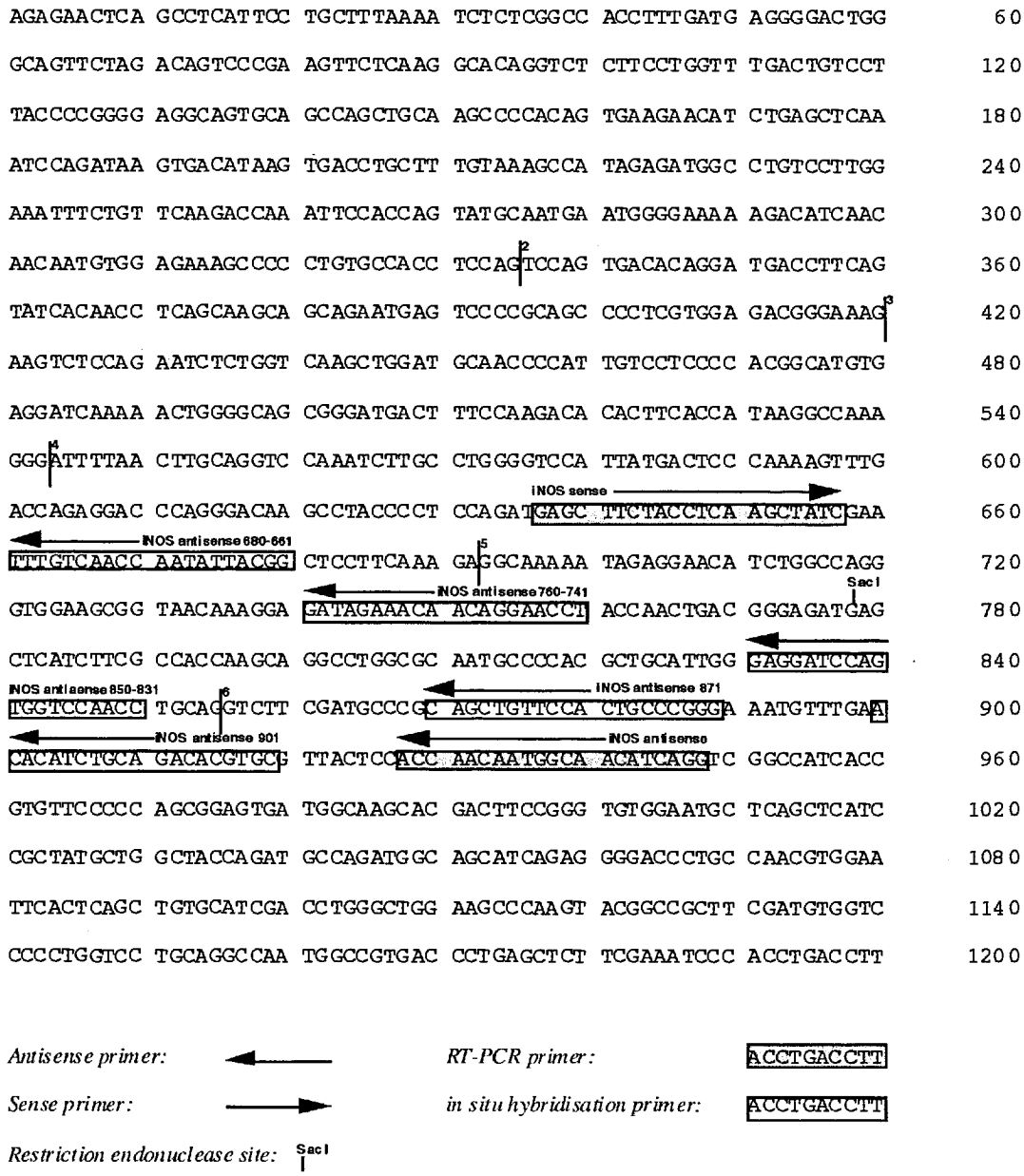
Slater DM, Berger LC, Newton R, Moore GE & Bennett PR (1995) Expression of cyclooxygenase types 1 and 2 in human fetal membranes at term. *Am J Obstet Gynecol*, 172, 77–82.

Turnbull A (1997) *The fetus and birth*. London: Elsevier.

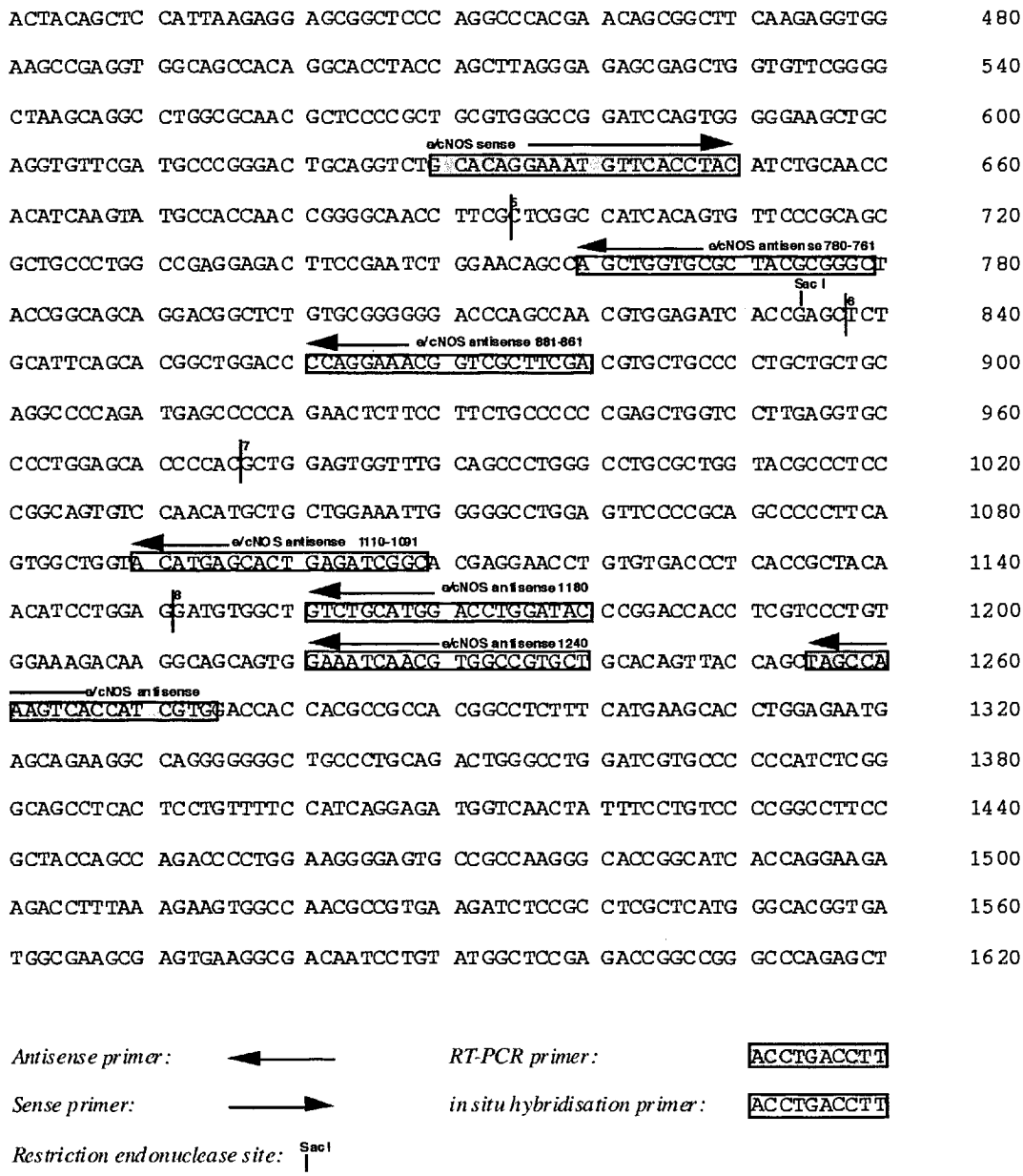
Van Meir CA, Sangha RK, Walton JC, Matthews SG, Keirse MJ & Challis JR (1996) Immunoreactive 15-hydroxyprostaglandin dehydrogenase (PGDH) is reduced in fetal membranes from patients at preterm delivery in the presence of infection. *Placenta*, 17, 291–297.

7.2 Gene Sequences

7.2.1 Arrangement of the iNOS gene



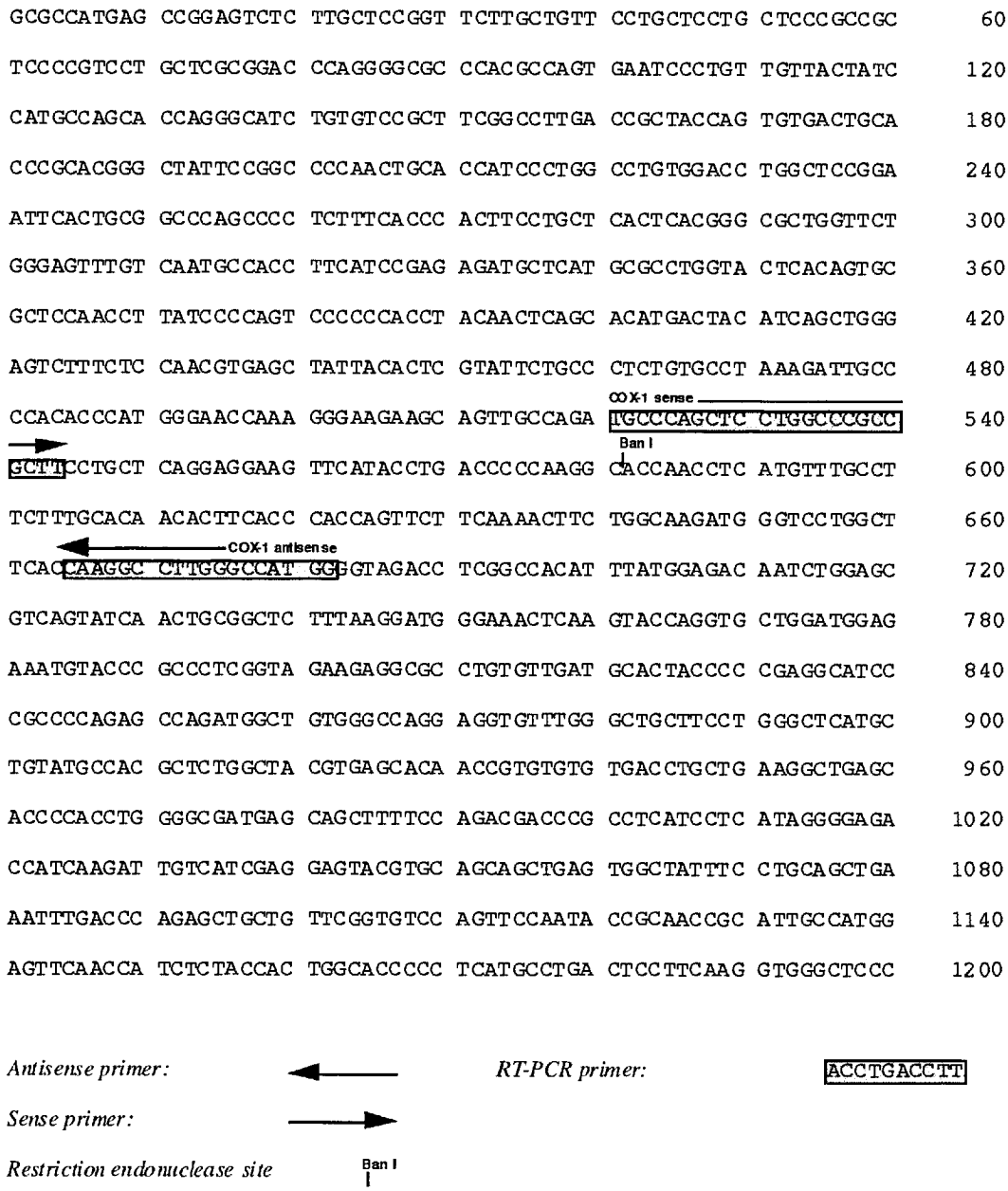
7.2.2 Arrangement of the e/cNOS gene



7.2.3 Arrangement of the nNOS gene



7.2.4 Arrangement of the COX-1 gene



7.2.4 Arrangement of the COX-2 gene

