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The role of hypoxia-inducible factor-1 alpha in xenon preconditioning versus hypoxic-ischaemic organ injury

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ABSTRACT

Background: The anaesthetic gas xenon provides long-lasting preservation of neuronal function when given several hours prior to neuronal injury: this phenomenon is described as preconditioning. However, little is known of the mechanisms by which xenon preconditioning mediates its protective effect. Interest has focused on the involvement of hypoxia-inducible factor-1α (HIF-1α) in preconditioning because:

(i) Hypoxia activates HIF-1α and is an effective preconditioning stimulus;

(ii) HIF-1α is a regulator of adaptive responses promoting cellular survival;

(iii) Several genes that have hypoxia-responsive elements (HRE) for HIF-1 in the promoter region are capable of mediating preconditioning (e.g., erythropoietin).

Therefore, this study speculates on the role of HIF-1α in xenon preconditioning.

Method: Separate cohorts of male adult C57 / BL6 mice were preconditioned by exposure to 75 % xenon / 25 % oxygen for 2 hours and thereafter immediately sacrificed for organ harvesting at 0 - 24 hours after xenon preconditioning. Semi-quantitative study of HIF-1α and EPO protein expression was performed by Western blot analysis, and RT-PCR was used as a measure of gene transcription.

Results: Xenon preconditioning provokes a time-dependent increase in HIF-1α protein in brain as well as kidney. Xenon preconditioning also caused a time-dependent increase in EPO (a HIF-1 target gene) transcription and protein expression in a corresponding time course to xenon-induced HIF-1α. To elucidate the mechanisms of xenon-induced HIF-1α accumulation, the effect of xenon preconditioning on HIF-1α transcription, translation and degradation was studied. Xenon preconditioning does not induce a change in HIF-1α mRNA expression, nor

does it significantly attenuate the expression of the PHD2 enzyme, required for HIF-1α degradation. To explore translation-dependent pathways, mice were treated with rapamycin before xenon preconditioning, to inhibit translation of HIF-1α through the mTOR pathway. Inhibition of this pathway prevented the xenon-induced increase of HIF-1α protein. To ascertain whether HIF-1α is required for xenon preconditioning, siRNA was used to knockdown HIF-1α expression in the kidney and xenon's renoprotective properties were shown to be abolished.

Conclusions: Over the same time course as xenon's protection against subsequent injury in both brain and kidney, xenon preconditioning induces expression of HIF-1α. Increased HIF-1α expression is also associated with increased activity as evidenced by enhanced transcription and translation of the downstream effector, EPO. Xenon preconditioning does not regulate HIF-1α at the transcriptional level, nor does it inhibit HIF-1α degradation. However, these results suggest that xenon preconditioning upregulates expression of HIF-1α through translation-dependent mechanisms. Furthermore, xenon's action on HIF-1α is shown to be causally related to its organ protective effect. If these data can be extrapolated to the clinical setting, exposure to xenon would be beneficial prior to procedures in which organ perfusion is interrupted, preventing hypoxic-ischaemic as well as ischaemic-reperfusion injury.

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TABLE OF CONTENTS

ABSTRACT 3
ACKNOWLEDGEMENTS 5

TABLE OF CONTENTS 6
LIST OF FIGURES 10
LIST OF TABLES 13
ABBREVIATIONS AND ACRONYMS 14

CHAPTER 1: GENERAL INTRODUCTION 18
1.1 HYPOXIC-ISCHAEMIC ORGAN INJURY 19
1.2 HYPOXIC-ISCHAEMIC CEREBRAL INJURY 19
1.2.1 The ischaemic penumbra: an intriguing paradox 20
1.2.2 The evolutionary phases of hypoxic-ischaemic injury 21
1.2.3 The molecular mechanisms of infarct progression 24
1.3 THERAPEUTIC STRATEGIES VERSUS HYPOXIC-ISCHAEMIC INJURY 31
1.3.1 Target-directed therapies 31
1.3.2 Clinical failure 32
1.4 PRECONDITIONING: A THERAPEUTIC STRATEGY VERSUS IMPENDING HYPOXIC-ISCHAEMIC INJURY 34
1.4.1 Terms and definitions 35
1.4.2 Examples of preconditioning in the clinical setting 37
1.4.3 The phases of preconditioning 38
1.4.4 The mechanisms of delayed preconditioning 38
1.5 HYPOXIA-INDUCIBLE FACTORs 40
1.5.1 Molecular structure 41
1.5.2 Regulation of hypoxia-inducible factors 44
1.5.3 Transcription of HIF target genes 52
1.5.4 Organ protective functions of HIF-1α 54
1.6 NON-ORGAN PROTECTIVE FUNCTIONS OF HIF-1 62
1.6.1 Critical role of HIF in embryogenesis 62
1.6.2 Paradoxical role for HIF–1α 62
1.6.3 Role in cancer 63
1.7 XENON 65
1.7.1 Physico-chemical properties of xenon 65
1.7.2 Medicinal uses of xenon 66
1.7.3 Possible utility for organ protection 68

1.8 AIMS 69

CHAPTER 2: MATERIALS AND METHODS 70

2.1 EXPERIMENTAL MODELS 71

2.1.1 ANIMAL MODELS 71
2.1.2 CELL MODELS 71
2.1.3 GAS EXPOSURE 73
2.1.4 ORGAN HARVESTING IN MICE 76

2.2 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION 76

2.2.1 Purification of total RNA 76
2.2.2 Determination of RNA concentration, purity and integrity 78
2.2.3 Reverse transcription 80
2.2.4 Polymerase chain reaction 81

2.3 WESTERN BLOTTING 82

2.3.1 Solutions 82
2.3.2 Protein extraction 82
2.3.3 Protein quantification using the Bradford assay 83
2.3.4 Electrophoretic protein separation 84
2.3.5 Electrotransfer 84
2.3.6 Immunodetection 86

2.4 IMMUNOHISTOCHEMISTRY 90

2.4.1 Solutions 90
2.4.2 Tissue fixation by transcardial perfusion 91
2.4.3 Immunostaining 93

2.5 RAPAMYCIN ADMINISTRATION 94

2.5.1 In vitro 94
2.5.2 In vivo 95

2.6 GENE SUPPRESSION THROUGH RNA INTERFERENCE 97

2.6.1 Preparation of the siRNA solution 100
2.6.2 Preparation of the mouse for surgery 100
2.6.3 Hydrodynamic tail vein injection in mice 101

2.7 RENAL ISCHAEMIC-REPERFUSION INJURY 101

2.8 HISTOLOGICAL ANALYSIS OF RENAL ISCHAEMIC-REPERFUSION INJURY 107

2.9 ANALYSIS OF RENAL FUNCTION 110

2.10 TUNEL STAINING 110

2.11 STATISTICAL ANALYSIS 111

CHAPTER 3: RESULTS 112

3.1 REGULATION OF HIF-1α BY XENON PRECONDITIONING 113

3.1.1 Nitrogen does not affect HIF-1α protein expression 113
3.1.2 Hypoxia increases HIF-1α protein expression 115
3.1.3 Xenon increases HIF-1α protein expression 117
3.1.4 Xenon increases expression of erythropoietin and vascular endothelial growth factor 124
3.1.5 Nitrous oxide does not affect HIF-1α protein expression 128

3.2 MECHANISM OF XENON-INDUCED HIF-1α PROTEIN EXPRESSION 130

3.2.1 Xenon increases HIF-1α through a degradation-independent mechanism 130
3.2.2 Xenon does not affect HIF-1α mRNA transcription 133
3.2.3 Xenon increases HIF-1α protein expression through a translation-dependent mechanism 133

3.3 NECESSITY OF HIF-1α IN XENON PRECONDITIONING VERSUS RENAL ISCHAEMIC-REPERFUSION INJURY 142

3.3.1 Delivery of siRNA intro the renal parenchyma by hydrodynamic tail vein injection 142
3.3.2 The functional effect of HIF-1α siRNA 142
3.3.3 The necessity of HIF-1α in xenon-induced erythropoietin expression .. 144
3.3.4 The necessity of HIF-1α in xenon preconditioning versus renal ischaemic-reperfusion injury 148
3.3.5 The necessity of HIF-1α in xenon-mediated attenuation of apoptosis in renal tubular cells 151
3.3.6 The necessity of HIF-1α in xenon preconditioning versus renal failure 155

CHAPTER 4: DISCUSSION 157

4.1 REGULATION OF HIF-1α BY XENON PRECONDITIONING 158

4.1.1 Xenon preconditioning regulates HIF-1α protein expression 159
4.1.2 Normoxic regulation of HIF-1α 160
4.1.3 Mechanisms of regulating HIF-1α protein expression 160
4.1.4 HIF isoforms 161
4.1.5 Anaesthetic regulation of HIF-1α 162
4.1.6 Sites of action of xenon 163
4.1.7 Xenon preconditioning in human-derived cells 164
4.1.8 Xenon preconditioning regulates erythropoietin expression 164
4.1.9 Mechanisms of erythropoietin-mediated protection 165
4.1.10 Xenon preconditioning regulates vascular endothelial growth factor expression 168

4.2 MECHANISM OF XENON-INDUCED HIF-1α PROTEIN EXPRESSION 169

4.2.1 Xenon preconditioning and HIF-1α gene transcription 169
4.2.2 Xenon preconditioning and HIF-1α post-translational degradation 170
4.2.3 Xenon preconditioning and HIF-1α translation 173

4.3 NECESSITY OF HIF-1α IN XENON PRECONDITIONING VERSUS RENAL ISCHAEMIC - REPERFUSION INJURY 180

4.3.1 Delivery and function of in vivo siRNA 181
4.3.2 Necessity of HIF-1α in xenon-mediated erythropoietin 181
4.3.3 Necessity of HIF-1α in xenon preconditioning against renal ischaemia-reperfusion injury 182
4.3.4 Regulation of erythropoietin 182
4.3.5 Apoptosis and HIF isoforms 183
4.3.6 siRNA technology 184
4.3.7 The requirement of anaesthesia for surgical experiments 186

CHAPTER 5: FINAL CONCLUSION 187

5.1 OVERVIEW OF FINDINGS 188

5.2 FUTURE DIRECTION 193

5.3 CLINICAL IMPLICATIONS 194

5.4 CONCLUSION 196

CHAPTER 6: REFERENCES 197

LIST OF FIGURES

Figure 1. The ischaemic penumbra 22

Figure 2. Evolution of the infarct core 23

Figure 3. Simplified schematic representation of molecular injury pathways that lead to cell death 30

Figure 4. Schematic representation of the domain structures of the hypoxia-inducible factor family and sites of regulation in normoxia 43

Figure 5. The mammalian target of rapamycin protein translation initiation pathway 47

Figure 6. Oxygen-dependent post-translational degradation of HIF-1α 50

Figure 7. HIF-1α accumulation results in HIF-1 transcriptional activity 53

Figure 8. Schematic illustration of in vivo gas exposure circuit 75

Figure 9. RNA integrity in the mouse brain and kidney 79

Figure 10. Electrotransfer of proteins, from gel to nitrocellulose membrane 85

Figure 11. Western blotting 89

Figure 12. Schematic diagram of the transcardial perfusion method 92

Figure 13. Time course and groups of rapamycin experiments 96

Figure 14. RNA interference in mammalian systems 98

Figure 15. Photograph of tail vein injection in mice 103

Figure 16. Time course and groups of HIF-1α siRNA in xenon preconditioning experiments in mice 104

Figure 17. Time course and groups of renal ischemia-reperfusion experiments in mice 105

Figure 18. Method of single-blinded histopathological analysis for renal ischaemic-reperfusion injury experiments 108

Figure 19. Nitrogen exposure does not affect HIF-1α protein expression in the mouse brain or kidney 114

Figure 20. Hypoxic exposure increases HIF-1α protein expression in the mouse brain and kidney 116

Figure 21. Hypoxic exposure transiently increases HIF-1α protein expression in the mouse brain and kidney 118

Figure 22. Xenon exposure increases HIF-1α protein expression in the mouse brain and kidney 119

Figure 23. Immunohistochemistry examples of xenon-induced HIF-1α expression in the mouse brain 121

Figure 24. Xenon exposure increases HIF-1α protein expression in primary neuronal-glial co-cultures 122

Figure 25. Xenon exposure increases HIF-1α protein expression in human kidney-2 cell cultures 123

Figure 26. Xenon exposure increases erythropoietin mRNA transcription in the mouse kidney 125

Figure 27. Xenon exposure increases erythropoietin protein expression in the mouse brain and kidney 126

Figure 28. Xenon exposure increases VEGF protein expression in the mouse kidney 127

Figure 29. Nitrous oxide exposure does not affect HIF-1α protein expression in the mouse brain or kidney 129

Figure 30. Xenon exposure increases HIF-1α through a VHL-independent mechanism 131

Figure 31. Xenon exposure does not affect PHD2 protein expression in the mouse brain or kidney 132

Figure 32. Xenon exposure does not affect HIF-1α mRNA transcription 135

Figure 33. Xenon exposure increases mTOR protein expression in RCC4+ cell culture..... 136

Figure 34. Rapamycin inhibits xenon-induced HIF-1α protein expression in RCC4+ cell culture 138

Figure 35. Rapamycin inhibits xenon-induced HIF-1α protein expression in the mouse brain and kidney..... 139

Figure 36. DMSO increases HIF-1α protein expression in RCC4+ cell culture..... 141

Figure 37. Verification of in vivo delivery of 3' fluorescein-labelled siRNA into the mouse renal parenchyma 143

Figure 38. Functional effect of HIF-1α siRNA in the mouse kidney 145

Figure 39. HIF-1α siRNA attenuates xenon-induced HIF-1α protein expression in the mouse kidney..... 146

Figure 40. HIF-1α siRNA attenuates xenon-induced erythropoietin protein expression in the mouse kidney..... 147

Figure 41. Histological assessment of HIF-1α in in vivo xenon preconditioning versus renal ischaemic-reperfusion injury 150

Figure 42. Functional assessment of HIF-1α in in vivo xenon preconditioning versus renal ischaemic-reperfusion injury 152

Figure 43. TUNEL + cells in the renal cortex after IRI 154

Figure 44. Kaplan-Meier survival curve..... 156

Figure 45. Possible mechanisms of erythropoietin-mediated protection..... 167

LIST OF TABLES

Table 1. List of PCR primers and amplification conditions used to analyse the expression of genes in this study 81

Table 2. List of primary antibodies used in Western blot immunodetection of protein expression 88

Table 3. Post operative assessment scoring assay for renal failure survival experiments 106

Table 4. Histopathological scoring system for renal ischaemic-reperfusion injury experiments 109

ABBREVIATIONS AND ACRONYMS

2-OG	2-oxoglutarate
4E-BP	eIF-4E binding proteins
A	adenine
ABC	avidin biotin peroxidase
ALT	alanine aminotransferase
Ap-1	activator protein-1
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
Bcl-xL	anti-apoptotic protein
BDNF	brain-derived neurotrophic factor
bHLH	basic-helix-loop-helix
C	cytosine
CBF	cerebral blood flow
CBP / p300	cAMP-responsive element binding (CREB) protein
cDNA	complementary DNA
CNLS	C-terminal localisation signal
CNS	central nervous system
CTAD	C-terminus transactivation domain
DAB	3,3 'diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
E	embryonic day
ECL	enhanced chemiluminescent
EDTA	ethylene di-ammine tetra acetic acid
EGF	epidermal growth factor
EGL-9	egg laying defect-9
Egr-1	early growth response factor - 1
EGTA	ethylene glycol tetra-acetic acid
eIF	eukaryotic initiation factor
eNOS	endothelial NOS
EPO	erythropoietin
FBS	foetal bovine serum
FIH	factor inhibiting HIF-1
FKBP-12	FK506 binding protein-12

g	acceleration due to gravity
G	guanine
GC	guanine-cytosine
Hep3B	hepatoma cell line
HER2	human epidermal growth factor receptor 2
HI	hypoxic-ischaemic
HIF-1	hypoxia-inducible factor-1
HIF-1α	hypoxia-inducible factor-1α
HIF-1β	hypoxia-inducible factor-1β
HIF-2	hypoxia-inducible factor-2
HIF-2α	hypoxia-inducible factor-2α
HIF-3α	hypoxia-inducible factor-3α
HK2	human kidney 2
HLH	helix-loop-helix
HRE	hypoxia-responsive element
IGF-1	insulin-like growth factor-1
IHC	immunohistochemistry
iNOS	inducible NOS
IP	intraperitoneal
IRES	internal ribosome entry site
JAK	janus kinases
L	litre
MAC	minimum alveolar concentration
MAPK	mitogen-activated protein kinases
MCAO	middle cerebral artery occlusion
MDM2	murine double minute-2
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NFκB	nuclear factor-kappa B
NGF	nerve growth factor
NMDA	N-methyl D-aspartate
NO	nitric oxide
NOS	nitric oxide synthases
nNOS	neuronal nitric oxide synthase
NTAD	N-terminus transactivation domain
ODD	oxygen-dependent degradation
OGD	oxygen-glucose deprivation

PAS	per-ARNT-sim
PEST	proline, glutamic acid, serine, threonine
PFD	paraformaldehyde
PHD 1, 2 and 3	prolyl hydroxylase domain-containing enzymes 1, 2 and 3
PI3K	phosphoinositide-3 kinase
PKC	protein kinase C
PND	post-natal-day
PTEN	phosphatase and tensin homolog
RCC	renal cell carcinoma
RISC	dsRNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
siRNA	small interfering RNAs
Smad	mothers against decapentaplegic homolog 3
SRC-1	steroid receptor coactivator-1
STAT	signal transducers and activators of transcription
T	thymine
TADs	transactivation domains
TAE	tris-acetate EDTA
TATA	thymine-adenine- thymine-adenine
TBS	tris buffered saline
TBS-T	tris buffered saline with Tween-20
TGF	transforming growth factor
TIA	transient ischaemic attack
TIF-1	transcriptional intermediary factor 1
Tris	hydroxymethyl aminomethane
UPS	ubiquitin-proteasome system
UTR	untranslated region
UV	ultraviolet
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau
VPF	vascular permeability factor

CHAPTER 1

GENERAL INTRODUCTION

1.1 HYPOXIC-ISCHAEMIC ORGAN INJURY

Hypoxic-ischaemic (HI) organ injury results in functional decompensation that contributes to major morbidity and mortality. Myriads of human disease states collectively converge on this common end-point that may occur through hypoxia, ischaemia, reperfusion following ischaemia, or combinations of these. Given that oxygen is an absolute requisite to any aerobic organism, living cells are vulnerable to HI injury and will respond in fundamentally similar ways; however, the brain, more so than other organs, is remarkably susceptible to HI injury. Stroke alone is the most common cause of disability worldwide (Murray, Lopez 1997) and the third most common cause of death in the UK . Furthermore, neonatal HI brain injury occurs with a worldwide incidence rate varying between 0.4 and 4.5 per 1000 live births, where 45 % will have permanent neurological disability or die (Finer et al. 1981, Wu et al. 2004). The indisputable clinical importance of HI injury has, through the decades, prompted intensive investigation to precisely identify the underlying pathogenic mechanisms in order that effective therapies may be developed. Accordingly, the description that follows focuses on the vulnerability of the brain to HI injury and those mechanisms that may be plausible targets of therapy.

1.2 HYPOXIC-ISCHAEMIC CEREBRAL INJURY

The healthy human brain is the master controller of body functions as diverse as bladder control to creative thinking. It derives its energy needs almost entirely through oxidation of glucose and, therefore, relies on the cerebral blood flow (CBF) to continuously deliver these substrates, for sustaining physiological cell function and metabolism (Back 1998). A decrease in CBF, *e.g.* through arterial occlusion, can result in damage distal to the obstruction, where compromises in neuronal oxygenation initiates a series of functional, structural and biochemical changes that conclude with cell death and brain damage (Bandera et al. 2006).

1.2.1 The ischaemic penumbra: an intriguing paradox

HI injury can result in a structure-less necrotic core of complete infarction that is irreversibly damaged. The surrounding peri-infarct area that adjoins dead with normal brain tissue receives a limited blood supply and is termed the ischaemic "penumbra," a description also used for the hazy halo of light around a full lunar eclipse (Astrup, Siesjo & Symon 1981).

The ischaemic penumbra presents itself as the more clinically important and intriguing area of interest in HI pathophysiology. Non-invasive imaging studies have shown that the infarct core, if left unhindered, grows to engulf the penumbra and worsen (Hossmann, Hoehn-Berlage 1995). Paradoxically, however, the experience of some patients having suffered from an HI insult has shown that incurred neurological deficits may sometimes resolve. A well-received postulation for this post-injury healing is based on observations that loss of structural integrity occurs with more severe hypoxia than that required for loss of function (Astrup, Siesjo & Symon 1981); therefore, the penumbral region is regarded as "structurally intact yet functionally paralysed" after the HI insult (Hossmann 2006) and may recover through plausible mechanisms, such as the restoration of blood flow through neighbouring collateral vessels, or modifications in neuronal metabolism that accommodates the lowered blood supply (Astrup et al. 1977). Though heavily constrained by time, it is the ischaemic penumbra that therapies target to mitigate the symptoms of HI injury.

Furthermore, a linear relationship between CBF and differential thresholds of cell viability has been established. Evaluation of perfusion imaging studies in animal models estimate that the threshold for the ischaemic penumbra correlates to a reduction in CBF to 40 % of normal, a level that corresponds to failure of cortical

evoked potentials that indicate loss of electrical function; irreversible brain damage from infarction occurs with CBF reduced below 15 % of normal, and is associated with a massive elevation in extracellular potassium, indicating membrane failure and loss of structural integrity; complete CBF interruption will culminate in cell death within only a matter of minutes (Back et al. 1996) (Figure 1).

1.2.2 The evolutionary phases of hypoxic-ischaemic injury

The process of HI injury is often described as evolutionary, progressing through three main phases. The initial pathogenic insult establishes the acute phase, causing immediate injury within minutes, directly inducing energy failure and cell membrane depolarisation to form a necrotic infarct core. The sub-acute phase lasts for the next 4 - 6 hours and is characterised by the expansion of the infarct core volume to engulf the adjacent ischaemic penumbra. Lastly, injury proceeds to a delayed, or reperfusion, phase that occurs during the recovery period lasting for days or weeks as injury further progresses, with secondary events such as inflammation and delayed cell death (Siesjo 2008) (Figure 2).

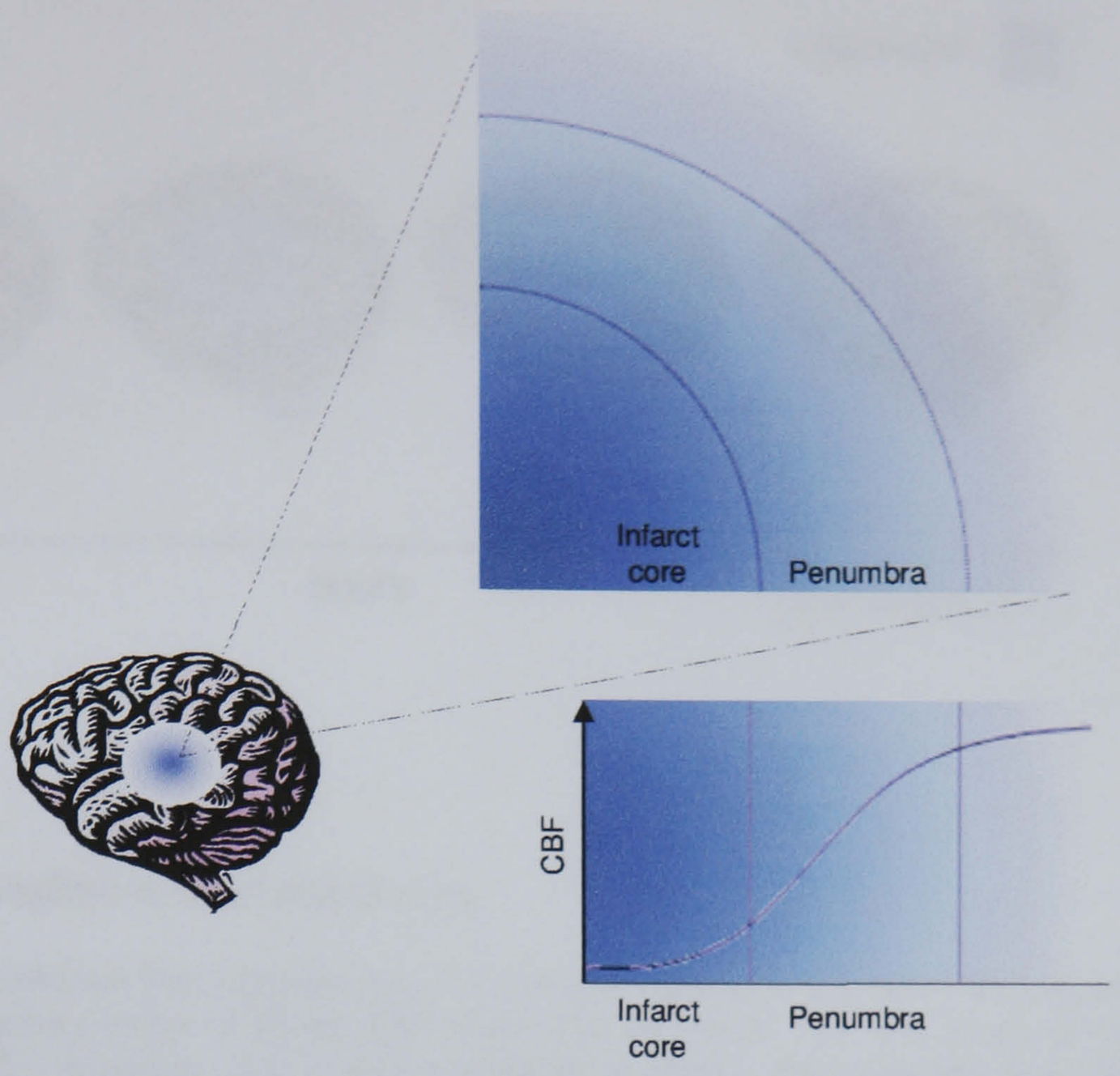


Figure 1. The ischaemic penumbra

This cartoon (not to scale) illustrates the regions of the infarct core and the ischaemic penumbra. Following hypoxic-ischaemic injury, the infarct core forms as a result of a critical reduction in CBF, leading to energy failure and cell death. The ischaemic penumbra forms as a result of mild to moderate reductions in CBF, which results in structurally intact, but functionally paralysed, tissue. **Abbreviations: CBF**, cerebral blood flow. Modified from (Lo 2008).

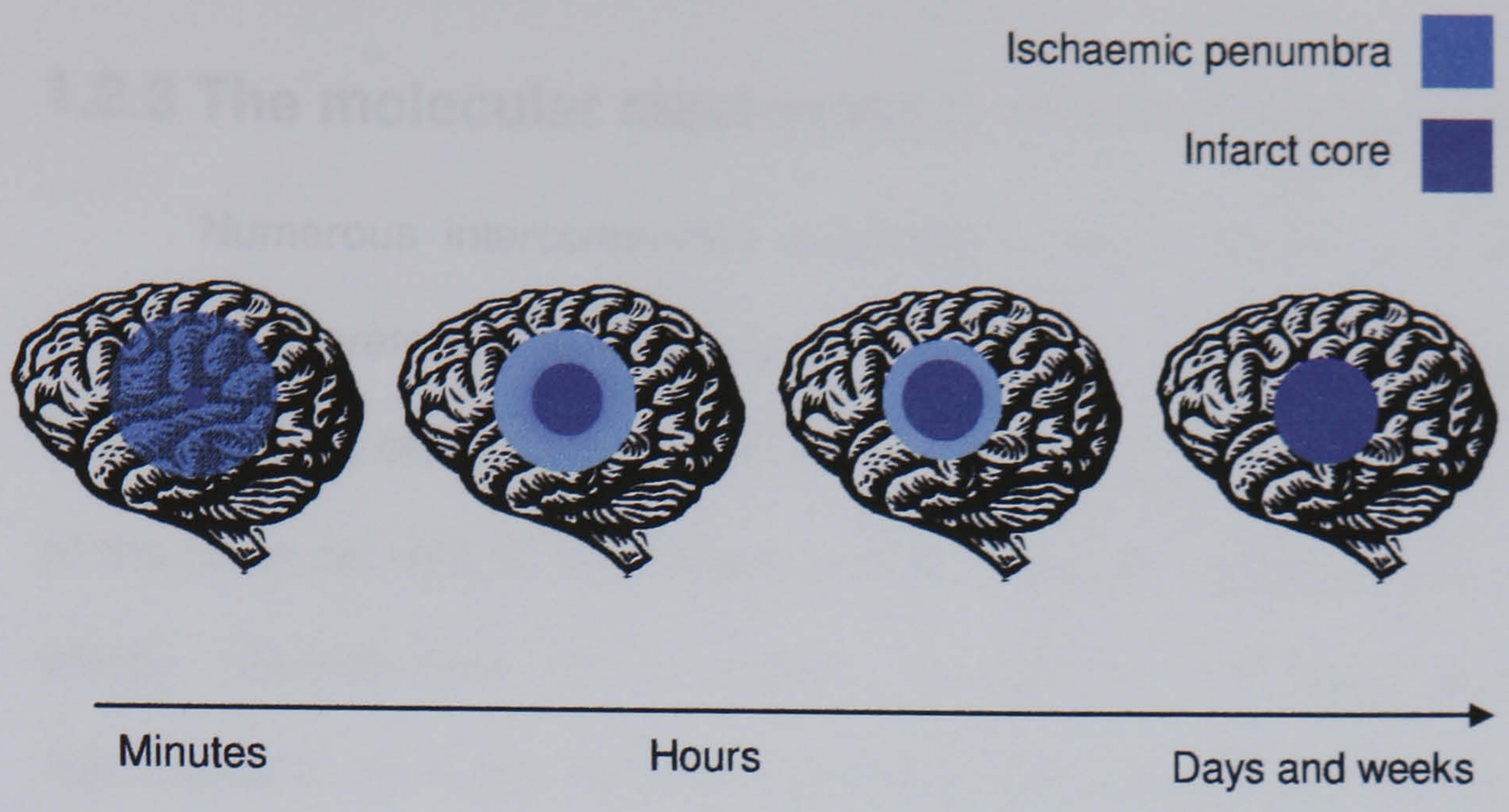


Figure 2. Evolution of the infarct core

This cartoon illustrates the progression of an infarct core after HI injury (not to scale). The acute phase occurs within minutes after the initial HI insult. The sub-acute phase occurs over the next 4 - 6 hours, with a rapid increase in infarct core volume, and decrease in penumbra volume. The delayed, or reperfusion, phase occurs over the following days and weeks, and is marked by inflammation and delayed cell death.

1.2.3 The molecular mechanisms of infarct progression

Numerous interconnected mechanisms take part in the processes of HI injury and, therefore, it is appreciated as a complex heterogeneous disorder whose development is characterised by the severity, extent and timing of the insult, the age of the brain, as well as the nature of the subsequent reoxygenation or reperfusion phase (Alvarez-Diaz et al. 2007). Therapeutic strategies addressing this heterogeneity are more likely to warrant further investigation, and may be in the form of a combination therapy as typified in certain cancer and HIV treatment regimes (Aronowski et al. 2003, Lyden et al. 2000), or as a single agent with widespread action as exemplified through the phenomenon of preconditioning (Ratan et al. 2004).

1.2.3.1 Excitotoxicity

At the cellular level, interruption in oxygen and glucose delivery prompts a complex cascade of deleterious biochemical events (a simplified diagram of the key events that take place is shown in Figure 3). Oxygen deprivation prevents oxidative phosphorylation and, therefore, rapidly obliges mitochondria to switch from aerobic to anaerobic metabolism, an energy inefficient process that rapidly exhausts adenosine triphosphate (ATP) reserves and accumulates lactic acid. This failure in energy, or ATP, generation results in cell membrane depolarisation as ATP-dependent transcellular sodium-potassium pumps cease to function; with loss of membrane polarity, excitatory and inhibitory neurotransmitters are released to act at their respective receptors, the most notable of these being glutamate (Shalak, Perlman 2004).

Glutamate, the principal excitatory amino acid in the mammalian central nervous system (CNS), is an agonist at post-synaptic ionotropic glutamate receptors, such as the N-methyl D-aspartate (NMDA) receptor. NMDA receptors have regions not only of agonist activity, but also of antagonist activity (*i.e.* magnesium) and are cation channels highly permeable to sodium (responsible for depolarisation), and calcium (capable of stimulating numerous enzyme systems) (Dirnagl, Iadecola & Moskowitz 1999). NMDA receptors play a dichotomous role where they are vital for brain function and underlie formation of memory and learning (Bliss, Collingridge 1993) and yet, by contrast, their intense and acutely unrelenting activation results in neuropathology and cell death (Lipton, Stamler 1994).

Glutamatergic neuronal activity has been shown by magnetic resonance spectroscopy to be tightly coupled to glucose metabolism (Sibson et al. 1998). Therefore, energy failure disables glutamate regulation and its excessive efflux onto the NMDA receptor facilitates depolarisation and intracellular calcium overload, producing glutamate neurotoxicity, also referred to as "excitotoxicity" (Olney 1971).

1.2.3.2 Calcium toxicity

Calcium is a universal intracellular second messenger that activates numerous enzyme reactions. Physiological cytosolic calcium concentration is extremely low with steep concentration gradients existing between the cytosol and the extracellular compartment, as well as between the cytosol and the endoplasmic reticulum (Hossmann 2006). During HI injury, cytosolic calcium accumulates to a major degree, by glutamate-mediated NMDA receptor stimulation, but also by interrupted calcium efflux from energy failure, as well as through mitochondrial and endoplasmic reticulum calcium release (Shalak, Perlman 2004). Elevation of intracellular calcium leads to a sharp rise in its activity in multiple calcium-dependent

effector pathways with formation of oxygen free radicals as by-products; these contribute to injury by fragmenting the cell membrane and disrupting DNA. inevitably triggering irreversible death of cells (Grow, Barks 2002).

1.2.3.3 Oxidative stress

Free radicals play an important role in the physiological control of cell function, for example, through participation in redox homeostasis as well as signal transduction pathways (Droge 2002). However, oxidative stress is a general term referring to pathological oxidative metabolism that, rather than generating ATP, produces excessive free radicals and compromise cell survival.

In physiologically functioning mitochondria, approximately 90 % of oxygen supplied to the cell will be involved in the production of ATP via the electron transfer chain in the inner mitochondrial membrane. The remainder of the free oxygen will form superoxide anions that are reduced to water through antioxidants, such as superoxide dismutases and glutathione peroxidase, and rendered non-damaging. Following hypoxia-ischaemia, energy failure and consequent calcium toxicity interferes with the mitochondrial electron transfer chain that, often with accompanying anti-oxidant depletion, results in accumulation of superoxides as well as more reactive species, such as the hydroxyl radical, by a Fenton reaction (Inder, Volpe 2000). Oxidative stress is pertinent to the reperfusion phase of hypoxia-ischaemia. The re-establishment of perfusion to a previously damaged cell does not bring back equilbirum but instead the oxygen floods into dysfunctional mitochondria and further contributes to free radical accumulation. High concentrations of free radicals promote cell damage by DNA fragmentation, lipid peroxidation of the cell membrane and intracellular organelles (Saito et al. 2005), and disruption of

membrane and pump function that leads to more glutamate efflux and, therefore, further perpetuation of excitotoxicity (Grow, Barks 2002).

1.2.3.4 Nitric oxide toxicity

Though the various molecular injury pathways in this account have been described as isolated entities they are inextricably related, as studies on nitric oxide further demonstrate. Nitric oxide (NO), or nitrogen oxide, is one of the few gaseous messenger molecules in the body and plays a role in functions such as blood vessel homeostasis. NO is produced from the reaction between L-arginine and nitric oxide synthases (NOS), of which three isoforms have been identified; neuronal and endothelial NOS (nNOS and eNOS, respectively) activation are dependent on intracellular calcium concentration, whereas inducible NOS (iNOS) is activated by cytokines in neurons and endothelial cells, as well as astrocytes and microglia (Samdani, Dawson & Dawson 1997).

Following hypoxia-ischaemia, NO exhibits physiological and pathological functions, acting to promote, as well as protect against, injury; the net effect, therefore, is dependent on the various isoforms of NOS activated, and this in turn is dependent on the location of insult, a situation that is unpredictable. Injury-mediated production of neuronal NO has been shown to modify the NMDA receptor to enhance calcium influx and excitotoxic damage (Sorrentino et al. 2004). In addition, the rise in intracellular calcium induces further NO generation that, in combination with superoxide, generates the potent peroxynitrite radical for further facilitation of glutamate release (Shalak, Perlman 2004). Peroxynitrite also undergoes further conversion to form the hydroxyl radical and nitrogen dioxide, enhancing oxidative stress through oxidation and nitration of DNA, proteins and lipids (Beckman, Koppenol 1996). Conversely however, calcium-dependent NO production in

endothelial cells via eNOS leads to vasodilation, blood flow, prevention of thrombosis and therefore alleviation of injury (Dalkara et al. 1994). Several days following reperfusion, iNOS expression has been shown to increase in glia and neutrophils with the consequential NO left to flood the ischaemic region and contribute to delayed neurotoxicity as well as potentiating pro-apoptotic pathways (Iadecola et al. 1995).

1.2.3.5 Inflammation

An inflammatory response has been shown to be evoked by HI injury, with interleukin-1β and tumour necrosis factor-α messenger RNA (mRNA) expressed from one hour after hypoxia-ischaemia in rats (Hagberg et al. 1996). Pro-inflammatory cytokines may contribute to injury progression directly, through stimulation of iNOS and free radical generation, as well as indirectly, by provoking cells to release further glutamate (Shalak, Perlman 2004). However, the role of inflammatory mediators is not straight forward and whether inflammation is beneficial or deleterious, may depend on the severity and extent of hypoxia-ischaemia, and the timing of the inflammatory response; an early response may promote injury and a later response may be beneficial for initiating repair processes (Wang, Tang & Yenari 2007).

1.2.3.6 Cell death

The inevitable end fate of unperturbed HI injury occurs through both necrotic and apoptotic mechanisms. A higher ratio of necrotic cell death is evident with more severe insults and also in the adult brain compared to the neonatal brain (Bonfoco et al. 1995) yet, great variability exists from one individual to another. Necrosis and apoptosis produce cell death through separate and distinct mechanisms. Necrotic cell death is characterised by passive cell swelling, disruption of cytosolic

organelles, loss of internal homeostasis, membrane lysis, release of cell contents, and activation of inflammation (Krysko et al. 2008). Apoptosis, by contrast, is an active process that is propagated through a well-orchestrated series of events defined with specific features such as cell shrinkage, membrane blebbing, nuclear pyknosis chromatin condensation and DNA fragmentation, without the occurrence of inflammation (Edwards, Mehmet 1996). Early excitotoxicity during the acute phase of injury has been shown to result in rapid necrotic cell death to form the initial infarct core. The surrounding ischaemic penumbra however, is subjected to milder excitotoxicity, oxidative stress and inflammation, resulting in delayed cell death that is more characteristic of apoptosis (Mergenthaler, Dirnagl & Meisel 2004). A simplified diagram of the key events is illustrated in Figure 3.

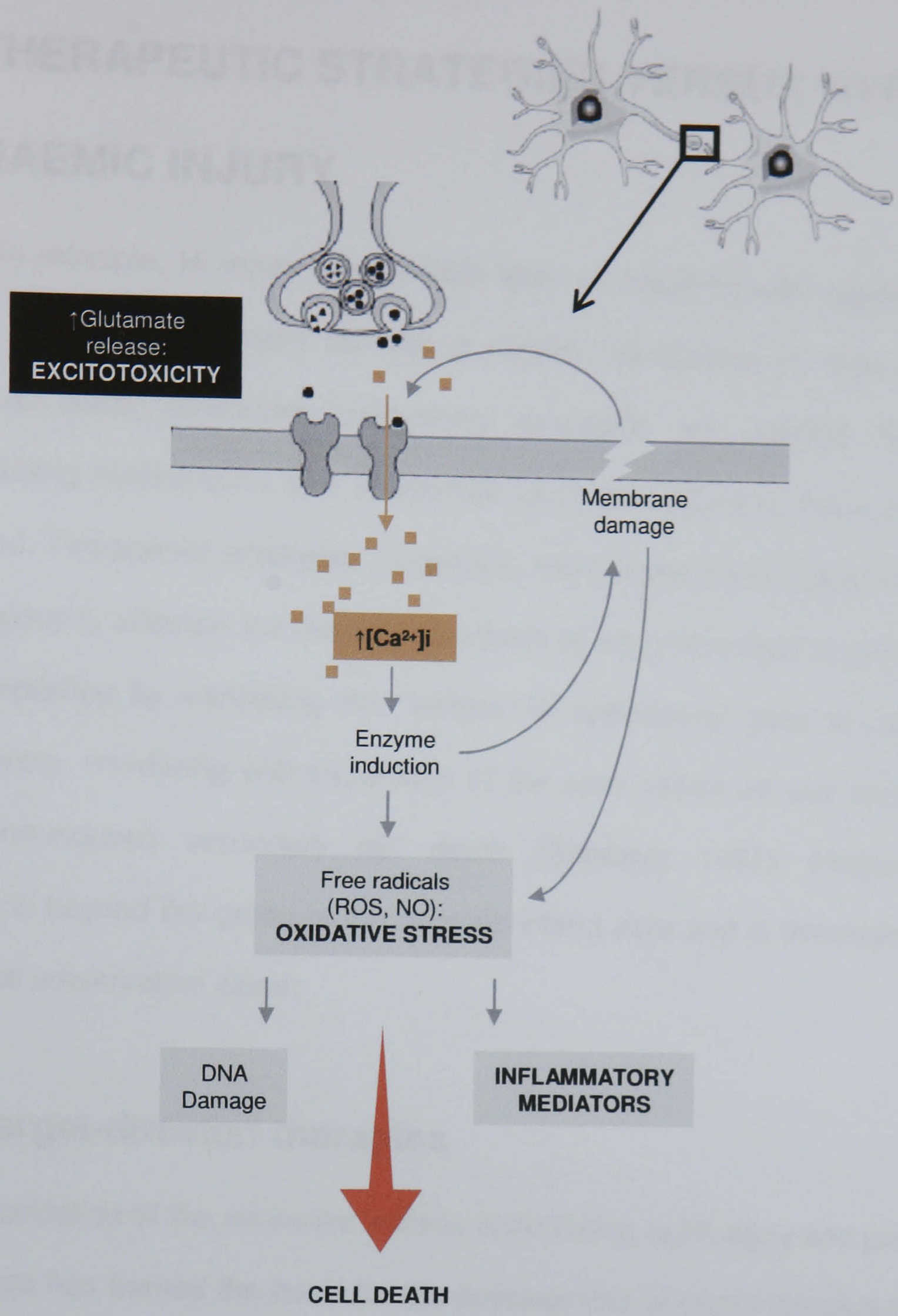


Figure 3. Simplified schematic representation of molecular injury pathways that lead to cell death

Energy failure from hypoxic-ischaemic injury disables glutamate regulation, where its excessive efflux onto the NMDA receptor facilitates depolarisation and intracellular calcium overload, resulting in excitotoxicity. Accumulation of intracellular calcium results in activity of numerous calcium-dependent effector pathways, which results in the formation of ROS and NO, contributing to oxidative stress. Free radicals promote cell damage by DNA fragmentation, lipid peroxidation of the cell membrane and intracellular organelles. Moreover, free radicals disrupt cell membrane pump function, which results in further glutamate efflux and excitotoxicity. An inflammatory response is initiated from 1 hour after HI injury and contributes to injury progress by further induction of ROS and NO production, as well as glutamate release. Left unperturbed, HI injury results in cell death. **Abbreviations: [Ca2+]I,** intracellular calcium concentration; **HI injury**, hypoxic-ischaemic injury**; NMDAR**, N-methyl-D-aspartate receptor; **NO**, nitric oxide; **ROS**, reactive oxygen species.

1.3 THERAPEUTIC STRATEGIES VERSUS HYPOXIC-ISCHAEMIC INJURY

In principle, HI injury is reversible after an insult through replenishment of oxygen and nutrient delivery but this is heavily constrained by time (Hossmann 2007); as such, alternative therapeutic strategies are needed. Endogenous counteracting mechanisms that ameliorate injury do exist and these will be later discussed. Therapeutic strategies, in general, refer to pharmacological interventions that temporarily alleviate the deleterious effects of injury that lead to cell death; this is accomplished by increasing the "window of opportunity" prior to restitution of blood supply, interfering with expansion of the core infarct as well as preventing reperfusion-induced secondary cell death (Ginsberg 1997). Pharmacological salvation is beyond the grasp of the necrotic infarct core and is therefore aimed at penumbral preservation alone.

1.3.1 Target-directed therapies

Elucidation of the molecular entities contributing to HI injury and progression to cell death has formed the basis for the development of target-directed therapies. Existing evidence demonstrates that the numerous molecular participants of injury contribute to cell death through mutually exclusive pathways (Won, Kim & Gwag 2002). Antagonists, inhibitors and blockers of glutamate, NMDA, calcium, NO, other free radicals (or antioxidant therapies), inflammatory mediators, apoptotic factors, as well as growth factors and trophic agents such as nerve growth factor (NGF) (Chiaretti et al. 2008), insulin-like growth factor-1 (IGF-1) (Hung et al. 2007) and brain-derived neurotrophic factor (BDNF) (Han, Holtzman 2000), have all been extensively examined as a means to relieve HI injury. The proven neuroprotective efficacy of therapies directed against their pathogenic target is regrettably also

compounded, with further investigation, to have detrimental effects on the other pathogenic pathways that take part in injury progression to cell death. This highlights the need for combinatorial therapies, or single agent therapies that exhibit multiple actions, for maximal prevention of cell death. Importantly however, regardless of therapy efficacy, alone or in combination, these interventions are of limited consequence or relevance if the tissue is not adequately reperfused in a timely fashion.

1.3.2 Clinical failure

Despite substantial experimental evidence of certain therapies acclaiming preclinical promise, their performance in the clinical arena has resulted in a litany of frustrating disappointments. These failures further exacerbate the difficulty in the hunt to provide therapies relevant to humans, as pharmaceutical industries are cautious to offer their financial support without proof of principle for a return on their investment. Several plausible explanations have been raised with respect to this translational difficulty, from animals to humans, at times even questioning whether basic understanding of injury concepts may be more fanciful than factual; it seems unlikely, however, that over thirty years of rigorous research into HI injury should be discounted as invalid. Reflecting on current limitations and flaws displays both preclinical and clinical experimental shortcomings; addressing these may offer a glimmer of cautious optimism for the long-suffering perseverance required in the search to attain future therapeutic success.

The use of animals, naturally, has its limitations. Findings in the ever popular rodent model may not be transferable in its entirety to humans because of interspecies variability. Even within the same animal species, the strain will affect outcome from the same model of injury; mild, moderate or severe injury is observed in SV129,

C57B or FVB mice subjected to the same insult, respectively (Fox, LeVasseur & Faden 1999). Therefore for experimental rigour, animals are constrained to be identical in strain, genetics and sex, whereas all strata of human population can be affected by hypoxia-ischaemia. Furthermore, animals may sometimes be excluded from analyses if the prescribed injury has fallen out of the experimental criteria or if they generate anomalous results. Anaesthetic use, a legal necessity in animals, can compound the action of the test therapy as well as injury-mediated cell death. Furthermore, consideration to the pharmacology of therapies has been poor or absent altogether, lacking in evaluation of dose response, therapeutic window, pharmacokinetics or pharmacodynamics (Wahlgren, Lyden 2000).

A key issue in human studies has been the delay in delivering therapies after injury. Glutamate antagonists, for example, may have been administered after the acute injury phase and excitotoxic damage would have already been established (Hoyte et al. 2004). Lastly, though by no means exhaustively, the outcome of some human trials may have detected a significant response to therapy if patient enrolment had been markedly increased for adequate statistical power (Green 2008).

1.4 PRECONDITIONING: A THERAPEUTIC STRATEGY VERSUS IMPENDING HYPOXIC-ISCHAEMIC INJURY

In most situations, the onset of injury is unpredictable but in those clinical settings in which it can be anticipated, for example, during brain or kidney surgery, pre-emptive measures may be considered. It has been proposed from as early as 1943 that rats subjected to small graded amounts of injury, induced by hypoxia, acquired a resistance enabling them to withstand an otherwise fatal degree of trauma (Noble 1943). It was not until 1986, however, that the concept of "preconditioning" was officially introduced to the scientific community, when Murry and colleagues demonstrated through a series of landmark experiments on canine hearts that, contrary to traditional and intuitive thinking, ischaemia is not always harmful; pre-treatment with repeated brief episodes of ischaemia interwoven with reperfusion protected the myocardium from a subsequent, prolonged ischaemic insult, able to reduce the size of myocardial infarct by as much as 75 % compared to controls (Murry, Jennings & Reimer 1986). In the 20 years since its first discovery, Murry's seminal paper is currently the most cited article published by Circulation, and a Pubmed search reveals that there are, to date, in excess of 2000 publications directly attributed to the profound effects of preconditioning. Interestingly, however, the experiments of Murry and colleagues somewhat overshadowed the work of Schurr, earlier that same year, demonstrating the adaptation of adult rat hippocampal slices to anoxia by hypoxia (Schurr et al. 1986). Since, the protective effect of preconditioning has been demonstrated in all organs, with the first demonstration of cerebral ischaemic preconditioning against ischaemic injury by Kitagawa (Kitagawa et al. 1990), as well as all species tested from bacteria to mammalian cells (Yellon et al. 1998).

1.4.1 Terms and definitions

1.4.1.1 Preconditioning

There is no established definition, as such, for preconditioning but it may be regarded as the brief presentation of a non-injurious, sub-lethal stressor to induce protection against a subsequent prolonged, injurious and potentially lethal stress (Gidday 2006). Preconditioning confers "tolerance" to living cells, employing their fundamental endogenous capability in resisting recurrent stress through evolutionarily conserved, natural adaptive processes (Dirnagl, Simon & Hallenbeck 2003).

1.4.1.2 Cross tolerance

While the original studies involved hypoxia or ischaemia, the early elucidation of the molecular mechanisms involved in the process of HI preconditioning raised the possibility of manipulating those targets pharmacologically. Therefore, a plethora of agents have been investigated; the most clinically promising of these are anaesthetic agents because of their possible application in elective, high-risk surgical patients. The putative therapeutic utility of anaesthetic preconditioning, especially for injury to the CNS, exceeds that induced by a strategy involving hypoxia and/or ischaemia because the latter strategy has a narrower safety margin. These studies introduced the notion of "cross tolerance," where preconditioning against HI injury may be induced not only by hypoxia or ischaemia but also by a diverse variety of metabolic or physical stressors, or pharmacological agents (Tauskela et al. 2001). However, the cross-induction of pharmacological-induced tolerance may differ in efficacy compared with hypoxic or ischaemic preconditioning and the protection is often governed by multiple mechanisms.

1.4.1.3 Postconditioning

Postconditioning is a relatively novel concept. Ischaemic postconditioning has been mainly experimentally demonstrated in the heart, where intermittently obstructing flow following post-ischaemic reperfusion leads to protection (in contrast to ischaemic preconditioning, which occurs before the onset of ischaemia). The appeal of postconditioning is its increased clinical applicability to patients and appears to occur through similar mechanisms as preconditioning. Clinical trials have shown postconditioning to be an efficacious therapy in patients with acute myocardial infarction (Thibault et al. 2008) and has more recently also been proven effective in the brain (Wang et al. 2008b).

1.4.1.4 Pharmacological preconditioning

Mizumura and colleagues in 1995 demonstrated that pharmacological agents are able to precondition (Mizumura, Nithipatikom & Gross 1995). This form of preconditioning generally refers to chemical compounds that mimic hypoxia through interference of cellular respiration and oxygen sensing mechanisms; these hypoxia mimetics include the respiratory chain inhibitor nitroproprionic acid (Wiegand et al. 1999), the HIF-1α inducers desferrioxamine and cobalt chloride (Jones et al. 2008), as well as mitochondrial potassium ATP channel openers such as diazoxide (Mayanagi et al. 2007). Importantly, pharmacological preconditioning is distinct from target-directed therapies administered prior to injury; while these therapies interfere with specific components of the injury cascade, pharmacological preconditioning reduces injury by adapting the whole organism to a more resilient phenotype.

1.4.1.5 Anaesthetic preconditioning

A notable form of pharmacological preconditioning is anaesthetic preconditioning where certain inhalational anaesthetics, such as halothane, isoflurane and sevoflurane, have been demonstrated as effective inducers of acute and delayed preconditioning (Cason et al. 1997). Anaesthetic preconditioning has also been successfully trialled in humans and is a promising therapy for surgical patients at high risk of developing ischaemic complications. Particularly relevant to this study, neuroprotection by preconditioning with xenon has recently been demonstrated in our laboratory (Ma et al. 2006), although the underlying molecular mechanisms remain to be elucidated.

1.4.2 Examples of preconditioning in the clinical setting

Several clinical trials exploring the role of preconditioning as a neuroprotectant have shown that transient ischaemic attacks (TIAs) protect the brain against stroke (Moncayo et al. 2000). However, these results have been challenged by the TIA study in North Carolina (Johnston 2004). In the heart, large clinical trials have displayed more robust evidence, where angina has been shown to reduce the size of myocardial infarcts, as well as decrease arrhythmias and improve left ventricular function, when compared to non-angina patients. Furthermore, the clinical exploitation of endogenous protection is demonstrated during angioplasty surgery, where brief balloon inflation has been shown to protect the heart from further inflations (Faries et al. 2008). The inhalational anaesthetic, isoflurane, has also been used during coronary artery bypass graft surgery, and was shown to be associated with improved long-term outcome (Yu, Beattie 2006).

1.4.3 The phases of preconditioning

Despite the array of stimuli able to induce preconditioning, certain hallmarks exist. Adaptation to a tolerant phenotype is biphasic, occurring as two temporally distinct types of preconditioning, namely acute and delayed preconditioning, that differ in their mechanisms. Acute preconditioning offers potent protection and occurs within minutes and persists for 1 - 3 hours after introduction of the preconditioning stimulus; it is short-lived and provides protection as a result of changes in ion channel permeabilities, protein phosphorylation and other post-translational modifications (Gidday 2006). Delayed preconditioning, first described by Marber and colleagues (Marber et al. 1993) is less potent than the acute form, but corresponds to at least a thirty-fold longer duration and broader level of protection (Stein et al. 2004), manifesting slowly from 4 - 24 hours after the preconditioning stimulus and protecting cells for up to 72 hours against injury (Marber et al. 1993). This phenomenon is best appreciated as one that may require changes in gene expression and activation and, in contrast to acute preconditioning, is dependent on *de novo* protein synthesis (Meldrum 1997). Therefore, delayed preconditioning does not merely compete to neutralise injury cascades but serves to genetically "reprogramme" the response to HI injury (Gidday 2006).

1.4.4 The mechanisms of delayed preconditioning

The endogenous protective processes that mediate delayed preconditioning are topical and have been the subject of many reports. The majority of researchers are in agreement that there is not one exclusive pathway leading to a preconditioned state. Evidence supports that preconditioning requires the concerted participation of numerous cell types, molecules and diverse effector mechanisms; these fundamentally end in reprogramming the genomic response to injury, from cell death to survival, with subsequent translation of proteins that promote oxygen

delivery and metabolism as well as cell function, thereby preventing the induction and the propagation of injury cascades. Bewildering assortments of molecules have been linked to preconditioning but no definitive or systematic understanding currently exists. However, it is possible to observe a degree of mechanistic similarity required to achieve a phenotype that is more resilient to hypoxia-ischaemia in different organs and even across several species.

The various preconditioning stimuli that have been identified may be loosely classified as non-pharmacological, such as hypoxia and ischaemia, or pharmacological, through noxious agents like inflammatory mediators; these stimuli cause energy and metabolic alterations that result in the generation of triggers, such as NO and reactive oxygen species (ROS), alerting the cell to the presence of "danger" and putting into action defensive measures that counteract any further harm. Triggers activate an exquisitely complex network of intracellular signaling pathways with numerous protein kinases and transcription factors, involved in cell growth and survival, implicated to play important transduction roles; these include protein kinase C (PKC) (Beguin et al. 2007, Weber et al. 2005a), mitogen-activated protein kinases (MAPKs) (Bu et al. 2007, Weber et al. 2005b), janus kinases (JAKs) (Xuan et al. 2001), signal transducers and activators of transcription (STATs) (Butler et al. 2006), nuclear factor-kappa B (NFκB) (Chen et al. 2008, Valen et al. 2000), and hypoxia-inducible factor-1 alpha (HIF-1α) (Eckle et al. 2008). Many of the downstream effector mechanisms of preconditioning directly counteract cell death by shifting the excitatory / inhibitory balance toward inhibition and generation of ROS scavengers, anti-apoptotic proteins and inflammatory protein antagonists. Effectors may also improve energy supply by improving energy metabolism and blood supply, through generation of vascular endothelial growth factor (VEGF) (Addya et al. 2005), erythropoietin (EPO) (Malhotra et al. 2006) and endothelium-

derived NO (Xuan 2007), as well as enhancing survival through growth factors and trophins such as BDNF (Lee et al. 2008, Ma et al. 2006).

Several extensive enquiries have demonstrated that reoxygenation or reperfusion, as occurs during hypoxia or ischaemia, with or without preconditioning, naturally activates an oxygen and redox sensitive family of transcription factors known collectively as hypoxia-inducible factors (HIFs) that may be implicated in ischaemic preconditioning. Whether HIFs play a role in xenon-induced preconditioning will be explored in this thesis.

1.5 HYPOXIA-INDUCIBLE FACTORs

The transcriptional activator HIF-1 is widely appreciated as the master regulator of oxygen homeostasis and plays a crucial role in the physiological response to counteract hypoxia; accordingly, modulation of its activity is a potent way to improve the outcome of a broad range of hypoxia-related pathologies, including HI injury. The discovery of HIF-1 arose through the observation that hypoxia, anaemia, polycythaemia or cobalt chloride administration induces transcription of the EPO gene as well as production of the biologically active protein, measured by an increase in serum EPO levels and accompanying elevation in oxygen carrying capacity (Goldberg, Dunning & Bunn 1988). Subsequent studies coordinated by Semenza et al. (1991) facilitated the identification of 50 base pair sites located in the 3'-flanking sequence of the EPO gene (now termed the hypoxia-responsive element (HRE)) that were able to bind hypoxia-inducible nuclear factors. Today, the hypoxia-inducible nuclear factor is known as HIF-1 (Semenza 1994). HIF-1 was later demonstrated not to be unique to the EPO gene but able to bind DNA and activate gene transcription in non-EPO producing cells, providing the initial line of evidence that HIF-1 DNA binding capacity is based on a common recognition

sequence of a ubiquitous cell response to hypoxia. HIF-1 transcription factor is fundamental to determining the life or death of various cells. It orchestrates not only the physiological response to counteract hypoxia, but also normal early embryonic vascular development (Iyer et al. 1998).

1.5.1 Molecular structure

HIF-1 has a heterodimeric structure, composed of a HIF-1α subunit that is sensitive to changes in oxygen tension, and a constitutively expressed, oxygen-independent HIF-1β subunit. Both subunits of HIF-1 are members of the basic-helix-loop-helix (bHLH) superfamily of eukaryotic transcription factors; the basic domain permits binding to DNA and the helix-loop-helix (HLH) structure, a common thread found in many transcription factors, mediates subunit dimerisation. HIF-1 also contains a region of amino acid homology termed the per-ARNT-sim (PAS) domain found in numerous species including prokaryotes, indicating that HIF-1 is highly evolutionarily stable and essential (Wang, Semenza 1995); it may function as an auxiliary DNA binding, dimerisation or transactivation domain. The acronym PAS represents the first three transcription factors identified in this superfamily from the *Drosophila melanogaster* fruit fly: period (per), a circadian rhythm-regulating protein; aryl hydrocarbon receptor nuclear translocator (ARNT), the dioxin receptor dimerisation partner; simple minded (sim), a regulator of neuronal development (). Incidentally, further elucidation of HIF-1β demonstrated it to be identical to ARNT differing solely on nomenclature (Semenza 1994).

In addition to the bHLH-PAS domains localised at the N-terminal half of the HIF-1 structure, HIF-1α also contains two transactivation domains (TADs), one at the N-terminus (NTAD) and the second located near the C-terminus (CTAD) of the protein structure, required for interaction with the gene transcription co-activators CBP /

p300 (Lando et al. 2002). Located between the TADs is the oxygen-dependent degradation (ODD) domain that is responsible for the five minute half-life of HIF-1α (Salceda, Caro 1997) as it contains "PEST" motifs (sequences rich in proline (P), glutamic acid (E), serine (S) and threonine (T)) that are frequently found in proteins that display a short half-life, often of less than 2 hours (Rechsteiner, Rogers 1996); deletion of the ODD domain results in HIF-1α stability in the presence of oxygen. HIF-1α additionally contains a C-terminal nuclear localisation signal (CNLS) that is crucial for its transportation into the nucleus (Kallio et al. 1998) (Figure 4).

HIF-1α and HIF-1β have been found in all human and mouse tissues tested with an additional variant of HIF-1β, also called ARNT2, found in brain and kidney. Three HIF-α isoforms have been identified so far, designated HIF-1α, HIF-2α and HIF-3α, and all dimerise with HIF-1β to bind to the hypoxia-responsive element (HRE) of target genes. HIF-3α, however, contains an inhibitory PAS domain that lacks transcriptional activation capacity and instead acts as a dominant negative regulator of HIF-1α, by binding to the HIF-1α NTAD and inhibiting its DNA binding activity (Makino et al. 2001). HIF-2α was discovered from the elucidation of the sequence of the human genome; HIF-1α and HIF-2α isoforms share similar amino acid identities within the bHLH-PAS and TADs and therefore both dimerise with HIF-1β and bind to DNA. Despite their structural and regulatory similarities, these two isoforms are likely to play unique and non-redundant roles in the organism. The HIF-2α structure shows an additional cysteine residue in the basic domain that may account for specific redox regulation of DNA binding (Lando et al. 2000), which may result in binding to its own unique set of target genes. Evidence suggests that

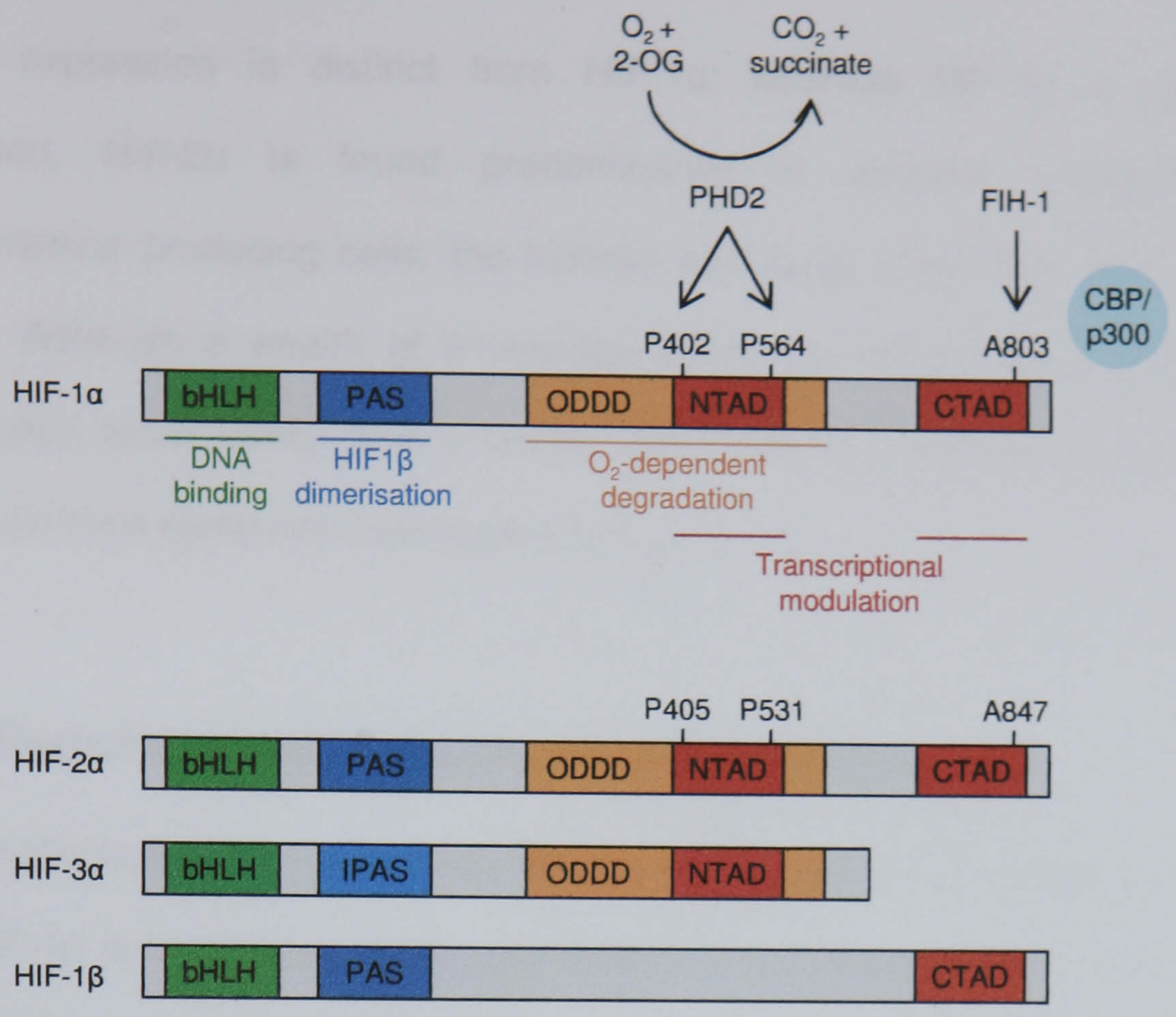


Figure 4. Schematic representation of the domain structures of the hypoxia-inducible factor family and sites of regulation in normoxia

All members of the HIF family of transcription factors contain bHLH and PAS domains, required for DNA binding and dimerisation to HIF-1β. Each of the 3 HIF-α isoforms, contain ODDDs and TADs, required for oxygen-dependent degradation and transcriptional activity, respectively. However, HIF-3α lacks transcriptional activation capacity, as it contains only one TAD and the PAS domain is inhibitory.

In the presence of oxygen the PHD2 enzyme hydroxylates HIF-1α proline residues P402 and P564, or HIF-2α proline residues P405 and P531, resulting in its degradation. Furthermore, asparaginyl hydroxylation by FIH-1 prevents HIF-α subunits from associating with transcriptional co-activators, such as CBP/p300.

Abbreviations: 2-OG, 2-oxoglutarate; **A**, asparaginyl residue; **bHLH,** basic helix-loop-helix; CBP/p300, CREB binding protein and p300; **CTAD,** C-terminus transactivation domain; **FIH-1,** factor inhibiting HIF-1; **HIF,** hypoxia-inducible factor; **IPAS,** inhibitory Per-ARNT-Sim; **NTAD,** N-terminus transactivation domain; **ODDD,** oxygen-dependent degradation domain; **P,** proline residue; **PAS,** Per-ARNT-Sim; **PHD2,** prolyl hydroxylase domain-containing 2 enzyme.

HIF-2α expression is distinct from HIF-1α; whereas HIF-1α is ubiquitously expressed, HIF-2α is found predominantly in vascular endothelial cells, catecholamine producing cells, the kidneys and lungs (Tian, McKnight & Russell 1997). Although a wealth of knowledge exists for HIF-1α, the founder of this transcription factor family, less is known about HIF-2α but these two isoforms are likely to perform some non-overlapping functions.

1.5.2 Regulation of hypoxia-inducible factors

Activation of HIF-dependent transcription is chiefly dependent on regulation of its HIF-1α subunit by synthetic and degradative pathways. HIF derives its name from hypoxia-mediated induction but many other factors, endogenous and exogenous, occurring during both normoxia and hypoxia, are involved in its regulation at different junctures.

1.5.2.1 Transcription of HIF-1α mRNA

HIF-1α is encoded by the *HIF1A* gene located on chromosome 14q21-q24 (Semenza et al. 1997), with human and mouse HIF-1α sharing 90 % amino acid, and 80 % nucleotide sequence, conservation (Iyer et al. 1998). Sequencing of the *HIF1A* gene has shown the promoter region to be rich in guanine-cytosine (GC) content but lacking in thymine-adenine-thymine-adenine (TATA) sequences, and the transcription initiation site to contain multiple points for binding of the nuclear factor Sp1; these are characteristics frequently observed in ubiquitously expressed genes, where Sp1 belongs to a family of transcription factors that maintain basal transcription of "house keeping" genes (Samson, Wong 2002).

HIF-1α mRNA transcription has generally been accepted as constitutively maintained regardless of oxygen availability (Kallio et al. 1997). More recently

however, HIF-1α mRNA levels have been reported to be transiently increased by acute but not delayed hypoxic preconditioning (Shao, Gao & Lu 2005). The reasons behind these discrepancies remain unclear but emphasises that though acute and delayed preconditioning may utilise similar biochemical components, they occur through distinct mechanisms. A possible mechanism for increasing HIF-1α mRNA expression may be via the transcription factor nuclear factor kappa B (NFkB), since hypoxia is able to activate NFkB (Koong, Chen & Giaccia 1994), and the p50 and p65 NFkB subunits have been demonstrated to bind to the promoter region of the *HIF1A* gene, that results in a transient increase in HIF-1α mRNA expression (Belaiba et al. 2007). Interestingly however, It has been postulated that the promoter region of the *HIF1A* gene, upstream from the transcription initiation site, may contain sequences that activate as well as inhibit its own transcription in a cell type-specific manner (Minet et al. 1999); furthermore, hypoxic upregulation may occur without stabilisation of the HIF-1α mRNA into translation (Wenger et al. 1997). To date, however, knowledge concerning the effect of anaesthetic preconditioning on HIF-1α mRNA expression is scarce; the use of isoflurane in the hepatoma cell line (Hep3B) was shown to induce HIF-1α protein but not HIF-1α mRNA (Li et al. 2006).

1.5.2.2 Translation of HIF-1α protein

Translation of most eukaryotic proteins requires the interaction of its mRNA 5' CAP sequence with the eukaryotic initiation factor (eIF) -4F translation initiation complex that on assembly, recruits ribosomal units to commence translation, after recognising the AUG initiation codon (Scheper, Voorma & Thomas 1992). eIF-4F consists of three components: the CAP binding protein eIF-4E, the scaffold protein eIF-4G and the ATP-dependent helicase eIF-4A. The rate-limiting factor of protein synthesis is dependent on eIF-4E binding proteins (4E-BPs) that sequester and strictly regulate eIF-4E availability for the translation initiation complex formation.

4E-BP phosphorylation is mainly controlled by signalling from the mammalian target of rapamycin (mTOR) pathway that lowers its affinity for eIF-4E, resulting in assembly of eIF-4F and facilitation of protein synthesis (Gingras, Raught & Sonenberg 2001) (Figure 5).

In aiming to reduce energy consumption, hypoxia is generally associated with inhibition in protein translation, which aims to sustain survival during low ATP production. This is achieved through a decrease in mTOR signalling and, therefore, hypo-phosphorylation of 4E-BP and associated eIF-4E sequestration (Richter, Sonenberg 2005). However, a decrease in HIF-1α mRNA translation is spared during hypoxia, through an additional mechanism independent of mTOR and eIF-4E binding to the mRNA 5' CAP sequence. This alternative mode is through an internal ribosome entry site (IRES), a nucleotide sequence located in the 5' untranslated region (UTR) that allows for CAP-independent translation; this occurs by allowing ribosome binding directly to the mRNA. Few mammalian mRNAs contain IRES except for those whose translation is required during stresses, such as hypoxia or glucose deprivation (Lang, Kappel & Goodall 2002).

Stimulation of HIF-1α protein translation has been shown to be inducible by growth factors, oncogenes and cytokines. Expression of epidermal growth factor (EGF) in human prostate cancer cells lacking the phosphatase and tensin homolog (PTEN) tumour suppressor has been observed to result in autocrine stimulation of tyrosine kinase receptor signalling with downstream induction of HIF-1α protein expression; this growth factor-induced HIF-1α is sensitive to the phosphoinositide-3 kinase

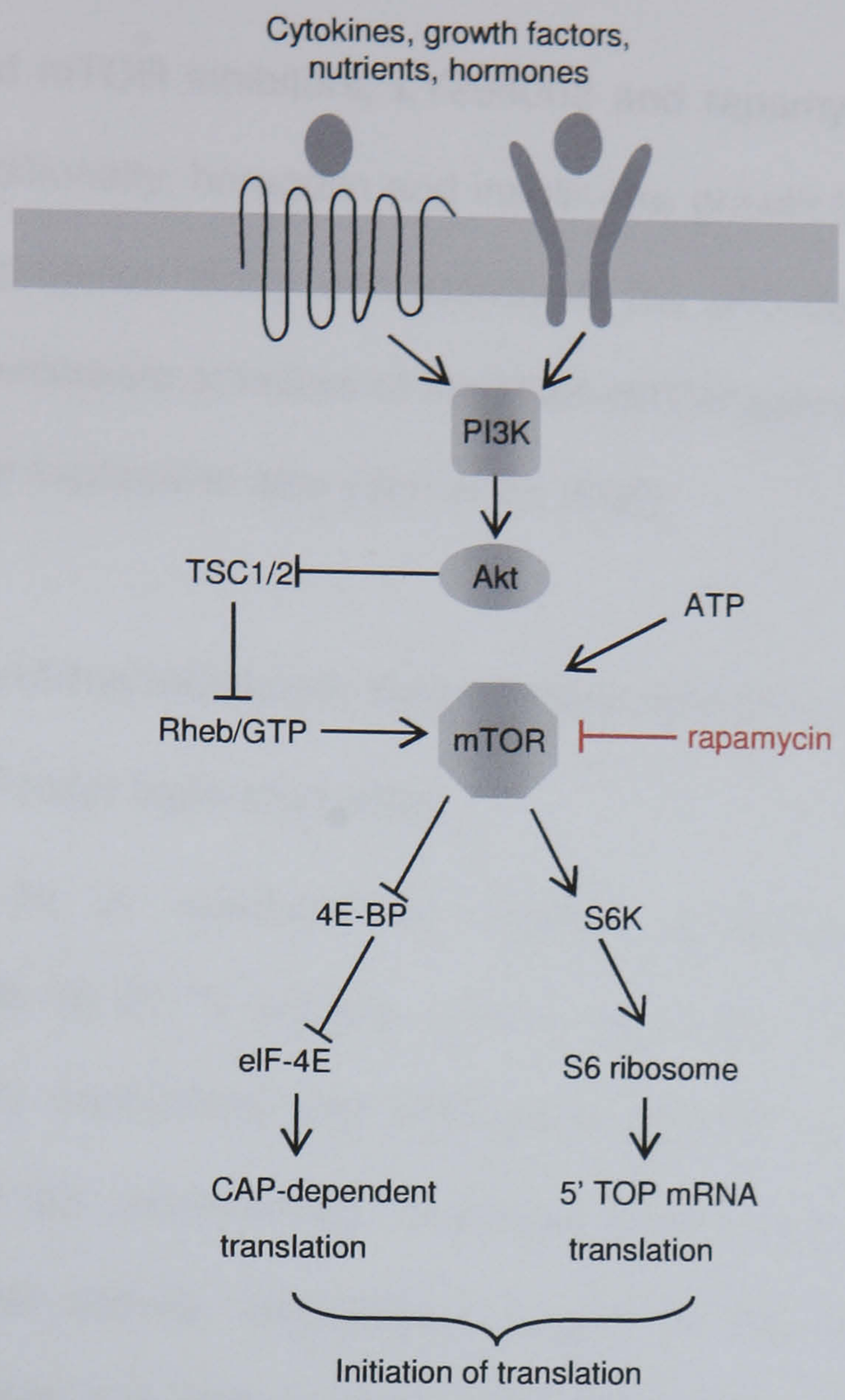


Figure 5. The mammalian target of rapamycin protein translation initiation pathway

This is a simplified schematic diagram illustrating the mTOR protein translation initiation pathway. mTOR-dependent signalling may be regulated by growth factors and nutrients, and protein kinase pathways such as the PI3K / Akt pathway. Akt activity phosphorylates TSC2, which inhibits the TSC1/2 complex and subsequently, disinhibition of Rheb/GTP. Modulation of mTOR leads to phosphorylation of 4E-BP and S6K. 4E-BP phosphorylation lowers its affinity for the CAP binding protein, eIF-4E. Availability of eIF-4E leads to the assembly of a translation initiation complex, facilitating protein synthesis. Through an independent pathway, S6K activity also leads to increased protein translation of 5' TOP mRNAs. HIF-1α contains both 5'CAP and 5'TOP sequences. **Abbreviations: PI3K**, phosphoinositide-3 kinase; **TSC**, tuberous sclerosis complex; **ATP**, Adenosine triphosphate; **mTOR**, mammalian target of rapamycin; **4E-BP**, eIF-4E-binding protein; **eIF-4E**, eukaryotic initiation factor-4E; **S6K**, S6 kinase; **TOP**, 5'-terminal oligopyrimidine-rich tract.

(PI3K) and mTOR inhibitors, LY294002 and rapamycin respectively (Zhong et al. 2000). Additionally, heregulin and insulin-like growth factor-1 (IGF-1) induce HIF-1α protein expression along with activity of the p70 S6 kinase and eIF-4E that are known downstream effectors of the PI3K-mTOR pathway, with an increase in HIF-1 target gene expression also (Semenza 2000).

1.5.2.3 Post-translational degradation of HIF-1α

1.5.2.3.1 Prolyl hydroxylation

HIF-1α is regulated by oxygen concentration where it is virtually undetectable at 21 % oxygen. During normoxia, both HIF-1α and HIF-1β are constitutively transcribed and translated, but HIF-1α is also post-translationally modified to be constitutively degraded, which results in abrogation of HIF-1 transcriptional activity. Degradation begins by the hydroxylation of two proline residues (P402 and 564) (Masson, Ratcliffe 2003) embedded in conserved amino acid motifs within the HIF-1α ODD domain. This prolyl hydroxylation reaction was first unearthed in the model organism *Caenorhabditis elegans* to be catalysed by the egg laying defect-9 (EGL-9) dioxygenase enzyme, where *egl-9* mutant worms displayed constitutive HIF-1α expression (Epstein et al. 2001). Since then, three mammalian *egl-9* homologues have been identified and named prolyl hydroxylase domain containing enzymes 1, 2 and 3 (PHD 1, 2 and 3) with a fourth PHD recently identified (Oehme et al. 2002). These enzymes are members of the 2-oxoglutarate (2-OG) -dependent dioxygenase superfamily and share biochemical similarities to the collagen prolyl-4-hydroxylases that also utilise molecular oxygen as a substrate for hydroxylation, as well as requiring iron and ascorbate as cofactors (Schofield, Zhang 1999). However, unlike PHDs, collagen prolyl-4-hydroxylases are unable to catalyse HIF-1α proline hydroxylation (Jaakkola et al. 2001). The half-life of HIF-1α has been shown to be increased by 2-OG analogues that inactivate PHDs with

PHD2 demonstrated to be the dominant enzyme controlling HIF-1α (Ivan et al. 2002), or iron chelators and metal ions that reduce or substitute iron from its binding site (Masson, Ratcliffe 2003). The PHD enzymes catalyse the splitting of molecular oxygen, transferring one oxygen atom to the HIF-1α proline residue, and reacting the other with 2-OG to generate succinate as a component of the Kreb's cycle, with carbon dioxide as a by-product (Masson, Ratcliffe 2003). Ascorbate is also important for optimising full PHD enzymatic activity by maintaining iron in the ferrous state (Bruick, McKnight 2001) (Figure 6).

1.5.2.3.2 Polyubiquitination

The conformational change of proline-hydroxylated HIF-1α represents a recognition signal to the von Hippel-Lindau (VHL) tumour suppressor protein, whose β-domain interacts directly with the HIF-1α ODD domain, targeting it for destruction by the ubiquitin-proteasome system (UPS) (Stolze, Mole & Ratcliffe 2006). Mutations of both alleles of the *VHL* gene are associated with the hereditary von Hippel-Lindau disease, a cancer syndrome marked by sporadic development of renal cell carcinomas and highly vascularised tumours that display constitutive expression of HIF-1α and aberrant production of its target genes (Iliopoulos et al. 1996). On binding to HIF-1α, VHL associates with the proteins elongin B and C, cullin-2 and Rbx-1 to form a multi-protein E3 ligase complex, which signals for the polyubiquitination of HIF-1α, irreversibly subjecting it to proteolytic degradation by the 26S proteasome (Kallio et al. 1999). To constitutively express and degrade HIF-1α is an energy consuming burden on the cell but this mechanism also allows for a rapid, energy-efficient response to hypoxia that may occur within minutes and even seconds. (Ravi et al. 2000) proposed the existence of a VHL-independent

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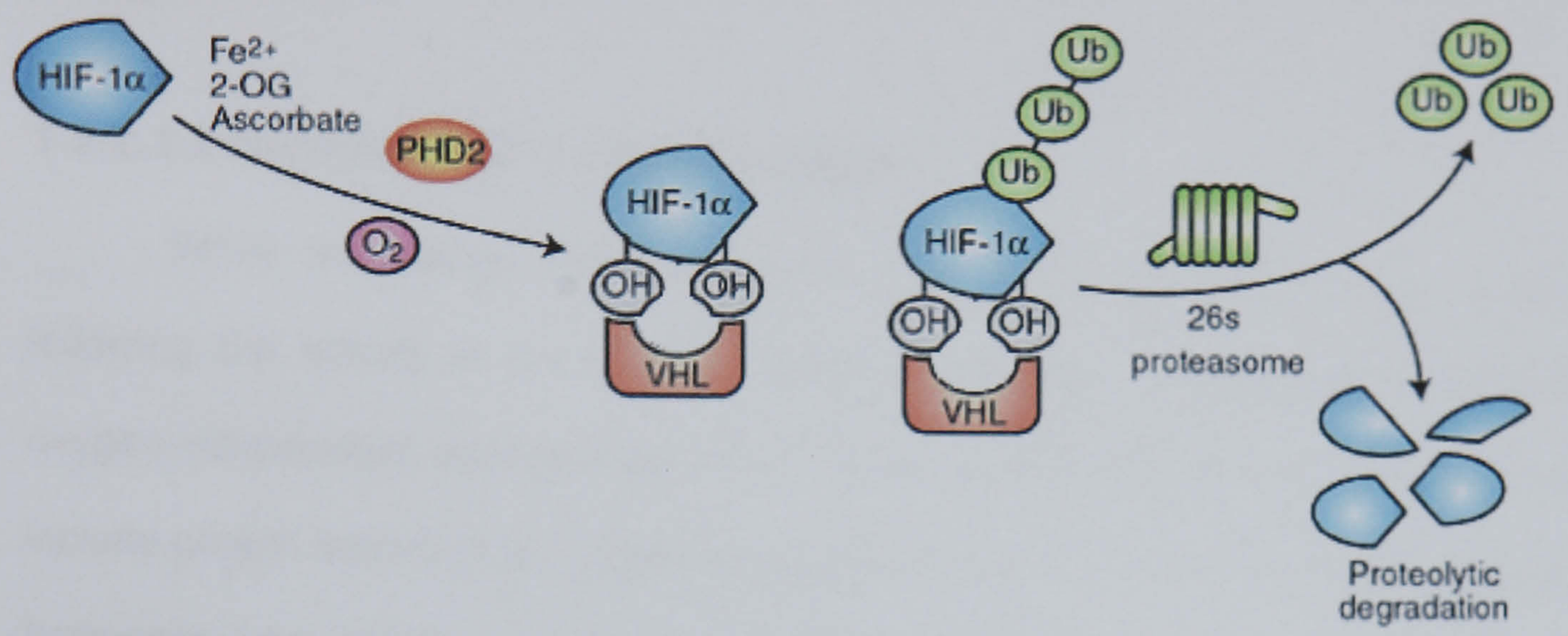


Figure 6. Oxygen-dependent post-translational degradation of HIF-1α

HIF-1α is classically regulated by oxygen availability. In normoxia, PHD2 enzymes hydroxylate HIF-1α proline residues (P402 and P564) (where PHD2 requires oxygen as a co-substrate, as well as iron, 2OG and ascorbate as cofactors). Hydroxylated HIF-1α is recognised by the VHL E3 ubiquitin ligase complex, which leads to the polyubiquitination of HIF-1α, irreversibly subjecting it to proteolytic degradation by the 26S proteasome. **Abbreviations: HIF-1α**, hypoxia-inducible factor-1 alpha; **HIF-1β**, hypoxia-inducible factor-1 beta; **HRE**, hypoxia-responsive element; **p300/CBP**, p300 and CREB binding protein; **PHD2**, prolyl hydroxylase domain-containing 2 enzyme; **vHL**: von Hippel-Lindau (modified from (Gidday 2006)).

pathway affecting HIF-1α stability, which depends on another tumour suppressor P53 enhancing the murine double minute-2 (MDM2) -mediated polyubiquitination and degradation of HIF-1α, where homozygous deletion of P53 in tumour cells enhances HIF-dependent gene transcription and neovascularisation of tumours (Ravi et al. 2000).

1.5.2.3.3 Normoxic HIF-1α accumulation

While knowledge regarding HIF-1 has been derived through studies following the effects of low oxygen, there is increasing evidence demonstrating oxygen-independent accumulation of HIF-1α by a variety of normoxic stimuli; these include growth factors (*e.g.* insulin-like growth factors (Feldser et al. 1999), anabolic hormones (*e.g.* insulin (Stiehl et al. 2002)), neurotransmitters (*e.g.* acetylcholine (Hirota et al. 2004)) and nitric oxide (Sandau et al. 2001) as well as pharmacological agents such as desferrioxamine (Wang, Semenza 1993). Although dependent on cell-type, signalling pathways shown to stimulate HIF-1α synthesis have involved MAPK (Zagorska, Dulak 2004) as well as PI3K (Li et al. 2004). Furthermore, many of the normoxic stimuli of HIF-1α are also known to activate other transcription factors such as NFκB; furthermore, the HIF-1α promoter contains NFκB binding sites, raising the possibility that cross-talk between transcription factors may occur. However, the mechanisms for HIF-1α accumulation under those settings are not yet fully elucidated.

1.5.3 Transcription of HIF target genes

HIF-1α accumulation, whether due to changes in synthesis or degradation, leads to its translocation to the nucleus for dimerisation to its HIF-1β subunit, forming the HIF transcription factor. For transcriptional activity, HIF binds to specific hypoxia-response elements (HRE) found in its target genes (5'-RCGTG-3'). This binding is necessary yet not sufficient for full transcriptional activity. HIF-1 related gene transcription also requires interaction with other transcription factors at adjacent DNA-binding sites such as hepatic nuclear factor 4 (Semenza 1994) as well as interaction with co-activators CBP / p300 histone acetyltransferases, SRC-1 and TIF-1, that are themselves regulated by oxygen concentration and redox state (Gray et al. 2005). Under normoxia, HIF-1α stability is further regulated by hydroxylation of the asparaginyl residue A803 by the asparaginyl hydroxylase factor inhibiting HIF-1 (FIH) that prevents its association with CBP / p300 and attenuates transactivation of HIF-1-target genes (Lando et al. 2002) (Figure 4). Hypoxia attenuates FIH-1 activity that allows for CBP / p300 association with HIF-1α and consequent transcriptional activity. Similar to PHDs, FIH-1 also utilises oxygen as a substrate and requires 2-OG, iron and ascorbate for enzymatic activity (Figure 7).

To date, HIF-1 has been reported to control more than one hundred target genes with diverse functions that collectively promote cellular adaptation to hypoxia (Semenza 2003). These genes may be nominally classified into three categories. Firstly, those that lead to cell proliferation and survival (*e.g.* insulin-like growth factors); secondly, those that decrease oxygen consumption within the cell (*e.g.* glycolytic enzymes for anabolic metabolism; and thirdly, those that enhance delivery of oxygen to cells, either swiftly through increased erythropoiesis (*e.g.* EPO, transferrin) or through angiogenesis (*e.g.* VEGF) for a more permanent oxygen delivery solution.

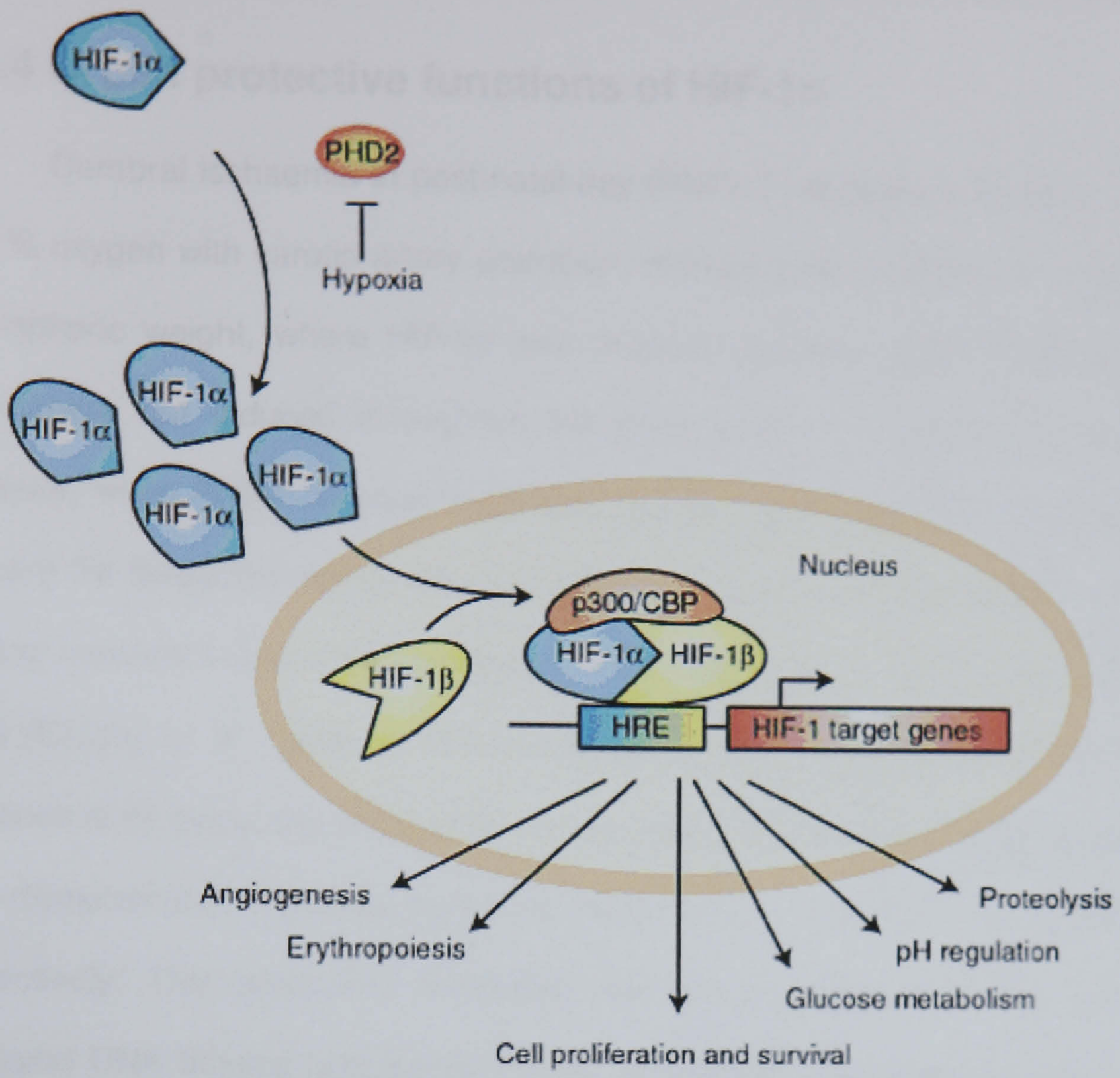


Figure 7. HIF-1α accumulation results in HIF-1 transcriptional activity

HIF-1α is classically regulated by oxygen availability. In hypoxia, PHD2 enzymatic activity is inhibited due to the absence oxygen as a co-substrate, resulting in HIF-1α accumulation rather than degradation. Accumulated HIF-1α translocates into the nucleus for heterodimerisation to HIF-1β. This results in formation of the HIF-1 transcription factor, which binds to the HRE of its target genes. Transcriptional co-activators, such as CBP/p300, interact with HIF-1, leading to transcription of the target genes. **Abbreviations: HIF-1α**, hypoxia-inducible factor-1 alpha; **HIF-1β**, hypoxia-inducible factor-1 beta; **HRE**, hypoxia-responsive element; **p300/CBP**, p300 and CREB binding protein; **PHD2**, prolyl hydroxylase domain-containing 2 enzyme. (Modified from (Gidday 2006)).

1.5.4 Organ protective functions of HIF-1α

Cerebral ischaemia in post-natal-day (PND) 7 rat pups, induced by 3 hours of 8 % oxygen with carotid artery occlusion, showed a 40 % reduction in ipsilateral hemispheric weight, where HIF-1α was found to be decreased in the ipsilateral hemisphere but induced throughout the brain in the contralateral hemisphere. However, when PND7 rat pups underwent hypoxic preconditioning (3 hours of 8 % oxygen) 24 hours preceding cerebral ischaemia, there was a dramatic protection against cerebral infarct that correlated with an induction of HIF-1α throughout the brain (Gidday et al. 1994). In further support to the concept that HIF-1α confers tolerance to HI injury, the proven HIF-1α inducers cobalt chloride or desferrioxamine were demonstrated to protect against cerebral HI injury by as much as 76 and 56 %, respectively. This protection correlated with induced HIF-1α in the brain that displayed DNA binding and transcriptional activity with expression of HIF-1 target genes (Sharp, Bernaudin 2004). Therefore, HIF-1α induction is relevant to both hypoxic as well as pharmacological preconditioning. However, iron chelators themselves, though effective, are unlikely to be pursued further for stroke therapy in the clinical setting, as iron is a cofactor for a host of important cellular functions; its inhibition is likely to have side effects that may be too toxic for patients (Ratan et al. 2004). HIF is believed to regulate the response to hypoxia and other stresses by orchestrating the transcription of its target genes where several have already been directly shown to protect against HI injury, including EPO and VEGF, which will now be discussed as possible mechanisms for the cytoprotective effects of HIF-1α.

1.5.4.1 Erythropoietin

Hypoxia increases the HIF driven expression of erythropoietin (EPO), a glycoprotein initially identified as a haematopoietic growth factor that stimulates the production of erythrocytes and oxygen delivery to tissues (Semenza et al. 1991). Foetal EPO production occurs in the liver but production in the adult takes place mainly in kidneys (Jelkmann 2004). Administration of exogenous EPO has an excellent safety profile and is used to treat anaemic patients with renal insufficiency in adults as well as infants; it is, however, also misused as a potent performance-enhancing drug amongst endurance athletes with the premise that it increases oxygen carrying capacity to exercising muscles. A growing body of evidence is mounting observing the widespread expression of EPO and its receptor in rodent as well as human tissue, including brain, provoking interest in a role for EPO beyond haematopoiesis (Chen et al. 2007, Grimm et al. 2006, Noguchi et al. 2007). EPO receptors have been found to be present in undifferentiated human embryonic neuroepithelial cells and, accordingly, EPO has been found to regulate neurogenesis through production of neuronal progenitors from neural stem cells (Shingo et al. 2001), as well as promoting progenitor cell differentiation into neurons, (Wang et al. 2004b), astrocytes (Lee et al. 2004) and oligodendrocytes (Sugawa et al. 2002). The centrality of EPO to successful brain development is exemplified when mice lacking EPO receptor expression exhibit severe anaemia and widespread neuronal apoptosis with death at embryonic day 13.5 (Yu et al. 2002). The adult brain displays only a low basal level of EPO expression but upon brain insult and hypoxia, a dramatic rise in transcription and translation of EPO and its receptor occurs (Digicaylioglu, Lipton 2001) that promote multi-factorial beneficial effects in the central nervous system, including anti-apoptosis (Masuda et al. 1994), anti-oxidation (Chattopadhyay et al. 2000), and neurotrophic effects (Campana, Misasi & O'Brien 1998). EPO is a clinically promising neuroprotective candidate

because induction of EPO is also found in preconditioned brains (Malhotra et al. 2006) and exogenous EPO administration is protective against HI injury *in vitro* (Meloni et al. 2006) and *in vivo* (Sakanaka et al. 1998) as well as being successfully used in pilot studies on patients with acute ischaemic stroke (Grasso et al. 2007).

1.5.4.1.1 EPO beyond haematopoiesis

The activity of EPO is mediated through its specific cell surface receptor that belongs to the superfamily of type 1 cytokine receptors (Ihle 1995). The EPO receptor exists as an inactive homodimer complex with the cytoplasmic portion in association with the Janus family tyrosine kinase JAK-2 (Tilbrook, Klinken 1999). Binding of EPO enables autophosphorylation of the EPO receptor-associated JAK-2 that consequently cascades to signal transduction pathways including STAT5 (Sola et al. 2005), MAPKs (Guillard et al. 2003), NFκB (Digicaylioglu, Lipton 2001)and PI3K (Zhande, Karsan 2007). EPO-induced activity of these pathways results in reduction in infarct volume after middle cerebral artery occlusion (MCAO) in rats with a dramatic reduction in apoptosis within the ischaemic penumbra, through increased expression of the anti-apoptotic protein Bcl-xL (Siren et al. 2001); furthermore, infusion of EPO neutralising antibodies after mild ischaemia results in apoptosis and neuronal degeneration (Sakanaka et al. 1998) and inhibition of MAPK and PI3K abrogate the observed EPO-induced survival against HI cell death (Siren et al. 2001). Beyond preventing apoptosis, EPO-mediated cytoprotection may include effects on transiently increasing intracellular calcium concentration; five minute administration of EPO 8 hours prior to injury dose-dependently increased neuronal cell viability from glutamate toxicity but this effect was lost on simultaneous addition of a calcium chelator, ethylene glycol tetra-acetic acid (EGTA), suggesting that calcium influx is a critical event in the mechanism of EPO-enhanced resistance to injury (Sakanaka et al. 1998). An anti-oxidant effect of EPO has also been

reported through restoration of cytosolic catalase and glutathione peroxidase anti-oxidant activity, that detoxify ROS and protect the cell membrane from lipid peroxidation (Chattopadhyay et al. 2000). Furthermore, brain endothelial cells have been shown to respond to EPO and express EPO receptors with consequent neovascularisation and capillary tube formation, that may increase perfusion and brain haemodynamics to aid metabolically compromised tissue after experimental stroke (Ribatti et al. 1999). Finally, EPO administration after brain injury leads to the increased expression of BDNF that may enhance neurogenesis (Wang et al. 2004a). Therefore, EPO is multi-dimensional in its possible cytoprotective mechanisms.

1.5.4.2 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a polypeptide belonging to a subfamily of angiogenic growth factors that exerts powerful mitogenic activity on endothelial cells from arterial, venous and lymphatic vessels (Leung et al. 1989). VEGF is well established to specifically stimulate vascular endothelial cell microvessel outgrowths and tri-dimensional angiogenesis as demonstrated in an *in vitro* model using rat aortic rings (Nicosia, Nicosia & Smith 1994) and *in vivo* on the rat cornea (Phillips, Knighton 1995), playing an essential role in embryogenic vessel formation, as well as physiological angiogenesis associated with metabolism, exercise and wound healing. The discovery of VEGF may be traced back to the late 1800s where tumours were first observed to be highly vascular (Ferrara 2005). The provocation of an angiogenic response by implantation of a tumour into a rabbit's ear raised the question as to whether tumour-derived vascular growth factors may exist, a speculation that consequently lead to the identification of vascular permeability factor (VPF) in 1983, as a soluble protein that induced vascular leakage (Senger et al. 1983); this was later cloned and shown to exhibit mitogenic

activity only to endothelial cells and accordingly renamed VEGF (Ferrara 2002). The pro-angiogenic mechanism of VEGF involves stimulating expression of plasminogen activators, collagenases and metalloproteinases that creates a pro-degradative environment for migration and tissue remodelling of endothelial cells. VEGF also increases vascular permeability that induces plasma protein leakage, which supports endothelial cell growth and energy demands (Ferrara, Davis-Smyth 1997).

1.5.4.2.1 VEGF in the nervous system

As a result of alternative mRNA splicing, there are at least six isoforms of VEGF and two tyrosine kinase VEGF receptors (VEGFR-1 and VEGFR-2) have been identified to date as the mediators of VEGF biological function (Terman et al. 1992). Additionally non-tyrosine kinase transmembrane receptors, called neuropilins, have more recently been identified as another VEGF binding site; first identified in Xenopus tadpoles, these cell-surface glycoprotein receptors are important mediators in guiding axonal extension in the developing nervous system (He, Tessier-Lavigne 1997), and are expressed in endothelial and tumour cells as well as dorsal root ganglion, optic and olfactory nerves and sympathetic neurons (Soker et al. 1998). Neuropilins play a role in embryonic development and angiogenesis; its overexpression leads to morphological anomalies of excessive capillary and nerve fibre sprouting and extra digits in mice (Kitsukawa et al. 1995), whereas neuropilin-deficient mice display impaired neural vascularisation and angiogenesis of major blood vessels (Kawasaki et al. 1999). Transcripts of VEGF are detectable from embryonic day (E) 7.0 in the murine endoderm with accompanying VEGF receptor expression, which increases vascular sprouting to the neuroectoderm in later development of the CNS from E11.5 (Dumont et al. 1995). Initially, VEGF is confined almost exclusively to cortical neurons, but as vascular beds mature, expression moves to glial cells that are in close proximity to vessels

(Ogunshola et al. 2002). In the adult, VEGF and its receptors are expressed at a minimal basal level throughout the whole organism but expression is inducible in the CNS by hypoxia through the activity of kinase signalling pathways, such as PKC (Ijichi, Sakuma & Tofilon 1995). Expression of VEGF in the CNS is necessary for angiogenesis and subsequent transport of oxygen and nutrients to the developing brain and nervous system, where it dose-dependently results in vasculo-genesis and branching of vessels, as well as an increase in vessel lumen size (Silverman et al. 1999). Beyond vascular effects, VEGF is directly neurotrophic and proliferative on neurons and glia throughout the brain, promoting the proliferation of neural stem cells and supporting axonal outgrowth, with its effects likely to be mediated through multiple signalling pathways, including PI3K and PKC (Zhu et al. 2003).

1.5.4.2.2 Neuroprotective and neurotrophic effects of VEGF

Interestingly, VEGF is also directly neuroprotective in an *in vitro* rat ischaemia model where its exogenous administration doubles the number of surviving cells after 24 hours of oxygen and glucose deprivation, an effect that was reversed in the presence of the PI3K inhibitors, wortmannin or LY294002 (Jin et al. 2000). Endogenous VEGF has also been found to rescue hippocampal neurons from glutamate-induced excitotoxicity (Matsuzaki et al. 2001) and its increased expression is observed after HI injury, with a contiguous increase in voltage-gated potassium channel expression also. In a rat model of middle cerebral artery occlusion (MCAO), VEGF was observed to attenuate cell death through PI3K and tyrosine phosphorylation of potassium channels; anti-sense VEGF suppression or PI3K inhibition during MCAO attenuated potassium channel activity, which was subsequently found to also be associated with decreased cell viability (Qiu, Zhang & Sun 2003). Evidence for PI3K-independent mechanisms of VEGF-mediated survival also exists. Cell viability of cultured neurons exposed to 24 hours of hypoxia may be

increased to a similar extent by administration of VEGF or a caspase-3 inhibitor: VEGF was found to inhibit caspase-3 activation and accordingly anti-sense VEGF increased the proportion of caspase-3 expressing neurons, that corresponded with an increase in cell death (Jin et al. 2001); as such, VEGF may also be regarded as an anti-apoptotic molecule.

HIF activity on the HRE of the VEGF gene has been shown to be crucial for protection against hypoxia-induced toxicity, but it is also critical in motor neuron function. Deletion of the VEGF HRE resulted in slow growth, decreased survival and impaired hypoxia-induced VEGF, that progressively resulted in motor neuron degeneration and muscle atrophy in an adult mouse model, similar to the symptoms of the rare neurodegenerative disease amyotrophic lateral sclerosis, alternatively known as Lou Gehrig's disease (Oosthuyse et al. 2001). The defects were observed to be due to a loss of myelination, caused primarily by a decrease in neural vascular perfusion and, therefore, chronic ischaemia. Exogenous administration of VEGF to the cerebrospinal fluid of these mice improved motor performance and decreased progression to paralysis with a significant increase in survival time also (Storkebaum et al. 2005).

The clear developmental and anatomical associations between the vascular and nervous systems naturally raise questions of whether, or how, they may be mechanistically coordinated. It remains difficult to resolve whether the stimulatory effects of VEGF on embryonic neurogenesis are independent of its angiogenic role since inactivation of a single VEGF allele in mice is embryonically lethal by day E11.0, stemming from severe anomalous vessel formation (Ferrara et al. 1996). In the adult, however, neurogenesis has strikingly been shown to occur within an endothelial cell-mediated angiogenic niche: the subgranule zone of the adult hippocampus is known to generate new neurons throughout life; proliferation of

neural stem cells within this zone were not only observed to be located in dense clusters that surrounded the vasculature, but additionally displayed immunoreactivity to endothelial cell markers (Palmer, Willhoite & Gage 2000). Furthermore, a causal coordination between angio- and neuro- genesis has been observed in the adult songbird brain where neurogenesis in the higher vocal centre proceeds through life. Testosterone in the brain was shown to induce expression of VEGF and its receptor, with associated local endothelial cell-mediated angiogenesis that also produced BDNF one week later, where BDNF is known for its neurotrophic role and support of neuronal differentiation; in addition, VEGF inhibition substantially diminished angio- and neuro- genesis in the songbird's vocal centre (Louissaint et al. 2002).

1.5.4.2.3 VEGF in disease

The discovery of VEGF is very much linked to its role in oncology where its expression is prevalent in many solid tumours including those in lung, breast and colon. Tumour cells able to secrete VEGF exhibit greater microvascular permeability and neovascularisation, which subsequently promotes tumour growth and metastasis (Folkman 1971) and, therefore, the size of a tumour is directly proportional to its vascularity. Accordingly, numerous anti-angiogenic agents are currently under development as potential treatments against solid tumours.

1.6 NON-ORGAN PROTECTIVE FUNCTIONS OF HIF-1

1.6.1 Critical role of HIF in embryogenesis

HIF-1α, HIF-2α and HIF-1β are all required for normal mouse development, where knockouts of either HIF-1α or HIF-2α are embryonically lethal. Homozygous HIF-1α deficient (-/-) mice arrest in development on day E9.0 and die by E10.5 from severe cardiovascular and neural tube defects, with massive and widespread cell death within the brain (Kotch et al. 1999). Heterozygous HIF1α +/- mice are, however, able to maintain normal development but are unable to respond to hypoxia showing impairment in erythrocytosis as well as in pulmonary vascular remodelling (Kline et al. 2002). HIF-2α -/- mice are also embryonically lethal (Tian et al. 1998). In the absence of HIF-1β, mouse development is stunted due to a lack of HIF gene transcription (Maltepe et al. 1997).

1.6.2 Paradoxical role for HIF–1α

HIF-1α is documented in literature to promote not only survival and protection of cells but paradoxically may also promote the death of cells. Its protective role in preconditioning is currently being extensively studied and its ability to induce neuronal survival factors, such as EPO and VEGF, is widely documented. It plays a pivotal role in metabolism, promoting the transcription of glucose transporters as well as glycolytic enzymes. Less documented is the ability of HIF-1α to promote death of cells. It is reported to mediate hypoxia-induced apoptosis through induction of the tumour suppressor P53 (Carmeliet et al. 1998) as well as being able to inhibit the Bcl-2 family member BNIP3 (Bruick 2000). What is unclear is whether the net effect of HIF-1α is to protect or promote infarction. Though evidence is pointing more towards its beneficial role for the organism in the context of preconditioning, the non-preconditioned ischaemic brain undergoes a HIF-1α related ischaemia-induced neuronal death (Halterman, Federoff 1999).

1.6.3 Role in cancer

Regions of poor oxygenation are a frequent feature of solid tumours. Chronic hypoxic exposure occurs as tumour cells proliferate and increase the distance for oxygen diffusion from a vessel to the tumour cell; acute hypoxia also occurs because of the disorganised circulatory system and consequential transient changes in blood flow and, as such, tumour survival is dependent on adaptation to such stresses. Clinical studies have observed that the degree of hypoxia correlates with poor prognosis with increased prevalence of metastasis (Brizel et al. 1997) as cells induced to survive in such an environment have adapted to exhibit reduced apoptotic potential (Sullivan, Graham 2007). The best described response to hypoxia is mediated by HIF-1α and its over expression is pathophysiologically implicated to facilitate tumour adaptation and survival; tumour grade, vascularisation, progression and aggressiveness is reported to be correlated to a high level of HIF-1α protein expression. This increased expression is further correlated with an increase in HIF-1α activity marked by induction of tumour vascularisation, as VEGF controls the angiogenic switch as well as metabolic transition to anaerobic glycolysis, through increased expression of glucose transporters and glycolytic enzymes.

The relevance of HIF-1α to tumour biology is displayed through its relationship with tumour suppressors such as P53 and VHL. In normal oxygen tensions, P53 is recognised by the ubiquitin protein ligase MDM-2 that results in the proteasomal degradation of P53 (Wawrzynow et al. 2007). During hypoxia the accumulation of HIF-1α results in HIF-1α binding to P53 that inhibits MDM2-mediated P53 degradation. Instead, MDM2 targets HIF-1α to proteasomal degradation, leaving P53 free to mediate apoptosis. However, tumours displaying P53 loss-of-function result not only in prevention of hypoxia-induced apoptosis but also decreased MDM-

2-mediated HIF-1α degradation. With VHL tumour suppressor loss-of-function in renal cell carcinomas or cerebellar haemangioblastomas, HIF-1α degradation is impaired and, therefore, the protein level is over expressed resulting in very well vascularised cancers (Kaelin 2007).

1.7 XENON

As mentioned earlier, xenon displays neuroprotective properties through a preconditioning phenomenon and before considering the putative role of HIF-1α in this action, more needs to be revealed about this noble gas.

1.7.1 Physico-chemical properties of xenon

The chemical family of primordial gaseous noble elements-helium, neon, argon, krypton, xenon and radon-has always been intriguing in that their most characteristic property is, rather, their lack of property in forming no compounds. Their choice of name reflects this circumstance as exemplified with xenon, deriving its name from the Greek for "strange one". Xenon gas is extremely rare and constitutes no more than between 0.00005 to 0.0875 parts per million of the earth's atmosphere (at the most, this amounts to 4 mL of xenon in a 50 m^3 room) (Marx et al. 2000).

Its discovery was made late in the history of chemistry, in 1898, by Scottish-born chemist William Ramsey with his student Travers working to understand the high density of atmospheric nitrogen. Deservedly, Ramsey was later awarded the Nobel Prize in chemistry, in recognition of his discovery of the noble gases and his determination of their position in the periodic table. Xenon is a naturally occurring, non-flammable, non-hazardous, colourless and odourless trace gas. It is extracted as a by-product of oxygen manufacture, from fractional distillation of liquefied air. The energy-intensive extraction process and the associated exorbitant cost required to produce xenon reflects on its rarity.

1.7.2 Medicinal uses of xenon

1.7.2.1 Anaesthesia

Interest in the anaesthetic properties of xenon arose in 1939 when the physician Behnke investigated deep sea divers in the United States Navy who displayed "drunkenness" from breathing mixtures of gas that had different narcotic potencies. Because of xenon's scarcity, Behnke could not demonstrate but, nonetheless, calculated that xenon should display anaesthetic effects even at normobaric pressure (Marx et al. 2000). In 1941, a Russian named Lahzarev may have assessed the effect of xenon in man but the work remained unpublished; the first published work was conducted by Lawrence in 1946 showing xenon to be a general anaesthetic in mice (Caffee et al. 1999). Five years later, xenon was demonstrated to possess anaesthetic properties in humans by (Cullen and Gross 1951), when they successfully conducted two surgical procedures under xenon anaesthesia; an orchidectomy in an 81-year-old male and fallopian tube ligation in a 37-year-old female. Loss of consciousness and an increased pain threshold was evident with inhalation of 50 % xenon and pronounced narcosis occurred with 80 % xenon. Despite its success, the prohibitive expense of xenon kept it out of the limelight until the development of the first closed-circuit anaesthetic machines made it potentially more economically viable. Technical improvements in delivering low flow xenon in a closed-circuit system (Dingley et al. 2001) or using xenon recyclers and scavengers (Dingley, Mason 2007), based on thermodynamic processes and molecular sieves, will further enhance the feasibility of clinically exploiting xenon.

Xenon displays many favourable properties of the ideal inhalational anaesthetic, with investigators noting many advantages over nitrous oxide, a routinely used anaesthetic gas; xenon is a small apolar atom with a low blood / gas partition coefficient, able to easily pass through the blood brain barrier for a rapid onset and

offset of action, which is independent of surgical duration (Goto et al. 1998), an advantageous property for an inhalational anaesthetic. It has a minimum alveolar concentration (MAC) of 63 % in humans, or 71 % in mice, with excellent analgesia at sub-anaesthetic concentrations. Xenon provides circulatory stability in that it does not affect heart rate, mean arterial blood pressure or cardiac contractility (Luttropp et al. 1993), nor does it impair the neurohormonal stress response to surgery, measured by an unchanged adrenaline level (Burov et al. 1995). Animal studies show xenon to be devoid of allergic, mutagenic, carcinogenic or foetotoxic properties (Lane et al. 1980). As well as being environmentally sound, by displaying no depletion of stratospheric ozone or contribution to the green house effect, its safety and efficacy profile in clinical anaesthesia remains unrivalled. Its scarcity in the atmosphere, and hence the substantial expense involved in its purification, is the only impediment limiting its widespread use (Sanders, Franks & Maze 2003).

With respect to xenon's mechanism of anaesthesia, a seminal paper by our laboratory demonstrated that xenon is a potent antagonist of the NMDA receptor (Franks et al. 1998), activation of which is pivotal in the propagation of acute neuronal injury, and this may lead to the inhibition of nociception in spinal dorsal horn neurons (Lynch, Guttmann 2002). Xenon has little effect on non-NMDA glutamatergic or $GABA_A$ receptors, nor is its actions affected by antagonists of α_2 adrenergic or opioid receptors; it seems likely, therefore, that xenon's mechanism of analgesia is also through NMDA receptor antagonism. The large size of xenon may contribute to its anaesthetic potency, where neighbouring molecules distort and polarise xenon to induce a dipole that facilitates its interaction with proteins (Trudell, Koblin & Eger 1998).

1.7.3 Possible utility for organ protection

Remarkably, neuroprotective effects of xenon have been demonstrated in various models. A series of *in vitro* studies showed that xenon reduced injury in a mouse neuronal-glial cell co-culture induced by either NMDA, glutamate, oxygen deprivation or oxygen-glucose deprivation (OGD) (Wilhelm et al. 2002), a finding that has been corroborated by other investigators in a different neural crest cell line (Petzelt et al. 2004). Protection by xenon has also been demonstrated in *in vivo* models of acute neuronal injury, involving administration of excitotoxins to rats (Ma et al. 2002), anaesthetic-induced apoptotic neurodegeneration in the neonate (Ma et al. 2007), cardiopulmonary bypass in rats (Ma et al. 2003), middle cerebral artery occlusion in mice (Homi et al. 2003), cardiac arrest in pigs (Schmidt et al. 2005), and hypoxia-ischaemia in neonatal rats (Ma et al. 2006). Xenon is a more efficacious neuroprotective agent than either gavestinel or dizocilpine (MK801) (Ma et al. 2005), two other NMDA antagonists that have been clinically tested, and lacks the toxicity that afflicts other NMDA receptor antagonists (Ma et al. 2002). Furthermore, the effect of xenon neuroprotection is synergistic with hypothermia and is likely to be mediated through inhibition of apoptosis (Ma et al. 2005).

1.8 AIMS

This body of research aims to better understand the mechanisms behind xenon's preconditioning capability by exploring whether HIF-1α may play a role, at least in part, in mediating the organ protection against HI injury that is afforded by xenon preconditioning. This research aims to answer the following specific questions:

I. Does xenon preconditioning affect the expression of HIF-1α?

   If xenon preconditioning affects HIF-1α expression, is the xenon-induced HIF-1α functionally active, and about to transcribe its target genes?

II. What are the mechanisms underlying xenon-induced HIF-1α expression? Specifically, does xenon preconditioning affect HIF-1α transcription, translation or degradation?

III. Is HIF-1α causally-related to xenon-induced organ protection against ischaemic injury?

CHAPTER 2

MATERIALS AND METHODS

2.1 EXPERIMENTAL MODELS

2.1.1 ANIMAL MODELS

This study was approved by the Home Office, United Kingdom, and all animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Every effort was made to minimise animal suffering and the number of animals used.

Ten-week-old C57BL6 male adult mice weighing 20 - 25 grams purchased from B & K Universal Limited (Grimston, Aldbrough, Hull, U.K.), were housed in a special pathogen-free facility at Imperial College London in an automated 12-hour-light / 12-hour-dark diurnal schedule colony room, in a temperature (20 - 22 °C) and humidity (23 %) controlled isolated ventilated chamber. Mice were given free access to irradiated food and sterile water *ad libitum* before the start of experimentation and allowed to familiarise to their new housing environment for three days.

2.1.2 CELL MODELS

2.1.2.1 Solutions

Media Stock (1 L)
100 mL 10X Minimum essential medium (MEM) (Sigma Aldrich)
83 mL Glucose bicarbonate stock
Top up to 1 L with dH_2O

Glucose bicarbonate stock (1 L)
44 g D-glucose
26.3 g $NaHCO_3$
Top up to 1 L with dH_2O

2.1.2.2 Neuronal-glial cell co-culture

The mixed cortical co-cultures containing both neuronal and glial elements were derived from foetal and early postnatal BALB/c mice, respectively. Whole cerebral neocortices were removed from post-natal-day (PND) 1 - 2 BALB/c pups. After trypsination and resuspension, cells were plated at a density of 6.25 x 10^4 cells / cm^2 on 4-multiwall plates (Costar, Cambridge, MA). These glia cells were grown in a culture medium of $PM_{5/5}$ (media stock with 5 % foetal bovine serum, 5 % horse serum, 2 mM L-glutamine, 100 U/mL anti-biotic anti-mycotic solution (Sigma Aldirch)) until reaching confluence, approximately one week after plating. Cortical neuronal cells were obtained from foetal BALB/c mice at gestation day 14 - 16 and plated at a density of 1.25 x 10^5 cells / cm^2 on the confluent monolayer of glia cells, derived from the corresponding genetic mouse strain. Neuronal-glial co-cultures were maintained in medium until reaching confluence one week after plating.

2.1.2.3 Human kidney-2 cell culture

Human kidney-2 (HK-2) cells (European Cell Culture Collection) are derived from a primary culture of human adult male kidney proximal tubular epithelial cells, immortalised by transfection with the E6 / E7 genes of a human papilloma virus-16 that does not change its phenotype or function. The E6 / E7 genes bind to its host's DNA regulatory proteins that promote cell proliferation, but without malignant transformation, as growth of the host's cells remain dependent on anchorage and under normal regulatory control (Ryan et al. 1994). HK-2 cells were maintained in a 75 cm^2 tissue culture flask at 37 °C in RPMI 1640 medium (Invitrogen) supplemented with 10 % foetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine (Invitrogen), and 100 U / mL penicillin-streptomycin (Invitrogen) in a humidified air with 5 % carbon dioxide atmosphere. After reaching confluence, cells were detached from the flask with 0.05 % trypsin / 0.6 mM EDTA solution (Invitrogen) for

2 minutes and activity stopped with the addition of RPMI 1640. The cell suspension was centrifuged at 1300*g* for 5 minutes, resuspended in supplemented RPMI 1640 and plated in 60 mm Petri dishes. Cells were used soon after reaching 80 % confluent.

2.1.2.4 Renal cell carcinoma cell culture

RCC4- and RCC4+ (kindly donated by Professor Patrick Maxwell) refer to two derivatives of the von Hippel-Lindau (VHL)-defective renal cell carcinoma (RCC) cell line RCC4. RCC4+ is stably transfected with an expression plasmid encoding the wild-type VHL tumour suppressor, and RCC4- is stably transfected with an empty vector. Both cell lines were cultured in parallel at 37 °C in RPMI 1640 medium supplemented with 10 % foetal bovine serum, 2 mM L-glutamine, and 100 U / mL penicillin-streptomycin (Invitrogen) in a humidified air and 5 % CO_2 atmosphere. They were used soon after reaching 80 % confluence.

2.1.3 GAS EXPOSURE

2.1.3.1 In vivo

C57BL6 male adult mice were preconditioned by exposure to 75 % xenon balanced with 25 % oxygen for 2 hours in a purpose-built closed circuit anaesthetic chamber (3 L volume) connected to a 3 L reservoir bag, a gas pump, a xenon monitor (439XE Analyser, Air Products, Surrey) and an oxygen monitor (Datex, Capnomac Ultima). A gas mixture of 75 % xenon and 25 % oxygen was delivered at a rate of 2 L / minute for 3 minutes to the air-tight 3 L gas chamber containing no more than 4 mice on a gridded platform, with silica gel and soda lime placed underneath to eliminate moisture and carbon dioxide, respectively. Any residual room air present in the circuit was then further displaced by emptying the 3 L reservoir bag 3 times. The circuit was then closed but replenished with fresh gas

mixture every 30 minutes. A thermometer was placed in the chamber with the mice and temperature maintained at 37 °C +/- 0.5 °C by the use of a heating pad. Negative control mice were exposed to 75 % nitrogen balanced with 25 % oxygen for 2 hours. Positive control mice underwent hypoxic preconditioning through an already established paradigm (Gidday et al. 1994) by exposure to 8 % oxygen balanced with nitrogen for 3 hours (Figure 8).

2.1.3.2 In vitro

Experimental medium was prepared by first bubbling gas mixtures of 75 % xenon, 20 % oxygen and 5 % carbon dioxide through fine sintered glass bubblers in Dreschel bottles for 45 minutes with continual stirring at 37 °C. The pre-bubbled experimental medium was used to replace the existing medium on the cell cultures that were then subsequently placed in an air-tight purposed-built gas exposure chamber set to 37 °C, equipped with inlet and outlet valves and an internal electric fan to evenly distribute gases over the cultures. Gases were delivered at a rate of 100 mL / minute and when connected to calibrated flow-meters, the desired gas composition could be obtained. After 45 minutes of gas equilibration, the gas circuit was closed to begin the preconditioning paradigm using gas concentrations of 75 % xenon, 20 % oxygen and 5 % carbon dioxide for 2 hours. Negative control cultures were bathed in experimental medium pre-bubbled with 75 % nitrogen, 20 % oxygen and 5 % carbon dioxide and then exposed to the same gas concentrations in the gas exposure chamber for 2 hours. Positive control cultures were bathed in experimental medium pre-bubbled with 1 % oxygen and 5 % carbon dioxide balanced with nitrogen and then exposed to these same gas concentrations for 4 hours, using an established paradigm for hypoxic preconditioning in cells (Gidday et al. 1994). Immediately after gas treatments, experimental medium was removed and replaced with standard supplemented growth medium.

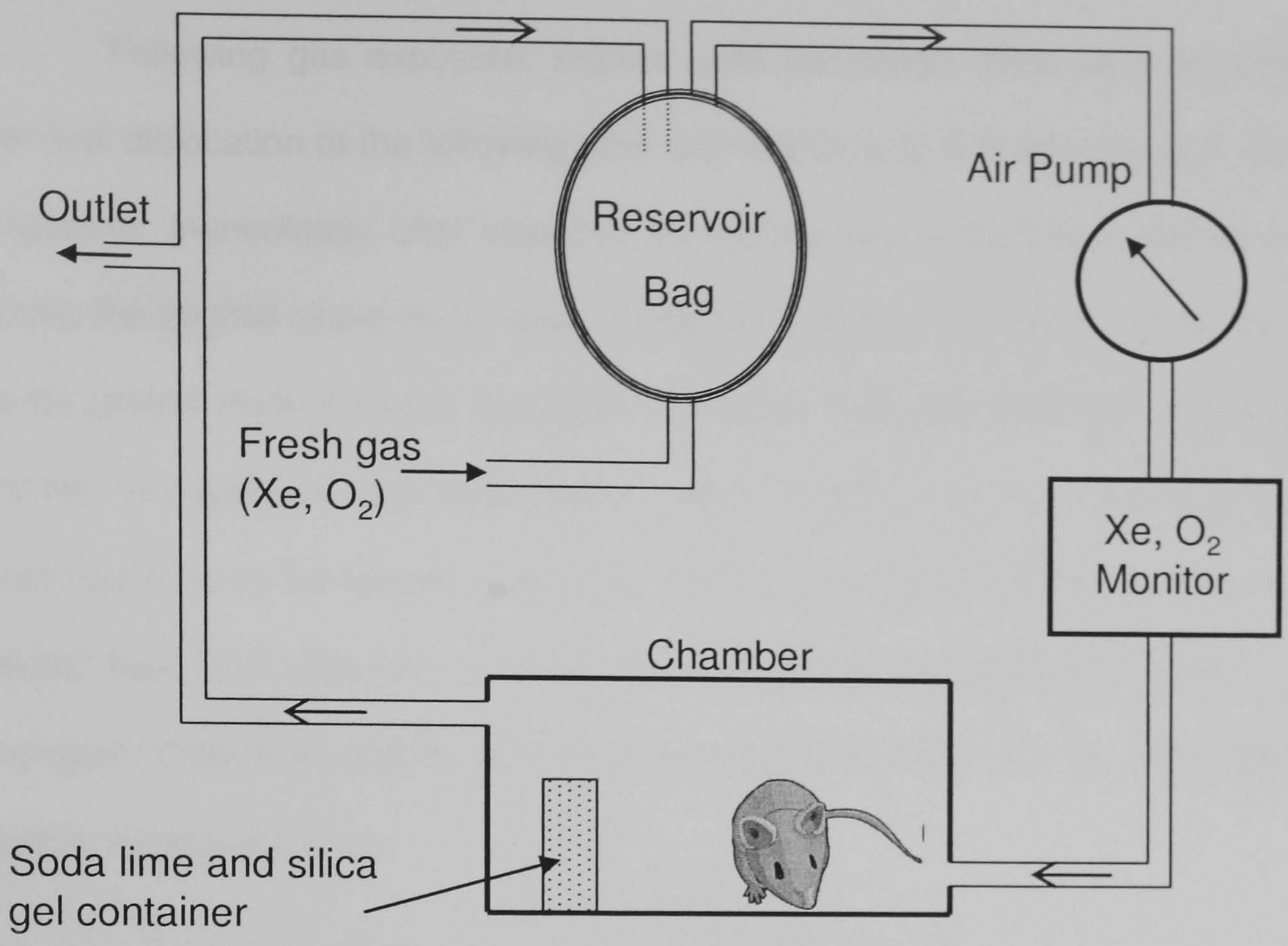


Figure 8. Schematic illustration of in vivo gas exposure circuit

A purpose-built closed circuit gas chamber was used for exposing mice to various gas preconditioning paradigms. The gas chamber (with a volume of 3 L) was connected to a 3L reservoir bag, an air pump, a xenon monitor and an oxygen monitor. Soda lime and silica gel were placed in the gas chamber, to absorb carbon dioxide and moisture, respectively.

2.1.4 ORGAN HARVESTING IN MICE

Following gas exposure, organs were harvested. Mice were sacrificed by cervical dislocation at the following time intervals of 0, 2, 4, 8 and 24 hours after gas exposure. Immediately after sacrifice, the mouse was decapitated and an incision along the sagittal plane was made, across the length of the skull that allowed for it to be peeled back to reveal the brain. The brain was dissected out, separated into its two hemispheres and immediately frozen in liquid nitrogen. A midline incision was made along the length of the body of the mouse and other organs including the heart, liver and kidneys were harvested and also immediately frozen in liquid nitrogen. Dissected organs were then archivally stored at -80 °C until required for further experimentation.

2.2 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

2.2.1 Purification of total RNA

2.2.1.1 In vivo

Total RNA was extracted from frozen organs using DNA- and RNA- free equipment and the RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. Briefly, after cleaning the work area with RNase erase (Qiagen) tissue was collected from archival storage at -80 °C, weighed and completely submerged in RNAlater solution with at least 1 mL of solution to 1 mg of tissue, to stabilise RNA expression in a small RNA-free Petri dish. The tissue was cut into small pieces of approximately 2 mm^3 each. 600 µL of RLT lysis buffer was added to a plastic mortar to which 30 µg of sample was added and then disrupted with a plastic pestle. Once the tissue was well homogenised, it was transferred to the QIAshredder homogeniser column, which was placed in a 2 mL collection tube and spun for 3

minutes in a microcentrifuge at maximum speed (6000*g*). The QIAshredder was removed and 600 µL of 70 % ethanol was slowly dripped into the collected homogenised lysate and then mixed well by pipetting. The sample was pipetted into a genomic DNA eliminating spin column placed in a collection tube and centrifuged for 30 seconds at 6000*g*. The flow-through was discarded and 700 µL of RW1 was pipetted into the spin column to wash it and centrifuged for 30 seconds at 6000*g*. The flow-through was again discarded and 500 µL of RPE was pipetted into the spin column to wash it and centrifuged for 3 minutes at 6000*g*. To elute the RNA, the spin column was then placed in a new collection tube and 50 µL of RNase-free water was pipetted in and centrifuged for 1 minute at 6000*g*. The extracted RNA may be stably stored for 1 year at -80 °C.

2.2.1.2 In vitro

The RNeasy Mini Kit (Qiagen) was used according to the manufacturer's instructions. Briefly, the cell culture medium was completely aspirated from the treated cell cultures and RLT lysis buffer was added directly to the Petri dish. Cells were then disrupted using a rubber cell scraper and pipetted up and down to remove visible cell clumps. The cell mixture was then transferred to the QIAshredder homogeniser column and subsequently processed using the same protocol as for animal tissues, described above.

2.2.2 Determination of RNA concentration, purity and integrity

The concentration of the total purified RNA was then quantified by measuring its ultraviolet (UV) absorbance at 260 nm wavelength in a spectrophotometer, where an absorbance reading of 1.0 corresponds to 44 µg / mL of RNA. The purity of RNA was also determined at the same time, by the ratios of the wavelengths at 260 nm and also at 280 nm, where 280 nm is the absorbance peak for protein contaminants. A ratio of 2:1 (260:280 nm) indicates the absence of protein contamination, with ratios above 1.7:1 deemed acceptable. The integrity and size distribution of the harvested RNA was checked by denaturing agarose gel electrophoresis with ethidium bromide staining. Briefly, a 2 % agarose gel, made with 1.2 g agarose in 60 mL of TAE buffer with 5 µL of the fluorescent dye ethidium bromide, was poured into a casting tray and set for 60 minutes at 4 °C. The gel was placed in an electrophoretic tank that was filled with Tris-acetate EDTA (TAE) buffer to cover the surface of the gel. Loading dye was mixed with RNA samples in a 1:5 ratio, respectively, and loaded into the gel for electrophoresis at 100 V for 25 minutes. The fluorescent bands were visualised using a Flour–S Multi–Imager imaging system (Bio-Rad). Good RNA integrity should give 2 sharp bands corresponding to the 28S ribosomal RNA and 18S RNA in an approximate ratio of 2:1 respectively. Smeared bands are an indication that the RNA sample is likely to have been degraded during extraction (Figure 9).



Figure 9. RNA integrity in the mouse brain and kidney

An RNA preparation from the mouse brain and kidney was electrophoretically separated on a 2 % agarose gel to assess its integrity. Crisp bands indicate intact RNA. **Abbreviations: B,** brain; **K,** kidney; **28S,** 28S ribosome; **18S**, 18S ribosome.

2.2.3 Reverse transcription

Reverse transcription is the synthesis of single-stranded complementary DNA (cDNA) using the isolated RNA as a template. The oligo dT primer (Promega) anneals to mRNA at its optimal temperature and the mRNA is subsequently reverse–transcribed into cDNA, in the presence of a reverse trancriptase enzyme (SuperScript II, Invitrogen). RNA samples were reverse–transcribed based on the manufacturer's (Invitrogen) guidelines. Briefly, olido dT primer and deoxynucleotide triphosphate (dNTP) mix (Promega) were added to a collection tube containing normalised amounts of RNA sample (approximately 1 µg of RNA per sample). In a PCR thermal mastercycler (Eppendorf), the mixture was heated to 65 °C for five minutes to denature RNA secondary structures and then quickly chilled to 4 °C for one minute, allowing the primer to anneal to the RNA. At this point, an RNase inhibitor (Superase-in, Ambion) and 5X first-strand buffer (Invitrogen) were added to the samples. Subsequently, the reaction was heated to 42 °C for two minutes before Superscript reverse transcriptase was added. The mixture was incubated at 42 °C for 50 minutes, allowing for the reverse transcription extension reaction, followed by 70 °C for 15 minutes to inactivate the reaction. The cDNA product was then kept at 4 °C for further use or storage at -80 °C.

2.2.4 Polymerase chain reaction

The following primers (Table 1) were designed and made by (MWG Biotech, Ebersberg, Germany) for use in PCR:

Primer name	Sequence (5' to 3')	T_a (°C)	Product size (BP)
β-ACTIN	AGCCATGTACGTAGCCATCC CTCTCAGCTGTGGTGGTGAA	57	228
EPO	CCACCCTGCTGCTTTTACTC CTCAGTCTGGGACCTTCTGC	57	166
HIF-1α	TCAAGTCAGCAACGTGGAAG TATCGAGGCTGTGTCGACTG	57	198
HIF-β	TGCCTCATCTGGTACTGCTG GAACATGCTGCTCACTGGAA	57	179

Table 1. List of PCR primers and amplification conditions used to analyse the expression of genes in this study

The internal control, HIF-1β or β-actin, was required to verify that similar levels of RNA was used in reverse transcription, allowing for comparison of gene expression between multiple samples. PCR amplification of the required cDNA was performed in 3 mM magnesium chloride, 0.2 μM primers, 0.25 U Taq DNA polymerase (Promega) and 0.8 mM deoxynucleotide mix (Promega) in a PCR thermal mastercycler (Eppendorf). The PCR program consisted of an initial incubation at 96 ℃ for two minutes that preceded 30 cycles of denaturation (96 ℃ for 30 seconds), annealing (57 ℃ for 1 minute), and extension (72 ℃ for 3 minutes). A final extension period of 7 minutes at 72 ℃ was also included. PCR products were subsequently separated by electrophoresis on a 2 % agarose gel containing the fluorescent dye ethidium bromide and visualized using a Fluor-S Multi-imager imaging system (Bio-Rad).

2.3 WESTERN BLOTTING

2.3.1 Solutions

Tris-Hcl solution (500 mL)
12.1 g Trizma base
0.5 L dH_2O
Titrate to pH 8.0 with Hcl

Tris-buffered saline (TBS) (1 L)
8.8 g NaCl
50 mL Tris-HCl
Top up to 1L with dH_2O

Tris-buffered saline with 0.05 % Tween-20 (1 L)
8.8 g NaCl
50 mL Tris-HCl
Top up to 1L with dH_2O
0.5 mL Tween-20 (very viscous so add after dH_2O)

2.3.2 Protein extraction

2.3.2.1 In vivo

Once mice were sacrificed, organs were rapidly excised and harvested and immediately fast frozen in liquid nitrogen prior to archival storage at -80 °C. The frozen tissue was subsequently weighed and bathed in cell lysis buffer (Cell Signalling Technology) at a concentration of 0.2 g / mL. Samples were immediately mechanically homogenised (Polytron Homogenise, Kinematica) on ice in short 3 second bursts interspaced with 3 second gaps for 30 seconds before centrifugation at 3000*g* for 10 minutes at 4 °C to remove cell debris. The supernatant was carefully pipetted out and removed into a new 1.5 mL collection tube and aliquoted to avoid multiple freeze-thaw cycles. Aliquoted samples were stored at -80 °C and thawed on ice when ready for use.

2.3 2.2 In vitro

On ice, growth medium was removed from the treated cell cultures and subsequently dissolved and homogenized in cell lysis buffer (Cell Signalling Technology). Samples were then sonicated on ice in short 3 second bursts interspaced with 3 second gaps for 30 seconds before centrifugation at 10 000*g* for 10 minutes at 4 °C to remove cell debris. The supernatant was carefully pipetted out and removed into a new 1.5 mL collection tube and aliquoted to avoid multiple freeze-thaw cycles. Aliquoted samples were stored at -80 °C and thawed on ice when ready for use.

2.3.3 Protein quantification using the Bradford assay

Bradford dye concentrate (Bio-Rad) was diluted with double distilled water (ddH_2O) in a 1:4 ratio respectively, following manufacturer's instructions. To make protein standard samples, a known concentration of bovine serum albumin (Bio-rad) was serially diluted with ddH_2O to range in concentration from 1000, 500, 250, 125, 12.5 and 1.25 µg / mL. On ice, 10 µL of protein samples and 10 µL of protein standard samples were loaded in triplicate into a 96-microwell flat-bottomed clear plate. Triplicates of cell lysis buffer (10 µL) were also loaded to remove its possible contribution to total protein concentration, and ddH_2O was used as blanks. Bradford dye (20 µL) was pipetted into each well and left to incubate for 5 minutes. The plate was then placed in a spectrophotometer to measure the optical density of each sample at 595 nm. The standard curve created from the protein standard samples allowed for determination of the protein sample concentration and their subsequent normalisation to each other.

2.3.4 Electrophoretic protein separation

Based on the Bradford assay determination of protein concentration, protein samples were normalised to the same concentration and volume. Denaturing sample buffer concentrate containing a tracking dye (Invitrogen) was added to the normalised protein samples in a 1 : 3 ratio respectively and subsequently heated at 70 °C for 10 minutes. 4 - 12 % gradient Bis-Tris gels (Invitrogen) were positioned in a gel holder and assembled into a Western blot tank that was then filled with running buffer (Invitrogen). Equal volumes of normalised protein samples were then loaded into the wells of the gel using a long fine-tipped pipette. Each well may hold a maximum of 25 µL of sample; 10 µL of mouse brain or kidney with sample buffer was loaded per well and this contained approximately 100 µg of total protein, where as 20 µL of cells with sample buffer were loaded per well and this contained approximately 60 µg of total protein. 5 µL of a biotinylated protein ladder (Cell Signalling Technology) was also loaded in an additional lane. The gel-loaded proteins were then fractionated by electrophoresis at 200 V for 35 minutes which was when the dye front had nearly reached the bottom of the gel.

2.3.5 Electrotransfer

The proteins separated by electrophoresis were then transferred onto a nitrocellulose membrane that acts as a solid support. Pre-cut nitrocellulose membranes, filter papers and sponges were soaked in 10 % methanol transfer buffer (Invitrogen) for 5 minutes before the end of the electrophoretic run. According to the manufacturer's instructions, a gel and membrane sandwich was then created from negative to positive (Figure 10).

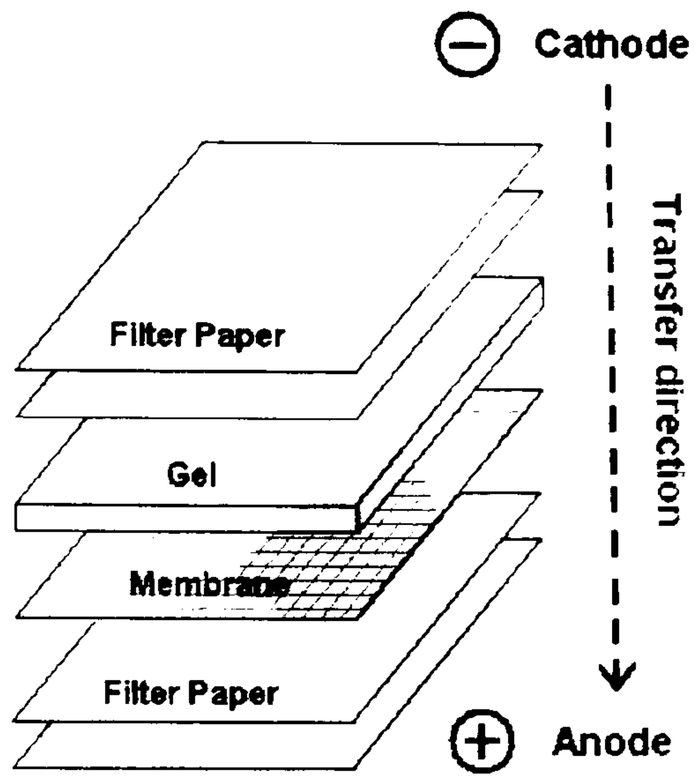


Figure 10. Electrotransfer of proteins, from gel to nitrocellulose membrane

As air pockets hinder the flow of electrical current, air bubbles were smoothed out of between the gel and nitrocellulose membrane by gently pressing and rolling together with a Pasteur pipette. The gel-membrane sandwich was then placed in a transfer box which was assembled into the Western blot tank. The transfer box was filled with transfer buffer and cool water was used to fill the rest of the tank to aid in heat dissipation. The transfer of proteins from gel onto membrane was run at 30 V for 60 minutes.

2.3.6 Immunodetection

After electrotransfer, the nitrocellulose membrane was incubated with a blocking solution composed of 5 % non-fat dry milk in Tween-20-containing Tris buffered saline (TBS-T) for 3 hours on a gyrating shaker (Fisher Scientific) at room temperature. Subsequently, the blocked membrane was incubated overnight for 16 hours at 4 °C on a gyrating shaker with the relevant primary antibodies directed against either HIF-1α (120 kDa) (rabbit polyclonal antibody, 1 : 500 (Novus Biologicals); or mouse monoclonal antibody, 1 : 1000 (Novus Biologicals); or rabbit polyconal antibody, 1 : 1000, (BD Biosciences)), EPO (34 kDa) (rabbit polyclonal antibody, 1 : 250 (Cell Signalling Technology); or rabbit polyclonal antibody, 1 : 500 (Santa Cruz)), PHD2 (46 kDa) (rabbit polyclonal antibody, 1 : 5000 (kindly donated by Professor Patrick Maxwell)), mTOR (289 kDa) (rabbit polyclonal antibody, 1 : 1000 (Cell Signalling Technology)), HIF-1β (87 kDa) (rabbit polyclonal, 1 : 1000 (Cell Signalling Technology)) or α-tubulin (55 kDa) (mouse monoclonal antibody, 1 : 5000 (Cell Signalling Technology)) (Table 2). The primary antibodies were incubated at optimised dilutions in 5 % non-fat dry milk solution. After removing the antibody, 4 washes in TBS-T lasting 5 minutes each were performed at room temperature on a rapidly moving gyrating shaker. The membrane was then incubated with the appropriate goat-derived horseradish peroxidase-conjugated

secondary antibody (Cell Signalling Technology) directed against the rabbit-derived primary antibodies at room temperature for 1.5 hours on a gyrating shaker. The secondary antibody was then removed and 5 washes in TBS-T lasting 5 minutes each were performed at room temperature on a rapidly moving gyrating shaker with an additional sixth wash for 5 minutes in TBS without Tween-20, as this detergent interferes with the chemiluminescent reaction. To maximise the visualising of immunoreactive bands, the enhanced chemiluminescent (ECL) reagent was mixed with the accompanying peroxidase reagent (Lumiglo kit (Cell Signalling Technology)) only at the very last minute. The membrane was then incubated for 1 minute in the ECL solution and then placed protein-side up between two transparent acetate sheets, with trapped air bubbles gently rolled out using a Pasteur pipette. The acetate-covered membrane was then placed inside a film cassette and in a completely dark room exposed to radiographic film (ECL Hyperfilm (Amersham Biosciences)) for optimised lengths of time (HIF-1α, 3 - 5 minutes; EPO, 5 - 10 minutes; PHD2, 5 - 10 minutes; mTOR, 3 - 5 minutes; α-tubulin, 15 - 30 seconds).

After radiographic film capture of the immunoreactive bands, the membrane was removed from the acetate and washed 3 times in TBS-T lasting 5 minutes each at room temperature on a rapidly moving gyrating shaker. The membrane was then subjected to the same protocol as outlined above for detection of the house-keeping protein, α-tubulin: it was re-blocked in blocking solution before incubation with the anti-α-tubulin antibody, washed, incubated with the secondary antibody, washed and visualised using the ECL system capture on radiographic film. The results were then quantified by densitometric analysis using Adobe Photoshop Elements 2.0 software, scaled relative to the control sample, and normalised with the house-keeping protein α-tubulin. A schematic representation of the steps involved in Western blotting is depicted in Figure 11.

Antigen	Primary antibodies	Dilution	MW (kDa)	Supplier
EPO	Rabbit PAb	1:250	34	CST
	Rabbit PAb	1:500	34	SC
HIF-1α	Rabbit PAb	1:500	120	NB
	Mouse MAb	1:1000	120	NB
	Rabbit PAb	1:1000	120	BD
HIF-1β	Rabbit PAb	1:1000	87	CST
mTOR	Rabbit PAb	1:1000	289	CST
PHD2	Rabbit PAb	1:5000	46	PM
α-tubulin	Mouse MAb	1:5000	55	CST

Table 2. List of primary antibodies used in Western blot immunodetection of protein expression

Abbreviations: BD; BD Biosciences; **CST**, Cell Signalling Technology; **EPO**, erythropoietin; **HIF-1α,** hypoxia-inducible factor-1 alpha; **HIF-1β,** hypoxia-inducible factor-1 beta; **kDa,** kilodaltons; **MAb,** monoclonal antibody; **mTOR,** mammalian target of rapamycin; **MW,** molecular weight; **NB**, Novus Biologicals; **PAb,** polyclonal antibody; **PHD2**, prolyl hydroxylase domain-containing 2 enzyme; **PM,** Professor Patrick Maxwell (a kind donation); **SC** Santa Cruz.

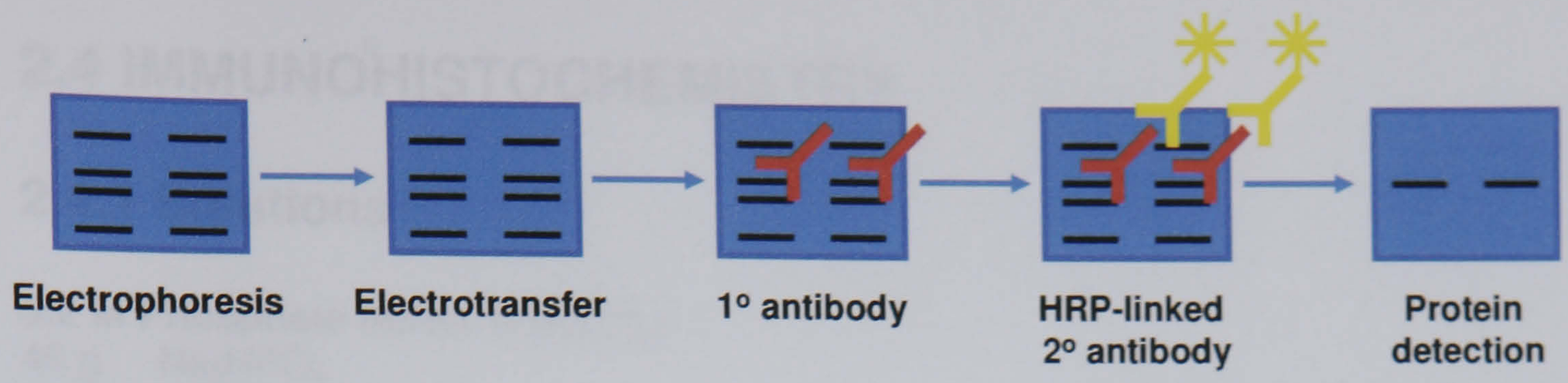


Figure 11. Western blotting

This cartoon illustrates the main steps involved in Western blotting. Protein samples, loaded onto a 4 - 10 % Bis-Tris gel, were **electrophoretically** separated. Proteins were then **electrotransferred**, from the gel to a nitrocellulose membrane. After blocking for nonspecific antibodies, the membrane was probed with a **primary antibody**, followed by a **secondary antibody** conjugated to horse radish peroxidase (HRP). The membrane was then incubated with chemiluminescent reagents and the **protein of interest** detected on radiographic film.

2.4 IMMUNOHISTOCHEMISTRY

2.4.1 Solutions

0.2 M Phosphate buffer (PB) (2 L)
46 g Na_2HPO_4
9.1 g $Na_2H_2PO_4$
2 L dH_2O

0.1 M Phosphate-buffered saline (PBS) (2 L)
1 L 0.2 M PB
1 L dH_2O
18 g NaCl

0.1 M Phosphate-buffered saline with 0.3 % triton (PBS-T) (2 L)
2 L PBS
6 mL Triton X-100 (Promega)

0.1M Phosphate-buffered saline with 0.3 % H_2O_2 / 70 % methanol (30 mL)
9 mL 0.1 M PBS
21 mL Methanol
300 µL 30% H_2O_2

Heparinised phosphate-buffered saline (PBS-Hep) (2 L)
2 L PBS
2 mL (1000 U / mL) heparin sodium solution (Sigma)

10 % paraformaldehyde (2 L)
Prepare under fume hood and store at 4 °C
1.6 mL dH_2O (heat to ~70 °C)
200 g paraformadehyde (dissolve in water)
1 mL NaOH (to aid dissolution)
2 L dH_2O
Cool solution to room temperature
Titrate to pH to 7.4 with HCl and/or NaOH
Filter and store at 4 °C

4 % Paraformaldehyde (2 L)
Prepare under hood, make immediately before use and store at 4°C
1 L 0.2 M PB
0.8 L 10 % paraformaldehyde
Top up to 2 L with dH_2O

30 % Sucrose solution (2 L)
600g sucrose
2 L 0.1 M PBS

1 M Sodium hydroxide (NaOH) (2 L)
50 g sodium hydroxide pellets
Dissolve to 2 L with dH_2O

2.4.2 Tissue fixation by transcardial perfusion

Transcardial perfusion is a common method for tissue fixation in immunohistochemical (IHC) techniques. This method uses the circulatory system to deliver the fixative solution throughout the body. Fixation is required to preserve the structure of tissues and polypeptides so that antibodies used during immunostaining can recognise the conformation of their antigen sites. Paraformaldehyde (PFD) is a commonly used and reliable fixative agent because it minimises non-specific staining and does not interfere with fluorescent dyes, allowing for optimal visualisation of the samples.

After gas exposure, mice were swiftly sacrificed by cervical dislocation and dissected to remove skin from the abdomen and chest. The thoracic cavity was opened by cutting the diaphragm from one lateral aspect to the other lateral aspect, and cutting through the lateral aspects of the rib cage, then horizontally below the neck to expose the heart.

The perfusion needle (blunted 15 gauge hypodermic needle) was inserted through the base of the apex (Figure 12) into the left ventricle and gently followed through into the ascending aorta. The right atrial chamber was lacerated to make a break in the circulatory system, allowing for effluent flow-out of blood and perfusion liquid. The heart and circulation were then transcardially perfused with 100 mL of heparinised normal saline, at a steady flow rate of approximately 25 mL / minute, to stop blood coagulation and to blanch the tissues of blood, followed by perfusion with 200 mL 4 % paraformaldehyde (PFD) in 0.1 M phosphate-buffered saline (PBS) solution to preserve the tissues (approximately 10:1 ratio of fixative : tissue); evidence of spontaneous "dancing" and tremors in the animal's extremities indicated that aldehyde had cross-linked with nerves and muscles and that the tissue was

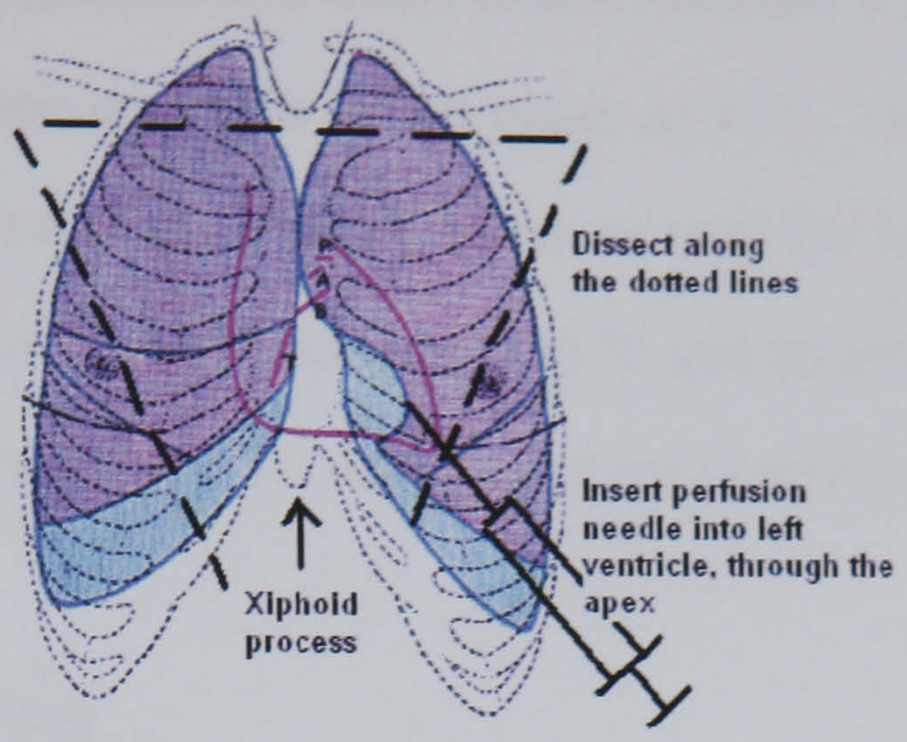


Figure 12. Schematic diagram of the transcardial perfusion method

fixed. Mice were then decapitated and whole brains removed and immersed in 4 % PFD in 0.1 M PBS solution overnight for 16 hours at 4 °C. This was followed by incubation in 30 % sucrose solution for 24 hours, or until the tissue sank, at 4 °C. The brain was then coronally cryosectioned at -20 °C into 30 µm sections, collected into sterile plastic dishes containing 0.1 M PBS and then mounted onto slides.

2.4.3 Immunostaining

The mounted sections were washed in PBS (washing was performed 3 times for 5 minutes). Endogenous peroxidase activity may be found in many tissues, for example, due to the presence of red blood cells and, therefore, sections were quenched of peroxidase activity by pre-treatment in 0.3 % hydrogen peroxide / 70 methanol in 0.1 M PBS for 30 minutes, and subsequently washed in PBS. Sections were then incubated with a 3 % blocking solution for 30 minutes to suppress non-specific binding, followed by washing. Sections were incubated with the primary antibody directed against HIF-1α (rabbit-derived polyclonal antibody, 1 : 1000 (New England Biolab) diluted in 1 % goat serum in PBS-T) overnight for 16 hours at 4 °C, followed by washing. To test for the specificity of the antibodies used, control slides were incubated with 1 % goat serum in PBS-T only, without a primary antibody present. Sections were then incubated with a biotinylated secondary antibody (goat-derived anti-rabbit IgG antibody, 1:200 (New England Biolab) for 1 hour at room temperature and washed in PBS-T. This indirect method employing a primary and a secondary antibody is used because signal from the protein of interest (HIF-1α) may be amplified through several secondary antibodies reacting to one primary antibody displaying several antigen binding sites. Subsequently, samples were incubated with avidin-biotin-peroxidase complex (ABC) solution (Vector Lab, Orton Southgate, Peterborough, United Kingdom) for 1 hour, followed by washing in PBS. Avidin, labelled with peroxidase, is a large glycoprotein that has

a high affinity for the biotin found conjugated to the secondary antibodies. The peroxidase reaction was developed by incubating the section in 0.02 % 3,3 diaminobenzidine tetrahydrochloride (DAB) solution (Sigma) containing 0.003 % hydrogen peroxide for 6 minutes, that results in a brown precipitate at the antigen site. Sections were washed with PBS and transferred to poly-lysine coated slides and allowed to dry overnight for 16 hours in darkness at room temperature.

2.5 RAPAMYCIN ADMINISTRATION

2.5.1 In vitro

Rapamycin (Tocris) was dissolved to a concentration of 40 mM in DMSO solvent and serially diluted in supplemented RPMI 1640 medium to the final concentrations of 100, 50 and 25 nM with the end volumes containing 0.0025 % DMSO. Supplemented RPMI 1640 medium containing rapamycin was incubated with the RCC4+ cell cultures for 30 minutes at 37 °C in humidified air containing 5 % carbon dioxide, after which it was removed and replaced with experimental medium pre-bubbled with pure gas mixtures of 75 % xenon, 20 % oxygen, and 5 % carbon dioxide, and preconditioned by exposure to the same gas concentrations for 2 hours at 37 °C, as previously described above. Experimental medium was then removed and replaced with supplemented RPMI 1640 culture medium and placed in the humidified air containing 5 % carbon dioxide incubator at 37 °C for 24 hours. Cells were then lysed, harvested and subsequently analysed by Western blotting. Harvesting at 24 hours after xenon preconditioning was chosen as HIF-1α protein expression was earlier demonstrated to occur maximally at this time point.

2.5.2 In vivo

Rapamycin (Tocris) was dissolved to a concentration of 40 mM in DMSO solvent and diluted in normal saline (0.9 % sodium chloride) to contain a final DMSO concentration of less than 0.1 %. C57BL6 male adult mice were then administered rapamycin by intraperitoneal injection at doses of 0.25 mg / Kg or 1.5 mg / Kg, 30 minutes prior to preconditioning by gas exposure to 75 % xenon balanced with 25 % oxygen for 2 hours. Mice were subsequently rested back in their cages for 24 hours in room air and then sacrificed by cervical dislocation. Organs were collected, frozen and subsequently analysed by Western blotting as described in the sections above. Figure 13 illustrates the time course and experimental groups of these rapamycin studies.

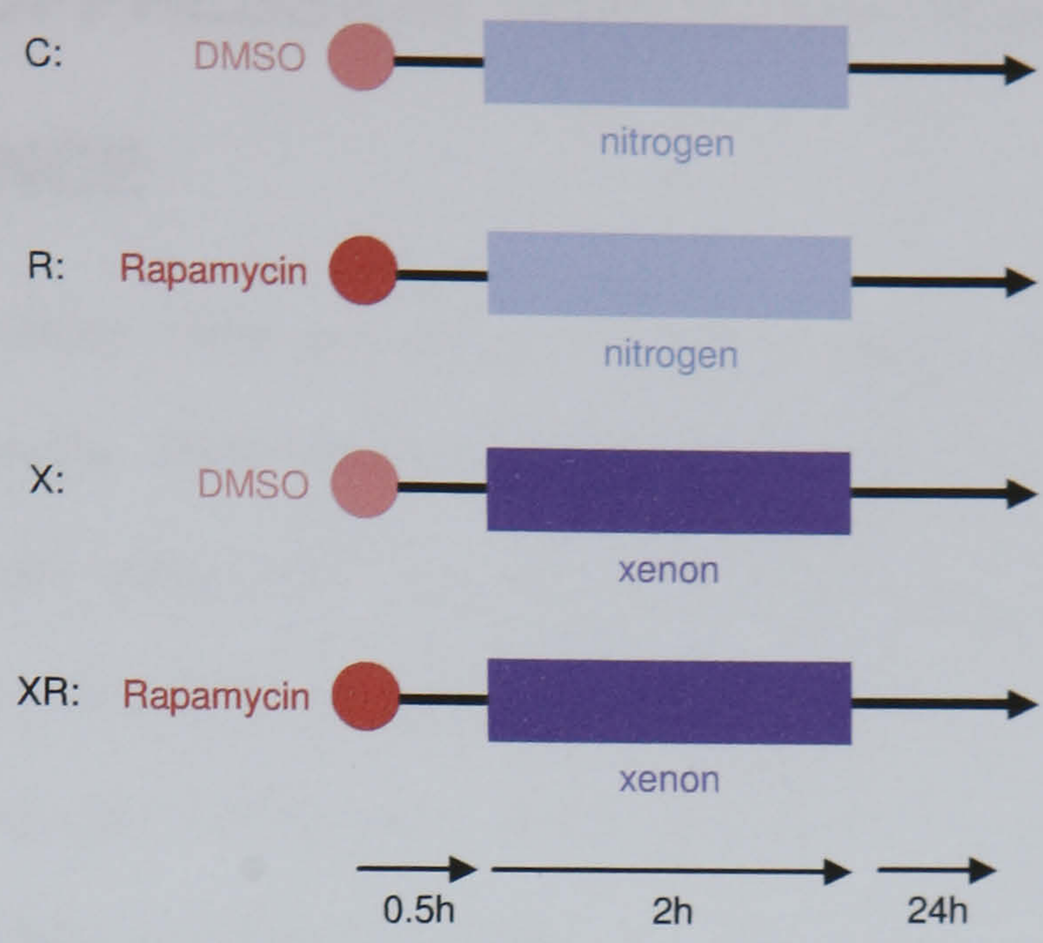


Figure 13. Time course and groups of rapamycin experiments

This schematic cartoon depicts the experimental groups and time course (not drawn to scale) of the rapamycin experiments. **C**, controls received volume-matched DMSO-containing medium (*in vitro)* or normal saline (*in vivo*) and were exposed to nitrogen; **R**, the rapamycin alone group received rapamycin and were exposed to nitrogen; **X**, the xenon alone received volume-matched DMSO-containing diluent and were exposed to xenon; **XR**, the xenon with rapamycin group received rapamycin and were exposed to xenon.

2.6 GENE SUPPRESSION THROUGH RNA INTERFERENCE

HIF-1α knockout mice are lethal mutants because HIF-1 is required for embryonic development. RNA interference (RNAi) is a relatively new method that results in "knockdown" rather than "knockout" of a specific gene and is afforded by a naturally occurring mechanism. Studies in *Caenorhabditis elegans* and the *Drosophila melanogaster* fruit fly have shown that RNAi is triggered when double stranded RNA (dsRNA) containing sequence homology to an endogenous mRNA is introduced into the cell (Fire et al. 1998). In the cell, an enzyme termed Dicer cleaves the dsRNA into 21 - 23 base pair fragments, termed small interfering RNAs (siRNAs) (Elbashir et al. 2001). siRNAs are then incorporated into a cytoplasmic ribonucleoprotein complex known as dsRNA-induced silencing complex (RISC) (Martinez et al. 2002). RISC uses the antisense strand of siRNA to associate with endogenous mRNA that displays sequence homology. The targeted mRNA is then cleaved and degraded by non-specific Rnases (Caudy et al. 2003). The result is post-transcriptional suppression of target mRNA expression (Figure 14).

In mammalian cell cultures, however, exposure to long dsRNA results in non-specific mRNA degradation and translation inhibition, through activation of interferons, and RNAi may only be achieved through administration of siRNAs directly (Elbashir, Lendeckel & Tuschl 2001). In mammals, delivery of siRNA into the cytoplasm of the target tissue has been a major hurdle because siRNAs, like all nucleic acids, are rapidly degraded by extracellular and intracellular nucleases. They are also not readily taken up by cells because they are not able to naturally

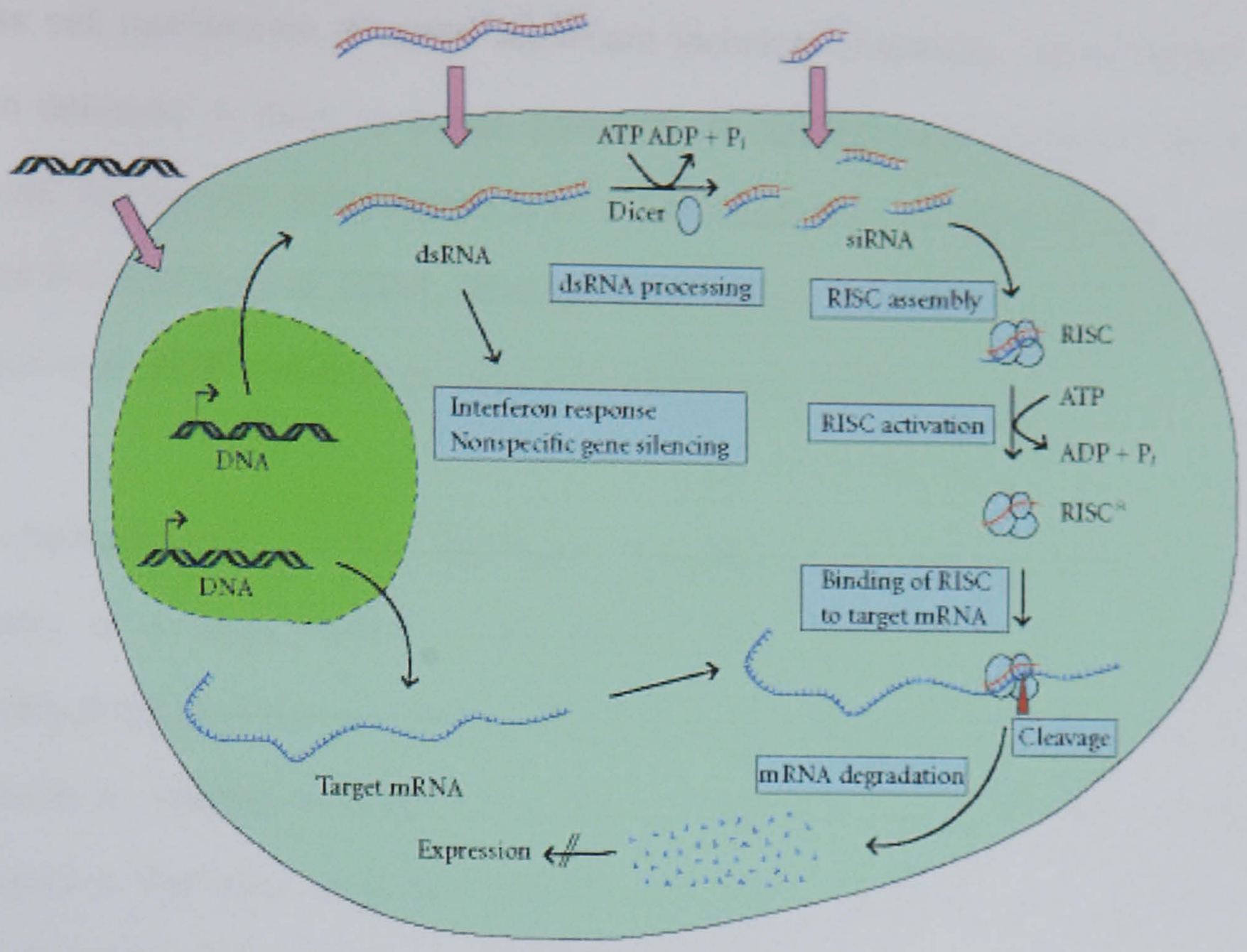


Figure 14. RNA interference in mammalian systems

This cartoon illustrates the mechanism of RNA interference (RNAi) in a mammalian cell. RNA may enter the cell in one of three ways. DNA vectors that express long double-stranded RNA may be introduced into the cell (left red arrow), or double-stranded RNA may directly enter the cell (centre red arrow). The double stranded RNA is then processed by the Dicer complex to form small interfering RNAs (siRNAs). Alternatively, siRNAs may be directly delivered into the cell (right red arrow). The siRNAs are then incorporated into a cytoplasmic ribonucleoprotein complex known as dsRNA-induced silencing complex (RISC). RISC uses the antisense strand of the siRNA to associate with the complementary target mRNA that results in its cleavage and destruction. (Reproduced from (Aigner 2006)).

cross cell membranes. Despite significant technical challenge, naked siRNA has been delivered to mice by a high pressure, or hydrodynamic, injection technique through the tail vein, that resulted in an 8-fold knockdown of target gene expression in the liver (Song et al. 2003), although more typical reduction is usually 20 - 40 % (Giladi et al. 2003, Lewis et al. 2002, McCaffrey et al. 2002).

The hydrodynamic injection technique, through the tail vein, involves the rapid delivery of a large volume of siRNA-containing solution into the vasculature, typically 2 mL of siRNA-containing solution is delivered within 7 seconds. This rapid increase in volume exceeds the capacity of the heart and instead, increases pressure in the vena cava that leads to retrograde flow and extravasation of fluid (Zhang, Hua 2004). This results in hepatocyte transfection and a smaller degree of transfection in other organs including the kidney (Zhang, Budker & Wolff 1999). This is also associated with a transient liver toxicity (measured by a rise in serum alanine aminotransferase (ALT)), but histological examination does not reveal significant pathology. The mechanism of siRNA uptake after hydrodynamic injection is not understood. Other macromolecules, such as proteins, may also be delivered by hydrodynamic injection and so the mechanism of cellular entry is likely to be non-specific. Although siRNA is dependent on delivery efficiency and siRNA efficacy, other gene suppression techniques, for example, small molecule inhibitors or antisense DNA oligonucleotides, may result in variable specificity or non-specificity of target gene suppression.

2.6.1 Preparation of the siRNA solution

Deprotected and annealed high quality (> 90 % pure) HIF-1α siRNA for mouse was predesigned and synthesised by Qiagen with the following sequences (NCBI accession number NM010431):

5'-GCUCAAUUUAUGAAUAUUAdTdT-3' (sense)

5'-UAAUAUUCAUAAAUUGAGCdGdG-3' (antisense)

siRNAs were designed using standard siRNA design guidelines, to avoid 5' or 3' untranslated regions (UTRs), in case other proteins bound to these regions prevented siRNA binding. A BLAST search was used to check that this sequence was not complementary to other non-target mRNAs, and was synthesised with a dTdT overhang. A scramble, or nonsense, siRNA without specific gene silencing activity was also provided by Qiagen as a negative control. A dose of 50 µg of HIF-1α siRNA was used for each animal, as recommended by the manufacturer. This was dissolved in siRNA suspension buffer (Qiagen) and added to RNase-free PBS, to a total volume of 2 mL, approximately equivalent to 10 % of the weight of the mouse. The siRNA solution was warmed to room temperature prior to delivery.

2.6.2 Preparation of the mouse for surgery

Animals were placed in a sealed gas chamber where anaesthesia was induced with 5 % isoflurane and maintained with 2 % isoflurane. Endotracheal intubation was performed with a 20 G cannula and tubings were connected to a mechanical ventilator set to 200 breaths / minute. Body temperature was measured by a temperature sensing probe inserted behind the left ear, and maintained at 37 °C +/- 0.1 °C by using a heated bed and a heating lamp. The lack of response to pain, by pinching the foot, indicated sufficient depth of anaesthesia.

2.6.3 Hydrodynamic tail vein injection in mice

A small incision was made midway in the dorsal aspect of the mouse tail, optimising visualisation of the tail vein. Upon exposure of the tail vein by blunt dissection, it was isolated with fine silk suture slings passed underneath, at the proximal as well as the distal end of the vein. The distal sling was tied to reduce the loss of injection substance distally. A cannula was then inserted for the administration of treatment. Back flow of blood into the cannula established that it was properly positioned for a clear injection pathway. The complete volume of siRNA solution was then rapidly dispensed into the vein within 10 seconds at a constant flow rate. Following closure of the wound, the mouse was emerged from anaesthesia and rested for 24 hours (Figure 15). The mouse was then preconditioned by gas exposure with 75 % xenon (or 75 % nitrogen as a control) balanced with 25 % oxygen for 2 hours as previously described. Twenty-four hours after gas exposure, mice were either sacrificed for kidney harvesting for Western blotting, or subjected to renal ischaemic-reperfusion injury (IRI) surgery (described below). Figure 16 illustrates the siRNA preconditioning experimental groups.

2.7 RENAL ISCHAEMIC-REPERFUSION INJURY

Twenty-four hours after gas exposure to either 75 % xenon or nitrogen with 25 % oxygen, mice were anaesthetised with 1 - 2 % isoflurane. Midline laparotomy was performed and renal IRI was induced by right renal pedicle clamping for 25 minutes, or by right renal pedicle clamping for 40 minutes with left nephrectomy in renal failure experiments. Renal ischaemia was confirmed by observing the change in colour of the kidneys from red to blanched. Sham-operated mice were dissected as above but with no occlusion of the renal vasculature. The intra-abdominal temperature was maintained at 37 +/- 0.1 ºC with a heating pad which was servo-adjusted by a temperature controller (Engineering Inc, Stamford, CT, USA)

throughout the experiment. The wound was closed with sutures and the skin treated with topical iodine solution. Twenty-four hours of reperfusion took place before animals were sacrificed and kidneys harvested for Western blotting, or fixed in 4 % PFD (Figure 17). For survival experiments in the model of renal failure, mice were not sacrificed after the reperfusion period, but monitored on a daily basis, for 7 days, with a scoring assay based on body weight, activity and general appearance. Any animals that scored > 7 were euthanized (Table 3).

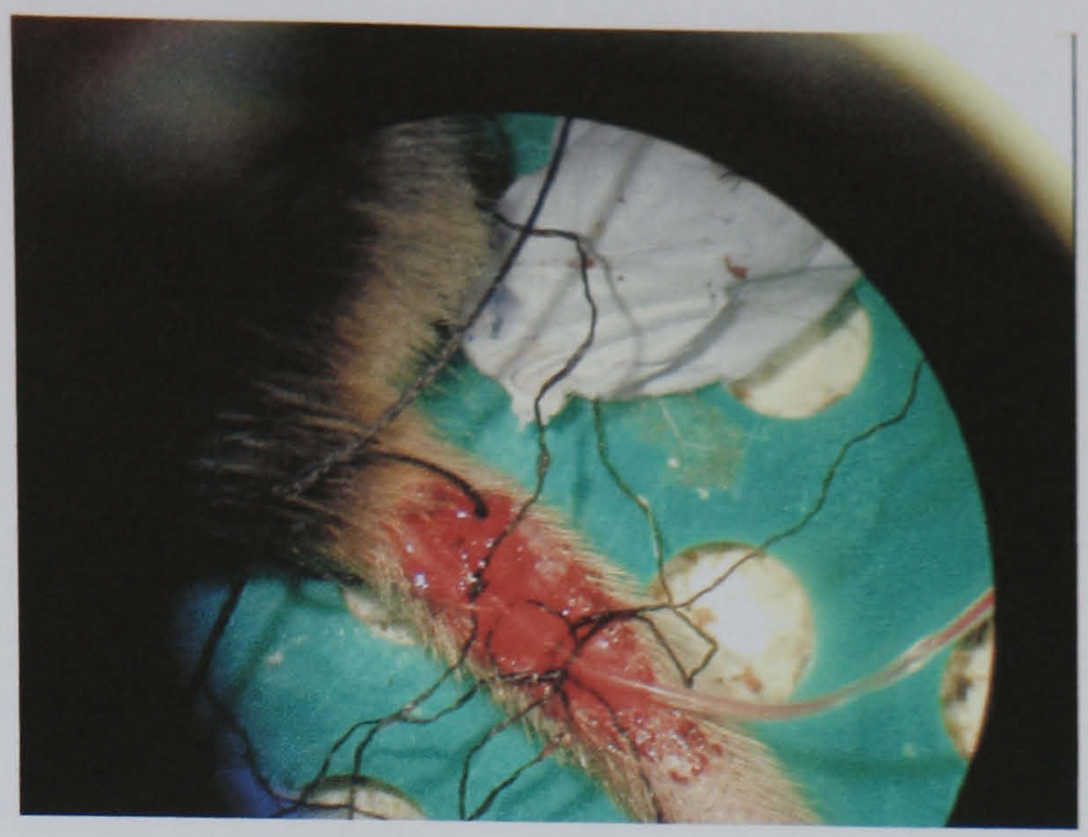


Figure 15. Photograph of tail vein injection in mice

Photograph of tail vein injection in mice, as seen through a light microscope to optimise visualisation.

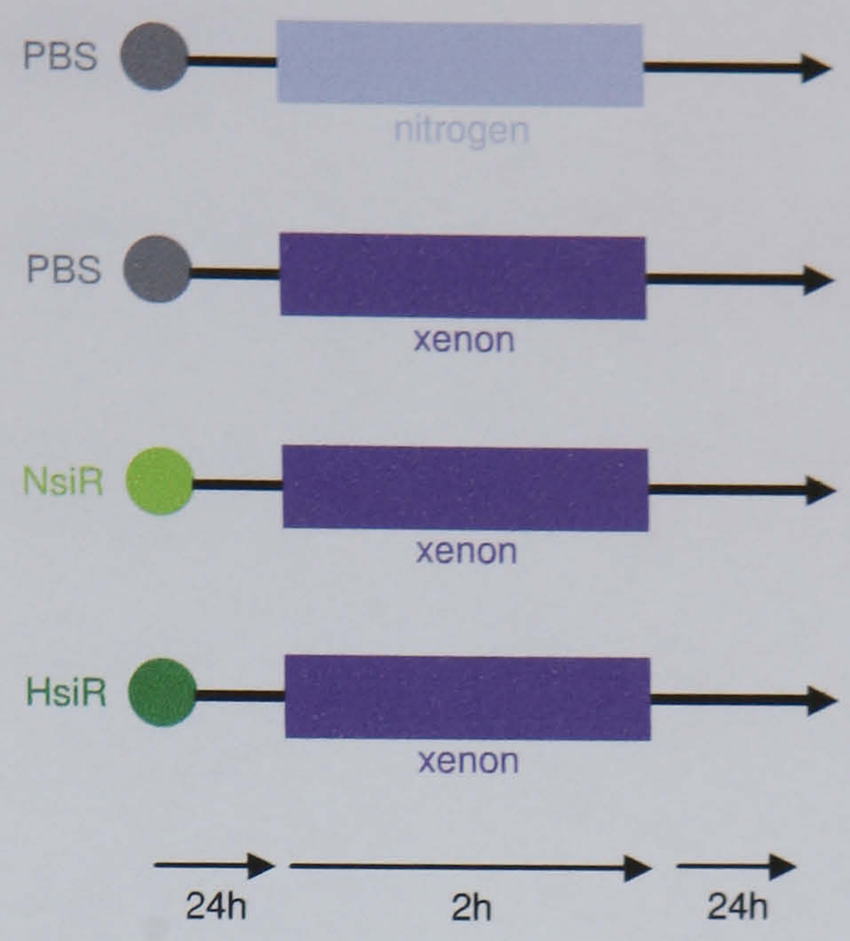


Figure 16. Time course and groups of HIF-1α siRNA in xenon preconditioning experiments in mice

This schematic cartoon depicts the experimental groups and time course (not drawn to scale) of HIF-1α siRNA in xenon preconditioning experiments in mice. Mice received a single hydrodynamic tail vein injection (50 µg siRNA in 2 mL PBS). Twenty-four hours later, they were exposed to 75 % xenon, or nitrogen, for 2 hours. After 24 hours, mice were sacrificed for analysis of gene and protein expression. **Abbreviations: PBS,** phosphate-buffered saline; **NsiR,** nonsense siRNA; **HsiR,** HIF-1α siRNA.



Figure 17. Time course and groups of renal ischemia-reperfusion experiments in mice

This schematic cartoon depicts the experimental groups and time course (not drawn to scale) of the renal ischaemic-reperfusion experiments. Mice received a single hydrodynamic tail vein injection (50 µg siRNA in 2 mL PBS). Twenty-four hours later, they were exposed to 75 % xenon, or nitrogen, for 2 hours. After 24 hours, renal ischaemia was induced by clamping the renal pedicle for 25 minutes (or for 40 minutes and left nephrectomy in the renal failure model). This was followed by 24 hours of reperfusion. **Abbreviations: PBS**, phosphate-buffered saline; **NsiR**, nonsense siRNA; **HsiR**, HIF-1α siRNA.

Post-operative assessment	Score
Body weight	
Greater than pre-operative score	0
90-100 % pre-operative score	1
80-90 % pre-operative score	2
Less than 80 % pre-operative score	4
Activity	
Normal	0
Moves spontaneous in cage, but reduced	1
Moves to stimulus, but not spontaneously	2
No movement to stimulus, huddled	5
General appearance	
Normal	0
Evidence of poor grooming	2
Animals to be killed if score > 7	

Table 3. Post operative assessment scoring assay for renal failure survival experiments

The severe renal ischaemic-reperfusion injury model of renal failure was induced with 40 minutes of right renal pedicle clamping for 40 minutes and left nephrectomy. Mice were subsequently monitored daily over 7 days with a scoring assay based on their body weight, activity and general appearance, and immediately euthanized if they scored more than 7.

2.8 HISTOLOGICAL ANALYSIS OF RENAL ISCHAEMIC-REPERFUSION INJURY

Kidneys were fixed in 4 % PFD as previously described and dehydrated with an automated dehydrator for 24 hours before embedding in paraffin wax. Coronal sections, 5µm thick, were cut at regular 500 µm intervals with a microtome, and then placed on glass slides (Super Frost). Samples were then dewaxed and rehydrated by running slides through xylene, 100 % ethanol, 90 % ethanol and 75 % ethanol and then water. Slides were then immersed in haematoxylin for 1 minute for staining of nucleic acid components, rinsed with water, and immersed in eosin stain for 1 - 2 minutes, for staining of cytoplasmic cell components, and rinsed again. Sections were dehydrated again by running slides through ascending ethanol washes and then cleared with xylene. Glass cover slips were then placed over the sections using mounting solution. For each kidney sample, 10 photomicrographs were taken from representative areas of the renal cortex with a digital camera (Olympus C 2020) attached to a light microscope (Zeiss Stemi 2000-C) with x 20 magnification. For each photomicrograph, arranged in a random order, ten cortical tubule cross-sections were chosen at random and scored 0 - 3 by a scorer who was unaware of the treatment groups, using a pathological scoring system modified from (Hamar et al. 2004), to obtain a maximum score of 300 per treatment group (Figure 18) and (Table 4).

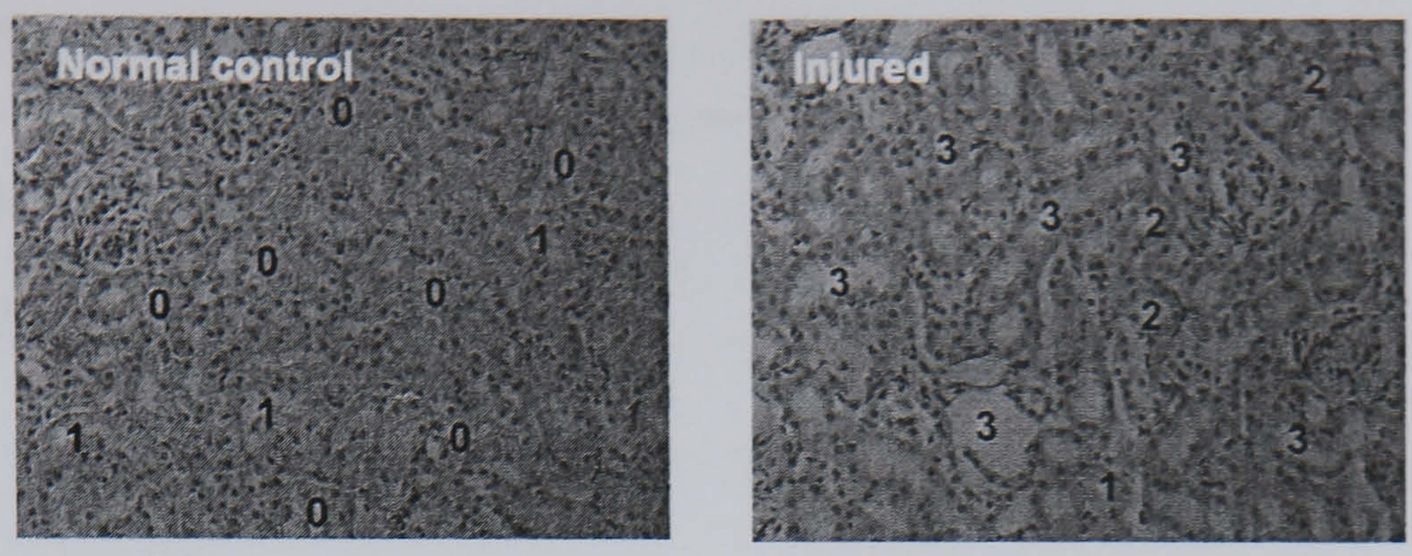


Figure 18. Method of single-blinded histopathological analysis for renal ischaemic-reperfusion injury experiments

Above, are photomicrograph examples of cross-sections of the normal and injured renal cortex, with scores from 0-3 assigned to 10 randomly chosen tubules. For each kidney, 10 photomicrographs were taken from the renal cortex. For each photomicrograph, 10 renal tubules were chosen at random by the scorer, who was unaware of the treatment groups. For each renal tubule chosen, a score of 0-3 was assigned, based on criteria described in Table 4, giving a maximum score of 300 per kidney. Mean scores were then calculated for each treatment group (magnification x20).

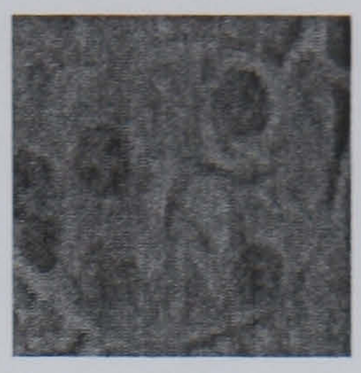
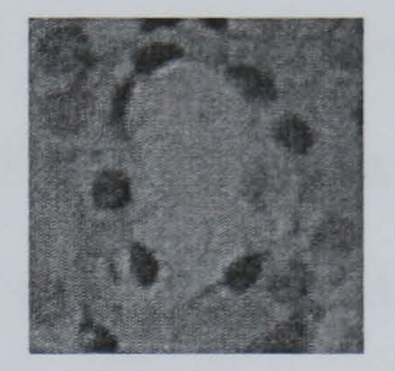
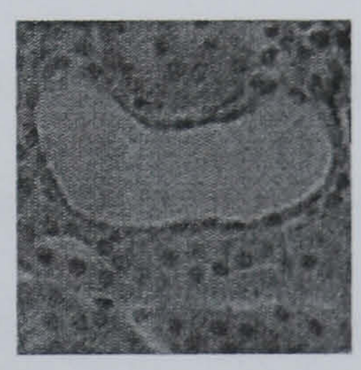
Histopathological scoring criteria	Photomicrograph (x20)
0 – No damage Normal renal tubular cells	
1 – Mild damage Slight dilatation of tubular lumen (50 % increase in diameter compared to normal)	
2 – Moderate damage Flattening of epithelial cells, or partial loss of nuclear staining, or moderately dilated lumen (50-100 % increase in diameter compared to normal)	
3 – Severe damage Flattening of epithelial cells and lacking nuclear staining or severely dilated lumen (>100 % increase in diameter compared to normal)	

Table 4. Histopathological scoring system for renal ischaemic-reperfusion injury experiments

This histopathological scoring system, based on a system devised by Hamar and colleagues (Hamar et al. 2004), assessed the degree of renal tubular dilatation, nuclear staining, and the presence of flattened epithelial cells, after renal ischaemic-reperfusion injury. The scorer was unaware of the treatment groups.

2.9 ANALYSIS OF RENAL FUNCTION

Blood samples from mice subjected to renal IRI were collected and centrifuged at 3000 rotations / minute for 10 minutes to obtain blood serum. As surrogate markers of renal function, creatinine and urea levels were measured in 100 µL of serum with an Olympus AU640 analyzer (Diamond Diagnostics, Watford, UK).

2.10 TUNEL STAINING

Apoptosis of renal tubular epithelial cells was detected by in-situ terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, a method that detects DNA fragments by labelling the terminal end of nucleic acids. The assay was performed according to the manufacturer's (Obiogene) instructions. Briefly, rehydrated 4 % paraformaldehyde-fixed cryostat sections were washed with 0.1 M PBS, twice for 5 minutes each, and then pretreated with 20 µg / mL proteinase K at room temperature for 15 minutes. For positive controls, sections were treated with nuclease (R&D system) at 37 ºC for 15 minutes. The sections were first quenched in 3 % hydrogen peroxide in 0.1 M PBS for 5 minutes. The quenched sections were then labelled with terminal deoxynucleotidyl transferase (TdT) enzyme at 37 ºC for 1 hour in a humidified chamber and subsequently incubated with anti-digoxygenin-dUTP, conjugated to horseradish peroxidase for 30 minutes at room temperature. They were then stained with DAB, followed by counterstaining with methyl green. The sum of TUNEL positive cells in an objective grid from ten areas of randomly selected renal cortex was counted under a 40x objective lens, by an investigator who was blinded to the experimental protocol.

2.11 STATISTICAL ANALYSIS

The results were analysed with Instat software and expressed as means +/- standard deviation of the mean. Statistical comparison was determined by one-way analysis of variance (ANOVA) followed by *post hoc* Student-Newman-Keul's multiple comparison test for parametric data, and Mann Whitney U test for non-parametric data. Survival was analysed by Kaplan-Meier test. Values with $p < 0.05$ were considered as statistically significant.

CHAPTER 3

RESULTS

3.1 REGULATION OF HIF-1α BY XENON PRECONDITIONING

3.1.1 Nitrogen does not affect HIF-1α protein expression

In establishing a suitable control for evaluating HIF-1α protein expression, which is sensitive to oxygen concentrations, a gas exposure paradigm of 75 % nitrogen balanced with 25 % oxygen for 2 hours was used and compared to mice exposed to normal room air, in order to be consistent with the 25 % oxygen used in the xenon preconditioning paradigm previously employed (Ma et al. 2006). Following exposure to either room air or 75 % nitrogen, mice were sacrificed over a time course of 0, 2, 4, 8 and 24 hours, and brains and kidneys harvested. Western blotting was performed to determine whether these two experimental groups differentially expressed HIF-1α.

HIF-1α protein expression was low and remained unaltered over the 24 hour time course in the mouse brain exposed to 75 % nitrogen with 25 % oxygen. These findings were comparable to mice exposed to room air. Results were normalised to the loading control, α-tubulin, to confirm that each sample contained equal concentrations of total protein. Similar findings were observed in kidney samples. Thereafter, control mice were exposed to 75 % nitrogen with 25 % oxygen for 2 hours, and sacrificed 24 hours after. This was to minimise the number of animals used in further experiments and to remain consistent with the xenon preconditioning paradigm previously established. There were no apparent signs of oxygen toxicity or distress to the mouse at 25 % oxygen exposure and this also avoided any risk of hypoxic exposure to mice, which would affect HIF-1α expression (Figure 19).

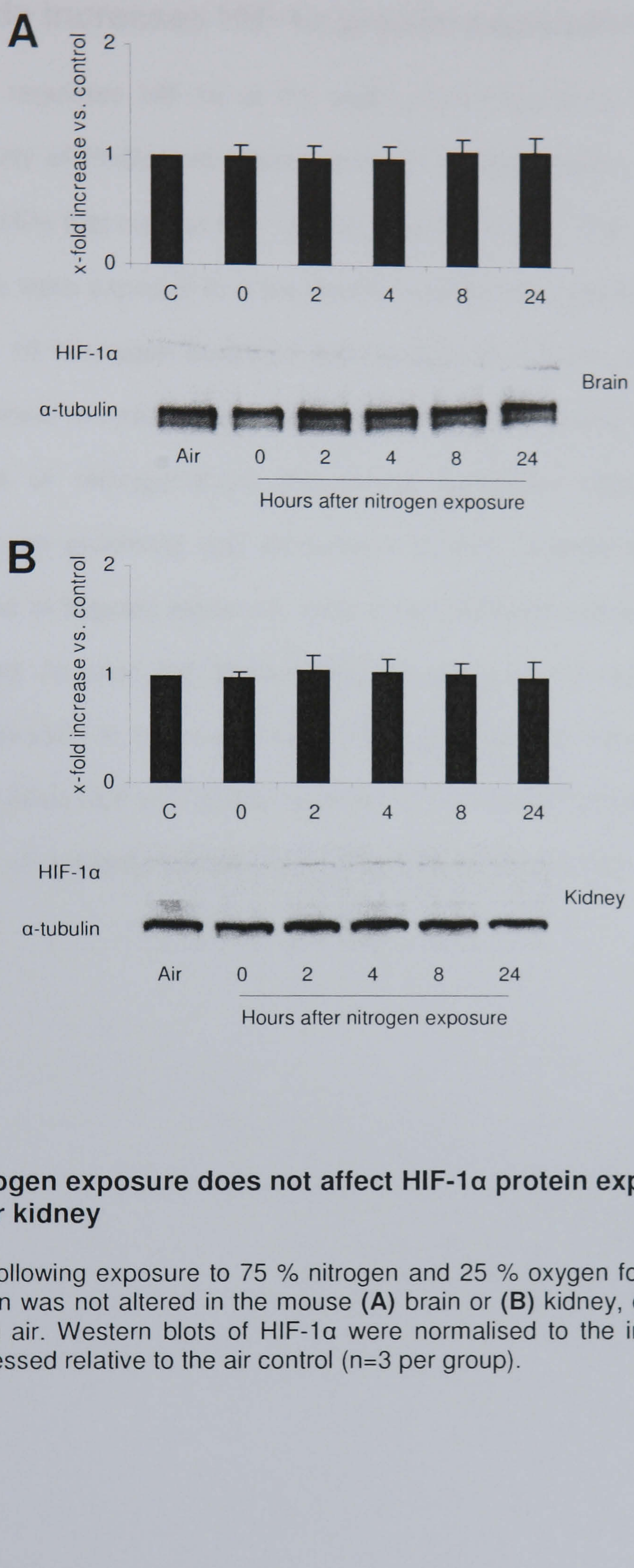


Figure 19. Nitrogen exposure does not affect HIF-1α protein expression in the mouse brain or kidney

In the 24 hours following exposure to 75 % nitrogen and 25 % oxygen for 2 hours, HIF-1α protein expression was not altered in the mouse **(A)** brain or **(B)** kidney, compared to mice exposed to room air. Western blots of HIF-1α were normalised to the internal control, α-tubulin, and expressed relative to the air control (n=3 per group).

3.1.2 Hypoxia increases HIF-1α protein expression

Oxygen regulates HIF-1α at the post-translational level, through oxygen-dependent activity of PHDs and subsequent HIF-1α degradation. Hypoxia inhibits activity of the PHDs that causes HIF-1α protein accumulation. For positive controls, male adult mice were exposed to a previously established hypoxic preconditioning paradigm of 8 - 10 % oxygen balanced with nitrogen for 3 hours. During exposure, mice were observed to reduce their spontaneous movements and were tachypneic. Within seconds of reoxygenation, the normal behaviour characteristics were displayed, such as grooming and exploration of their environment. Immediately following the end of hypoxic exposure, mice were sacrificed and brain and kidneys rapidly harvested. As expected, Western blot for expression of HIF-1α protein was significantly increased in hypoxia treated mice compared to nitrogen controls, in both the mouse brain (3.6 +/- 1.6 fold increase versus 1.0 of control (mean +/- SD)) and kidney (4.3 +/- 1.4 fold increase) (n = 3 per cohort) (Figure 20).

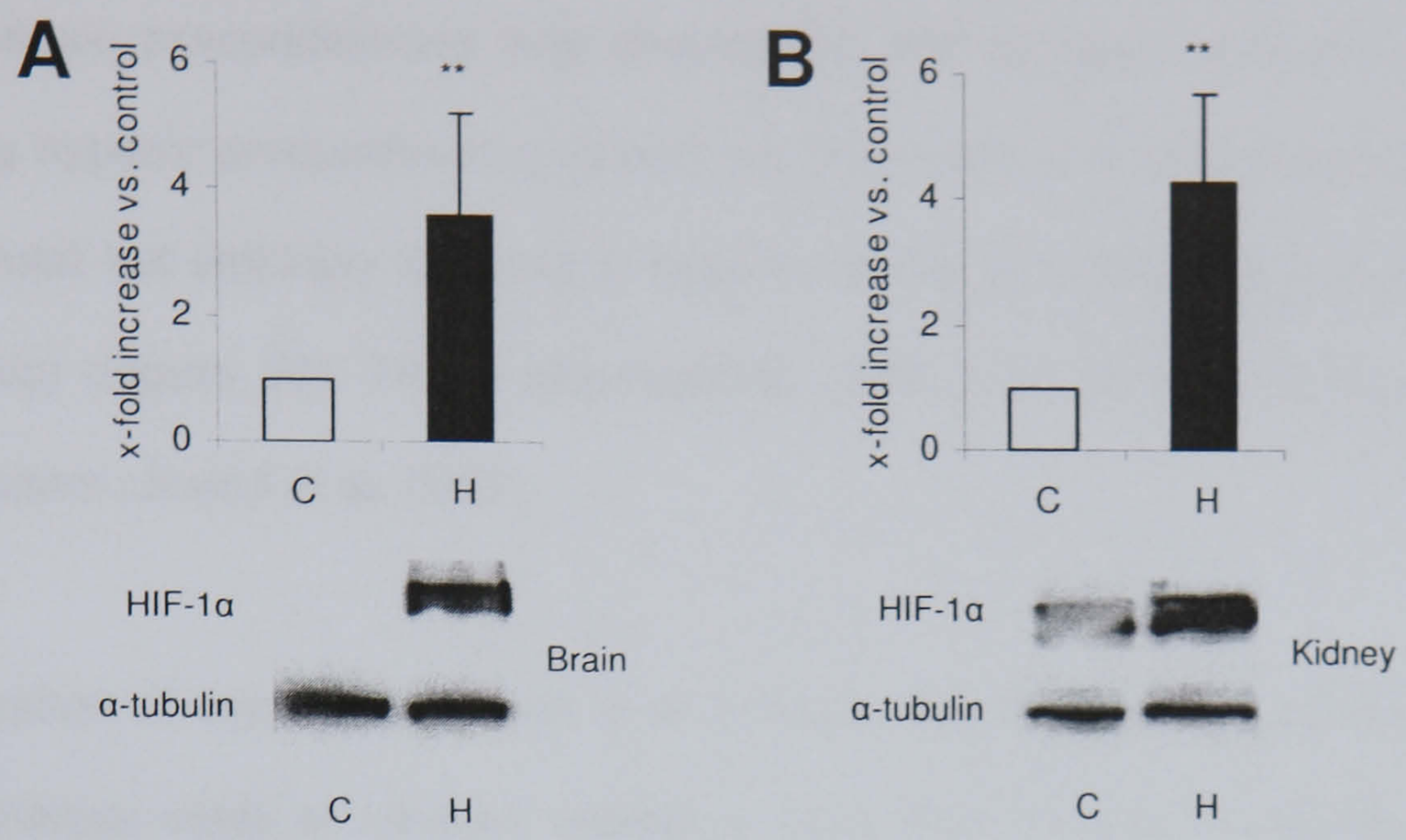


Figure 20. Hypoxic exposure increases HIF-1α protein expression in the mouse brain and kidney

Exposure to 3 hours of hypoxia (8 % oxygen) resulted in a striking increase in HIF-1α protein expression, the oxygen-sensitive subunit of the HIF-1 transcription factor, in the mouse **(A)** brain (3.6 +/- 1.6 versus 1.0 of control $p < 0.01$) and **(B)** kidney (4.3 +/- 1.4 versus 1.0 of control, $p < 0.01$). Western blots of HIF-1α were normalised to α-tubulin, and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. ** $p < 0.01$ vs. control (n=3 per cohort). **Abbreviations: C**, control; **H**, hypoxia; **HIF-1α**, hypoxia-inducible factor-1 alpha; **SD**, standard deviation.

Subsequently, HIF-1α expression over the time course of 0, 2, 4, 8 and 24 hours after hypoxic preconditioning was determined. HIF-1α was induced immediately following hypoxic preconditioning in both the brain (3.9 +/- 1.5 fold) and kidney (4.3 +/- 1.6 fold) but strikingly declined to baseline levels by 2 hours after hypoxia (n=3 per group) (Figure 21). These observations corroborate with the findings of other investigators (Jewell et al. 2001).

The duration of hypoxic exposure was 3 hours rather than 2 hours as used with xenon, nitrous oxide or nitrogen exposure. Less than 3 hours of hypoxic exposure has not been shown to protect against the noxious effect of subsequent HI injury, whereas 3 hours of hypoxia was able to provide robust protection against experimental stroke (Gidday et al. 1994). This paradigm also lacks detectable damage to the hippocampus or cortical neurons at the histological level (Prass et al. 2003) .

3.1.3 Xenon increases HIF-1α protein expression

To ascertain the effect of xenon preconditioning on HIF-1α expression, mice were exposed to the xenon preconditioning paradigm of 75 % xenon balanced with 25 % oxygen for 2 hours, and sacrificed over a time course of 0, 2, 4, 8 and 24 hours after. Western blots in brain and kidneys of mice were performed for detection of HIF-1α. In the brain, xenon preconditioning increased HIF-1α protein expression after 2 hours (1.80 +/- 0.2 versus 1.0 of control, $p < 0.05$) in a time-dependent manner, with further upregulation and maximal expression after 24 hours (2.91 +/- 0.62 versus 1.0 of control, $p < 0.01$). Similarly in the kidney, HIF-1α expression was increased 4 hours after xenon exposure (1.90 +/- 0.59 versus 1.0 of control, $p < 0.05$), with further upregulation and maximal expression after 24 hours (3.92 +/- 0.89 versus 1.0 of control $p < 0.001$) (n=4 per cohort) (Figure 22). This sustained

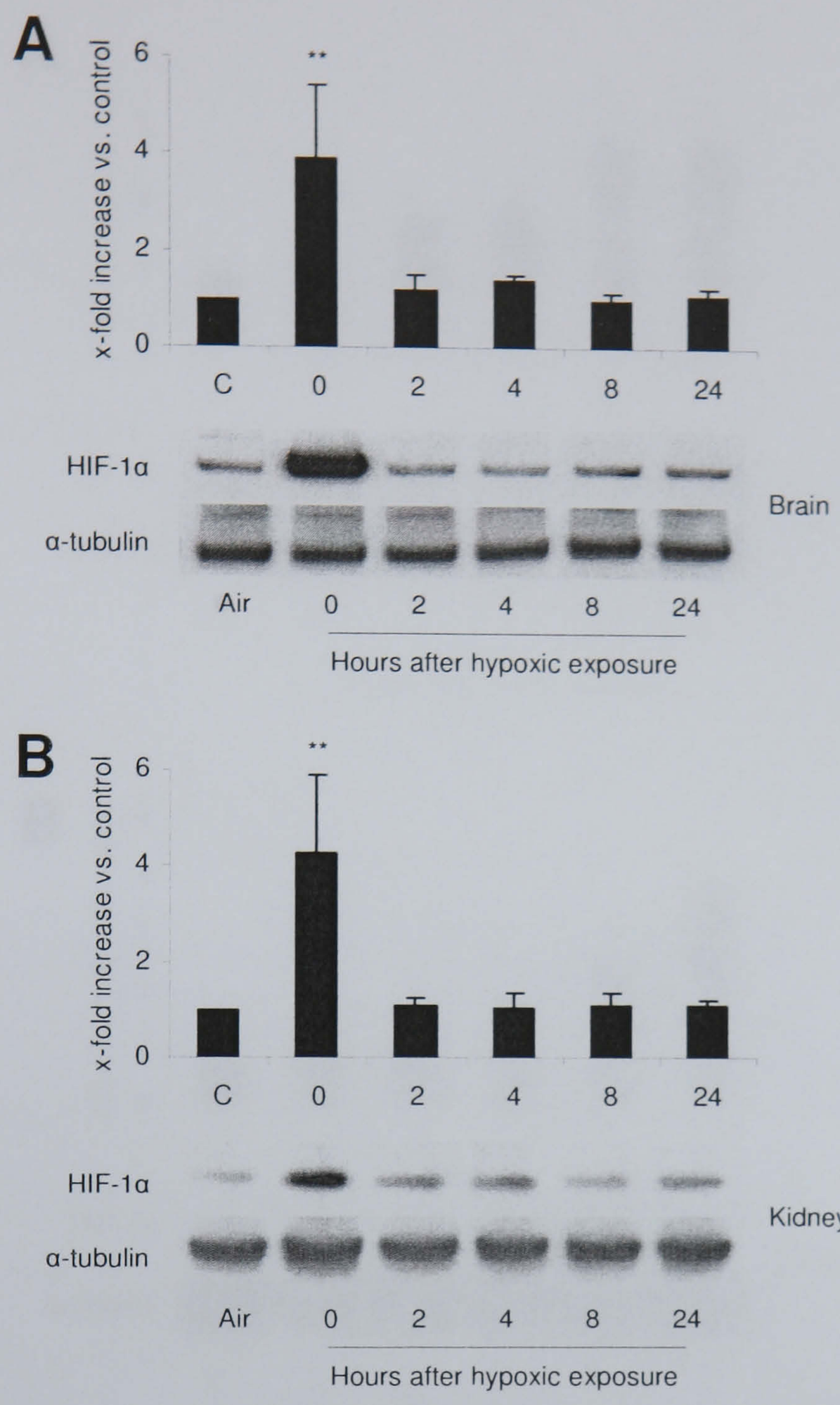


Figure 21. Hypoxic exposure transiently increases HIF-1α protein expression in the mouse brain and kidney

Over the time course of 0, 2, 4, 8 and 24 hours after exposure to hypoxia (8 % oxygen), an increase in HIF-1α protein expression was detectable immediately following hypoxia, at 0 hours, in the **(A)** brain (3.9 +/-1.5 fold increase) and **(B)** kidney (4.3 +/- 1.6 fold increase). Its expression was transient and declined to baseline levels by 2 hours after hypoxia. Western blots of HIF-1α were normalised to α-tubulin, and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. ** $p < 0.01$ vs. control (n=3 per group). **Abbreviations: C**, control; **H**, hypoxia; **HIF-1α**, hypoxia-inducible factor-1 alpha; **SD**, standard deviation.

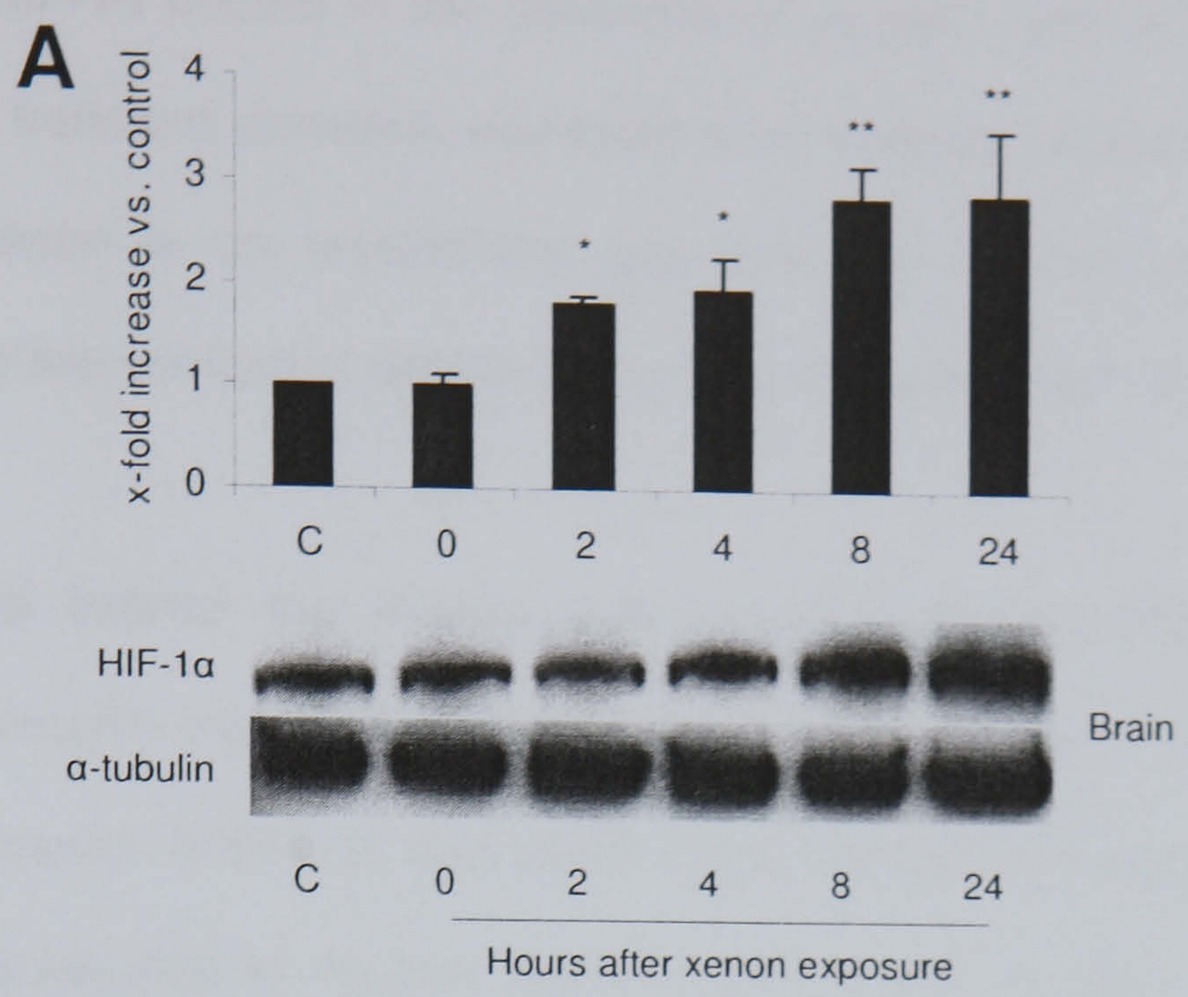
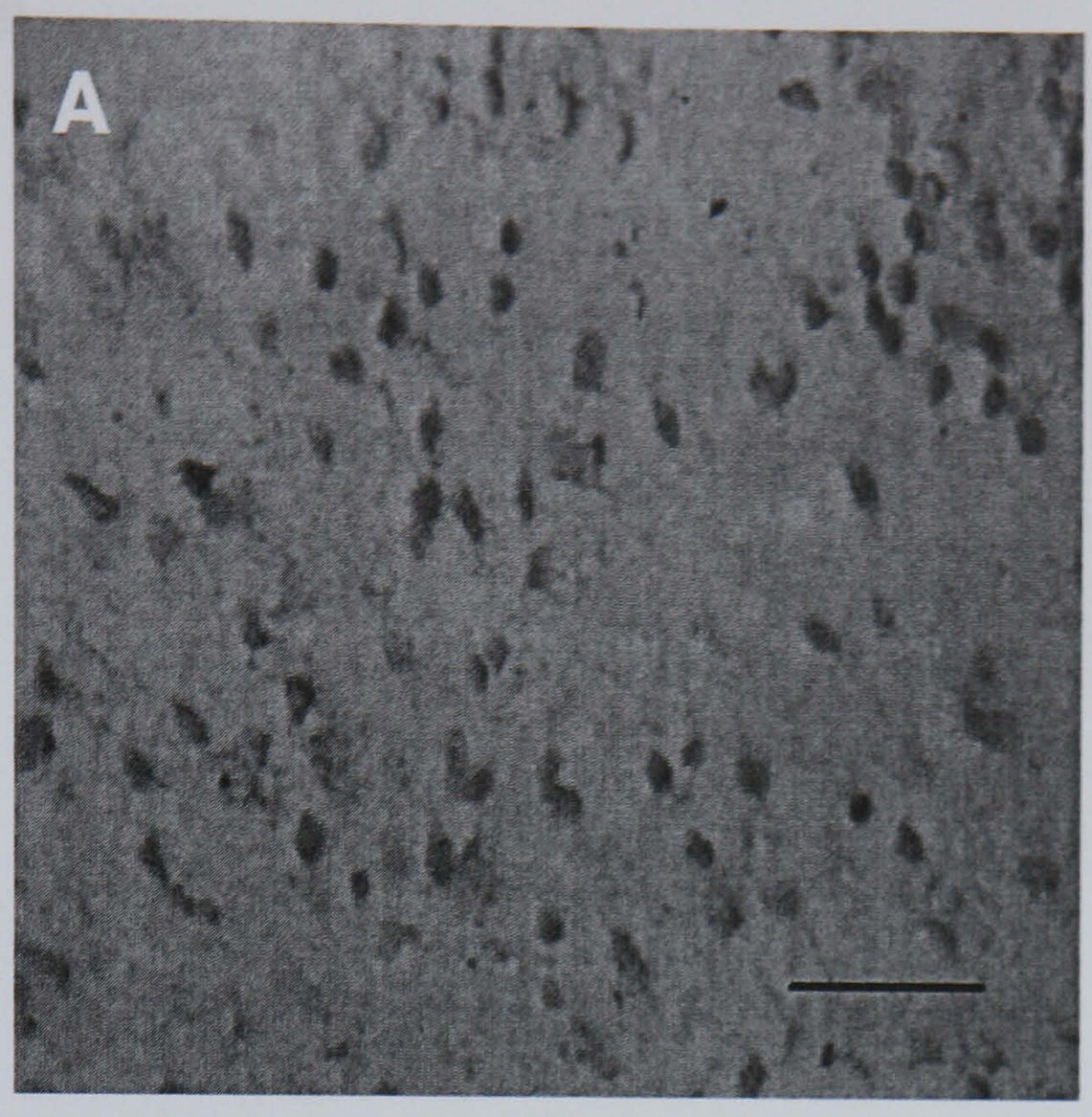


Figure 22. Xenon exposure increases HIF-1α protein expression in the mouse brain and kidney

Exposure to 75 % xenon and 25 % oxygen for 2 hours time-dependently increased HIF-1α protein expression in the mouse **(A)** brain after 2 hours (1.80 +/- 0.2 versus 1.0 of control, $p < 0.05$) in a time-dependent manner, with further upregulation and maximal expression after 24 hours (2.91 +/- 0.62 versus 1.0 of control, $p < 0.01$). In the **(B)** kidney, HIF-1α expression was increased 4 hours after xenon exposure (1.90 +/- 0.59 versus 1.0 of control, $p < 0.05$), with further upregulation and maximal expression after 24 hours (3.92 +/- 0.89 versus 1.0 of control $p < 0.001$). Western blots of HIF-1α were normalised to α-tubulin, and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control (n=4 per group). **Abbreviations: HIF-1α**, hypoxia-inducible factor-1 alpha; **SD**, standard deviation.

expression of HIF-1α occurs in the presence of oxygen, and is in contrast to the immediate and transient elevation observed after hypoxic exposure. These results indicate that xenon is an anaesthetic gas with HIF-1α inducing capability and suggest that the mechanism is different from that of hypoxic HIF-1α induction.

To confirm and extend the finding that xenon increases HIF-1α expression, immunohistochemistry was performed for HIF-1α in the mouse brain. While little or no nuclear expression of HIF-1α was observed in nitrogen-exposed control animals, xenon exposure resulted in nuclear accumulation of HIF-1α after 6 hours, in brain regions that are rendered tolerant by xenon preconditioning in acute neuronal hypoxic-ischaemic injury (Ma et al. 2006) (Figure 23). Additionally, primary neuronal-glial co-cultures were exposed to xenon. There was a trend towards an increase in HIF-1α expression from 4 hours after xenon exposure, with significant expression occurring after 8 hours (2.2 +/- 0.28 versus 1.0 of control, $p < 0.05$) (n=3 per group) (Figure 24). Human kidney 2 (HK2) cells were also exposed to xenon, where HK2 is a well characterised human kidney proximal tubular epithelial cell line. HK2 cells exhibited HIF-1α activation with similar kinetics to those observed in the *in vivo* mouse kidney. HIF-1α expression was increased after 4 hours (2.2 +/- 0.59 versus 1.0 of control, $p < 0.05$) with maximal expression after 24 hours (3.8 +/- 0.63 versus 1.0 of control, $p < 0.001$) (n=3 per group). Therefore, xenon preconditioning activates HIF-1α in isolated human renal epithelial cells (Figure 25).



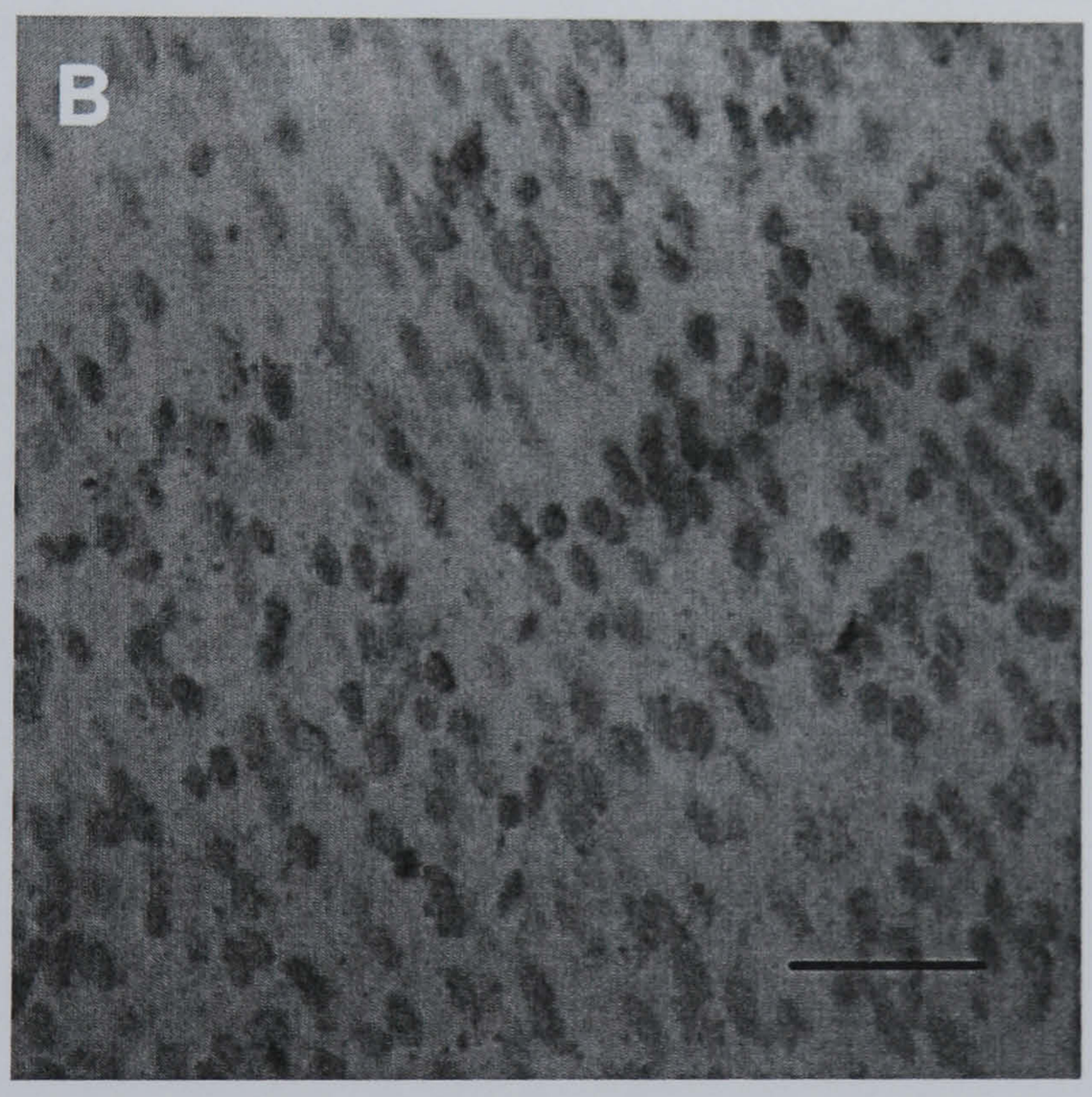


Figure 23. Immunohistochemistry examples of xenon-induced HIF-1α expression in the mouse brain

Above are representative immunohistochemical sample images, immunostained for HIF-1α. Panel **(A)** shows a section of the neuronal cortex from a control mouse, 6 hours after treatment with 75 % nitrogen and 25 % oxygen for 2 hours. Panel **(B)** shows a section of the neuronal cortex, 6 hours after treatment with 75 % xenon and 25 % oxygen for 2 hours. More HIF-1α positive cells were detected in xenon-treated mice, compared to controls. Scale bar represents 100 µm.

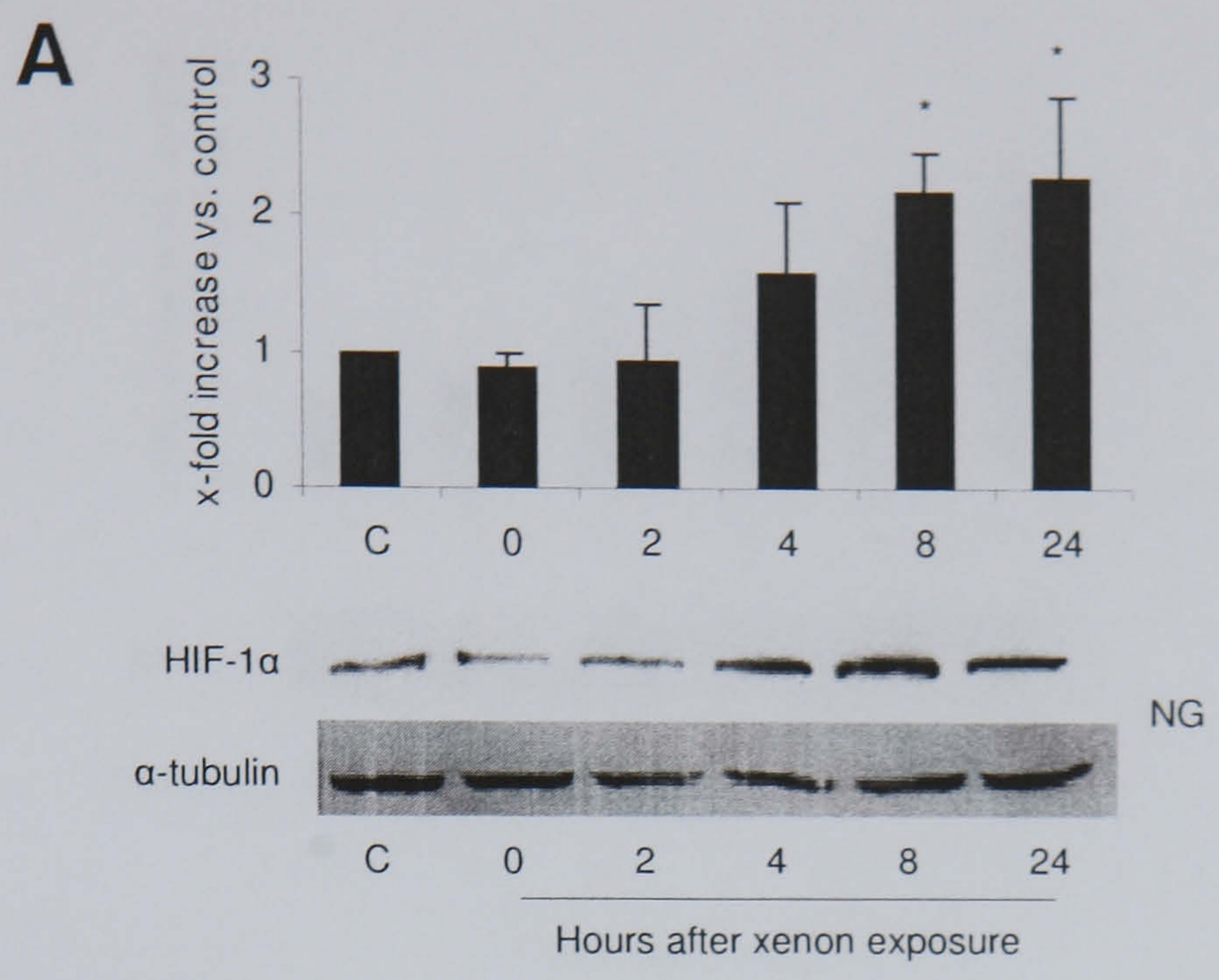


Figure 24. Xenon exposure increases HIF-1α protein expression in primary neuronal-glial co-cultures

HIF-1α protein expression was time-dependently increased in neuronal-glial co-cultures exposed to 75 % xenon, 20 % oxygen and 5 % carbon dioxide for 2 hours. Maximal xenon-induced HIF-1α expression occurred after 8 hours (2.2 +/- 0.28 versus 1.0 of control, $p < 0.05$). Western blots of HIF-1α, were normalised to α-tubulin, and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. * $p < 0.05$ (n=3 per group).

Abbreviations: HIF-1α, hypoxia-inducible factor-1 alpha; NG, neuronal-glial co-culture; SD, standard deviation.

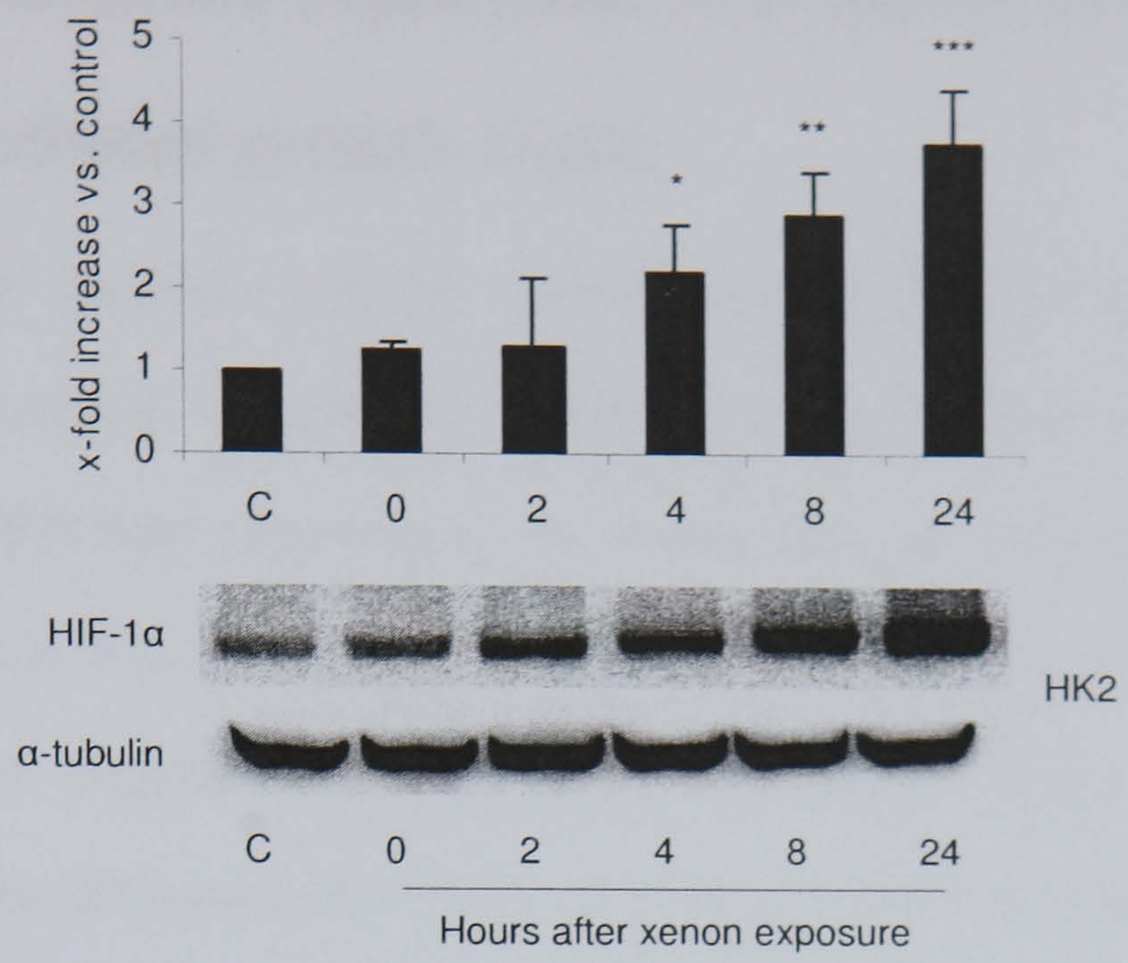


Figure 25. Xenon exposure increases HIF-1α protein expression in human kidney-2 cell cultures

Exposure to 75 % xenon, 20 % oxygen and 5 % carbon dioxide for 2 hours time-dependently increased HIF-1α protein expression in HK2 cell cultures from 4 hours (2.2 +/- 0.59 versus 1.0 of control, $p < 0.05$) with maximal expression after 24 hours (3.8 +/- 0.63 versus 1.0 of control, $p < 0.001$). Western blots of HIF-1α, were normalised to α-tubulin, and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control (n=3 per group). **Abbreviations: HIF-1α**, hypoxia-inducible factor-1 alpha; **HK2**, human kidney-2 cells; **SD**, standard deviation.

3.1.4 Xenon increases expression of erythropoietin and vascular endothelial growth factor

In order to ascertain whether xenon-induced HIF-1α was redundant or indeed biologically active, the effect of xenon preconditioning on expression of the HIF target gene EPO was examined in the mouse brain and kidney. In the kidney, RT-PCR revealed a time-dependent increase in EPO mRNA expression from 2 hours after xenon exposure (3.8 +/- 0.79, $p < 0.05$), with striking maximal expression occurring 24 hours after (6.32 +/- 1.98, $p < 0.01$) (n=3 per group) (Figure 26). Evidence of EPO mRNA translation was observed when Western blots for EPO protein detected a significant increase in expression 24 hours after xenon exposure (2.3 +/- 0.74 versus 1.0 of control, $p < 0.01$). In the brain, xenon–induced expression of EPO protein after 24 hours (2.23 +/- 0.60 versus 1.0 of control, $p < 0.05$) was also observed, occurring in a comparable fashion to that in the kidney (n=3 per group) (Figure 27).

It is not known whether xenon's effects are exclusive to EPO alone or more widespread, and therefore protein expression of another canonical HIF target gene, vascular endothelial growth factor (VEGF), in the mouse kidney was evaluated. Western blot detected an elevation in VEGF expression from 8 hours (1.9 +/- 0.22 versus 1.0 of control, $p < 0.05$) to 24 hours following xenon exposure (2.05 +/- 0.25 versus 1.0 of control, $p < 0.05$), in a similar manner to xenon–induced EPO. The time course of EPO mRNA elevation (occurring from 2 hours and preceding the protein expression of EPO and VEGF from 24 hours) is relevant to xenon-induced HIF-1α, where accumulation begins after 2 hours with maximal expression after 24 hours (Figure 28).

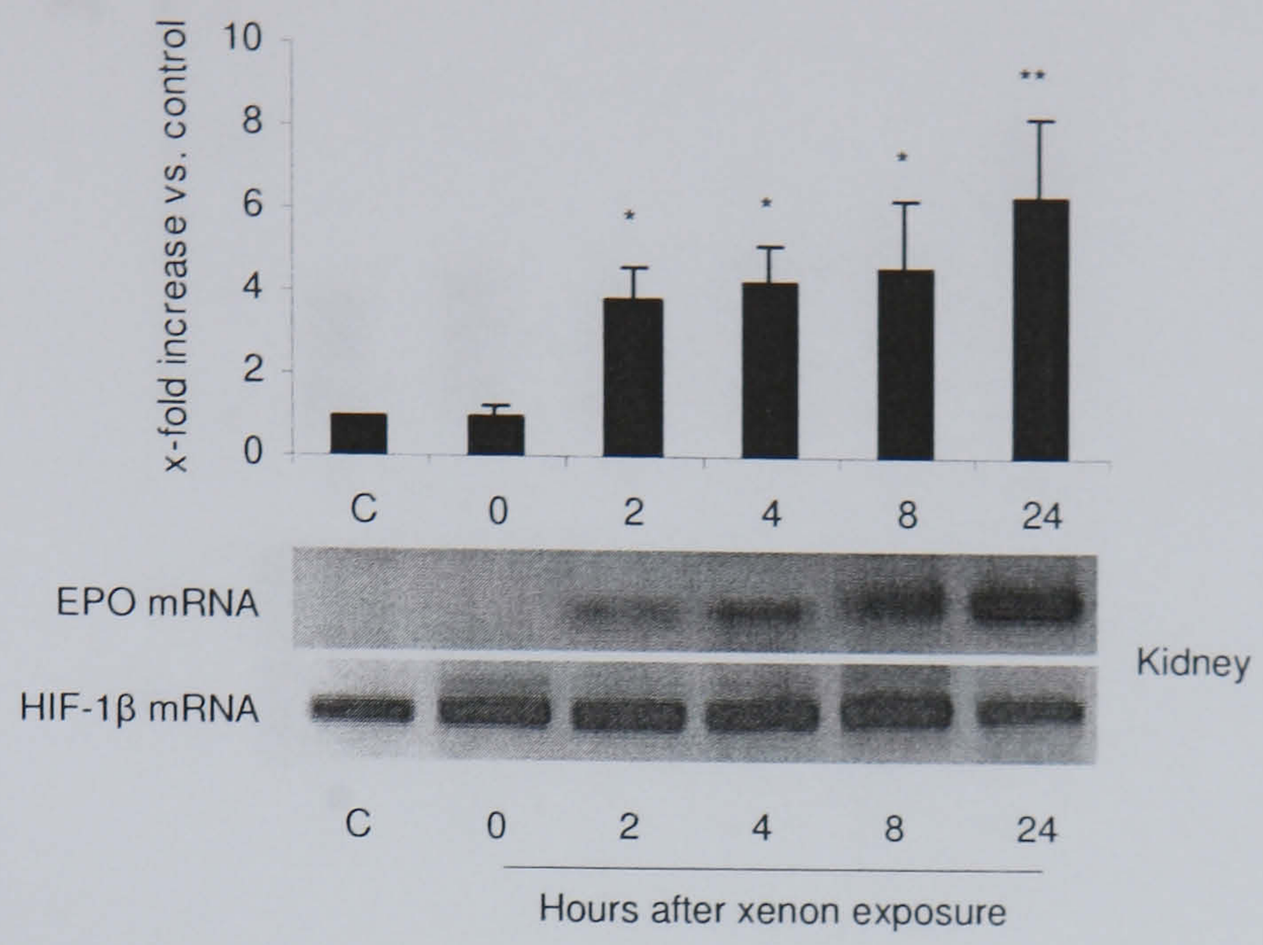


Figure 26. Xenon exposure increases erythropoietin mRNA transcription in the mouse kidney

RT-PCR experiments showed that exposure to 75 % xenon and 25 % oxygen for 2 hours time-dependently increased EPO mRNA expression in the mouse kidney from 2 hours after xenon exposure (3.8 +/- 0.79, $p < 0.05$), with maximal expression occurring 24 hours after (6.32 +/- 1.98, $p < 0.01$). EPO mRNA results were normalised to HIF-1β mRNA and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. * $p < 0.05$, and ** $p < 0.01$ versus control (n=3 per group). **Abbreviations: EPO,** erythropoietin; **HIF-1β,** hypoxia-inducible factor-1 beta; **SD,** standard deviation.

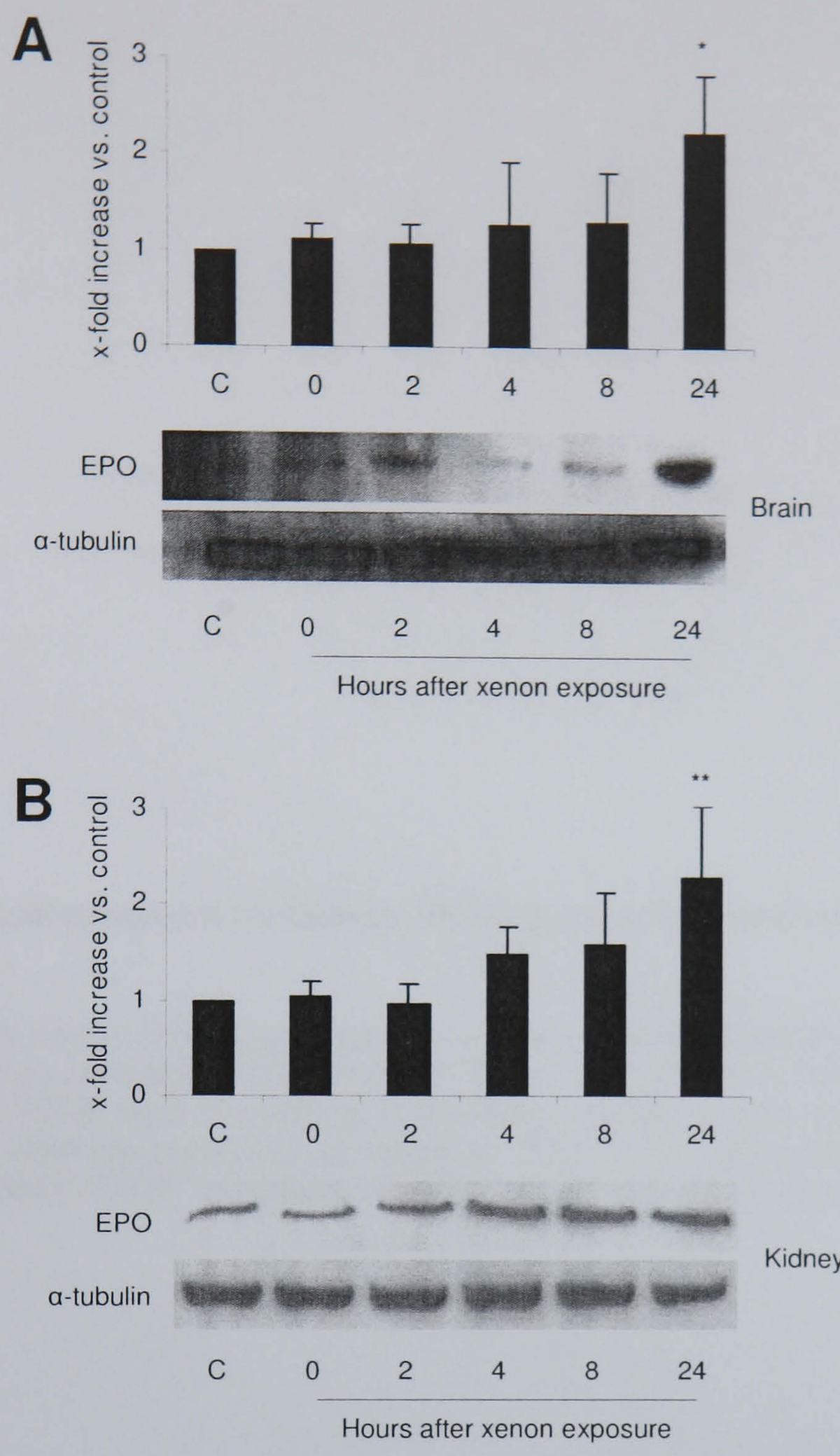


Figure 27. Xenon exposure increases erythropoietin protein expression in the mouse brain and kidney

Exposure to 75 % xenon and 25 % oxygen for 2 hours increased EPO protein expression in the mouse **(A)** brain after 24 hours (2.23 +/- 0.60 versus 1.0 of control, $p < 0.05$). Similarly, in the **(B)** kidney, xenon increased EPO protein expression after 24 hours (2.3 +/- 0.74 versus 1.0 of control, $p < 0.01$). Western blots of EPO were normalised to α-tubulin, and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. * < $p < 0.05$, ** $p < 0.01$ versus control (n=3 per group). **Abbreviations: EPO,** erythropoietin; **SD**, standard deviation.

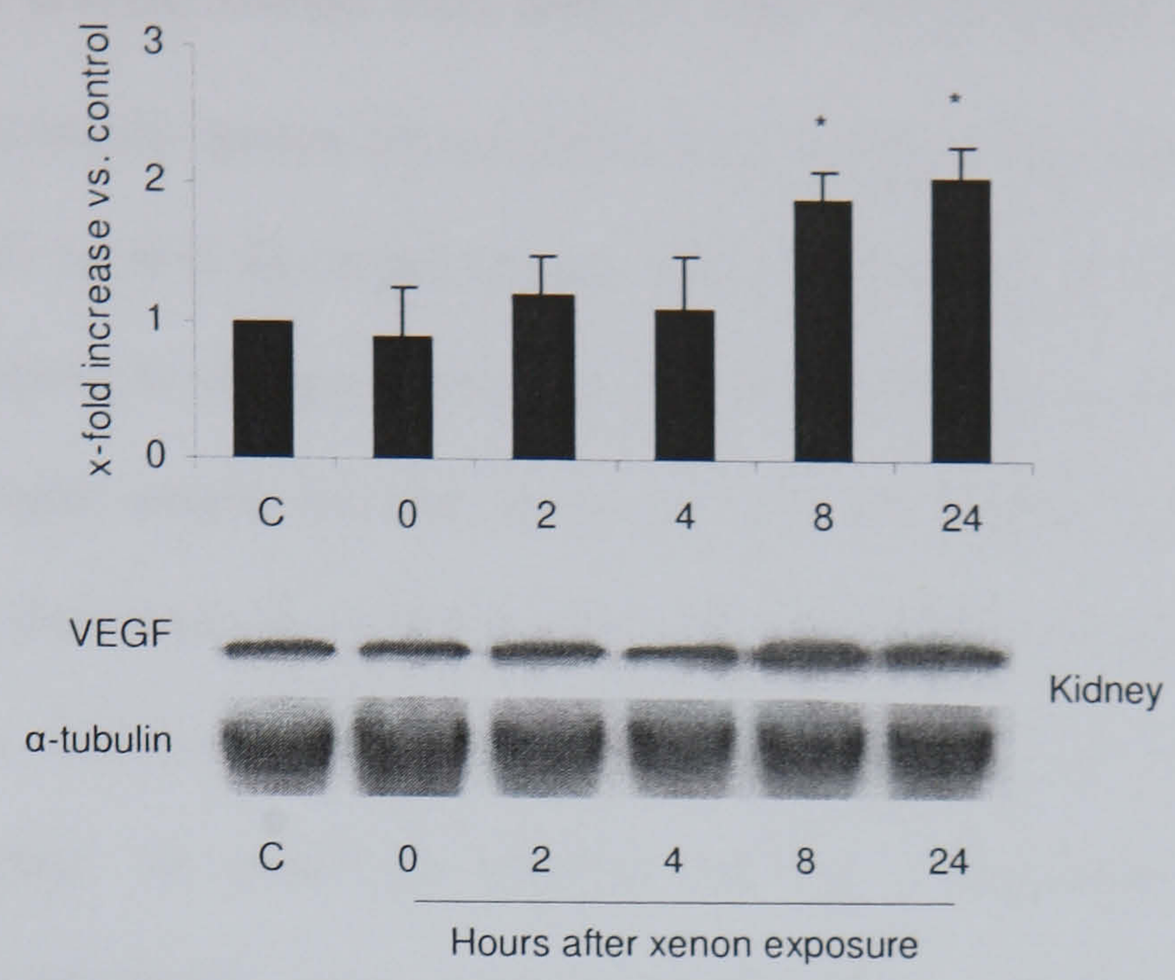


Figure 28. Xenon exposure increases VEGF protein expression in the mouse kidney

Exposure to 75 % xenon and 25 % oxygen for 2 hours increased VEGF protein expression in the mouse kidney from eight hours onwards (1.9 +/- 0.22 versus 1.0 of control, $p < 0.05$). Western blots of VEGF were normalised to α-tubulin, and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. * $p < 0.05$ versus control (n=3 per group). Abbreviations: VEGF, vascular endothelial growth factor; SD, standard deviation.

3.1.5 Nitrous oxide does not affect HIF-1α protein expression

The experiments above demonstrate that xenon preconditioning increases expression of HIF-1α and its target genes, EPO and VEGF. But whether xenon's preconditioning effect is related to its anaesthetic properties is not known. Xenon exerts its anaesthetic effects through antagonism of the excitatory NMDA receptor, thereby reducing downstream excitatory transmission. Nitrous oxide, also known as laughing gas, is another NMDA receptor antagonist that is routinely used in anaesthetic practice. To ascertain whether HIF-1α is regulated by anaesthetic NMDA receptor antagonism, mice were exposed to 75 % nitrous oxide for 2 hours and HIF-1α expression determined in the kidney from 0, 2, 4, 8 and 24 hours after. Western blot for HIF-1α revealed no effect of nitrous oxide on HIF-1α expression at any of the time points studied, compared to controls (n=4) (Figure 29). This suggests that the capability of xenon to induce HIF-1α is not simply due to its anaesthetic properties, and is independent from its blocking activity at the NMDA receptor.

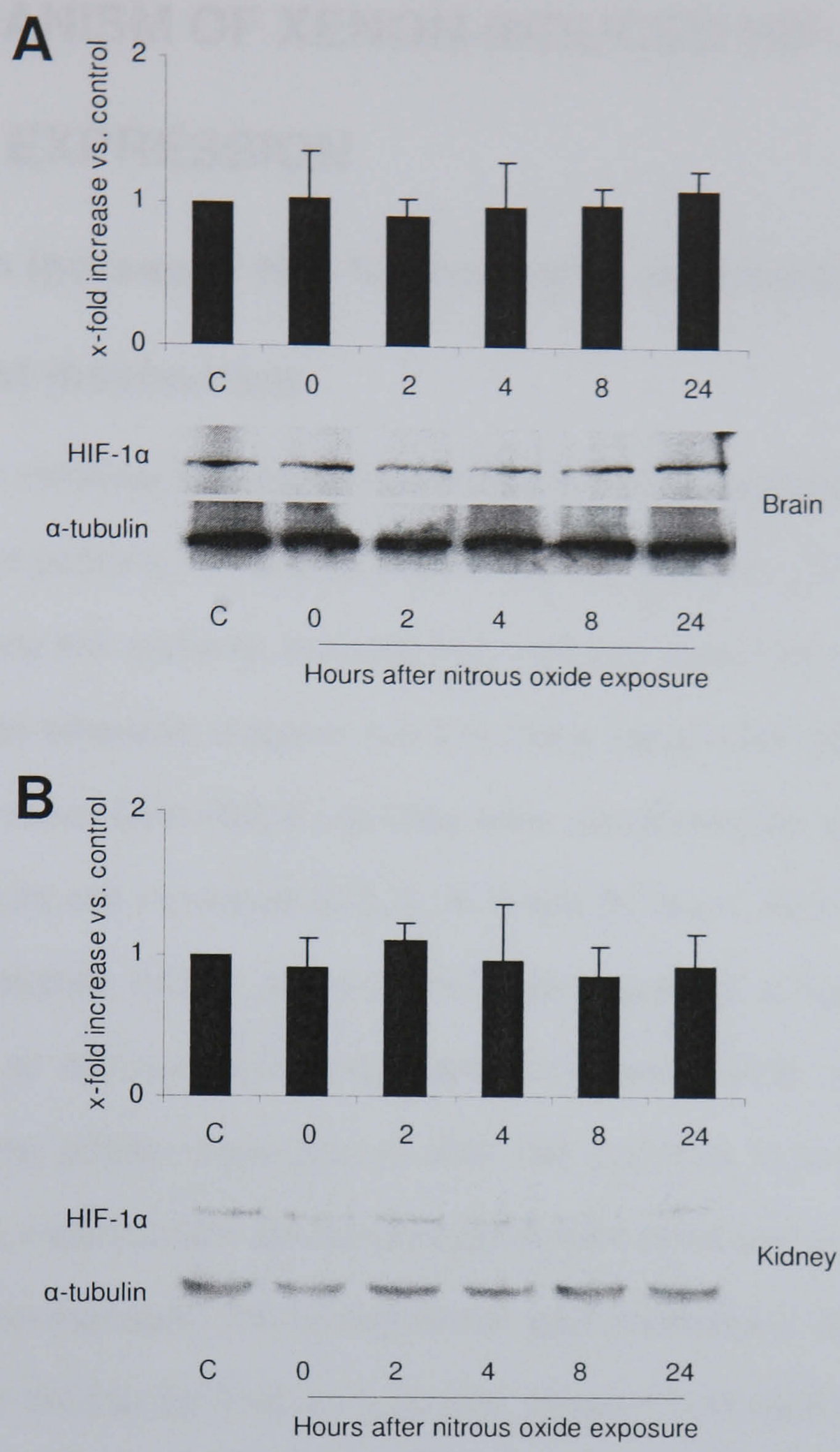


Figure 29. Nitrous oxide exposure does not affect HIF-1α protein expression in the mouse brain or kidney

Exposure to another NMDA receptor antagonist, 75 % nitrous oxide and 25 % oxygen for 2 hours, did not affect expression of HIF-1α over the 24 hour time-period studied, in the mouse **(A)** brain or **(B)** kidney, compared to control mice exposed to 75 % nitrogen. Western blots of HIF-1α were normalised to the internal control α-tubulin, and expressed relative to the nitrogen control (n=4 per cohort). **Abbreviations: HIF-1α,** hypoxia-inducible factor-1 alpha; **NMDA**, N-methyl D-aspartate.

3.2 MECHANISM OF XENON-INDUCED HIF-1α PROTEIN EXPRESSION

3.2.1 Xenon increases HIF-1α through a degradation-independent mechanism

Hypoxia stabilises HIF-1α through inhibition of its degradation in a PHD2 and VHL -dependent pathway. To answer whether xenon regulates HIF-1α by impairing its destruction via this pathway, parental VHL-negative renal cell carcinoma-4 cells (RCC4-), and an otherwise isogenic sub-line stably transfected with pcDNA3–VHL (RCC4+) were used. Both RCC4 sub-lines were simultaneously exposed to 75 % xenon for 2 hours and harvested at 0, 2, 4, 8 and 24 hours after for Western blot analysis. As expected, RCC4- (without VHL) cells expressed a higher level of HIF-1α compared to RCC4+ (with VHL) cells. However, xenon time-dependently increased HIF-1α protein expression in both cell sub-lines to the same order of magnitude, with a comparable rate of increase evident at all time points; twenty-four hours after xenon exposure, HIF-1α expression was increased in RCC4- by 3.31 +/- 0.89 fold, and in RCC4+ by 3.15 +/- 1.10 fold, versus 1.0 of control, $p < 0.01$ (n=4 per group). Therefore, xenon-induced increase in HIF-1α expression occurs through a VHL-independent mechanism (Figure 30).

The effect of xenon preconditioning on HIF-1α degradation was also investigated by measuring the expression of PHD2 protein by Western blot analysis. Mice were preconditioned with xenon and sacrificed at 0, 2, 4, 8 and 24 hours after. Xenon did not affect PHD2 protein expression compared to controls in the brain or kidney (n=3 per group) (Figure 31). Although HIF-1α is typically regulated through degradation, xenon's actions are not dependent on components of the canonical HIF-1α destruction pathway.

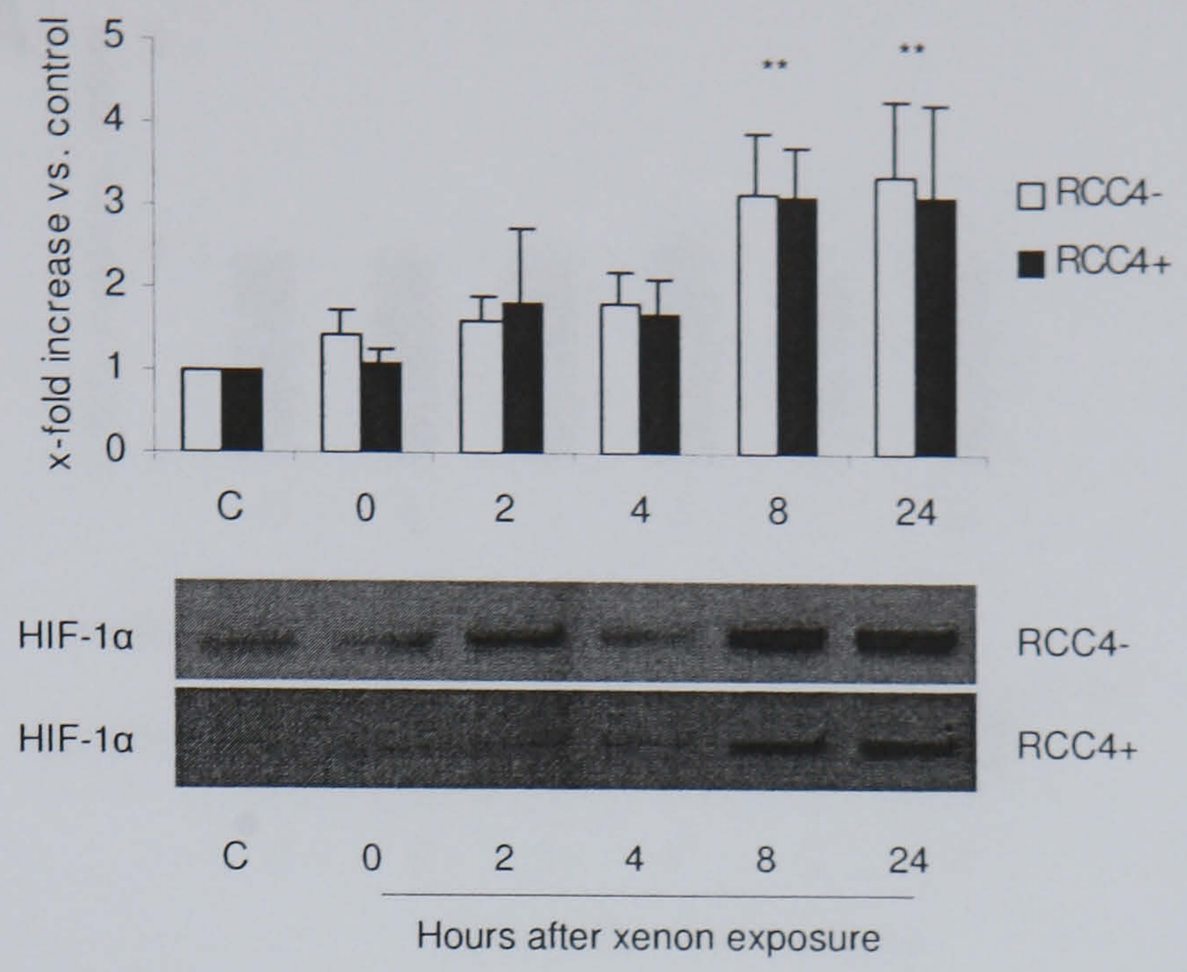


Figure 30. Xenon exposure increases HIF-1α through a VHL-independent mechanism

RCC4- and RCC4+ cells were simultaneously exposed to 75 % xenon, 20 % oxygen and 5 % carbon dioxide for 2 hours, and expression of HIF-1α protein determined by Western blot analysis. RCC4- cells expressed a constitutively higher level of HIF-1α compared to RCC4+ cells in the controls and at all time points after xenon exposure. Xenon exposure time-dependently increased HIF-1α protein expression in both cell sub-lines, to the same order of magnitude. Twenty-four hours after xenon exposure, HIF-1α expression was increased in RCC4- by 3.31 +/- 0.89 fold, and in RCC4+ by 3.15 +/- 1.10 fold, versus 1.0 of control, $p < 0.01$. Western blots of HIF-1α were normalised to α-tubulin, and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. ** denotes $p < 0.01$, versus control (n=4 per group). **Abbreviations: HIF-1α**, hypoxia-inducible factor-1 alpha; **RCC4-,** parental VHL-negative renal cell carcinoma-4 cells; **RCC4+,** renal cell carcinoma-4 cells with stable pcDNA3-VHL transfection; **SD**, standard deviation.

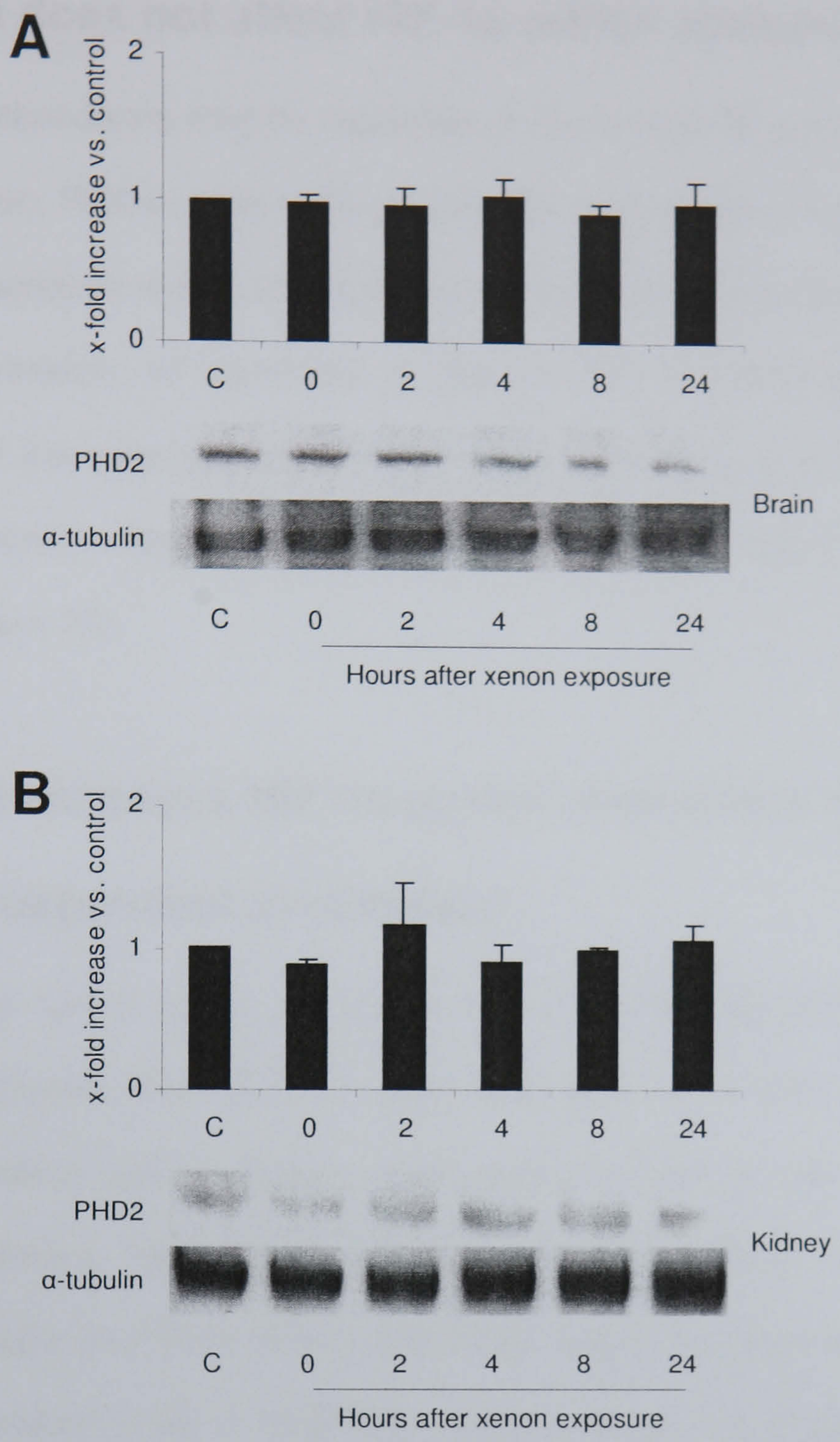


Figure 31. Xenon exposure does not affect PHD2 protein expression in the mouse brain or kidney

In the 24 hours following exposure to 75 % xenon and 25 % oxygen for 2 hours, expression of the PHD2 enzyme, required for HIF-1α prolyl hydroxylation, was not altered in the mouse **(A)** brain or **(B)** kidney compared to nitrogen controls. Western blots of PHD2, were normalised to the internal control α-tubulin, and expressed relative to the control (75 % nitrogen) (n=3 per group) **Abbreviations: PHD2,** prolyl hydroxylase domain-containing 2 enzyme.

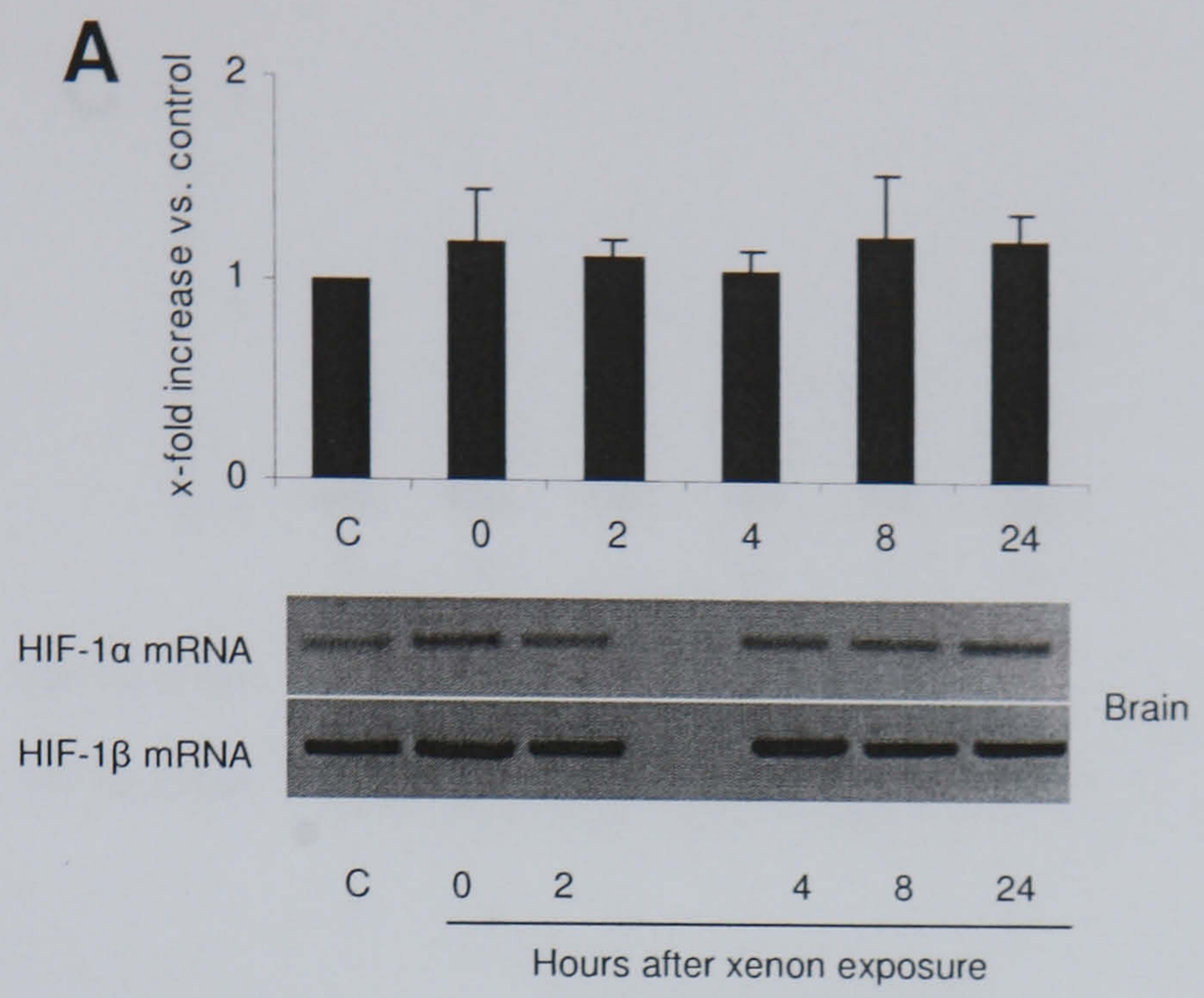
3.2.2 Xenon does not affect HIF-1α mRNA transcription

HIF-1α abundance may be regulated at the level of its gene transcription or mRNA translation. RCC4+ cells were preconditioned to explore the effect of xenon on HIF-1α transcription over a 24 hour time course. Although xenon increases HIF-1α protein expression, no significant changes in HIF-1α mRNA expression were detectable over the same period. Further *in vivo* studies in the mouse brain and kidney corroborated these findings that xenon did not affect HIF-1α mRNA expression (Figure 32).

3.2.3 Xenon increases HIF-1α protein expression through a translation-dependent mechanism

Next, the role of xenon in modulating the translational efficiency of HIF-1α mRNA was explored. The effect of xenon exposure on mTOR expression was studied by Western blot in RCC4+ cells, where mTOR is the best described regulator of protein translation, including HIF-1α synthesis. Interestingly, a detectable increase in mTOR protein expression could be observed from 2 hours after xenon exposure (1.48 +/- 0.39 fold increase versus 1.0 of control, $p > 0.05$), but striking and maximal expression occurred at 24 hours after xenon exposure (5.93 +/- 1.36 versus of control, $p < 0.001$) (Figure 33).

To explore the significance of the xenon–induced increase in mTOR protein expression, the specific mTOR inhibitor, rapamycin (25, 50 or 100 nM, dissolved in 0.00025 % DMSO), was administered 30 minutes prior to xenon or nitrogen exposure in RCC4+ cells. Cells were subsequently harvested 24 hours after, for detection of HIF-1α protein. Control cells received volume–matched culture medium containing 0.00025 % DMSO. Rapamycin alone did not affect HIF-1α expression; as expected xenon exposure increased expression of HIF-1α.



(Figure 32, legend overleaf)

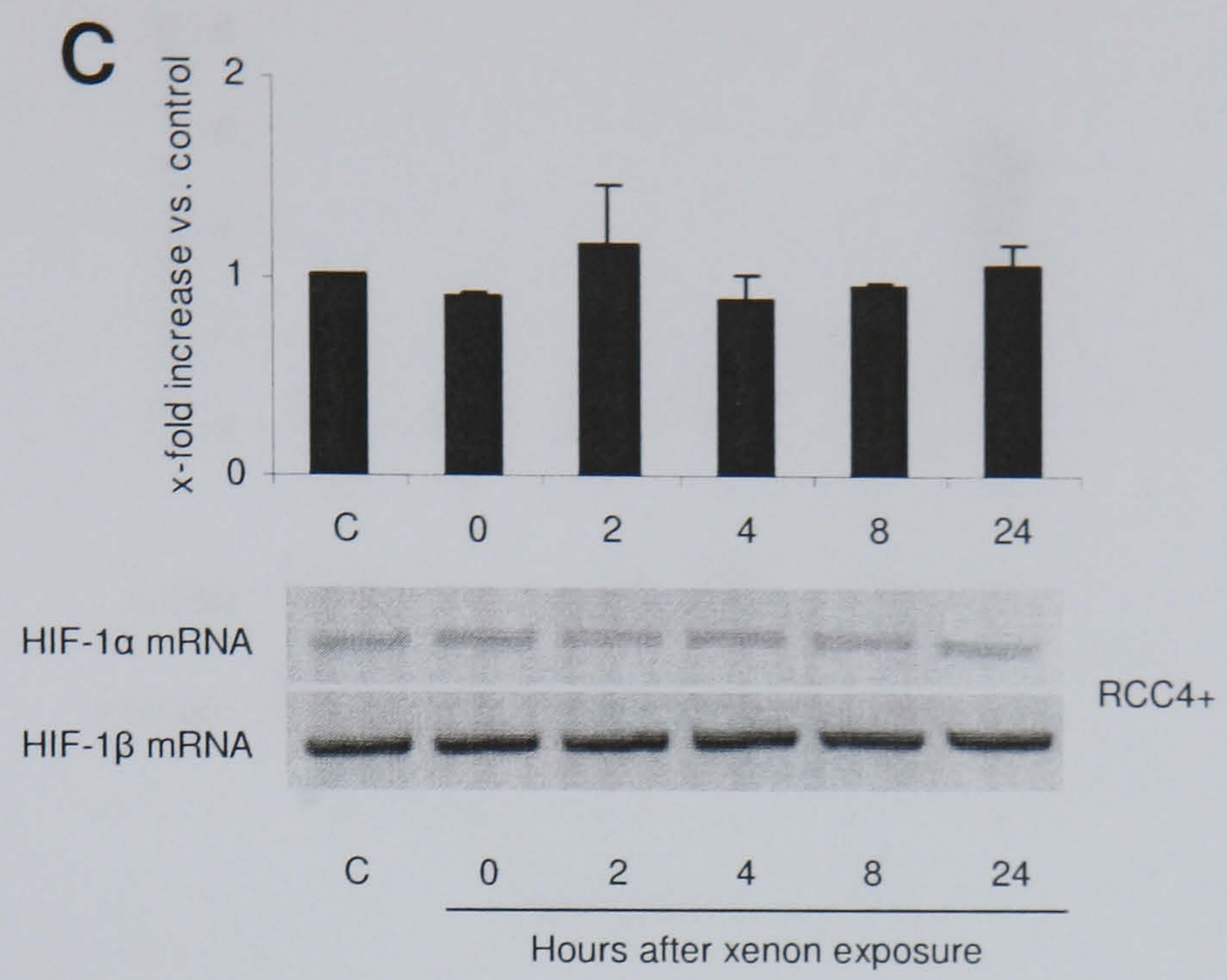


Figure 32. Xenon exposure does not affect HIF-1α mRNA transcription

Over the same 24 hour time period that exposure to 75 % xenon increases HIF-1α protein expression, xenon does not affect HIF-1α mRNA expression in the mouse **(A)** brain or **(B)** kidney, nor in **(C)** the RCC4+ cell sub-line, compared to nitrogen controls. RT-PCR results of HIF-1α mRNA were normalised to HIF-1β mRNA, and expressed relative to the control (75 % nitrogen) (n=3 per group) **Abbreviations: RCC4+,** renal cell carcinoma cell sub-line with stable pcDNA3-VHL transfection.

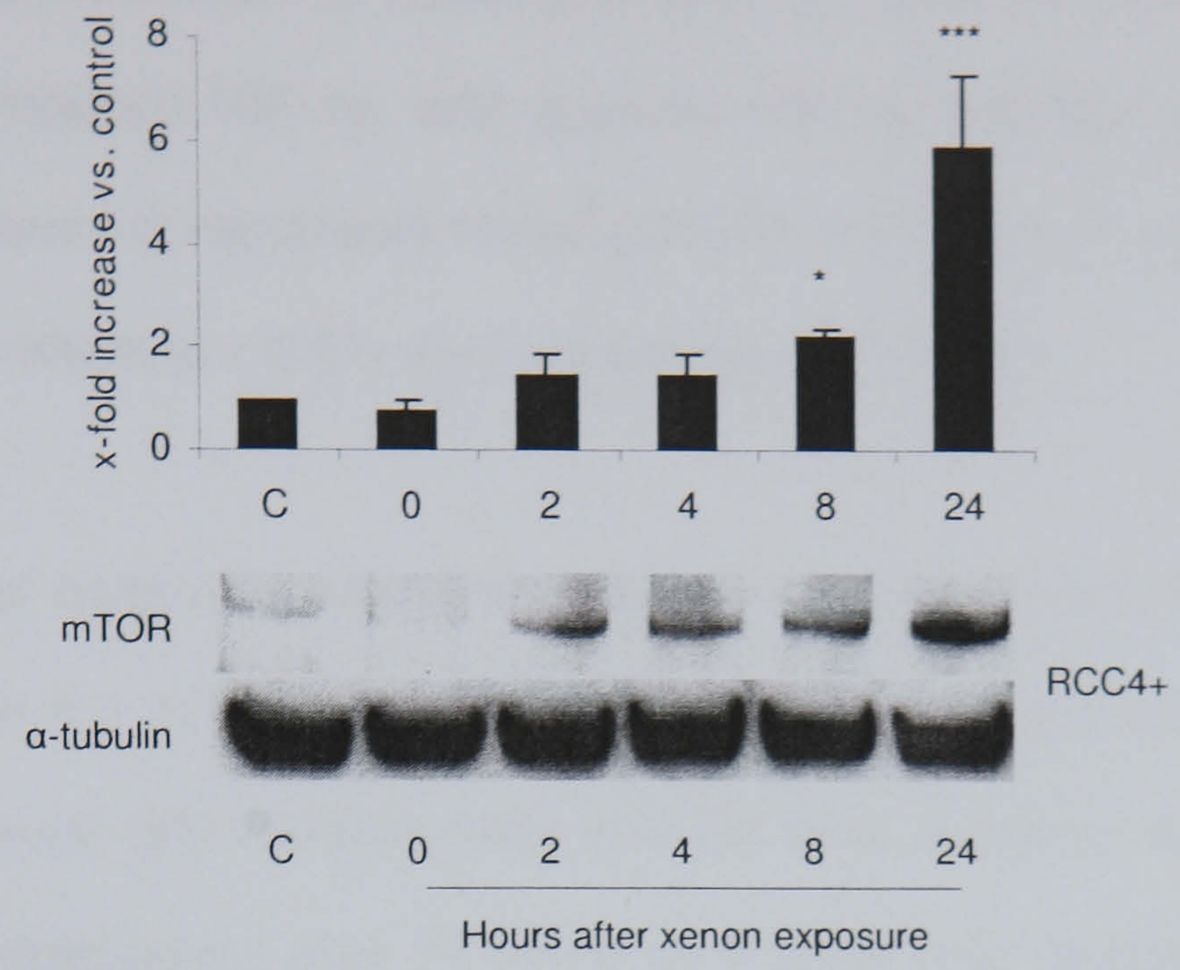


Figure 33. Xenon exposure increases mTOR protein expression in RCC4+ cell culture

The involvement of mTOR in xenon-induced HIF-1α protein expression was investigated by Western blot analysis. Exposure to 75 % xenon and 25 % oxygen for 2 hours induced a detectable, but not significant, increase in mTOR abundance after 2 hours. A striking increase occurred after 24 hours (5.93 +/- 1.36 versus of control, $p < 0.001$). Western blots of mTOR were normalised to α-tubulin, and expressed relative to the control (75 % nitrogen). Data are presented as means +/- SDs. * $p < 0.05$, *** $p < 0.001$ versus control (n=5 per group). **Abbreviations: mTOR,** mammalian target of rapamycin; **RCC4+,** renal cell carcinoma cell sub-line with stable pcDNA3-VHL transfection; **SD**, standard deviation.

However, the administration of rapamycin prior to xenon exposure significantly inhibited xenon–induced HIF-1α, with greatest HIF-1α inhibition occurring at the highest concentration of rapamycin tested (100nM; 0.89 +/- 0.21 compared to 2.89 +/- 0.93 of xenon alone, $p < 0.01$) (n=4 per group) (Figure 34).

Next, the effect of rapamycin was determined *in vivo*. Rapamycin (0.25 mg / Kg or 1.50 mg / Kg), in 0.1 % DMSO-containing normal saline, was administered by a single intraperitoneal (IP) injection thirty minutes prior to xenon exposure; HIF-1α expression was determined after 24 hours by Western blot analysis. In the brain, HIF-1α expression was not altered in mice treated with rapamycin alone at either doses and, as expected, xenon alone significantly increased HIF-1α protein expression. A rapamycin dose of 0.25 mg / Kg, IP, did not attenuate the xenon-induced expression of HIF-1α, but a dose of 1.50 mg / Kg, IP, did significantly attenuate HIF-1α (1.21 +/-0.58 versus 2.9 +/- 0.68 of xenon alone, $p < 0.01$). Similarly in the kidney, rapamycin 1.5 mg / Kg, IP, significantly attenuated the expression of xenon-induced HIF-1α (0.85 +/- 0.48 versus 2.86 +/- 0.58 of xenon alone, $p < 0.01$) (n=4 per group). Therefore, the inhibitory effects of rapamycin, *in vitro* and *in vivo*, provide further evidence that xenon increases HIF-1α protein expression through an mTOR-mediated pathway (Figure 35).

Dimethyl sulfoxide (DMSO) is a commonly used solvent, that dissolves both polar and apolar compounds, and is required in the preparation of rapamycin. A search of the open literature reveals no publications detailing its effect on HIF-1α. However, control experiments conducted as part of these rapamycin experiments showed that DMSO is able to increase expression of HIF-1α in a concentration-dependent manner. RCC4+ cell cultures were incubated in cell culture medium containing 0.0001 – 1.0 % DMSO (control cells received volume-matched medium without DMSO) for 30 minutes. After this time, the medium was removed and replenished

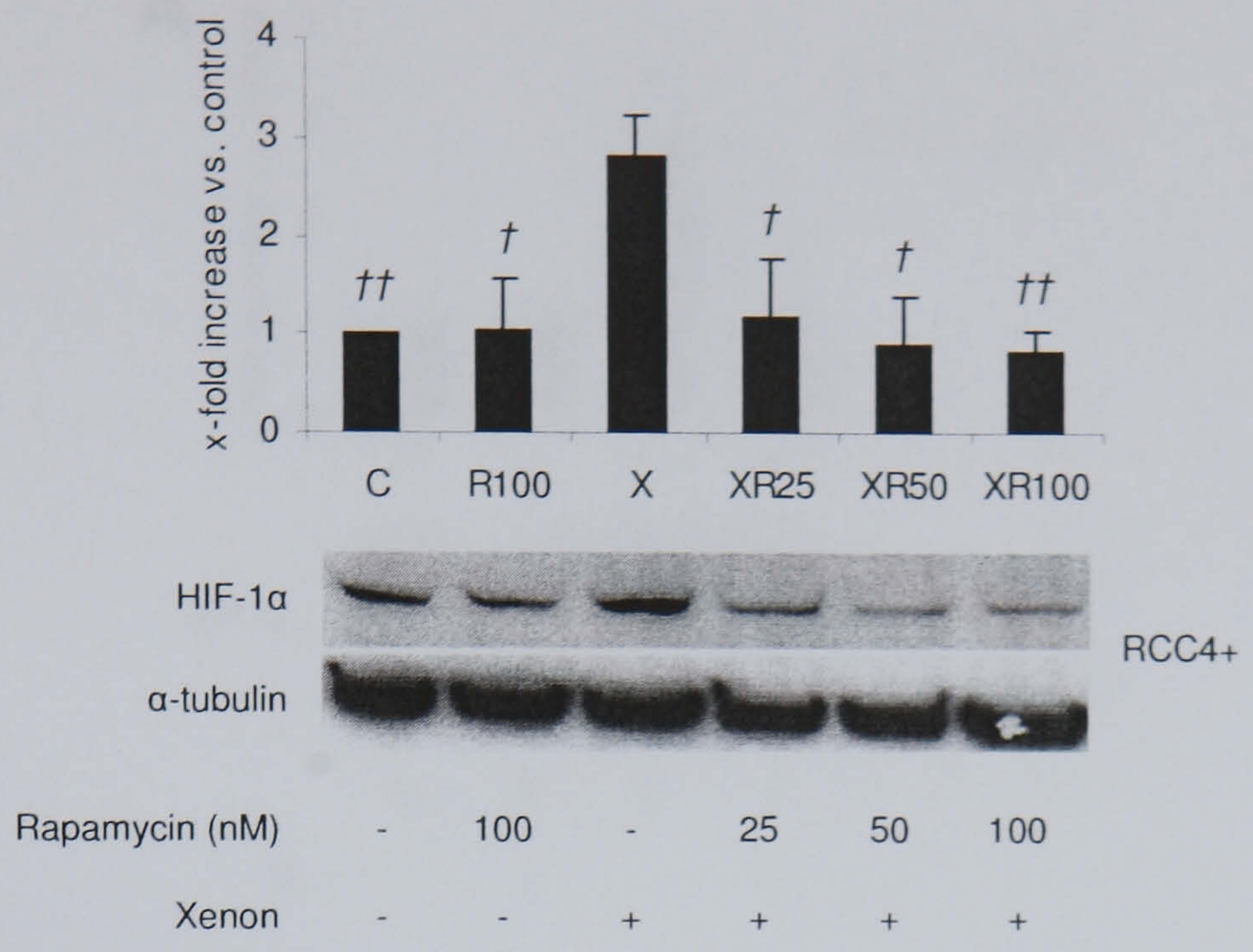


Figure 34. Rapamycin inhibits xenon-induced HIF-1α protein expression in RCC4+ cell culture

To determine the effect of rapamycin (25, 50, or 100 nM in 0.00025 % DMSO cell culture medium) on xenon-induced HIF-1α protein expression, RCC4+ cells were pre-treated with rapamycin (or volume-matched 0.00025 % DMSO medium) thirty minutes prior to 75 % xenon exposure (or 75 % nitrogen) for 2 hours, and HIF-1α protein expression determined 24 hours later. Rapamycin alone did not alter HIF-1α protein expression, compared to control cells, whereas xenon exposure increased HIF-1α by 2.89 +/- 0.93 fold (versus 1.0 of control, $p < 0.01$). Pre-treatment with each of the concentrations of rapamycin tested inhibited the induction of HIF-1α by xenon, with greatest HIF-1α inhibition occurring after 100 nM rapamycin pre-treatment (0.89 +/- 0.21 versus 2.89 +/- 0.93 of xenon, $p < 0.01$). Western blots of HIF-1α were normalised to α-tubulin, and expressed relative to the control. Data are presented as means +/- SDs. Single crosses denote $p < 0.05$, and double crosses $p < 0.01$ versus xenon alone (n=4 per group). **Abbreviations: C,** control; **DMSO,** dimethyl sulfoxide; **HIF-1α,** hypoxia-inducible factor-1 alpha; **R,** rapamycin; **RCC4+,** renal cell carcinoma cell sub-line with stable pcDNA3-VHL transfection; **SD**, standard deviation; **X,** xenon; **XR;** xenon and rapamycin.

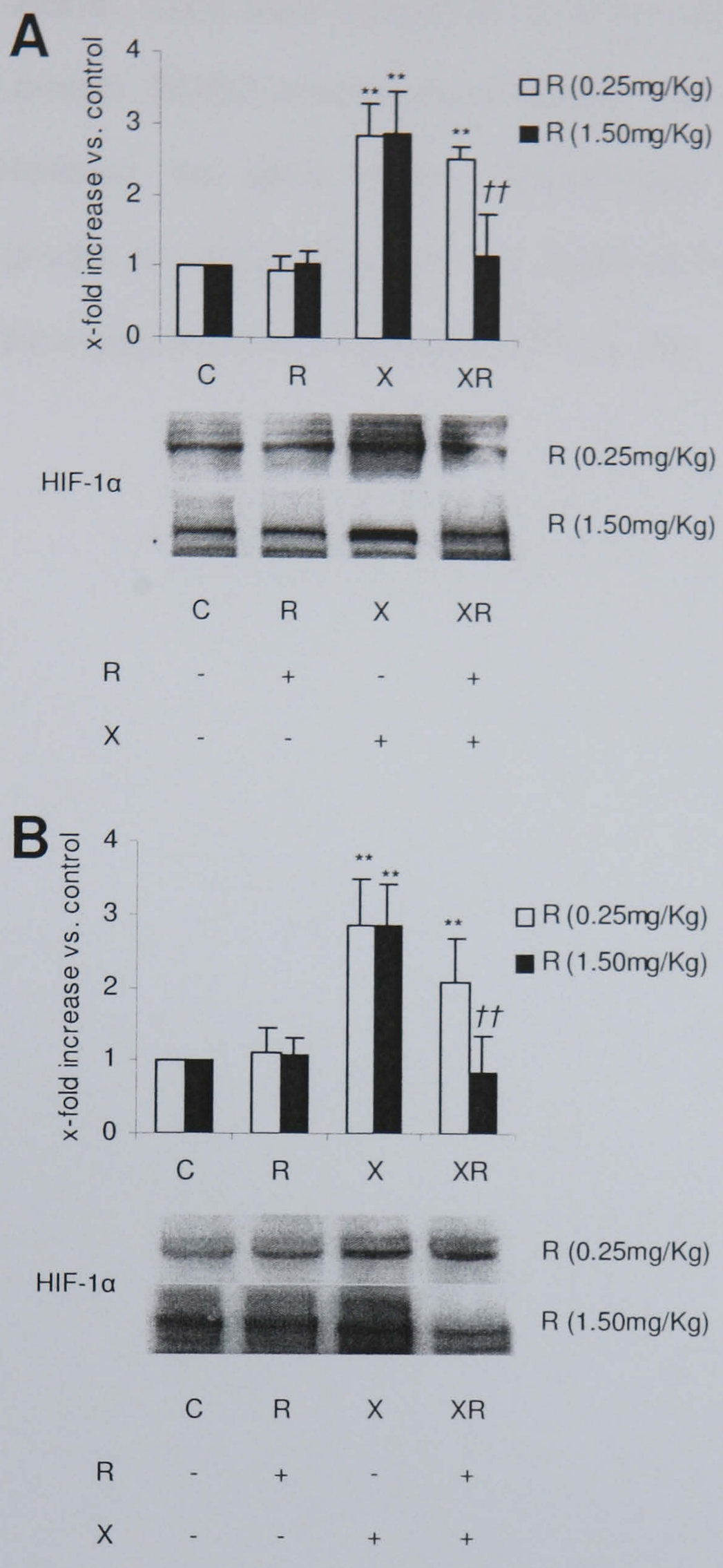


Figure 35. Rapamycin inhibits xenon-induced HIF-1α protein expression in the mouse brain and kidney

In both the mouse **(A)** brain and **(B)** kidney, rapamycin (0.25 mg / Kg, IP) thirty minutes prior to xenon exposure did not attenuate the expression of xenon-induced HIF-1α, assessed at 24 hours after xenon exposure. However, a higher dose of rapamycin (1.50 mg / Kg, IP) significantly inhibited the expression of xenon-induced HIF-1α in both the brain (1.21 +/- 0.58 versus 2.90 +/- 0.68 of xenon alone, $p < 0.01$) and kidney (0.85 +/- 0.48 versus 2.86 +/- 0.58 of xenon alone, $p < 0.01$). Western blots of HIF-1α were normalised to α-tubulin, and expressed relative to the control. Data are presented as means +/- SDs. * $p < 0.05$, ** $p < 0.01$, versus 1.0 of control. *††* $p < 0.01$ versus xenon alone (n=4 per group). **Abbreviations: C,** control; **HIF-1α,** hypoxia-inducible factor-1 alpha; **R,** rapamycin; **SD**, standard deviation; **X,** xenon; **XR,** xenon and rapamycin.

with DMSO-free medium. Cells were harvested 24 hours later for Western blot analysis of HIF-1α protein. DMSO concentrations greater than 0.1 % significantly increased HIF-α. However, the same DMSO concentration was used between different treatment groups and these effects do not appear to have confounded the results obtained in the experiments with rapamycin (Figure 36).

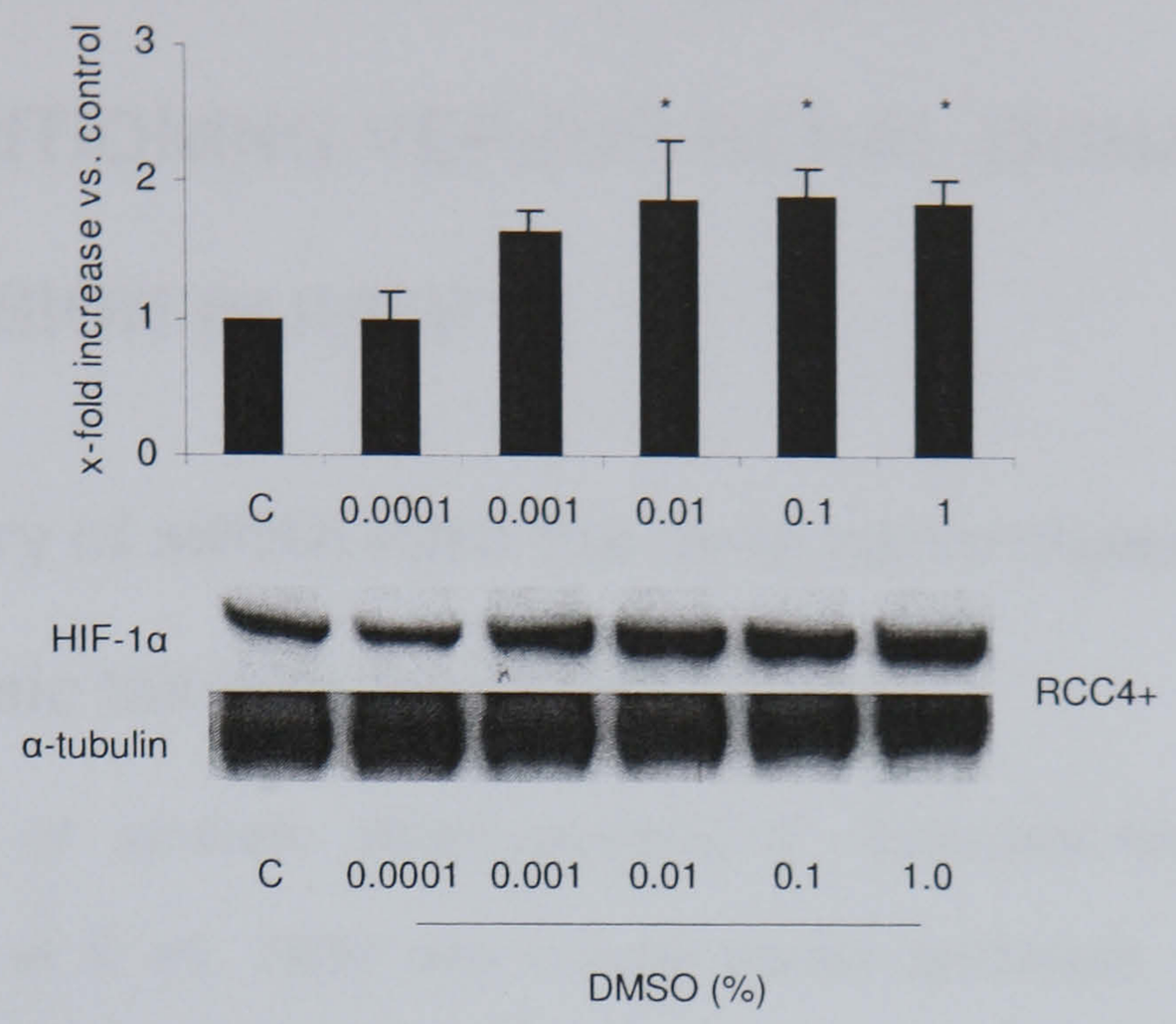


Figure 36. DMSO increases HIF-1α protein expression in RCC4+ cell culture

DMSO (0.0001-1.0 %) increased the expression of HIF-1α in a concentration-dependent manner in RCC4+ cell culture. Cells were incubated in DMSO-containing cell culture medium for 30 minutes. The medium was then removed and replenished with new DMSO-free medium for 24 hours. Western blots of HIF-1α were normalised to α-tubulin, and expressed relative to the control. Data are presented as means +/- SDs. * $p < 0.05$ versus 1.0 of control (n=3 per group). Abbreviations: **C**, control; **DMSO**, dimethyl sulfoxide; **HIF-1α**, hypoxia-inducible factor-1 alpha; **RCC4+**, renal cell carcinoma cell sub-line with stable pcDNA3-VHL transfection; **SD**, standard deviation.

3.3 NECESSITY OF HIF-1α IN XENON PRECONDITIONING VERSUS RENAL ISCHAEMIC-REPERFUSION INJURY

3.3.1 Delivery of siRNA intro the renal parenchyma by hydrodynamic tail vein injection

Delivery of synthetic double-stranded 3' fluorescein-labelled nonsense siRNA (50 µg in 2 mL PBS) into mouse tubular epithelium was verified, in comparison to volume-matched PBS injection in control mice. Intracellular fluorescent signals were detected in kidney tubular cells 24 hours after injection with scramble siRNA, while no such fluorescence was found in control mice 24 hours after PBS injection. This result corroborates with previous data that hydrodynamic siRNA injection is capable of accumulating in the kidney (van de Water et al. 2006) (Figure 37).

3.3.2 The functional effect of HIF-1α siRNA

The functional effect of HIF-1α siRNA delivery was verified. Twenty-four hours after hydrodynamic tail vein injection of HIF-1α siRNA, nonsense siRNA or PBS, mRNA expression in mouse kidney of HIF-1α and EPO was studied, and compared to an uninjected control mouse. RT-PCR detected no difference in HIF-1α or EPO mRNA expression in mice treated with scramble siRNA or PBS, compared to control. However, an attenuated expression of both HIF-1α and EPO mRNA was observed in mice treated with HIF-1α siRNA, indicating knockdown of HIF-1α and HIF-dependent EPO. Results were normalised to HIF-1β which is constitutively expressed. However, the effect of HIF-1α siRNA did not achieve complete

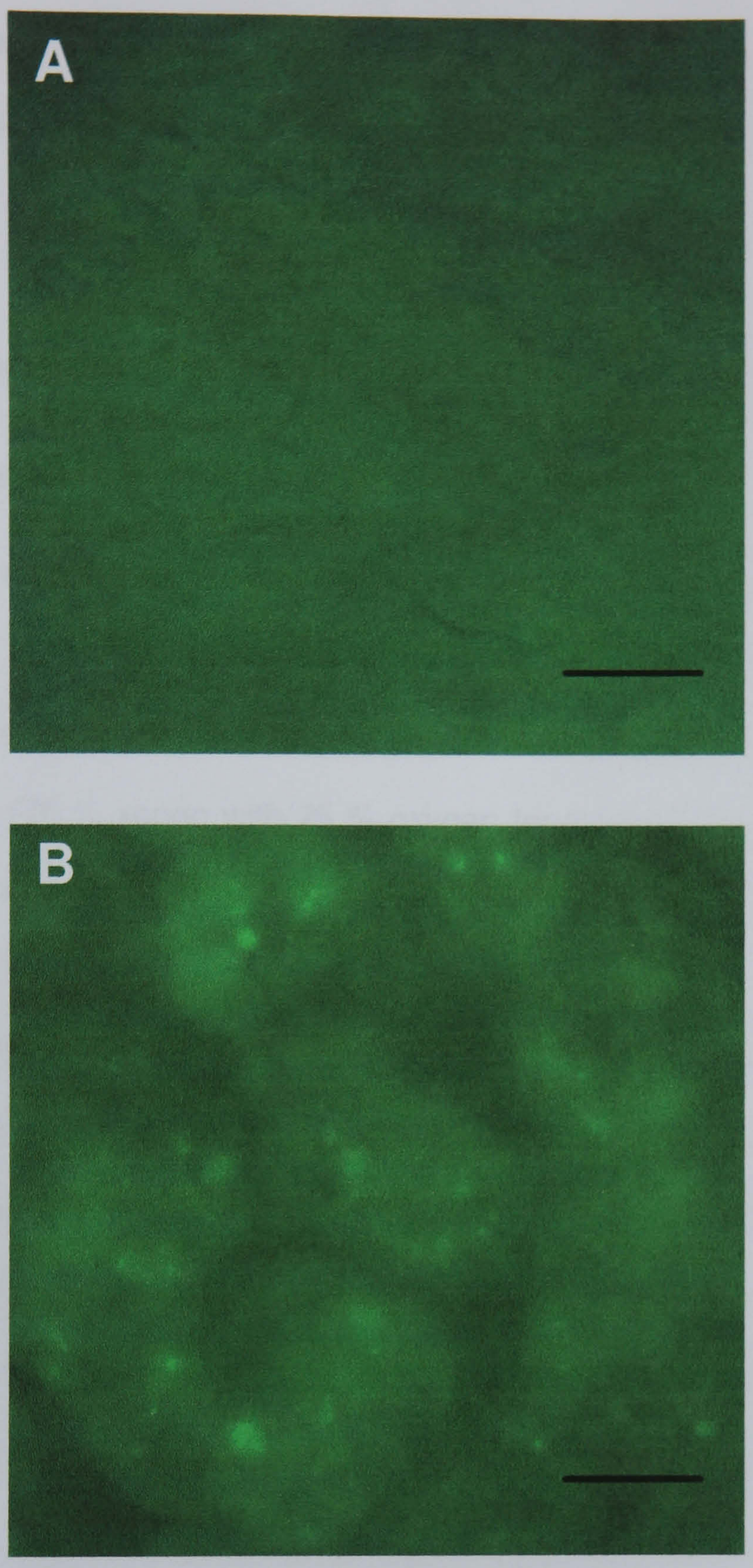


Figure 37. Verification of in vivo delivery of 3' fluorescein-labelled siRNA into the mouse renal parenchyma

Volume-matched **(A)** phosphate-buffered saline (PBS) or **(B)** 3' fluorescein-labelled nonsense siRNA (50 µg in 2 mL PBS) was hydrodynamically injected into the tail vein of mice, to verify its delivery into the renal parenchyma. Renal tubular cells took up the nonsense siRNA 24 hours after hydrodynamic injection, as shown by intracellular fluorescent signals, while no marker was found in PBS-injected control animals. Scale bar represents 100 µm.

knockdown, with faint bands for HIF-1α and EPO mRNA still visible; this may indicate that not all cells were transfected. Consistent with other laboratories, *in vivo* siRNA delivery does not result in 100 % uptake (McCaffrey et al. 2002) (Figure 38).

3.3.3 The necessity of HIF-1α in xenon-induced erythropoietin expression

Next, we investigated whether xenon-induced expression of EPO is dependent on the transcriptional activity of xenon-induced HIF-1α. Mice were injected with HIF-1α siRNA, scramble siRNA or PBS, 24 hours prior to xenon preconditioning (75 % xenon with 25 % oxygen for 2 hours). Control mice received PBS injection 24 hours before exposure to 75 % nitrogen for 2 hours. Mice were sacrificed 24 hours after, and expression of renal HIF-1α and EPO protein were detected by Western blot and compared to controls. HIF-1α expression in the kidney was increased 24 hours after xenon exposure in mice pretreated with PBS (2.11 +/- 0.42 versus 1.0 of control, $p < 0.05$, or nonsense siRNA injection (2.23 +/- 0.51 versus 1.0 of control, $p < 0.05$). However, xenon preconditioning failed to induce HIF-1α expression in mice pretreated with HIF-1α siRNA (Figure 39). Similarly, EPO expression was increased 24 hours after xenon preconditioning in mice pretreated with PBS (2.01 +/- 0.37 versus 1.0 of control, $p < 0.05$) or nonsense siRNA (1.9 +/- 0.49 versus 1.0 of control). EPO expression was not induced following xenon preconditioning in mice pretreated with HIF-1α siRNA (n=5 per group) (Figure 40).

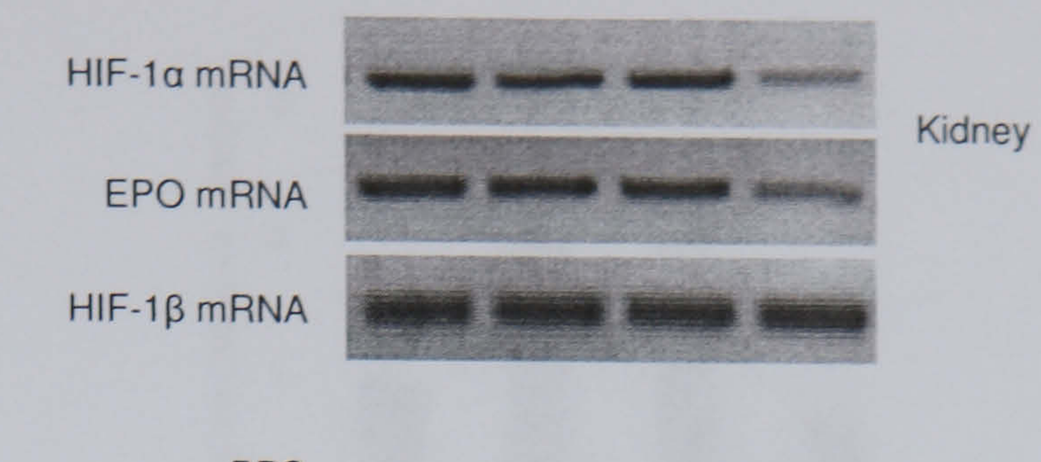


Figure 38. Functional effect of HIF-1α siRNA in the mouse kidney

Reverse transcription-polymerase chain reaction (RT-PCR) was used to assess the functional effect of *in* vivo HIF-1a siRNA delivery into the renal parenchyma. Twenty-four hours following a single hydrodynamic tail vein injection of HIF-1α siRNA (50 µg in 2 mL PBS, or volume-matched nonsense siRNA or PBS), expression of both HIF-1α mRNA and EPO mRNA were decreased, confirming the functional efficacy of HIF-1α siRNA. Results were normalised to the loading control, HIF-1β mRNA, which is constitutively expressed. **Abbreviations: EPO**, erythropoietin; **HIF-1α**, hypoxia-inducible factor-1 alpha; **HIF-1β**, hypoxia-inducible factor-1 beta; **PBS**, phosphate-buffered saline; **siRNA**, small interfering RNA.

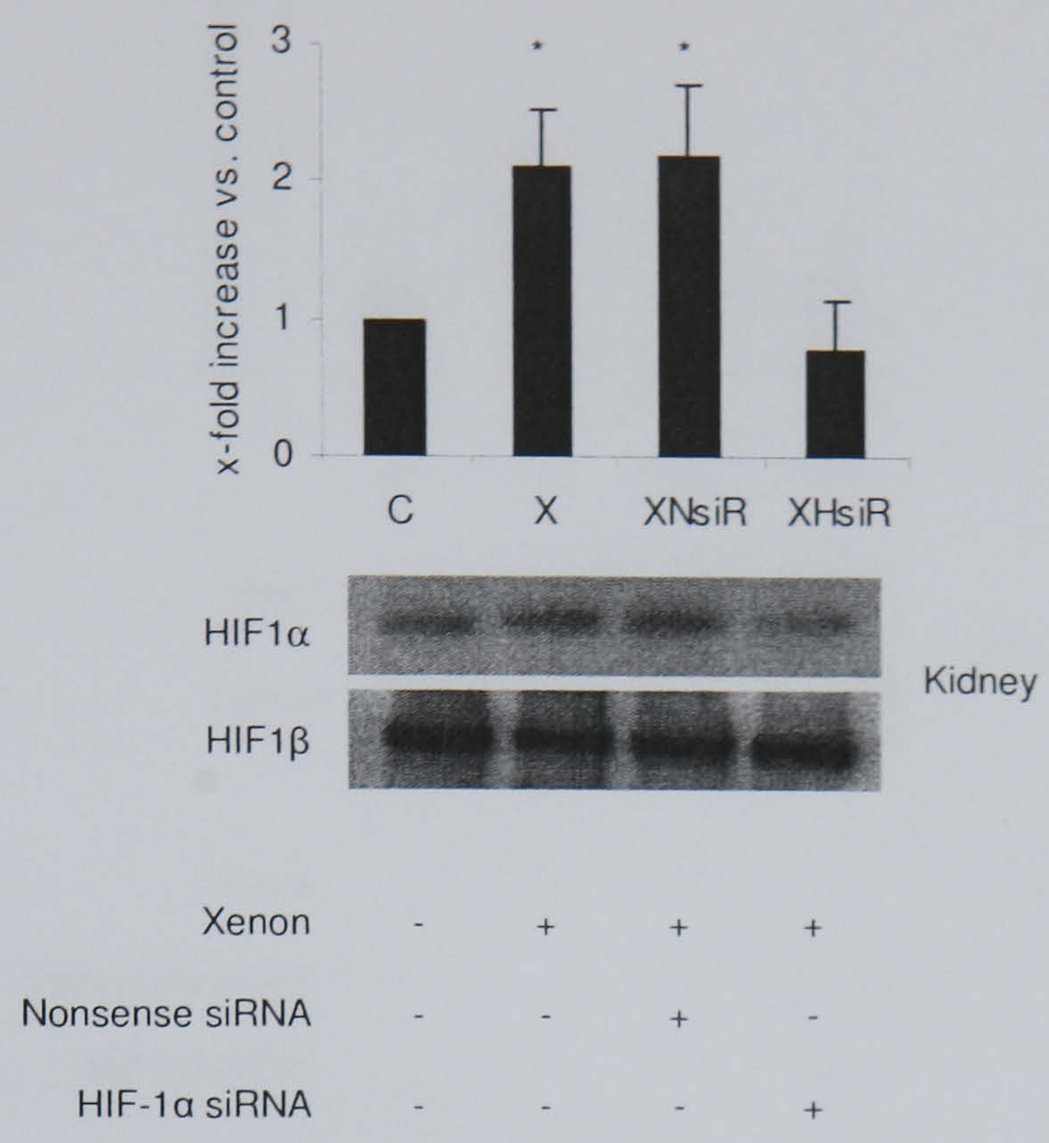


Figure 39. HIF-1α siRNA attenuates xenon-induced HIF-1α protein expression in the mouse kidney

Western blotting was used to determine the effect of HIF-1α siRNA on xenon-induced HIF-1α protein expression in the mouse kidney. Twenty-four hours following a single hydrodynamic tail vein injection of HIF-1α siRNA (50 µg in 2 mL PBS, or volume-matched nonsense siRNA or PBS), mice were exposed to 75 % xenon, or nitrogen, for 2 hours. Twenty-four hours following gas exposure, HIF-1α siRNA attenuated the protein expression of xenon-induced HIF-1α (0.78 +/- 0.36 versus 2.1 +/- 0.42 of xenon alone, $p < 0.05$). Western blots of HIF-1α were normalised to the internal control HIF-1β, and expressed relative to the control (PBS-injected mice exposed to 75 % nitrogen). Data are presented as means +/- SDs. *$p < 0.05$ versus control (n=5 per group). **Abbreviations: HIF-1α**, hypoxia-inducible factor-1 alpha; **HIF-1β**, hypoxia-inducible factor-1 beta; **PBS**, phosphate-buffered saline; **SD,** standard deviation; **siRN**A, small interfering RNA.

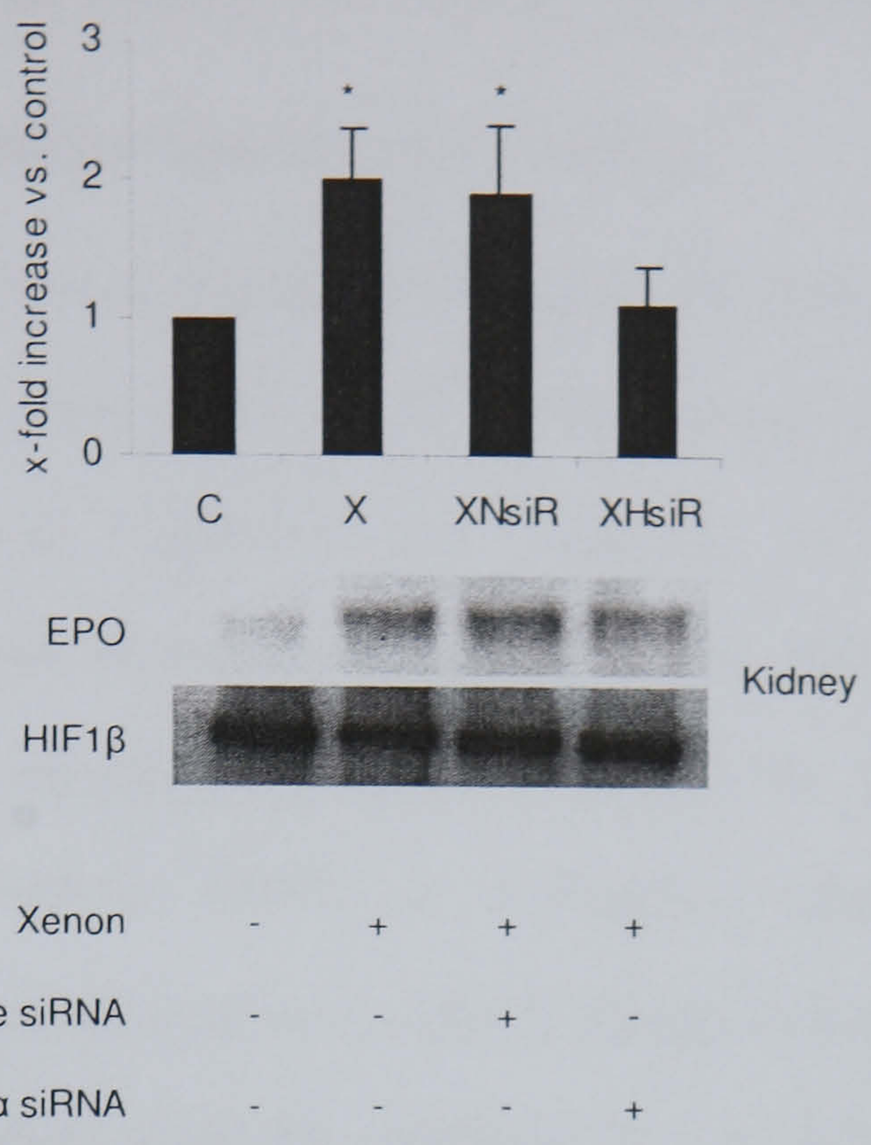
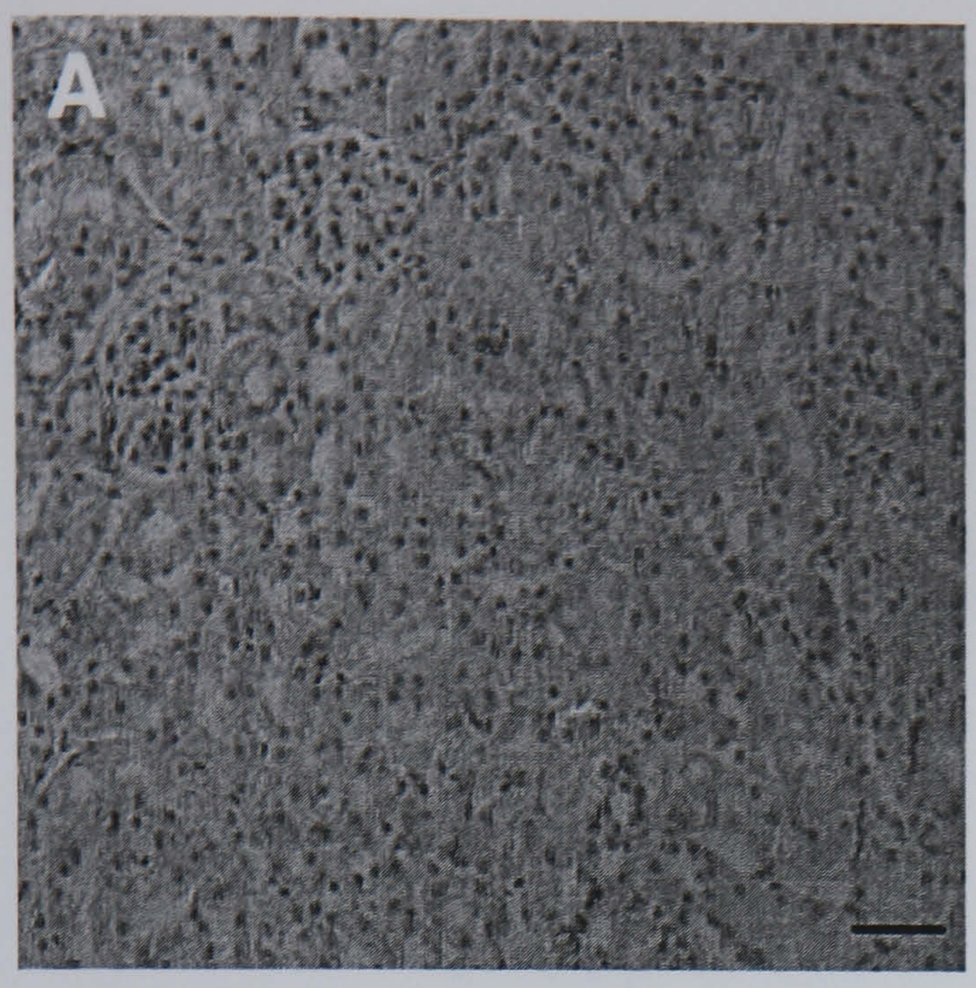


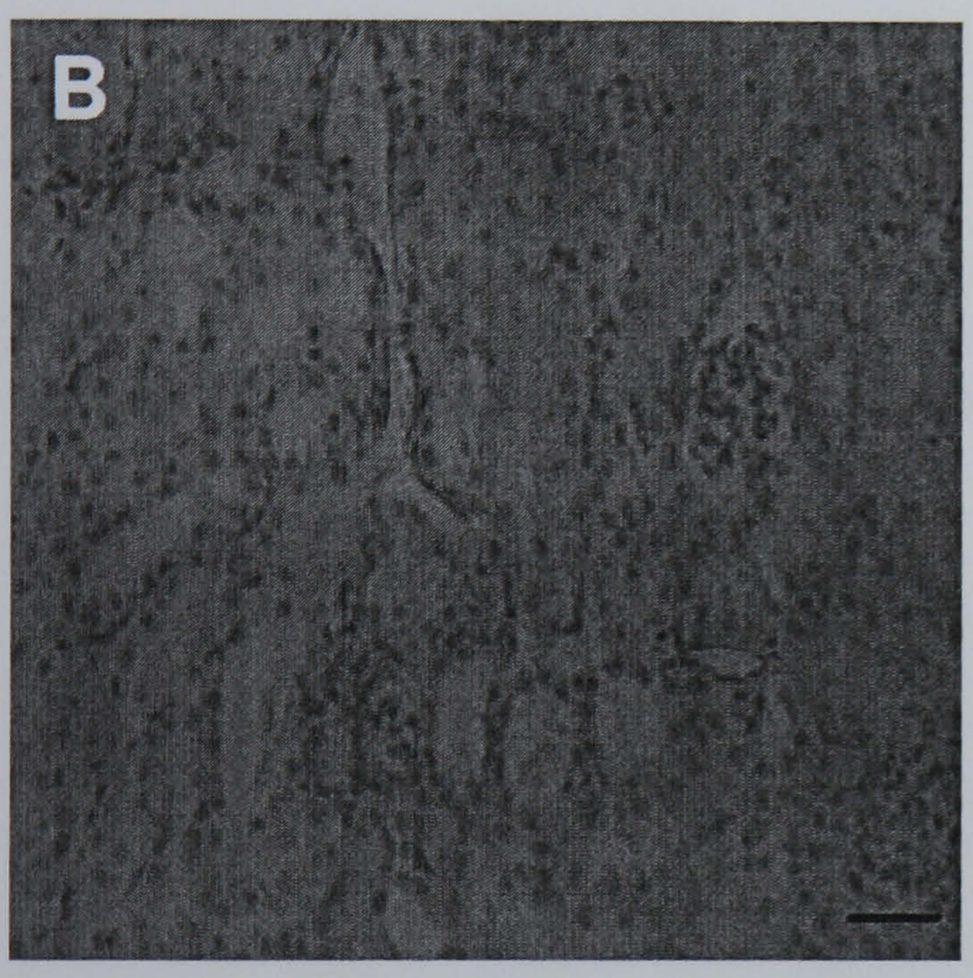
Figure 40. HIF-1α siRNA attenuates xenon-induced erythropoietin protein expression in the mouse kidney

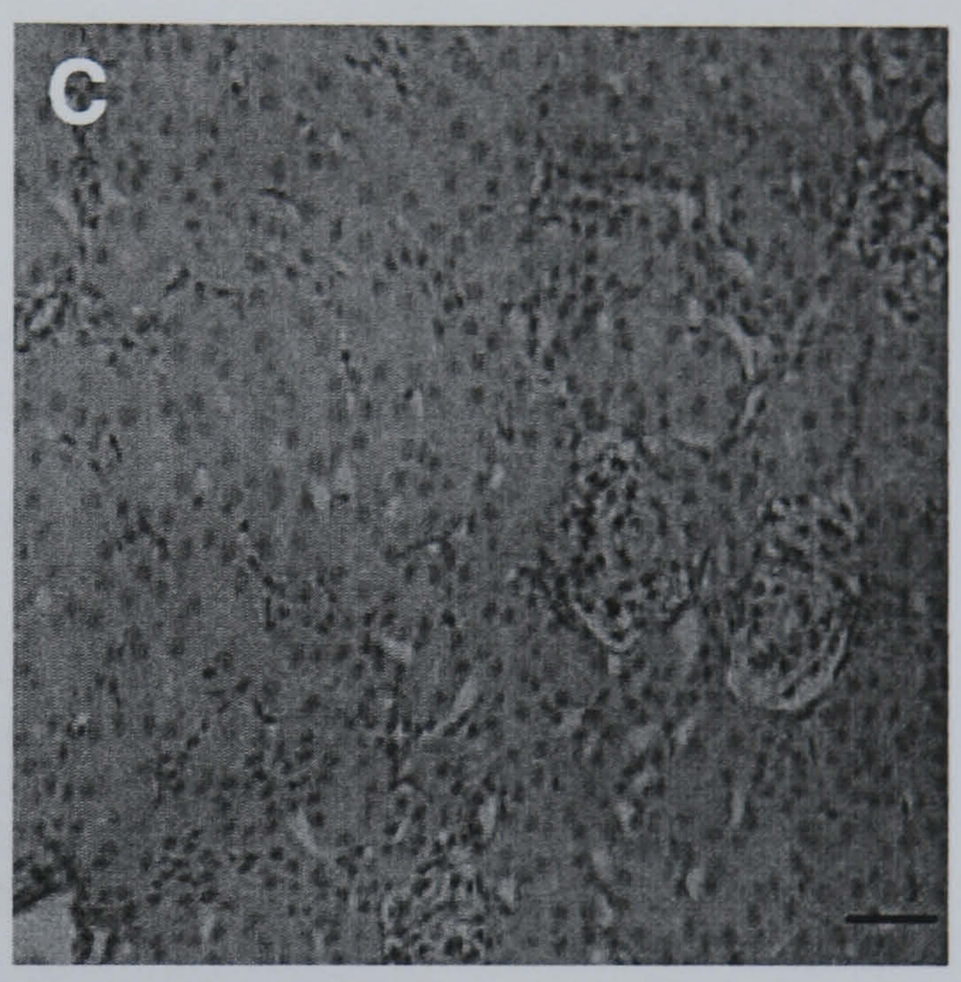
Western blotting was used to determine the effect of HIF-1α siRNA on xenon-induced EPO protein expression in the mouse kidney. Twenty-four hours following a single hydrodynamic tail vein injection of HIF-1α siRNA (50 µg in 2 mL PBS, or volume-matched nonsense siRNA or PBS), mice were exposed to 75 % xenon for 2 hours (or 75 % nitrogen). Twenty-four hours following gas exposure, HIF-1α siRNA attenuated the protein expression of xenon-induced EPO (1.1 +/- 0.29 versus 2.01 +/- 0.37 of xenon alone, $p < 0.05$). Results were normalised to the internal control HIF-1β, and expressed relative to the control (PBS-injected mice exposed to 75 % nitrogen). Data are presented as means +/- SDs. *$p < 0.05$ versus control (n=5 per group). **Abbreviations: EPO**, erythropoietin; **HsiR,** HIF-1α siRNA, **NsiR,** nonsense siRNA; **PBS**, phosphate-buffered saline; **SD,** standard deviation;

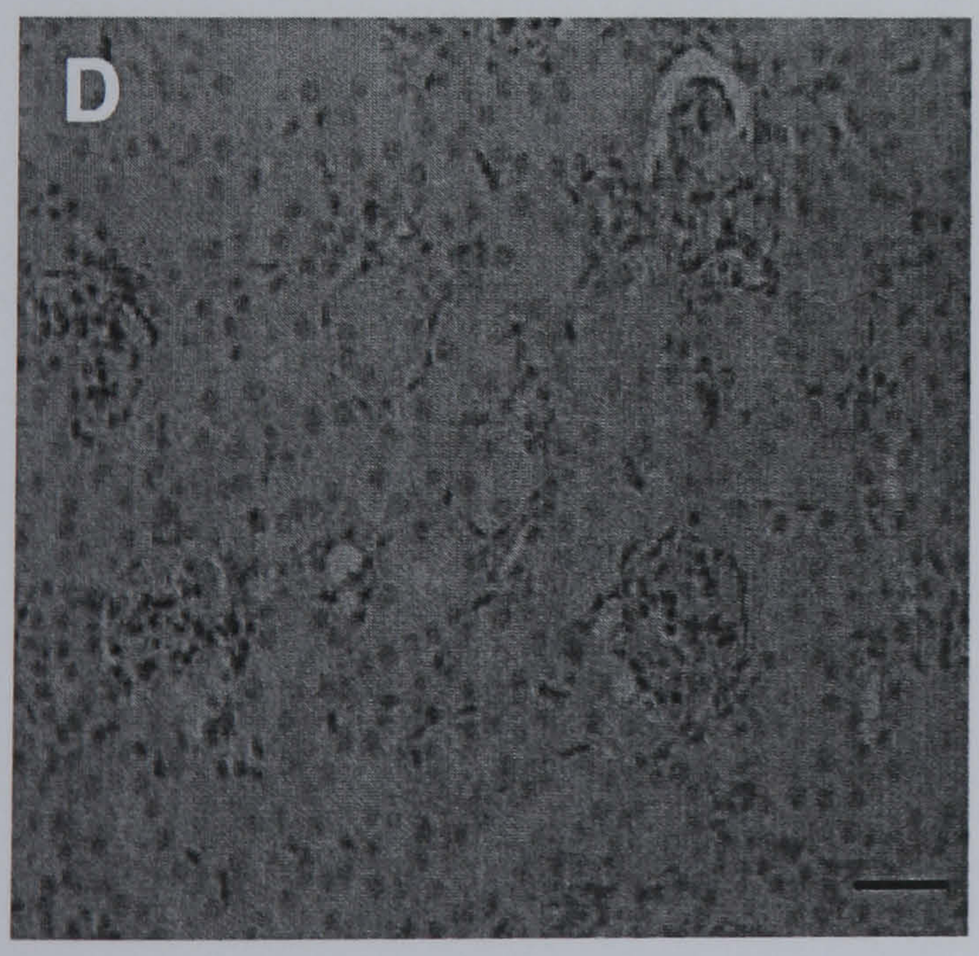
3.3.4 The necessity of HIF-1α in xenon preconditioning versus renal ischaemic-reperfusion injury

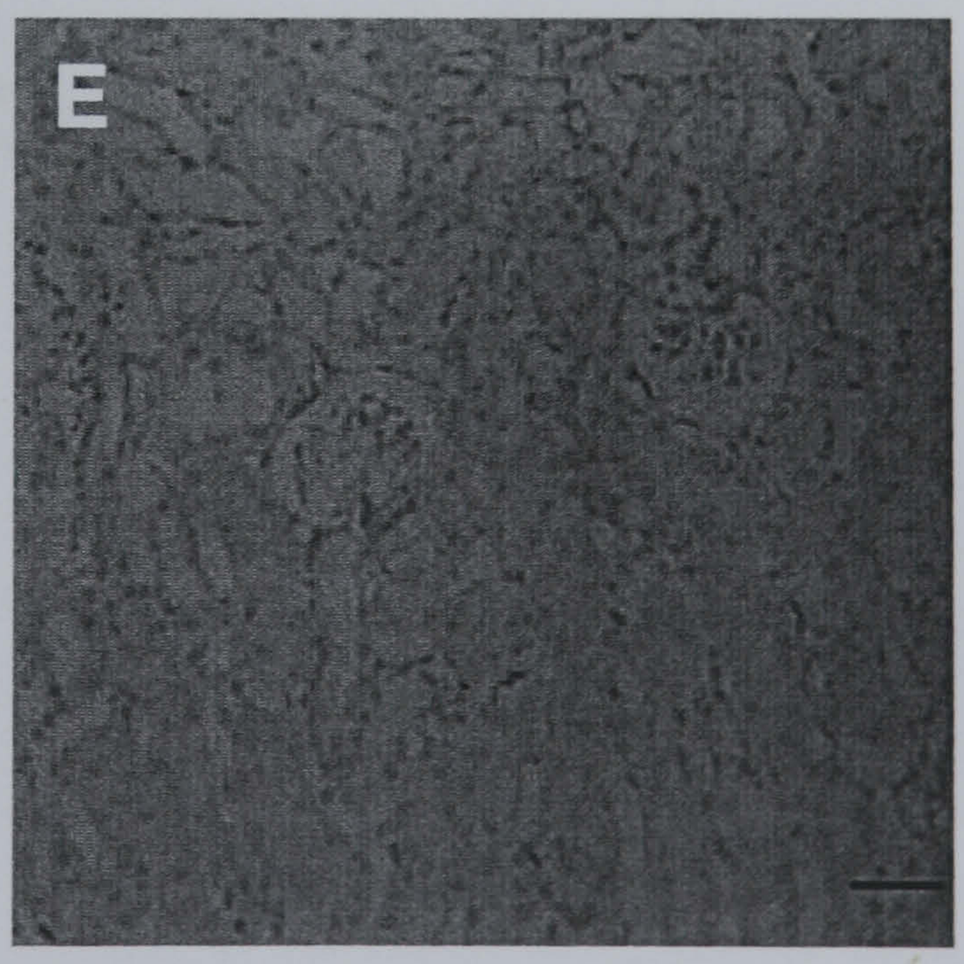
Previous experiments established the capability of xenon preconditioning to protect against cerebral HI injury (Ma et al. 2006). This study investigated xenon in the kidney, and aimed to determine the necessity of HIF-1α for xenon preconditioning against renal ischaemic-reperfusion injury (IRI) in mice, using an injury model of unilateral clamping of the renal pedicle for 25 minutes. Having shown the efficacy of HIF-1α siRNA at abrogating xenon-induced HIF-1α transcription, expression and downstream activity, it was investigated whether the renoprotective effect of xenon could be abolished by knocking down HIF-1α. As described previously, mice were hydrodynamically injected with HIF-1α siRNA (50 μg in 2 mL PBS), nonsense siRNA or PBS. Twenty-four hours later, mice were exposed to 75 % xenon or nitrogen for 2 hours. Twenty-four hours later, mice were injured by unilateral clamping of the renal pedicle for 25 minutes, or sham-operated without injury. Twenty-four hours after renal IRI, kidneys were harvested for histological assessment, and blood collected for determining serum creatinine and urea levels as a measure of renal function. Morphological evidence of renal IRI was assessed using a histopathological scoring assay of cortical tubular damage by an investigator who was unaware of the experimental protocol. Controls (PBS-injected, nitrogen-exposed, sham-operated) showed normal histology. Following IRI, histology revealed massively dilated tubular lumen, flattened epithelial cells that either lacked nuclear staining or exhibited nuclear chromatin condensation (histological score was 259 +/- 17). Renal ischaemic-reperfusion damage was attenuated in xenon preconditioned mice, injected with PBS (54 +/- 19, $p < 0.01$) or nonsense siRNA (69 +/- 20, $p < 0.01$). However, HIF-1α siRNA with xenon preconditioning resulted in abrogation of the previously observed renoprotection (284 +/- 6, $p > 0.05$) (Figure 41).



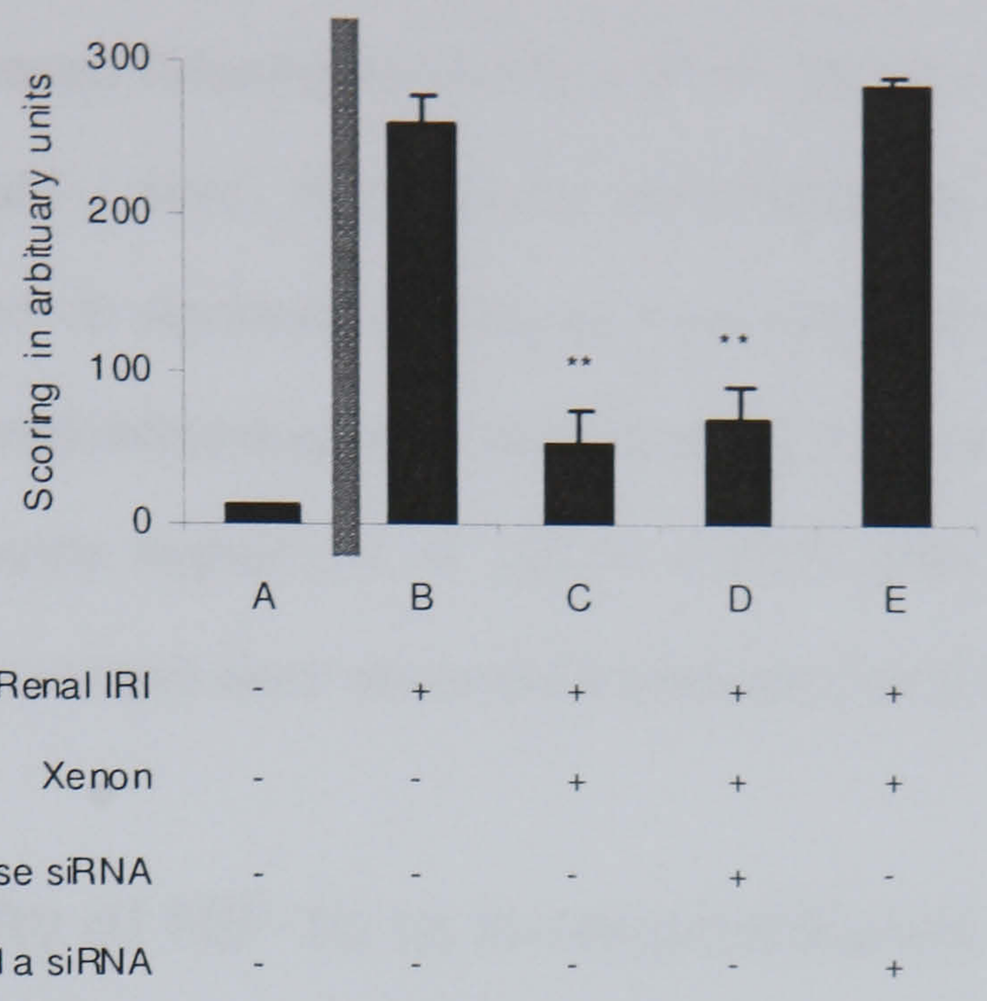








(Figure 41, legend overleaf)



Renal IRI	-	+	+	+	+
Xenon	-	-	+	+	+
Nonsense siRNA	-	-	-	+	-
HIF-1a siRNA	-	-	-	-	+

Figure 41. Histological assessment of HIF-1α in in vivo xenon preconditioning versus renal ischaemic-reperfusion injury

Morphological evidence of renal IRI injury was assessed from the analysis of 10 H&E-stained cortical tubular cross-sections per kidney, by using a histopathological scoring system ranging from 0 (no damage) to 3 (severe damage). The maximum score per kidney was 300 (severe damage). Photomicrographs from **(A)** uninjured controls displayed normal histology. **(B)** Renal IRI histology revealed dilated tubular lumen and flattened epithelial cells that lacked nuclear staining or chromatin condensation (histological score 259 +/- 17). **(C)** However, xenon preconditioning reduced the damage induced by renal IRI (54 +/- 19, $p < 0.01$ versus IRI), an effect that was not lost by (**D**) nonsense siRNA prior to xenon preconditioning (69 +/- 20, $p < 0.01$ versus IRI) . **(E)** HIF-1α siRNA however, attenuated the protective effect of xenon preconditioning (284 +/- 6, $p > 0.05$ versus IRI). Data are presented as means +/- SDs and ** denotes $p < 0.01$ versus IRI (n=6 per group). **Abbreviations: IRI,** ischaemic-reperfusion injury; **HsiR,** HIF-1α siRNA, **NsiR,** nonsense siRNA; **SD,** standard deviation. Scale bar represents 100 μm.

Qualitatively, similar changes were noted in renal function following IRI; notably, the plasma creatinine increased following renal IRI (162 +/- 18 μM / litre) compared to controls (35 +/- 1.2 μM / litre). With xenon preconditioning before renal IRI, creatinine levels showed no significant difference from mice not subjected to renal injury. When xenon preconditioning was preceded by HIF-1α siRNA injection, creatinine was significantly higher (91 +/- 58, $p < 0.05$) than that seen in the uninjured mice. Similar changes were observed in plasma urea (Figure 42).

3.3.5 The necessity of HIF-1α in xenon-mediated attenuation of apoptosis in renal tubular cells

In order to evaluate kidney damage at the cellular level, TUNEL staining was used to detect apoptotic tubular cells. TUNEL positive cells were almost undetectable in uninjured mice but were easily noted in every field in non-preconditioned mice following IRI (297 +/- 32); this was attenuated following xenon preconditioning and PBS (82 +/- 27, $p < 0.01$) or nonsense siRNA (155 +/- 16, $p < 0.01$) pretreated mice. In contrast, TUNEL positive cells in mice pretreated with HIF-1α siRNA injection followed by xenon preconditioning were as frequently observed as in renal IRI animals not exposed to xenon (272 +/- 28, $p > 0.05$) (Figure 43).

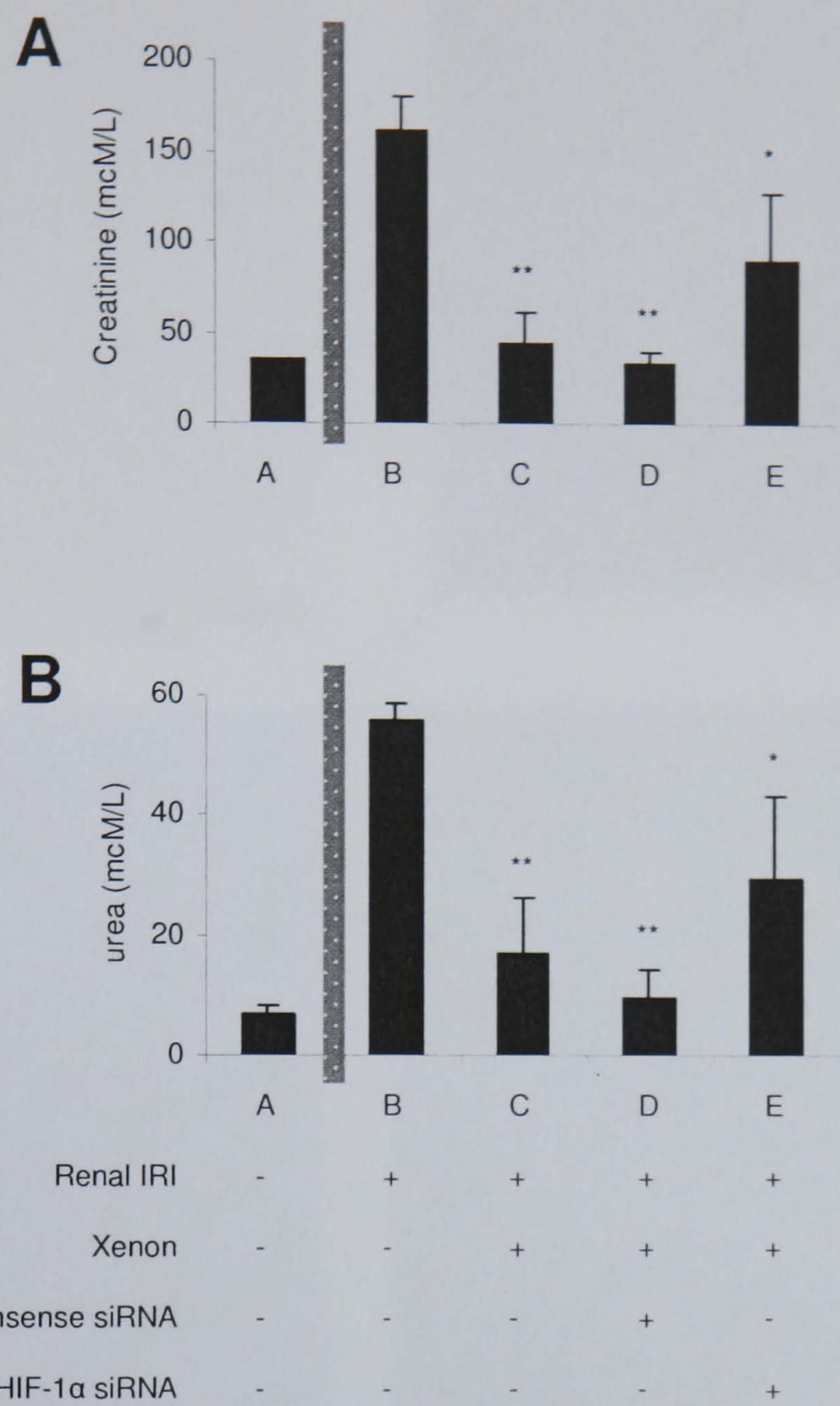
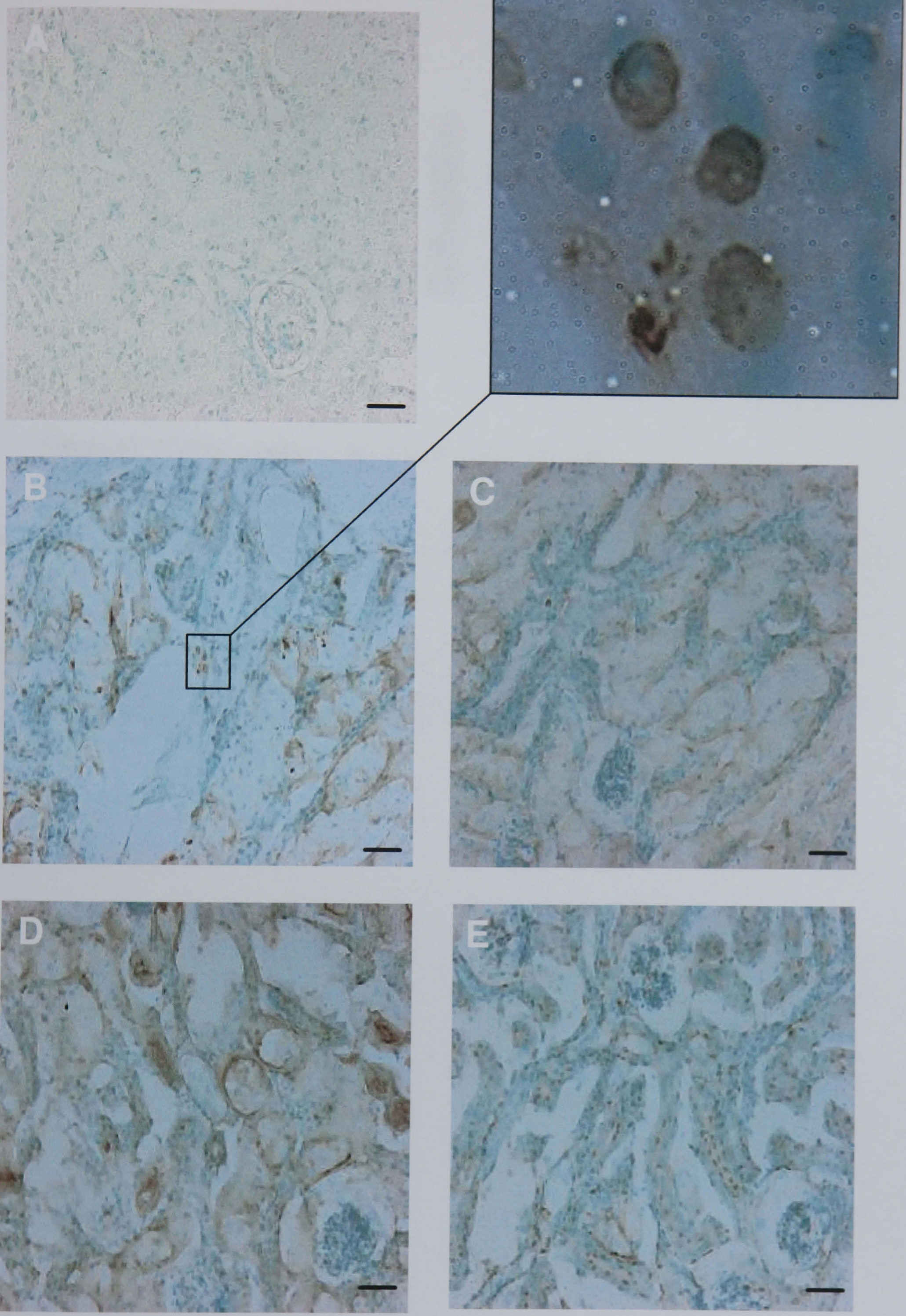


Figure 42. Functional assessment of HIF-1α in in vivo xenon preconditioning versus renal ischaemic-reperfusion injury

Renal function of IRI was reflected by **(A)** plasma creatinine and **(B)** plasma urea. Serum creatinine in uninjured mice (group A, 35 +/- 1.2 μM/L) increased after IRI (group B, 162 +/- 18 μM / L). However, xenon (with PBS or nonsense siRNA) prior to IRI decreased creatinine (group C and D, $p < 0.01$ versus IRI) to levels that were not significantly different from uninjured controls. HIF-1α siRNA attenuated the protective effect of xenon against IRI, reflected by an increased in creatinine (group E, 91 +/- 58, $p < 0.05$ versus control). Similar changes were seen in plasma urea. Data are presented as means +/- SD. * $p < 0.05$, ** $p < 0.01$ versus IRI (n=6). **Abbreviations: C**, control; **HsiR**, HIF-1α siRNA; **NsiR**, nonsense siRNA; **X,** xenon.



(Figure 43, legend overleaf)

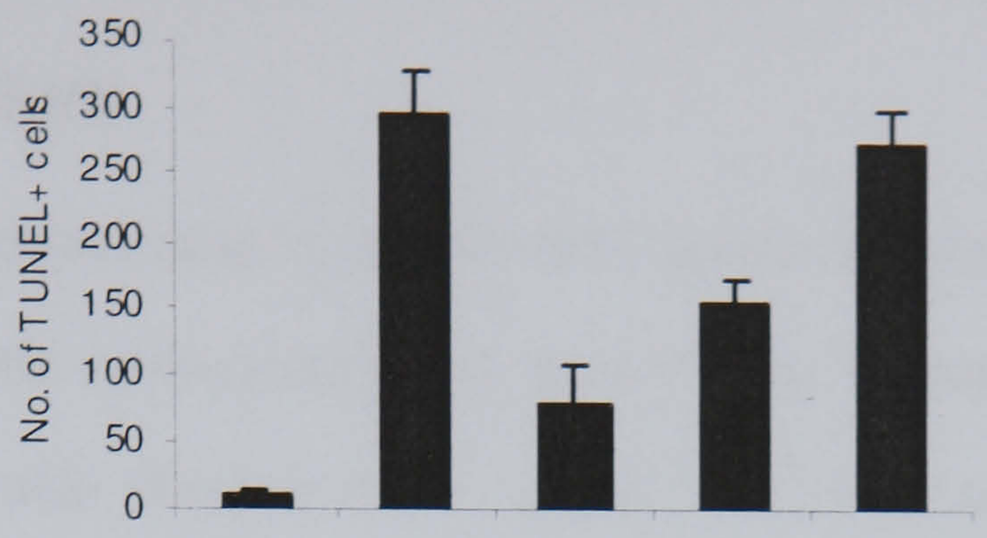


Figure 43. TUNEL + cells in the renal cortex after IRI

Apoptotic renal tubular cell death, following IRI was prevented by xenon preconditioning, as reflected by TUNEL staining. Photomicrographs from **(A)** uninjured controls showed no TUNEL+ cells. **(B)** Following renal IRI TUNEL+ cells were detected in the renal cortex (297 +/- 32). **(C)** Xenon preconditioning reduced the number of TUNEL+ cells (82 +/- 27 versus IRI, $p < 0.01$), an effect that was not lost by **(D)** nonsense siRNA prior to xenon preconditioning (155 +/- 16 versus IRI, $p < 0.01$). **(E)** HIF-1α siRNA, attenuated the protective effect of xenon (272 +/- 28 versus IRI, $p > 0.05$). Data are presented as means +/- SDs. ** $p < 0.01$ versus IRI (n=6 per group). **Abbreviations: IRI,** ischaemic-reperfusion injury; **HsiR,** HIF-1α siRNA, **NsiR,** nonsense siRNA**; SD,** standard deviation; **TUNEL,** terminal deoxynucleotidyl transferase biotin – dUTP nick end labelling.

3.3.6 The necessity of HIF-1α in xenon preconditioning versus renal failure

Following unilateral renal injury, the other kidney remained uninjured so mice did not suffer from the consequences of renal failure. To assess whether xenon preconditioning was also effective in the context of a more severe insult to renal function, further experiments were performed in which the right renal pedicle was clamped for 40 minutes and the left kidney was removed. Long-term survival (no signs of illness at 7 days) was noted in 75 % of mice exposed to xenon prior to severe renal IRI. In contrast, mice not exposed to xenon, or exposed to xenon combined with HIF-1α siRNA fared much worse. Within 2 days, 38 % of these mice had died (either spontaneously or euthanised because of severe ill health measured against defined criteria) and no mice survived beyond 5 days after renal IRI ($p < 0.001$ versus xenon preconditioning) (Figure 44). The mean value of plasma creatinine and urea rose more than 10-fold at day 2, just prior to termination, following renal IRI. Mice pretreated with xenon exhibited normal creatinine and urea values even at 7 days after IRI.

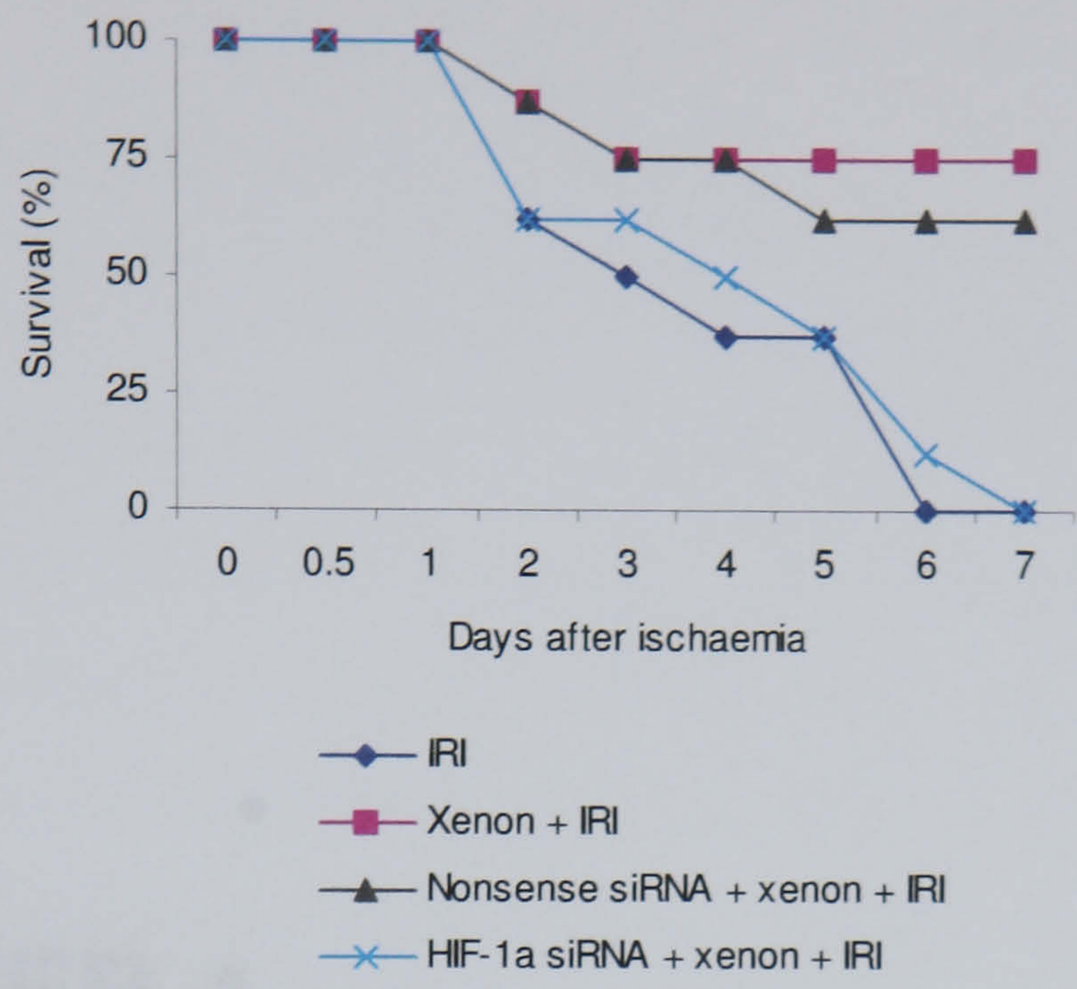


Figure 44. Kaplan-Meier survival curve

The effect of HIF-1α in xenon preconditioning in long-term survival was assessed in the more severe injury model of renal failure, with 40 minutes of ischaemia to the right kidney as well as left nephrectomy. In the injury alone group, only 62 % of mice survived beyond day 2 after injury, with no mice surviving beyond day 5. Xenon preconditioning with PBS or nonsense siRNA improved the survival (75 % and 62 % at day 7, respectively). The protective effect of xenon was lost in HIF-1α siRNA- treated mice, with 62 % surviving beyond day 2, but none surviving beyond day 6 (n= 8 per group). **Abbreviations: IRI**, ischaemia reperfusion injury.

CHAPTER 4

DISCUSSION

4.1 REGULATION OF HIF-1α BY XENON PRECONDITIONING

Xenon, derived from the Greek for "strange one," is so called to denote its rarity in the atmosphere, and its inability to take part in chemical reactions which renders it a noble gas in the periodic table of chemical elements. Of great fascination, however, is that although chemically inert, xenon exerts physiological effects of anaesthesia and analgesia, displaying many of the hallmarks of the ideal inhalational anaesthetic agent (Sanders, Franks & Maze 2003), for example, a rapid onset and offset of action and lack of haemodynamic and cardiovascular side effects (Sanders, Ma & Maze 2005). Further to this, there is increasing experimental evidence of the salutary effects of xenon in mediating organ protection, against acute neuronal injury (Homi et al. 2003, Ma et al. 2006) and against myocardial infarct (Weber et al. 2005a) through the phenomenon of preconditioning. Similarly, certain other inhalational anaesthetics display preconditioning capabilities, such as isoflurane (Raphael et al. 2008). However, the mechanisms of preconditioning remain to be unequivocally elucidated; in particular, the mechanism of xenon preconditioning is not known and has been little studied.

There is growing recognition for the importance of HIF-1α expression in the mechanism of hypoxic or ischaemic preconditioning against hypoxic-ischaemic or ischaemic-reperfusion injury, in the brain, heart, and kidney, as well as in other organs and tissues (Bernhardt et al. 2007). In these settings, HIF-1α appears to be the master controller of the endogenous adaptation to a more hypoxia-resilient phenotype orchestrating critical cell processes of survival signalling, glucose metabolism and transport, metabolic adaptation, vascularisation and increasing oxygen transport capacity. This occurs through the transcription of at least 100 target genes that alleviate the effects of hypoxia. Indeed, hypoxic preconditioning

induces expression of HIF-1α, where the level of expression positively correlates with the degree of protection afforded. Furthermore, several HIF-1 target genes are themselves capable of mediating the cytoprotective responses of preconditioning, such as EPO (Joyeux-Faure 2007).

4.1.1 Xenon preconditioning regulates HIF-1α protein expression

The molecular mechanisms at play in xenon-mediated adaptation to hypoxia-ischaemia, and the contribution of HIF-1α to xenon preconditioning have, so far, remained unknown. Therefore, this is the first study to provide evidence that xenon exposure increases HIF-1α protein expression. Firstly, hypoxia was demonstrated to increase HIF-1α protein expression in all experimental models used, a finding that is in line with that of other laboratories; this, therefore, necessarily validated the results obtained thereafter. Next, it was demonstrated that xenon exposure could increase HIF-1α protein expression, and this increase occurred *in vitro* as well as *in vivo*, which is of greater clinical relevance. The effect of xenon was not tissue-specific, inducing expression of HIF-1α in both of the organs tested, the brain and the kidney. Xenon exposure increased HIF-1α expression in a time-dependent manner, with maximal expression occurring after 24 hours. Importantly, the xenon-induced increase in HIF-1α occurred without hypoxia or ischaemia, and is sustained even after removal of xenon stimulation. This is dissimilar to hypoxic induction of HIF-1α, where expression is immediate but transient, disappearing upon reoxygenation. Furthermore, xenon increased expression of the HIF target genes EPO and VEGF, at the transcriptional and translational level, over a time course relevant to HIF-1α induction by xenon. In addition, these findings are not an effect of anaesthesia *per se,* since exposure to

another anaesthetic gas, nitrous oxide, did not induce changes in HIF-1α expression.

4.1.2 Normoxic regulation of HIF-1α

Interestingly, there is growing evidence displaying induction of HIF-1α by normoxic stimuli, in a cell-specific manner (Semenza 2005), and this occurs to a comparable degree to hypoxia-induced HIF-1α. These data reveal that xenon is a reliable and robust normoxic stimulator of HIF-1α protein expression, where induction of HIF-1α by xenon is applicable to different organs, including the brain and kidney. Although xenon preconditioning has been demonstrated in the rat heart, xenon-induced HIF-1α in the heart of mice or rats has not been studied.

4.1.3 Mechanisms of regulating HIF-1α protein expression

Although no other laboratories have investigated the effects of xenon preconditioning on HIF-1α induction, evidence of plausible mechanisms for induction of HIF-1α may be gleaned from other normoxic HIF-1α inducers, such as IGF-1 (Fukuda et al. 2002), insulin (Treins et al. 2002) and human epidermal growth factor receptor 2 (HER2) (Laughner et al. 2001). Another inhalational anaesthetic with preconditioning properties, isoflurane, has more recently also been demonstrated capable of inducing HIF-1α, in a hepatoma cell line, Hep3B (Li et al. 2006) and in the hearts of rats (Wang et al. 2006) and rabbits (Raphael et al. 2008). Evidence from these and other studies suggest the involvement of HIF-1α translational pathways, including mTOR-dependent (Raphael et al. 2008) and PI3K-dependent growth signalling pathways (Laughner et al. 2001, Uchiyama et al. 2004). Other studies on xenon preconditioning have suggested the involvement of MAPK signalling (Weber et al. 2005a, Weber et al. 2006a) in reducing myocardial infarct size in the rat. However, Weber and colleagues have not explored the

possible involvement of the MAPK pathways in the brain or kidney, nor looked at HIF-1α expression in their paradigms. Further investigation will be required to explore the relationship between xenon-induced HIF-1α and MAPK activity, and whether these pathways may cross-talk to exert their protective effects. A more detailed discussion on the mechanisms of xenon-induced HIF-1α may be found in discussion 4.2.

4.1.4 HIF isoforms

It should be stipulated that the effect of xenon on HIF-2α or HIF-3α has not been assessed in these studies. At the time of starting these experiments, the important role of HIF-1α for protection against hypoxia was apparent and, therefore, the investigation into its possible relevance to xenon preconditioning. However, the contribution of HIF-2α or HIF-3α at that time remained to be further elucidated. HIF-1α was the original HIF isoform identified, through its ability to regulate oxygen-sensitive EPO gene transcription. HIF-2α and HIF-3α were later identified, through homology screening for putative HIF-1β partners (Wiesener et al. 1998) (Talks et al. 2000). HIF-3α differs in function and expression pattern to either HIF-1α or HIF-2α; it appears to antagonise, rather than promote, HRE-dependent gene expression and, although it is believed to be present in all tissues, is expressed to a lesser degree (Makino et al. 2001). HIF-2α is closely related to HIF-1α, and interestingly, inactivation of each of these isoforms results in a distinctly different phenotype, indicating that they play non-redundant roles. Both isoforms are expressed in all tissues and are now believed to each have distinct transcriptional targets but, at this current point in time, remains to be more fully understood.

Additionally, it must be noted that a number of experiments conducted later in this thesis have employed HIF-1β as an internal control, rather than the more commonly used control, α-tubulin. Both HIF-1β and α-tubulin are suitable internal controls, as both are ubiquitously expressed by all cells and remain unchanged by experimental conditions. Therefore, the switch to using HIF-1β does not affect or jeopardise the reliability of the results but was intended to add to the completeness of the HIF story

4.1.5 Anaesthetic regulation of HIF-1α

This current study observed no changes in HIF-1α expression after nitrous oxide exposure and similarly, previous experiments demonstrated that nitrous oxide does not protect against acute neuronal injury (Ma et al. 2006). This result is not unexpected, but rather, concurs with the hypothesis that HIF-1α expression contributes to protection. Other laboratories have also reported on the inability of nitrous oxide to protect, where it may even exacerbate injury (Pagel, Warltier 2006). The anaesthetic properties of both xenon and nitrous oxide are attributed to their antagonism of the excitatory NMDA receptor. They share certain characteristics; both are gases that have low blood / gas partition coefficients (Goto et al. 1998) and display good analgesic properties. Yet, the physiological effects of xenon have long been observed as markedly different from other NMDA receptor antagonists. For example, xenon is a more potent narcotic agent (1.48-fold higher) than nitrous oxide, in mice and in humans; rapid induction and emergence is achievable with xenon, and it is non-toxic and does not undergo biotransformation (Preckel et al. 2006). As a further exemplification of the differential effects of xenon compared to other inhalational anaesthetics, where xenon increases HIF-1α, halothane is found to block HIF-1α protein expression (Itoh et al. 2001). Overall, these differences

indicate that normoxic regulation of HIF-1α is not a general property of anaesthetics, but is dependent on the anaesthetic molecule used.

4.1.6 Sites of action of xenon

It becomes increasingly clear that xenon does not act at the NMDA receptor alone. Other xenon sites of action have been reported that may contribute to its anaesthetic and analgesic, as well as its protective properties, although their relationship to xenon-induced HIF-1α is not known. Xenon may additionally reduce excitatory neurotransmitter release through AMPA and kainate receptors (Dinse et al. 2005); it favourably affects the ratio of pro-apoptotic and anti-apoptotic proteins (Ma et al. 2007); it is capable of opening potassium channels, including the recently described two-pore potassium channel; and decreases calcium influx (Weber et al. 2006b). Xenon is also able to affect cAMP-response element binding (CREB) protein expression (Ma et al. 2006). Furthermore, post-treatment with xenon is also able to reduce neuronal damage (Dingley et al. 2006), and sub-anaesthetic doses of xenon are able to mediate protection in neonatal rat models, when combined with hypothermia (Ma et al. 2005). However, in those clinical setting where the potential for injury may be pre-empted, for example, in a variety of elective surgical procedures in which the cessation of blood supply is inevitable, protection by xenon preconditioning is an attractive option, in that the tolerance of the tissue may be raised, endogenously equipped and prepared, rendering the forthcoming insult less damaging.

4.1.7 Xenon preconditioning in human-derived cells

Xenon neuroprotection by preconditioning of the neonatal rat brain demonstrated that most protection was afforded 24 hours after exposure (Ma et al. 2006). Although the *in vivo* model described in this thesis is in the male adult mouse, the time course of xenon protection correlates with maximal xenon-induced HIF-1α expression, and HIF-1 responsive genes. Other studies have demonstrated that the effects of preconditioning may last for up to 72 hours (Pagliaro et al. 2001). However, the current study did not explore the expression of HIF-1α beyond 24 hours after xenon. Furthermore, although the mouse is an excellent experimental model, the results obtained from them cannot be directly extrapolated to humans. Of interest however, is that a further experiment demonstrated xenon to reliably increase HIF-1α expression in HK2 cells, which are normal human kidney tubular cells, immortalised by transfection with the E6 / E7 genes of a human papilloma virus-16 that does not change its phenotype or function (Ryan et al. 1994). Again this cannot be considered equivalent, but suggests for the clinical potential of xenon-induced HIF-1α effects.

4.1.8 Xenon preconditioning regulates erythropoietin expression

A mechanism of acquiring tolerance may be achieved through HIF-1 activity and transcription of HIF-1 responsive genes, such as EPO. The current study demonstrated the capability of xenon to firstly induce EPO transcription, followed by translation at 24 hours after xenon. These data are in agreement with different models; ischaemic preconditioning induced EPO transcription and translation after 24 hours (Malhotra et al. 2006) and hypoxic preconditioning induced EPO at 48 hours (Jones, Bergeron 2001, Bernaudin et al. 2002). Previously, EPO produced by the kidney was thought unable to penetrate the uninjured blood brain barrier (Marti

et al. 1997), but more recent studies have demonstrated it may do so but at exceedingly inconsequential levels (Banks et al. 2004). Interestingly however, brain-derived EPO expression has been observed, and is thought to be produced in both neurons and astrocytes (Bergeron et al. 2000, Bernaudin et al. 2000). This current study demonstrated xenon-induced EPO in the brain, but did not look specifically at the sites of expression.

4.1.9 Mechanisms of erythropoietin-mediated protection

There is a growing appreciation for the multi-potential properties of EPO. Numerous *in vitro* and *in vivo* studies demonstrate that the detrimental sequelae of hypoxic-ischaemic injury may be ameliorated by administration of EPO (Semenza et al. 1991). As further evidence, administration of EPO receptor inhibitors reverses the protective effects of EPO (Ruscher et al. 2002). Binding of EPO to its receptor causes receptor homodimerisation and autophosphorylation of JAK-2 (Tilbrook, Klinken 1999), which initiates a cascade of downstream events through several pathways. Firstly, the PI3K-Akt pathway is activated by EPO, and is believed to participate in survival after injury through inhibition of Bad and suppression of apoptotic cell death (Noshita et al. 2001). PI3K signalling is generally considered necessary to EPO-mediated protection; inhibition of PI3K with exogenous administration of wortmannin partially inhibited EPO protection (Yano et al. 2001) and TUNEL staining revealed the reversal of PI3K-mediated reduction in apoptotic mechanisms (Siren et al. 2001). Secondly, the JAK2-STAT pathway is another means whereby EPO may be able to exert protection, through STAT5-mediated transcription of anti-apoptotic genes Bcl-2 and Bcl-xL (Siren et al. 2001), and differentiation of neural progenitor cells in the brain (Wang et al. 2004b). STAT5 knockout mice embryos are found to be severely anaemic, combined with increased apoptosis of erythroid progenitor cells (Socolovsky et al. 2001). Thirdly, EPO has

been demonstrated to protect against excitotoxin and nitric oxide–induced injury and apoptosis, through a JAK2 and NFkB cross-talking mechanism (Digicaylioglu, Lipton 2001); EPO-mediated activation of JAK2 was shown to result in phosphorylation of the NFkB inhibitor, IkB, and this was associated with NFkB-mediated transcription of protection genes. Additionally, inhibition of JAK2, or NFkB, attenuated the EPO-induced protection against apoptosis (Digicaylioglu, Lipton 2001). Finally, the ERK pathway may possibly be under EPO control, but its role in providing protection is uncertain. Ruscher and colleagues determined the unimportance of ERK in mediating brain protection (Ruscher et al. 2002); yet, Kilic demonstrated neuroprotection through activation of both Akt and ERK (Kilic et al. 2005) (Figure 45).

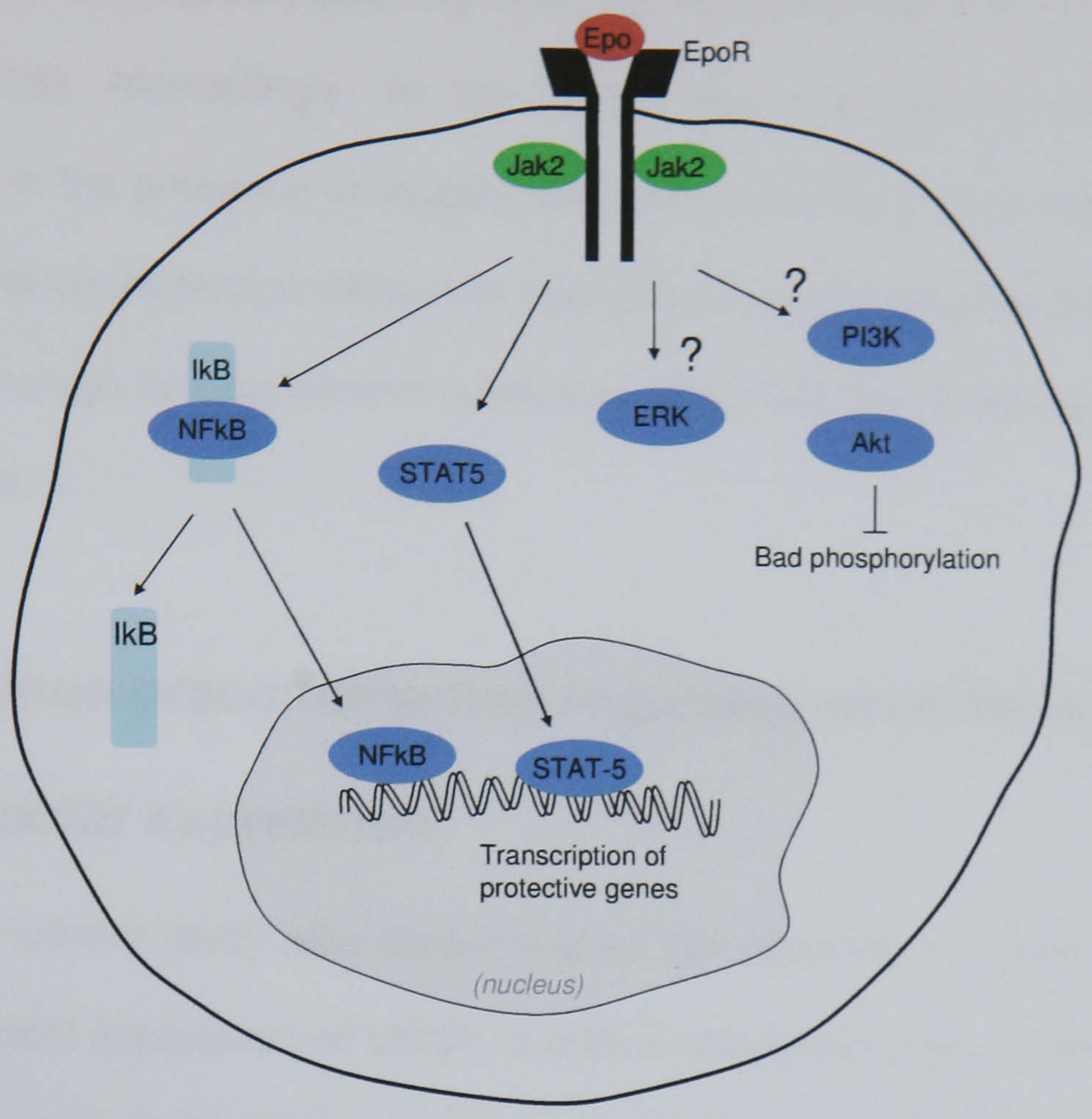


Figure 45. Possible mechanisms of erythropoietin-mediated protection

This cartoon illustrates several EPO-mediated signalling pathways that may contribute to protection against hypoxic-ischaemic injury. This includes EPO-mediated activation of the PI3K, ERK, STAT5 and NFkB pathways, which promote cell survival through increasing expression of anti-apoptotic factors as well as transcription of protective genes. **Abbreviations: ERK,** extracellular signal-related kinases; **IκB, inhibitor** of transcription factor NFκB; **Jak2,** Janus tyrosine kinase-2; **NFκB,** nuclear factor kappa B; **PI3K,** phosphatidylinositide-3 kinase; **STAT-5**, signal transducers and activators of transcription 5.

Furthermore, xenon preconditioning in the rat heart may occur through ERK (Weber et al. 2006a). Interestingly, in the same way that xenon increases HIF-1α expression in the presence of oxygen and after removal of xenon stimulation, EPO is able to induce protection without its sustained expression; once EPO has initiated signalling through its downstream pathways, EPO may be withdrawn and protection still afforded.

4.1.10 Xenon preconditioning regulates vascular endothelial growth factor expression

The current study also demonstrated the capability of xenon to induce the time-dependent expression of VEGF, a critical angiogenic factor and another HIF-1 responsive gene. Although identified as a regulator of proliferation, migration and permeability of endothelial cells, VEGF may also contribute to organ protection (Oosthuyse et al. 2001). Systemic hypoxia induces VEGF expression in all organs of the mouse, with greatest induction in the brain, followed by the kidney (Marti, Risau 1998). Ischaemia also induces VEGF, in the ischaemic penumbra (Leker et al. 2001). Similar to EPO, VEGF signalling is associated with the PI3K / Akt (Hofman et al. 1999) as well as the ERK pathway (Kilic et al. 2006) and may exert protection through activation of these pathways. However, the promoter region of the VEGF gene contains additional sites to the hypoxia-responsive element; this include sites for early growth response factor-1 (Egr-1) (Vidal et al. 2000), Sp1 (Koyama et al. 2002), mothers against decapentaplegic homolog-3 (SMAD3) (He, Chen 2005) and activator protein-1 (AP-1) (Shih, Claffey 2001), among others. VEGF is therefore inducible by various molecules. In light of this, although xenon-induces expression of two target genes of HIF-1α over a time course relevant to xenon-induced HIF-1α, it cannot be ruled out that their induction is through a HIF-1-

independent mechanism. However, later experiments in this thesis investigated this query with the use of HIF-1α siRNA in the mouse model of xenon preconditioning.

4.2 MECHANISM OF XENON-INDUCED HIF-1α PROTEIN EXPRESSION

This next study investigated the mechanism of HIF-1α protein induction by xenon preconditioning, exploring the effects of xenon on HIF-1α transcription, translation and degradation. The results show that in contrast to hypoxia, which is the classical HIF-1α inducer, xenon does not regulate PHD2 protein expression, and induces HIF-1α at a similar rate, in the presence or absence of VHL, implying that xenon does not affect HIF-1α through post-translational degradative mechanisms. Neither does xenon affect transcription of the HIF-1α gene. Instead, xenon increases expression of mTOR, a regulator of HIF-1α protein translation. Additionally, HIF-1α expression is abrogated by rapamycin, the specific mTOR inhibitor, when it was administered 30 minutes prior to xenon exposure. This demonstrates that xenon induces HIF-1α through mTOR-mediated translation-dependent mechanisms.

4.2.1 Xenon preconditioning and HIF-1α gene transcription

The possibility that xenon may induce HIF-1α expression through transcription was discounted, because of the lack of effect of xenon on HIF-1α mRNA expression in RCC4+ cells as well as in the mouse brain and kidney. This finding is not surprising considering that exposure to another anaesthetic preconditioner, isoflurane, does not affect HIF-1α mRNA either (Li et al. 2006). Furthermore, although there is discrepancy as to whether hypoxia, the major inducer of HIF-1α protein expression, may or may not induce HIF-1α mRNA

expression, it primarily regulates HIF-1α through degradative mechanisms (Huang et al. 1998).

4.2.2 Xenon preconditioning and HIF-1α post-translational degradation

Xenon was found not to affect the expression of PHD2, the prolyl hydroxylase believed to be the main contributor to HIF-1α degradation (Berra et al. 2003). As a caveat, PHD2 hydroxylation activity was not assessed. However, it is well explored that molecular oxygen is a critical co-substrate that results in PHD2 enzymatic activity, and this would allow for the degradation of HIF-1α during xenon exposure, as well as in the time period following removal of xenon. Furthermore, the PHDs are described as non-equilibrium enzymes, in that they do not catalyse the reverse reaction; the abundance of non-equilibrium enzymes is often reported as an important determinant of its activity (Schofield, Ratcliffe 2005). In light of this, and the lack of xenon-induced HIF-1α mRNA transcriptional effects, the rate of increase of HIF-1α protein expression may directly reflect the rate of its protein synthesis, induced by xenon. Nonetheless, it is still inappropriate to reject the possibility that xenon may increase expression and activity of other proteins that inhibit HIF-1α degradation, including other members of the prolyl hydroxylase family, PHD1 and PHD3.

4.2.2.1 PHD2-mediated HIF-1α degradation

The PHD enzymes belong to the non-haem iron (Fe (II)) and 2-oxoglutarate-dependent dioxygenease superfamily (Bruick 2001). To date the mammalian genome is found to encode three related proteins, termed PHD1, 2, and 3, although these are represented by a single gene in both *Caenorhabditis elegans* and *Drosophila melanogaster* (Epstein et al. 2001). Small interference RNA studies have

revealed a dominant role for PHD2 in regulation of HIF-1α degradation in normoxia, where PHD2 siRNA fully prevented HIF-1α degradation in the presence of oxygen, with little or no effect on HIF-1α expression observed with PHD1 or 3 siRNA (Berra et al. 2003). Additionally, PHD2, but not PHD1 or 3, contains an N-terminal zinc finger 'MYND' that is also found in the Caenorhabditis *elegans* PHD equivalent, EGL-9 (Taylor 2001). Interestingly, PHD2 is strongly induced by hypoxia, so that immediately upon reoxygenation, HIF-1α is rapidly degraded (Masson, Ratcliffe 2003). Furthermore, the hypoxic-induction of PHD2 is through HIF-1α-mediated transcription of the PHD2 gene. That PHD2 is a HIF-1 responsive gene is intriguing, since these data demonstrate that xenon is capable of increasing EPO and VEGF, yet it does not have an effect on PHD2 expression. The reasons for this are, at present, unclear. A possible explanation may be due to the limitation of the end-point RT-PCR approach used in our experiments; although it is a suitable and commonly used technique for mRNA detection, this technique is relatively insensitive and produces semi-quantitative data and, as such, pales in reliability when compared to real-time RT-PCR, the most sensitive technique for mRNA detection and quantitation currently available.

Alternatively, xenon's ability to differentially regulate HIF target genes may be due to the fact that xenon is capable of inducing other mechanisms, and EPO, VEGF and / or PHD2 may be additionally regulated by HIF-1-independent pathways. Firstly, this may be exemplified through the observations that HIF-1α siRNA decreased expression of xenon-induced EPO, but it did not result in complete abrogation; this may be because of less than 100 % HIF-1α siRNA efficiency, and / or indicative of HIF-1-independent EPO expression. Secondly, xenon may be capable of inducing other members of the HIF system, including HIF-2α and HIF-3α, since all the HIF isoforms are biologically active and play non-redundant roles, as demonstrated by mouse knockouts; to achieve this, xenon may be regulating other signalling

pathways that results in the coordinated activity of each of these isoforms, inducing a different phenotype to that induced by hypoxia. Thirdly, the different phenotypes produced by hypoxia or xenon may be due to effects wider than their effects on the HIF system, where the HIF system may form just a partial contribution to their overall phenotype. Lastly, HIF-1 transcriptional activity is also dependent on recruitment of other molecules to the HRE of genes, such as p300 (Gray et al. 2005), to form a transcription complex; xenon may cause the differential recruitment of these other molecules to the EPO, VEGF or PHD2 gene, that may result in their distinct expression profiles. Although the differential regulation of xenon-induced PHD2 and other HIF-1 target genes is currently enigmatic and requires further investigation, the current study corroborates findings from other laboratories, that normoxic HIF-1α induction occurs, not through degradation, but through protein synthesis.

4.2.2.2 VHL-mediated HIF-1α degradation

Another essential component of the HIF-1α degradation pathway is VHL. VHL recognises PHD2-mediated hydroxylated HIF-1α that results in formation of an E3 ubiquitin ligase complex and hydroxylated HIF-1α polyubiquitination (Kallio et al. 1999). To further understand the role of xenon in HIF-1α degradation, the effect of xenon-induced HIF-1α was explored using two RCC4 cell lines that were either VHL-deficient (RCC4-), or stably transfected with VHL (RCC4+). Experiments described in this thesis demonstrated that HIF-1α induction by xenon occurs at a similar rate in the absence or the presence of VHL. Therefore, the finding that xenon does not affect PHD2 expression and increases HIF-1α through VHL-independent mechanisms, suggests that xenon does not regulate HIF-1α at the post-translational degradation level. This is further supported by the observation that xenon increases

HIF-1α in normoxic conditions, and HIF-1α expression continues to rise, in the presence of oxygen, after the withdrawal of exposure to xenon.

4.2.3 Xenon preconditioning and HIF-1α translation

The results of the current study demonstrated that xenon exposure induces expression of the mammalian target of rapamycin (mTOR) in an *in vitro* model, using RCC4+ cells. mTOR expression occurs from 2 hours after xenon exposure, with striking induction occurring after 24 hours. This result is important as mTOR is a possible upstream regulator of HIF-1α protein expression and may be the signal transducer for xenon's effect on HIF-1α. Accordingly, these experiments also demonstrated that the specific mTOR inhibitor rapamycin was able to abrogate expression of xenon-induced HIF-1α, thereby showing an essential role for mTOR in HIF-1α induction. These results are in line with a recent investigation demonstrating that isoflurane preconditioning increases HIF-1α in the rabbit heart, through a rapamycin sensitive pathway; interestingly, rapamycin administration also resulted in abolishing the isoflurane-mediated cardioprotection (Raphael et al. 2008). Although our study is the first to demonstrate xenon preconditioning induces HIF-1α through mTOR, the recent elucidation from these isoflurane preconditioning studies lends support for the involvement of HIF-1α as a key mediator, not only in hypoxic preconditioning, but in other preconditioning paradigms also. The results obtained on our current study, however, are relevant to RCC4+ cells. Renal cell carcinomas are thought to be neoplastic due to VHL deficiency (Krieg et al. 2000), and therefore stable transfection of VHL (to form RCC4+) should restore the wild-type phenotype. However, the use of any cancer cell line still raises issues of translatability to the physiological mechanisms involved, because the processes occurring in neoplastic cells differ in many respects to normal primary cells in culture. Therefore, the involvement of mTOR was validated in the mouse brain and kidney, where

rapamycin administration to mice was also found to abrogate expression of HIF-1α following xenon exposure. Although these results cannot be directly extrapolated to the mechanisms involved in humans, this is a more clinically relevant model.

4.2.3.1 mTOR-mediated HIF-1α protein translation

Although evidence has begun to gather in the last couple of years, very little is still known about the molecular targets involved in xenon preconditioning. The serine-threonine protein kinase, mTOR, is a key component of the PI3K / Akt / mTOR pro-survival signalling pathway, where it plays a regulatory role in integrating signals from growth factors, nutrients and cellular energy levels, to stimulate cell growth and proliferation, through mRNA translation initiation and protein synthesis (Gingras, Raught & Sonenberg 2001). Through the inhibitory effects of rapamycin, the current study demonstrated that HIF-1α is a downstream target of mTOR signalling. Several preconditioning studies have demonstrated the recruitment and activation of mTOR in mediating protection, for example, in ischaemia- (Kis, Yellon & Baxter 2003) or insulin- (Jonassen, Mjos & Sack 2004) induced preconditioning; furthermore, rapamycin inhibited their protective effects.

4.2.3.2 Rapamycin inhibition of mTOR-mediated HIF-1α protein translation

Rapamycin, also known as sirolimus, is a macrolide compound obtained from *Streptomyces hygroscopicus* (Sehgal 2003), and is a relatively new immunosuppressant drug, clinically approved for use in prevention of organ rejection after transplant surgery. Additionally, it also possesses both anti-fungal and anti-neoplastic properties (Sehgal 2003), and has more recently been associated with anti-depressive-like effects in mice and rats (Cleary et al. 2008) . Mechanistically, however, rapamycin acts as the specific inhibitor of the serine-

threonine protein kinase, mTOR; to achieve its inhibition, rapamycin combines to the FK506 binding protein-12 (FKBP-12), that associates directly with the mTOR / p70 S6 kinase complex, and prevents downstream activity of mTOR (Asnaghi et al. 2004). The inhibitory effect of rapamycin on HIF-1α expression, as described in this thesis, is also exemplified in its recent approval for use as an anti-cancer treatment, in HIF-1α-expressing tumours(Belozerov, Van Meir 2005).

Curiously, a recent paper by Khan et al. have reported that rapamycin can itself confer preconditioning-like protection against ischaemia-reperfusion injury in isolated mouse myocytes (Khan et al. 2006). These findings are difficult to reconcile with data from other investigators (Hausenloy, Yellon 2006, Jonassen, Mjos & Sack 2004, Kis, Yellon & Baxter 2003) including ourselves. Although our study did not investigate the effect of rapamycin on xenon-mediated protection, we did demonstrate that rapamycin abrogates xenon-induced HIF-1α, and that HIF-1α plays an important role in xenon-mediated protection; this latter finding demonstrated that HIF-1α siRNA results in the loss of xenon's protective effects, and would suggest that rapamycin would worsen, rather than confer, xenon-mediated protection against injury. Khan's results may reflect differences in experimental models, where they used isolated mouse hearts rather than the intact animal. The results may also be dependent on the preconditioning and injury paradigm used: rapamycin was administered 30 minutes prior to removal of the heart, exposed to ischaemia for 20 minutes, and reperfused for 30 minutes before infarct size was assessed. Administering rapamycin 30 minutes prior to injury is an atypical preconditioning paradigm, as is the time used for reperfusion; as such, the observed reduction in infarct size does not reflect any later expansions of the infarct. Although these results are enigmatic, they are worth considering as they may provide insight into, as yet, novel and unexplored mechanisms of protection.

4.2.3.3 Administration of rapamycin

Several caveats need to be considered. Rapamycin at 25, 50 and 100 nM concentrations were used in the *in vitro* model because these doses have been shown to inhibit mTOR. However, the dose-response relationship to rapamycin in RCC4+ cells was not performed in this study, and these data suggest that lower concentrations of rapamycin may still be effective at inhibiting HIF-1α expression. Although rapamycin is described as a specific inhibitor of mTOR activity, it cannot be excluded that it may interact with the activity of other protein kinases that may also have an effect on HIF-1α expression. In the mouse model, rapamycin 1.5 mg / Kg, IP, significantly reduced xenon-induced HIF-1α expression in the brain and kidney. However, other preconditioning studies have observed mTOR inhibitory effects with rapamycin doses of 0.25 mg / Kg (Raphael et al. 2008). At this lower dose, our study observed a trend towards decreased HIF-1α expression, but without significance. Explanations for these differences may be related to the animal model and species, the organ of study, differing effects of preconditioning agents and paradigms, as well as the route of drug administration. Firstly, our study has used C57BL6 mice to investigate the effects of xenon preconditioning in the brain and kidney, whereas other studies demonstrating a role for mTOR in preconditioning have used rats or rabbits to study the effects of isoflurane in the heart. Furthermore, taking into consideration that the half-life of rapamycin in mice is 4.5 hours (Rivera et al. 1999) and over 5 hours in rats (Trepanier et al. 1998), the time course of our investigation, from rapamycin administration to organ harvesting was 26.5 hours (rapamycin administration, 30 minutes of stabilisation, 2 hours of xenon exposure, 24 hours rest, organ harvesting), compared to much shorter time courses employed by others, such as 5 to 6 hours (Raphael et al. 2008). Additionally, other preconditioning studies have administered rapamycin intravenously, compared to the intraperitoneal route chosen for use in experiments described in this thesis.

Doses of rapamycin 0.5 - 1.5 mg / Kg, IP, have previously been used and demonstrated effective at inhibiting mTOR (Lang et al. 2007). Nonetheless, these pharmacological data do suggest an important requirement for mTOR signalling in xenon-induced HIF-1α.

4.2.3.4 Effects of DMSO in vivo

Surprisingly, the current study observed the ability of the rapamycin solvent, DMSO, to increase expression of HIF-1α in a concentration-dependent manner, a finding that has not yet been reported by other investigators. The nanomolar concentrations of rapamycin used in the *in vitro* setting, with 0.0025 % DMSO, was not enough to alter expression of HIF-1α. However the higher concentrations of rapamycin used in mice, necessitated the use of higher concentrations of DMSO (0.1 %), which may account for the constitutively higher background expression of HIF-1α, observed in the Western blots of the rapamycin-treated mouse samples. Although DMSO-induced HIF-1α expression was a confounding factor, the ability of rapamycin to inhibit xenon-induced HIF-1α was still evident, occurring independently to the DMSO-induced HIF-1α expression. Another solvent of rapamycin, ethanol, was not chosen, as it may itself contribute to preconditioning (Wang et al. 2007) and induction of the HIF target gene, VEGF (Gu et al. 2001). Furthermore, rapamycin is less soluble in ethanol than DMSO, which would necessitate the use of even greater concentrations.

4.2.3.5 Upstream of the mTOR pathway

Importantly, it is still not known at what upstream juncture in the mTOR pathway xenon converges on. However, putative molecular targets may be gleaned from the increasing number of studies in hypoxic or ischaemic preconditioning. Activation of PI3K and Akt, molecules that are upstream of mTOR and stimulated via tyrosine kinase receptors, have been suggested to play protective roles in ischaemic preconditioning of the myocardium (Raphael, Rivo & Gozal 2005), as well as the brain, where the PI3K inhibitors LY294002 or wortmannin have been shown to reverse the protective effects of ischaemic preconditioning; these PI3K inhibitors blocked the reduction in myocardial infarct size in rats as well as the increase in phosphorylation and thus activation of Akt (Mocanu, Bell & Yellon 2002). Furthermore, consistent with the role of the PI3K / Akt / mTOR pathway in preconditioning, tyrosine kinase receptor agonists, such as insulin and IGF-1, are able to induce preconditioning-mediated protection (Fukuda et al. 2002). PI3K / Akt / mTOR signalling is thought to contribute to protection through inhibition of the pro-apoptotic proteins Bad, Bax and caspase-9 (Raphael et al. 2006), as well as through mediating eNOS-derived NO production (Zaugg et al. 2003). However, recent elucidations reveal that mTOR activity may also be mediated through PI3K-independent pathways including one that may involve ATP.

4.2.3.6 mTOR-independent pathways

Further adding to the complexity of preconditioning, HIF-1α may be regulated through mTOR-independent mechanisms, which instead relies on involvement of members of the MAPK family, such as ERK (Roux, Blenis 2004). ERK takes part in intracellular transduction pathways to relay extracellular signals within cells. It has been reported to contribute to ischaemic preconditioning (Fryer, Hsu & Gross 2001) and interestingly, both ERK and p38 MAPKs are demonstrated

to play a time-dependent role in xenon preconditioning of the rat heart. Furthermore, the role of ERK in isoflurane preconditioning of the rat heart was demonstrated to influence HIF-1α protein expression, where isoflurane-induced HIF-1α was abolished in the presence of the ERK inhibitor PD098059 (Wang et al. 2006). A more recent investigation studying both ERK and Akt, has demonstrated that hypoxic preconditioning results in phosphorylation and activation of both of these kinase pathways in mesenchymal stem cells, which is also associated with an increase in VEGF expression (Wang et al. 2008a); however, the effect of inhibition of either of these kinases on VEGF expression was not ascertained by these investigators. Alternatively, mTOR-independent yet PI3K-dependent pathways may also exist. PI3K / Akt signalling may additionally activate PKC, another protein kinase that is implicated in ischaemic (Chachami et al. 2004). and anaesthetic (Bergeron et al. 2000) preconditioning; moreover, PKC has also been shown to increase HIF-1α (Page et al. 2002).

It is believed that these different intracellular pathways are complementary to each other through cross-talking mechanisms, and preconditioning exerts its protection by manipulating multiple stress responsive pathways. However, the pathways employed and their actions on HIF-1α may be cell type-and-stimulus specific; furthermore, different cell types may express distinct combinations of each of these signalling proteins. Continued investigation will be required to unravel the intricacies of these intracellular signalling pathways and to ascertain their relevance to xenon preconditioning.

4.3 NECESSITY OF HIF-1α IN XENON PRECONDITIONING VERSUS RENAL ISCHAEMIC - REPERFUSION INJURY

The ability of xenon to precondition and protect the neonatal rat brain from acute neuronal injury has previously been demonstrated (Ma et al. 2006) but the mechanism of protection remains to be elucidated. It is known that the heterodimeric transcription factor HIF-1 plays an essential role in mediating tissue adaptation and tolerance to hypoxia, and is an important mediator of hypoxic preconditioning. This is by virtue of its ability to regulate transcriptional activity of over a hundred genes that take part in prosurvival mechanisms. Notably, it is the expression and stabilisation of the HIF-1α subunit that is the determinant of transcriptional activity of HIF-1. As such, the experiments aimed to explore whether HIF-1α is necessary for xenon preconditioning. Recently, HIF-1α has been shown to be upregulated by another anaesthetic gas, isoflurane, in a Hep3B cell line (Li et al. 2006) as well as by isoflurane preconditioning in the rabbit heart (Raphael et al. 2008). However, to the best of our knowledge, this current study is the first to demonstrate, under normoxic conditions, the capability of xenon to induce sustained expression of HIF-1α and its downstream effectors, EPO and VEGF compared to nitrogen controls, *in vitro* as well as *in vivo*, in the mouse brain and kidney.

Furthermore, we have investigated the necessity of HIF-1α in xenon preconditioning against ischaemia-reperfusion injury, through the use of siRNA technology. SiRNA was chosen to knockdown HIF-1α in mice, as HIF-1α knockout mice are not available, owing to embryonic lethality. Conditional HIF-1α knock-out mice are available, but were not chosen, due to unknown compensatory mechanisms that these mice may display. Additionally, siRNA technology is a relatively cost-effective

technique that results in specific knockdown of its putative target. The current study explored the necessity of HIF-1α participation in xenon preconditioning; as a proof of concept experiment we have addressed this question in the mouse kidney, in a previously established model of renal ischaemia-reperfusion injury.

4.3.1 Delivery and function of in vivo siRNA

Our experiments showed that administration of 3'-fluorescein-labelled nonsense siRNA through a single hydrodynamic tail vein injection in the mouse results in the presence of siRNA in the renal parenchyma 24 hours later, thereby demonstrating the efficient delivery of siRNA to the desired tissue under investigation. Furthermore, 24 hours following administration of HIF-1α siRNA, mRNA expression of both HIF-1α and its downstream target EPO were significantly decreased, when compared to non-treated control mice, as well as nonsense siRNA or PBS -injected control mice. This demonstrated that upon efficient siRNA delivery into the renal parenchyma, HIF-1α siRNA is capable of silencing HIF-1α gene expression. Additionally, the silencing of HIF-1α results in attenuation of EPO mRNA expression, thereby demonstrating that EPO is, at least in part, dependent on HIF transcriptional activity.

4.3.2 Necessity of HIF-1α in xenon-mediated erythropoietin

As described in discussion 4.1, xenon preconditioning increases expression of EPO at both the transcriptional and translational level, over a time course that is relevant to xenon-induced HIF-1α. Additionally however, the use of siRNA directed against HIF-1α further enhances knowledge, by showing that HIF-1α siRNA attenuates the xenon-induced expression of both HIF-1α and EPO, providing evidence that xenon-induced EPO occurs, at least in part, through HIF-1 transcriptional activity in the mouse kidney.

4.3.3 Necessity of HIF-1α in xenon preconditioning against renal ischaemia-reperfusion injury

In the context of renal ischaemia-reperfusion injury, xenon preconditioning resulted in a 4-fold reduction in the degree of ischaemic damage, according to histological data; importantly, HIF-1α siRNA injection prior to xenon preconditioning resulted in ischaemic damage that was not significantly different to the PBS-injected, untreated injury group. Similarly, renal function, as measured by urea and creatinine levels, showed significant improvement with xenon-mediated protection, but this functional protection was lost with HIF-1α knockdown. Furthermore, xenon preconditioning decreased apoptosis of renal tubular cells following injury, detected through TUNEL staining, whereas HIF-1α siRNA resulted in apoptosis to the same degree as non-preconditioned injured mice. In the more severe injury model of renal failure, xenon preconditioning not only improved the survival of these mice, but renal function was significantly better, as measured by urea and creatinine levels; HIF-1α siRNA also removed the xenon-mediated protection in this severe injury model. Overall, this current study demonstrated that the necessary contribution of HIF-1α is, at least in part, responsible for the phenomenon of protection induced by xenon preconditioning.

4.3.4 Regulation of erythropoietin

Attenuation of HIF-1α or EPO mRNA by HIF-1α siRNA is not absolute and several caveats must be considered. As previously mentioned, oxygen-sensitive induction of erythropoiesis, via the prosurvival growth factor EPO, led to the discovery of HIF-1α (Semenza 1994). At the time this study was initiated, EPO was considered a canonical HIF-1α target gene and evidence of EPO expression is still used by many investigators as a credible representative of HIF-1α activity. However, since the initiation of these studies, it has come to light that the regulation of EPO

may not be exclusively under the control of HIF-1α. HIF-1α and HIF-2α are both able to bind to the HRE of the EPO (Ratcliffe 2007), and indeed, recent studies using conditional knockouts of HIF-1α or HIF-2α have shown that EPO is predominantly dependent on HIF-2α in the hepatocytes of these mice (Rankin et al. 2007). The mechanisms driving either HIF-1α or HIF-2α -mediated EPO production, or perhaps even both, are not fully understood. The incomplete attenuation of xenon-induced EPO through HIF-1α siRNA may be due to xenon mediating HIF-1α-independent mechanisms, which have not yet been explored. Indeed, it is clear that xenon does not regulate HIF-1α in the same way as hypoxia-regulated HIF-1α; furthermore, hypoxia exerts distinct responses in the different HIF-α isoforms, which is further dependent on cell type and disease state of the tissue. Therefore, the mechanism of xenon-mediated protection is likely to extend beyond its effects on HIF-1α and the relevance of these interesting findings to xenon preconditioning requires further investigation. Nonetheless, these results show that xenon preconditioning induces EPO, at least in part, through HIF-1α dependent mechanisms, in the intact kidney.

4.3.5 Apoptosis and HIF isoforms

The results from the current study demonstrate the capability of xenon preconditioning to reduce the occurrence of apoptosis in renal ischaemia-reperfusion injury in mice; this requires HIF-1α activity, since HIF-1α siRNA abrogates the protective effect afforded by xenon. However, the role of HIF-2α has not been addressed in these studies, and a few remarks are worth considering. Interestingly, HIF-1α appears capable of playing a dichotomous role in the response to hypoxia, between pro-survival and proliferation on the one hand, and pro-apoptosis and anti-proliferation on the other (Aminova et al. 2005). It may be that these responses are not mediated by HIF-1α alone, but by a more complex, yet

coordinated, interaction between the different HIF-α isoforms, as non-redundancy of each isoform has been demonstrated in knockout mice. How these isoforms coordinate together is unclear, but examples from tumour biology may aid in better understanding. Non-cancerous renal tubular cells predominantly express HIF-1α; yet, in clear-cell renal carcinoma, hypoxia appears to drive, not HIF-1α. but HIF-2α to promote expression of anti-apoptotic proteins and proliferation for tumour growth (Rosenberger et al. 2002); conversely, HIF-1α in these advanced tumours, appears to promote apoptosis (Raval et al. 2005). These observations, and other findings, suggest for the existence of cell, condition, and disease -specific alterations in HIF-1α and HIF-2α activity that is, as yet, not well understood.

4.3.6 siRNA technology

RNA interference through siRNA is a powerful technique for post-transcriptional knockdown of a specific mRNA. Due to the properties of nucleic acids, siRNA is subject to rapid degradation by nucleases, and do not naturally cross cell membranes. However, the development of techniques, such as hydrodynamic injection, has greatly facilitated the use of siRNA in *in vivo* experimental models. Although delivery of the siRNA may be challenging, it results in gene silencing with greater specificity when compared to other techniques, such as small molecule inhibitors or anti-sense DNA oligonucleotides.

The use of siRNA in small mammals is relatively recent. In murine models of renal IRI, siRNA techniques have furthered the understanding of renal pathophysiology. For example, siRNA against Fas was shown to reduce renal tubular apoptosis and overall mortality from 90 % to 20 % (Hamar et al. 2004), and siRNA against caspase 3 and 8 also attenuated renal IRI (Zheng et al. 2006). Interestingly, PHD2 inhibition increased HIF-1α expression and activity and improved renal function (Bernhardt et

al. 2006). To date, no studies have investigated the effect of HIF-1α siRNA in preconditioning against HI or IR injury of any organ. However, a study on intermittent hypoxia in tumours, arising from disorganised tumour vasculature, demonstrated that the resistance of tumours to pro-apoptotic stress could be attenuated using HIF-1α siRNA; this underscores the role of HIF-1α in promoting cell survival (Martinive et al. 2006). Therefore, this current study using HIF-1α siRNA is the first to explore and provide evidence for the necessity of HIF-1α, in xenon preconditioning versus ischaemic organ injury.

Several caveats must be considered. A major barrier in *in vivo* RNAi experiments is the difficulty in delivering the siRNA to the tissue of interest; this is due to both the practical expertise required as well as the need for refinement of the manufactured reagents. It is known that naked siRNA, as used in these studies, is more prone to degradation by nonspecific proteases and, therefore, less effectively delivered compared to coated siRNA varieties. This may account for the incomplete suppression of HIF-1α and EPO expression that was observed after HIF-1α siRNA treatment. However, it may be argued that the reduction in expression, although incomplete, was enough to result in attenuation of HIF-1α function and activity; for example, the renoprotection afforded by xenon preconditioning was lost (despite incomplete HIF-1α suppression) in the presence of HIF-1α siRNA resulting in abrogation of the protective effect of xenon. Coated siRNAs constructs also have their disadvantages, where they are more likely to contribute to toxicity than naked varieties, due to "off-target" gene silencing (Jackson et al. 2003), and have been reported to induce components of the host's immune response, such as activation of the interferon system (Hornung (2005). This naturally raises concerns over their unknown and unanticipated effects.

4.3.7 The requirement of anaesthesia for surgical experiments

Anaesthesia was induced and maintained with 5 % and 2 % isoflurane respectively, in surgical experiment procedures; these included cannula implantation for hydrodynamic tail vein injection, clamping of the renal pedicle for induction of renal IRI, as well as nephrectomy. Isoflurane is one of a number of anaesthetic gases that is capable of inducing preconditioning, and protecting against ischaemic-reperfusion injury (Julier et al. 2003). It is possible that isoflurane may have contributed to the renoprotection observed with xenon preconditioning; however, animals in all experimental groups each received equipotent doses of isoflurane during surgery and as such, the observed differences in renal injury between the experimental groups is likely to be due to the effect of xenon preconditioning.

CHAPTER 5

FINAL CONCLUSION

5.1 OVERVIEW OF FINDINGS

By definition, aerobic organisms are entirely dependent on oxygen, for all metabolic processes, including ATP formation through oxidative phosphorylation. A disparity between oxygen supply and oxygen demand may result in deleterious consequences due to hypoxia and/or ischaemia. Interestingly, cells have evolved highly sensitive oxygen sensors and adaptive mechanisms to respond to hypoxic-ischaemic (HI) challenge, through increased HI tolerance and restoration of oxygen homeostasis. These mechanisms, termed preconditioning, are characterised by injury-induced protection from a subsequent more severe injury. Preconditioning is also inducible through pharmacological agents, including some anaesthetics, and as such, exploitation of this endogenous protective phenomenon may be applicable to humans and is putatively clinically important.

The noble anaesthetic gas xenon displays many of the hallmarks of the ideal anaesthetic agent, and its neuroprotective potential, through a preconditioning phenomenon, has been demonstrated in an *in* vivo model of acute neuronal HI injury (Ma et al. 2006). The mechanism for xenon-mediated adaptation to HI tolerance remains unclear. The transcription factor HIF-1 plays an important role in hypoxia, acting as a molecular switch for adaptation to a more hypoxia-resilient phenotype. HIF-1α activity induces expression of at least one hundred genes, of which several are capable of mediating the cytoprotective responses of preconditioning. Given the important roles of HIF-1α and its target genes for tolerance to HI injury, this current study investigated whether xenon preconditioning may be exerting its protective effects through HIF-1α.

Aim 1 of the experiments demonstrated the ability of xenon to induce the time-dependent expression of HIF-1α over a 24 hour time period in normoxic conditions, *in vitro* and *in vivo*, and in different tissues, such as the brain and kidney. Greatest HIF-1α induction occurred 8 - 24 hours after exposure to xenon, a time course that parallels that of xenon-mediated preconditioning. This provided evidence that HIF-1α accumulation may be a necessary component of the endogenous adaptation required for the protection conferred through xenon. Furthermore, this is different from the effect of acute hypoxia on HIF-1α expression, which rapidly returns to baseline levels on removal of the hypoxic stimulus.

Xenon exposure also time-dependently increased expression of EPO, firstly at the transcriptional level and subsequently at the translational level, over a time course that is relevant to xenon's induction of HIF-1α. This HIF target gene is itself a powerful neuroprotectant that is modulated by the binding of HIF to the EPO hypoxia-responsive element (HRE), and represents a candidate protein for transducing the preconditioning effects of xenon. Moreover, xenon exposure increased expression of VEGF, another HIF-1 target gene, providing further evidence that xenon-induced HIF-1α expression is functionally active and able to transcribe its target genes.

Plausible mechanisms of protection by xenon-induced EPO may be through a phosphorylation cascade of the JAK/STAT pathway, as reported by (Sola et al. 2005), the PI3K/Akt pathway (Ruscher et al. 2002), and/or the Ras/Raf/MEK/ERK pathway (Arai, Kanda & Miura 2002). Dirnagl and colleagues postulate an interwoven combination of these pathways to ultimately converge on end-effectors, such as the Bcl protein family, that inhibit apoptosis and promote cell survival, (Dirnagl, Simon & Hallenbeck 2003).

Exposure to nitrous oxide, another gas with anaesthetic properties through NMDA receptor antagonism, did not induce HIF-1α expression at any of the time points studied. This suggests that xenon's effects on HIF-1α are not simply due to its anaesthetic properties or to its blocking activity at the NMDA receptor alone.

Aim 2 explored the mechanism of xenon-induced HIF-1α, by investigating the effect of xenon on HIF-1α transcription, translation and degradation. Accumulation of HIF-1α may occur if its rate of synthesis is superior to its rate of degradation, either through increased transcription and/or translation, or decreased degradation.

Whereas hypoxia increases HIF-1α through PHD2 and VHL -dependent inhibition of its degradation, xenon does not affect PHD2 expression at any of the time points studied, and is able to induce HIF-1α by the same order of magnitude in the presence or absence of VHL. Therefore, unlike hypoxia, xenon does not inhibit HIF-1α degradation, and increases HIF-1α through PHD2 and VHL –independent mechanisms.

The possibility that xenon may induce HIF-1α expression through transcription was rejected, because of the lack of effect of xenon on HIF-1α mRNA expression in both *in vitro* and *in vivo* models. These data corroborate the finding in the hep3B cell line that isoflurane, another anaesthetic preconditioner, does not affect HIF-1α at the transcript level (Li et al. 2006). Furthermore, although there is discrepancy as to whether hypoxia, the major inducer of HIF-1α protein expression, may or may not induce HIF-1α mRNA expression, it primarily regulates HIF-1α through degradative mechanisms.

The best described regulator of translation of HIF-1α protein synthesis is the mammalian target of rapamycin (mTOR), where mTOR activation results in downstream phosphorylation, which activates as well as disinhibits translation initiation proteins, of which the HIF-1α mRNA is a target. Xenon is capable of increasing mTOR expression with a detectable, but not significant, increase occurring 2 hours after xenon exposure; 8 hours after, xenon significantly increases mTOR expression, with greatest expression after 24 hours. Additionally, rapamycin administration abrogates xenon's HIF-1α inducing capability, providing evidence that xenon increases HIF-1α through mTOR-mediated translation-dependent mechanisms.

Aim 3 investigated the necessity of HIF-1α in xenon preconditioning versus renal ischaemic-reperfusion injury in mice, through the use of siRNA technology directed against HIF-1α. First delivery of siRNA by hydrodynamic tail vein injection into the renal parenchyma was verified, and the functional effect of HIF-1α demonstrated, through the attenuation of both HIF-1α and EPO mRNA following siRNA treatment. Secondly, the necessity of HIF-1α in xenon-induced EPO was established; HIF-1α siRNA pre-treatment prevented xenon from inducing HIF-1α, as well as EPO, providing evidence that the induction of EPO by xenon occurs through a HIF-dependent mechanism.

Finally, a necessary role for HIF-1α in xenon preconditioning against renal IR injury as well as renal failure was elucidated. Xenon preconditioning ameliorates the detrimental effects of renal IR injury, both morphologically and functionally. Its anti-apoptotic effect following renal IR injury, measured by TUNEL staining, provides further evidence of xenon's powerful protective capabilities. Furthermore, xenon preconditioning significantly improves survival and function following renal failure. The role that HIF-1α plays in xenon preconditioning is an essential one. siRNA

directed against HIF-1α detrimentally attenuates the renoprotection afforded by xenon preconditioning, with morphological and functional outcome similar to injury alone. HIF-1α siRNA also abrogates the anti-apoptotic effects of xenon preconditioning against injury, and decreases survival after renal failure.

5.2 FUTURE DIRECTION

Several preconditioning mechanisms of organ protection have been postulated. The study into the role of HIF-1α in xenon preconditioning is novel and contributes to the knowledge that is gathering in this large field. The results of this body of work have suggested for a number of possible subsequent experiments in which to further explore the mechanisms of xenon preconditioning. Some questions that remain to be answered include:

- What is the effect of xenon on mTOR and HIF-1α beyond 72 hours?
- At what molecular juncture upstream of mTOR does xenon exert its effect?
- What is the effect of xenon on other HIF-α isoforms?
- What effect does xenon have on other signalling kinases and transcription factors, such as MAPKs and NFκB, and how do these pathways coordinate with xenon-induced HIF-1α?
- What are the similarities and differences in xenon preconditioning compared to postconditioning?
- What are the similarities and differences in xenon-mediated preconditioning compared to preconditioning by other agents, such as hypoxia or isoflurane?
- Is the administration of xenon in combination with other preconditioning agents additive or synergistic?
- Would the use of microarrays reveal molecules pertinent to the mechanism of xenon preconditioning? How would this compare to hypoxic preconditioning?
- What is the minimum exposure of xenon required, in terms of length of time as well as concentration, for the organ protective effect?

5.3 CLINICAL IMPLICATIONS

Despite intensive research over several decades, there are no satisfactory clinical strategies to diminish the vulnerability of tissues to injury. Mitigating the consequences of HI, IR injury with a safe, efficacious and non-toxic agent represents an unmet healthcare need.

Other than timely reperfusion, ischaemic preconditioning is acknowledged to be the most powerful mechanism for limiting ischaemic injury (Riksen, Smits & Rongen 2004), providing strong impetus to identify pharmacological agents capable of mimicking ischaemic preconditioning. Xenon is one such agent. Xenon has been used safely in the clinical setting as an anaesthetic in patients of all ages for more than 50 years and its potent preconditioning properties combined with its powerful organ protective capabilities, could be put to great use in several fields of medicine, where there is an anticipated risk of HI or IR organ injury. Applications may include the use of xenon intra-operatively for procedures that carry a substantial risk of HI injury, such as cardiopulmonary bypass surgery, renal transplantation surgery or neurosurgery. Xenon may also be pre-emptively administered to expectant mothers in labour who have a high risk of neonatal hypoxia, such as pre-term labour. In addition, it has a safety profile that is unrivalled (Sanders, Franks & Maze 2003). Xenon, therefore, represents a promising new therapeutic strategy for reducing the incidence of disorders arising through HI or IR injury. Elucidation of the organ protective mechanism of xenon as a preconditioner is essential if it is to progress for safe and efficacious use in the clinical setting.

Until recently, the high cost and scarcity of xenon have prevented its widespread clinical application; with refinements in production of xenon and the advent of xenon-conserving ventilation systems, such as closed-loop or low-flow systems,

financial concerns have been alleviated to a large extent. It is still probable at first, however, that its use will have to be reserved for high risk patients.

Clinical translation of findings from this project will facilitate the use of xenon in preventing hypoxia-related injury. By understanding the mechanisms involved in xenon's preconditioning action, it will be possible to prioritise the clinical settings that are most likely to benefit from this safe and efficacious anaesthetic agent. Moreover, results from these experiments have established a necessary role for HIF-1α in mediating xenon's preconditioning action; data from these studies will enhance our understanding of the interactions of the putative signalling cascades that are involved.

Even if xenon itself were not to be clinically exploited, date from the current study may facilitate the discovery and development of novel chemical entities for action at newly identified therapeutic targets to protect against impending injury, while avoiding those that may promote toxicity.

5.4 CONCLUSION

Xenon preconditioning time-dependently increases expression of HIF-1α and its target genes, EPO and VEGF, over a time course that is relevant to xenon-mediated protection against acute neuronal hypoxic-ischaemic injury. The xenon-induced accumulation of HIF-1α is sensitive to rapamycin, and occurs through an mTOR-mediated protein translation pathway. Xenon preconditioning is also capable of protecting against renal ischaemic-reperfusion injury as well as renal failure, reducing apoptosis, improving function after injury, as well as improving long term survival. HIF-1α plays a necessary role in this xenon-mediated protection. The sufficiency of HIF-1α alone in mediating xenon preconditioning remains to be seen.

CHAPTER 6

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