The Role and Regulation of Oxytocin/Oxytocin Receptor System in Human Amnion and Labour

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Institute of Reproductive and Developmental Biology
Division of Medicine
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Dedicated to my beloved family
Abstract

Preterm delivery occurs in 10% of all births. It is the major cause of infant death and handicap in the developed countries and accounts for 65% of neonatal deaths and 50% of childhood neurological disabilities. At the end of pregnancy, ‘pro-labour’ factors begin to mediate remodeling of the cervix resulting in cervical ripening and dilatation, uterine contractility and decidual/fetal membrane activation. The amnion plays an important role in the onset of human labour. It is a major site for prostaglandins (PG) and inflammatory cytokine synthesis which increases both before and during labour. Amnion derived inflammatory cytokines and prostaglandins contribute to the relaxation of the lower uterine segment and to cervical ripening. Oxytocin (OT) and oxytocin receptor (OTR) are classically considered to play a fundamental role in the mechanism of labour as OT stimulates uterine contractions and OTR antagonists are clinically used as tocolytics. The increase in OT and OTR expressions were observed in tissues other than myometrium, including the breast cells, decidua and amnion. However, amnion is not a contractile tissue and therefore the physiological role of the OT/OTR is less obvious. We hypothesised that the regulation of OT/OTR in human amnion is linked to NF-κB activity and plays an important role in the onset of labour.

We have demonstrated that in human amnion, labour induces expression of both OT and OTR expression. Using specific inhibitors and siRNA target knockdown studies, we have shown that unlike the myometrial OTR, OT binding to OTR in human amnion drives the receptor to couple with Gαi2 and Gαi3, but not Gαq. This subsequently triggers the sequential activation of ERK, p38 MAPKs and NF-κB signalling cascades leading to PG and proinflammatory cytokine/chemokine synthesis. This suggests that OT not only induces uterine contractions but also plays a role in triggering the onset of labour by mediating the proinflammatory effects in the amnion.

These proinflammatory effects of OT were suppressed by an OTR-specific antagonist, ornithine vasotocin (OVT), indicating that OT exerts its effects predominantly via OTR. However, the commonly used OTR antagonist, atosiban, had no effect on OT induced proinflammatory effects. Unexpectedly, atosiban treatment alone resulted in activation of inflammatory mediators such as MAPKs and NF-κB leading to downstream pro-labour gene expressions via Gαi. Activation of such inflammatory processes within the uterus initiates labor, whereas exposure to inflammation may be associated with fetal brain damage in preterm and term infants. Therefore, atosiban could exacerbate inflammation in the context of preterm birth and potentially have an effect on neonatal outcome. With this in mind the future development of OTR antagonists to prevent preterm birth will need to take into account the effects upon differential OTR G-protein coupling.
Statement of originality

All work presented in this thesis was performed by myself unless stated otherwise in the text.

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<tr>
<td>3'-UTR</td>
<td>3'-untranslated region</td>
</tr>
<tr>
<td>AA</td>
<td>Arachionic acid</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>AVPR1A</td>
<td>Arginine vasopressin receptor 1A</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor of the TNF family</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer-binding proteins</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CAP</td>
<td>Contraction associated proteins</td>
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<tr>
<td>CBP</td>
<td>CRE-binding protein</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<td>CHO cells</td>
<td>Chinese hamster ovary cell</td>
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<td>CK-2</td>
<td>p38-activated casein kinase II</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>CRH-R</td>
<td>Corticotrophin-releasing hormone receptor</td>
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<tr>
<td>CYP17</td>
<td>17α-hydroxylase/17,20-lyase</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles’ Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Electrochemical luminescence</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
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<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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<td>ERE</td>
<td>Estrogen response element</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>Go</td>
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<td>G-protein coupled receptor</td>
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<tr>
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<td>Histone acetyltransferase</td>
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<td>IκB kinase</td>
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<td>L-</td>
<td>Pre-labour</td>
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<tr>
<td>L+</td>
<td>Post-labour</td>
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<tr>
<td>LG</td>
<td>L-glutamine</td>
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<td>LiCl</td>
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<td>LPS</td>
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<td>Mitogen activated protein kinase</td>
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<td>MgCl2</td>
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<td>MLCK</td>
<td>Myosin light chain kinase</td>
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<td>MMP</td>
<td>Metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NES</td>
<td>Nuclear localisation sequence</td>
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<td>Nuclear export sequence</td>
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<td>NO</td>
<td>Nitrogen oxide</td>
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<td>NS</td>
<td>Non-stimulated</td>
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<td>OT</td>
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<td>Oxytocin receptor antagonist</td>
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<td>OVT</td>
<td>Ornithine vasotosin</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride</td>
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<td>PPROM</td>
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<td>PR</td>
<td>Progesterone receptor</td>
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<td>Abbreviation</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SON</td>
<td>Supraoptic</td>
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<td>SP</td>
<td>SP600125</td>
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<td>SP-A</td>
<td>Surfactant protein-A</td>
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<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TNFα</td>
<td>Tumour necrosis factor α</td>
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<td>U</td>
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<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
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1. Introduction
1.1. Pregnancy and parturition

1.1.1. Normal labour: phases of parturition

Human parturition is a complex sequence of events that involves four different physiologic phases [1]. For most of human pregnancy the myometrium remains in a state of functional quiescence, allowing development of the fetus. During this first parturitional phase putative inhibitors, such as progesterone, prostacyclin, relaxin, nitric oxide, parathyroid hormone-related peptide, calcitonin gene-related peptide, adrenomedullin, and vasoactive intestinal peptide, keeps the uterus in a quiescent state [2]. The mechanisms through which these agents act vary but in general, they lead to increased intracellular cyclic adenosine monophosphate (cAMP) levels resulting in a decrease in calcium release and reduction of myosin light chain kinase activity, which is required for contraction of myofilaments. At the end of the first trimester, progesterone production is taken over by the placenta and there is luteal regression. In many species, labour is initiated by progesterone withdrawal although this does not occur in the human [3]. Pharmacological inhibition of progesterone action however, increases myometrial contractility and causes cervical ripening [4, 5].

Towards the end of pregnancy, there is a relatively gradual change from myometrial quiescence to a more contractile state. The transformation of the uterine muscle from quiescent to contractile occurs during the second ‘activation’ phase in response to uterotropins and is thought to involve increased synthesis of contraction-associated proteins (CAP) including proteins encoding ion channels, connexin-43, prostaglandins (PG) and oxytocin receptors (OTR) [6, 7]. Activation prepares the uterus for labour and alters it from a relatively insensitive organ to a sensitive organ, primed for stimulated contractions. It is possible that there are two phases of upregulation of pro-labour proteins. Proteins such as OTR, PG receptors and gap junctions are upregulated during the third trimester whilst inflammatory-type mediators are upregulated in the 2-3 weeks prior to labour.

In the third ‘stimulation’ phase of parturition, the uterus can be stimulated by uterotropins including PGs, CRH and oxytocin (OT). It is accepted that the nonapeptide hormone oxytocin plays a key role in the stimulation process, and there is growing evidence for its contribution to the activation process as well. The biochemical events within the uterus resemble an inflammatory reaction [8]. Labour is associated with increased synthesis of cytokines and PGs and an influx of neutrophils into the uterus. Chemotactic cytokines, such as IL-8, attract protease secreting neutrophils into the fetal membranes and cervix. This leads to remodelling and ripening. Prostaglandins have been used to drive cervical ripening to induce labour. PGE₂ is able to promote cervical changes that resemble a normal physiologic ripening seen prior to labour. It is thought to involve stimulation of collagenolytic activity in cervical tissue [9]. This leads to relaxation of the lower segment of the uterus and stimulate fundally dominant contractions.
The fourth ‘involution’ phase of parturition includes the uterine involution that follows the delivery of the fetus and the placenta, which has been found to be primarily mediated by oxytocin.

1.1.2. Preterm labour

Preterm birth refers to delivery that occurs at gestational age less than 37 weeks. It occurs in 9% in high-income countries compared to 15% in the poorest countries [10]. Despite the advancing knowledge of the risk factors and mechanisms involved in preterm labour and increasing public health and medical interventions, there has been a marked rise in preterm birth rate in highly industrialised countries [11]. Preterm labour (PTL) is one of the main causes of perinatal morbidity and mortality. It accounts for 75% of perinatal mortality and more than half of morbidity (both short-term and long-term), including cerebral palsy, deafness, blindness and chronic lung disease [12]. However, most of neonatal disorders and deaths are accounted for by births before 32 weeks of gestation [13]. Preterm birth can be categorised into spontaneous preterm labour, spontaneous rupture of the membranes, and delivery for maternal or fetal indications [14], where approximately 30-35% of preterm labour are indicated, 40-45% with spontaneous preterm labour and 25-30% with preterm premature rupture of membranes (PPROM) [15].

1.1.2.1. Risk factors of preterm labour and methods of prediction

Predicting preterm labour takes various factors into consideration including risk factors, fetal fibronectin, cervical length, bacterial vaginosis, and presence of other biomarkers such as cytokines, chemokines, and oestriol [15, 16]. Risk factor scoring using standardised questionnaire has been utilised to identify women at risk of preterm birth and gauge the intensity of their prenatal care [17-19]. The use of risk factors for prediction of preterm labour has great potential as early identification of risk can lead to risk-specific treatments [20]. Risk scoring system has been successful at identifying women at higher risk of preterm labour on the basis of patient’s previous history of preterm labour. It has been estimated that the recurrence risk of preterm labour increases by 15-50% in women with previous preterm delivery depending on the gestational age of the delivery [15, 21]. There is an inverse correlation between the number of gestational age of the previous preterm birth and the risk of another preterm birth. Other maternal risk factors include low and high maternal age, race, and socioeconomic status. Black women have 2-3 fold higher risk of early preterm birth compared to women from other racial groups [22]. Low socioeconomic status and educational status have been shown to be associated with preterm birth [23, 24], but the mechanisms involved are unknown. In addition, there are evidences that suggest gene polymorphism associated with increased risk of preterm labour. It has been reported that maternal carriage of the rare tumour necrosis factor (TNF)-2 allele leads to approximately 2 fold increase in the risk of spontaneous preterm delivery with the mean gestational age at delivery of 32.9 weeks for cases compared to 39.3 weeks in control subjects [25].
Another risk factor is multiple pregnancies. In the case of twins, approximately 60% are born preterm, with 40% due to spontaneous labour or PPROM at gestational age less than 37 weeks and others due to maternal or fetal disorders. The relative risk for preterm birth is substantially higher for triplets than for twins as almost 90% of triplets are born preterm [26]. The underlying mechanism for this increase is preterm risk is not always clear, but uterine over-distension resulting in contractions and PPROM is one of the suggested causative mechanisms [27]. Bleeding in early pregnancy, specifically in second and third trimesters, has been associated with increase in spontaneous preterm delivery [28]. Smoking is also related to preterm birth as previous studies have demonstrated that tobacco use increases the risk of preterm birth by about 2-fold [29]. However, these risk factors have variable sensitivities and predictive values and thus the use of risk scoring system has limited success in reducing preterm birth [19].

Intrauterine infection is the most important mechanism leading to preterm birth and it accounts for 25-40% of all preterm births [30]. Systemic or intrauterine infection in several different pregnant animal models has been shown to result in preterm labour [31-33], which provides evidence supporting the role of infection in preterm labour. The mechanisms through which intrauterine infection leads to preterm labour resemble that of the innate immune response [27]. Microorganisms can gain access via multiple routes including accidental introduction during an invasive procedure, spreading through fallopian tubes, ascending from the vagina or cervix, and dissemination via haematogenous route through the placenta [30]. They are then detected by pattern-recognition receptors such as TLRs, which trigger a signalling cascade resulting in release of inflammatory cytokines and chemokines [15]. These in turn lead to PG synthesis and matrix-degrading enzymes to stimulate uterine contractions and PPROM [27].

Bacterial vaginosis has been associated with up to 3-fold increase in the risk of preterm birth [34]. It is a disorder characterized by changes in vaginal microbiome. Presence of clue organisms such as mobiluncus and bacteroides, high vaginal pH, and profuse white discharge are used for diagnosis [35]. There is a significantly higher incidence of bacterial vaginosis in black women than white women, this may contribute to the higher risk of preterm labour in black women [36].

Biological fluids have been used to predict preterm birth. There have been numerous studies to assess the value of biomarkers, however, only few have shown clinical usefulness [20]. One of the most powerful biochemical predictor/biomarker of preterm birth is fetal fibronectin. It is glycoproteins produced by the fetal membranes to maintain the attachment of placenta and membranes to uterine deciduas [37]. Presence of fetal fibronectin in cervicovaginal fluid of women undergoing routine screening at 24-26 weeks of gestation is normally considered an indication that they are at substantially higher risk of preterm delivery [38]. Increase in fetal fibronectin from under 40 ng/ml to 100 ng/ml and over has been associated with a progressive increase in the risk of preterm birth [38].
Meta-analysis confirmed that the fetal fibronectin screening has high negative predictive value, meaning that there is a strong correlation between absence of fetal fibronectin and lower risk of preterm labour [39].

Prior to the onset of labour, the cervix undergoes remodelling where it shortens, softens and dilates, which occurs in both term and preterm parturition [27]. In 1965, it was first reported that a short cervix is a risk factor for preterm labour and delivery [40] and since then there has been numerous studies confirming this finding [41-43]. Shortening of cervix is one of the earliest indicators of labour onset. Cervical length of less than 25 mm after 24 weeks of gestation increases the relative risk of preterm labour [44], and thus routine cervical length assessment is considered to be useful for prediction of preterm labour in high risk pregnancies. The combination of fetal fibronectin and cervical length assessment is thought to provide better predictive values [45].

1.1.2.2. Preventing preterm labour

Management of preterm birth has been developed target PTL before the onset of labour as well as established PTL. Preventing preterm labour before labour onset involves enhanced prenatal care in combination with nutritional interventions and patient education, screening and treatment of infections, progestin supplementation, and cervical cerclage [11].

There are contrasting evidences on the role of enhanced prenatal care in preventing preterm labour. Increasing the availability and frequency of prenatal care generally failed to reduce preterm births, however, few reported that enhancement of prenatal care by adding patient education and maternity care coordination result in lower premature labour and improved fetal outcome [46, 47]. Despite such promising results from initial observational studies, randomized, controlled trials showed that enhanced prenatal care is not effective in preventing preterm birth [48, 49]. These differences may be due to the variance in the enhancements to prenatal care, patient risk characteristics, and rate of hospital admission for preterm labour. With such inconsistencies in different reports, it is difficult to prove the effectiveness of prenatal care in preventing preterm labour.

As previously mentioned, infection has been shown to be strongly associated with preterm birth. Screening and treatment of intrauterine infections during early pregnancy has been used as preventative measures. There are several lines of evidence supporting the use of antibiotics in preterm birth. In the case of bacteriuria, analysis of randomized clinical trials showed that the antibiotic treatment led to significantly reduction in preterm delivery as well as occurrence of low birth weight [50]. Randomised controlled trials suggest that the use of antibiotics in bacterial vaginosis and abnormal vaginal flora resulted in a decrease in preterm birth [51]. However, administration of broad-spectrum antibiotics had conflicting results. A broad-spectrum antibiotic, erythromycin, had beneficial effects in women with bacterial vaginosis as well as PPROM [52, 53], but co-amoxiclav was found to
be associated with neonatal morbidity [53]. There are studies showing that antibiotics for bacterial vaginosis increase the risk of PTL. In recurring bacterial vaginosis, administration of intravaginal clindamycin has been shown to increase the rate of PTL which may be due to the selectivity of the antibiotics enabling virulent growth of *E. coli* and bother bacterial known to be associated with PTL [54]. The conflicting results on the use of antibiotics to prevent preterm birth are mainly due to the wide variety of antibiotics available. This would be particularly important finding among black women, who have higher incidences of bacterial vaginosis than women for other ethnicity.

Progesterone decline has been shown to precede the onset of labour. Thus a number of studies were designed to evaluate the effect of progestin supplementation in the prevention of preterm birth. The use of 17 alpha-hydroxyprogesterone caproate has been shown to significantly reduce the occurrence and recurrence of preterm birth [55, 56]. Progesterone has also been implicated in reducing the risk of spontaneous early preterm birth in women with short cervix [57]. In randomised trial, intravaginal progesterone administration to women with a sonographic short cervix led to 45% reduction in the rate of PTL before 33 weeks, 38% reduction in PTL at < 35 weeks and 50% reduction in PTL < 28 weeks [58, 59]. but not in women with a history-induced cerclage [60]. However, administration of 17 alpha-hydroxyprogesterone caproate seems less effective in prevention of prematurity in multiple pregnancy [61] and established preterm labour. The progestin supplement regime requires weekly injections and as the improvements were variable, it is no longer used regularly.

Cervical cerclage has been a traditional treatment for an incompetent or structurally weak cervix [11]. Several studies have demonstrated a clear association between short cervix and the risk of PTL. Placement of cervical suture was aimed to correct the weakness that occurs in the mechanisms that are required to retain the conceptus in the uterus [62]. Emergency cerclage has been reported to prolong pregnancy by 4-5 weeks (ranging from 1 day-18 weeks) [63], and history- or ultrasound-indicated cervical cerclage appears to have lower complication rates [64]. Highest efficacy of cervical cerclage reducing preterm birth was observed with a multifactorial approach, where cervical cerclage was used in combination with antibiotics, bed rest, and other tocolytics such as prostaglandin synthesis inhibitor, indomethacin [65, 66]. There are reports of contradictory conclusions which illustrated that placement of a cerclage is ineffective in reducing the rate of spontaneous preterm birth. Randomised controlled trials showed the insertion of Shirodkar suture in women with a short cervix at 22-24 weeks of gestation or McDonal cerclage of ultrasonographic short cervix does not reduce prematurity nor improve perinatal outcomes [67, 68].

Various tocolytic drugs for treatment of established preterm labour have been developed. Use of tocolytics aim to prevent preterm labour for women at risk of preterm birth and may postpone delivery long enough to improve neonatal outcome. Previous randomized trial data suggest that tocolytics can delay labour for up to 48 hours [69, 70]. This allows time for antenatal corticosteroid
administration for fetal lung maturity, which significantly reduces neonatal respiratory distress syndrome, intraventricular hemorrhage, and mortality [71]. It allows in utero transfer to a hospital that has facilities to provide neonatal intensive care which can potentially improve neonatal outcomes [72]. Tocolytics are also used in cases of uterine hyperstimulation, resulting in fetal distress and can facilitate external cephalic version at term. However, if aiming to prolong pregnancy for over a week, tocolytics have limited efficacy.

Currently available tocolytic drugs target preterm labour in a reactive way when uterine contractility and preterm labour have already started. The classes of drugs used as tocolytics include nitric oxide donors (glycerol trinitrate) [73], calcium channel blockers (such as nifedipine) [74], beta-mimetics (such as terbutaline) [75], magnesium sulphate [76], cyclo-oxygenase inhibitors [77] and oxytocin receptor antagonists [78]. Figure 1.1 shows their mechanism of action.

![Figure 1.1. Mechanisms of action by tocolytics. (modified from [79])](image)

Nitric oxide (NO) is produced during oxidation of amino acids catalysed by NO synthase. It acts as a strong vasodilator and has been suggested to be associated with cyclic guanosine monophosphate (cGMP) generation and uterine relaxation [80]. The ability of NO to suppress uterine contractility was more predominant in non-labouring tissues in comparison to labouring tissues, indicating that NO is
most potent during pregnancy and it loses its inhibitory effect with the onset of labour to enable uterine contractions [80]. Nitroglycerine is a typical nitric oxide donor, which has been used to relax the uterus. In the meta-analysis of randomised controlled trials, nitroglycerine had no effect at delaying labour or improving fetal outcome [73]. NO donors are not used in clinic despite its ability to reduce prematurity, due to significantly higher risk of side effects compared to other tocolytics [79].

Calcium-channel blockers are agents that interfere with the transfer of calcium ions in cell membranes. They are non-specific smooth muscle cell relaxants commonly used to treat hypertension. In myometrial cells, calcium-channel blockers decrease intracellular free calcium concentration by preventing influx of calcium ions and drive myometrial relaxation [81]. Human myometrium in vitro studies showed great promise in the use of calcium-channel blockers to manage preterm labour [82]. The most widely used calcium-channel blocker is nifedipine. A meta-analysis of randomised trials reported that nifedipine reduced the number of deliveries for 7 days and reduced neonatal morbidity [74]. Since then, there has been growing evidence supporting the efficacy of nifedipine in the management of preterm labour, which suggested that it is a promising tocolytic agent with easy route of administration and fewer side effects [83]. However, it is to be used with caution in patients with cardiovascular conditions as it may increase the risk of pulmonary oedema and cardiac failure [84].

Tocolytic betamimetics have been used routinely in clinical practice since 1980s [79]. They are used to activate adenyl cyclase to increase cAMP production. This increase in cAMP leads to a reduction of intracellular calcium and decrease in activity as well as phosphorylation of myosin light-chain kinase, thus inducing uterine relaxation [85]. Despite the initial studies reporting the efficacy of betamimetics in delaying preterm birth for two days, there were significant maternal side effects [75, 84]. Their use has been replaced by oxytocin receptor antagonists and nifedipine as they have similar efficacy with significantly less maternal side effects.

Magnesium sulphate has been reported to have relaxant effect in human uterine contractility. It is considered to be a calcium antagonist as it reduces intracellular calcium levels and the process of contraction [81]. However, a meta-analysis reported that it had no beneficial effect on preterm labour and its use in higher doses was associated with increased infant mortality [76]. There is lack of evidence to recommend this drug as a first-line tocolytic agent, but there is growing evidence that suggests magnesium sulphate provides fetal neuroprotection. This was first reported by Nelson et al. where they found that administration of magnesium sulphate protected premature infants against cerebral palsy [86]. Magnesium can reduce threats to preterm brain as it can reduce vascular instability and lessens hypoxic damage whilst providing protection against cytokine or excitatory amino acide damage [87]. Randomised, placebo-controlled trials followed to support the use of magnesium sulphate to lower the risk of cerebral palsy in infants [88-90].
Prostaglandins have been consistently reported to play a central role in the onset of labour. Prostaglandin-synthase inhibitors were designed to block the production of PGs by interfering with the activity of cyclooxygenase (COX) isoforms. The most widely used COX inhibitor is indomethacin, which is a non-specific COX inhibitor that has been reported to reduce birth before 37 weeks of gestation and increase gestational age compared to both placebo and other tocolytics [77]. However, its use is restricted due to its potential adverse effects on the fetus as well as the mother. The use of indomethacin has a potential risk of neonatal necrotising enterocolitis, premature closure of the ductus arteriosus and decrease in urine production [27, 77, 84]. Collectively, these studies indicate that indomethacin is an effective tocolytic drug for short-term effect in pregnancies below 32 weeks. Specific COX-2 inhibitors such as nimesulide or rofecoxib have been developed but are not recommended for use in clinical practice due to their side effects [91]. Rofecoxib has been shown to be associated with increased rate of PPROM as well as the risk of preterm delivery [92].

Oxtocin receptor antagonists (OTR-A) are another type of tocolytic agent currently available. The only OTR-A used in clinical practice is atosiban, which has been reported to have controversial efficacy. However, alternative OTR-A are still under development. More details about this particular type of tocolytic agent will be discussed further later on in this chapter.
1.2. Anatomy of tissues in pregnancy and labour

The gestational tissues are made up of tissues from both maternal and fetal origin. The maternal tissues consist of the uterus (myometrium, cervix and decidua), and fetal tissues consist of fetal membranes (amnion and chorion) and placenta (Fig. 1.2). The interactions between these tissues are important in maintenance of pregnancy as well as the onset of labour.

Figure 1.2. The gestational tissues of the human uterus. The gross anatomy of the intrauterine tissues comprised of the uterus, fetal membranes, fetus, and cervix (Modified from [93]).
1.2.1. The uterus

The uterus is a muscular organ which is capable of accommodating a growing fetus throughout pregnancy by hypertrophy and stretch. It then provides a contractile force towards the end of gestation to aid the expulsion of the fetus. There are three main parts of the uterus; the myometrium, the cervix and the decidua [94].

The myometrium is the major muscular layer of the uterus, predominantly composed of bundles of smooth muscle fibres connected by collagen fibres, which are called fasciulata. The seemingly random arrangements of these fibres provide the driving force behind the waves of contractions in every direction during labour, thus referred to as the engine room of parturition [95]. Therefore the processes involved in the onset of labour ultimately result in activation of this tissue. The myometrium can be divided into three topographically separate segments; the fundus (top), corpus (upper) and isthmal (lower) segments. At the time of active labour, the contractions begin at the fundal region travel downwards towards the lower, isthmal segment in a wave-like motion [96]. The spatial difference in this contractile activity is associated with the significant differences in gene expression between the fundal and isthmal segments [97]. There are increases in procontractile gene expression (such as oxytocin receptor and connexin-43) [98], whereas the lower segments has greater expression of corticotrophin-releasing hormone receptor R1 to aid relaxation of the uterus [99], and COX-1 and -2 to promote PG synthesis that subsequently results in cervical ripening [98].

The cervix is the lower cylindrical portion of the uterus which consists of collagen fibre, connective tissue and blood vessels. During most of pregnancy, the cervix remains rigid and tightly closed [100], whereas towards the time of labour, it undergoes a major remodelling process called cervical ripening. The ripening of the cervix occurs in both term and preterm parturition. It occurs concurrently with the activation of the myometrium, and it resembles a proinflammatory process with infiltration of macrophages and neutrophils [101]. This leads to cytokine release as well as production of enzymes necessary for cervical ripening. Collagenases, elastases, and matrix metalloproteinases release result in the breakdown of the connective tissue and collagen fibres, ultimately enabling the softening and shortening of the cervix prior to the passage of the fetus [102].

The outer layer of the uterus is referred to as the endometrium. During pregnancy, endocrine events driven by progesterone and other stimuli transform the endometrium to form the maternal component of the placenta, the decidua. It acts as an interface to the embryo, enabling the exchange of nutrients, gas and waste. Moreover, it is also considered to regulate the maternal-fetal immune-tolerance during pregnancy [103]. The decidua itself can be divided into three regional layers defined by their role in the implantation of the embryo. The region closest to the site of placentation, interacting with the trophoblast is called decidua basalis. The decidua capsularis is a layer that forms around the growing embryo within the uterine cavity, enclosing it to the endometrium. The third layer is called decidua
parietalis, which forms a lining inside the whole of uterine cavity. The decidua consists of a distinct population of leukocytes and specialised lymphocytes (uterine natural killer cells) that leads to production of cytokines and growth factors for angiogenesis, vascular stability and placental growth [104].

1.2.2. Fetal membranes and placenta

Fetal membranes are comprised of the amnion and the chorion (Fig. 1.3), and encapsulate the growing fetus and amniotic fluid. They act as a barrier against ascending infections and play a role in the exchange of nutrients along with the placenta. The amnion is the innermost layer of the fetal membranes, which is in contact with the amniotic fluid and helps provide the tensile strength required to the fetal sac. There are five different layers of amnion. The innermost layer of amnion is made of non-ciliated cuboidal epithelial cells attached to the amniotic basement membrane. The amniotic basement membrane combined with the fibroblast layer surrounded in a mass of reticulin form the bulk of the amnion and represent the strongest structure of the amnion. The innermost layer, amnion mesenchyme, is immediately apposed to the chorion but not fused. The amnion is devoid of muscular, vascular and innervated tissue, thus it contributes to the onset of labour via production of cytokines, chemokines and prostaglandins. The mechanical stretch of amnion is also thought to have a role in the onset of labour [105].

Figure 1.3. Schematic diagram of human fetal membranes. The amnion consists of a single epithelial cell layer and a mesenchyme with basement membrane and fibroblast layer. The chorion consists of reticular layer, basement membrane and trophoblast cells (Modified from [106]).
The chorion forms the outer layer of the fetal membranes. It is comprised of three layers; the trophoblast layer, the chorionic basement membrane and the reticular layer [107]. The trophoblast layer of chorion connects fetal membranes to the decidua via a mass of chorionic trophoblast cells loosely arranged with large intracellular spaces. These cells are attached to the chorionic basement membrane, a dense connective tissue, and then to the reticular layer that forms the main bulk of the chorion. As in the amnion, chorion contributes to the prostaglandin synthesis within the uterus associated with labour.

The placenta is a feto-maternal organ through which the mother supplies the nutrients and oxygen to the fetus [108]. As mentioned above, the maternal component of placenta develops from decidualisation of the endometrium and the fetal component from the chorionic sac. The placenta is a neuroendocrine organ, comprised of lymphoid and non-lymphoid cells and it contributes to the maintenance of gestation and the timing of parturition [109]. It is capable of producing high concentrations of hormones including progesterone, oestrogen, and gonadotrophins, which play significant roles in the maintenance of pregnancy [110]. The placenta also mediates the production of corticotrophin-releasing hormone (CRH) with advancing gestational age [111] and there is increasing evidence supporting the role of CRH in regulating the time of labour onset [112].
1.3. Inflammation – a hallmark of parturition

The processes involved in the onset of labour are not yet defined, however, there is increasing evidence supporting the significance of inflammation in both term and preterm parturition [113-115]. During the onset of labour, there is a global increase in a number of proinflammatory factors including prostaglandins, cytokines and manganese superoxide dismutase [116-118]. Previous microarray and suppression subtractive hybridisation studies demonstrated that this global increase is not only confined to the myometrium [119, 120] but it is also seen in the uterine cervix [121] and the fetal membranes [122].

Parturition has been typically associated with elevated levels of interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNFα) in amnion, myometrium, and choriodecidua [123, 124] and downregulation of anti-inflammatory cytokines such as IL-10 in placenta [125]. This may be due to the influx of inflammatory cells such as neutrophils, macrophages and T-lymphocytes into the labouring uterine tissues [114], which results in increase in production of proinflammatory cytokines. The role of IL-1 and TNFα in the onset of labour has been studied extensively. In several animal models, the levels of IL-1β has been shown to increase with advancing gestation [126] and systemic administration of IL-1β can drive both term and spontaneous preterm delivery, which is can be reversed with the use of IL-1β antagonist [127, 128]. The proinflammatory cytokines also trigger the activation of secondary mediators of inflammation such as prostaglandins and IL-8, which itself can contribute to the onset of labour. Other than promoting uterine activation during the onset of labour, proinflammatory cytokines may also promote inhibition of signalling pathways that are involved in maintaining uterine relaxation. For example, TNFα has been shown to inhibit cAMP production via G_αs expression and inhibit the relaxation of myometrium during pregnancy [129].

In addition, inflammation has been strongly implicated in the infection-driven preterm labour [130]. As discussed before, infection is a frequent cause of preterm delivery. Insults such as exposure to microorganisms trigger inflammation, which leads to delivery of macrophages, neutrophils and lymphocytes, similar to that in normal term labour. Recent findings demonstrated that a key signal for the initiation of uterine inflammatory response at term may be the increase in surfactant lipids and proteins released by the maturing fetal lung [131]. In mice, they have shown an increase in surfactant protein-A (SP-A) release from fetal lung and this was found to drive the influx of macrophages into the amniotic fluid. Subsequently the macrophages produce inflammatory cytokines, which migrate to the uterus to stimulate uterine contractions [132]. The findings from various studies over the last two decades have given rise to the notion that parturition is an inflammatory process.
1.3.1. Triggers of labour

1.3.1.1. Corticotrophin-releasing hormone (CRH)

CRH has been reported to play a role in the process of labour. CRH is a peptide hormone produced in the hypothalamus but also found in intrauterine tissues including placenta, chorio-decidua and amnion. In domestic ruminants such as sheep and cows, CRH is produced by the fetal hypothalamus to act as a primary factor in the onset of labour [133, 134]. This involves activation of fetal hypothalamic-pituitary-adrenal (HPA) axis during term, which leads to increased adrenal cortisol synthesis. The fetal cortisol then regulates the placental 17α-hydroxylase/17,20-lyase (CYP17) activity and catalyze the metabolism of pregnenolone to 17β-estradiol [135]. Increase in oestrogen alters the oestrogen and progesterone ratio at term, and promotes uterine prostaglandin production and labour onset. However, this was not observed in human parturition as the placenta lack the CYP17 enzyme [136]. Despite the fact the fetal-derived CRH are not associated with human labour, there were hypotheses proposed for the role of CRH in labour onset by acting as a ‘placental clock’. This was mainly due to the findings showing increases in placental CRH production and maternal CRH levels throughout advancing pregnancy, peaking at term [137].

The precise roles of CRH during pregnancy still remain undefined. However, the presence of specific CRH receptors (CRH-Rs) in various intrauterine tissues such as the myometrium, deciduas, and placenta has led to the speculation that CRH may directly target these tissues to exert important functions in the onset of labour [138-140]. CRH has been shown to result in different physiological outcomes depending on the type of receptor it activates [140]. In human myometrium, it has been demonstrated that CRH-R1 increases in expression in the lower segment during labour, but remain unchanged in the fundus [99]. The activation of CRH-R1, predominantly linked to Gαs proteins, lead to upregulation of myometrial intracellular signals such as cAMP, cGMP and NO, which generally believed to act as a relaxant in the myometrium [141, 142]. This ability of CRH to maintain uterine quiescence in pregnant myometrium was confirmed by isometric contractility studies on human myometrial strips [143]. This effect of CRH is suppressed with the onset of labour as there is a significant down-regulation of Gαs protein [144], and OT-induced protein kinase C (PKC) activation inhibits the activity to cAMP-linked CRH-R, which creates a microenvironment for myometrial contractility [145]. The specific activation of CRH-R2 by local CRH and CRH-related peptides such as uroco rtin-1 and -2 in the myometrium has been suggested to contribute to uterine contractility [146]. More recent findings reported that the onset of labour alters the expression of CRH-R1 splice variants in the myometrium, which subsequently impairs CRH-induced cAMP production to enable the induction of uterine contractility [147].

Other than directly contributing the uterine contractility, CRH may also affect uterine activity indirectly by regulating the expression of proinflammatory mediators. Local production of CRH has
been found to participate in promoting the production of prostaglandins by human placenta, deciduas and fetal membranes [148]. This increase in prostaglandins can subsequently contribute to the onset of labour as it has been reported to be involved in various stages of human parturition including cervical ripening and uterine contractions. Moreover, CRH has been suggested to inhibit production of progesterone by placenta trophoblasts [149], which supports the role of CRH in uterine transition from quiescent to contractile state.

1.3.1.2. Mechanical factors

Previous studies have suggested stretch as a factor contributing to the time of labour. Uterine distension in unilaterally pregnant animals is one of the key regulators of labour as it drives expression of CAPs, ultimately resulting in uterine contractions. Experiments in rats have provided evidence that preterm labour might result from mechanical stretch that induces expression of OTR and other genes activated within the pregnant uterus [150]. These changes were achieved with the use of an inert cylinder implanted into an empty horn of a pregnant rat. The expression of genes induced by mechanical stretch, such as CX-43 and CX-26, was not affected in the control, unstretched horns. In sheep, the increases in COX-2 and OTR mRNA were significantly higher in the gravid than the non-gravid horns [151]. Similar to this, studies in human myometrial cells have been shown to increase the expression of IL-8 [152], OTR [153] and prostaglandin synthetic enzyme, COX-2 [154], with mechanical stretch. Stretch also stimulated production of inflammatory cytokines and PGs in both cervical cells [155] and amnion epithelial cells [105, 156]. These data indicate that towards the end of pregnancy, the increase in fetal growth presses down into the lower segment of the uterus leading to stretch in the uterine walls, fetal membranes and the cervix. This subsequently results in the activation of contractile and labour-associated proteins (proinflammatory factors) at the time of labour.

1.3.1.3. Cytokines and chemokines

1.3.1.3.1. Cytokines

Cytokines are soluble mediators of the immune response that play a role in immune regulation, hematopoiesis and inflammation. Cytokines act as key communicators for immune cells and maintaining the delicate balance in their levels is essential for health. Due to the recent findings implicating significant role of cytokines in many chronic diseases, it has become a vital diagnostic, prognostic and therapeutic tool in human diseases [157]. Originally, cytokines were thought to be specific in their function and could be divided into functional classes; lymphocyte growth factors, pro- or anti-inflammatory, and others polarizing the immune response [157, 158]. However, studies using the recombinant molecules demonstrated that some cytokines are multifunctional where one can induce various effects depending on the target cell. Typical examples of multifunctional cytokines include IL-6, IL-1 and TNFα [159]. This multifunctionality can be explained by the ubiquitous expression of specific cytokine receptors, in which case, the cell type can define the effect of the
cytokine. For example, both IL-1 and TNFα have been known to activate transcriptional factors such as NF-κB to induce COX-2 and PGE₂ [160], but in T cells, which lack the expression of COX-2, IL-1 and TNFα demonstrate co-activating properties to drive IL-2 production [161].

Proinflammatory cytokines play a crucial role in both term and preterm parturition. Increase in the expression of cytokines is observed in both myometrium and cervix during labour [114], which is partly due to the influx of leukocytes expressing cytokines. The placenta and fetal membranes are also one of the major sources of a diverse number of cytokines [162]. Inflammatory mediator associated with preterm birth, such as IL-1β [163], IL-6 [164], IL-8 [116] and TNFα [165], have all been shown to increase in the fetal membranes and amniotic fluid during labour [166]. As mentioned above, IL-1β and TNFα work in a coordinated fashion to increase NF-κB activity [167], stimulate arachidonic acid release and upregulate COX-2, resulting in PG production in amnion, chorion, decidua and myometrium. The regulation of arachidonic acid metabolism and PG production in gestational tissues is vital in maintaining pregnancy and the onset of labour in women. Therefore, several studies focused on the prostanoid function, changes in their levels and their rate of production prior to and during labour. In addition, cytokines can directly alter myometrial function by modulating calcium influx, which can increase contractility [168]. IL-1β can also stimulate phosphodiesterase activity to increase cAMP metabolism and contribute to the reduction of the inhibition of myometrial contractility [169]. Furthermore, IL-1β and IL-6 drive OT synthesis in myometrial cells [170] and in turn, can enhance OT-induced contractility of myometrium [171]. Other than uterine contractility, IL-1β stimulates the production of metalloproteinases (MMPs) to drive cervical maturation and fetal membrane rupture [172], thus suggesting that cytokines play a crucial role in parturition by directly acting on myometrial contractility, cervical ripening and fetal membrane remodelling.

1.3.1.3.2. Chemokines

Chemokines are structurally related proteins that regulate the migration of cells to sites of inflammation. Chemokines can be divided into different families according to the relative position of their cysteine residues [173]. The characterizations of different chemokine families were concentrated on two; α- and β-chemokines. The α-chemokines have one amino acid separating the two cysteine residues (CXC), and β-chemokines have two cysteine residues besides each other (CC) [174].

Chemokines and their G-protein-coupled receptors promote trafficking of immune cells to target organs. In early pregnancy decidua, chemokine and chemokine receptors are expressed to orchestrate the complex cellular migration and interaction of immune cells and trophoblasts [175]. Similar to decidua, there is large infiltration of leukocytes, predominantly macrophages and neutrophils, to the cervical stroma [176] as well as myometrium during term and preterm labour [177]. Chemokines, such as CXCL-8, RANTES and IL-10, attract leukocytes to the site of inflammation and there is compelling evidence that the chemokine-driven infiltration of leukocytes contribute to the induction
of local inflammatory reactions leading to cervical ripening, decidual activation and myometrial and fetal membrane activation during labour [178].

1.3.1.4. Prostaglandins and cyclooxygenases

Prostaglandins are bioactive lipids, which belong to the eicosanoid family. They originate from arachidonic acids (AA) to exert their function in both paracrine and autocrine manner. Prostaglandins bind to their specific G-protein coupled receptors to trigger activation of downstream signalling pathways and mediate gene expression.

The process of PG synthesis is shown in Fig. 1.4. There are three major prostaglandin precursors, dihomogamma-linolenic acid, timnodenic acid and arachidonic acid, which comes from desaturation of the essential fatty acid linoleic and alpha-linolenic acid. The most abundant precursor is arachidonic acid and it is normally stored as complex lipids [179]. Phospholipases A$_2$ (PLA$_2$) exists in two different forms; cytoplasmic and secretory forms. These release arachidonic acids from phospholipids, which are then converted into prostaglandin H$_2$ (PGH$_2$) by cyclooxygenases (COX) particularly type 2. The subsequent conversion of PGH$_2$ to PGE$_2$ is mediated by PGES-1 and -2.

The levels of prostaglandin production increase within the uterus, myometrium and the amnion [1, 180, 181]. The single epithelial layer and the mesenchymal layer of amnion are considered to be the major site of PGE$_2$ synthesis [180]. COX-2 is the key rate-limiting enzymes for PGE$_2$ biosynthesis [182]. There are two major types of COX; constitutively expressed COX-1, and inducible COX-2. In mouse models, both *cox-1* and *cox-2* have been shown to be important in murine parturition [183]. Targeted disruption of the *cox-1* gene in mice led to delay in labour as well as neonatal death [184], whilst disruption of *cox-2* resulted in multiple failures in female reproductive processes including ovulation, fertilisation, implantation and decidualisation [185]. However, in infection-induced PTL, it has been suggested that *cox-2*, but not *cox-1*, is involved and that administration of a selective *cox-2* inhibitor can delay labour [186]. The prostaglandin synthesis in fetal membranes is predominantly mediated by COX-2 and previous studies have reported a steady increase in COX activity and mRNA expression throughout pregnancy in amnion epithelium [187]. Multiple studies have shown that COX-2 increases in both in term and preterm amnion epithelium. This increase in COX-2 and PG synthesis is also present in chorion, however, that increases in decidua occurs with advancing gestation [103, 188] and it is thought that increase in amnion-derived prostaglandins precedes the onset of labour and may cross the chorion to the decidua to facilitate cervical ripening, membrane rupture and uterine contractions [1, 189-191]. *In vitro* studies using radioactive PGE$_2$ demonstrated a significant increase in the membrane permeability to PGE$_2$, thus explaining the transfer of prostaglandins from the amnion to chorion and the cervix.
COX-2 expression is tightly regulated to allow rapid regulation of prostaglandin synthesis when required. COX-2 has also been shown to be upregulated by prostaglandins via a positive feedback loop [192]. It is thought to be regulated both transcriptionally [193] and post-transcriptionally [194]. Proinflammatory stimuli such as cytokines and chemokines can trigger COX-2 expression and the promoter region of COX-2 has been shown to contain putative transcriptional factor binding sites for CAAT/enhancer binding protein (C/EBP), NF-κB, and AP-1, which have been demonstrated to be active and induce COX-2 in various cell types [160, 195, 196]. These transcription factors have been shown to work in combination depending on the cell type, stimuli, and the signalling pathways involved in their activation. In human pancreatic cancer cells, basal COX-2 expression was found to be mediated by both CREB and NF-κB [197], whereas in human fibroblasts, TNF-α results in NF-κB binding to COX-2 promoter [198] and phorbol 12-myriate 13-acetate (PMA) induces binding of AP-1 and C/EBPβ [199].

Figure 1.4. Prostaglandin synthetic pathway. Arachidonic acids are mobilised from membrane phospholipids by phospholipase enzymes and cyclooxygenases convert free AA into prostaglandin intermediate, PGH₂. The PGH₂ intermediate is then converted to terminal prostaglandins by specific synthase enzymes.
The expression of COX-2 can also be regulated at a post-transcriptional level but there is lack of information on the protein factors that regulate the post-transcriptional metabolism of COX-2 mRNA. The 3′-untranslated region (3′-UTR) of COX-2 mRNA contains multiple adenylylate- and uridylylate-rich (AU-rich) elements (AREs), which have been implicated in posttranscriptional control of gene expression by altering mRNA stability [194], and typically, AREs mediate the rate of COX-2 mRNA degradation. Previous studies in mammary carcinoma cell line (MDA-MB-231) and human renal mesangial cells reported that stimuli such as serum-withdrawal or angiotensin II (AngII) can lead to recruitment of cytoplasmic HuR to the AREs in proximal 3′-UTR of COX-2 mRNA to increase its post-transcriptional stability [200, 201], thus suggesting that HuR binding to COX-2 is essential for its mRNA stabilisation.

The synthesis of prostaglandins is one of the key steps involved in the onset of human labour [202]. The prostaglandin receptors are present in myometrium and fetal membranes to transform the uterus from quiescent to active state. There are various mechanisms of action PGs utilise to trigger labour. PGs can lead to cervical ripening and dilation, placental separation [203] and fetal membrane rupture by inducing metrix metalloproteinase (MMP) activity [204]. PGs are currently used to induce labour and as mentioned in previous section, inhibitors of PG synthesis such as indomethacin, nimesulide and celecoxib have been implicated in the management of preterm labour. Knocking out cytosolic phospholipase A2 (cPLA2) in mice resulted in the failure to deliver at term [205, 206]. Collectively these data implicate that prostaglandins have a critical role in parturition.
1.4. Nuclear factor-κB (NF-κB) signalling

The nuclear factor-κB was first identified in 1986 as a transcription factor that regulates the expression of κ light-chain gene in murine B lymphocytes [207]. Since then, there have been extensive studies on NF-κB and its role. It has now been established that NF-κB is expressed ubiquitously and acts as a central regulator of multiple cellular processes throughout the tissue. The NF-κB signalling pathway can be activated by various stimuli such as infection and proinflammatory cytokines, which are associated with labour, and drives the expression of a cassette of inflammation-related genes including those that are pro-labour, suggesting that NF-κB may play a pivotal role in parturition.

1.4.1. Subunits of NF-κB

NF-κB is a complex of heterogeneous dimers of various subunits from the Rel/NF-κB family. The Rel/NF-κB family consists of RelA, RelB, c-Rel, p100/p52 and p105/p50, which share a Rel homology domain (RHD). The RHD is important in the process of NF-κB dimerization, DNA binding, nuclear translocation and IκB interaction. Each NF-κB subunit can form a homodimer or heterodimers with one another, except RelB, which does not dimerize with c-Rel and itself. There are a dozen different dimer variants, however, the classical and ubiquitously expressed dimer is the combination of RelA and p50, which form the primary inflammatory mediators [208]. The inactivated NF-κB subunits are present in the cytoplasm as homo- or hetero- dimers and they are tightly regulated by an inhibitory protein from IκB family, which prevents nuclear translocation of NF-κB. This family is comprised of four canonical members; IκBα, IκBβ, IκBε, and Bcl3, that contain multiple ankyrin repeat domains for protein/protein interaction [209]. Moreover, p105 and p100 precursor proteins can act as IκB-like proteins as they contain ankyrin repeat domains.

1.4.2. Different types of NF-κB signalling pathways

There are three distinct types of NF-κB signalling pathways, all of which involve sequential activation of kinases and nuclear translocation of NF-κB dimers (Fig. 1.5). The classical NF-κB pathway is typically triggered by proinflammatory stimuli such as cytokines and lipopolysaccharides (LPS). Following the ligand-specific receptor activation, the downstream signalling activates IκB kinase (IKK) complex. The IKK complex, comprised of IKKa, IKKβ, and NF-κB essential modulator (NEMO), phosphorylates IκBα on serines 32 and 36 [210]. IKKa and IKKβ are structurally similar, but have different functions. IKKa has been shown to phosphorylate IκB in response to morphogenic signals whereas IKKβ acts in response to proinflammatory stimuli and stress [211]. The phosphorylated IκBα undergoes tertiary structure changes, which expose motifs recognised by SCF ubiquitin ligases and becomes a target for ubiquination at lysines 21 and 22 [212]. This results in degradation of IκBα by 26S proteosome and frees NF-κB dimers to enter the nucleus.
The alternative pathway has been shown to be activated by several non-inflammatory stimuli, such as B cell activating factor of the TNF family (BAFF), lymphotoxin $\beta$ (LT$\beta$) and CD40 ligand [213, 214]. The alternative pathway is NEMO- and IKK$\beta$-independent, depending on the stimuli, RelB dimerize with p50 or p52 to drive the signalling pathway. In a simplified model, p100 precursor functions as IκB to maintain RelB/p52 or p52/p52 dimers in the cytoplasm of a resting cell. Receptor activation triggers increase in IKK$\alpha$ dimer activity, which phosphorylates p100. This elicits degradation of p100 to p52, thus releasing RelB/p52 or p52/p52 dimers for transcriptional activity [213, 214]. Notably, the alternative pathway activation is slower in comparison to the classical pathway due to the absence of strong negative feedback mechanism by the typical IκB proteins [208].

The third type of NF-κB signalling pathways is atypical pathway, which is IKK-independent. Stimuli such as DNA damage, hydrogen peroxide or pervanadate can phosphorylate IκB via p38-activated casein kinase II (CK-2) [215].
Figure 1.5. Three distinct NF-κB signalling pathways. NF-κB can be activated by classical, alternative or atypical pathways. In the classical pathway, extracellular signals such as TNFα, IL-1 or Lipopolysaccharide (LPS) enable the receptor to recruit the adaptor protein MyD88, and the kinases IRAK4 and IRAK1. These associate with IKK complex to stimulate activation. Degradation of IκB by phosphorylation and ubiquitination leads to the release of NF-κB p65/p50 heterodimers and nuclear translocation. The atypical pathway triggered by DNA damage results in phosphorylation of IκBα via casein kinase-II (CK2) and tyrosine kinase-dependent pathways. This results in dissociation of IκB from NF-κB. In the alternative pathway, LPS, lymphotoxin-β (LT-β) or CD40 activate IKKα via TRAF3/NIK-dependent pathway which then phosphorylates p100 to generate p52. RelB/p52 heterodimers translocate to the nucleus and regulate expression of target genes that are distinct from those regulated by the classical pathway (modified from [216, 217]).

Although these pathways have been studied extensively, the activation mechanism still remains somewhat unclear. This is mainly due to the fact the NF-κB response to a given stimulus may involve more than one NF-κB pathway. In particular, the classical and alternative pathways occur in a
completely independent way, requiring distinct signalling molecules [214], however, these pathways have been shown to be highly integrated as they can both contribute to the expression of a common set of genes. For example, in dendritic cells LPS-, IL-1β- and TNFα-activated classical NF-κB pathway can lead to late recruitment of RelB and this can subsequently exert redundancy or complete opposite effects [218]. This cross-talk between the different NF-κB pathways often occurs during long-lasting NF-κB activation, and it is more complex than initially thought. In addition, recent studies reported cross-talk between NF-κB and other signalling cascades as several kinases such as mitogen activated protein kinases (MAPKs) and protein kinase C (PKC) can directly activate NF-κB [219]. Such cross-talk can occur at various stages of NF-κB signalling cascade, including at the level of NF-κB subunit modification, coactivator recruitment and the target gene promote, thereby enabling cell type- and stimulus-specific NF-κB responses.

1.4.3. Multiple levels of NF-κB regulation

There are hundreds of substances and physiological conditions known to activate NF-κB and it regulates numerous genes involved in a plethora of cellular processes including cell-to-cell communications, cell survival, proliferation, apoptosis, cell adhesion, inflammation, immune responses and tissue remodelling [208]. Therefore the regulation and the specificity of NF-κB signalling pathway are crucial.

As mentioned before, IκB proteins are essential for NF-κB signal responsiveness as they are important in stabilizing DNA-bound NF-κB dimers and regulating the efficacy and duration of their transcriptional responses, whilst acting as transcriptional coactivators. The typical IκBs (IκBα, IκBβ and IκBε) are involved in the classical signalling of NF-κB. They have conserved serine residues, which get phosphorylated by IKKβ and targeted for ubiquitin ligases ultimately resulting in complete degradation by proteosomes. IκBα has been studied extensively and it is predominantly found associated with p65/p50 heterodimers to mediate the rapid, stimulus-induced response. Traditionally, it is thought that p65/p50/IκBα complex is retained in the cytosol when inactive. However, more recent crystallographic structure analysis studies have revealed that the actual events are more complex. The IκBα was only found to sequester the nuclear localisation sequence (NLS) of p65 but not p50 [220]. With the NLS of p50 and the nuclear export sequence (NES) of IκBα exposed, there is constant shuttling of p65/p50/IκBα complexes whilst the equilibrium appears to be exclusively cytosolic. This equilibrium is only shifted when IκBα is degraded, eliminating the effect of the NES and exposing the NLS of p65. IκBα is also important in the negative feedback regulation of NF-κB response. IκBβ has similar NF-κB binding specificity to IκBα and can completely replace IκBα functionally, but it also has some unique properties. It functions as a chaperone for NF-κB, indicating a possible regulatory function in the nucleus, and it can prolong stimulation by preventing IκBα association [221, 222]. Both IκBβ and IκBε have significantly delayed kinetics compared to IκBα.
when it comes to degradation and re-synthesis in response to NF-κB. The roles of IκBε are less well established, however, its expression and function has been reported to be limited to hematopoietic cell types.

Alternatively, NF-κB precursor proteins such as p100 and p105 can serve the function of IκB proteins due to their carboxy-terminal ankyrin repeats [223]. These precursor proteins undergo distinct processing mechanisms to generate p52 and p50. The precursor protein p105 is constitutively processed to p50, maintaining the p105:p50 ratio constant [224]. In case of p105 associated with NF-κB, it can be degraded completely upon phosphorylation by IKKβ [225]. In contrast to p105, p100 processing is stimulus-dependent and mediates the alternative NF-κB signalling pathway. As shown in Figure 1.5, p100 binds to RelB-containing dimers to maintain their stability. Following phosphorylation of p100 by IKKα, p100 is partially degraded to release active p52/RelB heterodimers. There is also evidence that p100 associates with p65-containing complexes to dampen the p65-dependent responses via a negative feedback loop, thus mediating not only the alternative but also the classical NF-κB signalling pathway [226].

The NF-κB response can be regulated via post-translational modifications of NF-κB subunits. Specific post-translational modifications can lead to changes in coactivator binding, nuclear localisation, and degradation of NF-κB dimers [225]. The most thoroughly investigated post-translational modifications are that of p65 subunit. There are diverse modification sites detected in p65, which can contribute to the fine-tuning of its transcriptional activity. Phosphorylation of p65 at Ser 276 is thought to promote acetylation at Lys310 by histone acetyltransferases (HAT) to facilitate DNA binding and the recruitment of transcriptional coactivators such as p300 and CBP (CREB-binding protein) [227]. Several kinases, including IKKα, have been shown to target Ser536 of p65 subunit to augment transcriptional activity [225]. In contrast to this, inhibitory effects were observed with acetylation of Lys122 and Lys123 in p65 [224]. Moreover, ubiquitination of p65 results in proteosomal degradation, thereby promoting transcriptional silencing [218]. Alternative mechanisms for NF-κB response termination include IKKα-induced acceleration of p65 and c-Rel turnover and removal from proinflammatory gene promoters [228]. There has been tremendous progress in understanding the complex network of multifactorial regulatory mechanisms involved in the NF-κB response, yet still there are areas that remain to be discovered and thus it will continue to attract future investigations.

1.4.4. Role of NF-κB in parturition

The downregulation of NF-κB in T cells of pregnant women throughout the gestation has been shown to be essential for maintaining the balance in the levels of cytokines required for successful pregnancy [229]. The expression of NF-κB is lower in the pregnant myometrium in comparison to non-pregnant controls [230]. Moreover, the activation of NF-κB has been reported to trigger the onset of labour as it
is highly inducible by multiple factors including proinflammatory cytokine/chemokines and infection. In addition, NF-κB regulates the expression of a cassette of genes including proinflammatory cytokines/chemokines, such as IL-1β, IL-6, TNFα, IL-8 and CCL5 [231], therefore creating a positive feedback loop that results in further NF-κB activation. The NF-κB-regulated cassette of genes also includes a number of other prolабour genes; OTR, COX-2 and MMPs (specifically MMP-9, MMP-3 and MMP-2), which can modulate uterine contractility. NF-κB can indirectly interfere with uterine quiescence by affecting the activity of progesterone receptor (PR) [132].

There is compelling evidence that the use of NF-κB antagonists in preterm labour is more effective in delaying labour as well as improving fetal outcomes compared to the currently available tocolytics [232]. Future studies on determining the components of the labour-specific NF-κB signalling, and selective targeting of this pathway may potentially provide new therapies for preterm labour.
1.5. Oxytocin/Oxytocin receptor in human parturition

1.5.1. OT

Oxytocin (OT) is classically considered to have a fundamental role in the mechanism of human labour. In 1906, it was first noted that the posterior pituitary extract had the ability to drive uterine contractions [233]. Subsequently, the active factor was identified to be oxytocin, which had a potent contractile activity in the pregnant uterus (from the Greek oxus, meaning sharp and tokos, referring to childbirth). Thus OT was soon introduced into clinical practice as a uterotonin to stimulate labour within 1940s [234]. By 1953, the biochemical structure of OT was determined [235], and the gene was cloned 30 years later [236].

OT is a nonapeptide hormone (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂) with a disulfide bridge between the two cysteines. As shown in Figure 1.8, the presence of a disulfide bridge results in a six-amino acid cyclic part and a COOH-terminal α-amidated three-residue tail. This structure of OT is very similar to other nonapeptide neurohypophysial hormones. These can be classified into two families; vasopressin and OT families, depending on their amino acid at position 8, where vasopressin family contains a basic amino acid (Lys, Arg) and OT family contains a neutral amino acid. It has been reported that OT and vasopressin genes are on the same chromosome locus in opposite directions and therefore arose as a result of common ancestral gene duplication. However, the variance in these key amino acids can promote differences in the polarity of the peptides, enabling them to bind to their respective receptors [237].

In mammals, it has been established that OT is synthesized in the magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus [238]. The produced peptide is then transported down the axons of the posterior pituitary gland where it is released to modulate its function. Rossoni et al. have demonstrated that some of the OT from the hypothalamus is transported to the dendrites in order to regulate the firing patterns of the oxytocin neuronal network [239]. Moreover, OT has been found to be synthesized locally in humans to contribute to the processes of labour [240].

The role of OT/OTR system in parturition will be discussed further later in this chapter, however, apart from mediating parturition, OT is also important for milk ejection during lactation [241], maternal behaviour, sexual receptivity and partnership bonding [242], thereby facilitating species propagation. OT is also involved in the regulation of a variety of social and non-social behaviours in both animal models and humans. The social behaviours influenced by OT include social recognition, aggression, and sexual, parental behaviours. Study in rats illustrated that OT infusion facilitates social recognition, where this effect was abolished by the use of an oxytocin receptor antagonist [243]. This was subsequently demonstrated in humans as OT was found to influence face recognition at early
stages of perceptual processing as well as previously seen faces [244, 245]. Aggressive behaviour seems to be highly dependent on the species, sexual status and age. There are contrasting evidences in the role of OT in controlling aggression in animal models but recent human studies have suggested that OT may act to decrease aggression by increasing recognition and feelings of affiliation [246], whilst decreasing anxiety. Increase in OT and OTR during pregnancy and nursing has been shown to elicit higher maternal behaviour responses in female rats as OT interact with the dopamine and serotonin systems [247]. Parental behaviour in males is much less known as only small number of species care for their young, however, there are reports of increase in plasma OT levels in expectant male California mice in comparison to virgin males [248]. The effect of increase in OT levels on paternal behaviour is yet to be assessed. In addition, OT is implicated in the regulation of sexual behaviours. In male rodents, OT can bring penile erections and accelerated ejaculation in the presence of testosterone [249] and this can be inhibited by injecting an oxytocin antagonist [250]. Studies in humans reported that there are increases in plasma OT levels during sexual arousal, indicating a role for OT in sexual behaviours [251, 252].

1.5.2. OTR

To date, only one type of oxytocin receptor (OTR) has been identified whereas a very similar nonapeptide, vasopressin, has been shown to have three difference subtypes of receptors. The human OTR gene is present in chromosome 3p25 - 3p26.2, and contains multiple putative binding sites for a variety of transcription factors including c-myb, AP-2, AP-1 and C/EBPβ in the 5’-flanking region [253]. There is an absence of complete estrogen responsive element (ERE) in human OTR, whereas mouse [254] and rat [255] OTR contains classical ERE along with other transcription factor binding sites. However, there are partial palindromic motifs of ERE present at the 5’-flanking region of human OTR gene [253], suggesting a role for estrogen in the regulation of OTR gene expression.

OTR is a 389-amino acid polypeptide with 7 transmembrane domains and belong to the rhodopsin-type (class I) G protein-coupled receptor family (Fig. 1.6). Similar to other GPCRs, activation of OTR drives conformational changes in the relative orientation of the transmembrane domains to enable G protein binding. On the basis of the amino acid sequence, the molecular mass of OTR can be calculated to be ~40-45 kDa. However, previous studies in guinea pig myometrial membranes identified OTR as a 68- to 80-kDa protein [256], and photoaffinity labelling experiements in rat mammary gland and rabbit amnion cells found OTR to be 65 kDa [257, 258]. Such discrepancy in the molecular mass of OTR in various tissues may be due to their differential glycosylation patterns, with each glycosylation core accounting for approximately 10 kDa. The OTR has been found to have two or three putative N-glycosylation sites depending on the species. Human OTR has three potential glycosylation sites as shown in Figure 1.6. Despite the differential glycosylation patterns, OTR
expression, ligand binding affinity and its function are not affected as recombinant deglycosylation mutants of OTR showed unaltered functional properties [259].

As discussed above, vasopressin is a nonapeptide that has a similar structure to OT. Due to the similar cyclic part of the hormones, vasopressin is able to act as a partial agonist on the OTR. It has been reported that OTR has only 10-fold higher affinity for OT in comparison to vasopressin [260]. Postina et al. demonstrated that the ligand selectivity of OTR is dependent on the NH$_2$ terminus and the first and second extracellular loops (shown in red Fig.1.6), with the cyclic part of OT interacting with the second extracellular loop [261].

**Figure 1.6. Schematic structure of the human oxytocin receptor.** The amino acid residues are shown in one-letter code, where the residues conserved between OT and vasopressin families shown in grey and those conserved for the whole GPCR family in black. The N-glycosylation sites are marked “Y” and domains interacting with OT are highlighted in red (modified from [262]).

Persistent stimulation of typical GPCR leads to receptor desensitisation, which can occur at transcriptional, translational and protein levels. The rapid desensitisation of GPCR involves phosphorylation of COOH-terminal tail of the receptor by GPCR kinases (GRKs) and recruitment of
arrestin proteins, subsequently resulting in G protein uncoupling and receptor internalisation, or sequestration. It has been suggested that OTR undergoes similar homologous receptor desensitization process following persistent stimulation [263]. Studies in human embryonic kidney (HEK) 293 cells expressing human OTR demonstrated that OTR internalisation occurs mainly via a clathrin-mediated pathway, with greater than 50% of total receptor loss after 5 minutes of OT stimulation [264]. In this model system, OTR internalisation involved phosphorylation by GRK-2 and facilitate internalisation via dynamin-driven formation of clathrin-coated vesicles. The fate of internalised OTR largely unknown, however, the receptor can be targeted for degradation by lysosomes or recycling to cell membrane. Transcriptional or translational suppression of OTR was shown in human myometrial cells where prolonged exposure to OT resulted in a significant decrease in OTR mRNA level and upto 10-fold reduction in OT binding capacity [265].

1.5.3. Control of OT and OTR expression

The regulation of OT gene expression has been suggested to involve estrogen; both rat and human OT promoter was found to contain a functional estrogen -responsive element (ERE) [266, 267] and increases in estrogen [268] as well as the rate of estrogen production [269] are observed at the time of parturition in humans. Previous study in amnion-chorion-decidua in vitro explants demonstrated that estrogen triggers the upregulation of OT gene expression [240] and increases in plasma OT concentrations in women [270].

Similar to regulation of OT gene expression, sex steroids such as estrogen and progesterone have been shown to be associated with OTR gene expression. Soloff et al. [271] demonstrated an increase in OTR levels in the rat uterus with estrogen stimulation, which can be suppressed by administration of progesterone [272]. Studies using estrogen receptor-α knockout mice failed to demonstrate an increase OTR expression by estrogen as observed in wild-type mice [273], suggesting a nonredundant role for estrogen in the control of OTR expression. Progesterone has been shown to inhibit the role of OTR via both genomic and nongenomic mechanisms as it reduces the uterine OT binding [274] and to downregulation of OTR without affecting its gene expression [275]. Unlike rat and mouse OTR, human OTR promoter does not contain any full consensus estrogen or progesterone response elements. However, it is possible that estrogen can act on the half-palindromic EREs present in the 5′-flanking region of OTR to mediate its expression [276] or involve additional factors that act or bind on alternative functional promoter elements [277].

The human OTR promoter contains several other putative transcription factor-binding sites in its promoter region, specifically for CCAAT/enhancer-binding protein (C/EBP) and NF-κB, which are frequently associated with inflammation. The direct regulatory effects of NF-κB and C/EBP have been demonstrated in previous studies [278, 279] whereas a regulatory role of sex steroids is yet to be understood. Both C/EBP and NF-κB are highly inducible by several inflammatory cytokines such as
IL-1β, IL-6 and TNFα, as well as lipopolysaccharides (LPS)-mediated bacterial infection [280]. Given that increases in these inflammatory cytokines are associated with both term and preterm labour when OTR expression is also upregulated, it has been postulated that C/EBP and NF-κB may mediate labour-associated increase in OTR expression. Previous studies in our group demonstrated that IL-1β increases OTR expression via C/EBP and NF-κB, which work in synergy to dramatically increase OTR promoter activity [281, 282]. Moreover, C/EBP has been found to stimulate c-fos expression [283]. In breast cells, activation of c-fos has been reported to be associated with GABP (GA-binding protein transcription factor) α/β-induced endogenous OTR gene expression [284], however this was not observed in cultured myometrial cells.

As discussed previously, another stimulus that contributes to the induction of OTR gene expression during the onset of labour is stretch. In rats, increase in OTR expression is observed only in the gravid horn of the uterus and the OTR expression in nongravid horn can be induced by stretch using a 3-mm plastic tube [150]. Similarly, Terzidou et al observed increased expression of OTR in stretched myometrial cells [153]. Mechanical stretch resulted in approximately 35% increase in C/EBP binding to OTR promoter which led to 4- to 5-fold increase in the promoter activity. It is hypothesized that the increased stretch in multiple pregnancies can increase uterine sensitivity to OT via OTR expression, which may account for the greater risk of preterm labour in multiple pregnancies.

1.5.4. Role of OT/OTR in parturition

There are several evidences supporting the role of OT in the initiation of parturition. Firstly, OT is one of the strongest uterotonin available and it is routinely administered to pregnant women to stimulate uterine contractions during late gestation. The key prolabour responses induced by OT are indistinguishable to that of normal spontaneous labour. Moreover, there is significant increase in uterine sensitivity to OT, with increase in OTR expression, in both rats and humans during the onset of labour [3, 285, 286]. Last but not least, recent studies in rats [287-289], non-human primates [290-292] and in humans [293, 294] reported that oxytocin antagonists can inhibit late gestational uterine contractions and the process of parturition. However, there are conflicting results on the role of OT in parturition. In rodents, OT is believed to play a significant role in the initiation and maintenance of parturition [285, 295], whereas in humans, circulating OT does not seem to be essential for the initiation and maintenance of labour [241]. Normal parturition has been observed in the absence of OT in mice [241] as well as in cases of clinical pituitary gland dysfunction [296]. There is no good evidence for an increase in maternal or fetal OT concentrations with the onset of, or during labour [297].

A hypothesis proposed to explain the absence of OT increase during labour was that it is not the peripheral OT but a local OT synthesis that initiates labour. As with the principal actions of OT in a paracrine system, any significant changes in the levels of OT may not be reflected in the maternal
circulation. The initial suggestion of OT synthesis in peripheral tissues was made with detection of high OT concentrations in ovine corpus luteal tissues [298] and subsequent studies have demonstrated synthesis of OT mRNA in luteal tissues [299]. In 1993, mRNA of OT was isolated in human uterine tissues in late gestation [240]. A semi-quantification analysis revealed that OT mRNA is principally localised/or identified in deciduas but also found in the chorion and amnion. Interestingly, significantly greater concentrations of OT mRNA were detected in the chorio-decidua after spontaneous onset of labour (at the expulsive stage of parturition) compared to before the onset of labour at term [240, 300, 301]. Post labour chorio-decidua samples showed similar levels of OT mRNA to that of spontaneous labour, indicating the maximal level of gene expression. However, prelabour samples showed substantial variation due to samples being taken at varying intervals before spontaneous labour [240]. These findings demonstrated that localised synthesis of mRNA encoding OT increased around the time of labour onset and may play a role in the physiology of human labour.

Moreover, the uterine sensitivity to OT markedly increases at the onset of labour. This is associated with an upregulation of OTR mRNA levels and a strong increase in the density of myometrial OTRs reaching a peak during early labour [302]. This was first described in rats [285], and subsequently demonstrated in rabbits and humans [3, 303]. In both species, OTRs are present in the endometrium as well as the myometrium, chorio-decidua and amnion [3, 304]. This increase in OTR is one of the most consistent findings in the study of parturition in several species, which introduces the possibility that the changes in the expression or function of the OTR, not only the peptide, are important for the onset of labour. In addition, a more recent gene association study showed that maternal genetic variation such as single nucleotide polymorphisms (SNPs) in OTR coding region may be associated with gestational age-dependent susceptibility to preterm birth [305]. This finding is in line with the hypothesis that OTR has a significant role in the process of labour onset.

1.5.4.1. OT/OTR in pregnant uterus

In general, the OT/OTR system within the pregnant uterus serves two distinct physiological functions, stimulation of contractions and production of PGs. Contraction and relaxation of myometrium are regulated by phosphorylation and de-phosphorylation of the 20kd light chain of myosin. Myosin is the primary protein of smooth muscle thick filaments and is composed of two heavy chain subunits (approximately 200 kd each) and two of each type of light chain subunits (20 and 17kd respectively). At least three distinct components can be discerned in the effect of oxytocin on human uterine smooth muscle; increase in frequency of contractions, initial transient increase in the base tone (incomplete relaxation), and long-lasting increase in the amplitude and duration of phasic contractions.

Activation of OTRs located on smooth muscle cells, such as uterine myometrial cells or mammary gland myoepithelial cells, induce contraction, which is triggered by an increase in intracellular Ca\(^{2+}\) that leads to a calmodulin-mediated activation of myosin light chain kinase (MLCK) [306] (details in
Fig. 1.7). Binding of OT to the OTR activates the heterotrimeric protein complex of a G-protein consisting of α, β and γ subunits [307]. OTR couples to G-proteins of the Gaq/11 subfamily [308], which stimulates phospholipase C, specifically, phospholipase Cβ (PLCβ) isoform. This enzyme hydrolyzes phosphatidylinositol-4, 5-bisphosphate (PIP₂) to inositol-1, 4, 5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ then releases Ca²⁺ from the sarcoplasmic reticulum into the cytoplasm. The increase in intracytoplasmic Ca²⁺ is further attenuated by a Ca²⁺ influx from the extracellular space through membrane Ca²⁺ channels [309]. The DAG generated by the actions of PPLCβ on PIP₂ stimulates protein kinase C (PKC). The mitogen-activated protein kinase (MAPK) system seems to be one of the important substrates for PKC. OT stimulation of cultured human myometrial cells induces activation of MAP kinases through a G-protein-mediated mechanism [310]. This effect is partially Gi dependent and is attenuated by β-adrenergic agonists [311]. Inhibition of MAPK activation does not affect OT-induced intracellular Ca²⁺ mobilisation, but it has been reported that it partly inhibits OT-induced contraction in the pregnant rat uterus [312].

The increase in intracellular Ca²⁺ results in a variety of cellular events. The formation of the Ca²⁺-calmodulin complex triggers activation of neuronal and endothelial isoforms of NO synthase. NO, in turn, stimulates the soluble guanylate cyclise to produce cGMP. In smooth muscle cells, the Ca²⁺-calmodulin system triggers the activation of MLCK activity, which initiates smooth muscle contraction. In neurosecretory cells, rising Ca²⁺ levels control cellular excitability and lead to transmitter release. Further Ca²⁺-promoted processes include gene transcription and protein synthesis.

In addition to regulating smooth muscle contractility by electromechanical coupling, membrane potential independent, pharmacomechanical coupling can also be accounted for the physiological mechanisms that regulate contractions [313]. Activation of OTR in human myometrium can convert inactive RhoA-GDP to its active form, RhoA-GTP through guanine nucleotide exchange factors (GEFs). RhoA-GTP acts via Rho kinase (ROK) to phosphorylate the regulatory subunit of MLC, leading to contractions. There have been records of RhoA and ROK upregulation in human myometrium during pregnancy [314] and ROK has been shown to mediate OT-induced myometrial contractions [315]. Another alternative pathway (and parallel) to induce myometrial contraction may be through activation of the enzyme CPI-17 by protein kinase C.
Figure 1.7. OT/OTR signalling pathways involved in myometrial contraction. Oxytocin binds to OTR and OTR coupled with Go
leads to activation of PLC, which hydrolyses PIP2 to DAG whilst causing release of Ca
from the sarcoplasmic reticulum. Both OTR and DAG activate PKC to initiate MAPK cascade which in turn causes increased cPLA
activity and PG synthesis. OTR also triggers RhoA/ROK cascade and results in increase in MLC kinase activation. Intracytoplasmic Ca
increases further via membrane Ca
channels and these combined with calmodulin activates MLC kinase. The increase in PG synthesis and MLC activation induce myometrial contractions. OT: oxytocin, OTR: oxytocin receptor, PLC: phospholipase, PIP2: phosphatidylinositol 4,5-bisphosphate, DAG: diacylglycerol, PKC: protein kinases type C, MLC: myosin light-chain, MAPK: mitogen-activated protein kinase, cPLA2: cytosolic phospholipase A2, ROK: RhoA associated protein kinase (modified from [316]).

It has been noted that OT stimulates an increase in cytoplasmic phospholipase A2 activity and induces COX-2 expression in Chinese hamster ovary cells [317] as well as in cultured human myometrial cells. These are two major enzymes required for PG synthesis (details in Fig. 1.3). In human myometrium, OTR coupling with Gq leads to activation of PLC and PKC which in turn phosphorylates ERK1/2 MAP kinase and promote PG synthesis.

A well described action of PGs is their role in promoting myometrial contractility. PGF2a induced contractions are partly mediated by electromechanical coupling as well as pharmacomechanical coupling, resembling that of OT-induced contractions [318]. Activation of decidual and OTRs induces PGF2a release, which results in enhanced uterine contractions whilst driving cervical ripening and
luteolysis. Inhibition of PG synthesis decreases the upregulation of OT binding sites as well as the effect of OT on promoting myometrial contractions [189].

1.5.4.2. OT/OTR in human amnion

Labour is preceded by changes in the fetal membranes [189], which are composed of amnion and chorion. The amnion is the innermost translucent structure surrounding the amniotic fluid and the chorion is the outer layer attached to the decidua [319]. Following the degradation of the extracellular matrix [320] and physical separation of the amnion from the choriodecidua, rupture of fetal membranes leads to labour onset [321]. Human labour is associated with increased PG synthesis and inflammatory cytokine release in the uterus [322], and amnion is a major source of inflammatory cytokines and PGs, especially PGE₂ which increases both before and during labour [181, 323].

A previous study in rabbit amnion has shown up-regulation of OTR at the end of pregnancy and OT-stimulated production of PGE₂ [324]. This was also observed in the primary amnion cells as PGE₂ release was increased significantly by 40-fold with OT stimulation [181], thus proposing a role for OT/OTR system in the activation of human amnion at the time of labour.

Increased PG synthesis occurs first in the region overlying the cervix where it is thought to mediate cervical ripening. The mechanism for this regionalization is not yet clear but it has been suggested that stretch through pressure from the fetal head plays a role [105]. Prostaglandin synthesis in the amnion is principally via cyclooxygenase type 2 (COX-2) whose expression is mediated by NF-κB [231], and the activation of NF-κB itself is regulated by inflammatory cytokines such as IL-1β.

There are three distinct NF-κB pathways described to date; classical, alternative and atypical, as mentioned above. The NF-κB signalling pathway relies on series of activated kinases, formation of DNA-binding homo- or hetero-dimers of the Rel family proteins such as p50, p65, c-Rel, p52 and RelB, and inducing transcription of NF-κB target genes. In general, basal NF-κB resides in association with a member of IκB protein family in the cytoplasm of resting cells [216, 325]. However, with the activation of NF-κB via inducers such as IL-1β, NF-κB is able to translocate into the nucleus to regulate expression of target genes [326]. It regulates a group of immune/inflammatory genes including IL-1β, IL-6, IL-8, PTX3 and chemokines such as CCL2, CCL5, CCL20, CCL11 and CXCL2 [231]. Increase in IL-8 has previously been associated with labour [327] which induces chemotaxis of inflammatory cells and trigger acute inflammatory response [328]. Increase in CCL20 synthesis in the uterus has also been associated with both term and preterm labour [329]. It binds specifically to CC-chemokine-receptor 6 to recruit dendritic cells and memory B lymphocytes. These, combined with the effects of other chemokines that are upregulated by NF-κB activity are responsible for the inflammatory infiltration in intrauterine tissues seen with labour [330].
Mechanical stretch of human amnion epithelial cells has been shown to lead to the activation of AP-1 and NF-κB systems and in turn increase COX-2 and PG expression [105]. AP-1 is a proinflammatory transcription factor that contributes to the initiation of the inflammatory response in several disease states [331, 332]. AP-1 binding sites are found in the promoter regions of many pro-inflammatory genes, including cytokines, adhesion molecules and cell proliferation growth factors. The AP-1 family of transcription factors function as either homodimers of the Jun family members or heterodimers of Fos and Jun family proteins. These homo- and hetero- dimers have different DNA-binding and transcriptional activation characteristic. The OTR, COX-2 and Cx-43 genes contain AP-1 binding sites in their promoters and temporal correlation has been demonstrated between the expression of c-fos and Cx-43 in rat myometrium during labour. Stretching of human amnion cells as well as myocytes in vitro can drive PG synthesis, which is one of the main factors driving labour onset. It is hypothesised that the increased stretch generated from a multiple pregnancy could stimulate greater expression of OTR, suggesting that stretch or other local factors can contribute in the regulation of local OTR concentrations.
1.6. Oxytocin/Oxytocin receptor antagonists

1.6.1. Peptide OTR-A

Selective inhibition of OTR has been shown to directly block contractions and decreases PGF$_{2\alpha}$ synthesis in human uterine smooth muscle cells [333, 334]. OTR antagonists may be peptides or non-peptides. Many OTR antagonists have been synthesized by alteration or substitution of various amino acid residues in the oxytocin molecule. This results in a wide variety of compounds with various degrees of antagonistic properties and various degrees of specificity for OTR and vasopressin receptors.

Atosiban is a combined oxytocin/vasopressin receptor antagonist registered for human use as a tocolytic in Europe and other countries. It is an oxytocin analogue (d[D-Tyr(Et)$_2$,Thr$_4$,Orn$_8$]-oxytocin) with modifications at tyrosine in position 2, threonine substitution at position 4 and substituting the basic arginine with less basic ornithine at position 8 [335]. Atosiban has high specificity for the uterus and its affinity to arginine vasopressin receptor 1A (AVPR1A) is approximately 100-fold greater than to oxytocin receptors (4.7 nmol/l and 397 nmol/l respectively) despite the fact that atosiban is often referred to as an ‘oxytocin antagonist’ [293]. The oxytocin antagonistic properties of atosiban rapidly decrease contractions and has been demonstrated to be effective in delaying delivery for at least 48 hours with minimal adverse effects [336]. Its AVPR1A antagonistic properties can add to the tocolytic effects. Clinically, atosiban is safer than beta-mimetics [337] and COX inhibitors [77], as it is associated with fewer maternal adverse drugs reactions requiring cessation of treatment [338]. However, the efficacy of atosiban is debatable as numerous studies have reported lack of reduction in preterm births with atosiban administration. There are several disadvantages of using atosiban as a tocolytic. Due to its limited bioavailability and high affinity to AVPR1A, atosiban requires hospitalization and can cause undesirable side effects such as nausea, headache, dizziness, tachycardia, hypertension, hyperglycemia and allergic reactions [339]. Moreover, there have been concerns about the effects of atosiban on neonatal outcome. In a randomised double blinded trial of OTR antagonists for tocolysis in the management of women in labour between 20 and 36 weeks gestation, atosiban resulted in lower birth weight and an increase in infant deaths at 12 months of age [78].

Limitations of atosiban have led to the discovery of new peptide OT specific antagonists for the management of preterm labour. Barusiban is a peptidyl OTR-specific antagonist (Fig. 1.8) with higher potency and longer duration of action than atosiban. It blocks OT-induced myometrial contractions of preterm and term myometrium in a concentration dependent manner [340]. Despite the promising results in non-human primates, in which barusiban inhibited OT-induced contractions and prolonged pregnancy successfully [341], a recent study in pregnant women did not show satisfactory clinical effectiveness. Although barusiban did not raise any safety concerns with respect to the mother, fetus, neonate, or infant, there was no reduction in uterine contractility or delay of delivery [342].
New generation OT peptide antagonists with significantly high affinity for human OTR receptor are still being evaluated at an experimental level. These include d(CH$_2$)$_5$[Tyr(Me)$_2$,Thr$_4$,Tyr-NH$_2$]OVT, desGly–NH$_2$-d(CH$_2$)$_5$[D-2-Nal$_2$,Thr$_4$,Tyr–NH$_2$]OVT, desGly–NH$_2$-d(CH$_2$)$_5$[D-2-Nal$_2$,Thr$_4$,Tyr–]OVT, and d(CH$_2$)$_5$[2-Nal$_2$,Thr$_4$,Tyr–]OVT as candidates for next generation tocolytic agents [343].

1.6.2. Non-peptide OTR-A

Since peptide antagonists have high affinity to AVPR1A and lack bioavailability, nonpeptide OTR-specific antagonists were developed. Retosiban, also known as GSK221149A (2′-methyl-1′,3′-oxazol-4′-yl morpholine amide derivative, Fig. 1.8), is a nonpeptide oxytocin antagonist developed by a pharmaceutical company which is more than 15-fold more potent at the human OTR compared to atosiban [344]. Administration of retosiban by i.v. and orally decreases both OT-induced and spontaneous uterine contractions in late-term rats in a concentration dependent manner [345]. Retosiban is currently in a Phase II clinical trial for the prevention of preterm labour.

Other nonpeptide oxytocin antagonists include L-368,899 (1-(((7,7-Dimethyl-2(S)-(2(S)-amino-4-(methylsulfonyl) butyramido) bicyclo [2.2.1]-heptan-1(S)-yl)methyl) sulfonyl)-4-(2methylphenyl) piperazine), SSR-126768A (4-Chloro-3-[(3R)-(+)-5-chloro-1-(2,4-dimethoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1H-indol-3-yl]-N-ethyl-N-(3-pyridylmethyl)-benzamide, Hydrochloride), and relcovaptan (SR49059) (Fig. 1.8). L-368,899 was shown to be a potent in pregnant rhesus monkeys by inhibiting spontaneous nocturnal uterine contractions [346], however, due to its brain penetrant properties, it demonstrated a change in maternal and sexual behaviour in non-human monkeys [347]. SSR-126768A acts as a competitive antagonist and oral administration delayed significantly delayed parturition of labouring pregnant rats by over 24 hours [333]. Being a competitive antagonist, its effects were dose-dependent. Unlike the previous nonpeptide OTR antagonists, relcovaptan is an orally active arginine vasopressin/OT antagonist with higher affinity to AVPR1A compared to OTR (7.2 nmol/l and 340 nmol/l respectively) [293]. Relcovaptan was reported to inhibit uterine contractions in vitro as well as in vivo, and demonstrated significant clinical effectiveness in decreasing contractions in preterm labour patients [348]. Relcovaptan has also revealed positive results against Raynaud’s disease, dysmenorrhoea, and ischemic brain damage, however it has not yet been approved for clinical use [349].
Figure 1.8. Structures of oxytocin and oxytocin receptor antagonists [350].
1.7. Hypothesis and aims

There are multiple factors associated with preterm birth, however there has not been a significant reduction in preterm birth due to the poor understanding of the precise mechanisms involved in human parturition. The role of myometrial OT/OTR system in the onset of labour has been studied extensively but recent reports indicate that this is not limited to the myometrium but also seen in amnion. As amnion is not a contractile tissue, the role of OT/OTR will be fundamentally different to that of the myometrium.

The aims of this study are;

- To investigate the changes in the expression of OT and OTR in human amnion during the onset of labour and their effect on the regulation of labour-associated genes such as PG. This will provide new insight into the role of OT/OTR system beyond uterine contractions.
- To elucidate the mechanisms initiated by OT/OTR system to drive inflammation and PG synthesis in human amnion.
- OTR is a GPCR which can activate a complex network of multiple downstream signalling pathways that can be regulated dynamically. We aim to explore the G protein signaling involved in the OT-induced pro-labour effects.
- OTR-A are anticipated to result in the down-regulation of OT-mediated effects. Therefore, it is proposed to determine the efficacy of currently available OTR-As, OVT and atosiban, in the prevention of the OT-induced pro-labour effects.
2. Materials and Methods
2.1. Materials
2.1.1. Chemicals and solvents

Absolute Ethanol
Acrylamide
Agarose
SYBR safe DNA gel stain
Ammonium Persulphate (APS)
β-Mercaptoethanol
Bovine serum albumin (BSA)
Bromophenol Blue
Dimethyl Sulfoxide (DMSO)
Dithiothreitol (DTT)
Glycerol
HEPES
Isopropanol
Methanol
NP-40 Alternative
Non-fat dried milk powder
TEMED
Triton X-100
Tween 20

VWR
Sigma-Aldrich
Invitrogen
Invitrogen
VWR
Sigma-Aldrich
Sigma-Aldrich
Bio-Rad
Sigma-Aldrich
Bio-rad
BDH
Sigma-Aldrich
Fisher Scientific
VWR
Calbiochem
Applichem
Sigma-Aldrich
Sigma-Aldrich
Sigma-Aldrich
## 2.1.2. Antibodies

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2.1.3. Treatments and inhibitors

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2.1.4. siRNA

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2.1.5. Reagents and buffers

**General buffers**

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<td>Tris-EDTA (TE) Buffer</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>pH 8.0</td>
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</tr>
</tbody>
</table>
**Cell culture materials and media**

Dulbecco’s Modified Eagles’ Medium (DMEM)  
DMEM/Nutrient Mixture F-12 HAM  
Penicillin/Streptomycin  
L-glutamine  
Fetal calf serum  
Trypsin/EDTA  
Collagenase X or 1A  
Dispase  
Nuclear Buffer B  
DMEM/F12  
Horse Serum  
EGF  
Hydrocortisone  
Cholera Toxin  
Insulin

Sigma-Aldrich

**Protein extraction buffers**

Cytosolic Buffer A  
10mM HEPES  
10mM KCl  
0.1mM EDTA  
0.1mM EGTA  
2mM DTT  
1% (v/v) NP-40  
Protease inhibitor tablet  
(Sigma-Aldrich)

Nuclear Buffer B  
10mM HEPES  
0.1mM EDTA  
0.1mM EGTA  
2mM DTT  
200mM NaCl
RIPA whole-cell protein extraction buffer

- 1% (v/v) NP-40
- Protease inhibitor tablet
  (Sigma-Aldrich)

- 1% (v/v) Triton X-100
- 1% (w/v) Sodium deoxycholate
- 0.1% (v/v) SDS
- 150mM NaCl
- 10mM Tris, pH 7.4
- 1mM EDTA
- 1mM PMSF
- Protease and phosphatase inhibitor cocktail

**Western Blotting reagents and buffers**

- Pre-stained protein markers
  (Fermentas)
- PVDF membrane
  (Millipore)
- ECL Plus Western blotting reagent
  (Fisher Scientific)
- ECL Western blotting reagent
  (Fisher Scientific)
- Supersignal Western blotting reagent
  (Fisher Scientific)
- ECL plus hyper film
  (Amersham Biosciences)
- Whatman 3MM paper
  (Bio-Rad)
- 30% Acrylamide/Bis
  (Sigma-Aldrich)

- Tris-Glycine running buffer
  - 25mM Tris-base
  - 250mM glycine
  - 0.1% (w/v) SDS
  - pH 8.3

- Transfer Buffer (TB)
  - 39mM glycine
  - 48mM Tris-base
  - 20% (v/v) methanol

- Blocking Buffer
  - 0.01% (v/v) Tween-20
  - 5% (w/v) non-fat milk
Washing Buffer (TBS-T) 0.01% (v/v) Tween-20 in TBS-T

**EMSA reagents and buffers**

- T4 polynucleotide kinase: Promega
- T4 kinase buffer: Promega
- MicroSpin sephadex columns: Amersham Biosciences
- poly(dI-dC): Amersham Biosciences
- $^{32}$P($\gamma$ATP) 9.25 Mbq: Amersham Biosciences
- BioMax MS-1 Kodak film: Sigma-Aldrich
- Sample loading buffer: Promega
- Consensus NF-κB: Promega
- Consensus Oct-1: Promega
- Annealing buffer: 10mM Tris-HCl, pH7.5, 5mM NaCl, 1mM EDTA
- Binding buffer: 20% (v/v) glycerol, 5mM MgCl$_2$, 2mM EDTA, 50mM Tris-HCl, 250mM NaCl, 2mM DTT
- TBE running buffer: 0.9M Tris-Borate, 20mM EDTA, pH8.0


### 2.1.6. Kits

<table>
<thead>
<tr>
<th>Kit</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein assay Kit</td>
<td>Bio-Rad</td>
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<tr>
<td>Human inflammation antibody array 3</td>
<td>RayBio</td>
</tr>
<tr>
<td>PGE(_2) ELISA Kit</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>IL-6 ELISA Kit</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>CCL5 ELISA Kit</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Amaxa Nucleofector Kit</td>
<td>Lonza</td>
</tr>
<tr>
<td>First strand cDNA synthesis</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>SYBR Green master mix</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>QIAquick PCR purification Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>IPOne HTRF assay Kit</td>
<td>CisBio</td>
</tr>
<tr>
<td>Fluo-4 Direct Calcium assay Kit</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
2.2. Methods

2.2.1. Patient selection

Samples were collected from patients who gave informed consent to the use of tissues for research purposes with the approval from the local ethics committee (RREC 2002-6283). Fetal membranes were obtained from two groups of patients. The first group consisted of women undergoing elective caesarean section at term (38°0 - 39°6 weeks of pregnancy), prior to the onset of labour (L-), and the second group after the onset of labour (L+). Women who had their labour induced were excluded in this study as well as those with pre-existing diseases, pre-eclampsia, or multiple pregnancies.

2.2.2. Primary amnion epithelial cell culture

Primary amnion cell cultures were established as previously described [351]. Amnion tissue was separated from the fetal membrane and washed in phosphate buffered saline (PBS) three times before cutting into small strips and incubating in 0.5mM EDTA in PBS for 15 minutes. The strips of amnion were washed twice in fresh PBS and digested in 2.5mg/ml dispase (Gibco) in PBS for 45-50 minutes at 37°C. Then, in order to dissociate the cells, the strips were shaken vigorously in Dulbecco’s Modified Eagles’ Medium (DMEM, Sigma-Aldrich) with 10% fetal calf serum (FCS, Sigma-Aldrich) for 4 minutes. The remaining strips were discarded and the cell suspension was centrifuged at 175 x g for 10 minutes. The pellet of amnion cells were re-suspended in fresh DMEM with 10% FCS, 2mM L-glutamine (LG), and 100U/ml Penicillin/streptomycin (PS), and cultured at 37°C, 5% CO₂.

2.2.3. Primary myometrial smooth muscle cell culture

Term human myometrial biopsies were collected from the lower segment of the uterine incision at the time of elective caesarean section. All specimens were obtained after fully informed and written patient consent with the approval of local ethics committee (RREC 1997-5089). Myometrial tissues were washed three times in PBS and dissected into fine pieces. Tissue samples were then digested in filter-sterilised collagenase solution with 1mg/ml collagenase 1A (Sigma-Aldrich), 1mg/ml collagenase X (Sigma-Aldrich), and 2mg/ml BSA (Sigma-Aldrich) in 50% serum-free DMEM and 50% DMEM/Nutrient Mixture F-12 HAM (Sigma-Aldrich) for 45 minutes at 37°C. DMEM containing 10% FCS was added to the collagenase solution to inactivate the enzymes. The cell suspension was filtered through a cell strainer (70µm) and centrifuged at 3000 rpm for 5 minutes. The pellet was resuspended in DMEM containing 10% FCS, 2mM LG, and 100U/ml PS and seeded into a T25 culture flask (Corning) to grow at 37°C, 5% CO₂. Once the cells reach ~95% confluence, they were washed in PBS and trypsinised in 0.25% trypsin containing 0.02% EDTA in PBS. DMEM containing 10% FCS was added to inactivate the enzyme and the cell suspension was centrifuged and diluted in fresh DMEM containing 10% FCS, 2mM LG and 100U/ml PS to be re-seeded in cell culture flasks or plates. Cells were used between passage numbers 1-4.
2.2.4. MCF-10A mammary epithelial cell culture

MCF-10A cells are common model system for investigating epithelial cell biology. These cells are an immortalized, normal breast epithelial cell line derived from human fibrocystic mammary tissue, which require exogenous growth factors for proliferation. The cells were kindly donated by Professor Bob Brown’s group of Imperial College London. MCF-10A cells were grown in growth media containing 5% (v/v) horse serum, 20ng/ml epidermal growth factor (EGF), 0.5mg/ml hydrocortisone, 100ng/ml cholera toxin, 10µg/ml insulin, and 1% (v/v) PS in DMEM/F12 (Sigma-Aldrich). The cells were passaged every 4 days and were used for experiments before reaching passage 20. Prior to any treatment, MCF-10A cells were serum depleted in DMEM/F12 containing 2% (v/v) horse serum and all the additives except EGF.

2.2.5. Protein extraction

2.2.5.1. Whole cell protein extraction with RIPA buffer

The monolayer of confluent primary amnion epithelial cells was scraped and lysed in modified RIPA buffer containing 1% Triton x 100, 1% Sodium Deoxycholate, 0.1% SDS, 150mM Sodium Chloride, 10mM Tris (pH 7.4) and 1mM EDTA with 1mM of PMSF and protease inhibitor cocktail (Sigma-Aldrich). Whole cell proteins were obtained by centrifuging the lysate at 13,000 x g for 30 minutes at 4°C and extracting the supernatant.

2.2.5.2. Nuclear/Cytosolic protein extraction

Primary amnion epithelial cells were grown to confluence and they were rinsed in PBS, then scraped in buffer containing 10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 2mM DTT and complete protease inhibitor (Sigma-Aldrich). The cells were lysed with addition of 1% NP-40 and cytosolic protein extracts were obtained by centrifugation of the lysate for 30 seconds at maximum speed at 4°C. For nuclear protein extraction, the pellet was re-suspended in a different buffer containing 10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 2mM DTT, 400mM NaCl, 1% NP-40 and complete protease inhibitor tablet (Sigma-Aldrich). The nuclear lysate was then shaken vigorously for 15 minutes at 4°C and centrifuged at maximum speed for 5 minutes.

2.2.5.3. Protein assay

All protein extracts were quantified by Lowry method using Bio-Rad protein assay reagents (Bio-Rad), following the manufacturers protocol. The protein samples were aliquoted appropriately to contain 50µg of protein and stored at -80°C in order to avoid freeze-thaw cycles.
2.2.6. Western Blot and Immunodetection

Proteins were denatured at 100°C for 10 minutes with loading dye which consists of 3% glycerol, 3% SDS, 1% Bromophenol Blue, and β-mercaptoethanol. Equal amount of proteins (50µg) were run on a 10% SDS-Polyacrylamide gel for 80 minutes at 120V, and were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) for 90 minutes at 300mA. The membranes were then incubated in 5% blocking buffer for 1 hour at room temperature and hybridized with primary antibody overnight at 4°C. Incubation with secondary antibody was carried out the following day and immunodetection using ECL, Supersignal, or ECL plus detection reagent (Fisher Scientific).

2.2.7. RNA extraction

Total RNA was extracted using a guanidium thiocyanate-phenol-chloroform extraction RNA STAT-60 (Tel-Test, Inc, Texas) according to the manufacturer’s instructions. RNA STAT-60 is known to be able to disrupt cells and dissolve cellular components whilst maintaining the stability of RNA during cell lysis or tissue homogenization. For RNA-mRNA isolation, firstly, cells were homogenized in 1ml of RNA STAT-60 per 5-10 X 10⁶ cells. Then RNA was extracted by adding 0.2 volume of chloroform to 1 volume of homogenate and precipitated using 0.5 volume of isopropanol. Following RNA precipitation, the pellet was washed in 75% ethanol, air-dried briefly and re-suspended in RNase-free water. The concentration and purity of RNA were analysed by measuring OD_{230}, OD_{260} and OD_{280} on a NanoDrop Nd-1000 spectrometer. The 260/280 ratio was expected to be between 1.8 and 2.0 and 260/230 ratio at 2.0-2.2 for RNA. If the ratios were below the expected value, it may indicate possible contamination. All RNA extracts were stored at -80°C until further analysis.

2.2.8. DNase treatment and first-strand cDNA synthesis

Prior to reverse transcription of total RNA to generate cDNA, any DNA contaminations in the RNA samples were eliminated through a DNase treatment. With total reaction volume of 10µl, 2µg of total RNA was digested with 1µl of DNaseI (Invitrogen) in 1x DNaseI reaction buffer and DEPC-treated water at room temperature for 15 minutes. Once the digestion is complete, the reaction was stopped by incubating at 65°C for 10 minutes with 1µl of 25mM EDTA, and the whole reaction was used for first-strand cDNA synthesis.

The DNase treated RNA was incubated at 65°C for 5 minutes with 1µl dNTP mix (10mM of dATP, dTTP, dGTP and dCTP each) and 1µl Oligo(dT)12-18 (0.5µg/µl), then incubated in ice for 1 minute. Following the addition of 1x reverse transcriptase buffer (Invitrogen), 2µl 25mM MgCl₂ 1µl 0.1M DTT and DEPC-treated water to attain the total reaction volume of 20µl, the reaction mix was then incubated at 42°C for 2 minutes. Reverse transcriptase SuperScript II (Invitrogen) was added to the reaction mix and incubated at 42°C for 50 minutes. Termination of reverse transcription was carried out by heating at 70°C for 15 minutes, and by incubating at 37°C for 20 minutes with 1µl RNase H.
(Invitrogen), any remaining RNA fragments were removed. Resulting cDNA was then stored at -20°C until further analysis.

2.2.9. Real-time PCR

Real time quantitative polymerase chain reaction (RTQ-PCR) was carried out using ABI StepOne Real Time PCR system (Applied Biosystems) for quantification of target cDNA. SYBR Green I dye was used for RTQ-PCR. SYBR Green generates fluorescence when incorporated into double-stranded DNA, thus an increase in the accumulation of PCR product leads to higher emission of SYBR Green fluorescence which is detected by the Real Time PCR system throughout the cycle. Amplification is carried out using specific primers (Table 2.1) for the target DNA, and then the software determines the baseline of fluorescence and generates a baseline-subtracted amplification plot by calculating a mathematical trend of the normalized fluorescent reporter signal. The cycle number at the point of amplification plot where it crosses the threshold, defined as the Ct value, is used as a quantitative measurement of the input target. A decrease in the Ct value is seen as an increase in the target cDNA. The target Ct values were normalised to L-19 for analysis and any significant changes in target cDNA level was determined using ANOVA.

Table 2.1. Sequences of PCR primers used in real-time PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>RefSeq number</th>
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<tr>
<td>L-19</td>
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2.2.10. Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were performed using Phusion high-fidelity DNA polymerase (New England Biolabs). PCR reaction mix prepared following the manufacturer’s protocol. Each 50µl reaction included 200µM of each dNTP (dATP, dCTP, dGTP, dTTP), 50nM of each forward and reverse primer, 50ng template DNA, 1xPhusion HF buffer containing 1.5mM MgCl$_2$ and 0.5µl of Phusion high-fidelity DNA polymerase. Template DNA were initially denatured for 30 seconds at 98°C, then the reaction was subjected to 30 thermo-cycles of 98°C for 10 seconds, primer annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. This was followed by the final extension step of 72°C for 10 minutes. PCR products were then analysed by agarose gel electrophoresis.

2.2.11. Agarose gel run

The final products of PCR were analysed using 1.5 – 2% agarose gels (w/v). Gels were prepared by dissolving appropriate amount of agarose in fresh 1 x TBE and heating until agarose goes into solution. The solution was then cooled to approximately 50°C, at which point, SYBR safe (Invitrogen) was added prior to pouring the gel into a suitable gel mould. Once the gel has set, it was submerged in 1 x TBE buffer and DNA samples containing 1 x DNA loading buffer (Promega) were loaded into the wells. Depending on the size of the gels, electrophoresis was carried out at 90 – 120V until the dye front reaches the end of the gel. The size of DNA fragments were estimated by using a 100bp or 1kb DNA markers (Bioline) which contain restriction fragments of known sizes. The bands were imaged using a dark reader (Dark Reader transilluminator, Clare Chemical Research).

2.2.12. DNA purification and sequencing

To prepare PCR products for sequencing analysis, they were purified using a QIAquick PCR Purification Kit (Qiagen). PCR purification was carried out using a microcentrifuge, following the manufacturer’s protocol. 1 volume of PCR sample and 5 volumes of Buffer PB were placed into a QIAquick spin column. To bind DNA, the columns were centrifuged for 1 minute at 10,000 x g. The columns were washed with 0.75ml of Buffer PE and centrifuged for another minute. In order to remove any residual ethanol from Buffer PE, discard the flow-through and for an additional minute at maximum speed. The purified DNA were then eluted from the column by adding 50µl of Buffer EB, containing 10mM Tris-Cl, pH 8.5, or RNase-free water to the centre of the QIAquick membrane and centrifuging for 1 minute. The resulting DNA sample concentrations were measured using NanoDrop Nd-1000 spectrometer. Sequencing was carried out by Beckman Coulter Genomics.
2.2.13. Transfection

2.2.13.1. Dharmafect transfection reagent

For transfection using Dharmafect 2 transfection reagent (Dharmacon), the amnion cells were cultured in PS- and LG-free, 10% FCS DMEM medium (Sigma-Aldrich) until 50-60% confluence. The transfection reagent was prepared for each well of a 6-well plate by mixing 4µl of Dharmafect transfection reagent with 196µl of serum-, PS- and LG-free DMEM without phenol-red (Sigma-Aldrich) and incubating at room temperature for 5 minutes. The mixture was then combined with siRNA (10µl of 20µM siRNA and 190µl of serum-, PS-, LG- and phenol-red-free DMEM) and incubated for 20 minutes at room temperature. The cells were grown in total of 400µl of the reaction mixture and 1600µl of 10% FCS DMEM (Sigma-Aldrich) for 72 hours for RNA extraction and 4-5 days for protein.

2.2.13.2. Amaxa electroporation

Transfection using electroporation was performed according to the manufacturer’s protocol (Amaxa Lonza, Germany). Primary amnion cells were seeded in culture flasks to reach 90-100% confluence, and then dissociated from the flasks using trypsin for 10-15 minutes. The cell suspension was centrifuged and diluted in PBS prior to electroporation. Transfected cells were washed after 24 hours and serum-depleted prior to treatment. The efficiency of transfection was tested using two different techniques described above using GFP (Figure 2.1). Transfecting by electroporation showed higher transfection efficiency in comparison to using Dharmafect transfection reagent despite the fact that it led to higher cell death post-transfection.
2.2.14. Fluorescent immunocytochemistry

Term, non-labouring primary amnion cells were grown on multi-chamber slides until fully confluent. Following overnight serum-depletion (16-18 hours), the cells were stimulated with IL-1β (1ng/ml) or OT (100nM) for 30 minutes. Treated cells were fixed with methanol and target proteins were incubated in primary antibody for 1-2 hours and washed thoroughly with PBS. Following the washing steps, cells were incubated with fluorescently conjugated secondary antibodies for 1 hour at room temperature. The slides were washed and mounted with Vectashield (Vector Laboratories) that contains 4’,6-diamidino-2-phenylindole (DAPI) for visualisation of the cell nuclei as it produces a blue fluorescence with excitation at ~360nm and emission at ~460nm when bound to DNA. IgG control was incorporated for each experiment as a negative control.

Slides were observed on a Leica SP5 confocal microscope (Zeiss) using x 60 objective. High resolution images of amnion epithelial cells were captured and saved in JPEG format using LAS-AF program.

Figure 2.1. Transfection efficiency comparison between Lonza Amaxa electroporation and Dharmacon transfection reagent. Pre-labour amnion cells were transfected with 3µg of GFP using electroporation or transfection reagent. Total of 5 different electroporation programs were tested as suggested by the manufacturer. The level of fluorescence was detected for two following days.
2.2.15. Electrophoretic mobility shift assay (EMSA)

Consensus and non-consensus double-stranded oligos were end-labelled with 0.37MBq $^{32}$P ($\gamma$ATP) by incubating for 30 – 60 minutes at 37°C with T4 polynucleotide kinase (Promega). The labelled oligos were cleaned by centrifugation at 3000 rpm for 5 minutes through MicroSpin G-25 sephadex columns. Total of 5µg nuclear proteins were incubated for 1 hour on ice with non-radiolabelled non-specific oligos (Oct-1) in an EMSA binding buffer containing 20% glycerol (v/v), 5mM MgCl$_2$, 2mM EDTA, 50mM Tris-HCl (pH 7.5), 250mM NaCl and 54mM DTT. Proteins were then incubated with 0.035pmol $^{32}$P ($\gamma$ATP)-end labelled probes for 40 minutes on ice. The resulting protein/DNA complexes were separated in a 4% non-denaturing acrylamide gel in 1 x TBE buffer. The gel was then dried and transferred to a filter paper under vacuum at 80°C, and exposed to X-ray film overnight at -80°C. For supershift analysis, 2µg p65 or p50 antibodies (Santa Cruz) were incubated with the samples prior to probe binding. Non-radiolabelled oligos were used for specific and nonspecific competition for DNA binding.

2.2.16. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of PGE$_2$, IL-6 and CCL5 were determined by a standard enzyme-linked immunosorbent assay (ELISA). Primary amnion epithelial cells were grown to confluence and treated for 1 hour, 2 hours, 4 hours, and 6 hours with OT (10$^{-7}$M) or Atosiban (10µM). Supernatant was collected and immediately frozen at -20°C for analysis by ELISA. PGE$_2$, IL-6 and CCL5 ELISA kits were used according to manufacturer’s instructions (R&D systems).

2.2.17. IPOne HTRF assay

Direct quantitative measurement of myo-Inositol 1 phosphate (IP1) was performed using IPOne HTRF assay kit (Cisbio). The principle behind this assay technique is illustrated in Fig 2.2. Total of 20,000 primary amnion epithelial cells were seeded in each well of a white, 384 well small-volume plate (Greiner). Prior to treatment, cells were washed in IP1 stimulation buffer which contains 10mM HEPES, 1mM CaCl$_2$, 0.5mM MgCl$_2$, 4.2mM KCl, 146mM NaCl, and 5.5mM glucose, in pH 7.4. The treatment compounds were diluted in 1 x StimB, provided by the kit. Cells were treated in duplicates for 1, 5, 15, 30, 45 and 60 minutes. After stimulation with compound, HTRF reagents were added (1$^{st}$ distribution IP1-d2 then 2$^{nd}$ distribution Ab-Crypt) then incubated for 1 hour at room temperature. Plate reading was carried out using an HTRF compatible reader, PHERAstar plus (BMG Labtech). As for all HTRF assay, results were calculated from the 665nm/620nm ratio to eliminate possible photophysical interference.
Figure 2.2. IPOne HTRF assay. The IPOne HTRF assay (Cisbio) is a competitive immunoassay based on a new HTRF technology involving antibody specific for IP1, labelled with Lumi4™-Tb cryptate. Presence of lithium chloride (LiCl) in the cell stimulation buffer causes the accumulation of IP1 in the cells when receptor is stimulated. The native IP1 accumulated in the cells competes with IP1 which is coupled to the dye d2 to bind to anti-IP1 cryptate Tb. The FRET signal is inversely proportional to the concentration of IP1.

2.2.18. Fluo-4 direct Calcium assay

Primary amnion epithelial cells were plated onto single chamber glass bottomed dishes (MatTek Corporation). Cells were grown to reach 80-90% confluence in DMEM containing 10% FCS, 2mM LG, and 100U/ml PS. Prior to Calcium assay, cells were serum depleted in DMEM containing 2% FCS, 2mM LG and 100U/ml PS overnight. Equal volume of Fluo-4 direct calcium reagent loading solution (Invitrogen) and culture medium were loaded directly to the wells containing cells, and incubated at 37°C for 30 minutes, then at room temperature for another 30 minutes. Culture dishes were then placed for live cell imaging on the Leica SP5 confocal microscope (Zeiss), using the LAS-AF program to capture images every 1.315 seconds. Cells were imaged at optimum gain and exposure points. Initially, the fluorescence was measured for 2 minutes without any treatment to obtain a basal value. After addition of the agonist/antagonist, the fluorescence was measured for 10 minutes. The data produced from the program provides histogram values based on the fluorescence emitted by the experiment. The average basal value was subtracted from these values and analysed in Excel. Control
experiments were done using 10µM BAPTA-AM, which is an intracellular calcium chelator, and 8mM EGTA, an extracellular calcium chelator.

2.2.19. Statistical analysis

All data were initially tested for normality using a Kolmogorov-Smirnoff test. Normally distributed data were analysed using ANOVA followed by Bonferroni or Dunnett’s post hoc test for three groups or more. Where appropriate, Student’s t-test for paired or unpaired data was used to calculate the significance of the differences of the means. Non-normally distributed data were analysed using Wilcoxon matched pars test for paired data and Mann-Whitney u test for unpaired data when comparing two groups. For three or more groups, Friedman’s Test with a Dunn’s Multiple Comparisons post hoc test was used. All data were presented with SE and p<0.05 was considered statistically significant.
3. Oxytocin/oxytocin receptor expression and its role in prostaglandin synthesis during the onset of labour
3.1. Introduction

Human labour strongly resembles an inflammatory reaction [113], with global increases in a number of proinflammatory factors including prostaglandins and cytokines [116-118]. Inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNFα, which increase during labour have been shown to activate MAPK, NF-κB, C/EBP and AP-1 [352-354]. There are series of published data which shows that coordinated upregulation of IL-1β, IL-8, PG, and COX-2 expression occur just prior to labour, suggesting they may play a role in the initiation of labour [137, 355-357]. Amnion is one of the major sites of PG and cytokines synthesis during human labour. Secretion of these factors by the fetal membranes increases with labour [356, 358-360] and in response to stimuli such as TNF-α, IL-6 and IL-1β [361, 362].

In most mammalian species, parturition is preceded by a rise in oestrogen, a decline in progesterone concentrations in maternal plasma [268] and upregulation of myometrial OTR expression [363], as well as an increase in neurohypophyseal OT in the maternal circulation [364]. However, there is considerable doubt about the role of OT in normal human parturition due to the lack of evidence in the increase in plasma OT before labour onset [297, 365] and the absence of apparent correlation between plasma OT levels and myometrial activity [366]. It was hypothesized that local synthesis of OT within the intrauterine tissues may play a role in regulating normal human parturition. The increase in local OT levels was demonstrated in amnion, chorion and deciduas at the time of labour onset, supporting the hypothesis that a paracrine system involving OT in intrauterine tissues can contribute to the onset of labour without being reflected in the maternal circulation [240].

The role of oxytocin and its receptor in the amnion during the onset of labour is not as well established as that in the myometrium, however, previous work in rabbit amnion showed that OT stimulates PGE₂ production, with OTR expression increasing up to 200-fold towards the end of pregnancy [367]. This marked increase in PGE₂ synthesis with OT stimulation was later demonstrated in pre-labour human amnion epithelial cells [181]. As mentioned previously, PG biosynthesis starts with the liberation of the substrate, which involves the release of arachidonic acid (AA) from membrane glycerophospholipids by the actions of phospholipases [368]. Cytosolic phospholipases A₂ (cPLA₂) is one of the most studied in human uterine tissues and has been postulated to play significant role in the process of parturition [369, 370]. The next step involves the conversion of substrate to product. In PGE₂ synthesis, PGES-1 and -2 activity coupled to that of COX-2 to metabolize arachidonate into prostanoids [371-373].
3.2. OT synthesis in human amnion during labour

Previous studies have demonstrated an increase in OT mRNA expression with labour, specifically in chorio-decidua. In this study, we have investigated whether there are any changes in OT mRNA levels in human amnion in respect to the onset of labour.

Pre-labour amnion cells were cultured from women undergoing elective caesarean section at term (38\(^{5}\) - 39\(^{6}\) weeks of pregnancy), prior to the onset of labour (L\(-\)), and labouring amnion cells were cultured from women undergoing emergency caesarean section or vaginal delivery after the onset of labour (L\(+\)). The expression of OT mRNA was analysed using real-time PCR. The result showed significantly higher basal expression of OT in labouring amnion cells compared to non-labouring (\(p<0.05\); Fig. 3.1). This indicates there is an increase in OT mRNA expression in human amnion with the onset of labour.

![Figure 3.1. Labour-associated increase in OT mRNA expression.](image)

**Figure 3.1. Labour-associated increase in OT mRNA expression.** Total RNA extracted from labouring (L\(+\)) and non-labouring (L\(-\)) amnion tissues were subjected to qRT-PCR analysis. Transcript levels were normalised to the housekeeping gene, L19, to quantify the relative abundance of OT mRNA (\(n=6\) from 6 independent samples; *\(p<0.05\) vs L\(-\), ANOVA).
3.3. Expression of OTR increases with labour in human amnion

The uterine sensitivity to OT increases markedly during the onset of labour, which is associated with increase in both OTR mRNA levels and myometrial OTR density during early labour [374]. This was first described in rats [285], and subsequently demonstrated in rabbits and humans [3, 303]. The role of OT/OTR system in human myometrium is well understood as OT is one of the major mediators of uterine contractions. The increase in OTR expression is not restricted to the myometrium. In both rats and humans, OTRs are present in the endometrium as well as the myometrium and chorio-decidua [3, 304]. In rabbit amnion, there is up to 200-fold increase in expression of OTR towards the end of pregnancy [324] and OTR binding to OT significantly increases in human fetal membranes during the onset of labour [375]. Also, previous study in human myometrial smooth muscle cells has illustrated an increase in OTR mRNA expression with IL-1β treatment, peaking at 4 hours [278]. Increase in OTR is one of the most consistent findings in the study of parturition across several species, which can imply that the changes in the expression or function of the OTR, as well as the peptide, are important for the onset of labour.

Pre-labour and labouring primary amnion epithelial cell cultures were established from samples taken at the time of Caesarean section from patients with informed consent. Basal OTR mRNA expression was significantly higher in the labouring samples compared to pre-labour (p<0.01; Fig. 3.2 A). OTR protein levels were also higher in labouring samples compared to non-labouring (p<0.05; Fig. 3.2 B). These results suggest increase in sensitivity to OT in human amnion during labour.

**Figure 3.2. Expression of OTR increases both with labour.** Total RNA and proteins extracted from labouring (L+) and non-labouring (L-) amnion tissues were subjected to qRT-PCR analysis (A) and western blot (B). Transcript levels were normalised to the housekeeping gene, L19, to quantify the relative abundance of OTR mRNA. Control with β-actin confirmed equal protein loading (n=3 from 3 independent samples; * p<0.05, ** p<0.01 vs L-, ANOVA).
3.4. OT increases expression of PG synthetic enzymes; cPLA₂, COX-2, PGES-1, and -2

To study the effect of OT on the expression of PG synthetic enzymes, cPLA₂, COX-2, PGES-1, and -2, in human amnion epithelial cells, cultured pre-labour primary amnion cells were treated with OT (100 nM) and total RNA were extracted at 1 h, 2 h, 6 h and 24 h. The changes in enzyme mRNA expressions were measured over time. Stimulation of amnion with OT induced significant increases of the enzyme mRNA levels compared to non-stimulated control (NS). Upon OT stimulation, the mRNA levels of cPLA₂ and PGES-2 increased by 3.8- and 6.4-fold respectively, peaking at 2 h (p<0.01; Fig. 3.3 A and D). PGES-1 increased by 6.9-fold after 6 h (p<0.05; Fig. 3.3 C), and COX-2 by 2.7-fold after 2 h (p<0.05; Fig. 3.3 B). By 24 h of OT stimulation, the mRNA expression of cPLA₂, COX-2, PGES-1, and PGES-2 returned to basal level.

Figure 3.3. Effects of OT on the expression of prostaglandin synthetic enzymes. Pre-labour amnion epithelial cells were treated with OT (100 nM) for 1 h, 2 h, 6 h and 24 h. The expression of cPLA2, COX-2, PGES-1 and -2 were analysed using qRT-PCR. Transcript levels were normalised to the housekeeping gene, L19 (n=6 sets of amnion epithelial cells isolated from 6 patients; * p<0.05 vs NS, ANOVA).
3.5. OT increases PGE$_2$ synthesis

As mentioned above, cPLA$_2$, COX-2, PGES-1, and -2 are PG synthetic enzymes. Therefore, it can be postulated that the increase of their mRNA expression with OT stimulation would be coupled with an increase of PGE$_2$ secretion. Pre-labour amnion cells were treated with OT (100 nM) for 1 h, 2 h, 4 h and 6 h, and the supernatants were collected to measure PGE$_2$ levels by ELISA. The PGE$_2$ ELISA showed that OT treatment significantly increased the synthesis of PGE$_2$, with approximately 15-fold increase after 6 h of stimulation ($p<0.01$; Fig. 3.4). Thus, suggesting that OT actives PG synthetic enzymes; cPLA$_2$, COX-2, PGES-1, and -2, to mediate PGE$_2$ production.

![Figure 3.4](image_url)

**Figure 3.4. Synthesis of PGE$_2$ increases upon OT stimulation.** Pre-labour amnion epithelial cells were serum depleted overnight and treated with OT (100 nM) for 1 h, 2 h, 4 h and 6 h. The culture medium was collected for PGE$_2$ EIA analysis (n=6; * $p<0.05$, ** $p<0.01$ vs NS, ANOVA).
3.6. Summary and discussion

There are several lines of evidence to support an important role for OT in the initiation of parturition. As discussed before, the first evidence being that OT is one of the most potent uterotonins currently available and is able to induce labour in pregnant women during late gestation that is indistinguishable from normal labour [376]. Second, OT antagonists designed to manage preterm labour have been demonstrated to inhibit late gestational uterine contractions in several species including humans [289, 291, 293]. There is a study which reported an increase in OT concentrations in maternal plasma during labour [301]. However, there are contrasting evidences as many investigators failed to show any significant changes in maternal plasma concentrations of OT [103, 297, 365], and no direct correlation has been shown between maternal plasma OT and myometrial activity [366, 377]. This led to the hypothesis that OT may be playing a role in parturition via a paracrine/autocrine system instead of acting as an endocrine hormone. A previous study demonstrated an increase in OT mRNA expression in intrauterine tissues during the onset of labour, which enables OT to aid the processes of labour via a paracrine/autocrine system [240]. Similar pattern of increase OT mRNA was observed in intrauterine tissues during late gestation in rats [378] and cows [379]. Our data complements previous findings as we have shown elevated basal expression of OT mRNA in labouring compared to non-labouring amnion (Fig. 3.1). This marked increase in OT mRNA expression in late gestation occurs in close proximity to the target tissues, including the endometrium and the myometrium, providing a strong support to the hypothesis that OT may act as a local mediator rather than a circulating hormone to regulate the timing of parturition.

Despite evidence supporting the role of OT in the initiation of parturition, the significance of its role is still in question. This is due to the fact that OT-knockout mice undergo normal parturition [241] and that uterine sensitivity to OT varies greatly between patients [380]. Appropriate timing of OT use is the key to successful induction of labour. One of the suggested explanations for this is that OTR expression may mediate the uterine responsiveness to OT. One of the most consistent findings in the study of parturition is the increase in OTR mRNA expression in intrapartum tissues before the onset of labour. The increase in OT binding in the uterus has been found to be associated with the increased sensitivity to OT via upregulation of OTR mRNA expression in both humans [3, 381] and rats [285, 363], which introduces a role for OTR in the initiation of labour. There is evidence in rats showing that progesterone inhibits OT binding by preventing OTR function via both genomic and non genomic mechanisms [274, 382, 383], thereby implying that the functional withdrawal of progesterone may contribute to increase in OT/OTR activity prior to initiation of labour. The increase in OTR mRNA expression before the onset of labour has also been found in deciduas and fetal membranes. The greatest increase in OTR expression with labour was seen in rabbit amnion cells with approximately 200-fold increase [384]. Subsequently, we have shown a 16-fold increase in OTR mRNA expression
as well as increase in OTR protein level with the onset of labour in human amnion epithelial cells (Fig. 3.2 A and B).

The production of PGs is one of the key steps in the biochemistry of human labour [202]. Prostaglandins have been studied extensively in their role in promoting myometrial contractility during labour onset [323]. There is increasing evidence supporting that PGs play a role at various stages of human parturition. They lead to increase in metalloproteinase-9 activity associated with rupture of fetal membranes [204], and there is large literature supporting the role of PGs in cervical ripening and dilation [190, 191, 385]. In addition, PGs have been reported to be associated with placental separation [386] and uterine involution [387].

Prostaglandins, specifically PGE$_2$, have been found to accumulate in the amniotic fluid with increasing gestation and the enzymes involved in its synthesis increase in both expression and activity in amnion cells prior to labour onset [355]. For biosynthesis of PGE$_2$, three major enzymatic reactions are required, which involves cPLA$_2$, COX-2, PGES-1 and -2. PGES-1 and PGES-2 (also known as microsomal PGE synthase-1 and -2). PGES-1 and PGES-2 are functionally coupled with COX-2 [373] and they have been shown to be upregulated in response to proinflammatory stimuli [388, 389] as well as labour in human myometrial cells [390]. In this study, OT stimulation resulted in the transient upregulation of multiple PGE$_2$ synthetic enzymes, including PGES-1, PGES-2, COX-2 and cPLA$_2$ (Fig. 3.3), and the consequent PGE$_2$ production. Stimulation with OT resulted in significant increase in the mRNA levels of PG synthetic enzymes after 2 h, which returned back to basal levels by 24 h. This transient response has been reported previously in HEK-293 and ECV304 cells, where a common proinflammatory stimuli, IL-1, induced rapid but transient activation of PGES-1, -2 and COX-2 transcription [373, 391]. Similar our data, IL-1β-driven upregulation of COX-2 in human myometrium rapidly declined after peaking at 4 h, returning to basal level by 24 h of treatment [392]. In addition, OT has been shown to upregulate cPLA$_2$ and COX-2 expression in both rat [393] and human uterus [394]. The transient increase in the expression of PG synthetic enzymes by OT may be explained by the functional desensitisation of OTR. G protein coupled receptors, such as OTR, are able to undergo desensitisation after a prolonged or repeated stimulation. Interestingly, studies in myometrial cells demonstrated a time-dependent decrease in OT binding and OTR mRNA expression during prolonged exposure to OT, with the significant reduction occurring from 6 h exposure [265, 395].

The data presented here demonstrated that the induction of OTR expression by OT in human amnion correlates with the increase in PG synthetic enzyme levels as well as PGE$_2$ production, suggesting a role for OT/OTR system in amnion during the onset of labour. Such correlation has also been demonstrated in rats, where the increase in the sensitivity to OT and the increase in PG production occurred in close temporal proximity [396]. Studies in rat models showed that PGs are important
stimulants of OTR expression [397, 398], and that OTR upregulation can be blocked by PG synthesis inhibitors [399]. Thus it is possible that OTR and PGs may work in a positive feedback system within the uterus to induce myometrial contractility during labour. Our data does not show whether it is OTR or PGs that is driving the positive feedback system, however, there is evidence that OTR is the primary driver of this positive feedback loop as Fang et al. showed increase in OTR peptide prior to PG synthesis [400].

In summary, the data suggests a pro-inflammatory role of amnion OT/OTR system in the onset of labour. During the initiation of labour, where there is an increase in OT as well as the sensitivity to OT, the OT/OTR system not only triggers uterine contractility but also mediate PGE$_2$ synthesis in amnion. The PGs produced mediate various physiological events of parturition including membrane rupture, cervical dilation, myometrial contractility, placental separation and uterine involution.
4. Effects of OT on mediators of inflammation and downstream pro-labour gene expression
4.1. Introduction

The production of PGs by amnion is fundamental in the onset and the continuance of labour. It has been established that PGE$_2$ is produced in amnion via the COX-2-dependant pathway [373, 401, 402]. The onset of labour promotes COX-2 mRNA and protein expression in human myometrium as well as amnion [403, 404]. In human amnion, OT has been shown to drive PGE$_2$ synthesis, suggesting a role for amnion OT/OTR system in the onset of labour. PGE$_2$ synthesis has been shown to be strongly associated with the upregulation of COX-2 [181]. COX-2 is highly regulated both transcriptionally and post-transcriptionally [405-407]. Previous work in both myometrial and amnion cells have implicated NF-κB, AP-1 and MAPKs in regulating COX-2 expression induced by mechanical stretch and inflammatory cytokines [105, 154, 231, 408, 409]. Here we investigated the signalling pathways involved in OT-induced COX-2 expression and PGE$_2$ synthesis.
4.2. OT activates NF-κB

The promoter region of the human COX-2 gene contains various putative transcriptional regulatory elements, including binding sites for NF-κB, cyclic AMP responsive element (CRE), SP-1, AP-1, AP-2 and NF-IL6 (CCAAT/enhancer binding protein β; C/EBPβ) [410]. Among these elements, the positive transcriptional regulators of COX-2 are CRE, C/EBPβ and NF-κB [160, 411, 412]. Our group has previously shown that NF-κB activity in human amnion increases with labour and that one of the most significantly upregulated genes is COX-2 [413], which suggests a role for NF-κB in regulating COX-2 expression in human amnion. To investigate whether NF-κB signalling pathway is involved in the OT-mediated expression of COX-2, we initially focused on the effects of OT on NF-κB.

4.2.1. OT leads to nuclear translocation of p65 subunit but not p50 or RelB

Nuclear/Cytosolic proteins were extracted from pre-labour amnion cells treated with OT (100 nM) for 15 min, 30 min, and 1h. The efficacy of nuclear and cytosolic protein separation was confirmed using TATA binding protein (TBP) and α-tubulin, which are only expressed in the nucleus and cytosol, respectively. We have demonstrated the translocation of the p65 NF-κB subunit from cytosol to nucleus after 30 min of OT stimulation ($p<0.05$; Fig. 4.1 A), however, there were no nuclear translocation of p50 or RelB subunits observed (Fig. 4.1 B). Fluorescent immunocytochemistry was used to confirm the nuclear translocation of p65 subunit upon OT stimulation. Pre-labour amnion cells were treated with OT (100 nM) for 30 min and subsequently fixed with methanol. Using a fluorescently-tagged secondary antibody specific for the p65 primary antibody, its subcellular location was studied. We have found an increase in fluorescence in the nucleus of OT treated cells compared to non-stimulated cells (Fig. 4.1 C), indicating nuclear translocation of p65 subunit upon OT stimulation.
Figure 4.1. Nuclear translocation of NF-κB subunits with OT treatment. Pre-labour primary amnion cells were stimulated with OT (100 nM) for 15 min, 30 min, and 1 h. Western blotting of nuclear and cytosolic protein extracts illustrates nuclear translocation of different NF-κB subunits, p65 (A), p50 and RelB (B). Membranes were probed with α-tubulin and TATA binding protein (TBP) to confirm separation of nuclear and cytosolic extracts (n=4; * p<0.05 vs NS, ANOVA). Cultures were treated with OT for 30 min and then fixed and immunostained for the NF-κB subunit, p65, and visualized with TRITC. IgG was used as the negative control. Scale bar, 25µm (C).
4.2.2. OT activates the canonical NF-κB signalling pathway involving p65 homodimers

There are three main NF-κB signalling pathways that have been described; classical, alternative and atypical, which involves a trigger, sequential activation of kinases leading to the release of NF-κB dimers from IκB proteins, nuclear translocation, and binding to NF-κB responsive genes. To determine which pathway is utilized by OT, we focused on looking at activation of different subunits of NF-κB signalling pathway. Pre-labour amnion cells were treated with OT (100 nM) for 15 min, 30 min, 1 h, 2 h and 4 h. Treatment with OT resulted in increased expression of phosphorylated IKKα/β and p65, and degradation of IκBα after 15 min and 30 min of stimulation (Fig. 4.2). This resembles the canonical NF-κB signalling pathway, which involves activation of IKKα/β, degradation of IκBα, phosphorylation of p65 and nuclear translocation of NF-κB dimers. However, as we have not seen nuclear translocation of p50 subunit with OT treatment (Fig. 4.1 B), we hypothesized that instead of classical p50-p65 heterodimer translocation, p65-p65 homodimers are involved in OT-induced NF-κB activation.

Further experiments using EMSA showed increase in NF-κB-DNA binding upon OT stimulation for 30 min (Fig. 4.3). Supershift analysis was used to examine the composition of the NF-κB complexes binding to its consensus probe. There was a supershift of NF-κB-DNA binding with the addition of p65 antibody, but no shift was observed in presence of p50 antibody (Fig. 4.3). This suggests that the NF-κB bound to the consensus probe consists only of p65 subunits, supporting the hypothesis that p65-p65 homodimers are predominantly involved in OT-induced NF-κB activation.
Figure 4.2. Oxytocin activates NF-κB subunits involved in the classical signalling pathway. Pre-labour primary amnion cells were grown to reach confluence and serum depleted overnight prior to treatments. The cells were stimulated with OT (100 nM) for 15 min, 30 min, 1 h, 2 h and 4 h. Whole cell lysates analysed by Western blot demonstrated activation of NF-κB p65 and IKKα/β, and degradation of IκBα upon OT stimulation. Control with β-actin confirmed equal protein loading (n=6; * p<0.05 vs NS, ANOVA).
Figure 4.3. Treatment with OT increases DNA binding of NF-κB p65 but not p50. Pre-labour amnion epithelial cells were treated with OT for 30 min and nuclear proteins were extracted for binding studies. EMSA study demonstrated increase in nuclear protein binding to DNA sequence containing binding sites for NF-κB upon OT stimulation. Specificity of NF-κB binding was confirmed by co-incubation with non-labelled non-competing (Oct-1 consensus; 5’-TGTCGAATGCAAATCACTAGAA-3’) and non-labelled competing (NF-κB consensus; 5’-AAGAGAAGGGGCTTGGCCCAAGG-3’) oligos. Unbound probes appeared as double bands possibly due to single and double stranded forms of the labelled probe. Co-incubation with antibodies to NF-κB p65, but not p50, resulted in a band supershift in OT treated samples, indicating increase in NF-κB p65 DNA binding.
4.2.3. OT drives the expression of proinflammatory NF-κB regulated genes

NF-κB has been found to regulate the expression of a cassette of proinflammatory genes [231, 413]. Having shown that OT leads to NF-κB activation in human amnion, we aimed to assess the effects of OT on the expression of downstream NF-κB-regulated genes. Pre-labour amnion cells were treated with OT for 1 h, 2 h, 4 h, 6 h, and 24 h, and total RNA was collected for qRT-PCR analysis. Stimulation with OT led to significant increase in IL-8 and CCL2 mRNA expression by 6- and 4-fold respectively, peaking after 2 h (p<0.05; Fig. 4.4 A and C). COX-2 and CCL5 mRNA levels were also upregulated within 2 h and 4 h of OT stimulation, respectively (p<0.05; Fig. 4.4 B and D). The mRNA levels of IL-6 and SOD2 showed a similar trend with an increase after 2 h stimulation (p<0.01; Fig. 4.4 E and F). The increase in protein levels of IL-6 and CCL5 with OT stimulation was further confirmed using ELISA, which demonstrated a significant increase in IL-6 after 4 h (p<0.01; Fig.4.4 G) of OT stimulation and CCL5 after 6 h (p<0.01; Fig.4.4 H).
Figure 4.4. Increase in the expressions of pro-labour, downstream NF-κB-regulated genes with OT stimulation. Pre-labour primary amnion cells were treated with OT (100 nM) for 1 h, 2 h, 4 h and 6 h and the expression of downstream NF-κB-regulated genes, IL-8 (A), COX-2 (B), CCL2 (C), CCL5 (D), IL-6 (E) and SOD2 (F) were analysed using qRT-PCR. Transcript levels were normalised to the housekeeping gene, L19 (n=6; *p<0.05, **p<0.01 vs NS, ANOVA). IL-6 (G) and CCL5 (H) protein levels were examined using ELISA from the collected culture media collected (n=3; **p<0.01 vs NS, ANOVA).
4.2.4. Activation of NF-κB upon OT stimulation is cell type-specific

It is well established that the components involved in the activation of NF-κB signalling cascade are specific to both the stimulus and the type of the cell or tissue. To determine whether the ability of OT to activate NF-κB is specific to amnion, the effect of OT was examined in two different cell types; MCF-10A mammary epithelial cells and primary myometrial smooth muscle cells. The MCF-10A cells were treated with OT for 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h and 6 h. Whole cell proteins were used to confirm expression of OTR in MCF-10A cells. Despite the fact that OT is capable of inducing NF-κB signalling in amnion, MCF-10A cells showed no significant changes in the levels of phospho-p65 upon OT stimulation (Fig. 4.5). This indicates that OT-mediated activation of NF-κB is cell type-specific.

Unlike in MCF-10A cells, OT treatment in primary myometrial smooth muscle cells led to nuclear translocation of p65 and p50 NF-κB subunits ($p<0.01$; Fig. 4.6 A), suggesting the involvement of p65-p50 heterodimers as seen in a typical classical NF-κB signalling pathway. Notably, the components involved in the NF-κB signalling cascade were different to that of the amnion. Stimulation with OT resulted in activation of p65 ($p<0.05$; Fig. 4.6 B), with no significant changes in OTR expression. The probability that OT may be driving tissue-specific NF-κB signalling cascades to regulate downstream gene expression indicates a complex proinflammatory role of OT.
Figure 4.5. The effect of OT on NF-κB p65 subunit in MCF-10A stably expressing OTR. The monolayer culture of MCF-10A mammary epithelial cells treated with OT (100 nM) for 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h and 6 h were extracted and Western blot analysis performed to study the effects of OT stimulation on p-p65 and OTR expression. Control with β-actin confirmed equal protein loading (n=3).
Figure 4.6. Activation of NF-κB in human myometrial smooth muscle cells upon OT stimulation. Pre-labour human myometrial smooth muscle cells were cultured from biopsies taken from patients undergoing elective caesarean section at term. Myometrial cells treated with OT (100 nM) for 15 min, 30 min, and 1 h were extracted and Western blotting of nuclear cytosolic extracts demonstrated nuclear translocation of NF-κB p65 and p50 upon OT stimulation (A). Membranes were probed with α-tubulin and TATA binding protein (TBP) to confirm separation of nuclear and cytosolic extracts (n=4; * p<0.05 ** p<0.01 vs NS, ANOVA). Western blot analysis of whole cell extracts was performed to study the effects of OT stimulation on p-p65 and OTR expression (B). Control with β-actin confirmed equal protein loading (n=6; * p<0.05 vs NS, ANOVA).
4.3. NF-κB activation is required for COX-2 upregulation

NF-κB has been described to be one of the key regulators of COX-2 expression in both human myometrium and amnion [105, 231, 409, 413]. We have shown above that OT activates IKK_{α/β}, and promote degradation of IκBα and phosphorylation of p65 to allow nuclear translocation of p65 homodimers to mediate gene expression. We then investigated the role of NF-κB pathway in OT-induced COX-2 expression using amnion cells. Pre-labour primary amnion cells were grown to confluence and pre-incubated in TPCA1, an IKK_{β} inhibitor, for 1 h. These cells were stimulated with OT for 30 min and 6 h. IL-1β treatments were used as a control. There was a significant decrease in both IL-1β- and OT-induced p65 activation in presence of TPCA1 (p<0.05; Fig. 4.7 A). Unlike IL-1β, OT-induced COX-2 expression was suppressed in presence of TPCA1 (p<0.05; Fig. 4.7 B), thus demonstrating that OT requires the activation of IKK_{β} to regulate COX-2 expression whereas IL-1β-induced COX-2 expression is not dependent on IKK_{β} activation.

To further confirm the role of NF-κB in COX-2 regulation, targeted siRNA knockdown studies were carried out. Pre-labour amnion cells were grown to confluence and transfected with NF-κB p65-target siRNA via electroporation prior to OT stimulation. The cells were treated with OT (100 nM) for 6 h and mRNA and proteins were extracted. The knockdown of p65 expression was confirmed using both qRT-PCR (p<0.01; Fig. 4.8 A) and Western blot analysis (p<0.01; Fig. 4.8 C). Only those samples with greater than 70% knockdown were used in this study. Successful knockdown of p65 subunit led to significant decrease in OT-mediated COX-2 expression at both mRNA and protein levels (p<0.05; Fig. 4.8 B and C), indicating that p65 NF-κB subunit is required in regulation of OT induced COX-2 expression. These data combined together demonstrate that OT drives COX-2 expression in human amnion via the canonical NF-κB signalling pathway.
Figure 4.7. IKKβ plays a role in OT-induced activation of NF-κB and COX-2 expression. Prelabour amnion epithelial cells were pretreated with IKKβ inhibitor (TPCA-1) for 2 h prior to OT (100 nM) or IL-1β (1 ng/ml) stimulation. Whole cell lysates were extracted for Western blot analysis. The presence of TPCA-1 inhibited both IL-1β- and OT-induced phosphorylation of NF-κB p65 (A), but only reduced OT-induced COX-2 expression (B). Control with β-actin confirmed equal protein loading (n=6; * p<0.05, ** p<0.01 vs NS, § p<0.05 vs OT, ≠ p<0.05 vs IL-1β, ANOVA).
Figure 4.8. NF-κB is essential in OT-induced COX-2 expression. Prelabour amnion epithelial cells were subjected to transfection with non-target siRNA or p65-target siRNA (30 pmol) via electroporation prior to OT treatment. QRT-PCR was used for gene expression analysis of p65 (A) and COX-2 (B) in cells transfected with non-target siRNA or p65-target siRNA, treated with OT (100 nM) for 6 h. Transcript levels were normalised to the housekeeping gene, L19 (n=3; * p<0.05, ** p<0.01, *** p<0.001, § p<0.05 non-target siRNA+OT, ANOVA). Western blot analysis for p65 and COX-2 (C). Control with β-actin confirmed equal protein loading (n=3; * p<0.05, ** p<0.01, *** p<0.001, § p<0.05 non-target siRNA+OT, ANOVA).
4.4. OT activates ERK1/2, p38 kinase, and JNK

MAPKs are important transcriptional and translational regulators. Numerous GPCRs, including OTR, have been found to result in downstream activation of MAPKs via both G-protein-dependent and -independent mechanisms [414]. OT has been shown to activate MAPKs in rabbit amnion to regulate PG synthesis [367] and with this in mind, our initial experiments were designed to study the effect of OT on MAPKs in human amnion. Cultured amnion epithelial cells were treated with OT (100 nM) for 15 min, 30 min, 1 h, 2 h, and 4 h, and whole cell lysates were analysed using Western blot. The levels of phosphorylated ERK1/2 and p38 kinase increased in response to OT after 15 min and 30 min, respectively ($p>0.001$, $p>0.05$; Fig. 4.9). Phosphorylation of JNK increased at 15 min and 30 min of OT treatment, peaking at 30 min ($p>0.05$; Fig. 4.9).

![Western blot images showing phosphorylation of ERK1/2, p38, and JNK](image)

**Figure 4.9. Oxytocin activates ERK1/2, p38 kinase and JNK.** Pre-labour primary amnion cells were treated with OT (100 nM) for 15 min, 30 min, 1 h, 2 h and 4 h, and Western blot analysis was performed to study the effects of OT stimulation on p-ERK1/2, p-p38 and p-JNK expression. Control with β-actin confirmed equal protein loading (n=6; * $p<0.05$ vs NS, ANOVA).
4.5. MAPKs are required for COX-2 upregulation

In amnion-derived WISH cells, MAPKs; p38 kinase and JNK in particular, have been shown to play a role in regulating COX-2 expression [415, 416]. In contrast to these findings, data from rabbit amnion and human amnion cells suggested that only ERK1/2 is involved in OT-mediated COX-2 expression [181, 367]. As we have previously shown that OT can activate ERK1/2, p38 kinase, and JNK, we aimed to determine the role of MAPK activation on OT-induced COX-2 expression using ERK1/2 inhibitor (U0126; 10 µM), p38 kinase inhibitor (SB203580; 10 µM) and JNK inhibitor (SP600125; 10 µM). Pre-labour amnion epithelial cells were treated with the different MAPK inhibitors for 2 h prior to 6 h OT stimulation. The presence of ERK1/2 and p38 kinase inhibitors attenuated OT-induced COX-2 protein expression, suggesting that ERK1/2 and p38 activation by OT/OTR signalling is essential for COX-2 expression (p<0.05; Fig. 4.10 A). The efficacies of the different MAPK inhibitors were confirmed by Western blot analysis (Fig. 4.11).

4.6. MAPKs play a role in OT-induced activation of NF-κB

Activation of MAPK pathways combined with NF-κB activation are involved in the regulation of proinflammatory cytokine synthesis [417]. Since we established a role for OT in activating both pathways, we then explored the association between the activation of MAPK and NF-κB in the regulation of COX-2 expression. Pre-labour amnion cells were pre-treated with MAPK inhibitors; U0126 (10 µM), SB203580 (10 µM) and SP600125 (10 µM), for 2 h and stimulated with OT (100 nM) for 30 min. We found a dramatic decrease in OT-induced p65 phosphorylation in the presence of ERK1/2 and p38 kinase inhibitors, U0126 and SB203580 (p<0.05; Fig. 4.10 B), indicating that OT-induced activation of NF-κB is MAPK-dependent. This demonstrates a cross-talk between the OT-induced activation of MAPKs and NF-κB signalling cascades.
Figure 4.10. OT-induced expression of COX-2 requires MAPK dependent NF-κB activation. Pre-labour primary amnion cells were incubated in the presence of the ERK1/2 inhibitor (U0126; 10 μM), p38 kinase inhibitor (SB203580; 10 μM) or JNK inhibitor (SP600125; 10 μM) for 2 h prior to OT (100 nM) stimulation for 30 min and 6 h. Whole cell lysates were extracted for Western blot analysis of COX-2 (A) and p-p65 (B). Control with β-actin confirmed equal protein loading (n=6; * p<0.05, ** p<0.01, *** p<0.001 vs NS, § p<0.05 vs OT, ANOVA).
Pre-labour primary amnion cells were incubated in the presence of the ERK1/2 inhibitor (U0126; 10 µM), p38 kinase inhibitor (SB203580; 10 µM) or JNK inhibitor (SP600125; 10 µM) for 2 h prior to OT (100 nM) stimulation for 30 min. Whole cell lysates were extracted for Western blot analysis of phospho-ERK, phospho-HSP27, and phospho-JNK. Control with β-actin confirmed equal protein loading.
4.7. Effect of IL-1β on NF-κB signalling and COX-2 expression

In this study we report a novel mechanism for OT activation of NF-κB, which involves activation of IKKα/β, degradation of IκBα, phosphorylation of p65 and nuclear translocation of p65 homodimers alone. OT-induced activation of NF-κB therefore resembles a classical NF-κB signalling pathway triggered by IL-1β stimulation in amnion cells [353]. To compare and contrast our findings, we used IL-1β stimulated pre-labour amnion epithelial cells as a control.

As reported previously, IL-1β led to nuclear translocation of NF-κB p65 and p50 subunits after 15 min and 30 min of stimulation (p<0.001; Fig. 4.12 A and B) and there was a delayed nuclear translocation of RelB after 1 h (p<0.05; Fig. 4.12 B). This was further confirmed by fluorescent immunocytochemistry (Fig. 4.12 C). To demonstrate that IL-1β activates the classical NF-κB pathway in human amnion, we focused on looking at activation of different subunits of NF-κB signalling pathway. Pre-labour amnion cells were treated with IL-1β (1ng/ml) for 15 min, 30 min, 1 h, 2 h and 4 h. IL-1β stimulation resulted increases in phosphorylation of IKKα/β and p65, and degradation of IκBα after 15 min, 30 min and 1 h, before returning to basal level at 2 h (p<0.05, p<0.001 and p<0.05; Fig. 4.13). This resembles the classical NF-κB signalling pathway, which involves activation of IKKα/β, degradation of IκBα, phosphorylation of p65 and nuclear translocation of p65/p50 heterodimers.

NF-κB is a key modulator of COX-2 expression in amnion [325]. Consistent with this we provided evidence that OT-induced COX-2 expression in the amnion is NF-κB-mediated, however, inhibition of IKKβ using TPCA1 had no effect on IL-1β-induced COX-2 expression. Previous reports have shown that IL-1β-mediated COX-2 expression involved MAPKs, specifically ERK1/2 and p38 kinases, in cardiac myocytes [418] as well as mesangial cells [419], therefore, we aimed to demonstrate the role of MAPKs on IL-1β-induced COX-2 expression. Pre-labour amnion epithelial cells treated with IL-1β showed significant increase in ERK1/2, p38 and JNK phosphorylation, peaking after 30 min (Fig. 4.14). Preincubating the cells with MAPK inhibitors; U0126 (10 µM) and SB203580 (10 µM), for 2 h showed significant reduction in IL-1β-induced COX-2 expression (p<0.05; Fig. 4.15 A). This was not observed in presence of JNK inhibitor, SP600125. Despite the evidence of cross-talk between NF-κB and MAPK pathways in OT-mediated COX-2 expression, this was not shown in IL-1β-treated amnion cells (Fig. 4.15 B).
Figure 4.12. Nuclear translocation of NF-κB subunits with IL-1β treatment. Pre-labour primary amnion cells were stimulated with IL-1β (1ng/ml) for 15 min, 30 min, and 1 h. Western blotting of nuclear and cytosolic protein extracts illustrates nuclear translocation of different NF-κB subunits, p65 (A), p50 and RelB (B). Membranes were probed with α-tubulin and TATA binding protein (TBP) to confirm separation of nuclear and cytosolic extracts (n=4; * p<0.05, ** p<0.01, *** p<0.001 vs NS, ANOVA). Cultures were treated with IL-1β for 30 min and then fixed and immunostained for the NF-κB subunit, p65, and visualized with TRITC. Scale bar, 25µm (C).
Figure 4.13. IL-1β activates NF-κB subunits involved in the classical signalling pathway. Pre-labour primary amnion cells were grown to reach confluence and serum depleted overnight prior to treatments. The cells were stimulated with IL-1β (1ng/ml) for 15 min, 30 min, 1 h, 2 h and 4 h. Whole cell lysates analysed by Western blot demonstrated activation of NF-κB p65 and IKKα/β, and degradation of IκBα upon IL-1β stimulation. Control with β-actin confirmed equal protein loading (n=6; * p<0.05, ** p<0.01 vs NS, ANOVA).
Figure 4.14. IL-1β activates MAPKs, ERK1/2, p38 kinase, and JNK. Pre-labour primary amnion cells were treated with IL-1β (1ng/ml) for 15 min, 30 min, 1 h, 2 h and 4 h, and Western blot analysis was performed to study the effects of IL-1β stimulation on p-ERK1/2, p-p38 and p-JNK expression. Control with β-actin confirmed equal protein loading (n=6; * p<0.05, *** p<0.001 vs NS, ANOVA).
Figure 4.15. IL-1β-induced expression of COX-2 requires independent activation of MAPKs and NF-κB. Pre-labour primary amnion cells were incubated in the presence of the ERK1/2 inhibitor (U0126; 10 µM), p38 kinase inhibitor (SB203580; 10 µM) or JNK inhibitor (SP600125; 10 µM) for 2 h prior to IL-1β (1 ng/ml) stimulation for 30 min and 6 h. Whole cell lysates were extracted for Western blot analysis of COX-2 (A) and p-p65 (B). Control with β-actin confirmed equal protein loading (n=6; * p<0.05, ** p<0.01, *** p<0.001 vs NS, ≠ p<0.05 vs IL-1β, ANOVA).
4.8. Summary and discussion

The simultaneous increase in the sensitivity to OT and PG synthesis in the amnion during the onset of labour has led to the concept that OT/OTR system in the amnion enacts events requisite to parturition that is separate from inducing uterine contractions. The physiology of human labour resembles that of an inflammatory process. PGs has been reported to play a key role in the generation of the inflammatory responses whilst orchestrating fetal membrane rupture, cervical ripening and uterine contractions [420]. It has been established that COX-2 is a key regulator of PG production and a central mediator of inflammation [421]. In human amnion, COX-2 is present at low levels throughout gestation and highly expressed during the onset of labour to drive PG synthesis [403]. Inflammatory cytokines, such as IL-1β, has been shown to upregulate the expression of COX-2 through MAPKs [367, 416] and NF-κB [409, 416, 422, 423] in various cell types. However, little is known about the detailed mechanisms involved in the regulation of the OT-mediated COX-2 expression in human amnion cells. Here, we utilised our primary human amnion epithelial cell model to demonstrate that both the OTR-dependant activation of MAPKs (ERK1/2 and p38 kinase) and subsequent activation of NF-κB signalling cascade are required for OT-induced COX-2 expression.

The NF-κB transcription factor family is a key regulator of inflammatory responses following exposure to extracellular stimuli, and it has been found to play an important role in the expression of a variety of genes involved in inflammatory responses as well as cell survival [208]. It has been established that the activation of NF-κB is strongly associated with parturition as it regulates expression of contraction-associated proteins whilst antagonising the relaxatory effects of progesterone [325]. There is evidence in mouse models that during the onset of labour, NF-κB is activated by the release of surfactant proteins from fetal lung, which indicate pulmonary maturity. Inhibition of NF-κB activity in mouse delays the onset of normal labour [131] and infection-associated preterm labour [424]. In human, there is increase in NF-κB activity in amnion during the onset of labour [325, 413], and NF-κB has been shown to play a critical role in the regulation of COX-2 expression and in the development of inflammatory response. Therefore, we investigated the effect of OT on NF-κB signalling pathway in human amnion. Treatment with OT resulted in nuclear translocation of p65 subunit, but not p50 or RelB (Fig. 4.1 A and B). This was further confirmed by immunofluorescence studies (Fig. 4.1 C). Moreover, we demonstrated that OT stimulates phosphorylation of IKKα/β and p65, and degradation of IκBα (Fig. 4.2). These data clearly indicate that OT activates the classical NF-κB signalling cascade, which resembles that activated by IL-1β (Fig. 4.12 and 4.13). However, the absence of p50 nuclear translocation and p50 supershift upon OT stimulation indicate that unlike IL-1β, OT-induced activation of NF-κB signalling cascade involves p65 homodimers and not p50-p65 heterodimers (Fig. 4.3).
The role of p65 homodimers in the regulation of gene expression has been suggested in previous studies. Early initial studies had suggested that only p50 subunit of NF-κB can bind to DNA [425], however, later investigations demonstrated that the p65 NF-κB subunit also possesses intrinsic DNA binding activity [426]. Fujita et al. showed that p50 and p65 homodimers are both transcriptional activators, but activation is dependent on different parameters [427]; where activation by p65 homodimers are dependent on their carboxy-terminal region and that of p50 is dependent on its conformation. The presence of p65 homodimers have been shown in human T cells where it was found to be capable of associating with IκBα under certain physiologic conditions [428]. Similar to our findings, a proinflammatory mediator, thrombin, has been shown to induce ICAM-1 expression in endothelial cells via NF-κB signalling pathway involving p65 homodimers binding to ICAM-1 promoter region [429].

Activation of NF-κB by OT is not confined to the amnion but also seen in the myometrium. Although we did not observe any activation of NF-κB in MCF-10A cells expressing OTR, human myometrial smooth muscle cells showed activation of p65 NF-κB subunit upon OT stimulation (Fig. 4.5 and 4.6). The components of the NF-κB signalling cascade activated by OT in amnion and myometrium had slight differences. OT resulted in the nuclear translocation of both p65 and p50 in myometrium whereas only p65 translocation was observed in amnion. The involvement of p65/p50 heterodimers in OT-induced NF-κB pathway in myometrium requires further analysis. However, the presence or absence of p50 NF-κB subunit activation can introduce a previously unsuspected element of specificity in regulation of gene expressions as the dimers of p65 and p50 (hetero- or homo-dimers) have been shown to possess variable DNA-binding affinity to NF-κB binding sites of different genes. For instance, in HeLa cell extracts, the IFNβ NF-κB binding site showed no transcriptional activity with p50 homodimers but responded to p65 homodimers and p65/p50 heterodimers [427]. Therefore, this tissue-dependent activation of NF-κB pathway by OT may enable it to control the expression of different cassette of genes in amnion compared to myometrium.

We have demonstrated that OT increases COX-2 expression in amnion. The OT-induced COX-2 expression was completely abolished in the presence of a selective IKKβ inhibitor TPCA1 (Fig. 4.7), which indicates that NF-κB is essential in OT-induced COX-2 expression. This was further confirmed via NF-κB p65 siRNA knockdown experiments (Fig. 4.8) where successful knockdown of p65 expression led to a marked decrease in both basal and OT-induced COX-2 mRNA and protein levels. These results were consistent with COX-2 expression induced by hypoxia, or LPS in various cell types including amnion cells [430, 431]. Opposite to previously studies that demonstrated a role for NF-κB in IL-1β-induced COX-2 expression, we found that IKKβ activation is not required for IL-1β-induced COX-2 expression [353, 423]. This discrepancy in the results may be due to the difference in the NF-κB subunits inhibited in each study as we have used IKK inhibitor whereas Lee et al. used siRNA p65-targeted knockdown and Ackerman et al. used inhibitor of IκBα degradation.
In addition to COX-2, we demonstrated that OT increases the expression of several downstream proinflammatory genes including IL-8, IL-6, CCL2, CCL5 and SOD2 (Fig. 4.4), which are included in the cassette of immune/inflammatory genes regulated by NF-κB in human pregnant myometrium [231]. The major genes upregulated at the time of labour are from inflammatory pathways [432] and these genes have considerable overlap with those upregulated by NF-κB pathway in human myometrium. The gene with the highest increase in expression in these studies was IL-8, which was most highly expressed cytokine in OT-treated amnion cells [417]. In both human and animal models, prolonged exposure to OT has been shown to drive systemic anti-inflammatory effects via depression of proinflammatory cytokine expression, especially TNFα and IL-6, in macrophages, lymphocytes and neutrophils [433, 434]. However, a recent study in human macrophages and monocytes has failed to observe any anti-inflammatory effects of OT [435]. Interestingly, our data demonstrate a novel role of OT in activating NF-κB signalling cascade through which it induces proinflammatory cytokines/chemokines and PG production that can contribute to the onset of labour.

There are several lines of evidence demonstrating that MAPKs can be activated by GPCR agonists [436]. MAPK activation has been implicated in the expression of COX-2 induced by various extracellular stimuli in different cell types. In macrophages, LPS activates ERK1/2 and p38 kinase to induce the transcription factor C/EBPβ and CREB and in turn, mediate the initial stages of COX-2 transcriptional activation [437, 438]. Several proinflammatory cytokines such as IL-1, IFNγ or TNFα have also been shown to induce COX-2 expression via MAPK activation. IL-1β and IFNγ-induced COX-2 expression involved p38 kinase activation [439] whilst TNFα-induced COX-2 expression was heavily regulated by ERK1/2 and NF-κB [440].

In this study, we demonstrated stimulus-specific activation of MAPKs in amnion cells. Both IL-1β and OT triggered the activation of ERK1/2, p38 kinase and JNK (Fig. 4.9 and Fig. 4.14); however, the MAPK response to IL-1β was more prominent. Recent study reported the significance of MAPK pathways in the onset of labour as they demonstrated that increase in activation of MAPKs in term pre-labour fetal membranes overlying the cervix play a role in the degradation of the ECM, promoting membrane rupture [441]. Pretreatment with the MAPK inhibitors U0126 or SB203580 both markedly reduced OT- and IL-1β-induced expression of COX-2 protein (Fig. 4.10 A and 4.15 A). These data suggest that in amnion cells, ERK1/2 and p38 kinase are required for OT- and IL-1β-induced COX-2 expression, consistent with those obtained from various cell types where the upregulation of COX-2 by OT or IL-1β was via ERK1/2 [394, 442]. For the role of p38 kinase, we are the first to demonstrate that p38 kinase plays a critical role in OT-induced COX-2 expression in human amnion epithelial cells.

OT-stimulated NF-κB p65 phosphorylation was significantly inhibited by the MAPK inhibitors U0126 or SB203580 (Fig. 4.10 B), indicating OT induced activation of NF-κB is mediated through
OT-dependent activation of ERK1/2 and p38 kinase. This cross-talk between MAPKs and NF-κB in the regulation of COX-2 expression was not seen with IL-1β stimulation (Fig. 4.15 B), but it has been demonstrated in a variety of other cell types. In endothelial [443] and vascular smooth muscle cells [444], COX-2 expression induced by thrombin were found to be mediated through MAPK; specifically ERK1/2 and p38 kinase, -dependent NF-κB activation. Similarly, Bradykinin (BK) induced COX-2 expression and PGE2 release in astrocytes was reported to be via ERK1/2-mediated NF-κB activation [445]. However, we are the first to report that OT-induced COX-2 upregulation in human amnion cells require sequential activation of MAPK (ERK1/2 and p38 kinase) and NF-κB pathways. The details of how MAPKs regulate NF-κB activation are not clear, but there are evidences suggesting that MAPKs can act directly on IKKα/β and in turn trigger the NF-κB signalling cascade [440]. JNK and p38 kinases have been shown to regulate phosphorylation of IκBα and NF-κB p65 subunit, repectively [446, 447]. Therefore, this interplay between MAPK and NF-κB pathways in the induction of COX-2 needs to be further investigated.

Several reports indicate that COX-2 expression can have both proinflammatory and anti-inflammatory effects depending on the type of prostaglandins it produces. Gilroy et al. [448] showed that during the early inflammatory process when there is induction of COX-2 expression and PGE2 levels are high, COX-2 functions in a proinflammatory manner. In human colon epithelial cells, it was suggested that COX-2-mediated PGE2 release can regulate the transcriptional activity of NF-κB, thus promoting inflammatory processes [449]. Therefore, it is possible that OT-induced COX-2 expression and PGE2 synthesis in human amnion at the time of labour onset can further enhance NF-κB activity and subsequently increase inflammation via a positive feedback system.

To summarise, we have illustrated that OT/OTR system in amnion exerts its effects on COX-2 expression through a sequential activation of ERK1/2, p38 kinase and NF-κB signalling cascades as shown in Figure 4.16. These findings provide new insights into the molecular mechanisms used by OT/OTR receptor system to induce proinflammatory responses via PGs and cytokines synthesis during the onset of labour in human amnion.
Figure 4.1. A model for OT/OTR signalling and action in amnion. OT binding to OTR drives the activation of MAPKs, ERK1/2 and p38 kinase, to activate the classical NF-κB signalling cascade involving p65 homodimers. The NF-κB dimers translocate to the nucleus and binds to κB sites to induce expression of pro-labour genes such as COX-2 and proinflammatory cytokines. This leads to the increase in PG and cytokine synthesis from human amnion cells during the onset of labour.
5. Oxytocin receptor/G-protein coupling in human amnion
5.1. Introduction

Oxytocin receptor (OTR) belongs to the group I G-protein coupled receptor family. It has been established that OT binding to its myometrial receptor leads to an activation of $G_{\alpha q/11}$ and phospholipase C$\beta$ (PLC$\beta$) pathway to generate inositol trisphosphate (IP$_3$), which results in increase in intracellular calcium levels [308]. This OT-stimulated phosphoinositide hydrolysis has been implicated in regulating COX-2 expression [450] as well as myometrial contractility and it has been found to involve both pertussis toxin-sensitive and -insensitive G-proteins, members of $G_{\alpha i}$ and $G_{\alpha q}$ family [451-454]. However, the OTR and G-protein coupling in amnion is yet to be understood. Previous work on rabbit amnion showed that, similar to human myometrium, OT binding to its receptor leads to activation of $G_{\alpha q}/$PLC pathway and increase in intracellular calcium, which in turn activates cPLA$_2$ and COX-2 for PGE$_2$ synthesis [367]. We have shown that in human amnion, OT regulates PGE$_2$ synthesis via COX-2 expression, which requires activation of both NF-$\kappa$B and MAPKs. Our aim was to identify the OTR and G-protein coupling involved in this process.
5.2. OT increases in intracellular calcium levels in human amnion cells

G-protein coupled receptors are capable of coupling to multiple G proteins, which is important for introducing variability in the mechanisms involved in response to a ligand between different cell types. The significance in understanding the receptor-G-protein coupling is becoming more apparent as promiscuity in receptor-G-protein coupling can give rise to great variability in the effects of receptor activation. Therefore the signalling pathways activated by a given GPCR need to be investigated in individual cell types. Since we have verified that OTR is present in human amnion and that OT stimulation results in PGE$_2$ synthesis, a series of experiments were carried out to determine the downstream G-protein signalling pathways involved in this process.

Increases in intracellular calcium levels by OT via G$_{\alpha q}$ coupling has been previously shown in CHO-OTR cells, amnion-derived WISH cells as well as human amnion [317, 455, 456]. Therefore we demonstrated the ability of OT to activate calcium signalling in amnion epithelial cells. Pre-labour amnion cells were grown to reach confluence and they were loaded with calcium dye Fluo-4 prior to OT treatment. Stimulation with OT resulted in a delayed (approximately 450 sec) increase in intracellular calcium levels, reaching a peak after 600 sec (Fig. 5.1 A).

![Graph showing calcium mobilisation by OT stimulation](image1)

**Figure 5.1. Oxytocin stimulates calcium mobilisation in human amnion.** Pre-labour amnion epithelial cells were loaded with calcium-sensitive dye, Fluo-4, before stimulation with OT (100 nM). The changes in the intracellular calcium levels with OT stimulation were determined by live cell imaging on the Leica SP5 confocal microscope (A). The addition of stimulus is indicated by an arrow. Snapshot of calcium loaded amnion cells treated with and without OT (B) (n=3).
5.3. Downstream G\textsubscript{aq} inhibitors and siRNA knockdown of G\textsubscript{aq} do not attenuate the proinflammatory effects of OT

In rabbit amnion, OTR has been shown to couple with G\textsubscript{aq} to induce PLC activity and calcium mobilisation leading to activation of PKC to regulate cPLA\textsubscript{2} phosphorylation and COX-2 expression via MAPKs, and in turn, mediate PGE\textsubscript{2} production [367]. Our work in human amnion epithelial cells demonstrated activation of MAPKs, ERK and p38 kinase, leading to increase in phosphorylation of cPLA\textsubscript{2} and COX-2 expression upon OT stimulation. To investigate the role of G\textsubscript{aq} in this regulatory mechanism, pre-labour amnion epithelial cells were pre-treated for 1 h with different downstream G\textsubscript{aq} inhibitors; U-73122 (PLC inhibitor; U7), GF109203X (PKC-\alpha and -\beta inhibitor; GFX), and Go6983 (broad spectrum PKC inhibitor; Go). The presence of U7 did not inhibit the OT-induced activation of p65, ERK and p38 kinase, but stimulation with U7+OT had additive effect on the activation of ERK and p38 kinase compared to OT alone in a dose-dependent manner (Fig. 5.2 A). This was also observed in the upregulation of COX-2 and activation of cPLA\textsubscript{2}, where U7 was insufficient to suppress the effects of OT but U7+OT resulted in an enhanced expression of COX-2 (Fig. 5.2 B). Similar results were observed with the PKC-\alpha and -\beta inhibitor, GFX (Fig. 5.3 A and B), as well as broad spectrum PKC inhibitor, Go (Fig. 5.4 A and B). Pre-treating the cells with all these inhibitors did not suppress the activation of NF-\kappaB and MAPKs, nor the upregulation of COX-2 and phospho-cPLA\textsubscript{2} upon OT stimulation. Our results indicate that activation of PKC and PLC are not required for OT-induced proinflammatory response in human amnion, thereby suggesting that G\textsubscript{aq} may not be involved in the OT-induced activation of NF-\kappaB, MAPKs, cPLA\textsubscript{2}, and COX-2.

These findings were further confirmed via G\textsubscript{aq} knockdown studies. Pre-labour amnion epithelial cells were grown to confluence in tissue culture flasks and trypsinised to obtain cell suspension. The cells were then transfected with 80 nmol G\textsubscript{aq} targeted siRNA by electroporation. Transfected cells were grown for another 3 days prior to any treatments. The efficiency of the knockdown was validated using Western blot analysis. Successful knockdown of G\textsubscript{aq} did not suppress the OT-induced activation of NF-\kappaB and MAPKs, nor upregulation of COX-2 and p-cPLA\textsubscript{2} (Fig. 5.5).
Figure 5.2. PLC inhibitor, U73122, does not suppress the proinflammatory effects of OT. Pre-labour primary amnion cells were incubated in a downstream G_{αq} inhibitor, PLC inhibitor (U73122; 5 µM/10 µM), for 2 h prior to OT (100 nM) stimulation. Whole cell lysates of cells treated with OT for 15 min and 30 min were subjected to Western blot analysis for p-p65, p-ERK1/2 and p-p38 (A). The expressions of COX-2 and p-cPLA₂ in amnion cells treated with OT for 4 h and 6 h were analysed using Western blotting (B). Control with β-actin confirmed equal protein loading (n=6; * p<0.05, ** p<0.01, *** p<0.001 vs NS, ANOVA).
Figure 5.3. PKC-α and -β inhibitor, GFX, does not suppress the proinflammatory effects of OT. Pre-labour primary amnion cells were incubated in a downstream $G_{\alpha_q}$ inhibitor, PKC-α and -β inhibitor (GFX; 5 µM/10 µM), for 2 h prior to OT (100 nM) stimulation. Whole cell lysates of cells treated with OT for 15 min and 30 min were subjected to Western blot analysis for p-p65, p-ERK1/2 and p-p38 (A). The expressions of COX-2 and p-cPLA$_2$ in amnion cells treated with OT for 4 h and 6 h were analysed using Western blotting (B). Control with β-actin confirmed equal protein loading (n=6; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs NS, ANOVA).
Figure 5.4. A broad spectrum PKC inhibitor, Go6983, does not suppress the proinflammatory effects of OT. Pre-labour primary amnion cells were incubated in a downstream G\(\alpha\)q inhibitor, broad spectrum PKC inhibitor (Go6983; 5 µM/10 µM), for 2 h prior to OT (100 nM) stimulation. Whole cell lysates of cells treated with OT for 15 min and 30 min were subjected to Western blot analysis for p-p65, p-ERK1/2 and p-p38 (A). The expressions of COX-2 and p-cPLA\(_2\) in amnion cells treated with OT for 4 h and 6 h were analysed using Western blotting (B). Control with β-actin confirmed equal protein loading (n=6; * \(p<0.05\), ** \(p<0.01\), *** \(p<0.001\) vs NS, ANOVA).
Figure 5.5. The role of G\(_{\alpha q}\) protein in the OT-mediated activation of NF-κB and MAPK, and up-regulation of COX-2 and p-cPLA\(_2\) in human amnion epithelial cells. Prelabour amnion epithelial cells were subjected to tranfection with non-target siRNA or G\(_{\alpha q}\)-target siRNA (30 pmol) via electroporation prior to OT (100 nM) treatment. Western blot analysis was used to examine the efficiency of G\(_{\alpha q}\) knockdown and its effect on OT-mediated activation of NF-κB and MAPKs, as well as COX-2 and p-cPLA\(_2\) expression. Control with β-actin confirmed equal protein loading. As with chemical inhibition, siRNA knockdown of G\(_{\alpha q}\) did not markedly alter p65 phosphorylation, MAPK activation or COX-2 protein levels (n=3; a \(p<0.05\) vs NS of Nt, b \(p<0.05\) vs NS of G\(_{\alpha q}\) siRNA, ANOVA).
5.4. Pertussis toxin, a $G_{\text{ai}}$ inhibitor, suppresses the OT-stimulated increase in intracellular calcium levels

In previous studies, OTR and $G_{\text{ai}}$ coupling has been shown to result in dissociation of $G_{\beta\gamma}$ subunits leading to activation of PLC$\beta$ and/or calcium channels directly and bring about calcium mobilisation [457, 458]. As data shown above indicate that OT/OTR system in human amnion may not require coupling with $G_{\text{aq}}$ to mediate its proinflammatory effects, we studied the role of $G_{\text{ai}}$ coupling. Pre-labour amnion epithelial cells were grown to confluence and incubated in pertussis toxin (PTX; 500 ng/ml) for 1 h. The cells were then loaded with the calcium dye Fluo-4. The presence of PTX completely inhibited OT-induced calcium response (Fig. 5.6 A and B), thus indicating that calcium mobilisation by OT is mainly via OTR/$G_{\text{ai}}$ coupling.

As mentioned previously, the coupling of OTR and $G_{\text{ai}}$ subunit can activate $G_{\beta\gamma}$ subunits to induce a calcium response. There are two alternative mechanisms that may be used by GPCR to initiate a calcium signal via $G_{\text{ai}}$; a distal activation of calcium via PLC$\beta$ and IP$_3$ [459] and a local activation of IP$_3$ receptors (IP$_3$Rs) with $G_{\beta\gamma}$ subunit via direct binding [457, 458]. In order to determine whether PLC$\beta$/IP$_3$ is involved in OT induced calcium response in human amnion, IPOne HTRF assay kit was utilised to measure accumulation of IP$_1$ with OT stimulation. Pre-labour amnion epithelial cells were plated into white small-volume 384 culture plates with approximately 20,000 cells per well. The cells were incubated in a buffer containing Lithium Chloride (LiCl) which leads to accumulation of IP$_1$, an IP$_3$ metabolite, upon receptor activation. Amnion cells were treated with a series of OT dilutions ranging from 1 pM to 1 µM for 1 h and stimulation with ATP was used as a positive control. We found no accumulation of IP$_1$ in presence of OT, even at the highest concentration of 1 µM (Fig. 5.6 C). This suggests that in human amnion, OT may activate a calcium response via an IP$_3$-independent pathway.
Figure 5.6. Calcium mobilisation mediated by OT is PTX-sensitive and independent of IP-1 increase. Pre-labour amnion epithelial cells were pretreated with a $G_{\alpha_i}$ inhibitor, pertussis toxin (PTX; 0.5 µg/ml), and loaded with calcium-sensitive dye, Fluo-4, before stimulation with OT (100 nM). The changes in the intracellular calcium levels with OT stimulation were determined by live cell imaging on the Leica SP5 confocal microscope (A). The addition of stimulus is indicated by an arrow. Preincubation with PTX resulted in complete inhibition of OT-induced calcium mobilisation (B). For IPOne HTRF assay, amnion cells cultured in a low-volume white 384-well plates were stimulated with increasing dose of OT (1 pM - 1 µM) over time, and the IP-1 concentrations were extrapolated from a standard curve (C). Treatment with ATP was used as a positive control ($n=3$).
5.5. The proinflammatory role of OT in human amnion is via OTR-\(\alpha_i\) coupling

The increased ability of human amnion cells to synthesise prostaglandins is accomplished by sequential activation of MAPKs, ERK and p38 kinase, and NF-κB. As amnion OTR couples with \(\alpha_i\) to initiate calcium mobilisation, which is considered a pro-labour response, it was postulated that this then might regulate the downstream proinflammatory responses exerted by OT. This potential link was tested by examining the effects \(\alpha_i\) inhibitor (PTX) and siRNA \(\alpha_i\)-target knockdown may have on the activation of NF-κB and MAPK signalling, and COX-2 expression in human amnion cells. Initially, the pre-labour amnion epithelial cells were incubated in PTX (0.2 µg/ml and 0.5 µg/ml) for 1 h prior to OT stimulation. Analysis using Western blot showed significant decrease in OT-induced activation of NF-κB and MAPKs in a dose-dependent manner (Fig. 5.7 A). The presence of \(\alpha_i\) inhibitor, PTX, also dampened the effect of OT on the expression of downstream genes including COX-2 and p-cPLA2 (Fig. 5.7 B), suggesting that OTR-\(\alpha_i\) coupling is required in the regulation of OT-mediated expression of COX-2 and p-cPLA2. In addition, pretreatment with PTX had downstream effect on PGE2 synthesis upon OT stimulation (Fig. 5.8), indicating that OTR and \(\alpha_i\) coupling may act as a key regulator of OT-induced proinflammatory effects in human amnion.

There are three different subtypes of human \(\alpha_i\) proteins identified to date; \(\alpha_{i1}\), \(\alpha_{i2}\) and \(\alpha_{i3}\) [460]. As \(\alpha_i\) proteins play a significant role in regulating the effect of OT in human amnion, the next set of experiments were conducted to identify the subtype of \(\alpha_i\) protein involved. Primers were designed for the three subtypes of \(\alpha_i\) proteins and their expressions were studied using standard PCR. Total of 5 pre-labour amnion samples from 5 different patients were used for the PCR, along with two pre-labour myometrial samples as positive controls. The result showed low expression of \(\alpha_{i1}\) in myometrial cells but none in amnion (Fig. 5.9 A) and a higher expression of \(\alpha_{i2}\) and \(\alpha_{i3}\) in both human myometrial and amnion cells (Fig. 5.9 B and C).

To determine which of the two subtypes expressed in amnion are involved in the regulation of OT induced proinflammatory effects, siRNA knockdown of \(\alpha_{i2}\) and \(\alpha_{i3}\) was used. Pre-labour amnion epithelial cells were grown to confluence and transfected with \(\alpha_{i2}\) and \(\alpha_{i3}\) -target siRNA using electroporation. Knockdown of \(\alpha_{i2}\) significantly inhibited the activation of NF-κB and both ERK and p38 kinase by OT (Fig. 5.10). This in turn resulted in suppression of COX-2 expression and cPLA2 activation (Fig. 5.10). Similar results were observed with \(\alpha_{i3}\) knockdown to a lesser extent, except for activation of p65, which was not affected (Fig. 5.11), implicating that these both \(\alpha_i\) subtypes expressed in amnion can couple with OTR to regulate the proinflammatory responses to OT in human amnion.
Figure 5.7. The proinflammatory effects of OT are PTX-sensitive. Prelabour amnion epithelial cells pretreated with PTX (0.2 µg/ml or 0.5 µg/ml) were stimulated with OT over time. Western blot analysis was used to examine the effect of PTX on OT-mediated activation of NF-κB and MAPKs (A), as well as COX-2 and p-cPLA₂ expression (B). Control with β-actin confirmed equal protein loading (n=6; * p<0.05, ** p<0.01, *** p<0.001 , ANOVA).
Figure 5.8. OT-induced PGE$_2$ synthesis is PTX-sensitive. Pre-labour amnion epithelial cells were serum depleted overnight and treated with PTX (0.5 µg/ml) prior to OT (100 nM) stimulation. The culture media were collected for PGE$_2$ EIA analysis (n=6; ** $p<0.01$ vs NS, ≠ $p<0.05$ vs OT 6h, ANOVA).

Figure 5.9. Human amnion expresses different subtypes of G$_{ai}$ proteins. Prelabour amnion epithelial cells were established from 5 different samples. cDNAs were synthesized using total RNA extracted from non-stimulated amnion cells. Products of PCR reactions with primers specific for G$_{ai1}$, G$_{ai2}$ and G$_{ai3}$ were analysed on 2% agarose gel. G$_{ai1}$ (A) was not detectable in amnion, however, G$_{ai2}$ (B) and G$_{ai3}$ (C) were detected. Myometrial cDNA was used as positive controls (+) (G$_{ai1}$ 115 bp, G$_{ai2}$ 212 bp and G$_{ai3}$ 154 bp).
Figure 5.10. The role of Gᵦᵢ₂ protein in the OT-mediated activation of NF-κB and MAPK, and up-regulation of COX-2 and p-cPLA₂ in human amnion epithelial cells. Prelabour amnion epithelial cells were subjected to tranfection with non-target siRNA or Gᵦᵢ₂-target siRNA (30 pmol) via electroporation prior to OT (100 nM) treatment. Western blot analysis was used to examine the efficiency of Gᵦᵢ₂ knockdown and its effect on OT-mediated activation of NF-κB and MAPKs, as well as COX-2 and p-cPLA₂ expression. Control with β-actin confirmed equal protein loading. As with chemical inhibition, siRNA knockdown of Gᵦᵢ₂ abated the OT-mediated activation of NF-κB, ERK and p38, and the consequent COX-2 and p-cPLA₂ levels (n=3; a p<0.05 vs NS of Nt siRNA, b p<0.05 vs NS of Gᵦᵢ₂ siRNA, c p<0.05 Nt siRNA vs Gᵦᵢ₂ siRNA, ANOVA).
Figure 5.11. The role of \( G_{\alpha3} \) protein in the OT-mediated activation of NF-\( \kappa \)B and MAPK, and up-regulation of COX-2 and p-cPLA\(_2\) in human amnion epithelial cells. Prelabour amnion epithelial cells were subjected to transfection with non-target siRNA or \( G_{\alpha3} \)-target siRNA (30 pmol) via electroporation prior to OT (100 nM) treatment. Western blot analysis was used to examine the efficiency of \( G_{\alpha3} \) knockdown and its effect on OT-mediated activation of NF-\( \kappa \)B and MAPKs, as well as COX-2 and p-cPLA\(_2\) expression. Control with \( \beta \)-actin confirmed equal protein loading (\( n=3 \); a \( p<0.05 \) vs NS of Nt siRNA, b \( p<0.05 \) vs NS of \( G_{\alpha3} \) siRNA, c \( p<0.05 \) Nt siRNA vs \( G_{\alpha3} \) siRNA, ANOVA).
5.6. Summary discussion

Oxytocin receptors are coupled to various G proteins including G\textsubscript{aq}, G\textsubscript{ai} and G\textsubscript{as} and they can regulate diverse cellular functions through multiple signalling pathways. Although the subtypes of G proteins involved in OT-induced COX-2 expression in certain target cells is well recognised, particularly in myometrium [451-454, 461], that in human amnion have not been investigated. The results in this study indicate that G\textsubscript{ai} and not G\textsubscript{aq} coupling, is involved in the OT-initiated signal transduction in human amnion epithelial cells.

In rabbit amnion, OT binding to OTR typically leads to G\textsubscript{aq} coupling and increase in PLC activity [367]. This increases both DAG and IP\textsubscript{3} formation, resulting in an increase in intracellular Ca\textsuperscript{2+} levels, subsequently favouring PKC activation to stimulate MAPK activation. In human amnion, we have shown an influx of calcium upon OT stimulation (Fig. 5.1). However, we found that pretreatment with U-73122 (PLC inhibitor; U7), GF109203X (PKC-\alpha and -\beta inhibitor; GFX), or Go6983 (broad spectrum PKC inhibitor; Go) was insufficient to interrupt OT-mediated MAPK and NF-\kappaB activation and the consequent COX-2 and phospho-cPLA\textsubscript{2} expression (Fig. 5.2-4). Interestingly, treatment with the inhibitors alone also resulted in increased activation of MAPK and NF-\kappaB, as well as downstream COX-2 and phospho-cPLA\textsubscript{2} expression. This may be due to the non-selective effects of the inhibitors.

Previous work in CHO cells have demonstrated that the PLC inhibitor, U-73122 can cause marked effects on cell morphology and integrity at higher concentrations of 4-15\muM [462]. It is possible that the ability for U-73122 to potentiate its effects may be associated to events that are involved in these morphological changes. As the cytolysis induced by U-73122 could be cell-dependent, it would be important to study these effects in the future. In addition to this, there has been an accumulation of reports that indicate undesired side-effects of U-73122. In pancreatic acinar cells and NG108-05 cells, it has been shown to activate cation channels and drive oscillations in intracellular Ca\textsuperscript{2+} levels [463, 464], and it has also been shown to activate tyrosine kinases or inhibit tyrosine phosphatases in platelets [465]. Non-specific effects have been reported with PKC inhibitors, GF109203X and Go6983. In human platelets, treatment with PKC inhibitors demonstrated enhancement of ligand-induced Ca\textsuperscript{2+} release as well as Ca\textsuperscript{2+} entry [466]. Therefore, it can be concluded that both PLC and PKC inhibitors can have multiple effects and thus using them as a tool to identify the role of PLC/PKC activity in cellular events may be limited.

The role of calcium in OT signalling needs to be investigated further, but these data do not support the role of G\textsubscript{aq}/PLC or PKC pathways in the regulation of OT-mediated effects. This complements the finding from PHM1 cells, where OT-induced activation of ERK1/2 was found to be independent of PLC or PKC pathways [467]. In human myometrial cells, OT-induced COX-2 expression and PG synthesis were also reported to be independent of PKC activation [394]. In contrast to this, CHO cells expressing OTR, demonstrate that activation of both PLC and PKC, as well as calcium increase are
essential for OT-induced ERK2 activation and PG synthesis [442]. Therefore, it is possible that the relative importance of PLC/PKC in OT signalling is cell type-dependent.

To further confirm that G_{aq} signalling is not required for OT to exert its effects in human amnion, G_{aq} targeted knockdown was performed. Under conditions where G_{aq} proteins were successfully downregulated by targeted siRNA transfection, OT-induced activation of MAPKs and NF-κB and the consequent COX-2 expression was not significantly affected (Fig. 5.5), suggesting that OTR-G_{aq} coupling may not be required to drive the effects of OT in human amnion. Krumins and Gilman reported that reductions in G_{αi} protein concentrations are associated with compensatory changes in the levels of other G proteins including G_{αi} [468]. Thus, although we have demonstrated that G_{aq} targeted knockdown has no effect on OT signalling, we cannot eliminate the potential role of G_{aq} due to the compensatory effects.

Previous report in cultured myometrial cells demonstrated that OT stimulated calcium response, ERK phosphorylation and COX-2 expression are PTX sensitive to an extent, indicating a possible involvement of G_{αi} [394, 451, 453, 469, 470]. Therefore, we used PTX (G_{αi} inhibitor) to disrupt G_{αi} signalling in amnion and studied its effects on OT responses. We observed an inhibitory effect of PTX on OT-induced increase in intracellular calcium was observed in human amnion (Fig. 5.6 B), consistent with previous findings in rats. However, despite evidence of OT stimulating PLCβ/IP_3 increase to drive accumulation of intracellular calcium in human myometrium [452, 453], such increase in IP_3 was not observed in amnion (Fig. 5.6 C). Our findings resemble the calcium signalling evoked by vasoactive intestinal peptide (VIP) binding to G_{αi} coupled receptor, which does not elicit a measurable increase in IP_3 [457]. It has been previously demonstrated that G_{αi} coupled receptors can mediate calcium signalling via G_βγ [459], independent of IP_3, as G_βγ can directly regulate multiple channels including IP_3R, inward-rectifying K^+ channels and the L-type Ca^{2+} channels [471]. G_βγ are able to regulate adenylyl cyclase [472], PLCβ [473] and cPLA_2 [474] directly, however, we did not see a PLC/PKC-dependent signalling with OT stimulation. Therefore, a potential explanation for our finding is that OTR-G_{αi} coupling in amnion uses a PLC/IP_3/PKC-independent pathway, mediated via G_βγ, to trigger increase in intracellular calcium levels. Pretreatment with PTX was successful in suppressing OT-induced MAPK and NF-κB activation (Fig. 5.7 A) as well as COX-2 and p-cPLA_2 expression at protein level (Fig. 5.7 B), resulting in a decrease in OT-induced PGE_2 production (Fig. 5.8). Activation of cPLA_2 requires calcium and phosphorylation at Ser505 by active MAPKs and both of which can be regulated by G_{αi} [475, 476]. In summary, our data indicate that in human amnion OT binds to OTR coupled to G_{αi}, which then activates a calcium response via a PLC/IP_3/PKC-independent pathway to contribute to the sequential activation of MAPK and NF-κB signalling cascades, ultimately mediating PGE_2 synthesis.
Although we have demonstrated a functional coupling of amnion OTRs to G\textsubscript{\textit{ii}} proteins using PTX, it is still a matter of debate as PTX treatment alone have been reported to affect cAMP levels in myometrial cells [467]. PTX acts by ADP-ribosylation, which irreversibly inhibits G\textsubscript{\textit{ii}} activation, thus PTX sensitivity is considered to be an indication for G\textsubscript{\textit{ii}} involvement. However, PTX treatment has been shown to lead to inhibition of PLC by increasing cAMP concentrations and activating PKA [311, 477]. It also inhibits MAPK activation by preventing transmission of signals from Ras to c-Raf [478-480]. Therefore, inhibition of OT-induced activation of MAPKs and NF-\textit{\kappa}B, and expression of COX-2 and p-cPLA\textsubscript{2} by PTX pretreatment does not necessarily constitute as proof of OTR-G\textsubscript{\textit{ii}} coupling [467]. To overcome this limitation, G\textsubscript{\textit{ii}} targeted siRNA knockdown studies were performed. Prior to siRNA knockdown, we first demonstrated expression of different G\textsubscript{\textit{ii}} isoforms in human amnion (Fig. 5.9). Keeping in mind that we only found expression of G\textsubscript{\textit{ii}}-2 and -3, the involvement of these subtypes were confirmed as G\textsubscript{\textit{ii}}-2 targeted knockdown reduced OT-induced activation of MAPKs and NF-\textit{\kappa}B, and expression of COX-2 and p-cPLA\textsubscript{2} (Fig. 5.10), and to a lesser extent by G\textsubscript{\textit{ii}}-3 targeted knockdown (Fig. 5.11). These data suggest that G\textsubscript{\textit{ii}}-2 predominantly mediates OT-induced responses in human amnion. Previous work in rat myometrium has established a strong association between OTR/G\textsubscript{\textit{ii}} coupling and cPLA\textsubscript{2} [461]. Cohen-Tannoudji \textit{et al.} showed up to 41% increase in the concentration of G\textsubscript{\textit{ii}}-2 in rat myometrium, second to G\textsubscript{\textit{iq}} increase of 2-folds, at the end of gestation whereas G\textsubscript{\textit{ii}}-3 decreased with advancing gestation [481]. These findings combined provide further support for a more prominent role of G\textsubscript{\textit{ii}}-2 compared to G\textsubscript{\textit{ii}}-3 during parturition, as they imply that other than G\textsubscript{\textit{iq}}, G\textsubscript{\textit{ii}}-2 is the main form of G\textsubscript{\textit{ii}} associated with OTR at the time of labour.

The summary of the mechanistic pathways involved in OT induced PG synthesis is shown in Fig.5.12. In human amnion, OT binds to OTR and with OTR/G\textsubscript{\textit{ii}-2} coupling, it drives the activation of ERK1/2 and p38 kinase, which subsequently trigger NF-\textit{\kappa}B activation. The p65-p65 homodimers of NF-\textit{\kappa}B transcriptional pathway translocate to the nucleus and result in an increase in COX-2 expression whilst activating cPLA\textsubscript{2} to promote PGE\textsubscript{2} production. The details of how G\textsubscript{\textit{ii}} drives MAPK and NF-\textit{\kappa}B activation is still not clear, the potential involvement of G\textsubscript{\textit{ii-3}} and direct association between G\textsubscript{\textit{ii}} and downstream effectors, such as calcium and cAMPs, need to be studied further.
Figure 5.12. A modified model for OT/OTR signalling and action in amnion. OT binding to OTR drives $G_{\alpha i2}$ signalling, which leads to sequential activation of MAPKs, ERK1/2 and p38 kinase, and NF-κB. The NF-κB p65 homodimers translocate to the nucleus and bind to κB sites to induce expression of COX-2. This leads to the increase in PG synthesis in human amnion cells during the onset of labour. It is hypothesized that the PLC/IP$_3$/PKC-independent calcium mobilisation induced by OT is via $G_{\beta/\gamma}$ proteins, which is capable of activating ion channels directly. The combination of calcium influx and MAPK activation triggers phosphorylation of cPLA$_2$, another key enzyme in PGE$_2$ synthesis. However, the link between calcium mobilisation and activation of MAPK and NF-κB signalling cascades requires further examination.
6. Effect of oxytocin receptor antagonists on OT-induced proinflammatory effects
6.1. Introduction

Although advances have been made in prediction and prevention of preterm birth in women identified as high risk based upon past history, the overall rate has not reduced. The 2012 WHO report “Born Too Soon” [482] highlighted the need for effective tocolytic drugs to delay preterm birth in women with preterm contractions. There are several classes of tocolytic agents developed including magnesium sulphate [76], calcium channel blockers [74], nitric oxide donors [73], COX inhibitors [77], betamimetics [75], and OTR antagonists [333].

Oxytocin is one of the strongest uterotonic agents known to stimulate uterine contractions and there is evidence to support that OT plays a key role in the initiation and regulation of both term and preterm labour. It has been shown that OT concentrations as well as sensitivity to OT increases markedly in both myometrium and fetal membranes prior to the onset of labour, which in turn leads to increase in PGF$_{2\alpha}$ and PGE$_2$ synthesis [181, 309]. Thus, there is a strong rationale for designing tocolytics that target OT/OTR system to inhibit premature uterine contractions. OTR antagonists are designed to block the increase in intracellular calcium levels leading to relaxation of the myometrium [483]. There are a number of peptide and nonapeptide OTR antagonists produced, with the most-studied OTR antagonist being atosiban. The effectiveness of OTR antagonists has been reviewed previously with conflicting results. A randomised, double-blind, placebo-controlled trial using an oxytocin receptor antagonist, atosiban, showed that atosiban prolonged the pregnancy for up to 7 days in women at a gestational age less than 28 weeks with minimal maternal-fetal adverse effects [336]. However, more recent reviews of multiple randomised controlled trials concluded that atosiban did not reduce the risk of preterm birth before 37 weeks, 32 weeks or 28 weeks [484], nor improve neonatal outcome [78].

We have shown in previous chapters that OT not only triggers uterine contractions but also activates proinflammatory responses in human amnion to initiate the onset of labour. The aim of the following chapter was to confirm that OT exerts its effect via OTR (not via AVPR1A), and to investigate the efficiency of different OTR antagonists in inhibiting the OT-induced proinflammatory responses. OTR antagonists were anticipated to result in down-regulation of PGE$_2$ and cytokine synthesis, by preventing activation of NF-$\kappa$B and/or MAPK pathways. Currently available OTR antagonists, atosiban and ornithine vasotocin (OVT) were employed to study their effectiveness. The proposed experiments aimed to expand the knowledge of human parturition and enable rapid progress in the management of preterm labour.
6.2. Human amnion epithelial cells express vasopressin receptor

OT binds not only to OTR, but also to vasopressin receptors [485], however, there is limited evidence suggesting a role for vasopressin receptors in the processes of parturition. The uterus contains AVPR1A, but its expression does not change during gestation or labour [486]. Similar findings were observed in ovine fetal membranes, where there were no significant changes in the AVPR1A mRNA levels throughout gestation [487]. A study by Czaja et al. showed that rabbit amnion membranes were completely devoid of AVPR1A [488], however, the presence or absence of AVPR1A in human amnion is yet unclear. As both OTR and AVPR1A are capable of coupling to G\textsubscript{iq} and G\textsubscript{ia} proteins [451, 485], we deemed it important to investigate whether human amnion expresses AVPR1A. Initially, cDNAs from amnion were subjected to GAPDH PCR amplification to verify their integrity. Then they were subjected to PCR amplification using primers targeting AVPR1A, followed by SYBR safe stained 1.5% agarose gel electrophoresis. The PCR products were visualised by UV light and a AVPR1A PCR amplicon of the expected size (452 bp) was observed in all amnion samples (Fig. 6.1 A). Placental cDNA were used as positive controls. The PCR amplicons from these samples were then purified using QIAquick PCR purification kit and sequenced to confirm specificity. The DNA sequence information confirmed the product as AVPR1A (Fig. 6.1 B).
Figure 6.1. Human amnion expresses arginine vasopressin receptor 1A. Prelabour amnion epithelial cells were established from 5 different samples. cDNAs were synthesized using total RNA extracted from non-stimulated amnion cells. Products of PCR reactions with primers specific for arginine vasopressin receptor (AVPR1A) were analysed on 2% agarose gel. PCR products of expected size, 473 bp, were detected (A). Placental cDNA was used as positive controls (P). The PCR product was purified and subjected to DNA sequencing. AVPR1A cDNA and PCR product sequences were aligned using BOXSHADE (B).
6.3. Ornithine vasotocin suppresses the proinflammatory effects of OT

There have been numerous studies on structural modification of OT to develop potent in vivo antagonists of OT. The du Vigneaud and Rudinger laboratories first reported in vitro OT antagonists with structural modifications [489, 490], which were then shown to be fully effective in vivo [491]. Later antagonists replaced the Tyr² by Tyr(Me)² and Leu⁸ by Orn⁸, and resulted in significant enhancement of in vivo oxytocic antagonism [492]. However, these modified OT antagonists lacked specificity with regard to V1 receptors. This led to development of new analogues which replaced the Gln⁴ to Thr⁴ and Gly-NH₂⁹ to Tyr-NH₂⁹, exhibiting similar in vivo oxytocic antagonism as [d(CH₂)₅,Tyr(Me)²,Orn⁸] vasotocin, but acting as a much weaker vasopressor antagonists [490]. A potent, highly specific OT antagonist, [d(CH₂)₅,Tyr(Me)², Thr⁴, Orn⁸, Tyr-NH₂⁹] vasotocin (OVT), was utilised in this study to verify whether the proinflammatory effects of OT in amnion are mediated specifically via OTR. Pre-labour amnion epithelial cells were treated with OVT (1 µM) for 30 min and stimulated with OT (100 nM) for 5 min, 15 min, 30 min, 1 h, 2 h, 4 h and 6 h. The presence of OVT significantly reduced the activation of MAPKs and NF-κB by OT (Fig. 6.2 A), as well as reducing OT-induced COX-2 and p-cPLA₂ expression to basal level (Fig. 6.2 B). These findings support our hypothesis that in human amnion, OT is binding to OTR to activate ERK, p38 kinase, and NF-κB, which in turn increases PGE₂ and cytokine production.
Figure 6.2. OT-induced proinflammatory effects are predominantly via OTR. Prelabour amnion epithelial cells pretreated with a potent OTR-A, OVT (1 µM), were stimulated with OT over time. Western blot analysis was used to examine the effect of OVT on OT-mediated activation of NF-κB and MAPKs (A), as well as COX-2 and p-cPLA₂ expression (B). Control with β-actin confirmed equal protein loading (n=6; * p<0.05, ** p<0.01, *** p<0.001, ANOVA).
6.4. Atosiban does not inhibit OT-induced proinflammatory effects

Atosiban is a currently available competitive OT and vasopressin antagonist and it is one of the most studied OT antagonists. Atosiban was developed specifically for the treatment of preterm labour and was designed to have uterine specificity thus minimizing multiorgan side-effects. Preliminary studies have shown atosiban to be effective in inhibiting uterine contractions in both animal [493, 494] and human [294, 495, 496] in vitro and in vivo models. Despite initial reports of atosiban as a promising tocolytic agent, more recent meta-analysis of various clinical trials concluded that atosiban does not have a significant effect on the risk of preterm birth, neonatal morbidity or perinatal mortality [78, 484]. The limited efficacy of atosiban may be due to its mechanism of action and its high affinity to vasopressin receptors [483].

Having shown that OT exerts its proinflammatory effects via OTR, we aimed to examine the effect of OTR-A, atosiban, on OT-induced activation of NF-κB and MAPKs, as well as upregulation of PG synthetic enzymes. Pre-labour amnion epithelial cells were pre-treated with atosiban (10 µM and 20 µM) for 30 min and then stimulated with OT (100 nM) for 5 min, 15 min, 30 min, 2 h, 4 h and 6 h. OT consistently activated NF-κB, ERK and p38 kinase after 15 min, 5 min and 30 min of stimulation respectively ($p<0.05$; Fig. 6.3 A). The presence of atosiban did not affect the effect of OT on NF-κB, ERK and p38 kinase (Fig. 6.3 A). The upregulation of COX-2 expression as well as the increase in activation of cPLA$_2$ by OT was unaffected by atosiban (Fig. 6.3 B). These findings suggest that atosiban may be effective in inhibiting uterine contractions to certain extent, but it is insufficient in inhibiting the proinflammatory effects of OT in human amnion.
Figure 6.3. Atosiban fails to inhibit the proinflammatory effects of OT. Prelabour amnion epithelial cells pretreated with atosiban (10 µM or 20 µM), were stimulated with OT over time. Western blot analysis was used to examine the effect of atosiban on OT-mediated activation of NF-κB and MAPKs (A), as well as COX-2 and p-cPLA₂ expression (B). Control with β-actin confirmed equal protein loading (n=6; * p<0.05, ** p<0.01 vs NS, ANOVA).
Atosiban activates MAPKs and NF-κB, and upregulates subsequent expression of COX-2 and cPLA₂

Pretreatment with atosiban was unable to inhibit the proinflammatory effects of OT in amnion. To study the effect of atosiban on its own, pre-labour amnion epithelial cells were treated with atosiban alone (10 μM) or atosiban and OT together for 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 6 h. Western blot analysis showed significant increase in phosphorylation of NF-κB p65, ERK1/2 and p38 kinase with both atosiban treatment alone \((p<0.01; \text{Fig. 6.4 A})\), and atosiban+OT \((p<0.05; \text{Fig. 6.4 A})\). However, there were no significant differences between the effects of atosiban alone and that of atosiban+OT treatments. The proinflammatory effects of atosiban were not limited to the activation of inflammatory mediators such as MAPKs and NF-κB but it also resulted in the upregulation of downstream pro-labour gene expression; COX-2 and p-cPLA₂, to similar extent as OT treatments (Fig. 6.4 B). This suggests that Atosiban may have a proinflammatory effect in human amnion.
Figure 6.4. Atosiban activates NF-κB and MAPK, and upregulates inflammatory genes in human amnion epithelial cells. Prelabour amnion epithelial cells were treated with atosiban (10 µM or 20 µM) and OT (100 nM) simultaneously over time. Western blot analysis was used to examine the effect of atosiban and OT on the activation of NF-κB and MAPKs (A), as well as COX-2 and p-cPLA2 expression (B). Control with β-actin confirmed equal protein loading. Atosiban treatment alone led to significant activation of p65, ERK and p38. Similarly, COX-2 and p-cPLA2 levels were increased in atosiban treated cells (n=6; * p<0.05, ** p<0.01, *** p<0.001 vs NS, ANOVA).
6.6. Atosiban stimulates expression of downstream NF-κB regulated genes as well as PGE$_2$ production

Our data implies that atosiban acts as a selective agonist to induce activation of proinflammatory pathways in human amnion. To determine whether atosiban has any effect on the downstream genes such as cytokine and prostaglandins, pre-labour amnion epithelial cells were grown to confluence, and treated with atosiban (10 µM) for 1 h, 2 h, 4 h, 6 h and 24 h. NF-κB and MAPKs were activated upon atosiban treatment to similar extent as OT treatment. The mRNA expression of downstream NF-κB regulated genes with atosiban was further studied. Atosiban increased mRNA expression of COX-2 and IL-6 by approximately 2-fold after 4 h, and CCL5 by 1.8-fold after 2 h ($p<0.05$; Fig. 6.5 A-C). We found increases in IL-6 and CCL5 with atosiban at protein level; IL-6 increased by 3-fold, reaching a statistical significance after 2 h ($p<0.05$; Fig. 6.5 D), and CCL5 increased by 2-fold ($p<0.05$; Fig. 6.5 E). The expression of IL-8, CCL2 and SOD2 were not affected by atosiban.

As previous result showed increase in the expression of PG synthetic enzymes, COX-2 and p-cPLA$_2$, with atosiban, the culture media from treated cells were collected and analysed for PGE$_2$ release. The PGE$_2$ ELISA showed an increase in PGE$_2$ concentration with atosiban treatment compared to NS controls, peaking after 4 h stimulation and reduced back to basal level by 6 h ($p<0.05$; Fig. 6.6). The details of how atosiban triggers activation of NF-κB and MAPK to regulate expression of different genes are yet unclear. Further investigations focusing on confirming the biased agonist effect of atosiban and determining the G-protein signalling involved in atosiban-OTR binding can aid the future development of tocolytic drugs.
Figure 6.5. Upregulation of inflammatory genes by atosiban in human amnion epithelial cells. Downstream NF-κB regulated genes, COX-2 (A), IL-6 (B), and CCL5 (C) all showed increased expression in response to atosiban (10 µM) treatment as assessed by qRT-PCR. Transcript levels were normalised to the housekeeping gene, L19 (n=6, *p<0.05 vs non stim, ANOVA). The collected culture media were used for IL-6 (D) and CCL5 (E) ELISA (n=3; *p<0.05 vs NS, ANOVA).
Figure 6.6. Increase in the production of PGE$_2$ upon atosiban treatment. Pre-labour amnion epithelial cells were serum depleted overnight and treated with atosiban (10 µM) over time. The culture media were collected for PGE$_2$ EIA analysis (n=6; * $p<0.05$ vs NS, ANOVA).
6.7. OTR-G$_{ai}$ coupling is involved in atosiban-mediated proinflammatory response

Recent studies have suggested biased agonist effect of OTR-A, specifically atosiban, where it has been shown to inhibit G$_{ai}$ signalling whilst acting as a G$_{ai}$ agonist in cell lines. To elucidate the G$_{ai}$ signalling involved in atosiban-induced proinflammatory effects in human amnion, pre-labour amnion cells were pre-incubated in PTX (0.5 µg/ml) and treated with atosiban (10 µM) for 5 min, 15 min, 30 min, 2 h, 4 h and 6 h. Both OT and atosiban significantly increased phosphorylation of NF-κB and MAPKs. Presence of PTX suppressed atosiban-induced activation of NF-κB p65 and ERK1/2 by 64% and 69% respectively, reaching basal level (p<0.05; Fig. 6.7 A). The activation of p38 kinase was inhibited to some extent but did not reach significance (Fig. 6.7 A). Similarly, atosiban-induced COX-2 and p-cPLA$_2$ expressions were suppressed in presence of PTX (p<0.05, p<0.001; Fig. 6.7 B), indicating a role for G$_{ai}$ in atosiban-mediated proinflammatory effects.
Figure 6.7. Atosiban-induced proinflammatory effects are PTX-sensitive. Prelabour amnion epithelial cells pretreated with PTX (0.5µg/ml) for 30 min and stimulated with atosiban (10 µM) over time. Western blot analysis was used to examine the effect of PTX on atosiban-mediated activation of NF-κB and MAPKs (A), as well as COX-2 and p-cPLA2 expression (B). Control with β-actin confirmed equal protein loading (n=6; * p<0.05, ** p<0.01, *** p<0.001, ANOVA).
6.8. Summary and discussion

It has been previously demonstrated that PGE$_2$ synthesised in amnion by inducible COX-2 is involved in the onset of labour. OT has been shown to upregulate COX-2 expression via MAPKs and NF-κB activation in various cell types. We are the first to demonstrate that OT mRNA level is elevated in amnion with labour and OT binding to OTR drives G$_{ai}$-2 signalling to activate MAPK-dependent NF-κB signalling pathway leading to subsequent cPLA$_2$ and COX-2 expression, and PGE$_2$ release.

OT principally activates OTR which is coupled to various G proteins including G$_{q}$ and G$_{i}$ [497]. It is known that OT binding to vasopressin receptor can trigger downstream signalling [485]. The signalling pathways activated by AVPR1A have been studied extensively in several different cell types [498-500]. Activation of AVPR1A coupled to G-proteins has been shown to drive calcium response and in turn increase PLC and PKC activity resulting in stimulation of transcription factors such as c-fos and c-jun [499]. We have shown that human amnion cells express AVPR1A (Fig. 6.1), and have confirmed that the downstream proinflammatory effects of OT during the onset of labour are through OTR and not AVPR1A. Pretreatment with OVT (specific OTR antagonist) reduced OT-induced ERK1/2 and p38 kinase activation and the subsequent activation of NF-κB (Fig. 6.2). COX-2 protein expression as well as phosphorylation of cPLA$_2$ was reduced in presence of OVT, suggesting that OTR predominantly mediates OT-induced PGE$_2$ release in amnion cells. Our data are consistent with the fact that OT effect is mediated principally by its receptor, OTR, whereas the effects of arginine vasopressin are mediated by both OTR and AVPR1A [497]. Thornton et al. has previously demonstrated that AVPR1A expression in human myometrium does not change with progressing stages of pregnancy or labour onset [500], indicating that AVPR1A is not involved in the activation of the pregnant human uterus.

As mentioned above, human OTR have the ability to couple to G$_{q}$, G$_{i}$ [451-454] and in a smaller extent to G$_{s}$ [461]. Only the signalling pathways induced by G$_{ai}$ have been studied extensively to be exploited for the development of tocolytic drugs. Atosiban, an OTR antagonist currently used for the treatment for preterm labour, works by blocking the G$_{ai}$ signalling pathway and subsequently inhibiting PLCβ activation and calcium mobilisation in myometrial cells, subsequently preventing uterine contractions [293, 497, 501]. In amnion cells, we have demonstrated that atosiban does not inhibit the OT-induced activation of MAPKs and NF-κB, or the expression of COX-2 and phosphorylation of cPLA$_2$ (Fig. 6.3).

We have found that despite being an OTR-A, atosiban activates MAPKs and NF-κB to induce COX-2 and p-cPLA$_2$ expression via G$_{ai}$ signalling. In addition, we have demonstrated that atosiban treatment alone is capable of stimulating PGE$_2$ synthesis whilst increasing the production of other proinflammatory chemokines and cytokines (Fig. 6.5 and Fig. 6.6). The effect of atosiban on the
mRNA level of proinflammatory chemokines and cytokines appeared to be similar to the transient effect of OT. However, Atosiban-induced PGE\(_2\) synthesis rapidly declined after reaching a peak at 4 h, whereas OT maintained high levels of PGE\(_2\) at 6 h. The significance of such temporal difference in PGE\(_2\) synthesis between OT and Atosiban is yet to be understood, however, it is possible that OT is capable of prolonging its effects further in comparison to Atosiban via post-transcriptional/translational regulation of PG synthetic enzymes. It would be important to understand the role of these differences in the OT and Atosiban effects as the induction of proinflammatory effects in human amnion could have detrimental consequences upon the fetus in preterm labour in the presence of chorionamnionitis if there is a proinflammatory effect on the fetal brain. Interestingly, the principal placebo controlled RCT of Atosiban\cite{336} showed that although Atosiban was ineffective in delaying preterm labour at gestational ages below 28 weeks its use was associated with an increase risk of fetal/neonatal death in infants below 26 weeks. This was explained in part by uneven recruitment at low gestational ages, but also by the presence of chorionamnionitis. The authors commented that adverse effects at these gestational ages, caused by Atosiban, could not be excluded.

As atosiban is a competitive antagonist to OTR-G\(_{\alpha q}\) coupling, the proinflammatory effects of atosiban may be due to a biased agonist effect. Biased agonism is a term introduced in 1998 to describe the behaviour of single ligands that can promote specific receptor conformations capable of differentially activating distinct signalling cascades\cite{502}. Recent evidence indicate that G protein-coupled receptor signalling does not involve a simple ‘on’ and ‘off’ switch but instead, different agonists/antagonists can activate multiple effectors with varying efficacies\cite{503}. The first report of biased agonism was seen in small cell lung carcinoma, where substance P (\(\text{D-Arg}^2, \text{D-Phe}^5, \text{D-Trp}^{7,9}, \text{Leu}^{11}\)) inhibited the G\(_{\alpha q}\) -mediated pathway whilst acting as an agonist to G\(_{\alpha i}\) resulting in activation of MAPKs, particularly JNK, and inhibition of cell growth\cite{502,504}. Another ligand shown to have biased agonist properties is the bradykinin antagonist dimer CU201, which blocks the G\(_{\alpha q}\)-mediated pathway and activates G\(_{12,13}\) leading to apoptosis in multiple cancer cell lines\cite{505}. Moreover, the OT/OTR system in various cell types has previously been suggested to activate multiple G protein signalling pathways which act in synergy\cite{506}. Biased agonist effect has been also observed in the OT/OTR system as activation of OTR can drive opposite effects via coupling to different type of G-proteins. In the case of neuronal cells, OT can inhibit or stimulate K\(^+\) conductance via PTX-resistant or -sensitive G-protein pathways\cite{507}. An analogue of OT, atosiban, has been shown to have biased agonist effect through OTR in neuronal cells as well as in other cell lines. Atosiban acts as a selective agonist upon OTR-G\(_{\alpha q}\) coupling to induce cell proliferation whilst acting as a competitive antagonist upon OTR-G\(_{\alpha i}\) coupling to inhibit cell growth in MDCK and HEK293 cell lines stably expressing OTR\cite{508,509}. Similar to this, inhibiting G\(_{\alpha i}\) by PTX resulted in a significant reduction in the proinflammatory effects of atosiban in human amnion (Fig. 6.7). These studies provide potential explanation for the agonistic effect of atosiban via G\(_{\alpha i}\) in human amnion, however, this hypothesis requires further investigation.
In conclusion, our data indicate that OT predominantly works through OTR to induce activation of MAPKs and NF-κB, to regulate COX-2 expression and cPLA₂ activity. Atosiban may possess a biased agonist property on amnion OTRs to act as a Gₐᵢ agonist, promoting proinflammatory effects similar to that of OT. Given that atosiban has already been approved as a tocolytic drug in the management of preterm labour, this ability of atosiban to drive proinflammatory effects in amnion is worth noting. Inflammation is strongly associated with labour and it is possible that whilst atosiban is blocking uterine contractions by acting as a Gₑᵦ antagonist, it may be activating an inflammatory response in the amnion via Gᵢᵣ signalling, which could potentially explain the limited efficacy of atosiban in delaying labour. The possibility that a drug designed for a therapeutic property can stimulate opposite effects in different tissue is a pharmacological oddity that requires attention in the future discovery of a target-specific drug. An ‘ideal’ OTR antagonist should block pro-contractile effects of OT without activating inflammation. The potential for differential effects of OT and OTR-antagonists has a major clinical implication in the use of OT in induction of labour, and OTR antagonists as tocolytics. It will therefore be critical that therapeutics designed to modulate the OT/OTR system for the management of term and preterm labour take into account the role of OT in the activation of inflammation and the effects of differential G-protein coupling of the OTR.
7. Summary and Final Conclusion
Oxytocin is widely recognised as playing a major role in promoting myometrial contractility [510]. It is the most potent uterotonin currently available and is extensively used in the clinical management of dysfunctional labor [234]. However, labor is not only characterised by myometrial contractions. The onset of human labor resembles an inflammatory reaction and a substantial body of evidence suggests that both preterm and term labor are linked with increased NF-κB activity in all gestational tissues [230, 352, 511]. Although labor onset is defined clinically by myometrial contractions, these are preceded by activation of NF-κB-mediated inflammatory pathways in gestational tissues leading to fetal membrane remodelling and cervical ripening. The fetal membranes (composed of amnion and chorion) undergo carefully regulated biochemical changes that modulate functional pathways precipitating labor and birth. Just prior to labor, there is an increase in inflammatory cytokine release from the amnion [322, 512] as well as increased prostaglandin (PG) synthesis, particularly PGE₂ [323]. Collectively these changes promote cervical ripening, lower uterine segment remodelling and initiation of myometrial contractions [190, 191, 204, 323, 385]. In murine parturition surfactant protein A (SP-A) produced by the maturing fetal lung has been suggested to represent the stimulus for a cascade of inflammatory signaling pathways leading to labor onset [131]. However in human pregnancy the endocrine or mechanical stimuli triggering inflammatory activation are obscure.

Our group has shown that NF-κB is activated in the human amnion prior to the onset of labour [413], leading to the upregulation of prolabour genes including PGs and COX-2. OT is an important regulator of PG production in the endometrium, amnion and decidua in several species including humans [324, 367, 513-516]. The NF-κB signalling pathway plays a critical role in the regulation of inflammation activation in both term and preterm gestational tissues [325]. Consistent with this, suppression of NF-κB activity inhibits LPS-induced preterm labour in mice [131] and IL-1β induced uterine contractions in Rhesus monkeys [517]. We have previously shown that both labour and inflammation increase amnion sensitivity to OT by inducing OTR expression and that this subsequently stimulates PGE₂ synthesis [181]. In this study we demonstrate a proinflammatory role for OT in the amnion whereby specific stimulation of OTR by its ligand drives the sequential activation of MAPKs and NF-κB via OTR-Gₐi coupling and subsequent prostaglandin and proinflammatory cytokine production. This suggests an additional and hitherto unrecognised role for OT in the onset of human labor via modulation of the inflammatory pathways leading to amnion activation.

It has been suggested that OT may play an additional role in prostaglandin production in amnion via an unknown mechanism. cPLA₂ and COX-2 mediate the committing and rate-limiting step of PG biosynthesis, generating the PGH2 intermediate that is converted to the terminal PGE₂ by PGES-1 and -2 [351]. We have demonstrated that OT treatment of amnion cells upregulates the gene expression of prostaglandin synthetic enzymes cPLA₂, COX-2, PGES-1 and PGES-2, as well as subsequent PGE₂ production. Consistent with these findings, OT has been reported to regulate the expression of cPLA₂.
and COX-2 genes in both rat [393] and in human myometrial cells [394] via the calcineurin/NFAT pathway [450]. Combined with our data demonstrating a local production of OT and OTR with the onset of labour, we can extrapolate that a paracrine/autocrine effect of OT drives to the synthesis of prostaglandins at term, facilitating downstream fetal membrane remodelling, cervical ripening and myometrial contractility.

OT has a well-defined role in stimulating uterine contractions via calcium signalling at term yet we have recently shown that labor is antedated by NF-κB activation and inflammatory stimulation in the amnion [413]. Amnion epithelial cells are easily isolated and cultured from fetal membranes collected after delivery. Sufficient cells can be obtained to undertake experiments without the need for passage and these cells retain their pre-labor non-activated, activated or post-labor phenotype for the duration of experiments and therefore represents a reasonable model for investigating OT stimulated NF-κB activation. It has been proposed that NF-κB plays a key role in the regulation of implantation, the menstrual cycle and parturition. However, it is noteworthy that NF-κB does not function in isolation from other intra-cellular signaling pathways. Studies have shown that there is cross talk of NF-κB with GPCRs [518, 519]. Our data illustrate a novel mechanism for OT in activating NF-κB, demonstrated by phosphorylation and nuclear translocation of the p65 subunit upon OT treatment. However, OT did not result in nuclear translocation of p50 or RelB, suggesting a possible involvement of p65 homodimers. This was confirmed using EMSA where only p65 supershift was observed with OT stimulation. In addition to p65 nuclear translocation, OT elicited activation of IKKα/β, degradation of IκBα, and phosphorylation of p65. Taken together, the OT-induced activation of NF-κB resembles that of the classical NF-κB signalling pathway triggered by IL-1β stimulation in amnion cells [353], with the exception that OT-induced activation of NF-κB involves nuclear translocation of p65 homodimers and not p65/p50 heterodimers. Pont et al. have recently reported that OT induces translocation of NFATC1-EFP in myometrial cells, leading to NFAT activation [450]. The NFAT transcription factors are also part of the Rel family and they have similar structure to the NF-κB family. They can bind to overlapping DNA sequence elements and have been previously shown to demonstrate interdependence in mediating cardiac hypertrophic gene expression as NF-κB nuclear translocation induced by IKKβ or p65 enhanced NFAT nuclear localisation [520].

NF-κB activation is a key modulator of COX-2 expression in amnion. Consistent with this we provide evidence that OT-induced COX-2 expression in the amnion is NF-κB mediated. The presence of IKKβ inhibitor (TPCA1) impeded OT-mediated expression of COX-2, implying that IKKβ activity is essential in the regulation of COX-2 by OT. Downregulation of the NF-κB p65 subunit using targeted siRNA inhibited the expression of OT-induced COX-2 at both mRNA and protein levels, further illustrating the significance of NF-κB in the regulation of COX-2. The OT-induced nuclear translocation of p65 homodimers also lead to increased expression of downstream NF-κB regulated genes including IL-8, CCL2, CCL5, IL-6 and SOD2, thus suggesting that OT can act as an
endogenous signalling molecule at labour onset triggering NF-κB activation in the amnion and the subsequent up regulation of key “pro-labour” genes involved in the inflammatory cascade preceding delivery.

Ligand activation of GPCRs, including OTR, often leads to rapid downstream activation of MAPKs via both G-protein dependent and independent mechanisms [414]. MAPKs are important regulatory components of IL-1β-mediated COX-2 expression and changes in their expression and activation are associated with the onset of labour [521]. Here we demonstrate that OT stimulation of human amnion epithelial cells increased levels of phospho-ERK1/2, phospho-p38 kinase and phospho-JNK. To investigate the role of MAPKs in COX-2 expression, different MAPK inhibitors were used. The presence of ERK1/2 inhibitor (U0126) and the p38 kinase inhibitor (SB203580), but not the JNK inhibitor (SP600125), resulted in suppression of OT-mediated NF-κB activation (measured as decreased phospho-p65) and COX-2 expression, thus suggesting that unlike IL-1β stimulation, where NF-κB and MAPK pathways function independently to trigger upregulation of COX-2, OT mediate activation of NF-κB via ERK1/2 and p38 kinases.

Typically, OTR couples with G_q and partly to G_i. The significance of G_q signalling was investigated using inhibitors for PLC and PKC. Pre-incubation of amnion cells with three different inhibitors of G_q (U73122, GFX, and Go6893) had no effect upon OT-mediated phosphorylation of p65, ERK1/2, p38 kinases nor COX-2 expression and similar results were observed following siRNA knockdown of G_q. To explore alternative G-protein signaling in human amnion, the effect of G_i inhibitor, PTX, on OT induced proinflammatory effects were examined. Pre-treatment with PTX reduced the effect of OT upon NF-κB, ERK1/2 and p38 kinases as well as the consequent COX-2 expression and PGE_2 production. Gene expression of G_{i2} and G_{i3}, but not G_{i1}, were detected and following siRNA knockdown of G_{i2} and G_{i3}, the proinflammatory effects of OT were significantly reduced. Our work indicates that OTR in human amnion couples to G_{i} and not G_q to regulate the downstream signalling cascade, ultimately leading to inflammatory cytokine/chemokine and PG synthesis.

We have investigated the hypothesis that OT may be working via the AVPR1A but that was quickly discarded as an OTR-specific antagonist, OVT, abolished the effect of OT completely. Subsequently, we used alternative OTR-A to examine their effects on OT-induced proinflammatory responses. The OTR antagonist, atosiban, has a well-described role in the modulation of myometrial contractility but its effect on other gestational tissues is unknown. Surprisingly, atosiban failed to inhibit the effect of OT in human amnion and atosiban treatment alone resulted in activation of NF-κB, ERK1/2 and p38 kinases, increased expression of COX-2, CCL5 and IL-6 to a similar extent as OT treatment alone. While the immediate effect of Atosiban in the myometrium may be OTR antagonism, subsequent suppression of G_q-mediated signalling and decreased uterine contractility, our results suggest atosiban concurrently activates inflammation in the amnion and downstream cytokine production via
Consistent with this notion, Busnelli et al. have recently reported that atosiban activates the $G_{\alpha i}$ but not $G_{\alpha q}$ subunit in HEK293 cell lines, stably transfected with the human OTR cDNA [503]. Our work has demonstrated that atosiban can act as a biased ligand which means that it can differentially activate different intracellular pathways. It is crucial that the ligand bias of OT and OTR antagonists are determined as antagonists that do not prevent intracellular coupling to all the contraction related pathways will not be effective tocolytics. Indeed the clinical effectiveness of OTR-A is markedly different, and our study highlights that understanding the alternative signaling pathways activated by different GPCR agonists/antagonists is key in designing ‘next generation’ tocolytics.

In conclusion, our results show a novel mechanism of inflammatory activation in human amnion mediated by the OT-OTR system, where OT stimulates PGE$_2$ and inflammatory cytokines/chemokines release via a mechanism involving specific binding of OT to OTR and the sequential activation of MAPKs and NF-$\kappa$B via the $G_{\alpha i2}$ signalling cascade. OT leads to translocation of NF-$\kappa$B into the nucleus and drives the expression of numerous prolabour genes including COX-2, cytokines and chemokines. The consequent upregulation of prostaglandins and inflammatory cytokines/chemokines contribute to fetal membrane remodelling, cervical ripening and myometrial activation. This provides compelling evidence that OT modulates key inflammatory pathways that promote the labouring phenotype and reveal a novel role for OT in the onset of labour.

Unexpectedly, OTR-A, atosiban, activates inflammatory pathways in amnion in a similar way to OT and increases cytokine/chemokine and prostaglandin secretion. This suggests that atosiban can drive inflammation in human amnion, which may have detrimental effects upon the fetus in preterm labour at early gestational ages. There is no evidence that current tocolytics, which targets uterine contractions, improve long-term outcome for preterm infants. It was previously thought that prematurity associated brain injury was mediated by hypoxia ischemia occurring during neonatal intensive care. It is now recognized that cytokine-mediated cerebral injury represents an important cause of prematurity linked handicap, thus infection/inflammation within the uterus is a major risk factor for fetal/neonatal CNS damage and poor outcome [522-524]. It was also previously thought that delaying preterm birth would be associated with improved neonatal outcome simply because of increased gestational age at delivery and the effect of in utero transfer and steroid administration to the mother. However, in the context of established chorioamnionitis, a perfect tocolytic drug could act to retain the fetus in an adverse environment and so do more harm than good. Therefore a tocolytic drug that stops contractions but does not inhibit infection-related inflammation in the fetal brain may delay delivery, but could worsen neonatal outcome. Furthermore, because activation of inflammatory pathways is central to the onset of labour, a drug inhibiting both contractions and inflammation is more likely to be effective, thus it is important to consider whether future therapeutics designed to modulate the OT/OTR system for the management of term and preterm labour stimulate the activation
of inflammation in the amnion by ligand directed signalling to minimise the risk of neonatal exposure to inflammation.
8. References


athology of adverse pregnancy outcomes associated
of an area of altered morphology overlying the


protein HuR regulates the expression of
cytosolic phospholipase A2 in allergic response and parturition.

Denny, W.G., and K.K. Wu, Role of p300 and PCAF in regulating
cyclooxygenase-2 promoter activation by inflammatory mediators.

binding and cyclooxygenase-2 promoter activity by sodium salicylate in quiescent human

Gorgoni, B., et al., The transcription factor C/EBPbeta is essential for inducible expression of the

Subbaramaiah, K., et al., Peroxisome proliferator-activated receptor gamma ligands suppress
the transcriptional activation of cyclooxygenase-2. Evidence for involvement of activator

Pino, M.S., et al., Prostaglandin E2 drives cyclooxygenase-2 expression via cyclic AMP

Kelly, R.W., Pregnancy maintenance and parturition: the role of prostaglandin in

Noort, W.A., et al., Changes in plasma levels of PGF2 alpha and PGI2 metabolites at and

McLaren, J., D.J. Taylor, and S.C. Bell, Prostaglandin E(2)-dependent production of latent

Uozumi, N., et al., Role of cytosolic phospholipase A2 in allergic response and parturition.

Bonventre, J.V., et al., Reduced fertility and postischaemic brain injury in mice deficient in

Sen, R. and D. Baltimore, Multiple nuclear factors interact with the immunoglobulin

Hoffmann, A. and D. Baltimore, Circuitry of nuclear factor kappaB signaling. Immunol Rev,


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and the effect of the synthetic oxytocin antagonist [1


172
lates myometrial guanosine triphosphatase and ion involved postanoid receptor isoforms leads to Rho on in human myometrium: changes in relation to nctional progesterone


