Development of new strategies for the inhibition of premature labour

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ADDENDUM

Page 9, paragraph 1, line 5 – insert paragraph;

A later study by Gibb *et al* (1996), used a specific antibody for PGHS-2 demonstrating ir-PGHS-2 was localized to the trophoblast epithelium and the mesenchymal core of the maternal component of the ovine placenta by day 125-127 of gestation. While at term, ir-PGHS-2 was localized predominately to the mononuclear cells in the trophoblastic epithelium, with little expression in the maternal syncytium.

Page 21, line 18 – insert before last sentence; "Administration of a PGHS inhibitor to pregnant rats has been shown to markedly reduce oxytocin receptor concentration in the uterus at the time of parturition (Chan *et al.*, 1988; Chan & Chen, 1992). This suppressive effect of PGHS inhibition on oxytocin receptors was reversed by the co-administration of $PGF_{2\alpha}$. In addition. a later study by Wu *et al.* (1998) demonstrated that selective PGHS-2 inhibition in fetal sheep was associated with a significant reduction in the expression of several of the key utero-placental labour-related genes, including the oxytocin receptor, oestrogen receptor and cytosolic PLA_2."

Page 40, paragraph 2, line 11 – insert paragraph; "Nimesulide was one of the first marketed drugs with preferential selectivity for PGHS-2 compared with PGHS-1 (Bennett, 1999). Numerous *in vitro* assay systems have been developed for testing and comparing the relative inhibitory activities of NSAIDs against PGHS-1 and PGHS-2 (Pairet *et al.*, 1998; Brideau *et al.*, 1996; Grossman *et al.*, 1995; Giuliano *et al.*, 1999 & Riendeau *et al.*, 1997). The most commonly used systems include human recombinant enzymes in whole cells and the human whole blood assay. Patrignani *et al.* (1997) evaluated the selectivity *in vitro* of various NSAIDs in inhibiting the cyclooxygenase activity of platelet PGHS-1 and monocyte PGHS-2 using a human whole blood assay. Nimesulide, meloxicam and diclofenac were approximately 10- to 20-fold more potent in inhibiting the PGHS activity of monocyte PGHS-2 than platelet PGHS-1. Similarly, Warner *et al.* (1999) found that nimesulide, along with meloxicam, etodolac and celecoxib were between up to 50-fold more selective for PGHS-2 over PGHS-1. Despite the variability in drug potencies among these different assay systems, a similar trend in non-steroidal anti-inflammatory drug selectivity is observed in most systems (Pairet *et al.*, 1998). Nimesulide is generally considered a preferential PGHS-2 inhibitor, estimated at being 10- to 20-fold more selective for PGHS-2, can also inhibit PGHS-1 at high concentrations (Hawkey *et al.*, 1999; Pairet *et al.*, 1998; Warner *et al.*, 1999). Many of the preferential PGHS-2 inhibitors like nimesulide, have also been tested for their PGHS-1 sparing effects *in vivo* using an *ex vivo* whole blood assay (Giuliano *et al.*, 1999; Shah *et al.*, 1999; Patrignani *et al.*, 1996b & Cullen *et al.*, 1998). Results from these studies indicate that nimesulide is a preferential inhibitor of PGHS-2 both *in vitro* and *ex vivo*. This suggests that preferential selective PGHS-2 inhibitors like nimesulide, might be useful in inhibiting the increase in PGHS-2 activity and thus uterine activity that is associated with term and premature labour. Indeed, studies using isolated human myometrium have shown nimesulide, meloxicam and celecoxib to be potent relaxant agents on myometrium obtained during pregnancy, before and after labour (Slattery *et al.*, 2001)."

Page 44 – delete Aim 1 and replace with;

1. Inhibition of premature labour

The aim of the study presented in Chapter 3 was to compare the effects of the selective PGHS-2 inhibitor nimesulide, alone or in combination with the oxytocin receptor antagonist atosiban, on the labour-associated changes in fetal and maternal prostaglandin concentrations and on the timing and progression of glucocorticoid-induced premature labour in sheep.

Page 44 – delete Aim 2 and replace with;

2. Prostaglandin H synthase mRNA expression during the inhibition of premature labour

The study presented in Chapter 4 aimed to determine the level of expression of PGHS-2 mRNA in placental tissues following the induction of premature labour with dexamethasone and during its inhibition with either nimesulide alone, or nimesulide in combination with atosiban.

Page 45 – delete Aim 4 and replace with;

4. Fetal responses to an inflammatory challenge during pregnancy

The aim of this study was to develop an ovine model to which an inflammatory state is induced by lipopolysaccharide administration to the maternal systemic, intra- or extra-amniotic compartments during pregnancy to mimic the processes thought to be involved in premature labour associated with infection in women. Then to use this model to investigate the mechanisms by which the fetus and mother respond to an inflammatory challenge during pregnancy.

Page 66, section 2.7.3.2, line 3 – insert sentence; "This Yeast RNA tube is negative controls for detection of protected probe bands and thus serve as a positive control for the function of the RNases. It shows if the probe is being protected in the absence of homologous sequence. Thus, no signal was observed in this lane of the gel."

Page 67, section 2.7.3.2, line 1 – insert sentence; "This no RNase control tube serves as a control for probe integrity. This control tube shows the gel migration of the full length probe. If any unexpected degradation of the probe occurs, it will be seen in this control sample. On the gel this showed a single band migrating at the expected probe sizes."

Page 71, paragraph 2, line 12 – insert paragraph; " The dose of atosiban was determined from a previous study by Jenkin *et al.* (1994). This study describes a dose response study to atosiban in pregnant sheep. At the doses of 100 and 200 μg/min for 4 h, fetal basal prostaglandin concentrations were not reduced, however, oxytocin-induced prostaglandin release was inhibited. From this study the dose of 200μg/min for 4 h (4.12 mg/kg/d) was selected. This dose is also comparable to that used in women. A pilot dose ranging study was conducted by Goodwin *et al* in 1996. This study describes a variety of dose regimes, with or without a bolus injection followed by infusion doses ranging from 30-300μg/min for a total infusion time of

6 h. This study showed that doses of 300 and 100 μg/min proceeded by a bolus injection resulted in a rapid control of uterine activity and may be useful in treating acute premature labour (Goodwin *et al.*, 1996)."

Page 90, paragraph 2, line 3 – insert paragraph; "Although not investigated in this study, atosiban alone treatment would not be expected to markedly delay preterm labour in sheep or have a similar inhibitory effect as nimesulide alone. Previously atosiban (up to500 μg/min), has been used in sheep to delay active labour (A/Prof G Jenkin; personal communication). Atosiban alone was inadequate to completely inhibit uterine activity due to the increasing prostaglandin concentrations at this time. The use of atosiban to supplement PGHS-2 inhibition therefore produces an additive effect as opposed to PGHS-2 inhibition alone. Using the combination of nimesulide with atosiban not only produces a dual prostaglandin and oxytocin inhibitory effect on uterine activity, but also an added effect of blocking local oxytocin-induced prostaglandin release just prior to delivery."

Page 94, section 4.2.1, line 3 – insert sentence "Prostaglandin concentrations were measured in these ewes, prior to, during and at the completion of maternal inhibitor treatment (refer to section 3.2.3)".

Page 97, figure description – delete first sentence and insert " PGHS-2 mRNA expression in whole cotyledon tissue collected from control ewes not in labour (GA140; n=4), during dexamethasone-induced premature labour (DEX; n=3), and during the inhibition of dexamethasone-induced premature labour with nimesulilde (NIM; n=4) or nimesulide and atosiban (NIM+ATO; n=4).

Page 98, paragraph 2, line 9 – insert sentence; "It is also possible that a type II error has been made with this statistical analysis which can be directly related to the power and sample size. To improve the power of the statistical analysis, and in turn decrease the likely hood of a type II error the sample size should be increased. Unfortunately there were inadequate tissue samples and I was unable to repeat these animal experiments, therefore further investigations should be done to confirm these preliminary results."

Page 100, paragraph 2 – delete paragraph and replace with; "The absence of a rise in PGHS-2 mRNA could have also resulted from prolonged glucocorticoid infusion. Glucocorticoids may exert both an up-regulation and down-regulation on the expression of the enzyme with prolonged use. As mentioned previously, the model of labour induction used in these studies, relies upon the administration of a synthetic glucocorticoid, dexamethasone. This advances the fetal cortisol surge and alters placental steroidogensis, resulting in the metabolism of progesterone to oestrogen, and the induction of PGHS-2 in the placenta. The altered placental steroidogensis, in this study is evident by the reduced progesterone concentrations observed in all animals (refer to section 3.3.7). Thus, dexamethasone treatment causes an initial up-regulation of PGHS-2 activity. After prolonged exposure to glucocorticoids, as seen in the NIM and NIM+ATO treatment groups, may cause a secondary phase of down regulation due to the observation of reduced PGHS-2 mRNA levels in the NIM alone and NIM +ATO treated ewes compared to that observed in DEX treated ewes. With the exception of the amnion, in which Zakar *et al* (1995) has shown an up-regulation of PGHS-2, glucocorticoids attenuate PGHS-2 expression in many different cell systems (DeWitt & Meade, 1993; Goppelt-Struebe, 1997), as described in Section 1.2.2.3. Glucocorticoids have been shown to attenuate PGHS-2 expression through altering mRNA stability in mitogen-induced fibroblast cells (Evett *et al.*, 1993). If indeed a down-regulation has occurred after the initial up-regulation of PGHS-2 in the present study, the precise mechanisms responsible for this secondary phase of down-regulation remains unclear. Further investigations are needed to determine if indeed a down-regulation is occurring and by what mechanism."

Page 100, paragraph 3 – delete paragraph and replace with; "These observations demonstrate the possible role of prostaglandins in the regulation of placental PGHS-2 mRNA expression in sheep, and that an increase in the expression of placental PGHS-2 may indeed rely, at least partially, on stimulation induced by an increase in intra-uterine prostaglandin concentrations. The current results, together with those of the previous study support the contention that prostaglandin synthesis is an important component of the positive feed-forward system in the processes of parturition."

Page 127-128, paragraph 1, line - insert paragraph; "An atosiban alone group was not included in this study as previously atosiban has been used to delay active labour in sheep without success (A/Prof G Jenkin; personal communication). Using doses up to 500 μg/min atosiban was administered to sheep at the commencement of labour, however atosiban alone was inadequate to completely inhibit uterine activity or significantly delay labour due to the increasing prostaglandin concentrations at this time. Thus atosiban was used in these studies to supplement PGHS-2 inhibition."

Page 156, paragraph 2, line 15 – insert sentence; "In contrast to when nimesulide and atosiban are combined, these drugs, when administered separately are not sufficient to block both prostaglandins and oxytocin, therefore complete inhibition of premature labour may be harder to achieve (Jenkin *et al.*, 1994; Poore *et al.*, 1999; Moutquin *et al.*, 2000; Romero *et al.*, 2000 & Valenzuela *et al.*, 2000)."

Page 165 – insert reference after Bejar *et al.* (1981);

Bennett, A. (1999). Overview of nimesulide. *Rheum* 38(Suppl-1), 1-3.

Page 165 – insert reference after Bottcher *et al.* (1987);

Brideau, C., Kargman, S., Liu, S., Dallob, A. L., Ehrich, E. W., Rodger, I. W. & Chan, C. C. (1996). A human whole blood assay for clinical evaluation of biochemical efficacy of cyclooxygenase inhibitors. *Inflamm Res* 45, 68-74.

Page 167 – insert 2 references after Challis *et al.* (2000);

Chan, W. Y., Berezin, I. & Daniel, E. E. (1988). Effects of inhibition of prostaglandin synthesis on uterine oxytocin receptor concentrations and myometrial gap junction density in parturient rats. *Biol Reprod* 39, 1117-1128.

Chan, W. Y. & Chen, D. L. (1992). Myometrial oxytocin receptors and prostaglandin in the parturition process in the rat. *Biol Reprod* 46, 58-64.

Page 168 – insert reference after Cook *et al.* (2000);

Cullen, L., Kelly, L., Connor, S. O & Fitzgerald, D. J. (1998). Selective cyclooxygenase-2 inhibition by nimesulide in man. *J Pharmacol Exp Ther* 287, 578-582.

Page 172 – insert reference after Gimpl *et al.* (2001);

Giuliano, F., Ferraz, J. P., Pereira, R., Nucci, G. & Warner T. D. (2001). Cyclooxygenase selectivity of non-steroidal anti-inflammatory drugs in humans: ex vivo evaluation. *Eur J Pharmacol* 426, 95-103.

Page 174 – insert reference after Group *et al.* (2001b);

Grossman, C. J., Wiseman, J., Lucas, F. S., Trevethick, M. A. & Birch, P. J. (1995). Inhibition of constitutive and inducible cyclooxygenase activity in human platelets and mononuclear cells by NSAIDs and COX-2 inhibitors. *Inflamm Res* 44, 253-257.

Page 174 – insert reference after Harding *et al.* (1982);

Hawkey, C. J. (1999). Cox-2 inhibitors. *Lancet* 353, 307-314.

Page 176 – insert reference before Kaga, N *et al* (1996);

Joseph, KS., Kramer, MS., Marcoux, S., Ohlsson, A., Wen, SW., Allen, A. & Platt, R. (1998). Determinants of preterm birth rates in Canada from 1981 through 1983 and from 1992 through 1994. *N Eng J Med* 339, 1434-1439.

Page 182 – insert reference before Nathanielsz, PW *et al* (1982);

Nassar, N. & Sullivan, EA. (2001). Australia's mothers and babies 1999. AIHW Cat No PER 19. Sydney. AIHW National Perinatal Statistics Unit (Perinatal Statistics Series no.11).

Page 184 – insert reference after Owen *et al.* (1995);

Pairet, M. & van Ryn, J. (1998). Experimental models used to investigate the differential inhibition of cyclo-oxygenase-1 and cyclooxygenase-2 by non-steroidal anti-inflammatory drugs. *Inflamm Res* 47(Suppl-2), S93-S101.

Page 184 – insert 2 references after Patel *et al.* (1999);

Patrignani, P., Panara, M. R., Sciulli, M. G., Santini, G., Renda, G. & Patrono, C. (1997). Differential inhibition of human prostaglandin endoperoxide synthase-1 and –2 by nonsteroidal anti-inflammatory drugs. *J Physiol Pharmacol* 48, 623-631.

Patrignani, P., Panara, M. R., Santini, G., Sciulli, M. G., Padovano, R., Cipollone, F., et al. (1996). Differential inhibition of cyclooxygenase activity of prostaglandin endoperoxide synthase isozymes in vitro and ex vivo in man. *Prostaglandins Leukot Essent Fatty Acids* 55(Suppl-1), P115.

Page 186 – insert reference before Robinson *et al.* (1978);

Riendeau, D.,Charleson, S., Cromlish, W., Mancini, J. A., Wong, E. & Guay, J. (1997). Comparison of the cyclooxygenase-1 inhibitory properties of nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors, using sensitive microsomal and platelet assays. *Can J Physiol Pharmacol* 75, 1088-1095.

Page 187 – insert reference after Sheldrick *et al.* (1983);

Shah, A. A., Murray, F. E. & Fitzgerald, D. J. (1999). The in vivo assessment of nimesulide cyclooxygenase-2 selectivity. *Rheum* 38(Suppl-1), 19-23.

Page 188 – insert reference after Slatter *et al.* (2002);

Slattery, M. M., Friel, A. M., Healy, D. G. & Morrison, J. J. (2001). Uterine relaxant effects of cyclooxygenase-2 inhibitors in vitro. *Obstet & Gynecol* 98, 563-569.

Page 188 – insert reference before Smith, GC *et al* (1998);

Smieja, Z., Zakar, T., Walton, JC. & Olson DM. (1993). Prostaglandin endoperoxide synthase kinetics in human amnion before and after labor at term and following preterm labor. *Placenta* 14, 163-175.

Page 189, reference 3 – the journal 'BMJ' should be in capital letters.

Page 191 – insert reference after Wang *et al.* (1998);

Warner, T. D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J. A. & Vane, J. R. (1999). Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full *in vitro* analysis. *Proc Natl Acad Sci* 96, 7563-7568.

Page 193 – delete reference;

Zakar, T. & Mitchell, B. (1996). The endocrinology of late pregnancy and parturition. In *Advances in organ biology.*, vol. 1, pp. 121-152. JAI Press Inc.

Page 193 – insert reference before Zuckerman, H *et al* (1974);

Zimmerman, EA. & Defendini, R. (1977). Hypothalamic pathways containing oxytocin, vasopressin and associated neurophysins. In *International Conference on the Neurohypophysis*, eds. Moses, A. M. & Share, L. pp. 22-29. New York: Karger.

ERRATA

Page VII, paragraph 2, line 7 – "meet" should be "met".
Page VIII, paragraph 3, line 7 – 'dexamethasone' spelt incorrectly.
Page XVI – insert abbreviation for COX-2 - Cyclooxygenase type-2.
Page XVII – insert abbreviations for $PPLA_2$ - phospholipase A_2 and OT - oxytocin.
Page 1, line 14 – delete Challis *et al* (2000) reference and replace with Joseph *et al* (1998) and Nassar and Sullivan (2001).
Page 9, paragraph 2, line 3 – in addition to the reference Teixeira *et al* (1994) insert reference Smieja *et al* (1993).
Page 11, paragraph 2, line 17 – should read "newborn circulation" not "fetal circulation".
Page 13, paragraph 1, line 19 – in addition to the reference Slater *et al* (2002) insert Lopez Bernal *et al* (1995).
Page 15, paragraph 3, line 9 – "been" should replace "be".
Page 17, paragraph 1, line 5 – delete Zakar & Mitchell (1996) reference and replace with Zimmerman & Defendini (1977).
Page 17, paragraph 1, line 10 – 'myoepithelial cells' spelt incorrectly.
Page 17, paragraph 1, line 11 – 'distension' spelt incorrectly.
Page 18, line 5 – should read "may be" instead of "maybe".
Page 21, line 22 – insert references after 'established' (Chan *et al*., 1988; Chan & Chen, 1992; Wu *et al*., 1998; Mitchell & Schmid, 2001).
Page 22, line 4 – the word "pressure" is missing.
Page 27, under table heading "Behavioural risks" insert "during pregnancy" instead of "after pregnancy".
Page 27, under table heading "Disorders of the placenta and membranes" – 'previa' spelt incorrectly.
Page 30, in figure description – 'chorionitis (B)' spelt incorrectly.
Page 34, paragraph 2, line 17, printing error - the word 'might' is superimposed upon itself.
Page 64, section 2.7.2, line 2 – insert after PGHS "(ORF 95-488, 393 nucleotides)" and after GAPDH insert "(260 nucleotides)"
Page 68, paragraph 1, line 5 – insert reference Smieja *et al* (1993).
Page 69, paragraph 2, line 25 – printing error, should read "vivo"
Page 70, paragraph 2, line 6 – 'analyzed' is spelt incorrectly
Page 91, paragraph 2, line 10 – 'PGHS-1' spelt incorrectly
Page 96, line 7 – "most prominent" changed to "highest".
Page 96, line 12 – "Its" should read "It is".
Page 97, figure description – "arbitrary" is spelt incorrectly.
Page 100, paragraph 3 – delete line 7 & 8.
Page 103, paragraph 2, line 4 – "experiments" spelt incorrectly.
Page 105, paragraph 2, line 1 – should read "all data are" instead of "all data is".
Page 105, paragraph 2, lines 2 and 3 – replace "was" with "were".
Page 123, paragraph 1, line 1 – 'principle' spelt incorrectly, should read 'principal'.
Page 137, paragraph 2, last sentence – insert "after LPS administration" before "into the extra-amniotic compartment".
Page 153, line 17 – sentence should read " The fetal sheep is much more sensitive to LPS"
Page 156, paragraph 2, line 2 – should read "cascade" not "cascaded".
Page 157, paragraph 3, line 2 – should read "was not different" not "were not different".
Page 158, paragraph 2, line 1 – "affecting" should replace "effecting".
Page 158, paragraph 2, line 3 – should read "detrimental side effect" no 's' on effect.
Page 158, paragraph 2, line 11 – should read "affect" no 's' on affect.
Page 158, paragraph 2, line 13 – 'PGHS-1' and 'PGHS-2' spelt incorrectly.
Page 162, paragraph 3, line 11 – 'cause' spelt incorrectly.
Page 163, line 5 – 'quite' spelt incorrectly.

I, that am curtailed of this fair proportion,
Cheated of feature by dissembling nature,
Deformed, unfinished, sent before my time
Into this breathing world, scarce half made up.

William Shakespeare (King Richard III)

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Summary

Premature labour is a significant problem in obstetric medicine. Neonates that survive premature delivery often have serious ongoing health problems. Intra-uterine infection has been recognised for over twenty years as a major contributor to the pathogenesis of premature labour and is now linked with several neonatal and infant complications such as periventricular leukomalacia, bronchopulmonary dysplasia and cerebral palsy. Attempts to develop appropriate tocolytic agents have been hampered by their potentially harmful side effects on the mother and more importantly, the fetus.

Prostaglandins are involved in both normal and premature labour and are produced by specific prostaglandin synthase enzymes. The rise in prostaglandin generating capacity at labour onset results from an increase in the expression of PGHS-2 in maternal intra-uterine tissue, whereas the patency of the fetal ductus arteriosus and other normal fetal functions are maintained by prostaglandins thought to be produced mainly by PGHS-1 within the respective tissue. The use of selective inhibition of uterine PGHS-2 for the prevention of premature labour has meet with variable success as labour is maintained by multiple stimulatory processes. The inclusion of a second drug that inhibits the action of another uterine stimulant, oxytocin, may markedly improve the effectiveness of blocking prostaglandin production. This may enable sufficiently low doses of the prostaglandin synthase inhibitors to be used to eliminate problems associated with fetal distress.

The central aim of this thesis was to investigate a potential new strategy for delaying premature birth by combining a selective PGHS-2 inhibitor, nimesulide, with an oxytocin receptor antagonist, atosiban. Secondly, to further our understanding of the consequences of uterine infections during pregnancy, the mechanisms by which the fetus and mother respond to an inflammatory challenge during pregnancy were also investigated.

The first study aimed to compare the effect of a selective PGHS-2 inhibitor, nimesulide alone, or in combination with the oxytocin receptor antagonist atosiban, on the progression of glucocorticoid-induced premature labour in sheep. Their effects on circulating maternal and fetal prostaglandin concentrations and fetal well-being were also examined. The progress of labour was continuously monitored by uterine electromyographic (EMG) activity.

No fetuses from nimesulide and atosiban-treated ewes were delivered during treatment. These animals were killed electively, 98.0 ± 6.8 h after the commencement of dexamethasone induction. This was significantly longer than the delivery time for those ewes treated with nimesulide alone (71.2 ± 3.9 h; n = 3) or vehicle-treated ewes (51.4 ± 1.7 h; n = 9). Both maternal and fetal plasma prostaglandin concentrations in nimesulide and atosiban-treated ewes and nimesulide-treated ewes decreased during treatment. In contrast, vehicle-treated ewes showed a significant increase in maternal and fetal plasma prostaglandin concentrations during dexamethasone induction of labour. Uterine EMG activity observed in nimesulide and atosiban-treated ewes was significantly suppressed compared to that in both vehicle and nimesulide-treated ewes during the treatment period. This treatment was markedly more effective than PGHS-2 inhibition alone and suggests a role for oxytocin in contributing to uterine activity once premature labour is established. The clinical use of atosiban, to prevent the oxytocin stimulated increase in uterine activity associated with labour, in combination with nimesulide, may permit reduction of the dose of PGHS-2 inhibitors used to a level that has minimal impact on fetal well-being.

The level of expression of placental PGHS-2 mRNA in the absence of prostaglandin release, when the parturient mechanism is activated, but when delivery is prevented has not been documented. In the second study, placental PGHS-2 mRNA expression was measured from control ewes that were not in labour (140 days gestational age), dexamethasone-induced labour, nimesulide alone and nimesulide and atosiban treated animals, along with GAPDH mRNA expression for the respective samples. Placental PGHS-2/GAPDH mRNA expression was most prominent in dexamtheasone-induced control ewes compared to control ewes that were not in labour. Placental PGHS-2/GAPDH mRNA expression in nimesulide alone and nimesulide and atosiban-treated ewes were not statistically different from control ewes. These findings further suggest that PGHS-2 may be regulated by one of its products, supporting the notion that prostaglandin synthesis is an essential component of the positive

feed-forward system in the processes of parturition. Thus, prostaglandins may also act as a positive signal for placental PGHS-2 mRNA expression *in vivo* during the progression of labour in sheep.

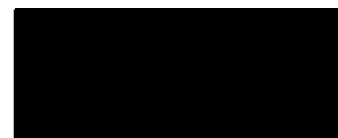
The aim of the third study was to establish a model that more closely relates to the clinical situation, that is, to block the progression of established premature labour. These studies evaluated the effectiveness of this combined treatment commencing after the onset of premature labour when prostaglandin concentrations and uterine activity are significantly elevated. The changes in uterine activity and prostaglandin concentrations after the cessation of nimesulide and atosiban infusion, but during continued induction of premature labour with fetal glucocorticoid infusion, were also determined. After the onset of active premature labour, ewes received a combined nimesulide and atosiban or vehicle infusion for 48 h. Four nimesulide and atosiban-treated ewes successfully completed the 48 h infusion period with no deliveries occurring during inhibitor treatment, or up to 6 h post-treatment. Delivery was delayed in 2 ewes, compared to vehicle-treated animals. Maternal and fetal prostaglandin concentrations and uterine EMG activity were significantly decreased in nimesulide and atosiban-treated ewes. The combination of nimesulide and atosiban treatment for a period of 48 h successfully inhibited the progression of active premature labour to delivery. This study further supports the use of a selective PGHS-2 inhibitor combined with an oxytocin receptor antagonist as an alternative treatment for delaying premature labour.

A link between intra-uterine infection and premature labour is widely accepted, yet the fetal responses to the inflammatory processes initiated by such infections are not well understood. The aim of the final study was to examine the effect of an inflammatory challenge on the fetus using sheep as a model. An inflammatory state was induced by lipopolysaccharide (LPS) administration to either the maternal systemic, intra- or extra-amniotic compartments during late pregnancy. Fetal and maternal blood gases and uterine EMG activity along with fetal and maternal circulating prostaglandin concentrations (PGE_2 and PGFM), cortisol and interleukin-6 (IL-6) concentrations were determined. Maternal systemic LPS treatment resulted in mild maternal hypoxemia, a rise in temperature and increased uterine activity. This treatment also caused fetal hypoxemia and a marked rise in fetal cortisol and PGE_2 concentrations that persisted for 48 h. Whereas, intra-amniotic administration of LPS, at doses 200 times greater than that used systemically only caused a small increase in fetal cortisol and PGE_2

concentrations. A transient rise in uterine activity was also observed. Extra-amniotic LPS administration caused no overt fetal or maternal inflammatory responses. The rise in fetal cortisol and PGE_2 concentrations after maternal LPS treatment may be a potential protective mechanism that aids the fetus in the event of premature delivery. The attenuated fetal response to intra-amniotic LPS treatment, despite the much higher dose used, may support a role for the amniotic fluid and or some of its components in providing a protective barrier for the fetus from endotoxin exposure during pregnancy.
The studies presented in this thesis clearly demonstrate the effectiveness of a combined treatment of PGHS-2 inhibition and oxytocin receptor antagonism for delaying premature labour. In addition, the combined tocolytic treatment was successful in delaying the progression to delivery after the commencement of active premature labour. This work outlines the advantages of such a combined drug therapy as a potential treatment for premature labour in women. Moreover, the present studies aimed to investigate the mechanisms by which the fetus and mother respond to an inflammatory challenge during pregnancy, in an attempt to further our understanding of the consequences of uterine infections during pregnancy. This knowledge will allow for the development of better approaches for the detection and treatment of premature especially when associated with an underlying infection. Thus, the model used in these studies may aid in our interpretation of the ambiguous responses to inflammation during pregnancy.

Declaration

I hereby declare that, to the best of my knowledge and belief, this thesis contains no material previous published or written by another person, nor maternal which has been accepted for the award of any other degree or diploma at Monash University or equivalent institution, except where due reference is made within the text of the thesis.



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Publications

PAPERS

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Abbreviations

ANOVA	Analysis of variance	IL-6	Interleukin-6
ATO	Atosiban	IL-8	Interleukin-8
CA	Carotid artery	IL-10	Interleukin-10
cAMP	Cyclic adenosine monophosphate	IP_3	Inositol triphosphate
CDNA	Complementary deoxyribonucleic acid	IU	International units
cm	Centimetre	i.v	Intravenous
CMP	Charcoal stripped maternal plasma	JV	Jugular vein
cpm	Counts per minute	kg	Kilogram
cRNA	Complementary ribonucleic acid	L	Litre
°C	Degrees Celsius	LPS	Lipopolysaccharide
d	Day	LSD	Least significant difference
DEPC-H_2O	Diethylprocarbonated water	m	Metre
DEX	Dexamethasone	M	Molar
DHEAS	Dehyroepiandrosterone sulfate	MAP	Mean arterial pressure
DNA	Deoxyribonucleic acid	MAPK	Mitogen-activated protein kinase
E.coli	Escherichia coli	mg	Milligram
EDTA	Ethylenediaminetetraacetic acid	min	Minute
ELISA	Enzyme linked immunosorbent assay	MIP-1α	Macrophage inflammatory protein-1α
EMG	Electromyograph	ml	Millilitre
g	Gram	mm	Millimetre
G-protein	Guanine nucleotide regulatory protein	mM	Millimolar
h	Hour	mmHg	Millimetres of mercury
H_2O	Water	ng	Nanogram
Hb	Haemoglobin	NIM	Nimesulide
5-HETE	5-Hydroxyeicosatetraenoic acid	nM	Nanomolar
HPA axis	Hypothalamic-pituitary-adrenal axis	nmol/L	Nanomol per litre
I.D.	Internal diameter	NO	Nitric oxide
IL-1	Interleukin-1	NSAIDs	Non-steroidal anti-inflammatory drugs
IL-4	Interleukin-4	O.D.	Outer diameter

O_2 sat	O_2 saturation	RPA	Ribonuclease protection assay
P	Probability	rpm	Revolutions per minute
P_4	Progesterone	sec	Seconds
$PaCO_2$	Partial pressure of carbon dioxide	SEM	Standard error of the mean
PaO_2	Partial pressure of oxygen	TAE	Tris-acetate-EDTA
PBS	Phosphate buffered saline	TBE	Tris-borate-EDTA
PGDH	15-Hydroxyprostaglandin dehydrogenase	TE	Tris-EDTA
PGE_2	Prostaglandin E_2	TEMED	N,N,N,N,tetramethylethylediamine
PGFM	13,14-Dihydro-15-keto-PGF (stable metabolite of $PGF_{2\alpha}$)	TxA_2	Thromboxane A_2
PGG_2	Prostaglandin G_2	TxB_2	Thromboxane B_2
PGH_2	Prostaglandin endoperoxide	TMB	Tetramethylbenzidine
PGHS	Prostaglandin H synthase	TNFα	Tumor necrosis factor-α
PGHS-1	Prostaglandin H synthase type-1	tRNA	Transfer ribonucleic acid
PGHS-2	Prostaglandin H synthase type-2	μCi	Microcurie
PGI_2	Prostacyclin	μg	Microgram
PLA_2	Phospholipase-A_2	μl	Microlitre
pmol	Picomole	μM	Micromolar
PLC	Phospholipase C	UOV	Utero-ovarian vein
QC	Quality control	v/v	Volume per unit volume
RIA	Radioimmunoassay	VEH	Vehicle
RNA	Ribonucleic acid	w/v	Weight per unit volume
RNase	Ribonuclease inhibitor	WISH	Wistar Institute Susan Hayflick

Chapter 1

Literature Review

Premature labour is the leading cause of perinatal mortality and morbidity among neonates worldwide (Lopez Bernal, 2001). Those that survive premature delivery often have serious ongoing health problems, which impose major financial costs on the family, health care systems and society. Despite advances in obstetric and neonatal care, the incidence of premature labour has changed little in the past 40 years (Challis *et al*, 2000), due in part to our inability to diagnose and prevent premature labour appropriately. Neonatal survival rates increase dramatically from less than 20% at 23 weeks gestation to nearly 100% at 30 weeks gestation (Lopez Bernal, 2001), suggesting that prolongation of pregnancy *in utero*, increases the chance of neonatal survival considerably. Unfortunately, presently available tocolytic treatments are often ineffective and have the potential to cause detrimental side effects to the mother and/or the fetus. Thus, the importance of finding a better tocolytic agent cannot be overemphasised.

Approximately 30-40% of premature births are associated with an underlying infective process (Romero *et al.*, 1989b; Lettieri *et al.*, 1993; Challis *et al.*, 2000). Identifying women with intra-uterine infections is therefore a major obstetric challenge, as intra-uterine infections are often asymptomatic with no signs of fever or uterine tenderness until labour begins (Romero & Mazor, 1988; Goncalves *et al.*, 2002). A greater understanding of the consequences of uterine infections during pregnancy and the mechanisms by which the fetus and mother respond to an inflammatory challenge is crucial to developing better approaches for the detection and treatment of premature labour.

1.1 INITIATION OF PARTURITION

Parturition is the result of complex interactions between maternal and fetal endocrine systems, biochemical and structural changes that facilitate activation and stimulation of the uterus. To ensure the successful transition and survival from an intra-uterine to an extra-uterine environment it is vital that the fetal cardiovascular, respiratory and neuro-endocrine systems are adequately mature (Rice *et al.*, 1987). It is therefore not unreasonable to assume that the trigger for labour is strongly linked to fetal maturation. In humans, it has been long debated the role of the maturing fetus in the initiation and timing of parturition. The most conclusive evidence for the fetus initiating parturition has stemmed from the intense research on the growth and development of fetal sheep (Liggins & Kitterman, 1979; Thorburn & Challis, 1979). In the sheep, a functional fetal hypothalamic-pituitary-adrenal (HPA) axis is required for the initiation of birth. Activation of the fetal HPA axis, results in enhanced cortisol secretion which provides the trigger to the subsequent maternal endocrine changes and is thought to act on the placenta to alter the pattern of steroidogenesis (Challis & Lye, 1994). By increasing placental 17_{α}-hydroxylase and $C_{17\text{-}20}$lyase enzyme activities, maternal progesterone concentrations fall with a concomitant increase in placental oestrogen concentrations (Challis *et al.*, 1971). Under the oestrogen dominance the myometrium sensitivity to contractile agents is enhanced and increased synthesis and release of oxytocin and prostaglandins by intra-uterine tissues occurs, causing myometrial contraction, cervical ripening and ultimately birth (Mijovic & Olson, 1996). Increased fetal adrenal output of cortisol has also been shown to result in the up-regulation of the prostaglandin synthase type-2 (PGHS-2) gene expression in the placenta, increasing the production of prostaglandins and further up-regulating fetal HPA cortisol output (Challis *et al.*, 2000).
In humans the role of the fetal HPA axis in the initiation of parturition is not clearly defined. The human placenta lacks the 17_{α}-hydroxylase and $C_{17\text{-}20}$lyase enzymes, which are critical to the pathway described in sheep. Consequently, the human placenta depends on androgen (dehydroepiandrosterone sulfate, DHEAS) derived from the fetal adrenals as the substrate for oestrogen synthesis (Siiteri & MacDonald, 1963). From these observations, in humans, co-operative organisation between the fetus and placenta must be maintained to facilitate the changing steroidal environment necessary during parturition. Although the initiation of parturition may vary among species, general consensus agrees that the production and release of prostaglandins underlies the major

physiological changes that occur leading to enhanced uterine contractility and the onset of parturition (Karim, 1972; Liggins *et al.*, 1973; Mitchell *et al.*, 1976; Silver *et al.*, 1979).

1.2 ENDOCRINOLOGY OF PREGNANCY AND PARTURITION

1.2.1 Oestrogens and progesterone

Uterine quiescence during mammalian gestation has been attributed to the steroid hormone progesterone (Challis *et al.*, 1971; Jenkin *et al.*, 1985; Haluska *et al.*, 1997). By acting on the smooth muscle of the myometrium and cervix, progesterone ensures that uterine activity is minimal and that the cervix remains indistensible (Liggins, 1994). These actions are mostly inhibitory and are mediated by the antagonism of the stimulatory effects of oestrogen (Challis & Lye, 1994). Progesterone causes excitation-contraction uncoupling, inhibition of myometrial gap junctions and the suppression of oxytocin and prostaglandin secretion (Mazor *et al.*, 1994; Haluska *et al*, 1997).

In many species, as in the sheep, a fall in maternal progesterone levels, together with a concomitant increase in oestrogens concentrations, is a necessary prerequisite for parturition (Fylling, 1970). However, the mechanism for this depends on whether the placenta or the corpus luteum is the major source of progesterone (Lopez Bernal, 2001). In rhesus monkeys there is no apparent decrease in maternal progesterone concentrations prior to parturition (Stanczyk *et al.*, 1986). Progesterone concentrations also show a similar pattern in the baboon (Albrecht, 1980), adding to the controversy associated with the concept of 'progesterone withdrawal' in human pregnancy. In contrast to sheep, progesterone withdrawal does not appear to be an essential component in human parturition (Mazor *et al.*, 1994). However, both oestrogen and progesterone are synthesised within human amnion, chorion and decidua (Romano *et al.*, 1986; Mitchell *et al.*, 1987), therefore local changes in oestrogen and progesterone may occur without being reflected in the peripheral circulation, and may still be able to significantly influence the process of human parturition (Mitchell & Wong, 1993).

During pregnancy, in the ewe, plasma progesterone concentrations are maintained by the corpus luteum up until 50-60 days of gestation, at which time the placenta becomes the major source of progesterone (Linzell & Heap, 1968). During the last week of pregnancy, circulating levels of progesterone decline in the ewe (Thorburn *et al.*, 1969;

Fylling, 1970; Elsner *et al.*, 1980). The importance of progesterone withdrawal in sheep was highlighted by Thorburn *et al.* (1982), demonstrating that extra-amniotic $PGF_{2\alpha}$ infusion, at concentrations comparable to those seen at term, were not sufficient to sustain increases in uterine activity that are required during labour, unless progesterone was inhibited. These observations indicated that the ovine myometrium is relatively unresponsive to prostaglandins when under progesterone dominance. The progesterone withdrawal towards the end of gestation may be related not only to the cortisol-induced metabolism within the placenta (Liggins, 1994), but also to the increased oestrogen levels that occurs at this time (Challis *et al.*, 1971).

In the sheep, an increase in oestrogens normally occurs approximately 24 h prior to spontaneous parturition (Challis & Lye, 1994). The increase in oestrogen/progesterone ratio leads to several changes that stimulate uterine contractility, including an increase in myometrial oxytocin receptors and, thus increased responsiveness of the uterus to oxytocin and an enhancement of myometrial gap formation (Mitchell & Wong, 1993). This results in a hormonal environment favourable for increased prostaglandin synthesis and the generation of uterine contractions.

1.2.2 Prostaglandins

Prostaglandins are a group of biologically active lipids that play an important role in human labour; stimulating uterine contractility, ripening of the cervix and enhancing fetal maturational processes (Challis & Lye, 1994). There is substantial evidence to implicate prostaglandins in the process of parturition. Firstly prostaglandin concentrations have been demonstrated in the amniotic fluid and plasma of pregnant women and animals and are elevated at the onset of parturition (Evans *et al.*, 1982; Mitchell *et al.*, 1995; McLaren *et al.*, 1996). Secondly, prostaglandins have been widely used to induce labour and cervical ripening (Karim, 1972; Calder & Greer, 1990), and thirdly, prostaglandin synthase inhibitors have been shown to reduce uterine activity and delay premature labour (Lewis & Schulman, 1973; Novy *et al.*, 1974), as discussed in Section 1.6.2. Oestrogens induce both oxytocin production and synthesis of uterine oxytocin receptors, and in turn, oxytocin augments the action of prostaglandins on the myometrium (Wimsatt & Nathanielsz, 1995). Similarly, prostaglandins induce oxytocin receptors, creating a positive feed-forward regulatory mechanism that produces the cascade of hormonal events seen during spontaneous term labour. Considerable evidence has accumulated to indicate that increases in prostaglandin concentrations

within the uterine compartment occurs during both normal and premature labour (refer to Section 1.5).

1.2.2.1 Prostaglandin biosynthesis

Arachidonic acid (cis-5,8,11,14-eicosatetraenoic acid) is the obligatory precursor for prostaglandins of the 2 series; those that contain two unsaturated double bonds (PGE_2, $PGF_{2\alpha}$, PGD_2) (Lopez Bernal & Watson, 1992). Arachidonic acid is a polyunsaturated fatty acid and is stored in neutral lipids and phospholipids in esterified form (Mitchell, 1994). Biosynthesis and release of prostaglandins from tissues readily occurs in response to a variety of physiological stimuli but, unlike many other biological substances, prostaglandins are formed immediately prior to use and are not stored in tissues (Funk, 2001). Figure 1.1 shows a schematic representation of the biosynthetic pathway of prostaglandin production, adapted from Williams and DuBois (1996). Arachidonic acid is liberated from cellular phospholipids either directly by the actions of phospholipase A_2 (PLA_2), or indirectly by phospholipase C (PLC) which catalyses the formation of diacylglycerol from phosphatidylinositol (Challis & Lye, 1994; Mitchell, 1994). Following liberation, free arachidonic acid is converted to prostaglandin endoperoxide (PGH_2), the upstream precursor of prostaglandins and thromboxanes via the key enzyme, prostaglandin H synthase (PGHS) (Smith & DeWitt, 1995). PGHS catalyses two separate reactions (Figure 1.1). The first is a cyclooygenase (*bis*-oxygenase) reaction, in which arachidonate is converted to PGG_2 and secondly a peroxidase reaction, in which PGG_2 undergoes a two-electron reduction to PGH_2 (Smith & DeWitt, 1995).

The cyclooxygenase reaction begins with the rate-limiting abstraction of the (13*S*)-hydrogen from arachidonate to yield an arachidonyl radical, followed by the oxygen additions at the C-11 and C-15 to yield PGG_2. Finally the peroxidase reaction reduces the 15-hydroperoxide group of PGG_2 to an alcohol yielding PGH_2 (Smith *et al*, 1996). The conversion of PGH_2 into prostaglandins and thromboxanes is then mediated by the action of specific isomerases as outlined in Figure 1.1.

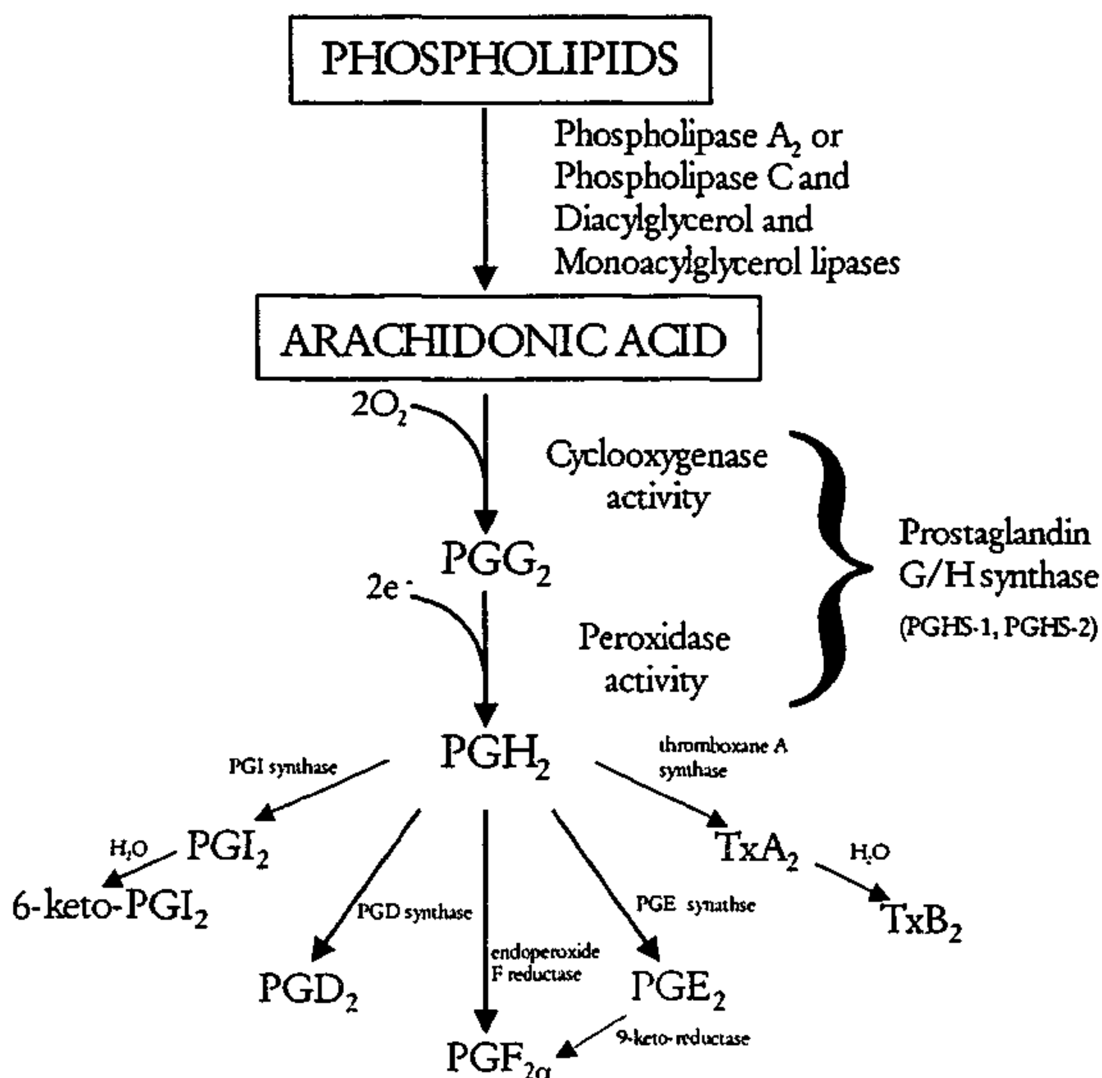

Figure 1.1 The biosynthetic pathway for prostaglandin production, adapted from Williams and DuBois (1996). Arachidonic acid is liberated from cellular phospholipids by the actions of phospholipase A_2 or phospholipase C. Free arachidonic acid is then converted to prostaglandin endoperoxide (PGH_2) via the enzyme, prostaglandin H synthase (PGHS). PGHS catalyses two separate reactions, the first is a cyclooygenase reaction, in which arachidonate is converted to PGG_2 and secondly a peroxidase reaction, in which PGG_2 undergoes a two-electron reduction to PGH_2. The conversion of PGH_2 into prostaglandins is mediated by the action of specific isomerases.

1.2.2.2 *Prostaglandin G/H synthase isozymes*

For many years, only one PGHS enzyme had been identified; PGHS type-1 (PGHS-1) or often referred to as cyclooxygenase-1 (COX-1) and was purified from ram seminal vesicles in the mid 1970's (Rome & Lands, 1975). This enzyme is encoded by a 2.8 kb mRNA transcript (Rice *et al.*, 1995). Subsequent work by Simmons *et al* (1989) identified a second, inducible form of PGHS, now known as PGHS type-2 (PGHS-2). Each isoform can convert arachidonic acid to PGH_2 (DeWitt *et al.*, 1993; Zhang *et al.*, 1996), as shown in Figure 1.1. PGHS-2 is encoded by a 4.0-4.5 kb mRNA transcript and displays 60% amino acid sequence homology with PGHS-1, with the major difference between the two, involving their regulation and expression (Smith & DeWitt, 1995), as

discussed in Section 1.2.2.3. These isozymes are also important pharmacologically, as they are targets of non-steroidal anti-inflammatory drugs (NSAIDs) (refer to Section 1.6.2). Importantly, these two isoforms show different selectivity for NSAIDs allowing for selective inhibition of either PGHS-1 or -2, offering a potential use in the inhibition of premature labour.

1.2.2.3 Regulation of the expression of PHGS-1 and PGHS-2 during parturition

PGHS-1 is constitutively expressed in most tissues, developmentally regulated, and performs maintenance functions, such as regulating vascular homeostasis, stomach function and renal function (Smith & DeWitt, 1995). PGHS-1 levels are maintained fairly constant, although small increases of two- to four-fold can occur following hormonal or growth factor stimulation (DeWitt, 1991). Unlike PGHS-1, PGHS-2 is nearly undetectable in most tissues under normal physiological conditions, but its expression can be dramatically up-regulated by growth factors, gonadotrophins, cytokines and endotoxins (O'Sullivan *et al.*, 1992; Smith *et al.*, 1996).

The control of prostaglandin production within intra-uterine tissues has received considerable attention and numerous substances have been implicated in the regulation of prostaglandin production. With the exception of the amnion, in most cellular systems glucocorticoids attentuate PGHS-2 expression. Reduced transcriptional activity by dexamethasone has been demonstrated in serum-stimulated fibroblast (DeWitt & Meade, 1993) and mesangial cells stimulated with platelet-derived growth factor (Goppelt-Struebe, 1997). PGHS-1 is constitutively expressed in most cells and only marginally regulated (DeWitt, 1991), however, when up-regulated, PGHS-1 mRNA expression can also be reduced by the addition of glucocorticoids (DeWitt & Meade, 1993). Conversely, in human amnion cells PGE_2 synthesis increases with the addition of glucocorticoids (Potestio *et al.*, 1988), and is now thought to occur via enhanced expression of PGHS-2 mRNA (Zakar *et al.*, 1995). Using a glucocorticoid receptor antagonist, the stimulatory effect of glucocorticoids can be inhibited, indicating that the effect of glucocorticoids in the human amnion are receptor mediated (Potestio *et al*, 1988; Zakar *et al.*, 1995). Indeed, glucocorticoid receptors have been localised within human fetal membranes and decidua (Sun *et al.*, 1996), indicating that these membranes are capable of responding to glucocorticoids. *In vitro* studies have also shown that oestradiol is capable of inducing prostaglandin output in human fetal membranes in culture (Schatz & Gurpide, 1983). Similarly, oestradiol has also been shown to up-regulate myometrial PGHS-2 expression *in vivo* in ovariectomised non-pregnant

sheep (Wu *et al.*, 1997). Elevated fetal and maternal plasma oestrogen concentrations have been documented at the end of pregnancy (Challis, 1971; Nathanielsz *et al.*, 1982) and therefore may be in part responsible for the induction of PGHS-2 expression with the onset of labour. Increased fetal adrenal output of cortisol has been shown to result in the up-regulation of the PGHS-2 gene expression in the placenta, increasing the production of prostaglandins and further up-regulating fetal HPA cortisol output (Challis *et al.*, 2000; Whittle *et al.*, 2000).

Many cytokines have been shown to act on the amnion, chorion and decidua to increase prostaglandin output via an up-regulation of PGHS-2 mRNA expression (Albert *et al*, 1994; Spaziani *et al.*, 1996; Denison *et al.*, 1998; Hansen *et al.*, 1998). Interleukin-1β (IL-1β) dramatically increased the production of PGE_2 in human amnion derived cell line WISH (Albert *et al.*, 1994; Hulkower *et al.*, 1997; Wang & Tai, 1998). In addition, TNFα has also been shown to stimulate the production of prostaglandins in amnion-derived WISH cells (Hulkower *et al.*, 1997; Perkins & Kniss, 1997; Hansen *et al*, 1998), both mediated by an increase in the expression of PGHS-2 mRNA with no change in the PGHS-1 mRNA expression. Very recently Johnson *et al* (2002) showed that PGHS-2 mRNA is transcriptionally regulated and constitutively stable in the human amnion and may accumulate in this tissue over days, causing a steady state rise in the prostaglandin biosynthetic capacity before and during labour. Figure 1.2 shows a schematic diagram showing some of the factors known to up-regulate or down-regulate PGHS-2 expression in human intra-uterine tissues, adapted from Challis *et al* (2000).

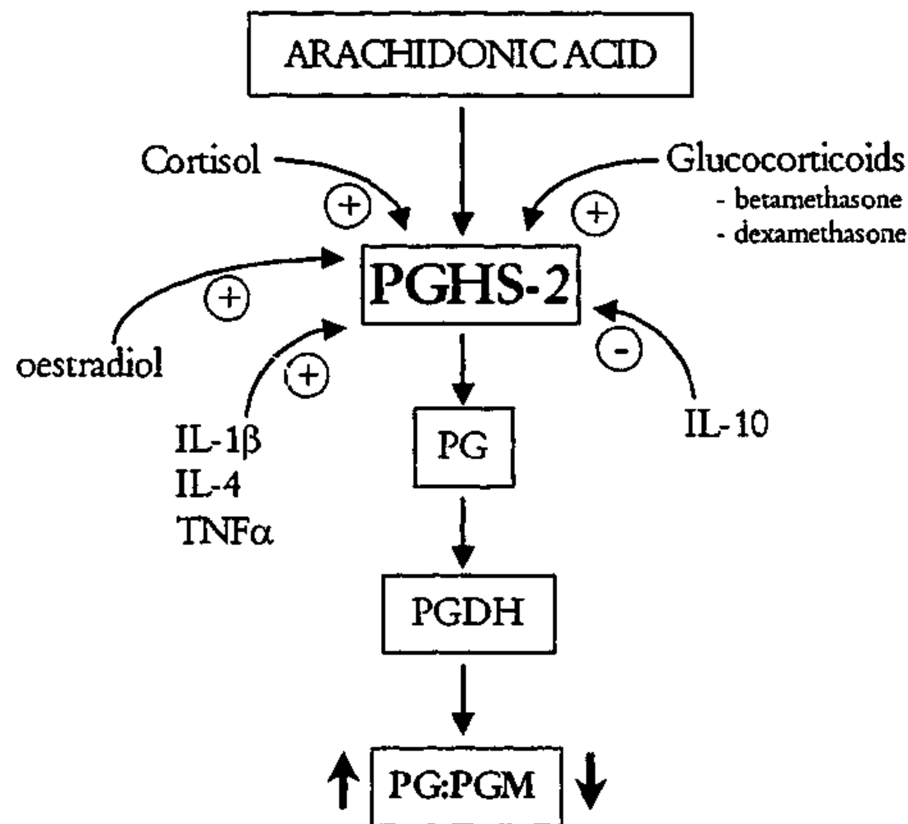

Figure 1.2 Schematic diagram showing the factors that can lead to the up-regulation or down-regulation of prostaglandin H synthase type-2 (PGHS-2) in human intra-uterine tissues, adapted from Challis *et al.* (2000). An increase in PGHS-2 mRNA expression alters the ratio of prostaglandins (PG) produced, to those metabolised (PGM). IL-1β, interleukin-1β; IL-4, interleukin-4; IL-10, interleukin-10; PGDH, 15-hydroxyprostagland dehydrogenase.

1.2.2.4 Expression of PGHS-1 and PGHS-2 during pregnancy and labour

Early investigations of the expression of immunoreacive (ir) PGHS in human gestational tissues showed PGHS was localised primarily in the cells of the amnion and chorion, and to a lesser extent in the cells of the cytotrophoblast and decidua (Bryant-Greenwood *et al.*, 1987), with similar distribution of PGHS during labour and premature labour (Divers *et al.*, 1995). Boshier *et al.* (1991), were the first to document the cellular localisation of PGHS enzyme in sheep placental tissue using immunohistochemistry; ir-PGHS cells were present throughout the trophoblastic epithelium in mononuclear cells, in maternal epithelial cells at the basal and apical regions of the placentome, the hemophagous zone, and in the chorionic fibroblasts and endothelial cells. However, these early studies were incapable of distinguishing between PGHS-1 and PGHS-2 isoforms due to the lack of specific polyclonal antibodies.

By examining the changes in specific activity of PGHS in human amnion tissue throughout gestation, PGHS activity increased three-fold over the final trimester of pregnancy before labour, and doubled with the onset of labour (Teixeira *et al.*, 1994).

Moreover, these investigators also demonstrated a four-fold increase in PGHS activity with the onset of idiopathic premature labour, suggesting that an up-regulation of PGHS is associated with both premature and term labour. Similarly, in sheep, increased cotyledon PGHS activity begins in the final third of gestation and increases dramatically in the last two weeks (Rice *et al.*, 1988).

With the identification of two distinct PGHS isoforms (Rome & Lands, 1975; Simmons *et al.*, 1989), it was then made possible to distinguish which isoform, PGHS-1 or PGHS-2, contributed to the increased PGHS activity seen at labour onset. A substantial amount of evidence now indicates that PGHS-2 is responsible for the increase in PGHS activity and prostaglandin production within intra-uterine tissues associated with normal and premature labour in humans and sheep. PGHS-1 mRNA expression although detectable, does not undergo parturition associated up-regulation in human fetal membranes, amnion, or chorion (Freed *et al.*, 1995; Hirst *et al.*, 1995b; Mijovic *et al*, 1997). Similarly, PGHS-1 mRNA is also constitutively expressed in the ovine placenta during pregnancy and does not significantly increase at the time of labour (Rice *et al*, 1995; Gibb *et al.*, 1996). Taken together, these studies support the contention that PGHS-1 is constitutively expressed during human and ovine pregnancy and that PGHS-1 may have a more important role in contributing to the maintenance of prostaglandin production involved in regulating vascular homeostasis, stomach and renal functions throughout gestation (McLaren *et al.*, 1996).

Conversely, PGHS-2 mRNA levels are increased after spontaneous labour and are positively correlated with enzyme activity in human amnion, chorion and decidual tissue (Hirst *et al.*, 1995a; Mijovic *et al.*, 1997). Therefore, it has been suggested that the increase in PGHS-2 mRNA is primarily responsible for the increased PGHS activity and amniotic fluid prostaglandin production seen at labour onset. Indeed this is supported by studies in the sheep, where specific up-regulation of PGHS-2 mRNA over PGHS-1 mRNA occurs during the last few days of gestation (Wimsatt *et al.*, 1993; Gibb *et al*, 1996). Using a well established model of induced premature labour in sheep by fetal glucocorticoid administration, McLaren *et al.* (1996) showed the induction of placental PGHS-2 protein in association with labour onset. Thus, strongly supporting the hypothesis that PGHS-2 is the enzyme responsible for the increased prostaglandin production at the time of labour in this species. It is likely then that this is responsible for the increase in PGHS immunoreactvity (Boshier *et al.*, 1991), and PGHS activity (Rice *et al.*, 1988; Wimsatt *et al.*, 1995) seen at this time.

1.2.2.5 *Prostaglandins and parturition*

Studies carried out in the mid 1960's first demonstrated the presence of prostaglandins in the amniotic fluid, with subsequent investigations identifying PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ within the amniotic fluid (Karim, 1966). The presence of PGE_2 and $PGF_{2\alpha}$ in amniotic fluid and circulation during labour and the ability of these prostaglandins to stimulate myometrial tissue *in vitro*, led to the idea that prostaglandins were involve in human parturition (Karim, 1972). In humans, the concentrations of PGE and PGF are higher in the amniotic fluid during labour than before, and increase progressively with labour (Mitchell, 1981; Mitchell *et al.*, 1995). This is also true for sheep, where amniotic fluid PGE and PGF concentrations are significantly elevated in late gestation compared to earlier gestation (Evans *et al.*, 1982) and, as labour approaches, PGF concentrations dramatically increase in the maternal utero-ovarian vein (Challis *et al.*, 1976). Also noted in these studies were increases in the intra-uterine tissue prostaglandin production, coinciding with the time when placental oestrogen production increases (Thorburn & Challis, 1979).

13,14-Dihydro-15-keto-PGF (PGFM) is the major metabolite of $PGF_{2\alpha}$ and circulates in 10 to 30-fold higher concentrations than $PGF_{2\alpha}$ (Mitchell, 1981). Many studies have used the measurement of PGFM as a reflection of $PGF_{2\alpha}$ concentrations in the maternal peripheral circulation. McLaren *et al.* (1996) showed that fetal plasma PGE_2, $PGF_{2\alpha}$ and PGFM concentrations were all significant increased after glucocorticoid-induction of premature labour in sheep. Moreover, an increase in maternal utero-ovarian vein $PGF_{2\alpha}$ and PGFM concentrations were observed with labour onset, as previously reported by Challis *et al.* (1976). In these studies, the level of PGFM in the peripheral circulation was substantially lower than that observed in the utero-ovarian vein. The arterio-venous difference in PGFM observed across the uterus suggests that the utero-ovarian vein PGFM concentrations originate from the metabolic degradation of $PGF_{2\alpha}$ delivered to the uterus from the peripheral circulation, as well as the intra-uterine production and metabolism of $PGF_{2\alpha}$ (McLaren *et al.*, 1996). Mitchell *et al.* (1978) demonstrated a significant arterio-venous difference for PGE in umbilical cord plasma, suggesting that the placenta is also an important source of PGE in the fetal circulation during later pregnancy. Indeed, after birth, prostaglandin concentrations in the fetal circulation decline rapidly in the sheep (Challis *et al.*, 1978) and human (Mitchell, 1981), most likely due in part to the increased metabolism through the

pulmonary circulation (Challis *et al.*, 1976), and mainly to the loss of placental prostaglandin production (Challis *et al.*, 1978).

1.2.2.6 Prostaglandins and the ductus arteriosus

During fetal life the ductus arteriosus diverts blood away from the fluid-filled lungs towards the descending aorta and placenta, while the ductus constricts rapidly after delivery following the initiation of breathing (Clyman, 1991). Patency of the fetal ductus arteriosus *in utero* is actively maintined by endogenously produced prostaglandins (Turner & Levin, 1984). Both locally generated and circulating prostaglandins are involved in the maintenance of ductal patency and closure (Friedman *et al.*, 1983). Prostaglandins, particularily PGE_2, seem to exert the major relaxing control on ductal patency *in vivo* (Guerguerian *et al.*, 1998). This is consistent with the findings from studies using isolated fetal lamb ductus arteriosus, which suggest that PGE_2 is the most potent ductus arteriosus relaxing agent (Clyman *et al.*, 1978; Coceani *et al.*, 1978). The formation of PGE_2 early in gestation appears to be dependent on PGHS-1, which is the dominant enzyme expressed in the ductus arteriosus at this time (Coceani *et al.*, 2001). Conversely, only at term does it appear that PGHS-2 contributes to prostaglandin production within the ductus arteriosus.

The notion that prostaglandins play a role in the maintenance of fetal ductus arteriosus patency has also stemmed from the demonstration that drugs interfering with the production of prostaglandins, such as indomethacin constrict this vessel. Indomethacin is capable of blocking all prostaglandin production, including those prostaglandins that act as pulmonary vasodilators (PGI_2 and PGE_2) and vasoconstrictors ($PGF_{2\alpha}$) in the newborn infant (Turner & Levin, 1984), lamb (Heymann & Rudolph, 1976) and rat (Sharpe *et al.*, 1974). Indomethacin used in late pregnancy for the prevention of premature labour (refer to Section 1.6.2), increases the risk of constriction of the ductus arteriosus and is therefore not recommended for use at or after 32 weeks gestation (Moise *et al.*, 1988; Norton *et al.*, 1993; Vermillion *et al.*, 1997; Guerguerian *et al.*, 1998). Chronic fetal ductal constriction can cause morphologic pulmonary vascular changes associated with fetal pulmonary hypertension, resulting in the syndrome persistent pulmonary hypertension (Levin *et al.*, 1979; Turner & Levin, 1984; Morin, 1989).

1.2.2.7 Prostaglandin receptors

Prostaglandin receptors have been pharmacologically classified according to their specificity for various agonists and antagonists in both animal and human tissue (Coleman *et al.*, 1994). There are five major prostanoid receptor subtypes (DP, EP, FP, IP, TP) named accordingly for their selectivity for each of the naturally occurring prostaglandins, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2, respectively (Coleman *et al*, 1994). The EP receptor is further subdivided into EP_1, EP_2, EP_3 and EP_4 subtypes according to their differential affinities for selective agonists (Coleman *et al.*, 1990). The prostanoid receptors are members of the guanine nucleotide regulatory proteins (G-protein) coupled receptor superfamily with seven transmembrane domains, characterised by seven hydrophobic regions that appear to represent membrane-spanning α-helices (Regan *et al.*, 1994). Prostaglandin receptors elicit biological responses by the stimulation or inhibition of second messengers, principally cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP_3) and intracellular calcium, acting through G-proteins (Coleman *et al.*, 1994; Regan *et al.*, 1994). G_s and G_i proteins have stimulatory and inhibitory effects, respectively, on the enzyme adenylate cyclase, which catalyses cAMP formation. Thus, coupling of prostaglandin receptors to G_s stimulates adenylate cyclase, causing relaxation, while, coupling of prostaglandin receptors to G_i inhibits adenylate cyclase, reducing cAMP, resulting in contraction (Slater *et al.*, 2002).

Prostaglandin receptors regulate uterine activity via the contractile (EP_1, EP_3, FP) or relaxatory (EP_2, EP_4) receptors (Ma *et al.*, 1999). The EP_1 and EP_3 receptors are excitatory, causing increases in phosphatidylinositol turnover and inhibition of adenylate cyclase, respectively (Coleman *et al.*, 1994). This classification is further complicated by the identification of isoforms of the receptors, which are generated by alternative splicing at the carboxy-terminal tail (Coleman *et al.*, 1994). EP_3 is generally coupled to G_i, however splice variants can couple to G_s and G_q, or both, leading to different cellular responses (Kotani *et al.*, 1997). The FP receptor is coupled with the G-protein mediated signal transduction pathway involving the stimulation of the PLC, IP_3 and the mobilisation of intracellular calcium (Coleman *et al.*, 1994). On the other hand EP_2 and EP_4 increase intracellular cAMP levels by stimulating adenylate cyclase activity.

In rat myometrium, EP_2 receptor mRNA expression is high before term and declines towards delivery (Brodt-Eppley & Myatt, 1998). Reduced expression of the EP_2 receptor mRNA has also been demonstrated in the myometrium of baboon (Smith *et al.*, 2001a),

sheep (Ma *et al.*, 1999) and with advancing gestational age in human myometrium (Brodt-Eppley & Myatt, 1999). Additionally, a decrease in the expression of EP_2 receptor mRNA has been demonstrated in the baboon decidua towards term (Smith *et al.*, 2001b). High expression of EP_2 receptor mRNA before term is consistent with the influence of EP_2 in activating intracellular adenylate cyclase, increasing cAMP and resulting in the maintenance of uterine quiescence (Brodt-Eppley & Myatt, 1999). Conversely, FP receptor mRNA expression is low before term and has been reported to dramatically increase at the onset of normal labour in the human myometrium (Brodt-Eppley & Myatt, 1999) and rat (Brodt-Eppley & Myatt, 1998; Dong & Yallampalli, 2000; Ou *et al.*, 2000). This is also consistent with the observation of increased expression of myometrial contractile FP receptor mRNA in several other species including the sheep (Ma *et al.*, 1999), mice (Cook *et al.*, 2000), and baboon (Smith *et al.*, 2001a). While in early human pregnancy, the contractile EP_3 and FP receptor mRNA are down-regulated (Matsumoto *et al.*, 1997). Furthermore, recent studies by Al-Matubsi *et al* (2001) demonstrated a significant increase in FP receptor protein in the rat myometrium with advancing gestational age, with maximal levels occurring at the time of delivery. These observations correspond to those changes previously observed with the expression of FP receptor mRNA in the rat (Ou *et al.*, 2000) and human (Brodt-Eppley & Myatt, 1999).

In the baboon myometrium, the contractile responses to prostaglandins has been shown to vary; PGE_2 contracts myometrial strips from the fundus but not the lower uterine segment (Smith *et al.*, 1998). This has been attributed to regional variations in prostaglandin receptor populations (Giannopoulos *et al.*, 1985; Smith *et al.*, 1998). Using northern blot analysis Smith *et al.* (1998) identified the prostaglandin receptor isoforms, EP_2, EP_3, FP_4 and FP receptor mRNA in the baboon myometrium. The EP_{2-4} receptor mRNA was also present in the cervix, decidua and fetal membranes including the chorion, whereas FP receptor mRNA was predominantly found in the myometrium and the cervix (Smith *et al.*, 1998; Smith *et al.*, 2001b). While other investigators have found the EP_3 receptor is more prominently expressed in the fundal part of the primate uterus, whereas the relaxatory EP_2 and EP_4 receptors dominate the lower segment (Giannopoulos *et al.*, 1985). These observations support the idea that varying receptor populations are responsible for the difference in contractile response of the fundus and lower uterine segment to prostaglandins. The specific reason for these regional variations in prostaglandin receptor populations within the uterus are yet to be fully

elucidated, but would appear to explain the differential control of regions of the uterus before and after delivery of the fetus (Smith *et al.*, 1998).

Thus, the change from a quiescent to a contractile uterus at parturition might occur because of the differential expression of relaxatory and contractile prostaglandin receptor isoforms (Brodt-Eppley & Myatt, 1999). It has been suggested that this process may be regulated by the steroid hormones, progesterone and oestrogen. Indeed, the expression of the relaxant EP_2 receptor mRNA in the rat uterus increases with pregnancy when progesterone concentrations are maximal and decreases with labour when circulating progesterone concentrations decline (Dong & Yallampalli, 2000). On the other hand, the contractile FP receptor mRNA expression is maximal at labour when the oestrogen concentrations are also increased in this species. This is in agreement with other similar findings observed by Ou *et al.* (2000). Thus, the steroid hormones appear capable of modulating the expression of relaxant and contractile uterine EP_2 and FP receptors that may participate in both the maintenance of uterine quiescence during pregnancy and the switch to contractions during labour (Dong & Yallampalli, 2000).

1.2.2.8 *Metabolism of uterine prostaglandins*

During pregnancy, at term, the amnion is the major source of prostaglandin synthesis (Mitchell, 1981). In the human, the chorion has a very high capacity to metabolise prostaglandins to their biologically inactive 15-keto metabolites due to the presence of type 1 NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase enzyme (PGDH), which is found predominantly within chorionic trophoblast and placental syncytiotropoblast cells (Van Meir *et al.*, 1997; Challis *et al.*, 2000). PGDH acts as an effective metabolic barrier that minimises the passage of unmetabolised prostaglandins derived from the amnion or chorion to the decidua and myometrium during pregnancy (Sangha *et al.*, 1994). Deficiency in PGDH enzyme activity within the chorion has be associated with idiopathic premature labour (Sangha *et al.*, 1994). Additionally, a reduction in PGDH expression and activity has also been associated with patients in premature labour with an underlying infection (Van Meir *et al.*, 1997), presumably due to the loss of extravillous trophoblast cells in the chorion in response to inflammation (Van Meir *et al.*, 1996). As a consequence, prostaglandins produced by the amnion and chorion can more easily reach the myometrium to stimulate myometrial contraction leading to premature labour (Sangha *et al.*, 1994). *In vitro* studies have shown basal PGDH expression and activity of chorionic trophoblast cells to be sustained by

progestogens, whereas glucocorticoids such as cortisol and dexamethasone decrease PGDH expression (Patel *et al.*, 1999), and can also up-regulate PGHS-2 expression in human fetal membranes, refer to Figure 1.2 (Challis *et al.*, 2000). Additional studies have reported that several cytokines (IL-1β and TNFα) can down-regulate PGDH enzyme activity (refer to Section 1.6.3) (Pomini *et al.*, 1999). These cytokines can also up-regulate PGHS-2 expression further increasing the availability of prostaglandins. Figure 1.3 shows a schematic diagram showing the regulation of PGDH by glucocorticoids, cytokines and progesterone within the human chorion, adapted from Challis *et al.* (2000). Hence, the net prostaglandin output within uterine tissues represents a balance between actions on prostaglandin synthesis and metabolising enzymes within intra-uterine tissues.

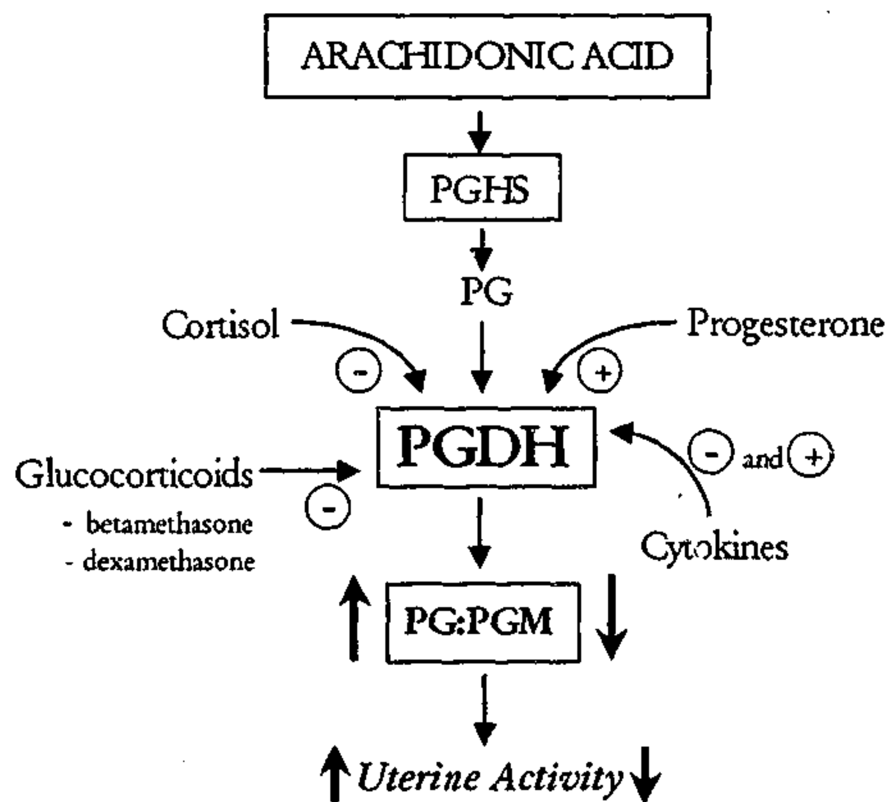

Figure 1.3 Schematic diagram showing the factors that can lead to the up-regulation or down-regulation of 15-hydroxyprostaglandin dehydrogenase (PGDH) in human chorion, adapted from Challis *et al.* (2000). Alteration in PGDH expression within the chorion alters the ratio of primary prostaglandin (PG) to prostaglandin metabolite (PGM), and the level of prostaglandins available to stimulate myometrial contractility. PGHS, prostaglandin H synthase.

1.2.3 Oxytocin and parturition

Oxytocin is the most potent known natural substance that stimulates uterine contractions, however, the role of oxytocin and its receptor in the initiation of parturition remains controversial (Mitchell & Schmid, 2001). Oxytocin is a nonapeptide that is mainly synthesised in the paraventricular and supraoptic nuclei of the hypothalamus (Zakar & Mitchell, 1996) and is transported along the axons of the supra-optic-hypophyseal nerve tract to the posterior pituitary. Oxytocin release into the circulation occurs in a pulsatile manner (Gibbens & Chard, 1976), and the frequency of these pulses increases as spontaneous labour progresses (Fuchs *et al.*, 1991). Oxytocin also plays an important role in lactation (Russell & Leng, 1998), causing contractions of the myoepothelial cells, which surround the alveoli, to cause milk ejection or the milk-let down reflex (Gimpl & Fahrenholz, 2001). In addition, distenstion of the cervix and vagina in the latter stages of labour, produces a neuro-endocrine reflex release of oxytocin, resulting in contractions of the smooth muscle of the uterus.

1.2.3.1 Oxytocin and the initiation of labour

There remains controversy in the literature over the role of maternal plasma oxytocin in the timing of oxytocin secretion at labour onset and delivery. This is partly attributed to the pusatile secretion of oxytocin and the action of circulating oxytocinase which has made it difficult to consistently show an increase in oxytocin secretion during labour in women. In humans, investigators have reported an increase in oxytocin levels with advancing gestation (Gibbens & Chard, 1976; Dawood *et al.*, 1979). While others have demonstrated that maternal plasma oxytocin concentrations were only significantly raised during the first stage of labour (Fuchs *et al.*, 1983a), and reach maximal levels during the second stage of labour (Gibbens & Chard, 1976; Dawood *et al.*, 1978). Similarly, in the ewe, Glatz *et al.* (1981) established that oxytocin concentrations were also elevated during the second stage of labour. Umbilical arterio-venous differences in oxytocin concentrations, has led to initial speculation that oxytocin may be of fetal origin and is transported into the maternal circulation, suggesting the fetus may play an active role in the initiation of labour (Chard *et al.*, 1971). Indeed, Dawood *et al* (1983) reported a change in fetal oxytocin levels at the time of labour onset, suggestive of fetal release of oxytocin at this time. This view, however, is not supported in other animal studies in which an increase in fetal plasma oxytocin concentrations was not evident (Glatz *et al.*, 1981; Hirst *et al.*, 1993). The sensitivity of the myometrium to oxytocin

increases with the advancement of pregnancy (Takahashi *et al.*, 1980; Burgess *et al*, 1992), and is directly related to the oxytocin receptor population within the uterus (Fuchs *et al.*, 1983b). Whether an increase in oxytocin release from the posterior pituitary triggers the onset of labour, or whether marked increases in the oxytocin receptors in both the endometrium and myometrium maybe more important at this time is not clear (Fuchs *et al.*, 1984).

Evidence supporting an additional extra pituitary source of oxytocin came from studies on the ovine corpus lutem (Wathes & Swann, 1982). In sheep, luteal oxytocin synthesis is initiated at the time of ovulation, peaks in the early to mid-luteal phases, then declines and remain low during gestation (Sheldrick & Flint, 1983). Additionally, human studies have shown that oxytocin can also be produced by both the endometrium and fetal membranes with concentrations increasing at term (Chibbar *et al.*, 1993). With this in mind, a paracrine loop between oxytocin and the receptors within the pregnant uterus may override the need for oxytocin, of posterior pituitary origin, to act as an initiating stimulus for parturition in both humans and sheep (Mitchell & Chibbar, 1995; Wathes *et al.*, 1996; Mitchell *et al.*, 1998). If the source of oxytocin that mediates uterine contractions is within the uterus itself, an increase in local oxytocin secretion could initiate labour without the increase being reflected in the maternal peripheral circulation (Mitchell & Schmid, 2001).

1.2.3.2 *Oxytocin sensitivity and receptor changes during pregnancy*

During the early stages of human pregnancy the uterus remains relatively unresponsive to oxytocin due to the antagonistic role of progesterone (Gimpl & Fahrenholz, 2001). In late gestation, oxytocin receptor numbers dramatically increase under the influence of higher circulating levels of oestrogens, at which stage myometrial contractility and uterine prostaglandin biosynthesis become enhanced (Fuchs *et al.*, 1982a). The oxytocin receptor is a member of the oxytocin-vasopressin receptor family and which in turn, is a member of the G-protein coupled receptor superfamily (Mitchell & Schmid, 2001). Figure 1.4 shows a schematic representation of the mechanism of action of oxytocin with its membrane bound receptor, from Mitchell and Schmid (2001). Oxytocin acts on the uterus via membrane-bound receptors of myometrial cells which, upon activation stimulate PLC, hydrolysing phosphatidylinositol 4,5-bisphosphate and causing the production of 1,2-diacylglycerol and inositol 1,4,5-triphosphate, which in turn release intracellular calcium from the sarcoplasmic reticulum, combined with a direct effect on the influx of extracellular calcium (Phaneuf *et al.*, 1993; Shojo & Kaneko, 2000). The rise

in intracellular calcium concentrations may then activate, in conjunction with calmodulin, myosin light chain kinase, leading to the activation of contractile proteins (Mitchell & Schmid, 2001). Recent evidence suggests oxytocin stimulation of culture human myometrial cells induces activation of mitogen-activated protein kinase (MAPK) through a G-protein-coupled mediated mechanism leading to PGHS-2 gene expression and an increase in uterine prostaglandin production (Molnar *et al.*, 1999).

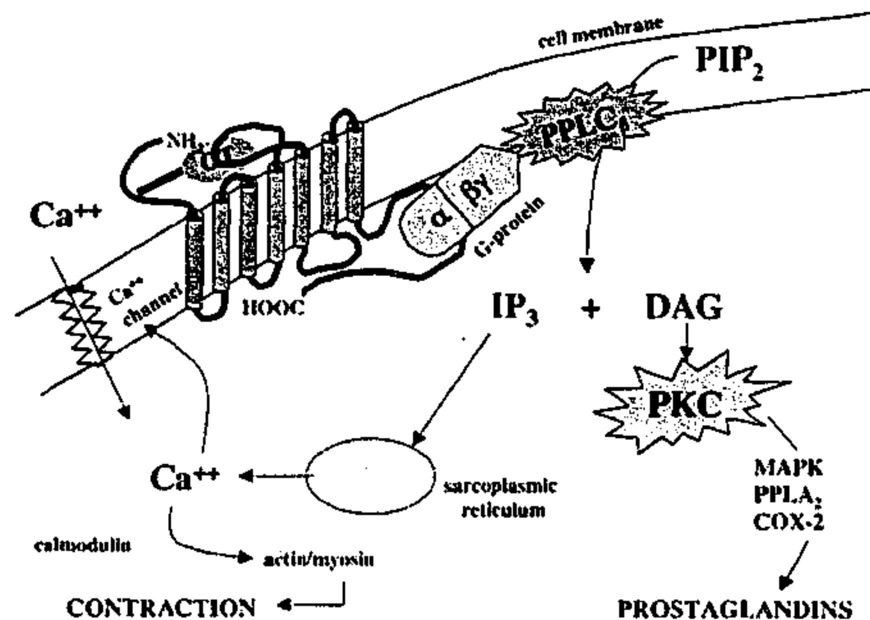

Figure 1.4 Mechanism of action of oxytocin (OT), from Mitchell and Schimid (2001). Interaction of oxytocin with its membrane bound receptor triggers G-protein (α/βγ) mediated activation of plasma membrane phospholipase C (PPLC) resulting in production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). The DAG then stimulates protein kinase C (PKC) which activate multiple mechanisms to increase contractility. The IP_3 stimulates intracellular calcium (Ca++) release from the sarcoplasmic reticulum. The oxytocin receptor may also be connected directly to a receptor-activated calcium channel. The increase in intracellular calcium stimulates actin/myosin coupling resulting in muscle fibre contraction.

The responsiveness of the myometrium to oxytocin greatly increases with advancing gestational age in many species (Takahashi *et al.*, 1980; Burgess *et al.*, 1992). This is associated with both an increase in myometrial oxytocin receptor mRNA and an increase in the density of myometrial oxytocin receptors, which was first demonstrated in rats (Soloff, 1979). This was later confirmed in humans (Fuchs *et al.*, 1982a; Kimura *et al.*, 1996), sheep (Wu & Nathanielsz, 1994; Wathes *et al.*, 1996) and cows (Fuchs *et al*, 1992), where it was noted that myometrial oxytocin receptors increases gradually throughout pregnancy and rise to maximal levels at the time of labour. In rhesus monkeys it was shown that, the maternal oxytocin concentrations were positively

correlated with nocturnal uterine activity and progressively increased during late pregnancy and delivery (Hirst *et al.*, 1993). In sheep, there is also a clear association between myometrial and endometrial oxytocin receptor mRNA and protein expression, with myometrial contractile activity (Wu *et al.*, 1996).

The increase in oxytocin receptors prior to labour is not confined to the myometrium. Using *in situ* hybridisation techniques, oxytocin receptor mRNA has also been localised in the amnion, chorion and decidua (Chibbar *et al.*, 1993; Takemura *et al.*, 1994), with the strongest mRNA expression in the decidual cells adjacent to the myometrium (Chibbar *et al.*, 1993). These observations are similar to the more recent findings of Wathes *et al.* (1999) describing the oxytocin receptor in human myometrium and fetal membranes throughout the second half of gestation. Oxytocin receptors found in the myometrium are presumed to be associated with contractile activity, whereas decidual oxytocin receptors may be involved in the stimulation of $PGF_{2\alpha}$ release (Fuchs *et al.*, 1982a; Fuchs *et al.*, 1984). Thus, the oxytocin receptor system has two important roles; the stimulation of uterine contractions and the production of prostaglandins.

1.2.3.3 *Interaction between oxytocin and prostaglandins*

The addition of oxytocin to cultured human amnion and myometrial cells results in a rapid and significant increase in PGE_2 production (Moore *et al.*, 1988), attributable to an up-regulation of PGHS-2 mRNA expression (Molnar *et al.*, 1999). It has been suggested that oxytocin stimulates hydrolysis of membrane phospholipids to liberate free diacylgycerol and other fatty acids that increase the availability of free arachidonic acid (Hirst *et al.*, 1993; Ivanisevic *et al.*, 2001). Therefore oxytocin may act at the substrate level of prostaglandin production, in addition to the stimulation of PGHS-2 expression. Elevated oxytocin receptor mRNA expression has been identified in the human decidua (Fuchs *et al.*, 1982a; Chibbar *et al.*, 1993), which has also been shown to possess high concentrations of PGHS (Hirst *et al.*, 1995a). It has also been proposed that oxytocin binding sites in the decidua are responsible for mediating the increase in prostaglandin synthesis which occurs at this time, and that these prostaglandins then diffuse to the adjacent myometrium and enhances oxytocin-induced concentrations in human labour (Fuchs *et al.*, 1982a). Oxytocin alone is unable to induce labour or cervical dilation unless there is a concomitant increase in $PGF_{2\alpha}$ production (Fuchs *et al.*, 1983a). In addition, oxytocin stimulation *in vitro* induces the production of $PGF_{2\alpha}$ in decidual cells (Fuchs *et al.*, 1981). There are considerable amounts of oxytocin found in the amniotic

fluid (Dawood *et al.*, 1979). Amniotic fluid oxytocin may pass through the amnion, and may continue through the chorionic cytotrophoblast to reach the decidua, hence it has been postulated that fetal oxytocin concentrations may be able to provide a stimulus to local production of prostaglandins at the onset of labour in humans (Fuchs *et al.*, 1982a). The interaction of oxytocin and prostaglandin is also well documented in sheep. $PGF_{2\alpha}$ release in response to oxytocin has also been observed in sheep by Burgess *et al.* (1990) and Meier *et al.* (1995), with maximal responsiveness at 145 days of gestation, supporting the role of oxytocin increasing the synthesis and release of $PGF_{2\alpha}$ via action on receptors in the myometrium at term. This is further supported by studies on ovine and bovine endometrium, where oxytocin induced a rapid increase in $PGF_{2\alpha}$ and PGFM and was associated with increased endometrial PGHS-2 expression (Asselin *et al.*, 1997; Burns *et al.*, 1997; Xiao *et al.*, 1999). Moreover, with the addition of a specific PGHS-2 inhibitor, oxytocin induced prostaglandin production is blocked, supporting the hypothesis that PGHS-2 is necessary for the stimulation of oxytocin-induced intra-uterine prostaglandin production in sheep (Asselin *et al.*, 1997) and humans (Molnar *et al.*, 1999). Alternatively, administration of an oxytocin receptor antagonist to pregnant sheep has also been shown to inhibit oxytocin-induced prostaglandin release (Jenkin *et al.*, 1994). It appears therefore that the coupling of oxytocin receptor activation to prostaglandins synthesis forms a positive feed-forward system within the decidua and uterine tissues that increases myometrial contractility and may be one of the critical steps in the mechanisms that contribute to the progression of labour once established.

1.3 UTERINE ACTIVITY

The uterus is a unique organ containing three functionally distinct layers and is capable of increasing approximately 20-fold in size during gestation (Jenkin & Nathanielsz, 1994). The smooth muscle component of the uterus, the myometrium, is subdivided into an inner circular layer of muscle fibres, and an outer layer of longitudinally aligned muscle fibres (Devedeux *et al.*, 1993). Periodic epochs of contractility, termed *contractures*, lasting several minutes, occur throughout the majority of pregnancy in a number of species including humans (Nathanielsz & Honnebier, 1992; Jenkin & Nathanielsz, 1994). Contractures are clearly distinguishable from *contractions* which occur at term, as they generally are low in amplitude with low frequency (1-3/h), and have a longer duration of 3-15 min (Figueroa *et al.*, 1985; Nathanielsz & Honnebier, 1992).

Contractures also generate a relative smaller rise in intra-uterine pressure. In contrast, uterine *contractions* are characterised by high amplitude and frequency (30/h) with a relatively short duration (0.5-1.0 min) and markedly greater increases in intra-uterine (> 5 mmHg).

In sheep, uterine activity has been recorded via electromyograph (EMG) electrodes sutured to the exterior surface of the myometrium, from the early stages of pregnancy up to and including the time of expulsion of the fetus, in combination with intra-uterine pressure, recorded by balloons placed directly in the uterus (Harding *et al.*, 1982; Sigger *et al.*, 1984; Jenkin & Nathanielsz, 1994). In early pregnancy, ewes display contractures with small rises in intra-uterine pressure, which are not synchronous over the entire uterus (Thorburn *et al.*, 1984), suggesting limited propagation of activity through the myometrium at this stage (Sigger *et al.*, 1984). Recordings of such activity also indicate that uterine activity is generated in localised areas of the uterus, and these regions may vary in position (Jenkin & Nathanielsz, 1994). The onset of labour in sheep is associated with increased frequency of uterine EMG activity and an increase in the pressure accompanying each burst, suggesting an increase in the coordination and spread of activity as delivery approaches (Thorburn *et al.*, 1984). The increased ability of activity to spread throughout the entire uterus during delivery may be explained by the change in electrical resistance of myometrial cells to allow the propagation of membrane currents that have been observed at term.

In sheep, the approach of labour can be observed by the switch in pattern of contractures seen during late gestation to a pattern charateristic of contractions, which occur in the last 24-36 h before delivery (Thorburn *et al.*, 1984; Nathanielsz *et al.*, 1995). The switch from contractures to contractions occurs progressively and only once during the process of labour in the sheep. The distinction between uterine contractures and contractions can be seen in Figure 1.5. In contrast to sheep, in non-human primates the switch from uterine contractures to uterine contractions occurs over several nights before the onset of labour (Honnebier *et al.*, 1989; Hirst *et al.*, 1991). When the switch initially occurs, uterine contractions last for 2 to 3 h before uterine activity reverts back to the contracture pattern. This pattern is repeated on several sequential nights, intensifying until eventually parturition occurs.

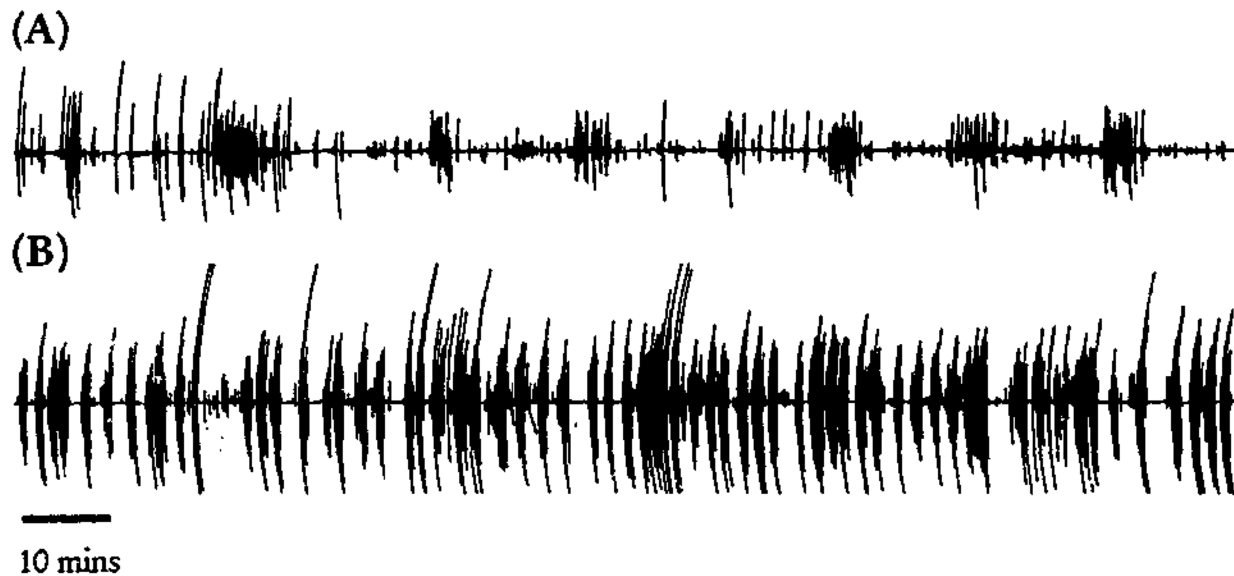

Figure 1.5 An example of uterine EMG activity recorded from a ewe during late gestation and labour. Panel (A) represents uterine EMG activity, characteristic of uterine *contractures*. Panel (B) shows the pattern of uterine EMG activity characteristic of uterine *contractions* observed during labour.

The development of uterine activity required during labour appears to be primarily the result of two events (Challis *et al.*, 2000). The initial activation of the myometrium involves a number of processes, particularly the appearance of oxytocin and oestrogen receptors (Wathes *et al.*, 1996), a lower resting potential of smooth muscle cells and gap junction formation, permitting cell-to-cell coupling (Garfield *et al.*, 1988). Further stimulation is produced by increases in stimulatory agonists, such as oestrogens, $PGF_{2\alpha}$, PGE_2 and oxytocin, to enhance contractile activity of the activated myometrium. Quiesence of the myometrium during pregnancy and the high levels of contractile activity during labour are largely controlled by an intricate interaction of steroids, peptides and prostaglandins. Oestrogens and progesterone seem to modulate the activity of the myometrium via changes in the synthesis of inhibitory or stimulatory regulators and their receptors. However, prostaglandins and oxytocin exert their effect via direct stimulation or inhibition of the myometrium through activation or inactivation of regulatory and contractile proteins. Oxytocin can stimulate the myometrium directly through membrane bound receptors, and indirectly through an increase in intra-uterine prostaglandin release (Meier *et al.*, 1995), and together they stimulate myometrial activity, possibly indicating a role in generation as well as maintenance of uterine activity in sheep (Nathanielsz & Honnebier, 1992), and non-human primates (Hirst *et al.*, 1993). Prostaglandins can also stimulate the myometrium directly by increasing calcium influx

into the cell and allowing calcium release from the sarcoplasmic reticulum, while also enhancing the production of myometrial gap junctions that are necessary for the production of co-ordinated uterine contractions (Garfield *et al.*, 1980; Garfield & Hayashi, 1981; Garfield *et al.*, 1988; Coleman *et al.*, 1994).

Episodes of nocturnal uterine activity in pregnant rhesus monkeys have been well characterised and increase as gestation advances (Hirst *et al.*, 1993). Nocturnal oxytocin concentrations also increase as gestation advances, and this increase is strongly correlated with uterine activity displayed in this species. Interestingly, the switching from contractures to a pattern of contractions in the rhesus monkey occurs at night, corresponding to increase nocturnal oxytocin concentrations seen at this time (Hirst *et al.*, 1991). The possibility that changes in maternal plasma oxytocin play a role in the periodicity of the switch from contractures to contractions is supported by studies in which oxytocin antagonists have successfully inhibited the switch of uterine activity in this species (Honnebier *et al.*, 1989). The oxytocin antagonist, atosiban, was found to inhibit the switch from contractures to contractions in the pregnant rhesus monkey (Giussani *et al.*, 1996), and pregnant baboons (Wilson *et al.*, 1990). These findings strongly suggest that oxytocin plays a central role in the switch from contractures to contractions and uterine activity during parturition (Nathanielsz *et al.*, 1995).

1.4 CERVICAL RIPENING

Prostaglandins appear to play a central role in cervical ripening (Bukowski *et al.*, 2001). Natural and synthetic prostaglandins can ripen the cervix at any stage in pregnancy and have therefore been used prior to labour and also before to the termination of a pregnancy (Calder & Greer, 1990). Cervical ripening is the result of very active and dynamic changes within the structure of the cervix; from a tightly closed structure designed to maintain the fetus with the uterus, to a soft more compliant composition that will facilitate the passage of the fetus during delivery (Danforth *et al*, 1974). Increases in hyaluronic acid and physiologic cell death are in part responsible for the structural alterations of the cervical connective tissue during pregnancy and parturition (Osmers *et al.*, 1993; Ludmir & Sehdev, 2000). The collagen bundles disperse and loose strength, cytokines, hyaluronic acid, collagenases and elastase work together to allow effacement (Ludmir & Sehdev, 2000).

1.5 PREMATURE LABOUR

Labour is considered premature when delivery occurs prior to 37 weeks gestation (Owen & Patel, 1995; Jeyabalan & Caritis, 2002). Premature labour is the major cause of neonatal morbidity and mortality, accounting for 65% of neonatal deaths and 50% of childhood neurological disabilities (Terzidou & Bennett, 2002). Some risk factors for premature labour have been defined, Table 1.1 (Lumley, 1987; Iams *et al.*, 1988; Meis *et al.*, 1995). However, about 50% of all premature births affect women that are thought not to be at risk (Amy & Cammu, 1992), and with the exception of multiple pregnancy, the predictive value of each of the individual risk factors is less than 30% (Iams *et al*, 1988).

Term labour results from physiological activation of multiple pathways, whereas premature labour is a pathological condition caused by multiple etiologies that activate one or more of the components of these pathways (Ivanisevic *et al.*, 2001). Manyof the endocrine changes observed in the time preceding premature labour in women, including increased prostaglandin biosynthesis, which leads to the synchronisation and stimulation of myometrial activity, closely resemble those observed prior to normal spontaneous labour (Fuchs *et al.*, 1983a; Rice *et al.*, 1987; Terzidou & Bennett, 2002). This has lead to the suggestion that premature labour results from the same sequence of events that occur in normal term pregnancy, but that are triggered too early. The plasma concentrations of PGFM and oxytocin have been measured in women during premature labour, and are higher than that measured in women of similar gestation age, but who are not in labour (Fuchs *et al.*, 1982b). In addition, early increases in the myometrial oxytocin receptors, similar to that observed during normal term labour, have been implicated in the uterine hyperactivity seen in premature labour (Fuchs *et al.*, 1982a), thus creating an early initiation of the hormonal events that normally occur at term. Including an increased responsiveness of the uterus to endogenous oxytocin and prostaglandins, which together can lead to active premature labour. More recently, a deficiency in prostaglandin metabolism within the chorion has been associated with premature labour without infection (Sangha *et al.*, 1994), and as a consequence, prostaglandins produced by the amnion can more easily reach the myometrium to stimulate myometrial contraction leading to premature stimulation of the uterus. While it is still unclear whether prostaglandins or oxytocin are involved in triggering premature labour, it is clear that these agents play an important role in its progression.

The fetoplacental unit may trigger premature labour if the intra-uterine environment becomes hostile and threatens the well-being of the mother or fetus (Norwitz *et al*, 1999). Many investigators consider that approximately 30-40% of premature births are associated with an underlying infective process (Romero *et al.*, 1989b; Lettieri *et al*, 1993; Challis *et al.*, 2000). Infection is now the only pathologic process for which a firm causal link with prematurity has been established, and for which a defined molecular pathophysiology is now known (Goncalves *et al.*, 2002).

Table 1.1 Principal risk factors associated with premature labour, adapted from Lumley (1987) and Iams *et al.* (1988).

Demographic risks
Age <17 or > 35
Ethnicity (black > nonblack)
Low socioeconomic and educational status
Behavioural risks
Deficient nutrition before and after pregnancy
Smoking during pregnancy
Excessive physical activity
Prepregnancy medical/obstetrical risks
Prior premature delivery
Prior second trimester loss
Uterine or cervical defects
Urinary tract infection
Current pregnancy
Viral pneumonia
Appendicitis
Low systolic/diastolic blood pressure
Anaemia
Pre-clampsia
Eclampsia
Disorders of the placenta and membranes
Polyhydramnios/oligohydramnios
Chorioamnionitis
Uterine distention-multiple gestation
1st or 2nd trimester bleeding
Abruptio placentae
Placenta pravia
Cervical dilation > 1 cm or effacement

1.6 INTRA-UTERINE INFECTION AND PREMATURE LABOUR

Intra-uterine infection has been recognised over the past twenty years as a major contributor to the pathogenesis of premature labour (Romero & Mazor, 1988) and is now linked to significant neonatal and infant complications such as periventricular leukomalacia, bronchopulmonary dysplasia and cerebral palsy (Dammann & Leviton, 1997; Romero *et al.*, 2001). Recent evidence suggests that intra-uterine infection may occur quiet early in pregnancy and remain undetected for months (Gravett *et al.*, 2000), thus exposing the fetus to the effects of infection for a considerable period. Identifying women with intra-uterine infections therefore, is a major obstetric challenge, as intra-uterine infection is often asymptomatic with no signs of fever or uterine tenderness until labour begins or membrane rupture (Romero & Mazor, 1988; Goldenberg *et al.*, 2000; Goncalves *et al.*, 2002).

1.6.1 Definition of intra-uterine infection

Intra-uterine infection has been classified into two broad categories, *intra-amniotic* and *extra-amniotic* infections and each have been extensively reviewed (Romero & Mazor, 1988; Romero *et al.*, 2001; Goncalves *et al.*, 2002). Detection of any organisms from the normally sterile amniotic fluid, constitutes evidence of microbial invasion (Romero & Mazor, 1988), which can then initiate a cascade of inflammatory mediators, resulting in prostaglandin production, uterine contractions and cervical changes culminating in premature birth (Greci *et al.*, 1998). Romero and Mazor (1988) defines intra-amniotic infection as the presence of a positive amniotic fluid culture regardless of the presence or absence of clinical symptoms of infection (i.e. fever, uterine tenderness and vaginal discharge). While the term *clinical chorioamnionitis* refers to the clinical syndrome associated with microbial invasion of the amniotic cavity (Gibbs *et al.*, 1982), only a small proportion of women with premature labour and a positive amniotic fluid culture have clinical symptoms of chorioamnionitis (Romero *et al.*, 1989b).

Extra-amniotic infections can be determined by culture of the chorio-amniotic compartment, however, surface culture of the membranes are often contaminated by the vaginal flora after the placenta and membranes have passed through the birth canal (Romero *et al.*, 2001). Therefore most studies have focused on the intra-amniotic infections where amniotic fluid is sampled by trans-abdominal amniocentesis.

1.6.2 Pathways of intra-uterine infection

Infections within the uterus can occur between the chorio-decidual compartment, within the fetal membranes (the amnion and chorion), in the placenta, amniotic fluid, or within the umbilical cord or fetus (Goldenberg *et al.*, 2000; Romero *et al.*, 2001; Goncalves *et al.*, 2002). The most common pathway of intra-uterine infection is thought to be ascending from the vagina and/or cervix. This is supported by the finding that most organisms associated with premature labour are commonly found in normal vaginal flora (McDonald *et al.*, 1991). Figure 1.6 shows a schematic representation of the proposed pathway of intra-uterine infection in women, adapted from Romero and Mazor (1988) and Goldenberg *et al.* (2000). Romero and Mazor in 1988 first proposed a four stage process during ascending uterine infection. The first stage consists of the presence and overgrowth of organisms in the vagina and cervix (Figure 1.6 A). The micro-organisms then ascend from the vagina into the chorio-decidual compartment leading to deciduitis and further extension to chorionitis (Figure 1.6 B). In women, the micro-organisms are then thought to cross the intact chorio-amniotic membranes into the amniotic fluid (Figure 1.6 C) (Romero & Mazor, 1988). The infected amniotic fluid then provides a conduit through which the infection, or the infective agents can then gain access to the fetus (Figure 1.6 D). The fetus may swallow or aspirate the infected amniotic fluid, which can result in congenital pneumonia, or direct contact with infected amniotic fluid may lead to localised infections, and once in the fetal circulation can lead to fetal sepsis (Romero & Mazor, 1988).

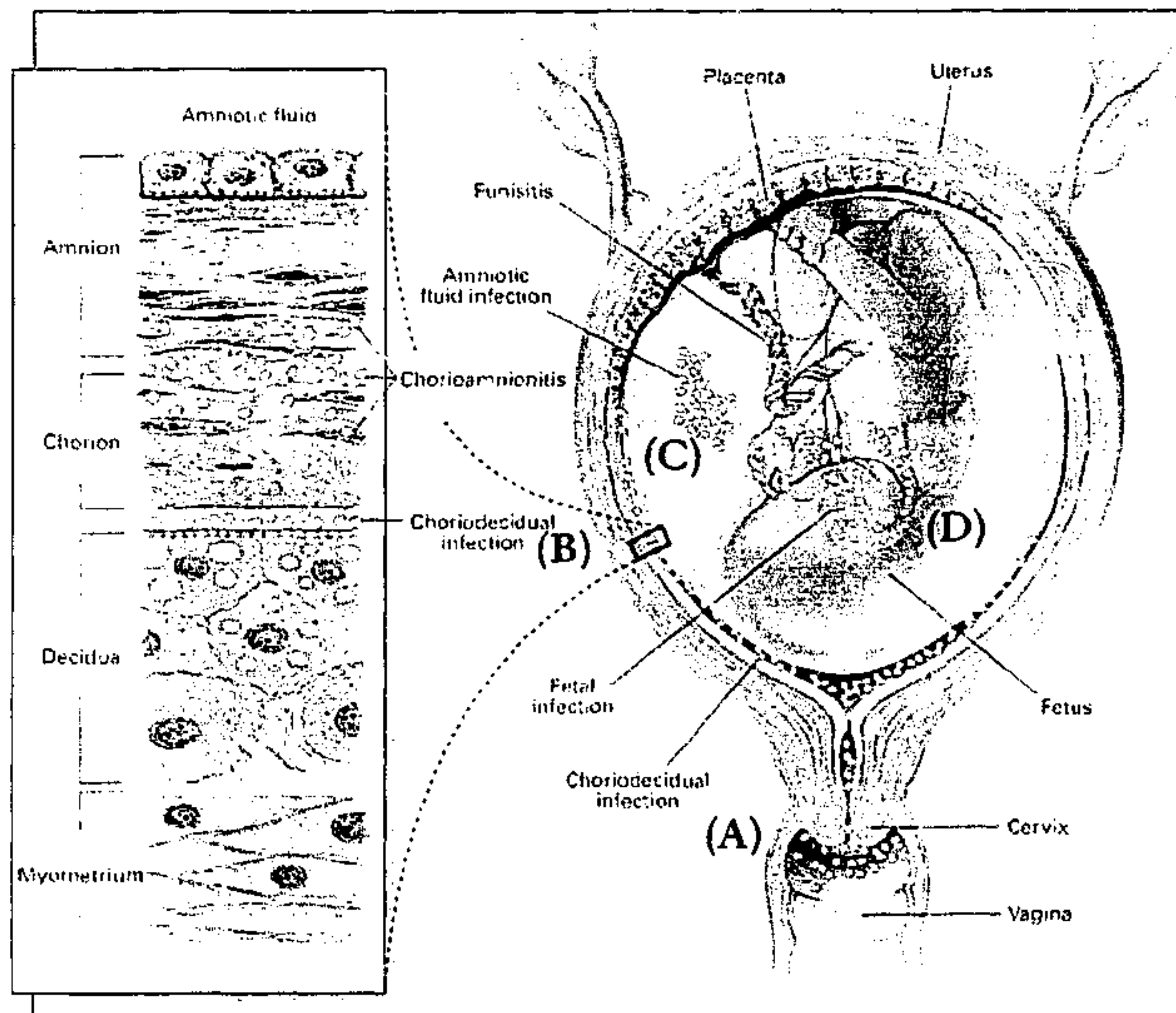

Figure 1.6 A schematic representation of the proposed pathways of intra-uterine infection, adapted from Romero and Mazor (1988) and Goldenberg *et al.* (2000). Organisms first ascend from the vagina (A), through the cervix and reside within the chorio-decidual space leading to deciduitis and chonrionitis (B). The micro-organisms are then thought to cross the intact chorio-amniotic membranes, infecting the amniotic fluid (C), leading to fetal sepsis (D).

1.6.3 Mechanisms responsible for the initiation of labour associated with intra-uterine infection

The presence of lipopolysaccharides (LPS), a pyrogenic component of gram-negative bacteria cell walls in the chorio-decidual compartment, stimulates macrophages to produce cytokines, such as TNF-α, IL-1 and IL-6 as well as nitric oxide (NO) in response to LPS (Romero *et al.*, 1989b; Dziegielewska *et al.*, 1998; Garnier *et al.*, 2001). These cytokines then stimulate prostaglandin synthesis and release while also initiating neutrophil infiltration. These cells synthesise and release metalloproteases and other bioactive substances that are involved in cervical ripening, Figure 1.7 (Goldenberg *et al*, 2000). The formation of phospholipid metabolites in human gestational tissues is also,

in part, due to the release of the primary phospholipase isozyme secretory type II phospholipase-A_2 (PLA_2) (Rice, 1995). This enzyme accounts for more than 80% of total tissue PLA_2 enzyme activity in the term placenta (Rice *et al.*, 1998). Its release is enhanced by bacterial endotoxin (Farrugia *et al.*, 1999), and has been shown to stimulate prostaglandin production by human amnion (Bejar *et al.*, 1981; Bennett *et al.*, 1987b). In addition, chorionic infections decrease the metabolism of prostaglandins by decreasing prostaglandin dehydrogenase expression and activity within uterine tissues, therefore allowing increased amounts of prostaglandins to reach and stimulate the myometrium, Figure 1.7 (Hahn *et al.*, 1998; Goldenberg *et al.*, 2000).

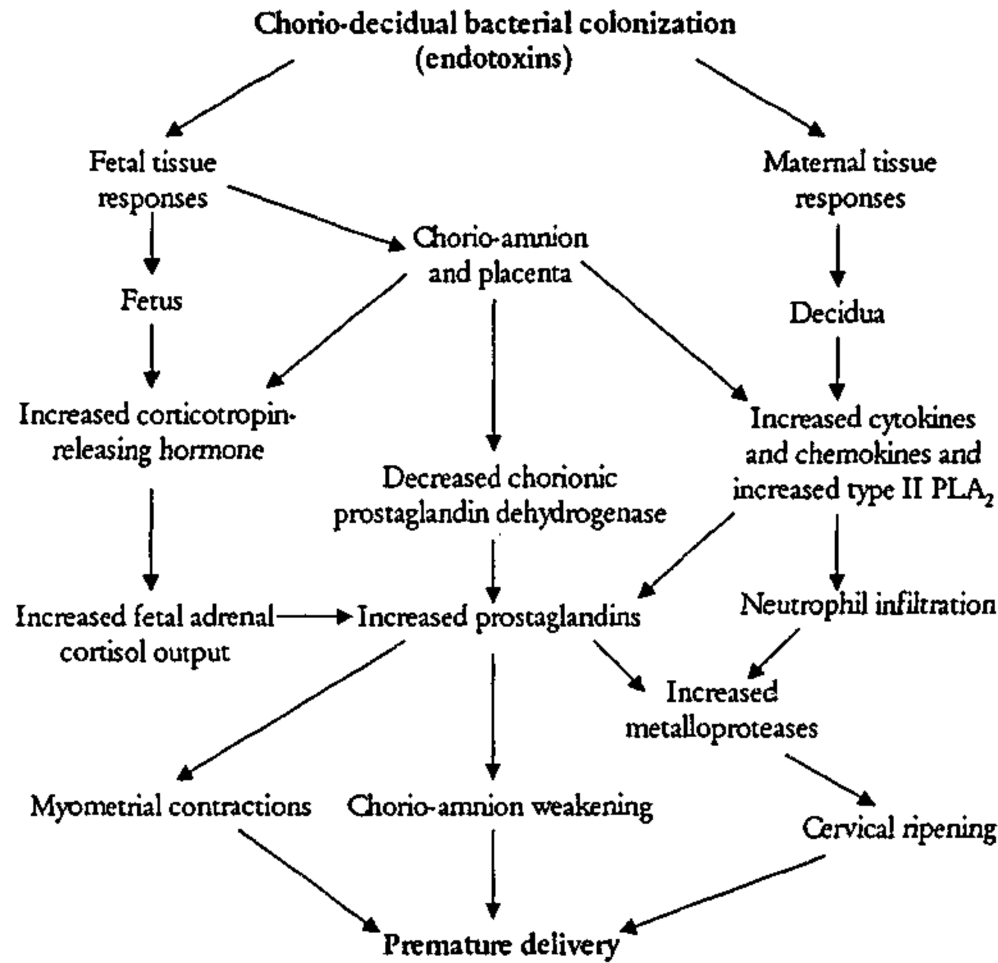

Figure 1.7 A schematic diagram representing the proposed pathways from chorio-decidual bacterial colonization to premature delivery, adapted from Goldenberg *et al.* (2000).

The fetus itself may also play a role in premature labour associated with infection. Intra-uterine infections increase fetal hypothalamic and placental production of corticotropin-releasing hormone, causing an increase in fetal corticotropin secretion, which in turn increases fetal adrenal production of cortisol (Figure 1.8; Korebrits *et al.*, 1998; Gravett *et al.*, 2000). Increased levels of cortisol compete with the action of

progesterone (Karalis *et al.*, 1996), and therefore by increasing corticotropin-releasing hormone, DHEAS and oestrogens are also increased (Siiteri & MacDonald, 1963). Alternatively, it has been suggested that the increase in fetal cortisol concentrations may be a component of the acute-phase response to tissue damage induced by pro-inflammatory cytokines (Yoon *et al.*, 1998).

1.6.4 Cytokines and parturition

There is substantial clinical and experimental evidence to support the role of cytokines in normal term labour (Opsjon *et al.*, 1993), as well as premature labour in the absence (Steinborn *et al.*, 1999), and presence of infection (Romero *et al.*, 1990; Hillier *et al.*, 1993; Fidel *et al.*, 1994; Hirsch *et al.*, 1999). Pro-inflammatory cytokines such as TNF-α, IL-1α and -1β, IL-6 and IL-8, function as part of a cascade that orchestrates the recruitment and activation of inflammatory cells (Hirsch *et al.*, 1999), and are capable of stimulating arachidonic acid metabolism in human gestational tissues (Lundin-Schiller & Mitchell, 1991; Mitchell *et al.*, 1991; Romero *et al.*, 1992). Almost any cell type can release inflammatory cytokines after tissue damage, however, macrophages, monocytes and fibroblasts are most likely the key cells which initiate and promote inflammatory responses (Dudley & Trautman, 1994).

1.6.4.1 Cytokines during spontaneous labour

Cytokine levels in the amniotic fluid and cervical secretions have been shown to increase towards term in normal pregnancies, and may play a regulatory role in parturition by stimulating the local production of prostaglandins (Laham *et al.*, 1993; Steinborn *et al.*, 1995; Gunn *et al.*, 1996). Using immunohistochemistry, positive staining for IL-1β, IL-6 and TNFα has been demonstrated in fetal membranes obtained from women in premature labour in the absence of chorioamnionitis, showing a similar pattern of localisation described after spontaneous labour (Steinborn *et al.*, 1999). In addition, IL-1β, IL-6 and IL-8 mRNA are commonly expressed in human gestational tissues after normal labour and premature labour with and without intra-uterine infection (Dudley *et al.*, 1996). Activation of inflammatory mediators therefore appears pivotal in the initiation and propagation of events leading to parturition especially when associated with infection (Greci *et al.*, 1998), suggesting that term and premature labour, may indeed be a host-mediated inflammatory response (Romero *et al.*, 1989b; Laham *et al.*, 1993; Fidel *et al.*, 1994; Keelan *et al.*, 1999).

1.6.4.2 *Cytokines during premature labour associated with infection*

Several studies have shown that human amnion, chorion and decidual tissue can synthesise and release a number of cytokines in response to inflammatory stimuli and bacterial products (Dudley & Trautman, 1994; Laham *et al.*, 1996; Gomez *et al.*, 1997). In addition, human gestational tissues can also produce anti-inflammatory cytokines, such as IL-10, a potent inhibitor of inflammatory cytokine production, which may act as a braking system to aid in the resolution of the inflammatory process (Simpson *et al*, 1998).

Microbial invasion of the amniotic fluid in women with premature labour is associated with an 8-fold increase in the amount of TNF-α concentrations with respect to that observed in women without premature labour (Maymon *et al.*, 1999). Pro-inflammatory cytokines, including IL-1, IL-6, IL-8 have also been found to be significantly elevated in the amniotic fluid of women with infection associated premature labour (Romero *et al*, 1990; Hillier *et al.*, 1993; Dudley & Trautman, 1994; Hsu *et al.*, 1998). Elevated amniotic fluid IL-6 concentrations are more likely to be associated with clinical chorioamnionitis and the presence of bacteria in the chorio-amnion compartment (Hillier *et al.*, 1993). Amniotic fluid IL-6 concentrations have also been shown to be associated with premature delivery in the absence of clinical evidence of infection (Romero *et al*, 1993), therefore it has been suggested that the measurement of amniotic fluid IL-6 concentrations may help to identify women with subclinical infections who might be at risk of premature delivery (Greci *et al.*, 1998; Hirsch *et al.*, 1999; El-Bastawissi *et al*, 2000). Similarly, in a non-human primate model, intra-amniotic inoculation with group B *Streptococcus*, increases amniotic fluid cytokines, particularly IL-6, which occur in parallel with increases in amniotic fluid prostaglandins (Gravett *et al.*, 1994b). Both these changes precede increases in uterine contractility. The elevation in amniotic fluid cytokines observed after experimental infection are consistent with those reported in women, and are supported by other animal models where infection-associated premature labour is preceded by a dramatic increase in maternal serum TNF-α, IL-6 and IL-1α and amniotic fluid IL-6 and IL-1α concentrations (Fidel *et al.*, 1994).

Figure 1.8 shows a schematic representation of the proposed inflammatory cytokine network within human gestational tissues, leading to myometrial activation and uterine activity, adapted from Dudley and Trautman (1994). Bacterial colonisation (A), activates decidual macrophages to produce TNF-α and IL-1 (Figure 1.8 B). TNF-α is a 17-kd polypeptide pro-inflammatory pleiotropic cytokine and is produced in response to

bacterial endotoxins, viruses and immune complexes (Maymon *et al.*, 1999). TNF-α plays a crucial role in initiating the production of immunomodulatory cytokines (IL-6), chemokines (IL-8), macrophage inflammatory protein-1α (MIP-1α) and also immunosuppressive cytokines (IL-10) (Dudley & Trautman, 1994), thus TNF-α has been considered an early marker of infection (Gravett *et al.*, 1994b). *In vivo* studies using preparations of decidual cells isolated from term gestational membranes respond to IL-1α, IL-β and TNF-α by producing significant amounts of IL-6 (Dudley *et al.*, 1992a), IL-8 (Dudley *et al.*, 1993) and MIP-1α (Dudley *et al.*, 1995). While stimulation of chorion cells by IL-1α, IL-1β and TNF-α produces large amounts of IL-6 (Dudley *et al.*, 1992b), chorion cells, like decidual cells, do not produce IL-1α, IL-1β or TNF-α after direct stimulation with LPS (Dudley & Trautman, 1994), suggesting that both decidual and chorion cells are involved in the propagation of the inflammatory response, by producing other immunomodualatory cytokines.

Infection of gestational tissues is histologically characterised by cellular infiltrates of neutrophils, monocytes and macrophages (Dudley & Trautman, 1994). IL-8 is a potent chemotactic and activating factor for neutrophils, and is produced in large amounts by both decidual cells (Figure 1.8 C) and chorion cells (Figure 1.8 D) in response to IL-1-α, IL-1β and TNF-α (Dudley *et al.*, 1993). Amnion cells are also capable of producing IL-8 (Trautman *et al.*, 1992), and this chemokine has been shown to stimulate production of proteinases in the cervix, which subsequently causes collagen degradation (Ogawa *et al*, 1998). The amnion is also a large source of arachidonic acid metabolites, with PGE_2 and 5-hydroxyeicosatetraneonic acid (5-HETE) two of the primary metabolites that are produced and that act on myometrial cells (Dudley & Trautman, 1994). Amnion cell PGE_2 production occurs via the stimulatory action of many cytokines, including IL-6 (Mitchell *et al.*, 1991), IL-1β (Romero *et al.*, 1989a) and TNF-α (Bry & Hallman, 1991). These cytokines have been shown to significantly enhance the release of arachidonic acid from human myometrial cells in culture (Molnar *et al.*, 1993). IL-1β also dramatically up-regulates PGE_2 production in cultured amnion cells, which results in an increase in synthesis of the EP_1 receptor (Spaziani *et al.*, 1999). These observations suggest that cytokine stimulation of amnion cells *in vivo* might produce an increase in PGE_2 without a concomitant down-regulation of the EP_1 receptor thus providing an irreversible drive stimulating the myometrium. The end result of prostaglandin production, uterine contractions and cervical changes that eventually lead to premature

labour, involve a complex interaction of cytokines with the arachidonic acid metabolic pathway.

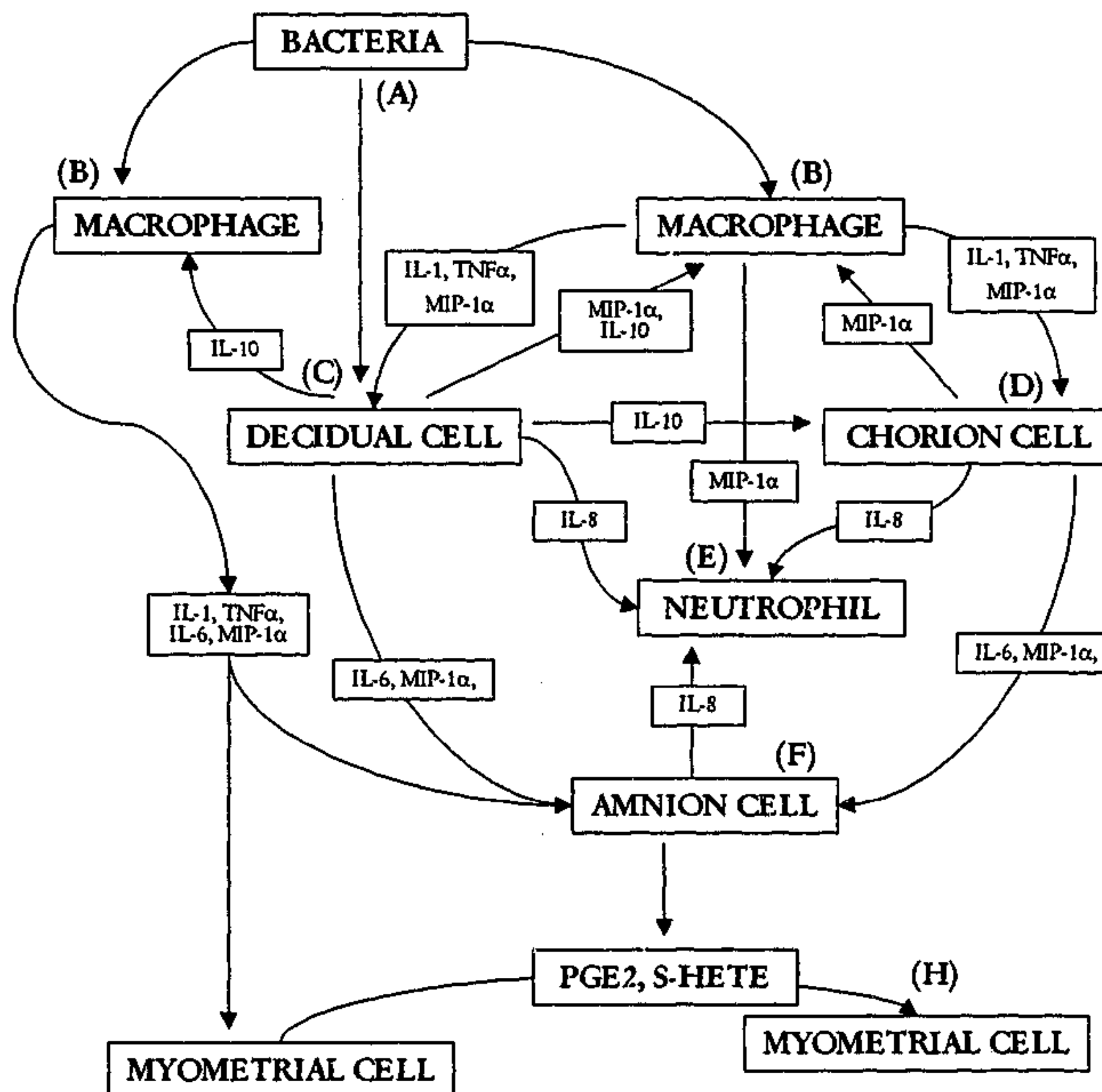

Figure 1.8 Proposed inflammatory cytokine network in human gestational tissues, adapted from Dudley and Trautman (1994). (A) bacterial infection of human gestational tissue; (B) decidual macrophage activation; (C) decidual cell cytokine production; (D) chorion cell cytokine production; (E) neutrophil recruitment and activation; (F) amnion cell prostaglandin production; (G) myometrial cell contractility and uterine activity.

1.6.5 Animal models of infection and premature labour

A number of studies have used the administration of bacteria or bacterial products to pregnant animals as a model of human premature labour associated with intra-uterine infection (Gravett *et al.*, 1994a; Schlafer *et al.*, 1994; Kaga *et al.*, 1996; Fidel *et al.*, 1998). Using a non-human primate model, Gravett *et al.* (1994b) demonstrated that

intra-amniotic inocculation with group B *Streptococcus*, is associated with increases in amniotic fluid cytokines and prostaglandins, followed by increases in uterine activity, cervical effacement and dilation. Despite evidence of clinical chorio-amnionitis, no animal was febrile or displayed clinical symptoms of infection, which is often the case with women (Goncalves *et al.*, 2002). A study by Fidel *et al.* (1998) showed intra-peritoneal LPS administration (50 μg) in mice at 70% gestation results in an 87% incidence of premature labour, yet these authors did not describe fetal or neonatal outcome. Similarly, Kaga *et al.* (1996) demonstrated a 100% incidence of premature delivery in mice intra-peritoneally treated with LPS (50 μg, twice at 3 h intervals), however, all fetuses that delivered prematurely were dead and appeared to have died *in utero*. Fetal death was also observed in a study by Schlafer *et al.* (1994), where LPS administration (1 μg/kg) to the ewe resulted in fetal death and the onset of premature labour.

Collectively, these data support the view that the presence of microbial products leads to increased concentrations of pro-inflammatory cytokines and can lead to premature labour. However, because most of these studies have been undertaken in species in which it is not possible to monitor the fetal effects of chronic infection, little is known of the fetal responses during this inflammatory process or of the mechanisms by which these processes influence fetal well-being and neonatal outcome. Detecting the presence of infective organisms in the amniotic fluid may not be an adequate predictive measure of premature birth. This may be best achieved by measuring the response of the fetus and/or mother to infection by measuring the amniotic fluid cytokine concentrations, particularly IL-6 (Hillier *et al.*, 1993; Greci *et al.*, 1998). A greater understanding of the consequences of uterine infections during pregnancy and the mechanisms by which the fetus and mother respond to bacterial infections is crucial to developing better approaches for the detection and treatment of premature labour associated with infection.

1.7 INHIBITION OF PREMATURE LABOUR

Because the aetiology of premature labour is complex and multi-factorial, the ability to predict women who will eventually develop premature labour has proved difficult. Approximately 10-30% of patients with premature labour are eligible for tocoltyic therapy (Sullivan & Morrison, 1995). Factors that may exclude the use of tocolytic agents include, advanced cervical dilation, fetal distress, severe intra-uterine growth

retardation and significant maternal complications such as pre-eclampsia (Jeyabalan & Caritis, 2002). Although tocolytic therapy is limited to a small percentage of patients, there are benefits to this treatment. Generally, short term (24-48 h) tocolysis attempts to prolong gestation to achieve optimal benefit from the administration of corticosteroids for enhancement of fetal organ maturation (Jeyabalan & Caritis, 2002). Tocolytic treatment also provides the necessary time required for transport of the mother to a suitable care centre with the specialist skills and facilities required for the birth and immediate intensive care of the expected premature infant (Keirse, 1995). A range of tocolytics have been shown to be efficacious in randomized-controlled clinical trials, with varying success and toxicity (Zuckerman *et al.*, 1974; Papatsonis *et al.*, 1997; Romero *et al.*, 2000). Despite this, the incidence of premature deliveries has not changed considerably over the past 40 years.

1.7.1 β-Adrenergic receptor agonists

β-adrenergic receptor agonists have been used for tocolysis in the setting of premature labour for more than three decades (Caughey & Parer, 2001). There are two types of β-adrenergic receptors, β_1-adrenergic receptors predominate in the heart, small intestine and adipose tissue; β_2-adrenergic receptors are located in the smooth muscle of the uterus, blood vessels, diaphragm and bronchioles (Rang *et al.*, 1995). Stimulation of β_2-adrenergic receptors can therefore effect multiple organ systems, thus the lack of specificity of these drugs during pregnancy can result in many adverse side effects.

β_2-adrenergic receptor agonists inhibit myometrial contractility by the activation of adenylate cyclase, via a G-linked regulatory protein (Litime *et al.*, 1989). Prolonged β_2-agonist exposure has been shown to cause desensitisation due to down regulation of the β_2-adrenergic receptors, thereby reducing the effects of these drugs very quickly (Caritis *et al.*, 1987). As a result, β_2-adrenergic receptor agonists, including ritodrine and terbutaline are not adequate for long term use. Treatment with ritodrine, a commonly used β_2-adrenergic receptor agonist, has had various success in delaying premature labour with some clinical trials demonstrating no difference in the prolongation of pregnancy compared to placebo (Spellacy *et al.*, 1979; Larsen *et al*, 1980), while in other clinical trials, ritodrine treatment significantly delayed delivery for up to 24 h. In addition, these drugs readily cross the placenta, consequently, chronic exposure to β_2-adrenergic receptor agonists can adversely alter both maternal and fetal

cardiovascular and metabolic status (Spellacy *et al.*, 1979; Larsen *et al.*, 1980). The numerous maternal and fetal cardiovascular and metabolic alterations which occur as a result of treatment justifies the use of alternative drug therapy.

1.7.2 Calcium channel blockers

Calcium channel blockers have been used for tocolysis since the early 1980's (Elliott, 1983; Ulmsten, 1984). Magnesium sulfate exerts its tocolytic effect by competing in the smooth muscle for calcium, altering the actin-myosin interaction and impairing contractility (Elliott, 1983). Magnesium sulfate rapidly equilibrates across the placenta and fetal plasma concentrations are comparable to those in the mother. When magnesium sulfate treatment is maintained in the nontoxic ranges adverse side effects are few and include; nausea, vomiting, visual blurring and shortness of breath. However, excessive levels of serum magnesium have been associated with respiratory depression, cardiac arrest and death (Higby *et al.*, 1993). In addition to the well known maternal side effects, there is accumulating evidence showing an increased frequency of adverse outcomes in the fetus and neonate and has lead to the recommendations to abandon its use entirely (Pryde *et al.*, 2001; Mittendorf *et al.*, 2002).

Nifedipine is also used for tocolysis and is an effective smooth muscle relaxant with low toxicity (Ferguson *et al.*, 1990). Many studies have compared ritodrine with nifedipine in the management of premature labour, demonstrating a similar tocolytic efficacy with nifedipine treatment and much fewer maternal and fetal side effects (Ferguson *et al*, 1990; Meyer *et al.*, 1990; Kupferminc *et al.*, 1993). While Read and Wellby (1986), and more recently Papatsonis *et al.* (1997), found nifedipine to be significantly more effective than ritodrine in delaying premature labour and was also associated with fewer side effects. Although adverse side effects are less common with nifedipine treatment, its effectiveness in inhibiting premature labour is short-lived, and like β-adrenergic receptor agonists there is no specificity for the myometrium (Lopez Bernal *et al.*, 1995).

1.7.3 Prostaglandin synthesis inhibitors

The prominent role of prostaglandins in the initiation and progress of labour have lead to the investigations of methods to inhibit their synthesis during premature labour. In the 1970's, human and animal experiments showed that prostaglandin synthase inhibitors could prolong the duration of labour and length of gestation (Lewis & Schulman, 1973; Novy *et al.*, 1974). Indomethacin was the first non-steroidal anti-inflammatory drug to be widely used for tocolysis (Terzidou & Bennett, 2002). This class of tocolytic drug, blocks prostaglandin synthesis by inhibiting the enzymes (PGHS-1 and PGHS-2) involved in the synthetic pathway (Figure 1.9; Macones *et al.*, 2001), and appear more effective in inhibiting premature labour than other tocolytic agents.

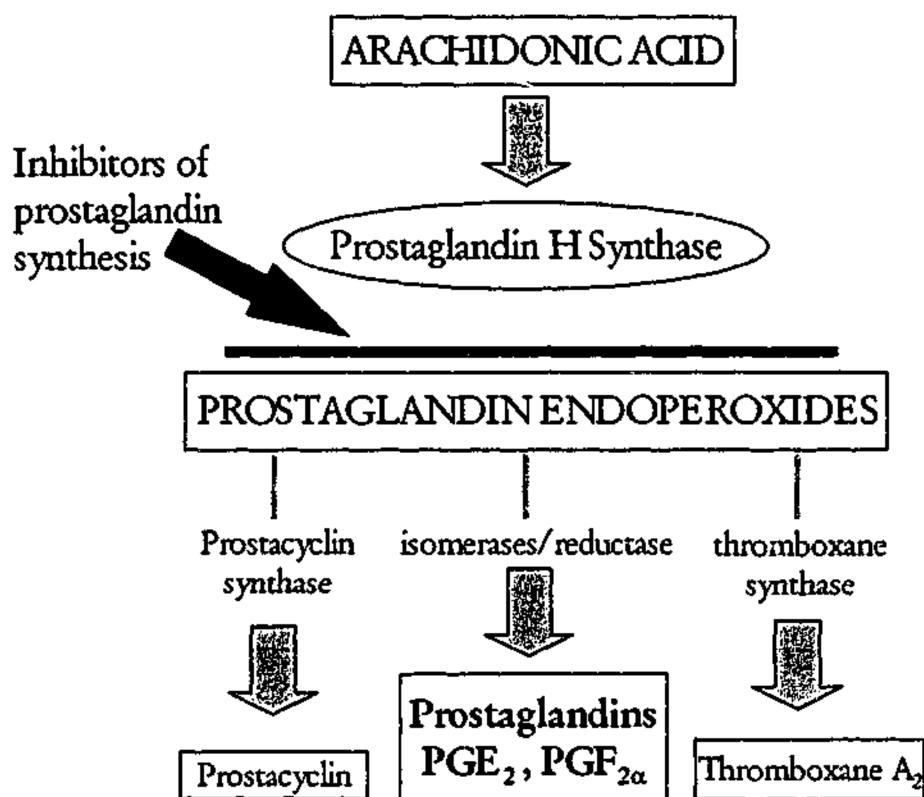

Figure 1.9 Mode of action of prostaglandin synthase inhibitors, adapted from Keirse (1992).

Indomethacin is generally well tolerated by the mother with minimal maternal side effects (Niebyl *et al.*, 1980; Sullivan & Morrison, 1995; Terzidou & Bennett, 2002). Nausea and vomiting are seen in approximately 4% of women treated with indomethacin (Jeyabalan & Caritis, 2002). Many physiological effects of indomethacin that can occur in the mother parallel those found in the fetus and neonate. Thus, the

potential fetal and neonatal side effects, particularly the associated fetal cardiovascular effects have limited the use of this drug as a tocolytic agent. Indomethacin used in late pregnancy increases the risk of constriction of the ductus arteriosus (Moise, 1993; Norton *et al.*, 1993; Guerguerian *et al.*, 1998), with diversion of the right ventricular outflow into the pulmonary bed (Moise *et al.*, 1988) and therefore indomethacin treatment is not recommended for use at or after 32 weeks gestation (Vermillion *et al*, 1997; Steer & Flint, 1999). Increased pulmonary blood flow causes an increase in pulmonary artery pressure, leading to right ventricular dilation with subsequent tricuspid regurgitation (Van den Veyver & Moise, 1993). These effects are likely due to the reduction of circulating prostaglandins and/or suppression of local prostaglandin production within ductal tissue (Poore *et al.*, 1999). Other fetal complications from indomethacin include impaired renal function with resultant oligohydramnios (Itskovitz *et al.*, 1980; Sullivan & Morrison, 1995).

It is now well established that there are two prostaglandin producing systems during late pregnancy; one involving the rapid production of $PGF_{2\alpha}$ via increased expression of PGHS-2 within the placenta, leading to parturition, and the other involving PGE_2 production via consitutive PGHS-1 expression, for maintaining ductal patency (Poore *et al.*, 1999), refer to Section 1.2.2.6. PGHS inhibitors such as indomethacin, are relatively non-selective for the two isoforms of PGHS, whereas nimesulide, (4-nitro-2-phenoxymethanesulfonanilide), an NSAID of the sulfonanilide class (Bevilacqua & Magni, 1993), has been shown to selectively inhibit PGHS-2 (Futaki *et al*, 1994). Nimesulide is a compound with potent anti-inflammatory, antipyretic and analgesic properties (Bottcher *et al.*, 1987) and has few of the side effects commonly associated with other non-steroidal anti-inflammatory agents (Magni, 1993). The use of a selective PGHS-2 inhibitor in pregnancy was reported by Sawdy *et al* (1997). Ductus arteriosus velocity and amniotic fluid index remained normal throughout the 18 weeks of nimesulide treatment, with spontaneous delivery of a healthy infant occurring 6 days after nimesulide was discontinued. These observations strengthened the idea that selective inhibition of PGHS-2 may be useful in the prolongation of pregnancy. In sheep, nimesulide has been shown to delay glucocorticoid-induced premature labour for at least 18 h (Poore *et al.*, 1999). These investigators did however, find that there was a depression of fetal circulating prostaglandin concentrations during nimesulide treatment which is in agreement with previous reports (Wu *et al.*, 1998), suggesting there may be some fetal effects of this treatment. Some of this effect may be the result of PGHS-1

inhibition. Thus these findings indicate that a more highly selective PGHS-2 inhibitor may be required to spare the fetus from the effects of inhibition of PGHS-1 and thus ensure fetal prostaglandin production that is essential for fetal well-being. Nonetheless, the development and use of highly selective inhibitors of PGHS-2, may prove more beneficial in the suppression of uterine activity and premature labour, since they are more likely to act on intra-uterine rather than fetal prostaglandin production (refer to Section 1.2.2.3).

1.7.4 Oxytocin receptor antagonists

An increase in myometrial oxytocin receptor concentrations has been shown to be associated with term and premature labour (Fuchs *et al.*, 1984). Oxytocin receptors are primarily localised to the myometrium and mammary glands, therefore blocking the action of oxytocin has greater uterine specificity than alternative tocolytic drugs. As a consequence, the major advantages with oxytocin receptor antagonists are the reduced maternal and, more importantly, fetal side effects. Oxytocin has a direct action on uterine activity by stimulating the myometrium, via intracellular IP_3 and the release of calcium from the sarcoplasmic reticulum (Carsten & Miller, 1987). Additionally, oxytocin has an indirect role by acting to increase the production of prostaglandins within the decidua and fetal membranes, Figure 1.4 (Moore *et al.*, 1988), as described in Section 1.2.3.1. Therefore treatment with an oxytocin antagonist would have a dual action that may be more effective than agents that are limited to either effects on myometrial smooth muscle or to the synthesis of prostaglandins (Figure 1.10).

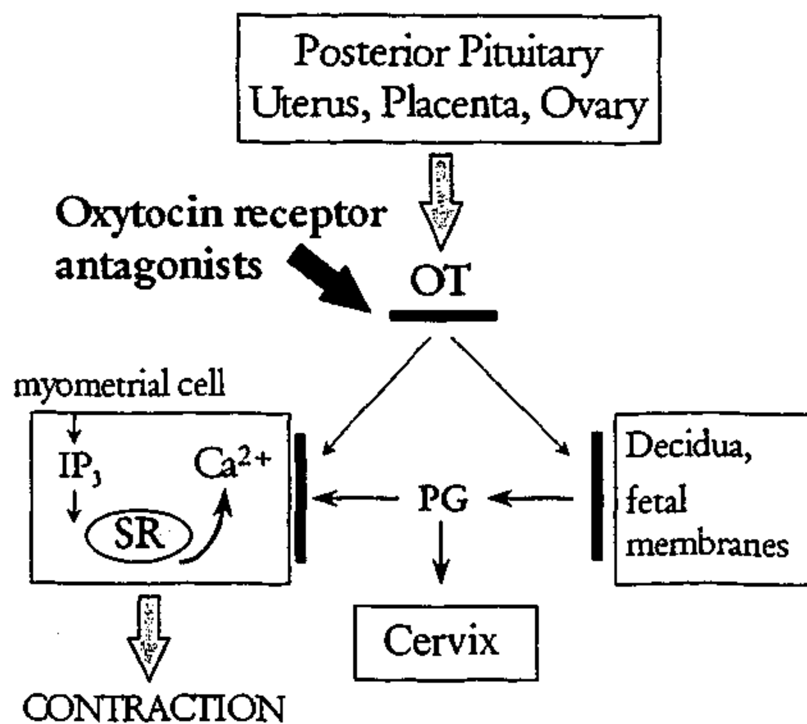

Figure 1.10 Schematic representation of the action of oxytocin in pregnancy and the mode of action of oxytocin receptor antagonists, adapted from Melin (1993). Ca^{2+}, cytosolic calcium; IP_3, inositol triphosphate; OT, oxytocin; PG, prostaglandins; SR, sarcoplasmic reticulum.

Atosiban (1-deamino-2-D-Try-(oET)-4-Thr-8-orn-oxytocin) is a competitive oxytocin receptor antagonist (Keirse, 1995; Goodwin *et al.*, 1996b). Atosiban inhibits the second messenger process that normally leads to intracellular calcium increase and contraction (Melin, 1993). Blocking oxytocin receptors of the decidua and fetal membranes may also act to inhibit oxytocin-mediated prostaglandin release from these membranes (Jenkin, 1992). Atosiban has been shown to decrease uterine contractility in women threatened with premature labour (Akerlund *et al.*, 1987; Moutquin *et al.*, 2000). Most importantly, atosiban does not reduce basal fetal prostaglandin release in late gestation (Jenkin *et al*, 1994). Placental transfer of atosiban in humans is relatively low, with fetal plasma concentrations of atosiban reaching only 12% of maternal plasma concentrations (Valenzuela *et al.*, 1995). In lactating animals, oxytocin antagonists have been observed to have an inhibitory effect of milk let-down (Soloff *et al.*, 1989). In studies to date, there appears to be little risk of such an effect in humans, due to the reversal of the effect of the drug within a comparatively short time (Melin, 1993). Recent clinical studies have focused on atosiban as a potential new tocolytic agent (Moutquin *et al.*, 2000; Romero *et al.*, 2000; Valenzuela *et al.*, 2000; Group, 2001a). Romero *et al.* (2000) conducted a large multicentre, double-blind, placebo controlled trail with approximately 500 women. This study found that atosiban resulted in significant prolongation of pregnancy compared to

placebo and this occurred with a low rate of maternal-fetal adverse effects. Further supporting this, Valenzuela *et al.* (2000) reported that the use of atosiban as a maintenance treatment for preventing the recurrence of premature labour was well tolerated. In these studies atosiban was administered for up to 33 days with no difference in adverse maternal effects compared to placebo, and importantly no fetal adverse effects were observed. Both these studies clearly show that atosiban treatment had marked tocolysis action compared to placebo.

The efficacy and safety of atosiban has also been compared to that of the β-adrenergic receptor agonists (Moutquin *et al.*, 2000; Group, 2001a; Group, 2001b). These studies were performed with a total of 730 women between 23-33 week of gestation using either, ritodrine, terbutaline or salbutamol as comparators. These β-adrenergic tocolyic agents are very similar in their mechanism of action and consequently have very similar adverse effects on the mother and fetus (refer to Section 1.7). Several of the maternal side effects are serious and can limit the dose and effectiveness of all of these agents. Moutquin *et al.* (2000) found that compared to ritodrine that atosiban was clinically comparable in tocolytic affect however, the maternal side effects were substantially lower in the atosiban treated group and was generally much better tolerated. Fetal tachycardia was observed almost exclusively in the ritodrine group due to the effect of the compound on the fetal heart after transfer to the fetal circulation.

The European (Group, 2001b) and the French/Australian (Group, 2001a) Atosiban Study Groups have compared atosiban with terbutaline and salbutamol, respectively. These groups reported essentially similar findings in terms of tocolytic effectiveness and similar maternal adverse effects. Discontinuance of treatment due to maternal side effects ranges from 11-16% with the β-adrenergic receptor agonists but in contrast was < 2% in the atosiban treated groups. The incidence of fetal tachycardia was much higher with terbutaline (44%) and salbutamol (21%) compared to atosiban (5%) and was the major difference between the groups in terms of fetal adverse effect. Together these extensive clinical trial studies indicate atosiban is equal in effectiveness as β-adrenergic receptor agonists for delaying delivery. Compared to the comparators, however, atosiban was well tolerated by mother and fetus, in agreement with earlier studies (Goodwin *et al.*, 1996a; Goodwin *et al.*, 1996b). Atosiban therefore might be a useful drug that can be used in combination with another drug to better suppress premature uterine activity and minimise the potential detrimental fetal side effects that have accompanied several other tocolytic agents.

1.8 SPECIFIC AIMS

The central aim of this thesis was to investigate the mechanisms that contribute to premature labour and to examine methods to better suppress premature uterine activity. Specifically there were two major areas of investigation; the development of a potential new strategy for delaying premature birth by combining a selective PGHS-2 inhibitor, with the oxytocin receptor antagonist, atosiban, as well as evaluating the fetal effects of this treatment. Secondly, to investigate the fetal responses to an inflammatory challenge during pregnancy in order to gain a greater understanding of the consequences of uterine infections during pregnancy and the mechanisms by which the fetus and mother respond to an inflammatory challenge. This may provide an indication as to when it may be appropriate to delay cases of premature labour that may be associated with infection.

1. Inhibition of premature labour

The aims of the study presented in Chapter 3 were to increase the effectiveness of nimesulide treatment, while also reducing its potential detrimental effects on the fetus, by determining if the blockade of oxytocin receptors with atosiban improves the effectiveness of nimesulide treatment for delaying premature labour in sheep.

2. Prostaglandin H synthase mRNA expression during the inhibition of premature labour

The study presented in Chapter 4 aimed to determine the level of expression of PGHS-2 mRNA in placental tissues following the induction of premature labour, in the absence of prostaglandin release and when delivery is prevented.

3. Inhibition of active premature labour

The aims of the study presented in Chapter 5, were to assess the potential usefulness of the combined nimesulide and atosiban treatment in a situation that more closely resembles that which would occur in clinical practice. Specifically investigating the effectiveness of nimesulide and atosiban following the onset of active premature labour, at a time when uterine activity and prostaglandin concentrations are already significantly elevated.

4. Fetal responses to an inflammatory challenge during pregnancy

The study presented in Chapter 6, aimed to investigate the fetal effects of an inflammatory challenge during pregnancy using different sites of inflammation by maternal systemic, intra- or extra-amniotic administration of LPS, thus mimicking the processes thought to be involved in premature labour associated with infection in women.

Chapter 2

Methodology

The general protocols for experiments performed to complete this thesis are described within this chapter. Specific procedures relating to individual sections are expanded within the appropriate experimental chapter. All experiments conducted in relation to this thesis had the approval of the Deartment of Physiology Animal Ethics Committee (AEC) and complied with the Code of Practice for use of animals for scientific purposes.

2.1 EXPERIMENTAL ANIMALS

Border Leicester-Merino cross-bred ewes were used in all the animal studies. All ewes were housed in individual metabolism cages and fed between 0900 h to 1200 h daily, water was provided *ad libitum*. A constant environmental temperature was maintained at 20-24 °C with a 12 h light/dark cycle (0700 h-1900 h).

2.2 SURGICAL PROCEDURES

2.2.1 Pre-operative animal preparation and anaesthesia

Animals were fasted for 24 h prior to surgery and allowed at least a 3 day recovery period before any experimentation commenced. General anaesthesia was induced by an intravenous injection of 20 mg/kg thiopentone sodium in water (Pentothal, Bomac Laboratories Ltd., Asquith, New South Wales, Australia), followed by immediate intubation with an endotracheal tube (cuffed I.D. 9.0 mm, O.D. 11.0 mm, Portex Laboratoire, Berck Sur Mer, France). Anaesthesia was maintained by inhalation of 1.5-2.0 % halothane (Fluothane, ICI, Villawood, New South Wales, Australia) in oxygen/nitrous oxide mixture (50/50 v/v), achieved by a closed circuit Midget 3 anaesthetic apparatus (CIG Midget 3, Medishield, Alexandira, New South Wales, Australia) connected to a positive pressure Campbell anaesthetic ventilator (Ulco Engineering, Marrikville, New South Wales, Australia). Surgical preparation began by

shaving the right flank, front neck region and entire ventral side of the ewe. Shaved areas were initially cleaned with savlon antiseptic solution (ICI, Australia). Starting with the operative sites and working outwards in a circular fashion, 7.5% w/v povidone-iodine solution was liberally applied and removed twice, followed by 10% w/v povidone-iodine solution (Betadine Surgical Scrub Antiseptic, F.H Fauldings & Co. Ltd., South Australia, Australia). Animals were then transferred to the operating table and secured in a supine dorsal recumbent position. Finally, a 5% Chlorhexidine Hibitane in 70% alcohol (ICI, Australia) was applied to all incision sites. Just prior to the completion of surgery, administration of halothane was ceased, with mechanical breathing maintained until the ewe was spontaneously breathing.

2.2.2 Surgery

All surgery was performed under strict aseptic conditions. Surgeons wore face masks and hair caps and scrubbed their hands with Hibiclens Surgical scrub (Chlorhexidine Gluconate 4% w/v, isopropyl alcohol 4% w/v, ICI, Australia) or Betadine Surgical scrub (7.5% w/v povidone-iodine, F.H Fauldings & Co. Ltd., Australia). Hands were dried with a sterile towel before surgeons dressed in sterile surgical gowns and fitted sterile gloves. All gowns, drapes and instruments were sterilised by autoclaving (Department of Physiology, Monash University, Melbourne, Victoria, Australia), while catheters and electromyographic leads were sterilised by ethylene oxide (Alfred Hospital, Melbourne, Victoria, Australia). Animals were draped such that only incision sites were exposed.

An 8-10 cm midline incision was made in the abdomen, extending from the area of the umbilicus towards the mammary gland. Bleeding was controlled by use of a diathermy (SSE2-K Electro-surgical Generator, Valeylab Inc., Colorado, U.S.A). The pregnant uterus was exposed after the incision through the *linea alba* and peritoneum. The fetus was located within the uterus and the head exposed by a 5-6 cm incision in the uterine wall. To minimise amniotic fluid loss babcock clamps were placed around the incision site in the uterine wall.

2.2.2.1 Cannulation of fetal blood vessels

The fetal head was positioned to expose the ventral side of the neck. A 3 cm incision was made lateral to the trachea and blunt dissection was used to locate and expose 2 cm of the fetal carotid artery (CA) and jugular vein (JV). Two pieces of 3/0 silk suture (Dynek Surgical Products, Hendon, South Australia, Australia), were placed under each

vessel. The vessels were occluded proximal to the fetal head by securing a silk suture. A bulldog clip was placed at the distal end where a small transverse incision was made in each vessel with iris scissors to allow insertion of a heparinised saline-filled polyvinyl catheter (I.D. 0.81 mm, O.D. 1.52 mm, 155 cm length, Dural Plastics and Engineering, Silverwater B.C, New South Wales, Australia; 0.9% NaCl; 25 000 IU heparin/5 ml, Baxter Healthcare Pty. Ltd., New South Wales, Australia). The catheter was inserted approximately 7.5 cm in the direction of the heart and was secured in place. Patency of each catheter was ensured before wound closure. The fetal neck incision was closed with 3/0 silk suture using a continuous mattress stitch.

An intra-amniotic fluid catheter (basket) with a perforated end to avoid surrounding membranes from occluding the terminal ports when sampling, was sutured to the nape of the fetal neck and secured on the ear along with the CA and JV catheters. These catheters were made from silicone rubber tubing (I.D. 2.62 mm, O.D. 4.88 mm, 100 cm length; Sil-Med Corporation, Taunton, Massachusetts, U.S.A). A 10 cm length of one end was perforated with small 3 mm diameter holes and encased by a larger perforated 10 cm length of silicone rubber tubing (I.D. 9.5 mm, O.D. 12.7 mm), which was attached to the smaller tubing with silicone (Silastic RTV adhesive/sealant; Dow Corning Corporation Pty. Ltd., Blacktown, New South Wales, Australia). In some animals an extra-amniotic polyvinyl catheter (I.D. 1.50 mm, O.D. 2.70 mm, 100 cm length), was required (refer to Chapter 6).

Before returning the fetus to the uterus, an intra-muscular injection of 2.5 ml Depomycin (200 mg/ml procaine penicillin and 250 mg/ml dihydrostreptomycin, Intervet Pty. Ltd., Castle Hill, New South Wales, Australia) was administered. The uterine incision was closed using an inverted mattress stitch (Chromic 2/0 cat gut, Ethicon, Ethnor Pty. Ltd., Sydney, New South Wales, Australia).

2.2.2.2 *Cannulation of the utero-ovarian blood vessels*

Utero-ovarian veins (UOV) were catheterised as described in Section 2.2.2.1. Two secondary branches of the utero-ovarian vein, ipsilateral and contralateral to the uterine horn containing the fetus, were localised and a catheter, approximately 7.5 cm in length, was introduced and positioned into the left and right utero-ovarian vein, respectively. The uterine incisions were closed with a 3/0 silk suture using a continuous stitch.

2.2.2.3 *Uterine electromyograph leads*

Uterine electromyograph (EMG) leads were sutured to the external surface of the uterus in a triangular orientation approximately 2 cm apart. These electrodes were made using 3 strands of teflon-coated stainless steel wire (Cooner Wire Company, Chatsworth, California, U.S.A) sealed in polyvinyl tubing (I.D. 1.50 mm, O.D. 2.70 mm, 150 cm length) with silicone (RTV adhesive/sealant). For clear signal detection the wires were bared of the teflon coating at the point of contact with the uterus.

2.2.2.4 *Exteriorisation of all catheters*

All catheters and EMG leads were passed to the exterior through a small (~2 cm) incision in the right flank of the ewe. The incision was closed by a continuous stitch placed around the catheters in the peritoneum and the muscle layer of the flank using chromic cat gut suture (2/0 cat gut, Ethicon, Australia). The skin was closed with Vetafil Bengen synthetic suture (Clements Stansen, North Ryde, New South Wales, Australia) using a continuous everting mattress stitch. The *linea alba* and subcutaneous fat of the midline incision were closed using a continuous locking stitch with chromic 2/0 cat gut suture. The skin was closed with Vetafil Bengen synthetic suture using an everting mattress stitch.

2.2.2.5 *Cannulation of maternal blood vessels*

The right maternal CA was catheterised, as described in Section 2.2.2.1 (I.D. 1.50 mm, O.D. 2.70 mm, 100 cm length). The right JV was also catheterised in a similar fashion to that of the fetus, with a polyvinyl catheter (I.D. 1.50 mm, O.D. 2.70 mm 100 cm length). In some ewes an additional JV catheter was required for drug administration (refer to Section 3.2.2 and Section 5.2.1), therefore a silastic catheter (Sil-Med Corporation, U.S.A) was also inserted into the maternal JV. The maternal neck incision was closed with Vetafil Bengen synthetic suture using a continuous everting mattress stitch (silk 0).

2.2.3 Post-operative care and maintenance

All catheters were fitted with three way stopcocks and placed in sealable plastic bags and positioned under elasticised netting (Setonet, size 7, Seton Healthcare Group, U.K) which was extended over the entire torso of the ewe. The endotracheal tube was removed once the swallowing reflex had returned. Ewes then recovered in their metabolic cages. Catheters were flushed daily with sterile heparinised saline to ensure

patency was maintained. Fetal and maternal well-being was assessed daily by measurement of the partial pressure of oxygen (PaO_2), carbon dioxide ($PaCO_2$), pH, haemoglobin (Hb) and oxygen saturation (O_2 sat). These were measured using an ABL5 acid-base analyser and OM2 haemoximeter (Radiometer, Copenhagen, Denmark), with fetal temperature corrected to 39 °C.

2.3 FETAL AND MATERNAL BLOOD SAMPLING

Blood samples were collected into chilled sterile EDTA tubes (BDH, Port Fairy, Victoria, Australia), containing indomethacin (10 µM/ml blood) and centrifuged at 3000 rpm for 5 min at 4 °C. Plasma aliquots for prostaglandin FM (PGFM; 13,14-dihydro-15-keto $PGF_{2\alpha}$, the stable metabolite of $PGF_{2\alpha}$), progesterone, cortisol and interleukin-6 (IL-6) were stored at -20 °C until further assay. Plasma aliquots for prostaglandin E_2 (PGE_2) were diluted 1:1 with 0.12 M methyloxyamine hydrochloride (Sigma Chemical Co., St Louis, Missouri, U.S.A) in sodium acetate buffer (1 M, pH 5.6) containing 10 % ethanol and incubated overnight at room temperature then stored at -20 °C. Amniotic fluid samples were also taken for hormone and cytokine analysis. Samples were collected into chilled sterile EDTA tubes containing indomethacin (10 µM/ml blood). Aliquots of amniotic fluid for PGFM and IL-6 were immediately stored at -20 °C. Amniotic fluid samples for PGE_2 assay were diluted 1:1 with 0.12 M methyloxymamine hydrochloride before storage at -20 °C.

2.4 POST MORTEM PROCEDURES

All ewes and their fetuses were killed by an intravenous overdose (25 ml) of the barbiturate Lethabarb (active constituent pentobarbitone sodium 325 mg/ml, Virbac Pty. Ltd., Peakhurst, New South Wales, Australia). Immediate opening of the maternal abdomen and uterus allowed for visual assessment of the fetus. Uterine tissues were separated into myometrium, endometrium and amnion layers and frozen in liquid nitrogen and stored at -70 °C for future analysis. Maternal and fetal cotyledon tissue were also collected and immediately frozen in liquid nitrogen, then stored as above until needed for RNA analysis (refer to Chapter 4).

2.5 RADIOIMMUNOASSAY (RIA) PROTOCOLS

2.5.1 General

2.5.1.1 Solvents, chemicals and buffers

All chemicals used in these studies were of analytical grade and were obtained from Sigma Chemical Company (St Louis, Missouri, U.S.A) unless otherwise stated. n-Hexane was purchased from Rhone Poulenc Chemicals Pty. Ltd., (Victoria, Australia) and ethanol from BDH Chemicals, Australia. Bovine γ-Globulin (Calbiochem-Novabiochem Corporation) and polyethylene gylcol 6000 was supplied by BDH Chemicals.

The diluent buffer used in the PGFM, PGE_2 and progesterone assays was a phosphate-buffered saline (PBS; pH 7.0), containing sodium phosphate (1.0 M, pH 7.0) in sodium chloride (0.9%), gelatin (0.1%) and sodium azide (0.1%). A sodium phosphate assay buffer (0.5 M, pH 7.4) was used in the cortisol assays.

2.5.1.2 Charcoal-stripped plasma

Hormone-free ovine plasma samples were used for inclusion in standard curves and were prepared by charcoal treatment. Plasma was incubated with 10% w/v distilled water-washed charcoal (BDH Chemicals, Australia) for 3-4 h at 37 °C. The charcoal/plasma mixture was then centrifuged at 3000 rpm for 30 min at 4 °C to sediment the charcoal. The supernatant was carefully decanted, filtered twice and stored at -20 °C. For use in the PGE_2 assay, charcoal stripped plasma was first methyl-oximated as described in Section 2.5.2.1.

2.5.1.3 Quality control samples

Aliquots of ovine plasma containing the hormone of interest at a concentration similar to those normally measured in plasma were stored at -20 °C and used as quality controls in each assay and to calculate the intra- and inter-assay co-efficient of variation.

2.5.2 Prostaglandin RIA protocol

The concentration of PGFM in fetal and maternal plasma and amniotic fluid were measured by direct RIA, as previously described by Burgess *et al.* (1990). Plasma PGE_2 concentrations were also measured by direct RIA of methyl-oximated plasma samples as described by Kelly *et al.* (1986) and Fowden *et al.* (1986).

2.5.2.1 *Standards*

Standard PGFM and PGE_2 were obtained from Upjohn Company and the Sigma Chemical Company, respectively. Stock solutions (1 mM) in ethanol were prepared for PGFM and PGE_2 and subsequently diluted to 2 μM with ethanol. These solutions were then stored at -20 °C. Working dilutions of 40 nM and 2 nM were prepared from these standard solutions. The standard PGE_2 (1 nM) in ethanol (0.5 ml) was methyl-oximated by overnight incubation with 2.5 ml of 0.15 M methoxylamine hydrochloride in sodium acetate buffer (1.2 M, pH 5.1). Following the incubation period, the PGE_2 standard was extracted with ether: ethyl acetate (3:1, v/v) and then evaporated with air at 37 °C. The residue of the solution was then reconstituted in 0.5 ml absolute ethanol and stored at -20 °C.

2.5.2.2 *Tracers*

Radiolabelled prostaglandin 13,14-dihydro-15-keto-[5,6,8,9,11,12,14 (n)-^{3}H] $PGF_{2\alpha}$ (Amersham/NEN Pty. Ltd., Australia) was diluted 1:4 in ethanol and stored at -20 °C. PGE_2 (50 μCi of [5,6,8,11,12,14,15 (n)-^{3}H] PGE_2) was methyl-oximated as described in Section 2.5.2.1, and stored at -20 °C in ethanol. In all prostaglandin assays, working dilutions containing approximately 5000 cpm tracer/100 μl phosphate buffer were used.

2.5.2.3 *Antisera*

The PGFM antiserum (#0462) was used at a working dilution of 1/7500 in all assays. The PGE_2 antiserum (#9183), raised in sheep against the methyl oxime of PGE_2 conjugate to bovine serum albumin was used at a final dilution of 1/37,500. Both antisera were kindly supplied by Dr. R. I. Cox (CSIRO, Division of Animal Production, Prospect, New South Wales, Australia) and stored at -20 °C. The cross reactivities of the PGFM (Burgess *et al.*, 1990) and PGE_2 (Fowden *et al.*, 1986) antisera with related compounds are shown in Table 2.1 and 2.2.

Table 2.1 Percent cross-reactivity of the PGFM antisera (#0462) with closely related compounds, from Burgess *et al.* (1990).

Compound	PGFM antiserum cross-reactivity (%)
$PGF_{2\alpha}$	< 0.01
13,14-dihydro-$PGF_{2\alpha}$	6.90
PGE_2	< 0.01
6-keto-$PGF_{1\alpha}$	< 0.01
15-keto-PGE_2	0.20
15-keto-$PGF_{2\alpha}$	4.50
PGD_1	< 0.01
PGD_2	< 0.01
PGB_2	< 0.01
TxB_2	< 0.01

Table 2.2 Percent cross-reactivity of the PGE_2 antisera (#9183) with closely related compounds from Fowden *et al.* (1987).

Compound	**PGE_2 antiserum cross-reactivity (%)**
PGE_2 methyl oxime	100.00
15-keto-PGE_2	0.30
15-keto-$PGF_{2\alpha}$	< 0.01
13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.10
13,14-dihydro-15-$PGF_{2\alpha}$	< 0.01
6-keto-$PGF_{1\alpha}$	< 0.01
PGE_1	270.00
PGB_2	< 0.01
PGD_1	< 0.01
PGD_2	< 0.01
$PGF_{2\alpha}$	< 0.01
$PGF_{2\beta}$	< 0.01
TxB_2	< 0.01

2.5.2.4 *General PGFM and PGE_2 direct RIA procedure*

Standard PGFM and PGE_2 (0.02-2.0 pmol) in 100 µl ethanol were evaporated under air at 37 °C, and then reconstituted in a volume of charcoal-stripped maternal plasma (PGFM) or methyl-oximated charcoal-stripped maternal plasma (PGE_2) equal to the plasma sample volume. Plasma was assayed in duplicate at volumes of 20-200 µl. For both assays, tracer (5000 cpm: 100 µl) and antisera (100 µl) diluted in phosphate buffer were added to the standard curve and samples before incubation overnight at 4 °C.

To separate the antibody-bound hormone from free hormone, 50 µl of bovine γ-globulin and 0.8 ml 30 % polyethylene gylcol 6000 (PGFM) or 1.0 ml 22 % polyethylene glycol 6000 (PGE_2) were added to all tubes. After centrifugation at 3000 rpm for 15 min at 4 °C, the supernatant was aspirated and discarded. Water (PGFM) or absolute ethanol (PGE_2) was added to resuspend the pellet that remained.

Scintillation fluid (EcoscintA, National Diagnostics, Atlanta, Georgia, U.S.A), 1.5 ml or 1.0 ml for PGFM and PGE_2 respectively, was then added to all tubes. Tubes were vortexed and sonicated for 30 min to ensure that the pellet was dislodged and disaggregated. The beta radioactivity was determined in each tube using a liquid scintillation counter (Beckman LS 3801; Beckman Instruments Inc., Irvine, California, U.S.A).

2.5.3 Progesterone RIA protocol

The concentration of progesterone in maternal plasma were measured by extracted RIA, previously described by Rice *et al.* (1986).

2.5.3.1 Standards

Standard progesterone was obtained from Sigma Chemical Company. Stock solution (4 mM) in ethanol was prepared and subsequently diluted to 10 μM with ethanol and stored at -20 °C. Working dilutions of 200 nM and 10 nM were prepared from the standard solution and stored at 4 °C.

2.5.3.2 Tracer

[1,2,6,7-^{3}H(N)]-Progesterone in benzene (250 μCi) was purchased from Amersham/NEN Pty. Ltd., Australia and stored at -20 °C.

2.5.3.3 Antisera

Progesterone antisera (S23), raised in sheep against progesterone-11-α-BSA, was generously supplied by Dr. J Malecki (Regional Veterinary Centre, Department of Agriculture and Rural affairs, Bairnsdale, Victoria, Australia). The cross reactivity of this antisera with closely related compounds is presented in Table 2.3, from Rice *et al.* (1987).

Table 2.3 Percent cross-reactivity of progesterone antiserum (S23) with closely related compounds (Rice *et al.*, 1986).

Compound	**Progesterone antiserum cross-reactivity (%)**
Progesterone	100.00
11α-OH-progesterone	100.00
5α-pregnane,3,20-one	15.90
5β-3pregnane,3α-ol,20-one	10.00
Corticosterone	1.05
Cortisol	< 0.20
11-Deoxycortisol	< 1.00
Dehydroepiandrosterone	< 0.40
17α-OH-progesterone	0.70
20α-OH-pregnane,3-one	< 0.30
5α-pregnane,3β-ol-2-one	< 1.00
5α-pregnane,3α,17α-diol,20-one	< 1.00
5β-pregnane,3α,17α,20α-triol-20-one	< 1.00
5β-pregnane,3α,17α,20α-triol	< 1.00

2.5.3.4 *General progesterone extracted RIA procedure*

Standard progesterone (0.1-10.0 pmol) in 100 μl of ethanol were evaporated under air at 37 °C and reconstituted in 50 μl charcoal-stripped maternal plasma. Maternal plasma samples (50-200 μl) were assayed in duplicate in 12 x 75 mm glass tubes (Neutrex, H.L.S Scientific Pty. Ltd., Collingwood, Victoria, Australia). In standards and samples, progesterone was extracted with n-hexane (2.0 ml), tubes were vortexed for 1 min and left for 5 min, this was then repeated twice. The aqueous phase was frozen using an ethanol freezing bath (Hetofrig, Birkerood, Denmark) and the n-hexane containing the extracted progesterone was decanted into 10 x 50 mm polypropylene tubes. The solvent phase was evaporated under air at 37 °C and the samples resuspended in 50 μl

phosphate buffer. Tracer (10 000 cpm/100 µl) and antisera (100 µl) diluted in phosphate buffer, were added to all tubes before incubation overnight at 4 °C.

The antibody-bound hormone and free hormone were separated by addition of 50 µl bovine γ-globulin and 30 % polyethylene glycol 6000 (0.8 ml). The pellet was resuspended in 50 µl of absolute ethanol and vortexed. Scintillation fluid (1.5 ml) was added to all tubes then vortexed and sonicated for 30 min. Beta radioactivity was measured in each tube by a liquid scintillation counter (Beckman LS 3801; Beckman Instruments Inc., U.S.A).

2.5.4 Cortisol RIA protocol

Cortisol was measured in fetal and maternal plasma after extraction with dichloromethane (CH_2Cl_2; Merck, Darmstadt, Germany) as previously described by Bocking *et al.* (1986).

2.5.4.1 Standards

Cortisol standard (hydrocortisone; H-4001, Sigma Chemical Company) was used at concentrations of 0.0195-5 ng/100 µl in absolute ethanol, evaporated with air at 37 °C.

2.5.4.2 Tracer

250 mCi of [1,2,6,7-^{3}H] cortisol was purchased from Amersham Pty. Ltd., Australia and was made up to 5 ml in toluene:ethanol (9:1) and stored at -20 °C.

2.5.4.3 Antisera

The cortisol antiserum (#3368) was raised in sheep and kindly supplied by Dr. R. I. Cox (CSIRO, New South Wales, Australia). The cross reactivity of this antisera with closely related compounds is presented in Table 2.4 (Dr R. I. Cox, personal communication).

Table 2.4 Percent cross-reactivity of cortisol antiserum (#3368) with related compounds (Dr R. I. Cox, personal communication).

Compound	**Cortisol antiserum cross-reactivity (%)**
Cortisol	100.00
Cortisone	20.50
Corticosterone	1.00
Progesterone	0.57
17α-OH-progesterone	3.90
Testosterone	0.02
4-Androstene-3,17-dione	0.03
Betamethasone	0.50
Dexamethasone	< 0.01

2.5.4.4 *General cortisol extracted RIA protocol*

Duplicate aliquots of plasma (50-100 μl) were extracted with 2.0 ml dichloromethane and distilled water (100 μl) in glass tubes. Tubes were vortexed for 1 min every 5 min over a period of 10 min. The aqueous phase was aspirated and 1.0 ml of the organic phase transferred into polypropylene tubes. The organic layer was then evaporated to dryness at 37 °C under air. The standards and samples were incubated overnight at 4 °C with antiserum (1:24 000 final dilution in phosphate buffer, 100 μl), bovine γ-globulin (0.8 mg, 100 μl; Commonwealth Serum Laboratories, Melbourne, Australia), and ^{3}H-cortisol (10 000 cpm diluted in phosphate buffer, [1,2,6,7-^{3}H (n)]-cortisol, Amersham, 2.92 TBq $mmol^{-1}$, S.A. 79 Ci $mmol^{-1}$). After overnight incubation, bound and free hormones were separated by addition of 22% polyethylene glycol 6000 (1 ml) and centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was aspirated and discarded and the pellet resuspended in 200 μl phosphate buffer. Scintillation fluid (1 ml; Aqueous Counting Solution, Amersham Pty. Ltd., Australia) was added to each tube and the beta radioactivity was determined using a liquid scintillation counter (Beckman LS 3801; Beckman Instruments Inc., U.S.A).

2.6 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) PROTOCOL

2.6.1 General

2.6.1.1 Chemicals and buffers

All chemicals used in these assays were of analytical grade and were obtained from Sigma Chemical Company unless otherwise stated. Phosphate-buffered saline (10x PBS; pH 7.0), containing sodium phosphate (0.5 M) in sodium chloride (1.5 M), at pH 7.4 was used in these assays unless stated otherwise. The coating buffer (pH 9.6) contained sodium carbonate (15 mM, Na_2CO_3), sodium hydrogen carbonate (30 mM, $NaHCO_3$) and sodium azide (3 mM, NaN_3). Wash buffer (pH 8.0) containing 10 x PBS and 20% Tween 20 (Polyoxyethelene sorbitan monolaurate) was used to wash the ELISA plates. Blocking buffer was 10 x PBS and supplemented with 1% skim milk powder (Diploma skim milk powder, Bonlac Foods Ltd., Victoria, Melbourne, Australia), which was filtered before use. The diluent used in these assays was 5% (w/v) bovine serum albumin (BSA) made with 1 x PBS.

2.6.1.2 Quality control samples

One Border-Leicester-Merino cross-bred ewe was used to obtain quality control samples for the interleukin-6 (IL-6) ELISA. This ewe underwent surgery on 125 days of gestation to implant maternal vascular catheters as previously described in Section 2.2.2. On 130 days of gestation the ewe was administered 2 μg/kg maternal body weight of lipopolysaccharide (LPS), derived from *Escherichia coli* (serotype: 0127:B8; Sigma Chemical Company), diluted in sterile saline. Aliquots of maternal plasma were taken prior to LPS administration (time 0) and at 4 h after LPS administration and immediately stored at -20 °C. These plasma aliquots were then used as low and high IL-6 quality controls samples in each assay, and to calculate the intra- and inter-assay co-efficient of variation.

2.6.2 Interleukin-6 (IL-6) ELISA protocol

IL-6 concentrations in the fetal and maternal plasma and amniotic fluid samples were measured as previously described by McWaters *et al.* (2000), with minor modifications as follows.

2.6.2.1 *Standards*

The standard ovine recombinant IL-6 (1 μg/ml) was generated and supplied by Dr. Jean-Pierre Scheerlinck (Centre for Animal Biotechnology, University of Melbourne, Victoria, Australia). Working dilutions of the ovine IL-6 standard (0.007-5.0 ng/ml) were prepared in 400 μl of diluent and used immediately in each assay.

2.6.2.2 *Original IL-6 ELISA protocol*

The ELISA plates (Microtiter 96 well plates, Thermo Labsystems, Helsinki, Finland) were coated with monoclonal anti-ovine IL-6 antibody (Mouse IgG1, Epitope Technologies Pty. Ltd., Southbank, Victoria, Australia) diluted in coating buffer (1:200) and incubated overnight at room temperature. Following this incubation period, the quality control, plasma and amniotic fluid samples were assayed in duplicate (50 μl) and incubated for 1-2 h. Quality control plasma samples were positioned at the beginning, middle and end of the ELISA plate to ensure consistent measurements across the plate. However, it was noted that there was a 50% decrease in the quality control IL-6 concentrations from the beginning to the end of the plate, indicating that the incubation period had not reached equilibrium. Therefore, a time course experiment was conducted to determine the optimal incubation period for the plasma and amniotic fluid samples.

2.6.2.3 *Incubation Time course experiments*

Following the protocol of McWaters *et al.* (2000), quality control plasma samples were incubated for 1, 2, 4 and 24 h and the IL-6 concentrations determined (Figure 2.1). The mean IL-6 concentrations measured after the 1, 2, 4 and 24 h incubation period were, 1.17 ± 0.11, 2.24 ± 0.28, 3.93 ± 0.25 and 7.68 ± 0.08 ng/ml, respectively. Incubating the quality control plasma samples for 24 h resulted in the most consistent IL-6 concentrations across the plate with an intra-assay co-efficient of variation of 2.6 %, as opposed to 24 %, 30 % and 16 % for the incubation times of 1, 2 and 4 h, respectively. It was therefore considered that the incubation period had reached equilibrium, thus a

24 h incubation period was used for all quality control, plasma and amniotic fluid samples.

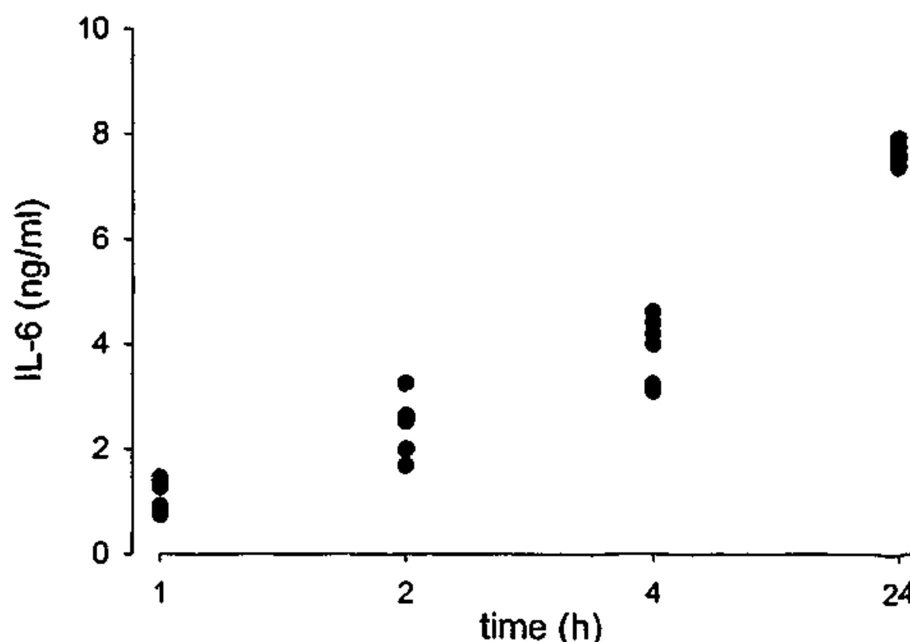

Figure 2.1 Incubation time course experiment. ELISA plates were coated with monoclonal anti-ovine IL-6 antibody (50 µl per well) and incubated overnight. Quality control samples were then assayed in duplicate and incubated for 1, 2, 4 and 24 h to determine the optimal incubation period for the plasma and amniotic fluid samples. The incubation period of 24 h resulted in the most consistent IL-6 concentrations across the plate, therefore this incubation time period was used in all subsequent IL-6 ELISA assays.

2.6.2.4 *General IL-6 procedure*

ELISA plates (Thermo Labsystems, Finland) were coated with 50 µl monoclonal anti ovine IL-6 antibody (Epitope Technologies Pty. Ltd.) diluted in coating buffer (1:200) in each well, covered and incubated for 1-2 h at room temperature. After tipping off the coating buffer, each well was filled with blocking buffer (300 µl) and incubated for 1-2 h at room temperature. Plates were then washed 4 x with ELISA wash buffer using a wellwash 4 MK2 plate washer (Thermo Labsystems, Finland), then 25 µl of diluent was added to each well immediately after washing.

Ovine IL-6 standard (0.007-5.0 ng/ml), quality control plasma, sample plasma and amniotic fluid samples were added in duplicate and were incubated overnight at room temperature in a sealed humid container. On day two, plates were washed 4 x withwash buffer. Rabbit anti-ovine IL-6 polyclonal antibody (Thermo Labsystems, Finland) was added (1:1000; 50 µl) to each well, then incubated for 2 h in a sealed humid container at room temperature. At the completion of this incubation, plates were washed 4 x with wash buffer, then 50 µl of sheep anti-rabbit-HRP antibody (1:1000) (Anti-rabbit Ig

affinity isolated peroxidase conjugated, Silenus Labs Pty. Ltd., Boronia, Victoria, Australia) was added to each well and left for 1 h in a sealed humid container at room temperature.

After a final wash of 4 x with wash buffer, 100 µl zymed tetramethylbenzidine (TMB, Zymed, San Francisco, California, U.S.A) was added to each well and then incubated for 10 min at room temperature. This reaction was then stopped by the addition of 50 µl 0.4M H_2SO_4. The plasma and amniotic fluid IL-6 concentration were determined by quantification of colour absorbency using a Mulitskan RC plate reader (Thermo Labsystems, Finland) read at 450 nm, using 630 nm as a reference and analysed using Genesis v.3.04 (Thermo Labsystems, Finland).

2.7 PROSTAGLANDIN H SYTHASE TYPE-2 (PGHS) EXPRESSION ANALYSIS

2.7.1 Tissue isolation and extraction

Whole cotyledon tissues were collected at *post mortem* (refer to Section 2.4) and immediately stored at -70 °C until needed, at which time they were moved to dry ice. Each tissue was pulverised on dry ice using an air hammer and anvil and placed in a sterile 15 ml tube (Falcon; Beckton Dickinson Labware, New Jersey, U.S.A), then kept at -70 °C until needed for RNA extraction.

2.7.1.1 Ribonucleic acid (RNA) extraction

The RNA from each tissue was isolated using the Tri-reagent method (Sigma Chemical Company), a variation on the method of Chomcznski and Sacchi (1987). Samples were homogenised in 1 ml of Tri-reagent per 50-100 mg of tissue (total 300-400 mg) in sterile 15 ml polypropylene tubes (Falcon, U.S.A) using a homogeniser (Ultra-turax T25; IKA Labortechnik, Staufen, Germany). After homogenisation, samples were centrifuged (Sorvell RC-5B, DuPont, Delaware, U.S.A) at 12, 000 x g for 10 min at 4 °C to remove the insoluble material (extracellular membranes, polysaccharides, and high molecular weight DNA). The clear supernatant containing RNA and protein was then transferred into a new sterile 15 ml tube and incubated for 5 min at room temperature. Chloroform (BDH Chemicals, Australia) was added to the samples at 0.2 ml per ml of Tri-reagent originally used, vortexed for 15 sec and allowed to stand for 10 min at room temperature. The samples were then centrifuged at 12, 000 x g for 15 min at 4 °C.

Following this centrifugation, the colourless upper aqueous layer, containing the RNA was again transferred into new 15 ml tubes. Isopropanol (BDH Chemicals, Australia) was added at 0.5 ml per ml Tri-reagent originally used and allowed to stand for 10 min at room temperature before being centrifuged at 12, 000 x g for 10 min at 4 °C. The supernatant was removed and discarded, while the pellet was washed in 75% ethanol (1.0 ml per ml Tri-reagent originally used), vortexed then centrifuged again at 7, 500 x g for 5 min at 4 °C. The pellet was then resuspended in 200 µl diethylpyrocarbonate (Sigma) treated water (DEPC-H_2O).

2.7.1.2 *Acid/Phenol Chloroform extraction*

To further purify RNA samples, an additional acid/phenol chloroform extraction was performed. An equal volume of acid phenol/chloroform/isoamyl alcohol (Ambion Inc., Austin, Texas, U.S.A) was added to the RNA samples in a sterile 1.5 ml microfuge tube, and vortexed briefly before centrifugation at 12, 000 x g for 1 min at 4 °C. The upper aqueous layer was then removed and transferred to a new 1.5 ml microfuge tube where 1/10 volume of 3 M sodium acetate (NaAc, pH 4.0) was added and mixed. Ice-cold 100% ethanol was added at 2.5 x volume (calculated *after* salt addition) and placed in -70 °C for 20 min. Following precipitation, samples were centrifuged at 12, 000 x g for 5 min at 4 °C. After removing and discarding the supernatant, 1 ml of 70% ethanol was added to wash the pellet, samples were then centrifuged as above. The supernatant was removed and the pellet dried for 5 min before being resuspended in 50 µl DEPC-H_2O.

2.7.1.3 *Agarose gel analysis*

Each RNA sample was analysed by electrophoresis by adding 2 µl of RNA to 2 µl DEPC-H_2O and 2 µl of Northern Loading Buffer. The samples were boiled for 3 min and stored on ice prior to loading on the gel. A 1% agarose gel in 1 x tris-acetate-EDTA (TAE) solution was prepared and each sample was loaded onto the gel. The gels were run for 1 h on a Submarine Agarose Gel Unit (Hoefer Scientific Instruments, San Fransisco, California, U.S.A.) at 70 V (Multidrive XC; Amrad, Pharmacia Biotech, Boronia, Australia). Following the electrophoresis, the gel was viewed under ultraviolet light (Polaroid transilluminator, Kemp Keuer, Cambridge, U.S.A) for the presence of discrete RNA bands. Only samples showing discrete bands, indicative of the absence of degradation were used for analysis of PGHS expression.

2.7.1.4 *Spectrophotometrical analysis*

Each RNA sample was analysed in a spectrophometer (UV/VIS 918; GBL Scientific Equipment, Dandenong, Victoria, Australia) at 260 and 280 nm. The spectrophometer was calibrated to zero by reading the optical density of DEPC-H_2O at 260 nm and 280 nm. Samples routinely displayed ratios of 260/280 optical densities ≥ 1.7. From the readings obtained, the concentration, and total amount of RNA and yield of each sample was calculated for future use.

2.7.2 Ovine PGHS-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes

The ovine PGHS-2 and GAPDH probes were transcribed from the cDNA sequence encoding ovine PGHS and ovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously reported by Zhang *et al.* (1996) and Phillips *et al.* (1997), respectively. The GAPDH probe has been extensively used as a reference probe in ovine tissue (Phillips *et al.*, 1997; Phillips *et al.*, 2001). Linear templates for PGHS-2 and GAPDH probe transcription were provided by Dragan Illic (Physiology Department, Monash University), and Dr. Ian Phillips (Cytopia Pty. Ltd., St. Vincents Hospital, Melbourne, Victoria, Australia).

2.7.2.1 *Transcription reaction*

The RNA PGHS-2 and GAPDH probes were transcribed using the transcription reactions presented in Table 2.5.

Table 2.5 Summary of the reagents and concentrations used in the labeling of the RNA probe for the PGHS-2 and GAPDH transcription reaction.

Reagents	PGHS-2 (μl)	GAPDH (μl)
5x transcription buffer (Gibco, Life Technologies, Maryland, U.S.A)	5.0	5.0
Dithiothreitol (DTT; Gibco)	2.0	2.0
RNasin (RNA inhibitor, Promega)	0.5	0.5
Nucleotide mix C (Promega)	4.0	4.0
Cold α-32^{P}-CTP (Amrad, Australia)	2.4	2.4
cDNA template	2.0	3.0
α-32^{P}-CTP (Amrad)	5.0	5.0
Transcription enzyme (T3, Gibco)	1.2	1.2
Sterile milli Q H_2O	2.9	2.9

Once labelled, the probes were incubated for 60 min at 37 °C in a water bath. At the completion of this incubation, 0.6 μl Rnasin (Promega Corporation, Annandale, New South Wales, Australia) and 4 μl Dnase I (Gibco, Invitrogen Life Technologies, Carlsbad, California, U.S.A) were added and incubated for another 20 min at 37 °C in a water bath. Once the DNA template was digested, 100 μl Tris-EDTA (TE) buffer, 4 μl tRNA (Sigma Chemical Company) and 150 μl phenol/chloroform (Sigma Chemical Company) were added, vortexed and microfuged at 10, 000 x g for 2 min. The upper aqueous layer was then removed to a new 1.5 ml tube, to which 50 μl TE buffer, 50 μl ammonium acetate (10 M) and 800 μl 100% ethanol were added and left to incubate overnight at -20 °C.

2.7.2.2 *Gel purification of the probe*

The probes were microfuged at 10, 000 x g for 15 min and the ethanol removed. The pellets were dried for 10 min upon which 3 μl TE buffer and 16 μl loading buffer were added, boiled for 10 min and left on ice while the mini vertical gel was prepared. A mini vertical gel was prepared by adding 70 μl ammonium persulphate (BioRad Laboratories, Hercules, California, U.S.A) and 18 μl TEMED (BioRad, U.S.A) to 25 ml 6% acrylamide solution. The probes were loaded onto the minigel (Miniprotean II; BioRad, U.S.A) and run at 120 V (Hoefer Scientific Instruments, U.S.A) for 1.5 h. Following the

electrophoresis, the gel was exposed on Kodak XRP X-ray film and developed (100 plus developer, Jacobs Medical Supplies, St. Albany, New South Wales, Australia), the exposed band was then cut and removed from the gel. To elute the labelled probes, the gel fragments were incubated with 350 µl gel elution buffer (RPA Kit III, Ambion Inc., Austin Texas, U.S.A) for 4 h at 37 °C. After this incubation the amount of radioactive label was determined from 2 µl in 4 ml scintillant (Ecoscint A, National Diagnostics) using a liquid scintillation counter (LS 3801; Beckman Instruments Inc., U.S.A), and the probes were then stored at -20 °C.

2.7.3 Ribonuclease Protection Assay (RPA)

The level of PGHS-2 and GAPDH mRNA expression in whole cotyledons was determined by performing ribonuclease protection assays using an RPA Kit III (RPA Kit III, Ambion Inc., U.S.A). The method used was provided in the RPA kit and was as follows.

2.7.3.1 *Hybridisation procedure*

RNA samples 20-40 µg, were combined with 4-8 x 10^4 cpm per 10 µg total sample RNA of labelled probe. Two control tubes containing the same amount of labelled probe plus Yeast RNA, equivalent to the amount of sample RNA, were included in each assay. To each sample, the concentration of NH_4OAc was adjusted by adding 1/10 volume of 5M NH_4OAc, followed by the addition of 2.5 x volume of 100% ethanol and mixed thoroughly. Each tube was then incubated for 15 min at -20 °C. Following the co-precipitation of the probe and RNA, samples were centrifuged at 12, 000 x g for 15 min. The ethanol supernatant was removed, taking care not to dislodge the pellet. Pellets were then resuspended in hybridisation buffer (10 µl). After adding the hybridisation buffer to each pellet, samples were vortexed for 5-10 sec, then centrifuged to collect the contents at the bottom of the tube. To denature the RNA, samples were incubated for 3 min at 90-95 °C, followed by incubation overnight at 42 °C tohybridise the probe to its complement in the RNA.

2.7.3.2 *RNase digestion of hybridised probe and sample RNA*

A working dilution of RNase in RNase Digestion buffer (1:100; RNase A/RNase T1 mix: RNase Digestion III buffer), was prepared and added (150 µl) to each sample RNA tube and one of the yeast control RNA tubes. To the other yeast control RNA tube, 150

μl of RNase digestion III buffer without RNase was added. All sample RNA and control yeast samples were then incubated for 40 min at 37 °C.

Following the incubation, 225 μl of RNase Inactivation/Precipitation III Solution was added to each sample RNA and control yeast RNA sample, vortexed and then briefly microfuged before a further incubation for 15 min at -20 °C. Samples were then centrifuged at 12, 000 x g for 15 min. The supernatant was carefully removed and the pellet resuspended in 6 μl Gel Loading buffer II. Before loading the samples on to the gel, samples were incubated for 3 min at 90-95 °C. Undigested probe (1 μl) and mass ladder (1 μl; Probase50™, Progen Industries Ltd., Queensland, Australia) plus Gel loading buffer (5 μl) were also prepared.

All RNA samples and undigested probe were loaded on a sequencing gel made from 70 ml 6% acrylamide solution with 210 μl ammonium persulphate and 49 μl TEMED, pre-run for 1 h at 1500 V (Hoefer Scientific Instruments, U.S.A). The sequencing gel (Model S2; Gibco) was then run for 1.5 h at 1550 Volts. The gel was then transferred onto 3 mm Whatmann filter paper and covered with plastic wrap. The covered gel was dried for 50 min at 80 °C in a gel drier (Model 583; BioRad). Once dry, the gel was placed in a phosphor imager cassette (Molecular Dynamics, Sunnyvale, California, U.S.A) and developed for 24 h. Following the developing period the phosphor imaging sheet was analysed on a phosphor scanner (STORM; Molecular Dynamics) for the detection of probe that was protected by mRNA fragments. The analysis of the protected bands and densitometry were performed using the computer software, Imagequant (Molecular Dynamics, U.S.A.).

Chapter 3

Inhibition of premature labour

3.1 INTRODUCTION

Prostaglandins play a major role in human and ovine parturition; stimulating uterine contractility, ripening of the cervix and enhancing fetal maturational processes (Challis & Lye, 1994). An increase in intra-uterine tissue prostaglandin concentrations has been associated with both term and premature labour in all species examined to date (Rice, 1990). Prostaglandin H synthase (PGHS) catalyses a critical step in the prostaglandin synthetic pathway and exists in two iso-forms, PGHS-1 and PGHS-2 (Xie *et al*, 1993; Smith *et al*., 1996). An increase in the activity of this enzyme is observed with advancing gestation in human amnion and ovine placental tissue and there is a further increase with the onset of labour (Rice *et al*., 1988; Teixeira *et al*., 1994). The expression of PGHS-1 is developmentally regulated and appears to perform maintenance functions in the fetus (Smith *et al*., 1996). Conversely, PGHS-2 expression is induced by several growth factors and cytokines in cells derived from gestational tissues (O'Sullivan *et al*, 1992). Elevated PGHS-2 expression has been observed in intra-uterine tissues following spontaneous term and premature labour in humans (Hirst *et al*., 1995b). Similarly, PGHS-2 expression increases in intra-uterine tissues at the onset of normal and glucocorticoid-induced premature labour in sheep (McLaren *et al*., 1996).

Prostaglandin synthase inhibitors have been used to delay the onset of normal and premature labour in primates (Novy *et al*., 1974) and sheep (Mitchell & Flint, 1978). While prostaglandins are critical in the initiation of parturition, they also have a central role in maintaining the fetal circulation and regulating fetal renal function. Indomethacin, a non-selective PGHS inhibitor is more effective in prolongation of pregnancy than the commonly used β-adrenergic receptor agonists (Keirse, 1992). However, the use of indomethacin is also limited by its potential fetal side effects, and its use in late pregnancy increases the risk of constriction of the ductus arteriosus (Moise *et al*., 1988; Norton *et al*., 1993; Guerguerian *et al*., 1998), making

indomethacin unsuitable for use at or after 32 weeks of gestation (Steer & Flint, 1999). Each of these side effects are likely due to inhibition of constitutively produced prostaglandins in the fetal compartment which would therefore be products of PGHS-1 activity (Moise *et al.*, 1988; Norton *et al.*, 1993). The use of selective PGHS-2 inhibitors to inhibit premature labour has therefore, been proposed (Keirse, 1995), since they are more likely to act on intra-uterine rather than fetal prostaglandin production.

It has previously been reported that selective inhibition of PGHS-2 with the non-steroidal anti-inflammatory drug nimesulide, significantly reduces circulating maternal and fetal prostaglandin concentrations and delays, but does not inhibit the onset of glucocorticoid-induced premature labour in sheep (Poore *et al.*, 1999). Previously, it was found that nimesulide did not totally suppress the glucocorticoid-induced increase in uterine activity. The residual uterine activity may be a result of oxytocin secretion and this, coupled with heightened sensitivity to oxytocin at this time (Meier *et al.*, 1995), may lead to delivery despite suppression of circulating prostaglandin production by nimesulide. Oxytocin plays a critical role in the progression of labour by coordinating myometrial contractions (Fuchs *et al.*, 1984) and increasing decidual production of prostaglandins, particularly $PGF_{2\alpha}$ (Andersen *et al.*, 1989). Conversely, prostaglandins can also up-regulate oxytocin receptors, creating a positive feed-forward process during parturition (Burgess *et al.*, 1990). Although oxytocin directly stimulates uterine contractility in late pregnancy, labour will not be maintained unless there is a concomitant increase in prostaglandin biosynthesis by the decidua (Fuchs *et al.*, 1983a). In the study by Poore *et al.* (1999) delivery occurred in nimesulide treated animals despite significant suppression of circulating prostaglandin concentrations, suggesting that local decidual prostaglandin production may not have been sufficiently blocked by the PGHS-2 inhibitor. This small prostaglandin release, reflecting an increase in production in gestational tissues may have facilitated cervical relaxation and initiated oxytocin release, allowing adequate uterine activity for delivery. Therefore, inhibition of uterine $PGF_{2\alpha}$ production via oxytocin receptor blockade (Jenkin *et al.*, 1994) may thus contribute to inhibiting the uterine activity and further delaying premature labour. Atosiban ([1-deamino-2-d-Tyr(Oet)-4-Thr-8-Orn]-oxytocin) is a competitive antagonist of oxytocin that inhibits oxytocin-induced uterine contractions in both *in vitro* and *in vivo* animal models (Melin, 1993). Atosiban has also been shown to decrease uterine contractility in women threatened with premature labour (Akerlund *et al.*, 1987; Moutquin *et al.*, 2000). Most importantly, atosiban does not reduce basal fetal

prostaglandin release in late gestation (Jenkin *et al.*, 1994). Therefore, oxytocin receptor antagonists like atosiban, are an attractive alternative to currently used tocolytic drugs because of their high specificity and lack of serious maternal and fetal side effects (Melin, 1993). Despite this, a recent clinical study, demonstrated that atosiban was only comparable in effectiveness to therapy with ritodrine, but had much less side effects (Moutquin *et al.*, 2000). These findings suggest that atosiban may lower uterine activity and further suppress the release of prostaglandins if administered together with nimesulide, therefore improving the ability to inhibit premature labour considerably.
Therefore, the aim of this study was to increase the effectiveness of nimesulide treatment while also reducing its potential detrimental effects on the fetus. The principle aim of the study, was to determine if the blockade of oxytocin receptors with atosiban improves the effectiveness of nimesulide treatment for delaying premature labour in sheep. The overall advantage of this new approach is that it may not only block the direct stimulatory effects of prostaglandins and oxytocin on uterine activity, but also the indirect effect of local oxytocin-induced uterine prostaglandin release. To investigate this concept an established ovine model of premature labour, in which the hormonal changes that occur at term in this species are advanced by fetal glucocorticoid administration was used.

The work presented in this chapter is an extension to work conducted in partial fulfilment of my Bachelor of Science Honours degree with the Fetal and Neonatal Research Group, Department of Physiology at Monash University in 1998. The work presented for my BSc (Hons) was incomplete with respect to the hormone and fetal well-being analysis required for statistical significance. Additional animal results were thus studied and further analysised together with analysis and recordings of results obtained from the animals used in the original study during my Ph.D. research to complete this study for publication.

3.2 METHODOLGY

3.2.1 Induction of premature labour

Labour was induced by continuous infusion of dexamethasone (DEX; 1 mg/d diluted in sterile heparinised saline, 1 ml/h; Baxter Healthcare Pty. Ltd., Australia) via the fetal jugular vein (JV) catheter, commencing on 138 days of gestation.

3.2.2 Inhibition of premature labour

Nineteen Border Leicester-Merino cross-bred ewes of known gestational age (GA) were used in this study. Ten of these ewes had been used in a previous study in which the effect of nimesulide alone on glucocorticoid-induced labour was examined (Poore *et al*, 1999). Some parameters of the previous study were further investigated and analysed in the current study. Nimesulide (NIM; Cayman Chemicals, Ann Arbor, Michigan, U.S.A) was administered by continuous intravenous infusion via the maternal jugular vein (JV) silastic catheter (20 mg/kg/d maternal body weight, in n-Methyl-2-Pyrrolidone; ISP Australasia Pty. Ltd., Victoria, Australia, 1 ml/h; n = 5). Ewes treated with NIM and atosiban (ATO; Ferring Polypeptide Laboratories AB, Sweden) received a continuous intravenous infusion of both NIM (20 mg/kg/d maternal body weight) via the maternal silastic JV catheter and ATO (4.12 mg/kg/d maternal body weight in sterile saline, 1 ml/h; n = 5) via the maternal polyvinyl JV catheter. Control ewes (VEH; n = 9) were administered a continuous intravenous infusion of the vehicles only via the maternal JV catheters. All maternal infusions (NIM, NIM and ATO or VEH) commenced 30 min prior to the onset of DEX labour induction.

The infusion of NIM, NIM and ATO or VEH continued until immediately prior to delivery of the fetus or elective *post mortem*, as determined by (a) the appearance of the fetus at the vagina (given as delivery; *D*), or (b) at least 95 h, approximately twice the time from the commencement of glucocorticoid induction to delivery of VEH-treated ewes but within 24 h thereof, or (c) no delivery 12 h after membrane rupture, or (d) deterioration of maternal condition requiring termination of the experiment for ethical considerations. The progress of labour was continuously monitored by uterine EMG activity and final blood samples were collected immediately before delivery or the completion of the experiment as described above, after which time ewes and their fetuses were humanely killed by barbiturate overdose (refer to Section 2.4). At *post mortem*, the patency of the ductus arteriosus and the amount of meconium staining were

visually examined to assess fetal condition. Fetal body weight was also recorded. In addition, whole cotyledon tissue was collected and frozen immediately in liquid nitrogen and stored at -70 °C for RNA analysis (refer to Chapter 4).

3.2.3 Sampling regime

Blood samples were obtained from the fetal and maternal carotid artery (CA), and both utero-ovarian vein (UOV) catheters at 15 min prior to and immediately before commencing maternal inhibitor treatment (time 0; 10 am) and at 0.5, 1.0, 1.5, 2.0, 6.0, 12.0 h after the onset of infusion. Samples were then collected 3 times daily at 10 am, 4 pm and 10 pm, until delivery or elective *post mortem*. PGFM and PGE_2 concentrations in fetal and maternal plasma were measured by RIA, as described in Section 2.5. In this study the intra- and inter- assay co-efficient of variance of the PGFM RIA were 10% and 21%, respectively. The mean sensitivity of the assay was 0.43 ± 0.07 nmol/L. The intra- and inter-assay co-efficient of variation of the PGE_2 RIA were 10% and 24%, respectively, and the mean sensitivity of the assay was 0.24 ± 0.05 nmol/L. The intra- and inter-assay co-efficient of variation of the progesterone RIA were 16% and 32%, respectively, and the mean sensitivity of the assay was 0.3 ± 0.01 nmol/L. At 6 h intervals, beginning at time 0 until the end of treatment, maternal and fetal arterial blood was collected for analysis of blood respiratory gases (PaO_2 mmHg, $PaCO_2$ mmHg, O_2 sat) and pH were measured as described in Section 2.2.3. Plasma aliquots for measurement of glucose and lactate concentrations were also taken and stored immediately at -20 °C for later analysis using a YSI 2300 STAT glucose lactate analyser (Yellow Springs Instruments, Ohio, U.S.A).

3.2.4 Uterine EMG analysis

Signals from myometrial EMG electrodes were amplified and recorded using a polygraph data recording system (Grass Instruments, Quincy, Massachusetts, U.S.A), during the experimental period. For analysis, the study period was divided into 2 h sections commencing 24 h prior to the onset of NIM, NIM and ATO or VEH infusions, and throughout the entire experiment until delivery or elective *postmortem*. For each animal, the number of discrete *bursts* of uterine EMG activity occurring within each 2 h period were measured. A burst was defined as having a minimum duration of 0.5 min and a minimal interval of 2.0 min between each burst.

3.2.5 Blood pressure analysis

Fetal blood pressure was measured continuously throughout the experimental period in all NIM and ATO-treated ewes (n = 5) and VEH-treated ewes (n = 3). Fetal blood pressure was corrected for amniotic pressure and amplified using a Grass 7P3-amplifier (Grass Instruments, U.S.A) with a calibrated range of 0-100 mmHg. For analysis, three systolic and diastolic pressure measurements were taken every 6 h from the onset of DEX infusion and averaged at each time point. The mean arterial pressure (MAP) at 6 h intervals was then calculated.

3.2.6 Statistics

All results are presented as mean ± standard error of the mean (SEM). Data were first tested for homogeneity of variance using Bartlett-Box F and Cochran's C tests. Data found heterogeneous was rendered homogeneous by square root or logarithmic transformation. The effects of treatment, time and individual animal were tested using multifactorial analyses of variance (ANOVA) for repeated measures (Statistical Packages for Social Sciences, SPSS-X Data Analysis System, SPSS Inc., Illinois, U.S.A). Where significant treatment or treatment-time interactions were found, NIM alone and NIM and ATO treatment were compared to VEH treatment individually. NIM and NIM and ATO treatments were then tested against each other without the VEH treatment group. To test the initial effectiveness of the inhibitor treatments, the data was divided into the first 6 h of maternal treatment, which was then analysed against the first 6 h of VEH treatment. Secondly, data from both inhibitor groups was analysed from 48 h prior to delivery or elective *post mortem* against VEH treatment to determine the effectiveness of each inhibitor group on inhibiting the increase in prostaglandin concentrations, which occurred towards delivery in VEH animals. The time to delivery or elective *post mortem* of the three treatment groups was compared by one way ANOVA. To analyse the time of onset of increased uterine EMG activity in each animal, the mean number of uterine bursts during the 24 h prior to treatment and 3x standard deviation was calculated. The time at which uterine EMG activity increased above this set value was considered to be the onset of increased uterine EMG activity for that animal. These values were then analysed using ANOVA as above. To analyse the duration of labour for VEH and NIM treatment, the initial 8 h after the onset of increased uterine EMG activity were compared. The NIM and ATO treatment group was not included in this analysis as

none of the ewes were in active labour or delivered their fetuses. Significance is reported at the 5% level ($P < 0.05$).

3.3 RESULTS

3.3.1 Outcome of animals

All fetuses from ewes treated with VEH infusions delivered within 51.4 ± 1.7 h after the onset of DEX infusion. Membrane rupture occurred in all ewes treated with NIM, however, only three ewes progressed to delivery. The mean time to delivery (n = 3) or elective *post mortem* (n = 2), of NIM-treated ewes was significantly longer than the time to delivery of VEH-treated ewes (n = 9, Table 3.1). In contrast, none of the NIM and ATO-treated ewes progressed to delivery during treatment (n = 5), therefore these ewes and their fetuses were electively killed. One ewe showed no signs of delivery or membrane rupture and was killed at the completion of the infusion time period as described in the experimental protocol (refer to Section 3.2.2). Membrane rupture occurred in two NIM and ATO-treated ewes with no further progress to delivery after 12 h. Maternal distress was observed in the two remaining ewes, therefore the experiment was discontinued at 80.5 h and 90.5 h after the commencement of DEX infusion. It has been observed that sheep are relatively sensitive to treatment with PGHS inhibitors and tend to lose appetite with prolonged administration. A reduction in food intake in pregnant ewes can lead to the onset of the early stages of pregnancy toxemia (Marteniuk & Herdt, 1988), requiring the termination of the experiment for ethical considerations. However, these animals were killed at times significantly longer than the delivery times for VEH-treated animals. The times to the end of the experiment, whether delivery, or elective *post mortem*, were significantly different between the three treatment groups with ewes treated with NIM alone longer than VEH-treated ewes, and ewes treated with NIM and ATO longer than both VEH-treated ewes and ewes treated with NIM alone (Table 3.1).

Table 3.1 Outcome of animals. Time to delivery following fetal dexamethasone infusion (1 mg/d in saline, 1ml/h; n = 19) and maternal treatment with either vehicle (n-Methyl-2-Pyrrolidone and saline, 1 ml/h; n = 9), nimesulide (20 mg/kg/d maternal body weight; n = 5) or nimesulide and atosiban (20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5).

Dexamethasone plus vehicle			*Dexamethasone plus nimesulide*			*Dexamethasone plus nimesulide and atosiban*		
Animal number	*Time to delivery or elective post mortem (h)*	*Delivery before elective post mortem*	*Animal number*	*Time to delivery or elective post mortem (h)*	*Delivery before elective post mortem*	*Animal number*	*Time to delivery or elective post mortem (h)*	*Delivery before elective post mortem*
463	64.0	Yes	522	69.5	Yes	030	80.5[§]	No
523	51.5	Yes	602	69.8	Yes	031	94.5[f]	No
663	49.0	Yes	666	73.3	Yes	062	119.5	No
603	53.8	Yes	590	59.5	No	146	107.5[f]	No
757	51.0	Yes	481	84.0	No	147	90.5[§]	No
179	51.5	Yes						
200	48.3	Yes	Mean for all	71.2 ± 3.9*		Mean for all (no animals delivered)	98.5 ± 6.8†	
202	46.5	Yes						
006	46.5	Yes	Mean for animals that delivered only	70.9 ± 1.2*				
Mean for all (all animals delivered)	51.4 ± 1.7							

Delivery before elective post mortem, undelivered fetuses and ewes were electively killed according to the experimental protocol, refer to Section 3.2.2. Dexamethasone infusion commenced at time 0; vehicle, nimesulide or nimesulide and atosiban infusions commenced 30 minutes prior to dexamethasone infusion. *Significantly different from vehicle-treated group. † Significantly different from both vehicle-treated and nimesulide-treated groups. § Deterioration of maternal condition requiring discontinuation of the treatment for ethical considerations, f Membrane rupture with no other signs of delivery after 12 h, therefore these ewes and their fetuses were electively killed.

3.3.2 Uterine EMG activity

Uterine EMG activity recorded from 24 h prior to the onset of DEX infusion and throughout the treatment period is shown in Figure 3.1. A significant increase in uterine EMG activity in VEH-treated ewes was first observed at 40.9 ± 2.0 h after the start of DEX infusion. In NIM alone and NIM and ATO-treated ewes, the increase occurred at 48.9 ± 3.0 h and 56.0 ± 2.0 h after the start of DEX infusion, respectively. These times were not different from each other, but were significantly longer than those for VEH treatment (Figure 3.1). During the first 8 h after the initial increase in uterine EMG activity, there was a further rise in activity in all treatment groups. The rate of this increase was, however, significantly greater in VEH-treated ewes than in the NIM alone and NIM and ATO-treated ewes. The rate of increase in NIM alone and NIM and ATO-treated ewes were not different from each other, however, uterine EMG activity observed in NIM-treated ewes continued to rise until delivery or elective *post mortem*. In contrast to ewes treated with NIM alone, uterine activity in NIM and ATO-treated ewes declined after the initial rise, and by 84 h uterine EMG activity began to fall to similar levels seen prior to treatment with none of the ewes delivering their fetuses (Fig. 3.1 C). The duration of labour associated with increased uterine EMG activity for NIM-treated ewes was significantly longer than that observed in VEH-treated ewes.

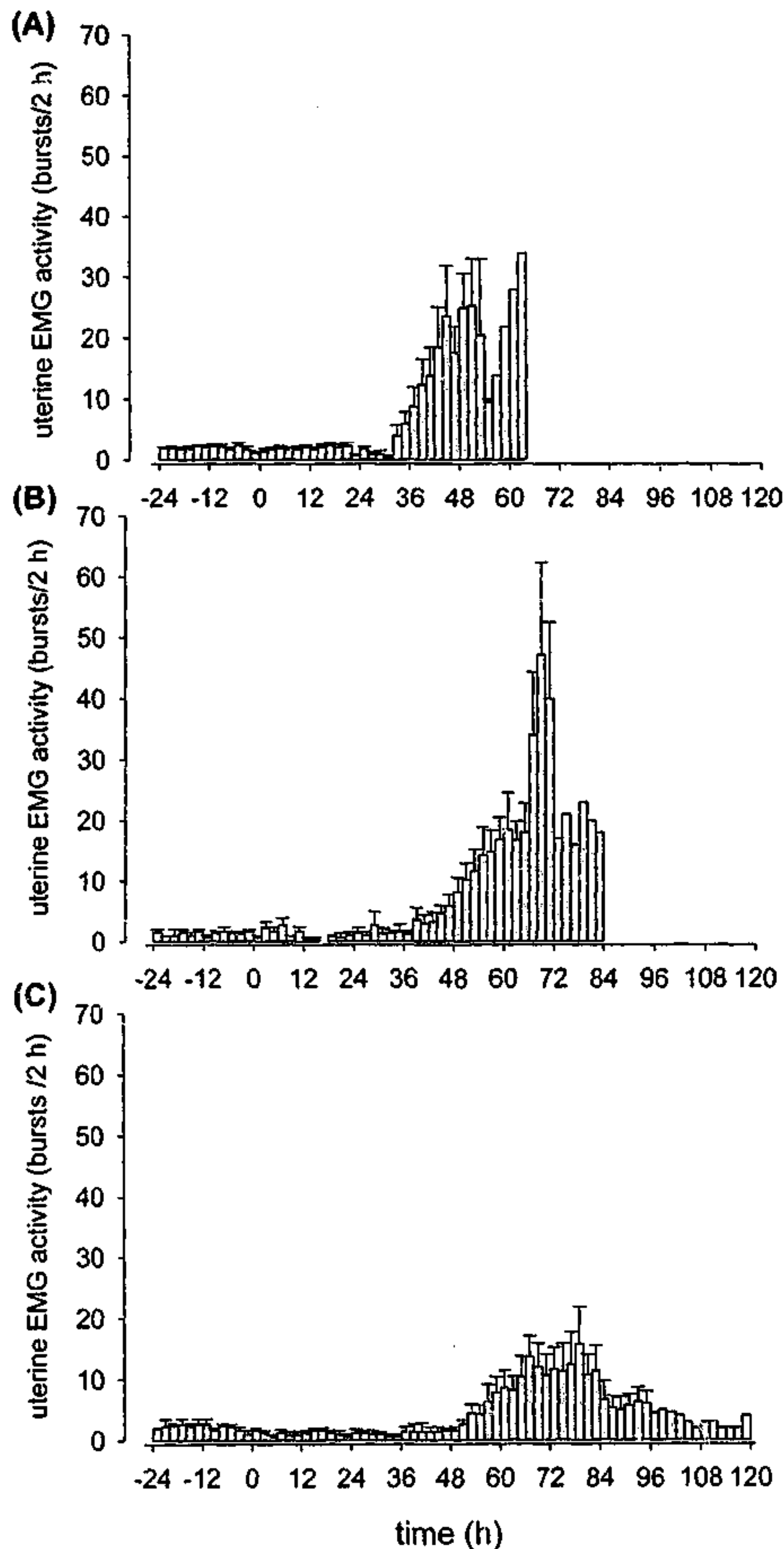

Figure 3.1 Uterine electromyographic (EMG) activity during dexamethasone-induced premature labour. Values are from 24 h before the onset (time 0) of vehicle (A, n = 9), nimesulide (B, 20 mg/kg/d maternal body weight; n = 5), and nimesulide and atosiban (C, 20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5) infusions until delivery or elective *post mortem*.

3.3.3 Maternal and fetal prostaglandin concentrations

PGFM and PGE_2 concentrations in the maternal and fetal circulations during labour induction are presented for the first 6 h after the commencement of VEH, NIM or NIM and ATO infusions and then from the last 48 h before the time of delivery or elective *post mortem* (Figure 3.2-3.4). Maternal PGFM and PGE_2 concentrations (CA, UOV) in VEH-treated ewes steadily increased prior to delivery, reaching maximum levels at delivery (Figure 3.2 and 3.3). Prior to the commencement of treatment, maternal PGE_2 concentrations (CA) in NIM and ATO-treated ewes were slightly higher than in VEH- and NIM-treated ewes. However, once treatment began, maternal PGE_2 concentrations in both NIM- and NIM and ATO-treated ewes decreased to similarly low levels within 6 h (Figure 3.2 C and D). Maternal (CA) PGFM and PGE_2 concentrations remained significantly suppressed, and in most cases, below the sensitivity of the assays, in ewes treated with NIM alone and NIM and ATO throughout the infusion period until delivery or elective *post mortem*. Maternal UOV PGFM and PGE_2 concentrations were also significantly reduced in both treatment groups, however, PGFM concentrations observed in NIM alone-treated ewes increased slightly at delivery or elective *post mortem*. These values were not significantly different from pre-infusion values but were markedly different from concentrations observed in VEH-treated ewes at delivery (Figure 3.3 A)

In VEH-treated ewes, PGFM and PGE_2 concentrations in the fetal circulation (CA) significantly increased prior to delivery and continued to rise to maximum levels at delivery (Figure 3.4). Fetal circulating PGFM and PGE_2 concentrations from both NIM alone and NIM and ATO-treated ewes were markedly reduced within 6 h after the commencement of treatment and remained significantly suppressed until delivery or elective *post mortem* (Figure 3.4). Fetal PGE_2 concentrations from ewes treated with NIM alone increased to levels that were not significantly different from pre-infusion values at delivery or elective *post mortem*, though these levels were significantly lower than levels observed in fetuses from VEH-treated ewes at this time (Figure 3.4 C).

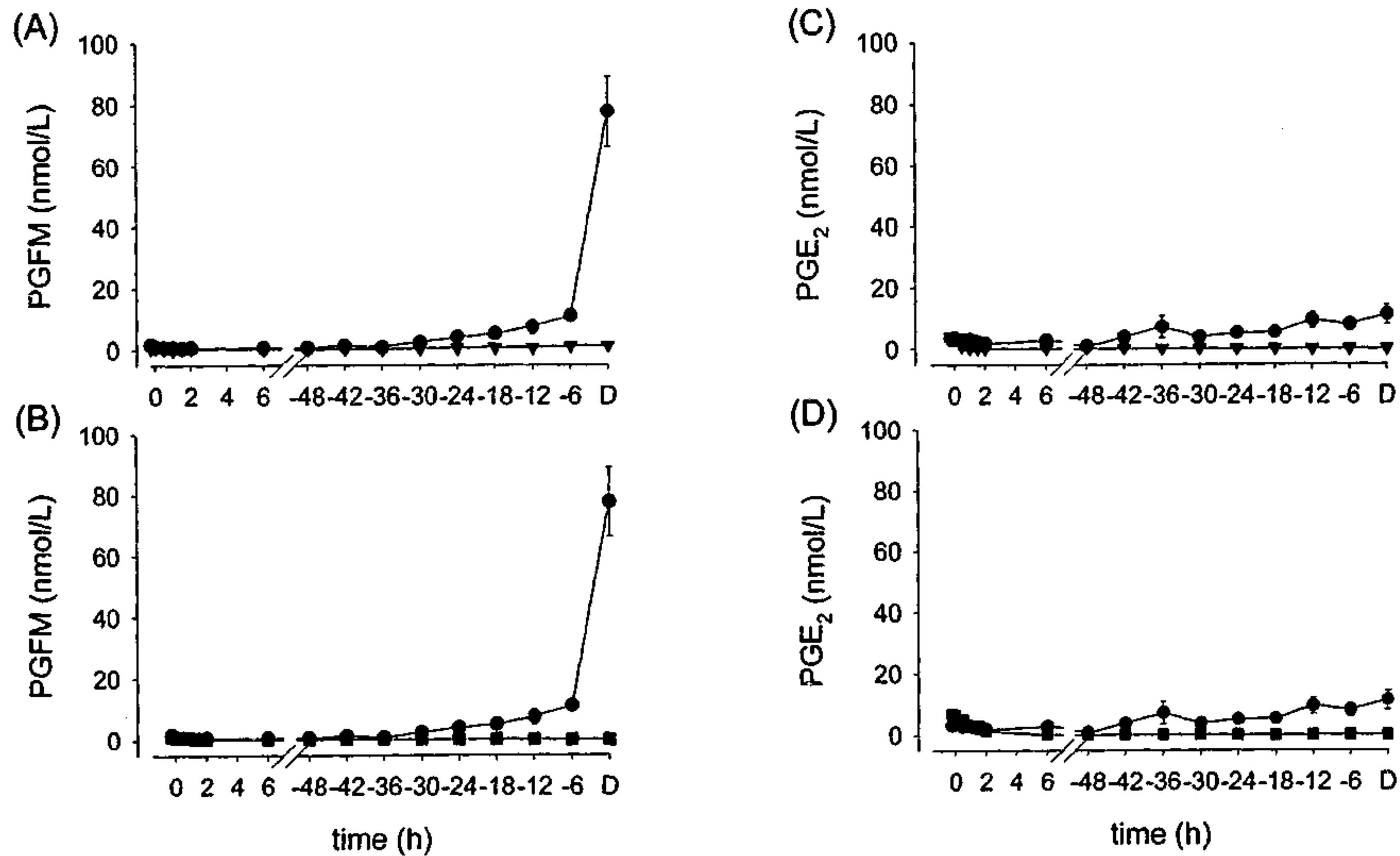

Figure 3.2 Maternal (CA) PGFM and PGE_2 concentrations after dexamethasone-induced premature labour from ewes treated with vehicles only (●; n = 9), or (A) and (C) nimesulide (▼, 20.0 mg/kg/d maternal body weight; n = 5) or (B) and (D) nimesulide and atosiban (■, 20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5), during the first 6 h after maternal treatment onset (time 0) and the last 48 h before delivery or elective *post mortem* (*D*).

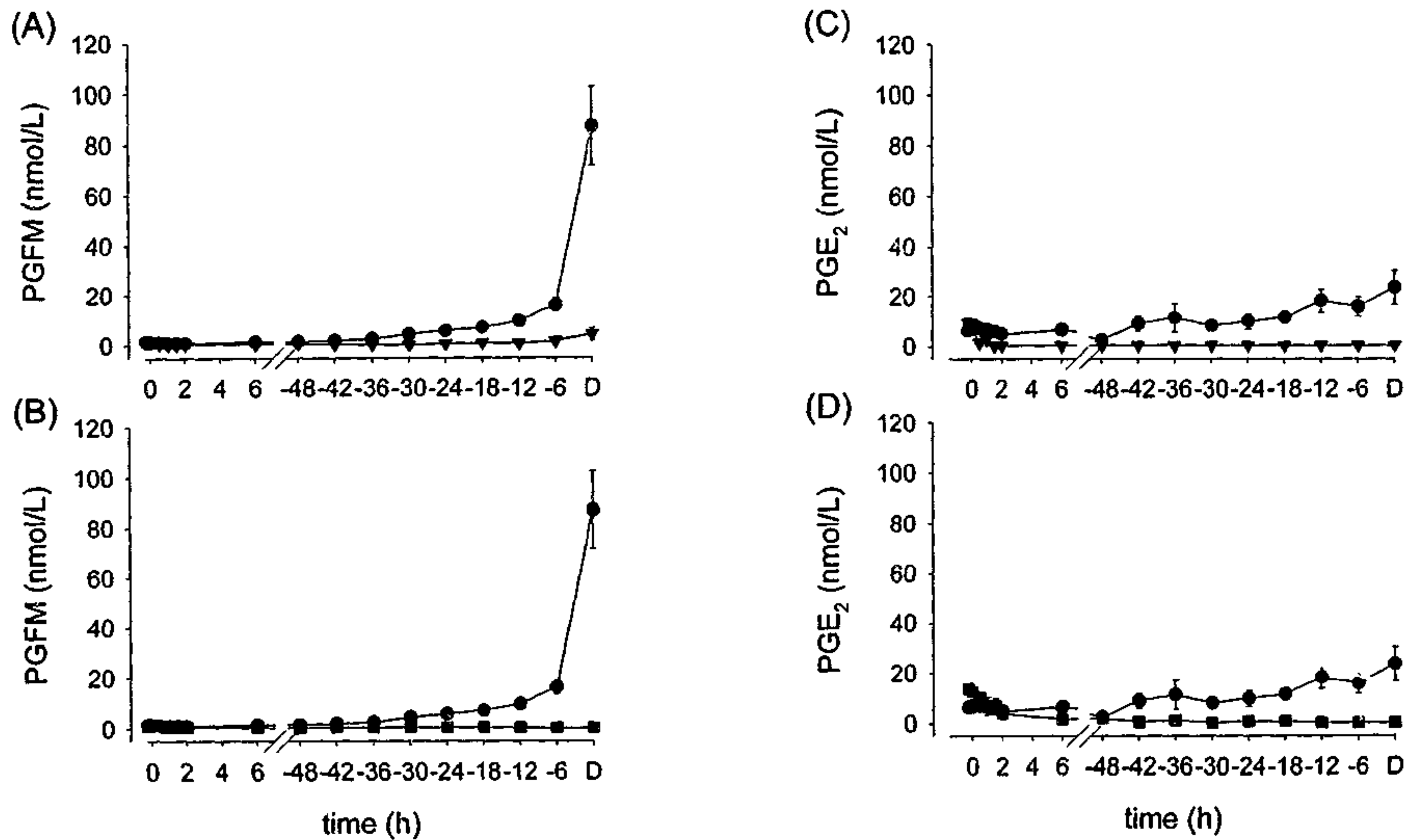

Figure 3.3 Maternal (UOV) PGFM and PGE_2 concentrations after dexamethasone-induced premature labour from ewes treated with vehicles only (●; n = 9), or (A) and (C) nimesulide (▼, 20.0 mg/kg/d maternal body weight; n = 5) or (B) and (D) nimesulide and atosiban (■, 20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5), during the first 6 h after maternal treatment onset (time 0) and the last 48 h before delivery or elective *post mortem* (*D*).

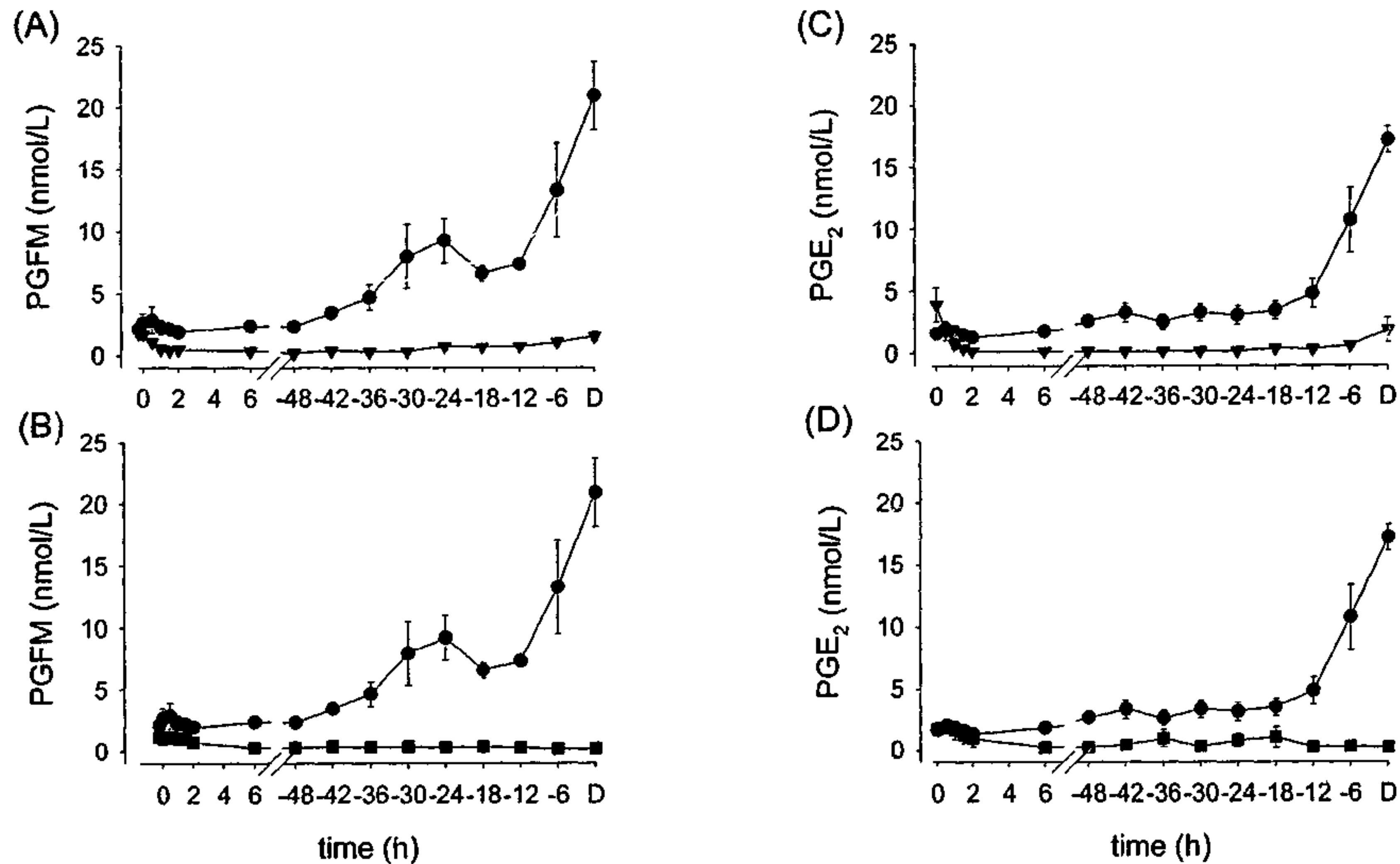

Figure 3.4 Fetal (CA) PGFM and PGE_2 concentrations after dexamethasone-induced premature labour from ewes treated with vehicles only (●; n = 9), or (A) and (C) nimesulide (▼, 20.0 mg/kg/d maternal body weight; n = 5) or (B) and (D) nimesulide and atosiban (■, 20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5), duringthe first 6 h after maternal treatment onset (time 0) and the last 48 h before delivery or elective *postmortem* (*D*).

3.3.4 Fetal and maternal blood gas parameters and fetal blood pressure

Fetal arterial oxygen saturation and PaO_2 was reduced in all treatment groups at delivery or elective *post mortem* when compared to pre-infusion values (Table 3.2). The greatest reduction was observed in fetuses from ewes treated with NIM alone while a smaller reduction was observed in fetuses from ewes treated with NIM and ATO. There were no significant differences in fetal $PaCO_2$, and pH (Table 3.2), or maternal arterial blood gas parameters between the three treatment groups during the experimental period (Table 3.3). Maternal VEH or NIM and ATO treatment had no effect on fetal mean arterial blood pressure (Figure 3.5).

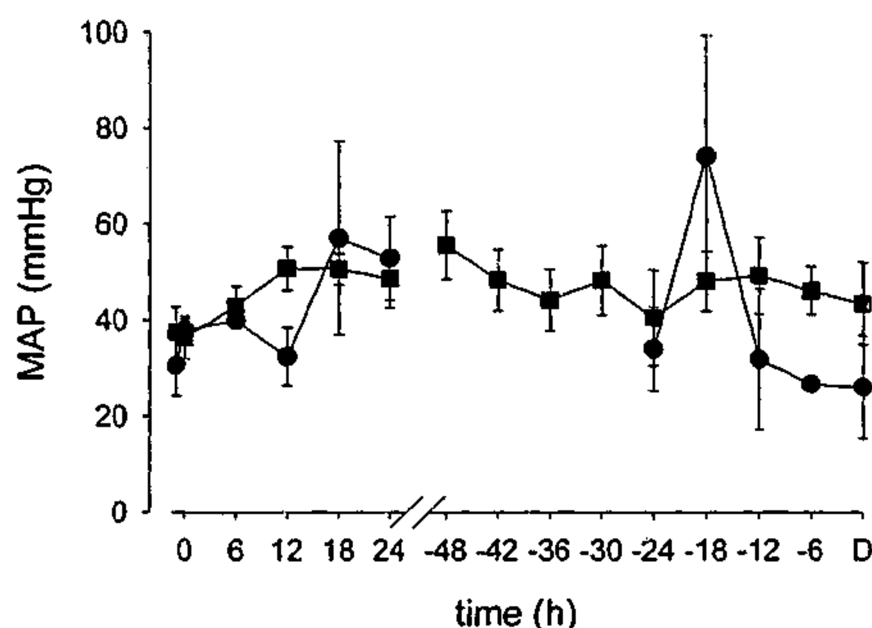

Figure 3.5 Fetal mean arterial blood pressure (MAP) from ewes treated with vehicles (●; n = 3) or nimesulide and atosiban (■, 20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5), during the first 6 h after maternal treatment onset (time 0) and the last 48 h before delivery or elective *post mortem* (*D*).

3.3.5 Fetal glucose and lactate concentrations

Glucose and lactate concentrations were measured in fetuses from VEH-treated ewes (n = 4) and NIM and ATO-treated ewes (n = 5) every 6 h during the treatment period until delivery or elective *post mortem* (Figure 3.6). The mean fetal glucose concentrations during VEH- and NIM and ATO-treatment were 1.82 ± 0.18 and 1.67 ± 0.34 mmol/L, respectively. While the mean fetal lactate concentrations during VEH- and NIM and ATO-treatment were 2.70 ± 0.62 and 3.31 ± 0.88 mmol/L, respectively.

There were no significant differences in either fetal glucose or lactate concentrations from ewes treated with NIM and ATO to the fetal glucose and lactate concentrations from fetuses of ewes treated with VEH only. There was however, a significant effect of time in both the VEH- and NIM and ATO-treated ewes, increasing above pre-treatment glucose and lactate concentrations just prior to labour.

Table 3.2 Fetal arterial blood gases from ewes treated with vehicles (n-Methyl-2-Pyrrdidone and saline, 1 ml/h; n = 9), nimesulide (20 mg/kg/d maternal body weight; n = 5) or nimesulide and atosiban (20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5), during premature labour induction (0-54 h) and at delivery or elective *post mortem*

	PaO_2 (mmHg)			*$PaCO_2$ (mmHg)*			*pH*			*O_2sat*		
Time (h)	*Vehicle*	*Nimesulide*	*Nimesulide and Atosiban*	*Vehicle*	*Nimesulide*	*Nimesulide and Atosiban*	*Vehicle*	*Nimesulide*	*Nimesulide and Atosiban*	*Vehicle*	*Nimesulide*	*Nimesulide and Atosiban*
0 h	22.8±1.06	22.0±0.58	24.4±1.03	49.7±0.93	48.6±0.75	46.8±1.11	7.34±0.01	7.36±0.01	7.36±0.01	57.3±2.95	56.8±2.47	69.3±1.70
24 h	22.1±1.09	19.8±1.65	22.0±1.3	47.8±1.32	48.2±1.24	47.4±0.68	7.35±0.02	7.34±0.03	7.38±0.01	56.8±2.47	47.4±3.15	62.4±2.87
Delivery	17.5±2.08*			51.6±1.67			7.39±0.02			41.5±5.69*		
54 h		16.6±0.68	20.0±1.38		50.8±1.20	48.0±2.30		7.41±0.01	7.39±0.01		42.4±2.98	55.4±3.61
Delivery or elective *post mortem*		12.5±1.32*†	15.0±1.47*		52.5±2.10	53.8±11.88		7.35±0.03	7.31±0.09		27.1±2.49*†	33.3±5.75*

Delivery before elective post mortem, undelivered fetuses and ewes were electively killed according to the experimental protocol, refer to Section 3.2.2. Dexamethasone infusion commenced at time 0; vehicle, nimesulide or nimesulide and atosiban infusions commenced 30 minutes prior to dexamethasone infusion. * Significantly different from pre-infusion values, † Significantly different from vehicle-treated ewes at delivery.

Table 3.3 Maternal arterial blood gases from ewes treated with vehicles (n-Methyl-2-Pyrrdidone and saline, 1 ml/h; n = 9), nimesulide (20 mg/kg/d maternal body weight; n = 5) or nimesulide and atosiban (20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5), duringpremature labour induction (0-54 h) and at delivery or elective *post morten*

	PaO_2 (mmHg)			*$PaCO_2$ (mmHg)*			*pH*			*$O_2 sat$*		
Time (h)	*Vehicle*	*Nimesulide*	*Atosiban and Nimesulide*	*Vehicle*	*Nimesulide*	*Atosiban plus Nimesulide*	*Vehicle*	*Nimesulide*	*Atosiban plus Nimesulide*	*Vehicle*	*Nimesulide*	*Atosiban plus Nimesulide*
0 h	99.7±3.11	96.3±4.18	100.6±4.53	31.8±1.01	33.2±0.86	33.2±0.58	7.48±0.01	7.48±0.01	7.45±0.01	96.7±0.68	95.0±0.84	94.6±0.86
24 h	102.7±3.09	97.8±7.77	100.0±3.15	32.1±0.67	33.5±0.87	31.2±0.58	7.49±0.02	7.47±0.01	7.47±0.01	96.7±0.81	93.8±2.05	94.6±0.75
Delivery	98.3±4.88			30.8±0.92			7.49±0.02			95.4±0.98		
54 h		106.8±1.16	98.8±3.34		34.0±1.52	33.0±2.05		7.48±0.02	7.43±0.02		95.8±0.71	93.7±0.85
Delivery or elective *post mortem*		96.5±5.32	96.3±5.75		31.8±1.38	30.8±1.03		7.50±0.01	7.39±0.06		95.1±0.57	93.3±1.39

Delivery before elective post mortem, undelivered fetuses and ewes were electively killed according to the experimental protocol, refer to section 3.22. Dexamethasone infusion commenced at time 0; vehicle, nimesulide or nimesulide and atosiban infusions commenced 30 minutes prior to dexamethasone infusion.

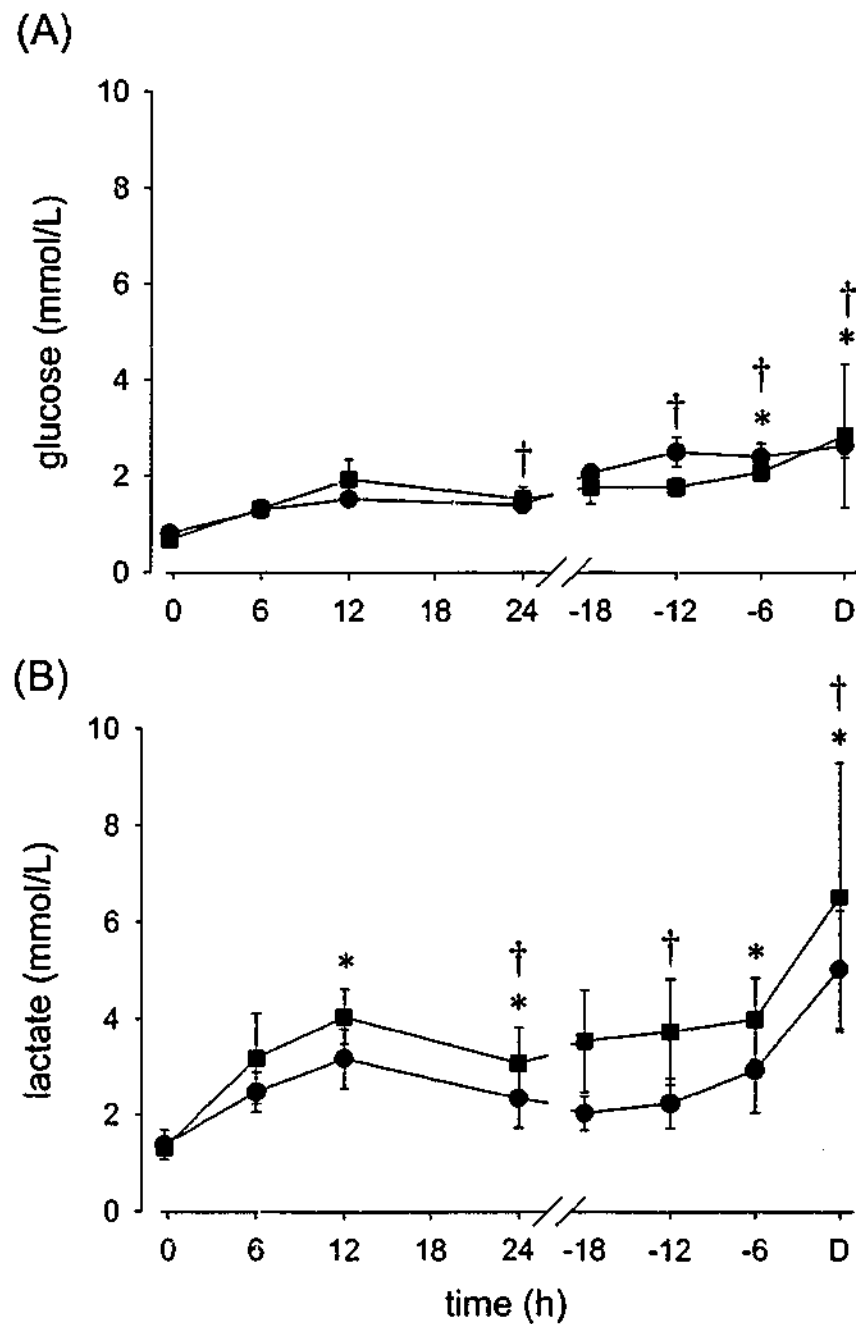

Figure 3.6 Glucose concentrations (A) and lactate concentrations (B) from fetuses from ewes treated with vehicles only (●; n = 4) or nimesulide and atosiban (■, 20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5), during the first 6 h after maternal treatment onset (time 0) and the last 18 h before delivery or elective *post mortem* (*D*). VEH and NIM and ATO infusions (time 0) commenced 30 minutes prior to dexamethasone infusion. Symbols represent significantly increased glucose and lactate concentrations, compared to those observed prior to maternal treatment onset (time 0) for *VEH and †NIM and ATO-treated ewes.

3.3.6 Fetal condition at post mortem

The diameter of the fetal ductus arteriosus was examined visually immediately after the fetuses were killed at *post mortem*. Normal patency appeared to have been maintained and there were no observable differences in the diameter of the lumen between the NIM-, NIM and ATO- and VEH-treated animals. There appeared to be no differences in the small amount of meconium staining observed at *post mortem* of fetuses from NIM- or NIM and ATO-treated ewes when compared to fetuses from ewes treated with VEH alone. There were also no significant differences in fetal body weights between the three treatment groups. The mean body weights of fetuses from ewes treated with VEH, NIM alone and NIM and ATO were 4.57 ± 0.26, 4.63 ± 0.43 and 5.01 ± 0.36 kg, respectively.

3.3.7 Maternal progesterone concentrations

Progesterone concentrations were measured in the maternal circulation (CA) from all animals studied. Data is presented for the first 48 h after VEH, NIM or NIM and ATO infusion onset (Figure 3.7). Maternal progesterone concentrations in NIM-treated ewes ($n = 4$) were not different from VEH-treated ewes ($n = 5$) and maternal progesterone concentrations in NIM and ATO-treated ewes ($n = 5$) were not significantly different from NIM-treated ewes. Significant reductions in maternal progesterone concentrations were observed in all treatment groups at 30 h after infusion onset (time 0) and remained reduced at 48 h after maternal treatment onset.

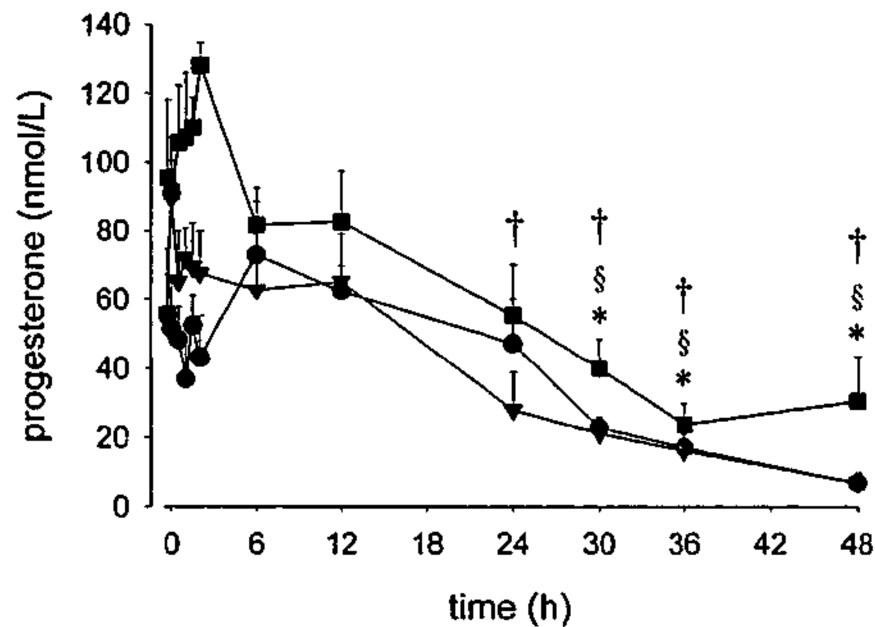

Figure 3.7 Maternal (CA) progesterone concentrations after dexamethasone-induced premature labour from ewes treated with vehicles only (●; n = 5), nimesulide (▼, 20.0 mg/kg/d maternal body weight; n = 4) or nimesulide and atosiban (■, 20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 5), during the first 48 h after maternal treatment onset (time 0). Symbols represent significantly reduced maternal progesterone concentrations, compared to those observed prior to maternal treatment onset (time 0) for * VEH-, § NIM- and † NIM and ATO-treated ewes.

3.4 DISCUSSION

The principal finding of this study was that the combined treatment of nimesulide, a selective PGHS-2 inhibitor, with atosiban, an oxytocin receptor antagonist, was markedly more effective in delaying glucocorticoid-induced premature labour in sheep when compared to treatment with nimesulide alone. In addition, fetal well-being appeared not to be compromised during treatment with nimesulide and atosiban, adding to the potential advantages of this drug regime over previously available treatments.

Circulating PGFM and PGE_2 concentrations were reduced to very low levels within two hours after treatment and remained significantly reduced throughout nimesulide and atosiban treatment, indicating the effectiveness of nimesulide in suppressing PGHS activity. Alternatively, circulating PGFM concentrations in ewes treated with nimesulide alone were increased at the time of delivery or elective *postmortem*. This rise was modest, and the level achieved was not greater than that observed in pre-infusion samples. These observations suggest that the rise in prostaglandin concentrations in ewes treated with nimesulide alone was due to oxytocin-induced PGHS activity, which was not totally inhibited at the dose of nimesulide used. This small increase in prostaglandin concentrations, reflecting an increase in production in gestational tissues, may have facilitated cervical dilation and initiated oxytocin release during treatment with nimesulide alone, allowing adequate uterine activity for fetal expulsion in three out of the five animals in this group. In the remaining two nimesulide-treated ewes, labour did not progress to delivery, despite membrane rupture 12 h earlier. Prostaglandin and or oxytocin concentrations in these animals, whether circulating or at a local level, were clearly not sufficient for delivery. Membrane rupture without delivery was also observed in two ewes in the nimesulide and atosiban treatment group. Since circulating prostaglandin concentrations were very low in these animals and in most cases below the sensitivity of the assays, these findings support a role for local prostaglandin production that might be too low to be detected in utero-ovarian vein plasma samples. Such local production may have allowed membrane rupture but may not have supported adequate uterine activity to facilitate delivery.

The recently developed PGHS-2 inhibitors that have even greater selectivity than nimesulide, may prove more successful in suppressing residual PGHS activity and local tissue prostaglandin production. The present findings support the use of these inhibitors in combination with an oxytocin receptor antagonist in future studies that may delineate a treatment that will suppress labour indefinitely in the face of potent stimulation by

glucocorticoids in sheep and, potentially, premature labour in women. In addition, by combining selective PGHS-2 inhibitors with atosiban, it may be possible to titrate the amount of PGHS inhibitor needed to delay labour adequately, thus further reducing the potential detrimental effects on the fetus.

Treatment with nimesulide alone clearly delayed the onset of increased uterine activity and prolonged the time to delivery, however, once established, the pattern of uterine EMG activity in these animals was similar to that seen in vehicle treated ewes. In contrast, uterine EMG activity in ewes treated with both nimesulide and atosiban showed a very different pattern. In these animals, uterine EMG activity achieved during dexamethasone administration was lower in magnitude and the onset of increased uterine activity was delayed. These findings suggest that oxytocin may make an appreciable contribution to uterine activity once active labour has been initiated and are consistent with the inhibition of an oxytocin-induced rise in uterine activity during premature labour in the nimesulide and atosiban treated ewes. The generation of the uterine EMG activity that was observed in nimesulide and atosiban treated ewes (Figure 3.1 C), may be due to an alternative mechanism or factor(s) in this group given the significantly reduced circulating prostaglandin concentrations and the reduced ability of oxytocin to bind to its receptors. Previously, it has been suggested that oxytocin may play a role in the switching of *contractures* to *contractions* at term in non-human primates (Nathanielsz & Honnebier, 1992). A similar switching has been reported in ewes at term (Jenkin & Nathanielsz, 1994) and was observed in vehicle and nimesulide treated ewes in this study. In contrast, the uterine EMG activity observed in the nimesulide and atosiban treated ewes did not change from a *contracture* pattern of activity to one characteristic of *contractions* at any time during the treatment period, supporting a role for oxytocin in this switching mechanism during labour. An increase in glucocorticoids associated with premature labour has been suggested to be responsible for placental separation in this species (Jack *et al.*, 1975). The reduction in progesterone concentrations observed in all animals (Figure 3.7), is an indication that dexamethasone administration is affecting placental steroidogenesis, and although initially stimulating prostaglandin production, no such detachment was seen in animals treated with nimesulide and atosiban, indicating that placental separation may not be due to a direct effect of glucocorticoids but may also require a rise in prostaglandin production together with the associated increase in contractile strain. Thus, the effectiveness of

nimesulide and atosiban in inhibiting uterine activity likely also contributes to the lack of placental separation observed in these studies.

An important finding of this study was that all fetuses from the two treatment groups were alive at the time of delivery or elective *post mortem* andthe ductus arteriosus in each fetus appeared to have remained patent. Circulating PGE_2, as well as local prostaglandin production within the wall of the ductus, is thought to be the major contributor to ductal patency (Coceani *et al.*, 1975). Circulating prostaglandin levels were significantly suppressed, suggesting that local production of prostaglandins within ductal tissue itself may be more important for maintaining ductal patency than circulating prostaglandin levels. The finding that nimesulide does not adversely affect fetal survival supports the proposal that a selective PGHS-2 inhibitor, may suppress placental PHGS-2 activity, but may have little or no effect on PHGS-1 activity in the premature ductus (Coceani *et al*, 2001). There were no significant differences in fetal blood gas parameters between groups except for values taken immediately before delivery or elective *post mortem*. At this time there was a significant decrease in fetal oxygen saturation in the nimesulide alone treated ewes, perhaps resulting from the prolonged period of elevated uterine activity seen in these animals (Figure 3.1 B). Uterine EMG activity in nimesulide and atosiban-treated ewes did not increase to such high levels or for the duration seen in nimesulide alone treated ewes. It was observed that fetal oxygen saturation in the nimesulide and atosiban-treated group was not reduced to the same extent as nimesulide alone treatment at the end of the study period. A reduction in fetal PaO_2 and O_2 sat was observed in vehicle treated ewes, an effect similar to that normally observed prior to delivery in sheep (Comline & Silver, 1972). If glucocorticoids play a major role in placental separation, independent of prostaglandin concentrations, a greater reduction in fetal oxygen might be observed in the nimesulide or nimesulide and atosiban-treated animals, due to prolonged placental degradation and reduced placental blood flow. This was however, not the case, further indicating that placental malfunction was not occurring in these animals. Furthermore, these findings indicate that inhibition of oxytocin-induced uterine activity further delays premature labour and reduces the potential adverse effects on fetal blood gas parameters.

In summary, this study has clearly demonstrated the effectiveness of a combined treatment of PGHS-2 inhibition and oxytocin receptor antagonism for delaying premature labour in sheep. This treatment was markedly more effective than PGHS-2 inhibition alone and suggests a role for oxytocin in contributing to uterine activity once

premature labour is established. With the observation of maintained fetal well-being during this treatment, and the possibility that nimesulide may suppress placental PGHS-2 activity, with little effect on PGHS-1 activity in ductal tissue, this work outlines the advantages of such a combined drug therapy as a potential treatment for premature labour in women.

Chapter 4

Prostaglandin H synthase type-2 mRNA expression during the inhibition of premature labour

4.1 INTRODUCTION

It is well established that an increase in PGHS-2 expression occurs at the onset of term and premature labour in humans (Hirst *et al.*, 1995a) and sheep (Wimsatt *et al.*, 1993; McLaren *et al.*, 1996), and prostaglandins are central in this process (refer to Chapter 3). However, the level of expression of placental PGHS-2 mRNA in the absence of prostaglandin release, when the parturient mechanism is activated, but when delivery is prevented has not been fully characterised. In the previous study it was demonstrated that selective inhibition of PGHS-2 with the non-steriodal anti-inflammatory drug, nimesulide, combined with the oxytocin receptor antagonist, atosiban, successfully inhibited premature labour in sheep (Grigsby *et al.*, 2000). This treatment was effective in suppressing both prostaglandin concentrations and uterine activity, while normal parameters of fetal well-being were maintained.

Many studies have shown an up-regulation of PGHS-2 mRNA expression in human amnion tissue by several cytokines, IL-4 (Spaziani *et al.*, 1996) and IL-1β (Albert *et al.*, 1994), and glucocorticoids (Zakar *et al.*, 1995), as described in Section 1.2.2.3. Interestingly, recent evidence suggests that indomethacin and other non-steroidal anti-inflammatory drugs can not only block PGHS activity, and thus reduce the formation of prostaglandins, but can also act as potent ligands for the peroxisome proliferator-activated receptor γ (PPARγ) (Lehmann *et al.*, 1997; Meade *et al.*, 1999). PPARγ is a ligand-dependent transcription factor belonging to the family of nuclear receptors that includes the oestrogen receptor, thyroid hormone receptor, and

glucocorticoid receptor (Mangelsdorf *et al.*, 1995). In addition, prostaglandins of the A, D and J series, and particularly the non-enyzmically formed metabolites, 15 hydroxy-PGJ$_2$, are also potent ligands for PPARγ (Kliewer *et al.*, 1995). Activation of PPARγ by prostaglandins and several non-steroidal anti-inflammatory drugs, such as meclofenamate, ibuprofen and NS-398, have been shown to increase the expression of PGHS-2 mRNA and protein levels in both mammary epithelial cells and colonic epithelial cells (Meade *et al.*, 1999). Induction of PGHS-2 expression by non-steroidal anti-inflammatory drugs has also been reported by Lu *et al.* (1995). In addition, other peroxisome proliferators, thapsigargin and okadaic acid activate PPARγ in immortalised mouse liver cells, resulting in elevated PGHS-2 mRNA expression (Ledwith *et al.*, 1997). These observations suggest that PGHS-2 may be transcriptionally regulated via a direct effect mediated through the PPARγ signalling pathway. Therefore it is possible that a feed-forward pathway exists between PPAR activation and PGHS gene expression, resulting in enhanced prostaglandin production. Whether or not this signalling pathway contributes to the increase in the PGHS-2 activity observed at the onset of labour remains speculative. The potential effect of prostaglandin inhibition by non-steroidal anti-inflammatory drugs, such as nimesulide on PGHS-2 expression *in vio* during the inhibition of premature labour has not been examined. This is important clinically, because if the levels of PGHS-2 mRNA increases markedly during inhibition of prostaglandin release by nimesulide treatment, and this treatment was discontinued, there may be a rebound, over production of prostaglandins once tocolytic treatment has ceased. This may have the opposite effect to the intended therapeutic effect, leaving the fetus at the risk from increased contractile activity.

Therefore the aim of this study was to determine the level of PGHS-2 mRNA expression in placental tissues following the induction of premature labour and during the suppression of PGHS activity by tocolytic treatment.

4.2 METHODOLOGY

4.2.1 Animals and cotyledonary tissue

Whole cotyledon tissue was collected from fifteen Border Leicester-Merino cross-bred ewes in which the effect of nimesulide alone and nimesulide and atosiban on dexamethasone-induced labour were examined (refer to Chapter 3). Tissue was collected at the completion of each experiment as described in Section 3.2.2. For inclusion in this

study, cotyledonary tissues were also collected from four control ewes not in labour at 140 days of gestation (GA140) as described in Section 3.2.2.

4.2.2 Ribonuclease Protection Assay (RPA)

The level of PGHS-2 and GAPDH mRNA expression in whole cotyledons was analysed by performing a ribonuclease protection assay. The assay was performed using a RPA kit (RPA Kit III, Ambion Inc., U.S.A), as described in Section 2.7.3. GAPDH mRNA levels in the cotyledon samples were determined in order to obtain a reference point for each sample, so as to ensure equal abundance of mRNA, and allow mRNA expression to be quantified as a ratio of PGHS-2 and GAPDH mRNAlevels. For every cotyledon collected two RPA were preformed for the detection of PGHS-2 and GAPDH.

4.2.3 Statistics

The densitometric values for PGHS-2 and GAPDH mRNA were calculated by computer analysis of the fluorescence generated by β-emission from the ^{32}P-labeled protected cRNA band on each gel. The densitometric values obtained for the GAPDH mRNA were used as a reference value, and PGHS-2 mRNA expression was quantified as a ratio of the GAPDH cRNA signal for each respective sample. All data are presented as mean ± standard error of the mean (SEM). Data were first tested for homogeneity of variance, with heterogeneous data rendered homogeneous by square root or logarithmic transformation. Statistical analysis was performed by identifying differences between means using a one-way analysis of variance (ANOVA; SPSS Inc., U.S.A.). Significance is reported at the 5% level ($P < 005$).

4.3 RESULTS

4.3.1 Placental PGHS-2 mRNA expression

PGHS-2 mRNA expression measured from control ewes not in labour (GA140), dexamethasone-induced labour (DEX), nimesulide (NIM) and NIM and atosiban (NIM+ATO) treated animals, along with GAPDH mRNA expression for the respective samples and densitomentric analysis is presented in Figure 4.1. From the gel image it can be seen that PGHS-2 mRNA is strongly expressed in all treatment groups. Subsequent densitometric analysis revealed that PGHS-2/GAPDH mRNA levels were most prominent in DEX treated animals, however, there were no significant differences found between each treatment group. Although the PGHS-2/GAPDH mRNA levels observed from DEX-induced animals showed a 142% increase from control GA140 animals, an additional analysis by t-test revealed no significant differences between these two groups ($P = 0.094$). Importantly there was no increase in PGHS-2 mRNA expression following inhibition of delivery with NIM alone and NIM+ATO. Its noteworthy that expression following inhibition of delivery appeared somewhat lower than following DEX treatment alone.

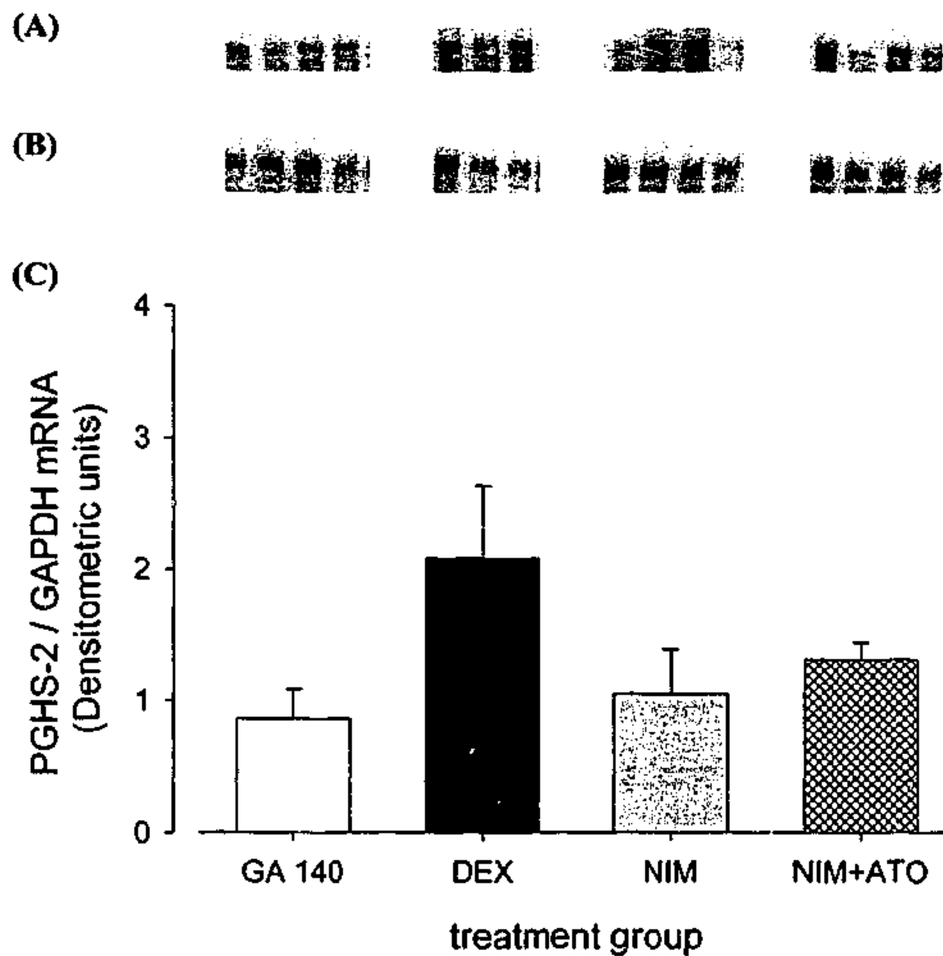

Figure 4.1 PGHS-2 mRNA expression in whole cotyledon tissue during the inhibition of premature labour. RNA samples were hybridised with PGHS-2 and GAPDH specific radiolabelled RNA probes and was analysed by ribonuclease protection assay as described in Section 2.7.3. The RNA protected fragments panels (A) PGHS-2 and (B) GAPDH were visualised by autoradiography and quantified by densitometric scanning. Results of the densitometric analysis are shown in panel (C) as arbitary densitometric units of PGHS-2/GAPDH mRNA.

4.4 DISCUSSION

The aim of this study was to assess the effect of prostaglandin inhibition on placental PGHS-2 mRNA expression following the induction of premature labour in sheep. Placental PGHS-2 mRNA expression was most prominent in ewes treated with dexamethasone and in which labour had occurred, compared to that in control ewes not in labour. Previous studies in sheep have reported a significant elevation of placental PGHS-2 mRNA with the onset of labour (Wimsatt *et al.*, 1993). Furthermore, glucocorticoid-induced parturition in sheep also results in a significant elevation of placental PGHS-2 protein, whereas PGHS-2 protein levels remain very low in control ewes not in labour (McLaren *et al.*, 1996). In the current study, however, there were no significant differences in the placental PGHS-2 mRNA expression found between any of the treatment groups.

In the study by McLaren *et al.* (1996), control cotyledon tissue was collected from ewes at 133 days of gestation, whereas in the current study control tissue was collected from ewes at 140 days of gestation. Therefore, the control samples were closer to the onset of parturition and PGHS-2 mRNA expression may have begun to rise in some of these tissues. This variability may have reduced the difference between the groups and thus the chance of observing any significant differences between the control ewes not in labour and the dexamethasone-induced animals. Nonetheless, it is evident that there is a trend for increased PGHS-2 expression in the dexamthesone-treated ewes which is consistent with previous studies.

The present finding that placental PGHS-2 expression in nimesulide alone and nimesulide and atosiban-treated ewes were not statistically different from that in control ewes not in labour is most interesting as well as reassuring for the potential clinical use of these treatments for delaying premature labour. Although not reaching significance in this study, there was a trend to lower PGHS-2 mRNA expression in these animals compared to that observed in dexamethasone-treated ewes, despite their having received dexamethasone for a significantly longer period. Thus, the stimulatory effect of dexamethasone on PGHS-2 expression observed in previous studies was, in some way, inhibited during nimesulide and atosiban treatment. One explanation is that this enzyme is positively regulated by one of its products, a concept which has been suggested previously (Tsai & Wiltbank, 1997; Wu *et al.*, 1998). Tsai and Wiltbank (1997) proposed that a positive feedback loop exists with prostaglandin production such that PGHS-2 expression is up-regulated due to prostaglandins binding to prostaglandin receptors

within luteal cells and causing activation of PGHS. Similarly, Wu *et al* (1998) reported that fetal prostaglandin concentrations and PGHS-2 mRNA expression were decreased in ewes treated with nimesulide, 30 mg bolus, followed by a 6 h infusion at 30 mg/h. Along with the altered prostaglandin concentrations, there were also significant decreases in the abundance of several key utero-placental labour-associated genes including, the oestrogen receptor, oxytocin receptor, and cytosolic PLA_2. These observations suggest that prostaglandins may be an essential link in a positive feed-forward loop leading to myometrial activation during labour (Wu *et al*, 1998). It was demonstrated in the previous study (refer to Chapter 3), that the production of circulating prostaglandins were significantly reduced by nimesulide treatment as well as with the combined treatment of nimesulide and atosiban. The present findings indicate that, while prostaglandin production was decreased to basal levels resulting in a decrease of a potential positive feed-back on PGHS-2, the expression of placental PGHS-2 mRNA in nimesulide alone and nimesulide and atosiban-treated ewes was not different to that observed in placental tissue collected from control ewes that were not in labour. These findings support the notion of a positive feed-back loop between prostaglandin production and placental PGHS-2 mRNA expression during parturition. These findings also suggest that a rebound production of prostaglandins is unlikely to occur immediately after the cessation of prostaglandin inhibition due to the lack of up-regulation of the PGHS-2 mRNA at this time. To investigate this concept further, it would be useful to study the time required for PGHS-2 mRNA expression and prostaglandin concentrations to increase after tocolytic treatment has ceased. The average circulating half-lives for nimesulide and atosiban are approximately 3 h (Bernareggi, 1998) and 18 min (Goodwin *et al.*, 1995), respectively, therefore, an increase in the placental PGHS-2 mRNA expression and thus an increase in prostaglandin production, might occur after the cessation of tocolytic treatment in a relatively short time. Although this may result in delivery, the timely inhibition of premature labour, sufficient for the beneficial maturational effects of glucocorticoid treatment on the fetus, would already have occurred and labour induction would be expected.

The model of labour induction we have established (refer to Chapter 3), relies upon the administration of a synthetic glucocorticoid, dexamethasone, to fetal sheep. This advances the fetal cortisol surge which is the physiological signal for labour in this species (Challis *et al.*, 2000). The glucocorticoid signal is transduced by the placenta, resulting in the metabolism of progesterone to oestrogen, with induction of PGHS-2 in

the placenta (Gyomorey *et al.*, 2000) and oxytocin receptors within the uterus (Meier *et al.*, 1995). These changes then lead to labour and delivery within 54 h (Grigsby *et al*, 2000). The decrease in maternal progesterone concentrations measured from all ewes, is indicative of altered steroidogensis within the placenta (refer to Section 3.3.7). When myometrial activity is blocked by using this model the tissues are exposed to a high oestrogen/progesterone ratio for a much longer than usual period. Oestradiol has been shown to up-regulate myometrial PGHS-2 expression *in vivo* in ovariectomised non-pregnant sheep (Wu *et al.*, 1997). In other species oestrogen has been shown to exert a dose related biphasic effect on prostaglandin production. In the guinea pig oestrogens initially stimulate the expression of factors that enhance uterine activity. Conversely, at high concentrations oestrogens reduce prostaglandin production (Schellenberg & Kirkby, 1997). The lack of up-regulation of PGHS-2 mRNA expression observed in this present study may reflect the prolonged exposure to unopposed oestrogen and a suppressive action on PGHS-2 expression.

The absence of a rise in PGHS-2 mRNA could also have resulted from the prolonged glucocorticoid infusion. Glucocorticoids may exert both an up-regulation and down-regulation on the expression of the enzyme with prolonged use. With the exception of the amnion, in which Zakar *et al.* (1995) has shown an up-regulation of PGHS-2, glucocorticoids attenuate PGHS-2 expression in many different cell systems (DeWitt & Meade, 1993; Goppelt-Struebe, 1997), as discussed in Section 1.2.2.3. In addition, glucocorticoids have also been shown to reduce PGHS-2 mRNA stability in mitogen-induced fibroblast cells (Evett *et al.*, 1993). While there is sufficient evidence to indicate that glucocorticoids can interfere with prostaglandin synthesis by diminishing PGHS-2 mRNA, the precise mechanisms responsible for the secondary phase of down-regulation in the present study remains unclear.

These observations demonstrate the importance of prostaglandins in the regulation of placental PGHS-2 mRNA expression in the sheep, and that an increase in the expression of placental PGHS-2 may indeed rely, at least partially, on stimulation induced by an increase in intra-uterine prostaglandin concentrations. The current results, together with those of the previous study, strongly suggest that prostaglandin synthesis is an essential component of the positive feed-forward system in the processes of parturition, and that prostaglandins may act as a positive signal for increased placental PGHS-2 mRNA expression *in vivo* during the progression of labour in sheep. The finding that PGHS-2 expression is not up-regulated during the suppression of

prostaglandins by nimesulide treatment, suggests that a rebound production of prostaglandins is unlikely to occur clinically subsequent to treatment, this is reassuring for the potential use of these treatments for delaying premature labour in women.

Chapter 5

Inhibition of active premature labour

5.1 INTRODUCTION

The experimental study outlined in Chapter 3 showed that premature labour, induced by fetal glucocorticoid infusion, can be successfully inhibited by using a combined treatment of nimesulide and atosiban in late pregnant sheep. In addition, placental PGHS-2 mRNA expression was found to be no different to the levels of expression to that observed in control animals that were not in labour (refer to Chapter 4). Together these studies have clearly demonstrated the importance of prostaglandin synthesis in the positive feed-forward system in the processes of parturition. However, tocolytic treatment in the previous studies commenced prior to the induction of premature labour, before the associated rise in uterine activity and prostaglandin concentrations were observed. The success of the combined treatment in preventing glucocorticoid-induced premature delivery warranted further investigation of the effectiveness of this treatment regime *once the onset of premature labour had become established*, in order to establish a model that more closely relates to the clinical situation. In many clinical trials, successful tocolysis is dependent on the degree of cervical dilation, thus women with advanced cervical ripening and uterine activity are often excluded from current tocolytic treatment (Amon *et al.*, 2000). By this stage of active labour it is thought that many of the endocrine-controlled biochemical and structural changes have begun without the possibility of inhibition or reversal.

Therefore the aim of the current study was to investigate the effectiveness of a 48 h infusion of nimesulide and atosiban following the onset of premature labour, at a time when uterine activity and prostaglandin concentrations are already significantly elevated. A 48 h infusion period was chosen to more closely mimic a clinical situation, where tocolytic treatment is used in order to delay labour and allow adequate time for antenatal

corticosteroid therapy (Jeyabalan & Caritis, 2002). Given that the average circulating half-lives for nimesulide and atosiban are approximately 3 h (Bernareggi, 1998) and 18 min (Goodwin *et al.*, 1995), respectively, uterine activity and prostaglandin concentrations might be expected to increase after the cessation of maternal inhibitor treatment, which may not only result in delivery, but also influence fetal well-being. Therefore, the change in uterine activity and prostaglandin concentrations after the cessation of nimesulide and atosiban infusion, but during continued induction of premature labour with fetal glucocorticoid infusion, were also examined.

Some of the work presented in this chapter was performed by Ms Jennifer Scott, in fulfilment of her Bachelor of Science Honours degree with the Fetal and Neonatal Research Group, Department of Physiology at Monash University in 1999. The planning of the expreiments and the development of the protocols was made by myself together with my Ph.D. supervisors A/Prof Graham Jenkin and Dr. Jonathan Hirst. Ms Scott was present, at the commencement of her BSc (Hons) year, in the latter stages of this process. The experiments included in the chapter that were performed by Ms. Scott include; animal experiments (nimesulide and atosiban treatment group only), hormone assays (nimesulide and atosiban treatment group only), and initial statistical analysis. The experiments included in this chapter that were performed by myself include; animal experiments (control group), hormone assays and analysis (control group), uterine EMG activity analysis (control group and nimesulide and atosiban treatment group). In addition, I was involved in the initial animal surgeries and all post-operative animal care. The publication that has arisen from these experiments was prepared and written by myself together with editorial advice from my Ph.D. supervisors. With the aggreement of all authors, the equal contribution as chief investigators and joint first authors of myself and Ms Scott was acknowledged in the publication.

5.2 METHODOLOGY

5.2.1 Establishment of premature labour

Activity of the uterus was measured by the frequency of discrete uterine EMG bursts as previously described (refer to Section 3.2.4). Monitoring of uterine activity began 24 h prior to dexamethasone infusion to establish basal activity for individual animals. In each ewe, active premature labour was defined as having begun once uterine EMG

activity had consistently increased above twice that of basal levels during at least two consecutive 2 h recording periods. NIM and ATO-treated ewes (n = 6) received a continuous intravenous infusion of both NIM and ATO via the maternal JV catheters as described in Section 3.2.2, once the establishment of active labour was confirmed. Control ewes (VEH; n = 4) were administered a continuous intravenous infusion of the vehicles only, via the maternal JV catheters, at the same rates as for the NIM and ATO-treated ewes.

5.2.2 Sampling regime

Blood samples were obtained from the fetal carotid artery (CA), maternal CA and both utero-ovarian vein (UOV) catheters in all ewes, 24 h, 12 h and immediately prior to NIM and ATO or VEH infusions. Upon induction of labour and VEH infusion, samples were taken every 30 min for 2 h, followed by samples at 6 h and 12 h after labour induction. Samples were then collected 3 times daily at 10 am, 4 pm and 10 pm, until delivery. Blood sampling for NIM and ATO-treated animals continued daily as for VEH-treated ewes until the establishment of premature labour and the commencement of inhibitor infusions. Blood samples were then collected immediately prior to NIM and ATO treatment (time 0), then every 30 min for 2 h, followed by 4 h, 6 h and 12 h after the start of inhibitor infusions. Frequent blood samples were collected in these animals to determine the immediate effects of NIM and ATO treatment. Sampling then continued every 6 h until completion of the 48 h treatment period or earlier if delivery occurred. Further samples were then taken every 2 h for up to 10 h after the completion of NIM and ATO treatment to determine any rapid changes in prostaglandin concentrations after the cessation of treatment, after which time ewes and their fetuses were humanely killed by barbiturate overdose (refer to Section 2.4). Maternal and fetal blood respiratory gases (PaO_2 mmHg, $PaCO_2$ mmHg, O_2 sat) and pH were measured from all VEH- and NIM and ATO-treated animals as described in Section 2.2.3. In addition, at *post mortem*, the health of the fetus was further assessed by visual examination of the patency of the ductus arteriosus and the presence and amount of meconium staining. The placenta was also carefully examined for any sign of degradation or separation. Fetal body weight was recorded. In these experiments, the intra- and inter-assay co-efficient of variation of the PGFM RIA were 15% and 23%, respectively. The mean sensitivity of the assay was 0.43 ± 0.07 nmol/L. The intra- and inter-assay co-efficient of variation of the PGE_2 RIA were 9% and 12.5%, respectively,

with a mean sensitivity of 0.24 ± 0.05 nmol/L. The intra- and inter-assay co-efficient of variation of the progesterone RIA were 16% and 32%, respectively, with a mean sensitivity of 0.3 ± 0.01 nmol/L.

5.2.3 Statistics

All data is presented as mean ± standard error of the mean (SEM). Data were first tested for homogeneity of variance, data found heterogeneous was rendered homogeneous by square root or logarithmic transformation. Data was analysed relative to the onset of NIM and ATO treatment at the establishment of active premature labour. Control animals were similarly analysed from the establishment of labour in each individual animal. All results were grouped into 12 h time periods and the data averaged for collective overall analysis. Differences in uterine activity were also identified by comparison with basal values, 24 h prior to VEH or NIM and ATO infusions.

Statistical analysis was performed by identifying differences between means using a one-way repeated measures analysis of variance (ANOVA), time being the variable factor (Statistical Packages for Social Sciences, SPSS Inc., U.S.A.). Least significant difference (LSD) tests were used to identify significant differences between pairs of mean values. Significance is reported at the 5% level ($P < 005$).

5.3 RESULTS

5.3.1 Outcome of animals

The outcome of each individual animal studied is presented in Table 5.1. Time to established active premature labour relative to the onset of VEH or NIM and ATO treatment was similar in all animals (n = 10) with an overall mean of 39.3 ± 2.2 h. Delivery occurred in all control animals (n = 4) within 50 h from onset of DEX infusion (48.6 ± 1.2 h). Four of the ewes treated with NIM and ATO completed the 48 h treatment and 10 h post treatment period without delivery, and were killed 98.8 ± 1.9 h after the commencement of DEX infusion. This time was significantly longer than the time taken for VEH-treated animals to deliver. Delivery also occurred in two of the NIM and ATO-treated animals (052 and 133) prior to the completion of the 48 h treatment period. However, delivery was delayed in these ewes by 22.3 h and 25.6 h compared to the mean delivery time observed for VEH-treated ewes (Table 5.1). Three fetuses from the animals that completed the inhibitor treatment were alive upon exposure of the uterus, appearing healthy with minimal meconium staining. One fetus (017) died immediately prior to the completion of the 10 h post-NIM and ATO treatment, but appeared normal at the time of *post mortem*. Of the two ewes that failed to complete the 48 h NIM and ATO treatment, one fetus was alive and normal after delivery, while the remaining fetus (052) died just prior to *postmortem* examination.

5.3.2 Uterine EMG activity

Uterine EMG activity recorded prior to premature induction of labour and throughout the experiment is shown in Figure 5.1. Uterine EMG data is expressed relative to the onset of established premature labour. Activity rose steadily in VEH-treated animals after the onset of premature labour until delivery (Figure 5.1 A). The activity of ewes that completed the 48 h of NIM and ATO treatment (n = 4), showed an initial significant increase in activity 4-6 h prior to the onset of NIM and ATO treatment, supporting the appropriateness of the criteria for the recognition of established premature labour. Uterine EMG activity in NIM and ATO-treated ewes declined significantly 4-6 h after the commencement of NIM and ATO treatment compared to levels observed immediately before inhibitor treatment and compared to those observed in VEH-treated animals at the equivalent time. Uterine EMG activity increased during the subsequent 24 h after the commencement of NIM and ATO treatment. This

increase was, however, only transient and was followed by a decline to levels similar to those observed at the time of established premature labour. Importantly, uterine EMG activity observed in NIM and ATO-treated animals was markedly suppressed over the entire study period when compared to the level of uterine activity reached in VEH-treated animals at delivery. Furthermore, uterine activity in ewes treated with NIM and ATO remained suppressed during the post-treatment period (Figure 5.1). Separate analysis of the two animals (052 and 133), that failed to complete the 48 h of NIM and ATO treatment, revealed heightened uterine activity similar to that observed in control animals at delivery (Figure 5.2). The progressive increase in uterine activity in animal 052, from the onset of treatment was characteristic of uterine *contractions* (Nathanielsz & Honnebier, 1992) and reached a maximum 16 h after the establishment of labour. There was then a decline in uterine activity during the subsequent 14 h, with, the pattern of uterine activity varying between *contractures* and *contractions* (Figure 5.3).

Table 5.1 Outcome of animals. Time to delivery following fetal dexamethasone infusion (1 mg/d in saline 1.0 ml/h; n = 10). Vehicle-treated animals received a continuous infusion of vehicles only (n-Methyl-2-Pyrrolidone and saline, 1.0 ml/h; n = 4). Nimesulide and atosiban-treated animals received vehicle infusion until the establishment of labour and then received infusions of nimesulide and atosiban (20.0 and 4.12 mg/d/kg maternal body weight, respectively; n = 6).

Dexamethasone plus vehicle				*Dexamethasone plus nimesulide and atosiban*			
Animal number	*Time to established active labour (h)*	*Treatment completed*	*Time to delivery or elective post mortem (h)*	*Animal Number*	*Time to established active labour (h)*	*Treatment completed*	*Time to delivery or elective post mortem (h)*
006	36.0	-	46.5*	017	26.7	Yes	93.2
179	38.0	-	52.0*	052	40.7	No	70.9*
200	32.0	-	48.8*	053	38.4	Yes	99.9
202	38.0	-	47.0*	074	45.8	Yes	100.1
				123	46.8	Yes	100.8
				133	50.3	No	74.2*
Mean for all	36.0 ± 1.4	-	48.6 ± 1.2	**Mean for all**	41.5 ± 3.4	4/6 completed treatment	89.9 ± 5.6
				Mean for animals completing treatment only	39.4 ± 4.6	4/4 completed treatment	98.8 ± 1.9

Dexamethasone infusion commenced at time 0; vehicle infusions commenced 30 minutes prior to dexamethasone infusion. Asterisks (*) indicates ewes that delivered.

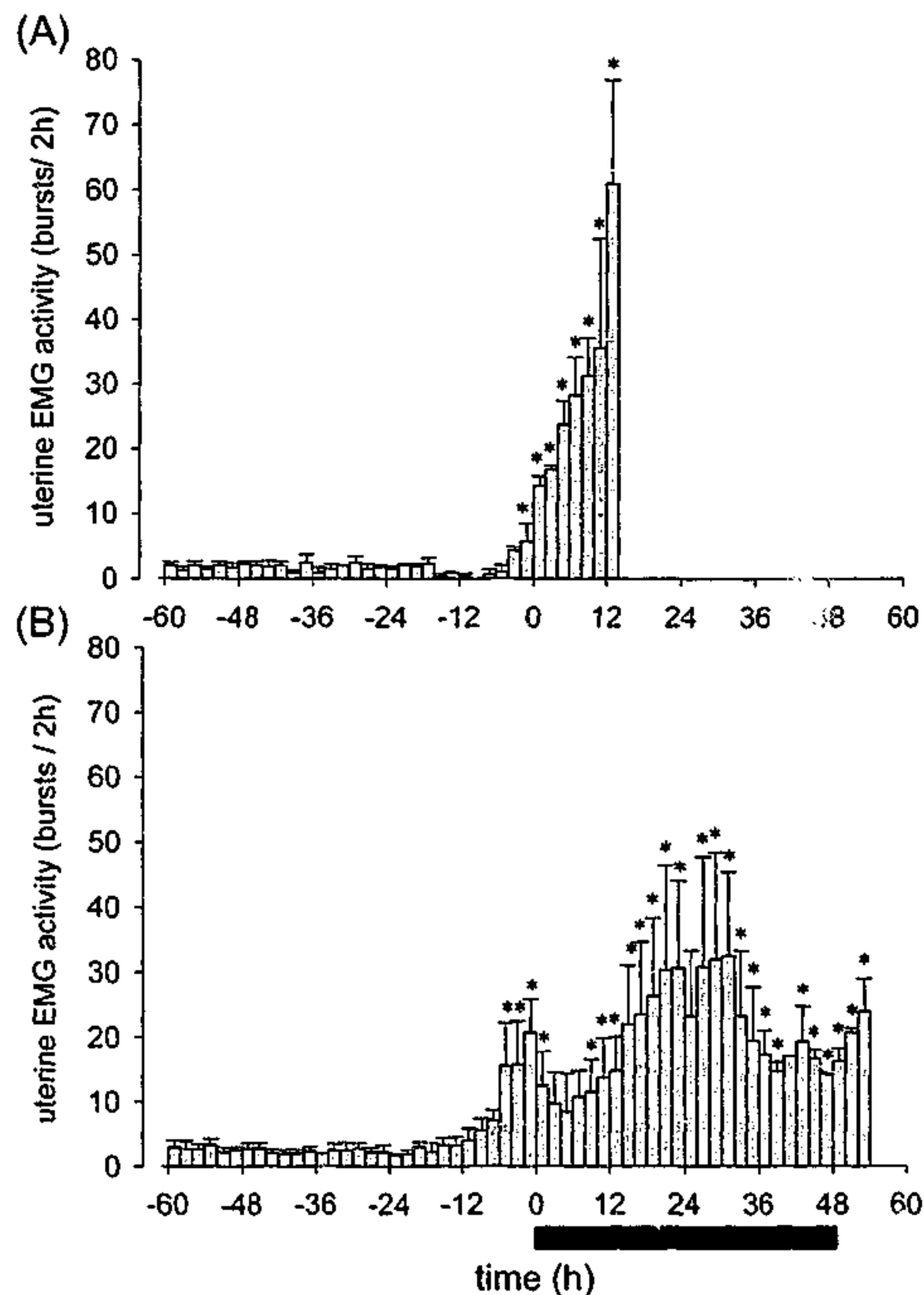

Figure 5.1 Uterine electromyographic (EMG) activity, measured as the frequency of discrete uterine bursts, from 60 h before (-60 h) the onset of active labour (time 0) until delivery or elective *post mortem* in (A) vehicle (n = 4) and (B) NIM and ATO (20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 4) treated animals. Active labour was defined as a rise in uterine EMG activity twice that of basal levels during two consecutive 2 h recording periods. Solid bar represents maternal infusion of NIM and ATO, asterisks (*) indicates values significantly different from basal activity, prior to induction of labour.

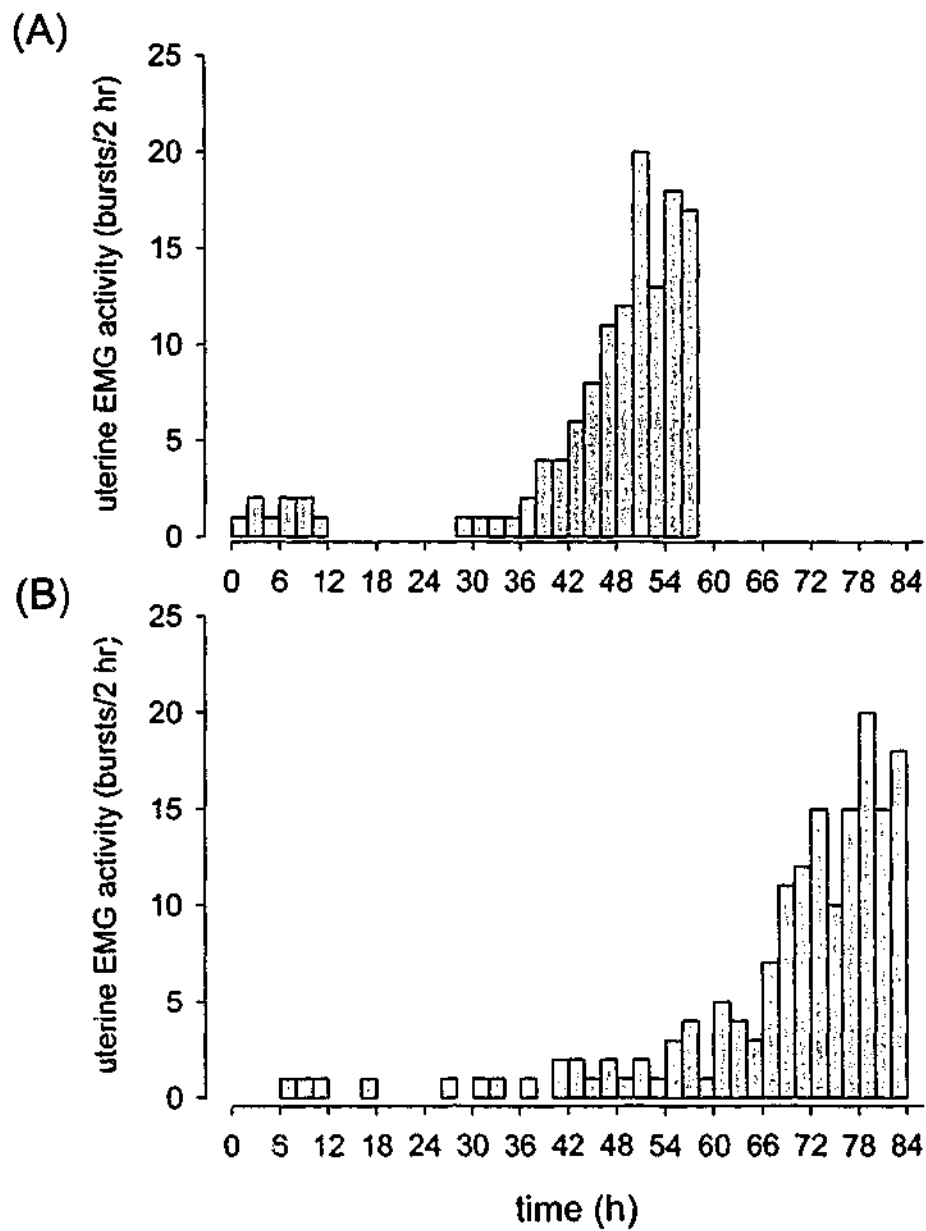

Figure 5.2 Uterine electromyographic (EMG) activity, measured as the frequency of discrete uterine bursts, from the onset of dexamethasone-induced premature labour (time 0) in ewes treated with NIM and ATO (20.0 and 4.12 mg/kg/d maternal body weight, respectively) in (A) animal 052 and (B) animal 133, that delivered during NIM and ATO treatment.

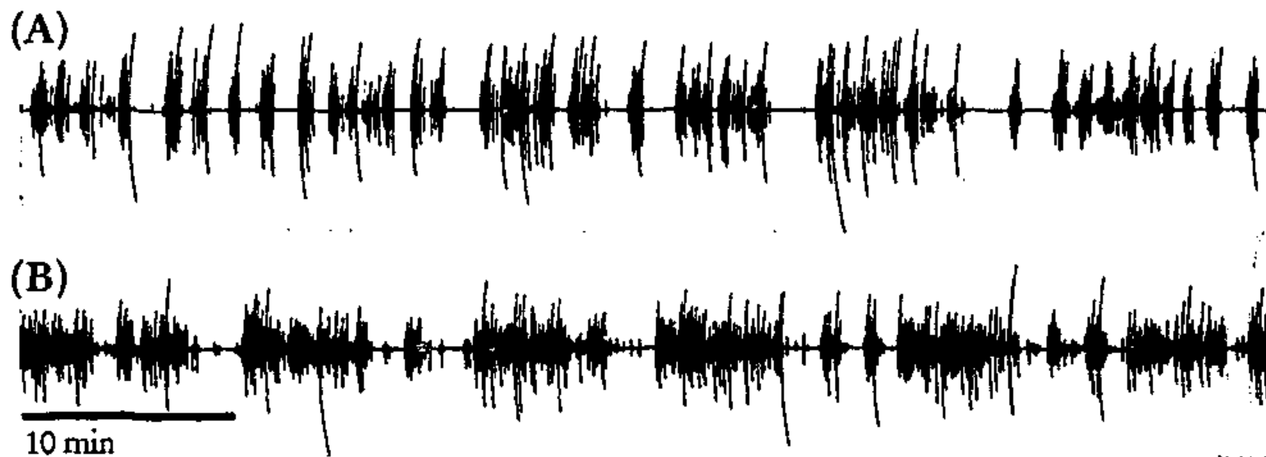

Figure 5.3 An example of the uterine EMG activity recorded from animal 052. Panel (A) represents uterine activity characteristic of uterine *contractions*, observed in this animal at 16 h after the establishment of labour. Panel (B) shows the uterine activity 21 h after the establishment of labour, at which time the pattern of uterine activity varied between *contractures* and *contractions*.

5.3.3 Fetal and maternal prostaglandin concentrations

PGFM and PGE_2 concentrations in the maternal (CA) and fetal (CA) circulation of VEH- (n = 4) and NIM and ATO-treated ewes (n = 4) during labour induction are presented relative to the establishment of active premature labour (time 0) in Figures 5.4-5.7. The expanded inserts represented in Figures 5.4-5.7 (B) were included to demonstrate the changes observed during frequent sampling at the commencement, and at the completion of NIM and ATO infusions. PGFM concentrations in both the maternal circulation and fetal circulation of VEH-treated animals increased significantly prior to delivery and continued to rise to maximum levels at delivery (Figures 5.4 A and 5.5 A). Maternal and fetal PGFM concentrations in NIM and ATO-treated ewes significantly increased during dexamethasone induction of premature labour, beginning 18 h prior to the commencement of NIM and ATO treatment (Figure 5.4 B and 5.5 B). Upon commencement of maternal NIM and ATO treatment, circulating maternal PGFM concentrations were reduced by 0.5 h after the commencement of maternal treatment (Figure 5.4 B). During the NIM and ATO treatment period, maternal PGFM concentrations were not significantly different to basal levels, however when compared to the concentration at time 0, a significant decrease was noted in all samples collected during the initial 6 h of NIM and ATO treatment (Figure 5.4 B insert). Surprisingly, following the cessation of NIM and ATO treatment, circulating maternal PGFM

concentrations failed to increase above basal levels during the post-treatment period of up to 10 h (Figure 5.4 B insert). Fetal PGFM concentrations were decreased within 0.5 h and remained significantly below PGFM concentrations observed prior to NIM and ATO treatment (Figure 5.5 B), and at time 0 (Figure 5.5 B insert). Furthermore, concentrations of fetal PGFM remained suppressed until at least 6 h after the cessation of NIM and ATO treatment (Figure 5.5 B insert).

Maternal and fetal PGE_2 levels appeared to rise slightly during the induction of premature labour in both NIM and ATO- and VEH-treated animals, however this rise was found not to be significantly different compared to basal levels (Figures 5.6 and 5.7). This was followed by a marked increase in concentrations up until delivery in VEH-treated animals (Figure 5.6 A and 5.7 A). In contrast, the commencement of NIM and ATO treatment caused an immediate and significant reduction in both maternal and fetal circulating PGE_2 concentrations within 0.5 h (Figures 5.6 B and 5.7 B inserts). Concentrations remained significantly below pre-inhibitor treatment values during the remainder of NIM and ATO treatment. After the completion of the 48 h inhibitor treatment period, there were no significant increases in PGE_2 concentrations in either the maternal or fetal circulations at 6 h after NIM and ATO treatment (Figures 5.6 B and 5.7 B inserts).

The two animals that did not complete the 48 h NIM and ATO treatment period (052 and 133) were individually analysed for differences in prostaglandin concentrations. PGFM concentrations in both animals followed the same progressive increase, prior to the establishment of active premature labour, as seen with other NIM and ATO-treated ewes at this time (Figure 5.8). Following the onset of NIM and ATO treatment, maternal and fetal circulating PGFM concentrations in ewe 052 continued to rise with a delay in the inhibition of prostaglandin concentrations by 24 h and 12 h in the maternal and fetal circulations, respectively, compared to other NIM and ATO-treated animals (Figure 5.8 A and B). Upon the commencement of NIM and ATO treatment, circulating PGFM concentrations in ewe 133, were reduced in the maternal and fetal circulations within 6 h, but this inhibition failed to continue, resulting in marked increases above basal PGFM concentrations until delivery (Figure 5.8 A and B).

Maternal and fetal circulating PGE_2 concentrations from animal 052 were decreased below basal PGE_2 concentrations by NIM and ATO treatment (Figure 5.8 C and D). Fetal PGE_2 concentrations in animal 133 showed a transient decrease after the commencement of NIM and ATO treatment, followed by a rise just prior to delivery,

closely resembling the profile of PGE_2 concentrations observed in VEH-treated ewes (Figure 5.8 D). The maternal PGE_2 concentrations observed in ewe 133 decreased below basal levels, however, a slight increase in PGE_2 concentrations 24 h prior to delivery was noted (Figure 5.8 C). Because the profile of prostaglandins concentrations were different in these animals and inhibition failed to prevent a further increase in prostaglandin concentrations and uterine activity, these two animals were treated as a separate group from those in which delivery was inhibited, and were excluded from further analysis.

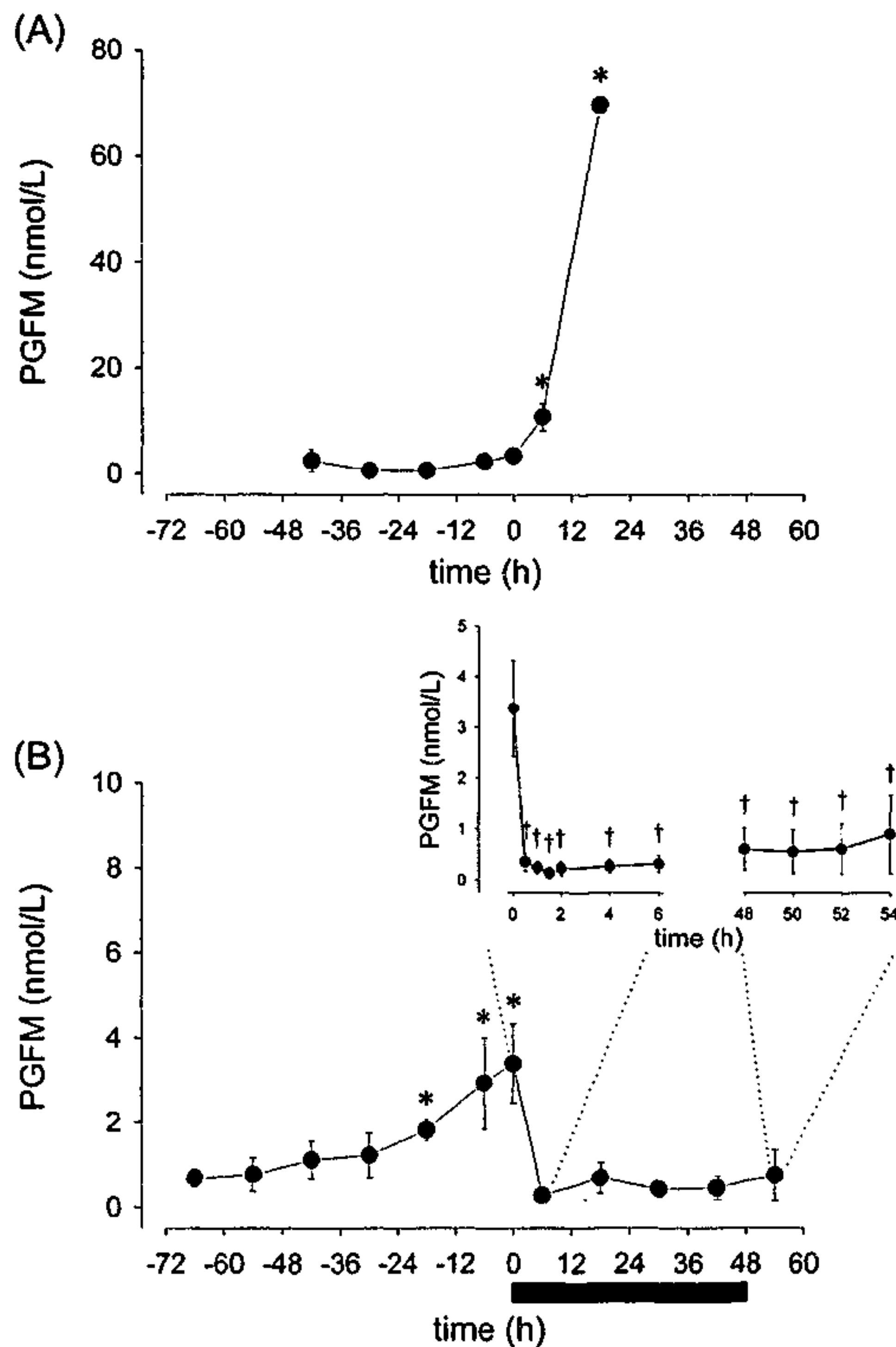

Figure 5.4 Maternal PGFM concentrations measured from before the start of induction of premature labour with dexamethasone infusion, prior to, during and post treatment in (A) vehicle treated ewes (n = 4) and (B) NIM and ATO (20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 4) treated ewes, note the reduced scale used for the y-axis. Values shown are presented relative to the onset of active labour and the beginning of NIM and ATO or vehicle infusion onset (time 0). Bar represents the NIM and ATO infusion period. Asterisks (*) indicates values significantly different from basal PGFM concentrations, prior to labour induction. Left hand insert in (B) represents expanded time scale, during treatment, while right hand insert represents post-treatment period. Crosses (†) indicate values significantly different from PGFM concentrations prior to NIM and ATO treatment (time 0 insert).

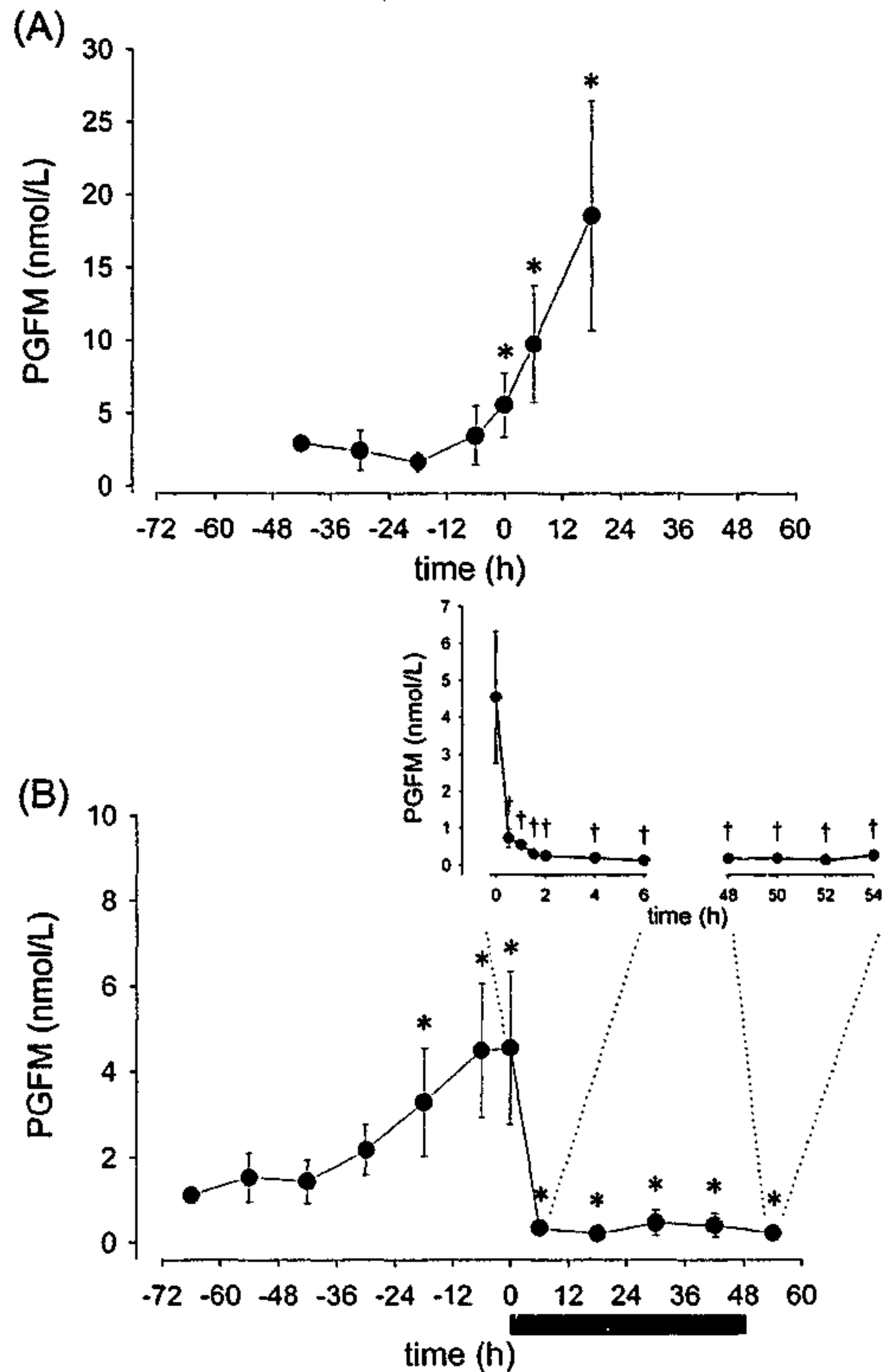

Figure 5.5 Fetal PGFM concentrations measured from before the start of induction of premature labour with dexamethasone infusion, prior to, during and post treatment in (A) vehicle treated ewes (n = 4) and (B) NIM and ATO (20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 4) treated ewes. Values shown are presented relative to the onset of active labour and the beginning of NIM and ATO or vehicle infusion onset (time 0). Bar represents the NIM and ATO infusion period. Asterisks (*) indicates values significantly different from basal PGFM concentrations, prior to labour induction. Left hand insert in (B) represents expanded time scale, during treatment, while right hand insert represents post-treatment period. Crosses (†) indicate values significantly different from PGFM concentrations prior to NIM and ATO treatment (time 0 insert).

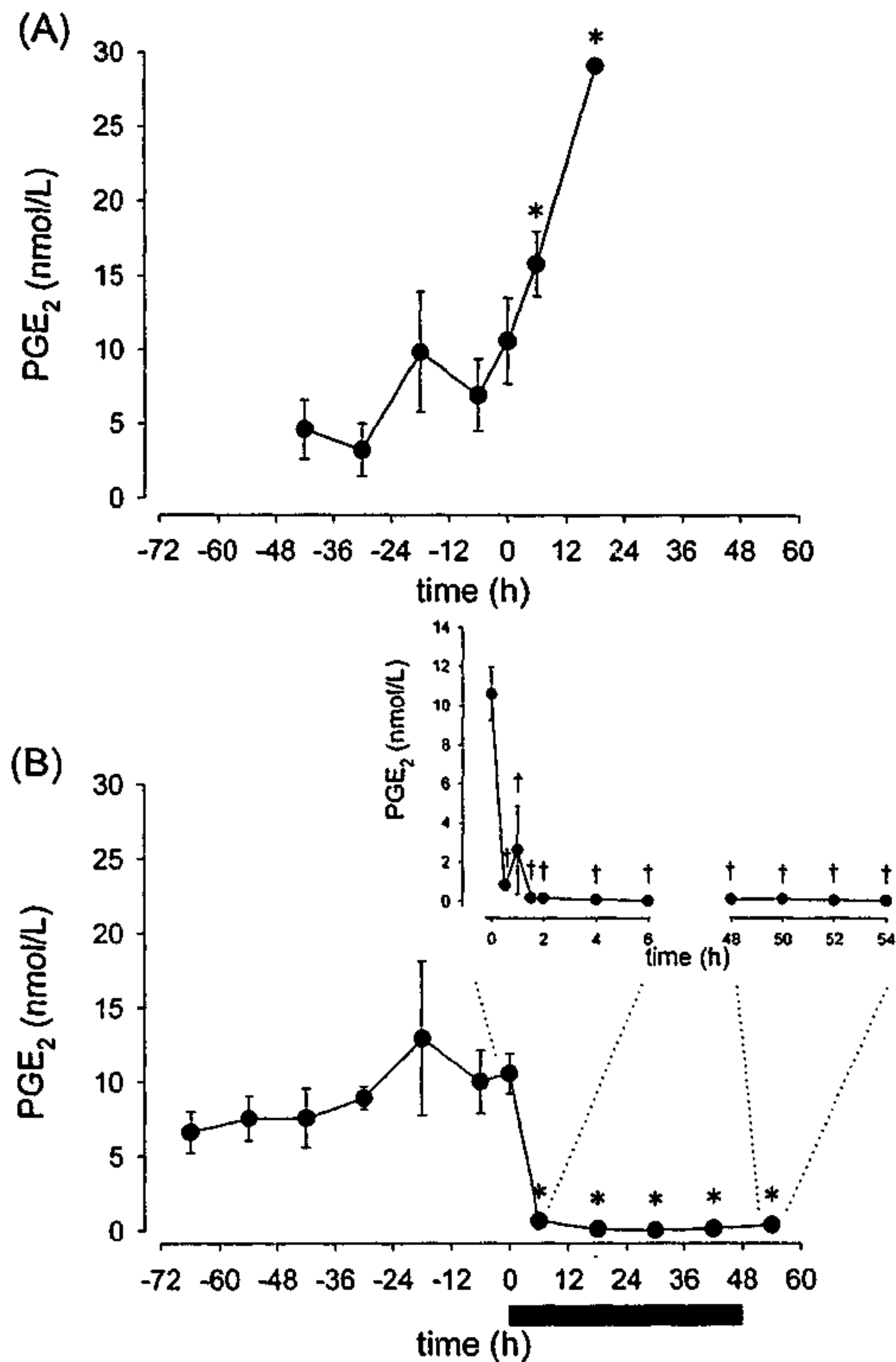

Figure 5.6 Maternal PGE_2 concentrations measured from before the start of induction of premature labour with dexamethasone infusion, prior to, during and post treatment in (A) vehicle treated ewes (n = 4) and (B) NIM and ATO (20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 4) treated ewes. Values shown are presented relative to the onset of active labour and the beginning of NIM and ATO or vehicle infusion onset (time 0). Bar represents the NIM and ATO infusion period. Asterisks (*) indicates values significantly different from basal PGE_2 concentrations, prior to labour induction. Left hand insert in (B) represents expanded time scale, during treatment, while right hand insert represents post-treatment period. Crosses (†) indicate values significantly different from PGE_2 concentrations prior to NIM and ATO treatment (time 0 insert).

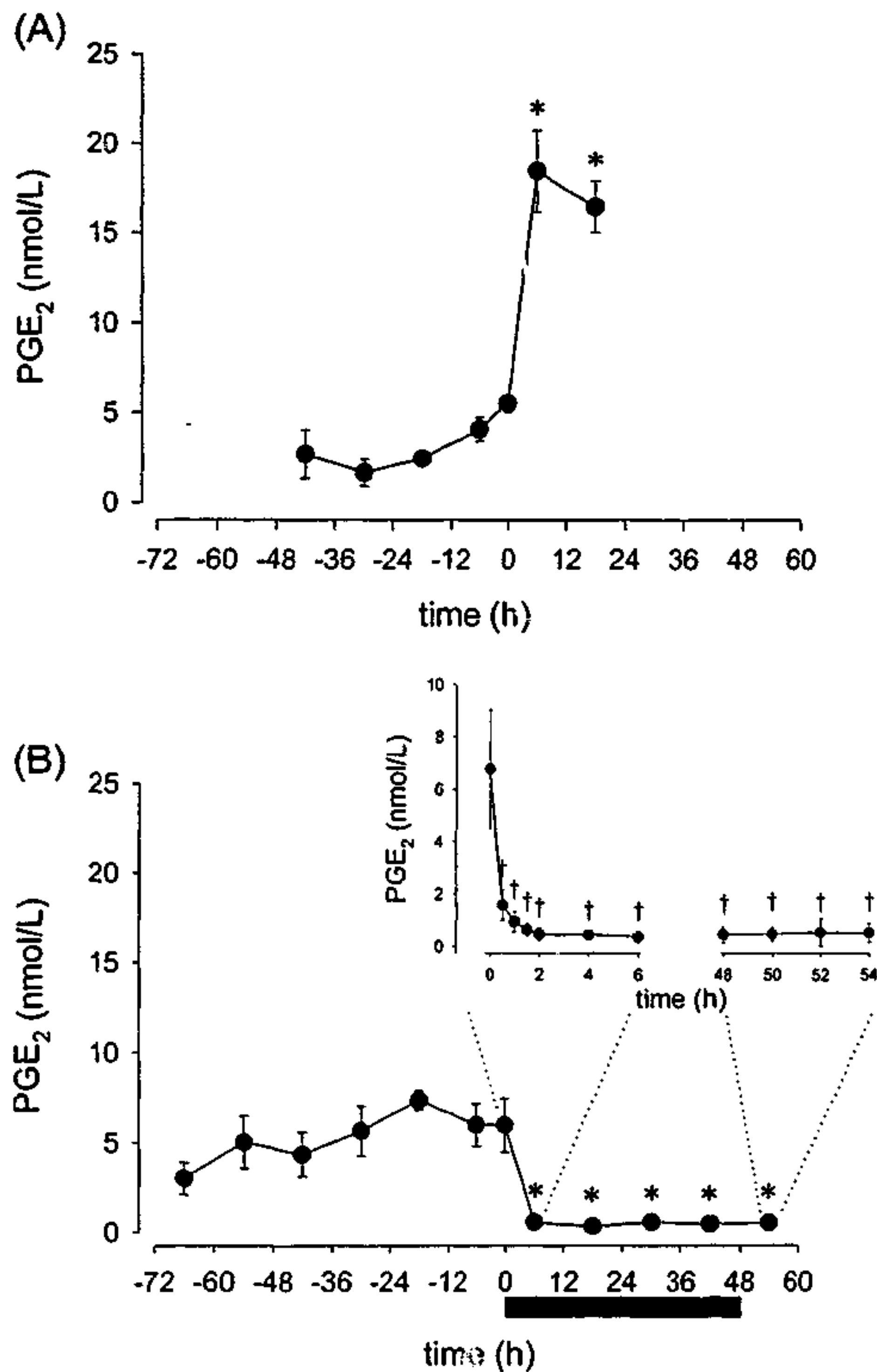

Figure 5.7 Fetal PGE_2 concentrations measured from before the start of induction of premature labour with dexamethasone infusion, prior to, during and post treatment in (A) vehicle treated ewes (n = 4) and (B) NIM and ATO (20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 4) treated ewes. Values shown are presented relative to the onset of active labour and the beginning of NIM and ATO or vehicle infusion onset (time 0). Bar represents the NIM and ATO infusion period. Asterisks (*) indicates values significantly different from basal PGE_2 concentrations, prior to labour induction. Left hand insert in (B) represents expanded time scale during treatment, while right hand insert represents post-treatment period. Crosses (†) indicate values significantly different from PGE_2 concentrations prior to NIM and ATO treatment (time 0).

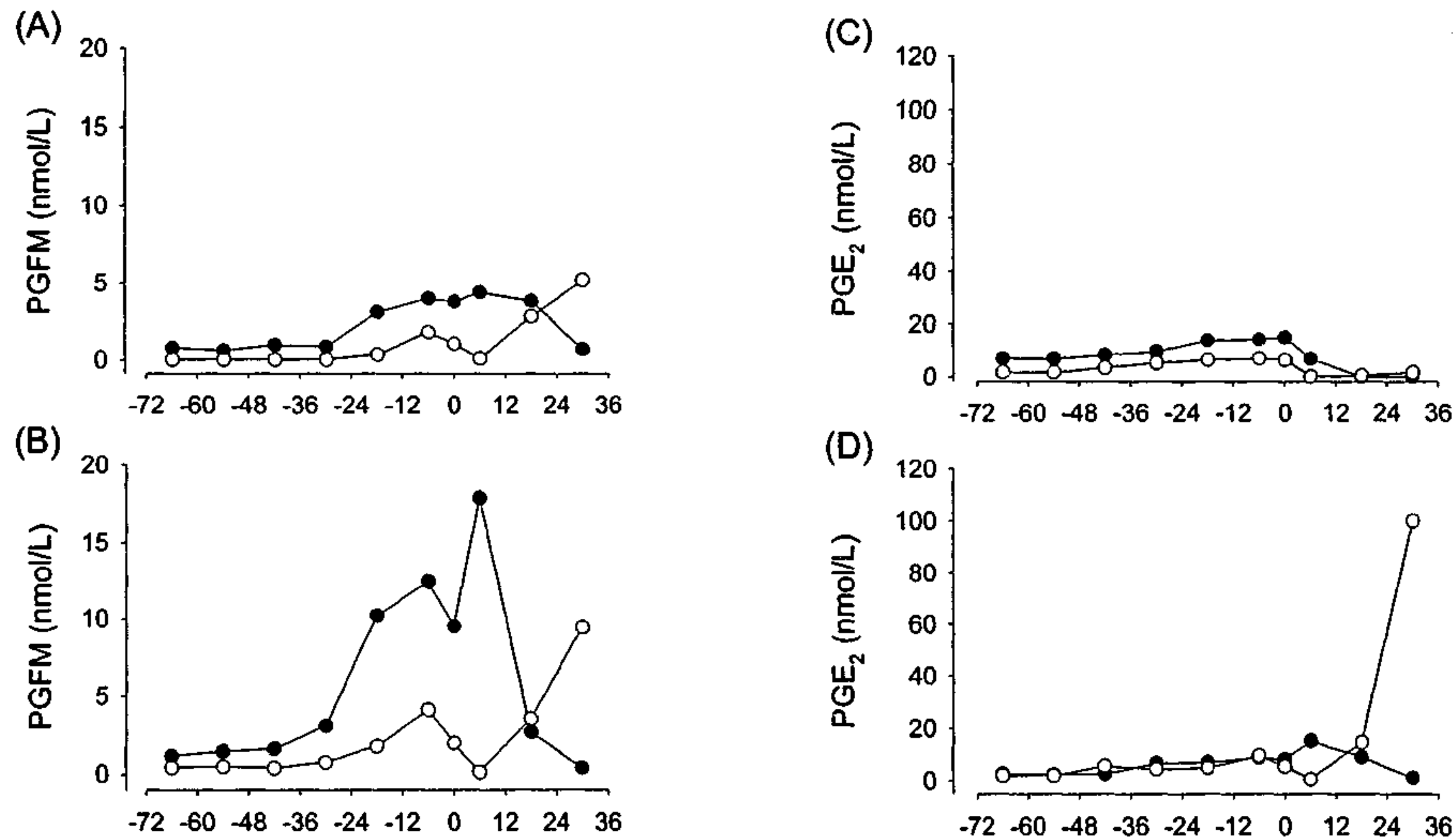

Figure 5.8 PGFM and PGE_2 concentrations in NIM and ATO-treated ewes (20.0 and 4.12 mg/kg/d maternal body weight, respectively), animal 052 (●) and animal 133 (O), values are for (A) and (C) maternal CA or (B) and (D) fetal CA. Values shown are presented relative to the onset of active labour and the beginning of NIM and ATO infusion onset (time 0) until delivery.

5.3.4 Fetal and maternal blood gas parameters

There were no significant differences found between fetal blood gas parameters in VEH- or NIM and ATO-treated ewes, during the induction of premature labour prior to the commencement of NIM and ATO infusions (Table 5.2). There were no differences found in fetal $PaCO_2$ or pH during the entire experimental period until delivery. There was, however, a reduction in fetal PaO_2 at the completion of NIM and ATO treatment (48 h) and during the post-treatment period. Fetal O_2 saturation was reduced compared to VEH-treated ewes, after 24 h of NIM and ATO infusions and remained reduced at 6 h after NIM and ATO treatment. There were no significant changes in maternal blood gas parameters measured prior to, during or after the completion of NIM and ATO infusions (Table 5.3).

5.3.5 Fetal condition at post mortem

Normal ductal patency appeared to have been maintained and there were no observable differences in the diameter of the lumen between the NIM and ATO- and VEH-treated animals. There appeared to be no differences in the small amount of meconium staining observed at *post mortem* of fetuses from NIM and ATO-treated ewes when compared to fetuses from ewes treated with VEH alone. There were also no significant differences in fetal body weights between the two treatment groups. The mean body weights of fetuses from ewes treated with VEH and NIM and ATO were 4.24 ± 0.38 and 4.20 ± 0.13 kg, respectively. In addition, careful examination of the placenta revealed no signs of degradation or separation.

5.3.6 Maternal progesterone concentrations

Progesterone concentrations were measured in the maternal circulation from VEH- and NIM and ATO-treated ewes throughout the entire experimental period (Figure 5.9). The fall in maternal progesterone concentrations previously reported to occur during glucocorticoid-induced premature labour (McLaren *et al.*, 1996), was observed in all animals in this study. The maternal progesterone concentrations in the VEH- and NIM and ATO-treated ewes were 67.4 ± 29.3 and 44.4 ± 14.8 pmol/ml, respectively, before the commencement of labour induction. Progesterone concentrations fell significantly in both groups after the start of dexamethasone infusion to 28.1 ± 13.7 and 26.2 ± 15.9 nmol/L, respectively, 6 h prior to the establishment of active premature

labour. Concentrations declined further in these groups to 5.52 ± 1.77 and 8.69 ± 7.31 nmol/L, respectively, at the time of delivery or elective *post mortem*.

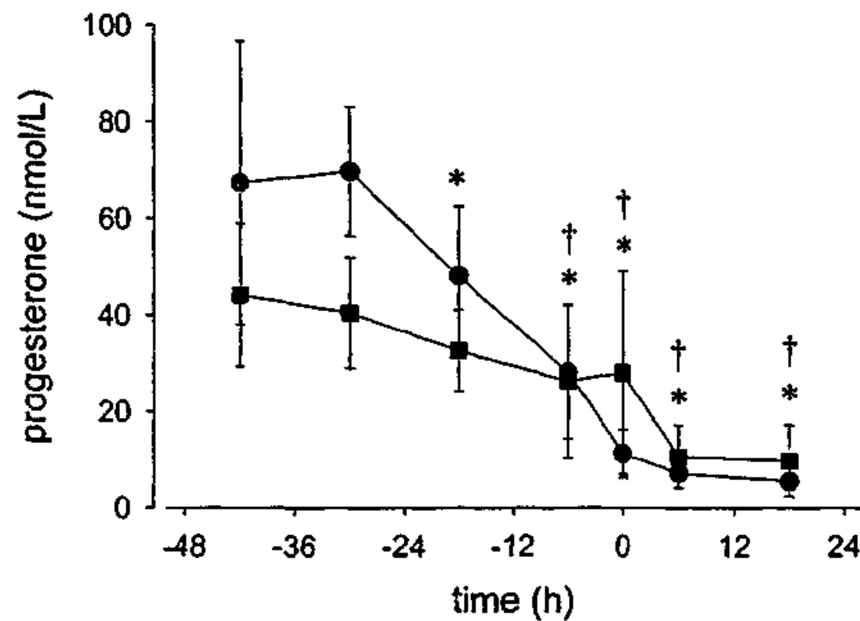

Figure 5.9 Maternal progesterone concentrations measured from before the start of induction of premature labour with dexamethasone infusion, prior to and during NIM and ATO or VEH treatment in control ewes (●, n = 3) and NIM and ATO (■, 20.0 and 4.12 mg/kg/d maternal body weight, respectively; n = 4) treated ewes. Values shown are presented relative to the onset of active labour and the beginning of NIM and ATO or vehicle infusion onset (time 0). Symbols indicate maternal progesterone concentrations significantly reduced compared to those observed prior to onset of maternal treatment (time 0) for *VEH and †NIM and ATO-treated ewes.

Table 5.2 Fetal arterial blood gas parameters from ewes treated with vehicles (n-Methyl-2-Pyrrolidone and saline, 1 ml/h; n = 4), or nimesulide and atosiban (20 and 4.12 mg/kg/d maternal body weight, respectively; n = 4) during established premature labour until delivery or elective *post motem*.

	PaO_2 (mmHg)		$PaCO_2$ (mmHg)		pH		O_2sat	
Time (h) relative to the onset of premature labor	*Vehicle*	*Nimesulide and Atosiban*	*Vehicle*	*Nimesulide and Atosiban*	*Vehicle*	*Nimesulide and Atosiban*	*Vehicle*	*Nimesulide and Atosiban*
Before DEX (time -42)	21.50±2.36	24.25±1.75	50.83±2.09	44.88±2.52	7.34±0.02	7.35±0.01	54.97±7.70	65.03±1.58
Inhibitor or vehicle onset (time 0)	22.0±3.21	24.00±1.00	48.00±2.65	47.25±1.31	7.38±0.02	7.39±0.01	56.10±5.59	63.78±2.30
6-12 h after inhibitor or vehicle infusion	20.50±4.92	20.50±1.50	49.67±4.26	45.75±1.89	7.38±0.03	7.38±0.01	49.37±14.13	55.30±2.87
Delivery	19.50±3.88	-	50.33±2.91	-	7.38±0.03	-	49.20±10.00	-
24 h after inhibitor infusion	-	21.00±1.00	-	48.63±1.84	-	7.30±0.09	-	52.70±6.88*
48 h after inhibitor infusion	-	16.88±0.13*	-	46.50±12.6	-	7.43±0.02	-	49.48±3.80*
6-12 h post inhibitor treatment	-	13.75±0.25*	-	46.75±0.92	-	7.41±0.03	-	45.54±4.75*

Vehicle or nimesulide and atosiban infusions commenced after the established premature labour (time 0), as determined by uterine activity, refer to section 5.2.1. Asterisks (*) indicate values significantly different from pre-inhibitor treatment values.

Table 5.3 Maternal arterial blood gas parameters from ewes treated with vehicles (n-Methyl-2-Pyrrolidone and saline,1 ml/h; n = 4), or nimesulide and atosiban (20 and 4.12 mg/kg/d maternal body weight, respectively; n = 4) during established premature labour until delivery or elective *post mortem*.

	PaO_2 (mmHg)		*$PaCO_2$ (mmHg)*		*pH*		*O_2sat*	
Time (h) relative to the onset of premature labour	*Vehicle*	*Nimesulide and Atosiban*	*Vehicle*	*Nimesulide and Atosiban*	*Vehicle*	*Nimesulide and Atosiban*	*Vehicle*	*Nimesulide and Atosiban*
Before DEX (time -42)	97.2 ± 1.66	99.50 ± 0.50	33.2 ± 0.12	32.0 ± 0.71	7.47 ± 0.02	7.45 ± 0.01	97.87 ± 1.04	98.44 ± 1.51
Inhibitor or vehicle onset (time 0)	99.0 ± 1.08	102.0 ± 5.00	32.5 ± 0.61	34.5 ± 1.85	7.46 ± 0.01	7.46 ± 0.01	97.24 ± 0.84	98.03 ± 1.51
6-12 h after inhibitor or vehicle infusion	92.5 ± 3.06	104.5 ± 2.50	33.0 ± 0.82	30.0 ± 1.47	7.46 ± 0.01	7.47 ± 0.01	98.46 ± 0.27	98.45 ± 1.53
Delivery	100.3 ± 1.75	-	33.0 ± 0.00	-	7.48 ± 0.00	-	94.50 ± 0.00	-
24 h after inhibitor infusion	-	110.8 ± 8.25	-	30.9 ± 1.56	-	7.48 ± 0.02	-	98.61 ± 1.09
48 h after inhibitor infusion	-	113.0 ± 0.00	-	29.4 ± 0.63	-	7.48 ± 0.03	-	97.92 ± 1.38
6-12 h post inhibitor treatment	-	102.3 ± 1.75	-	31.3 ± 1.03	-	7.46 ± 0.03	-	96.63 ± 1.66

Vehicle or nimesulide and atosiban infusions commenced after the established premature labour (time 0), as determined by uterine activity, refer to Section 5.2.1.

5.4 DISCUSSION

The principle finding of this study was that glucocorticoid-induced active premature labour in sheep, was successfully delayed in all six, and delivery prevented in four out of six, ewes that were treated using the combination of nimesulide and atosiban. The increase in uterine activity and prostaglandin concentrations observed in control ewes, was markedly suppressed in nimesulide and atosiban treatment. Interestingly, these ewes failed to show a dramatic increase in concentrations after the cessation of nimesulide and atosiban infusions despite continued fetal glucocorticoid treatment for the induction of premature labour.

The previous experiments outlined in Chapter 3, showed that a continuous infusion of nimesulide and atosiban commencing before dexamethasone treatment delayed the induction of premature labour in sheep. The findings of the present study, indicate that the combined treatment with these compounds was effective in blocking the interplay between $PGF_{2\alpha}$ and oxytocin before labour onset as well as in suppressing this positive mechanism after premature labour is initiated. These findings further support the use of selective inhibitors of PGHS-2 as a potential approach for delaying premature delivery. Previous studies have shown that elevated PGHS-2 expression is primarily responsible for the rise in PGHS activity at labour in women (Mijovic *et al.*, 1998) as well as in the sheep model (Hirst *et al.*, 1995b; McLaren *et al.*, 1996). Intra-uterine PGHS-1 expression does increase during gestation, but does not appear to substantially contribute to prostaglandin synthetic capacity at labour onset (Mijovic *et al.*, 1998). However, this iso-form may have a more important role in rodent species in which labour is initiated following the demise of the corpus luteum (Reese *et al.*, 2000).

Previous studies have demonstrated that dexamethasone, a synthetic glucocorticoid, effectively induces premature labour in sheep with a tightly controlled time span (McLaren *et al.*, 1996) and is an appropriate model for studying the efficacy of inhibitors of premature labour. The endocrine changes induced by this treatment are equivalent to those observed during normal labour (Challis *et al.*, 1978) and these changes were seen in this study as indicated by a fall in progesterone concentrations. In all 10 animals used in this study, the onset of labour, based on the criteria of at least 2 x baseline uterine EMG activity, was achieved, 39.3 ± 2.2 h after the commencement of fetal dexamethasone infusion.

Concurrent with a decrease in circulating prostaglandin concentrations, uterine EMG activity in treated animals was significantly reduced compared to basal activity for 6 h

after the commencement of nimesulide and atosiban. Despite the continued reduction in circulating prostaglandin concentrations, uterine EMG activity increased slightly over the following 24 h in these animals. Importantly, uterine EMG activity seen in these animals at this time was at levels far less than that observed in vehicle treated animals at delivery. PGFM and PGE_2 may still have had a local direct stimulatory effect on myometrial cells at this time despite being suppressed in the circulation. This may be due to enhanced prostaglandin receptor sensitivity or concentration at this time (Adelantado *et al.*, 1988; Coleman *et al.*, 1990). Prostaglandin receptors are linked to distinct intracellular pathways that regulate uterine activity via contractile (EP_1, EP_3, FP) or relaxatory (EP_2, EP_4) receptors (Ma *et al.*, 1999). Differential up-regulation of contractile EP subtypes and FP has been reported at the onset of normal labour particularly in the myometrium (Ma *et al.*, 1999). *In vivo* administration of nimesulide has been shown to lower *in vitro* spontaneous myometrial contractility and sensitivity to oxytocin while also increasing the sensitivity to prostaglandins, suggesting an up-regulation of prostaglandin receptors (Baguma-Nibasheka *et al.*, 1998).

Oestrogens were not measured in this study, but a glucocorticoid-induced alteration in placental steroidogenic enzyme activities, causes maternal progesterone concentrations to fall with a concomitant increase in placental oestrogen concentrations (Challis *et al*, 1971). Maternal progesterone concentrations measured in all animals decreased after the induction with dexamethasone, therefore indicating that placental oestrogen levels had increased. Analysis of the effect of an increasing oestrogenic environment on prostaglandin receptor subtype expression would be required to determine if prostaglandin sensitivity changes are involved in this model of premature labour. In addition to potential changes in prostaglandin sensitivity, some of the residual uterine activity may also have resulted from the inability of atosiban to completely block all oxytocin receptors. As labour progresses into the final stages, oxytocin sensitivity is heightened due to a rapid increase in the number of uterine oxytocin receptors in the endometrium and myometrium associated with increased oestrogen concentrations (Fuchs *et al.*, 1984; Andersen *et al.*, 1989; Wathes *et al.*, 1996). This may have resulted in a consequential increase in oxytocin binding and the transient rise in uterine activity that was observed during nimesulide and atosiban treatment.

An alternative explanation for unblocked uterine activity may include the metabolism of arachidonic acid through a PGHS independent pathway. During treatment with non-steroidal anti-inflammatory drugs, arachidonic acid metabolism has been shown to

continue through the lipoxygenase pathway leading to the formation of uterine contractile agents such as 5-hydroxyeicosatetraenoic acid (5-HETE) (Edwin *et al*, 1996). Previous studies have shown that 5-HETE stimulates contractions in human myometrial strips obtained prior to labour, suggesting a direct role of lipoxygenase metabolites in eliciting uterine contractions (Bennett *et al*., 1987a). Thus, residual uterine activity observed in these animals may in part, be the result of arachidonate metabolites such as 5-HETE directly stimulating the myometrium.

Although the uterine activity profiles for the four animals completing the treatment were similar. The data obtained from the two animals that delivered during the 48 h nimesulide and atosiban treatment period revealed heightened uterine activity resembling the level of activity observed in control animals during delivery. Maternal and fetal prostaglandin concentrations in both these animals were similar to those in the other treated animals prior to inhibitor treatment onset. However, upon commencement of nimesulide and atosiban treatment, prostaglandin concentrations in these two animals were not immediately reduced. Therefore increased uterine activity and lack of immediate inhibition of the elevated prostaglandin concentrations may account for the failure to block delivery for the entire 48 h treatment period in these animals. Although failing to complete nimesulide and atosiban treatment, delivery was delayed by more than 20 h after the mean delivery time for vehicle treated animals.

In the one fetus that died after treatment, the fetal blood gas values were normal during the nimesulide and atosiban infusion period but declined rapidly thereafter. Upon *post mortem* examination there was evidence of interference of umbilical blood flow by the vascular catheters, which were found in close proximity to the umbilical cord. This may have, and most likely, accounted for the demise of this fetus. Similarly, in animal 052, the fetal blood gas parameters were normal during the nimesulide and atosiban treatment period, however, the subsequent reduction in PaO_2 and O_2 sat in this fetus was during the time when the level of uterine activity was just sufficient to allow alternate switching between a *contracture* and *contraction* type pattern without delivery. This suggests that the reduction in prostaglandin concentrations at this time, together with oxytocin receptor blockage may have resulted in insufficient stimulation for conversion of *contractures* to *contractions*, leading to inadequate coordination of uterine activity and thus contributing to fetal stress. Again the reduction in fetal PaO_2 and O_2 sat observed in animals completing the 48 h inhibitor treatment may be a result of the sustained submaximal uterine activity seen in these animals. Examination of the ductus arteriosus

of each fetus at *post mortem*, indicate patency was maintained in all fetuses completing the nimesulide and atosiban treatment.

Uterine activity declined in the initial 6 h of inhibitor treatment, compared to vehicle-treated animals, however, uterine activity was not completely blocked. Complete suppression of this residual uterine activity may be required to minimise the reduction in fetal well-being caused by the prolonged uterine activity. In this study glucocorticoid stimulation was continued throughout the entire study period. The glucocorticoid treatment protocol used in this study is a continual drive to prostaglandin synthesis in the sheep. The successful blockade of glucocorticoid-induced premature labour requires higher inhibitor doses than may be needed with less potent stimuli. Consequently, lower doses of inhibitors with less impact on the fetus may be needed, for example, in women where the rise in intra-uterine PGHS-2 mRNA expression at premature labour is relatively small and of similar magnitude to the rise seen at normal labour onset (Mijovic *et al.*, 1998).

Recent studies have shown that PGHS-2 is expressed in the fetal kidney as well as the fetal placenta (Olson *et al.*, 2001). Prostaglandin production by this enzyme may have important roles for fetal development (Olson *et al.*, 2001). These findings suggest while short term exposure to selective PGHS-2 inhibitors may not be detrimental to the fetus, that longer term treatment may have serious adverse effects. By combining an oxytocin antagonist such as atosiban, which shows little placental transfer to the fetal circulation (Greig *et al.*, 1993), and less maternal side effects than other currently used tocolytic agents (Moutquin *et al.*, 2000), may allow lower doses of selective PGHS-2 inhibitors to be used. This treatment regime would reduce uterine activity, minimise adverse fetal side effects, yet adequately delay premature delivery. The reduction in uterine activity as a result of atosiban treatment may also contribute to the lowering of prostaglandin concentrations observed in this study. Thus, although the exact contribution of each of the two inhibitors to the overall suppression of uterine activity cannot be directly delineated, this effect may represent a further benefit of the combined treatment approach. In women, once premature labour is initially suppressed and potential cyclic stimulatory processes broken, uterine activity may decline and the doses of the inhibitors may be able to be reduced to minimise potential side effects to the fetus. Clearly further studies are required using a primate model to address these possibilities.

It was hypothesised that prostaglandin concentrations and uterine activity might dramatically rise after the cessation of nimesulide and atosiban treatment in the presence

of continued fetal glucocorticoid infusion in treated animals. However, no significant rise in maternal or fetal prostaglandin concentrations were observed for more than 6 h after the cessation of nimesulide and atosiban treatment. This was surprising considering the relatively short half-lives of nimesulide and atosiban. Whether this ongoing suppression of prostaglandin synthesis is a consequence of continued glucocorticoid exposure or loss of the stimulus for PGHS-2 expression requires further investigation. However, the present findings indicate that a possible rebound rise in prostaglandin production and uterine activity following the cessation of the use of selective PGHS-2 inhibitor treatment is unlikely.

It has been previously suggested that the increase in glucocorticoids associated with premature labour is responsible for placental separation in this species (Jack *et al*, 1975). Despite dexamethasone administration initially stimulating prostaglandin production, no such detachment was seen in animals completing the nimesulide and atosiban treatment, in both inhibitor studies, indicating that placental separation may not be due to a direct effect of glucocorticoids but may also require a rise in prostaglandin production together with the associated increase in contractile strain. Thus, the effectiveness of nimesulide and atosiban in inhibiting uterine activity likely also contributes to the lack of placental separation observed in the current study and the previous study outlined in Chapter 3.

At *post mortem* examination, nimesulide and atosiban treated ewes showed some softening of their cervixes but not proceeding to full dilation. Increases in hyaluronic acid and physiologic cell death are in part responsible for the cervical remodelling process (Ludmir & Sehdev, 2000). The collagen bundles disperse and lose strength, cytokines, hyaluronic acid, collagenases and elastase work together to allow effacement (Ludmir & Sehdev, 2000). Prostaglandins appear to play a central role in cervical ripening (Bukowski *et al.*, 2001), and the reduced cervical dilation observed with nimesulide treatment suggests that the inhibition of prostaglandin synthesis was adequate to block this process. The recent finding that cervical administration of nimesulide can prevent cervical ripening in late pregnant rats (Bukowski *et al.*, 2001) is consistent with the present observations. Uterine activity also contributes to cervical ripening (Ludmir & Sehdev, 2000), thus, the suppression of uterine activity in the nimesulide and atosiban treated ewes, combined with the inhibition of prostaglandin production may contribute to the reduced cervical dilation observed in these animals.

In this study, it was demonstrated that a combined treatment of nimesulide and atosiban, after the establishment of premature labour, successfully delayed the progression to delivery. Future studies are now needed to investigate reducing the dose of PGHS-2 inhibitor, when used in combination with an oxytocin receptor antagonist, to achieve the same reduction in uterine activity and to ensure fetal well-being. The current study supports the potential benefit of an alternative treatment for delaying premature delivery using a selective prostaglandin synthase type-2 inhibitor in combination with an oxytocin receptor antagonist.

Chapter 6

Fetal responses to an inflammatory challenge during pregnancy

6.1 INTRODUCTION

Intra-uterine infection is recognised as a major contributor to the pathogenesis of premature labour (Romero & Mazor, 1988), yet the fetal inflammatory responses to maternal systemic and intra-uterine infection remain unclear. The incidence of sepsis in premature neonates has been attributed to intra-uterine infection during pregnancy, and appears to increase with decreasing gestational age at delivery (Martius & Roos, 1996). Bacteria found in the uterus in association with premature labour are mainly vaginal in origin (Gravett *et al.*, 2000), and it is likely that the fetal effects are induced by inflammatory responses to the abnormal flora rather than specific effects of any one organism (Elder, 1992).

It is thought that infections within the uterus can occur between the chorio-decidual space, within the fetal membranes (the amnion and chorion), in the placenta, amniotic fluid, or within the umbilical cord or fetus (Romero & Mazor, 1988). It has been proposed that organisms first ascend from the vagina into the chorio-decidual space. In women, the organisms are then thought to cross the intact chorio-amniotic membranes into the amniotic fluid (Romero *et al.*, 2001). Recent evidence suggests that intra-uterine infection may occur quite early in pregnancy and remain undetected for months (Goldenberg *et al.*, 2000), thus exposing the fetus to the effects of endotoxin for a considerable period. Identifying women with intra-uterine infections therefore, is a major obstetric challenge, as intra-uterine infection is often asymptomatic (Goncalves *et al.*, 2002). A number of studies have used the administration of bacteria or bacterial products to pregnant animals as a model of human premature labour (Gravett *et al.*, 1994b; Fidel *et al.*, 1998 ; Kaga *et al.*, 1996 ; Schlafer *et al.*, 1994). In a non-human primate

model, intra-amniotic inoculation with group B *Streptococcus*, increases amniotic fluid cytokines, particularly interleukin-6 (IL-6), in parallel with increases in amniotic fluid prostaglandins (Gravett *et al.*, 1994b). Both these changes precede increases in uterine contractility. The elevation in amniotic fluid cytokines observed after experimental infection are consistent with those reported in women where IL-6, IL-1 and tumor necrosis factor α (TNFα) have been found in elevated concentrations in the amniotic fluid of women with intra-amniotic infection and premature labour (Romero *et al*, 1990; Hillier *et al.*, 1993).

Lipopolysaccharide (LPS), a pyrogenic component of gram-negative bacteria cell walls, induces a downstream cascade of inflammatory responses, stimulating macrophages to produce large amounts of cytokines, such as TNF-α, IL-1 and IL-6 (Dziegielewska *et al*, 1998; Garnier *et al.*, 2001), similar to that seen with infection, but without causing invasion or multiplication of micro-organisms within the body. Most previous studies have focused on endotoxin exposure within the intra-amniotic cavity, where the amniotic fluid is thought to provide a conduit through which the infection, or the infective agents, then gain access to the fetus, initiating responses that may either harm or protect the fetus (Newnham *et al.*, 2002). Work by Kallapur *et al* (2001), showed that intra-amniotic LPS administration in sheep resulted in chorioamnionitis, an elevation in pro-inflammatory cytokine expression in the amnion/chorion, and lung inflammation, similar to that observed in premature infants with associated chorioamnionitis. Thus, LPS was considered as an ideal experimental tool to investigate inflammatory responses during pregnancy, but the effect on uterine activity and fetal endocrine parameters were not measured in this study. Many other studies have used LPS as an agent to induce premature labour, which have also been accompanied by fetal death. A study by Fidel *et al.* (1994) showed that intra-peritoneal LPS administration (50 μg) in mice at 70% gestation results in an 87% incidence of premature labour, yet these authors did not describe fetal or neonatal outcome. Similarly, Kaga *et al.* (1996) demonstrated a 100% incidence of premature delivery in mice intra-peritoneally treated with LPS (50 μg, twice at 3 h intervals), however, all fetuses that delivered prematurely were dead in utero. Fetal death was also observed in a study by Schlafer *et al.* (1994), where LPS administration (1 μg/kg) to the ewe resulted in fetal death and the onset of premature labour. Collectively, these data support the view that an inflammatory challenge induced by LPS can lead to increased concentrations of pro-inflammatory cytokines and, subsequently, premature labour. However, because most of these studies have been undertaken in

species in which it is not possible to monitor the fetal effects of chronic endotoxin exposure, little is known of the fetal responses during this inflammatory process or of the impact of these responses on fetal well-being and neonatal outcome. In addition, previous studies administering LPS have used doses in excess of those that would be severely toxic if administered to the mother (O'Brien *et al.*, 1981) or the fetus (Kallapur *et al.*, 2001). We hypothesise that the inflammatory effects of endotoxins that are produced as a result of ascending intra-uterine infections during pregnancy are suppressed, in comparison with that observed in response to a vascular insult, through the barrier provided by the amniotic fluid.

The aim of this study, therefore, was to use an ovine model that allows chronic fetal monitoring to investigate the fetal effects of an inflammatory challenge during pregnancy using maternal systemic, intra- or extra-amniotic administration of LPS, thus mimicking the processes thought to be involved in premature labour associated with infection in women (Romero & Mazor, 1988). A greater understanding of the consequences of uterine infections during pregnancy and the mechanisms by which the fetus and mother respond to an inflammatory challenge is crucial to developing better approaches for the detection and treatment of premature labour. This may also provide an indication as to when it may be appropriate to delay cases of premature labour that may be associated with infection.

6.2 METHODOLOGY

6.2.1 Animals and surgery

Surgery was performed on pregnant ewes at 123 ± 1 days gestation (GA) to insert vascular catheters in the maternal and fetal carotid artery (CA) and jugular vein (JV) as previously described in Section 2.2. An intra-amniotic fluid catheter was also sutured to the nape of the fetal neck to allow sampling directly from the amniotic fluid. A separate intra- or extra-amniotic catheter was inserted for LPS administration (Figure 6.1). The extra-amniotic catheter was placed in between the chorio-amnion and the endometrium (Wintour *et al.*, 1986) and secured to the uterine wall. Uterine electromyographic (EMG) leads were sutured to the external surface of the myometrium for continual monitoring of uterine activity prior to, and during LPS treatment.

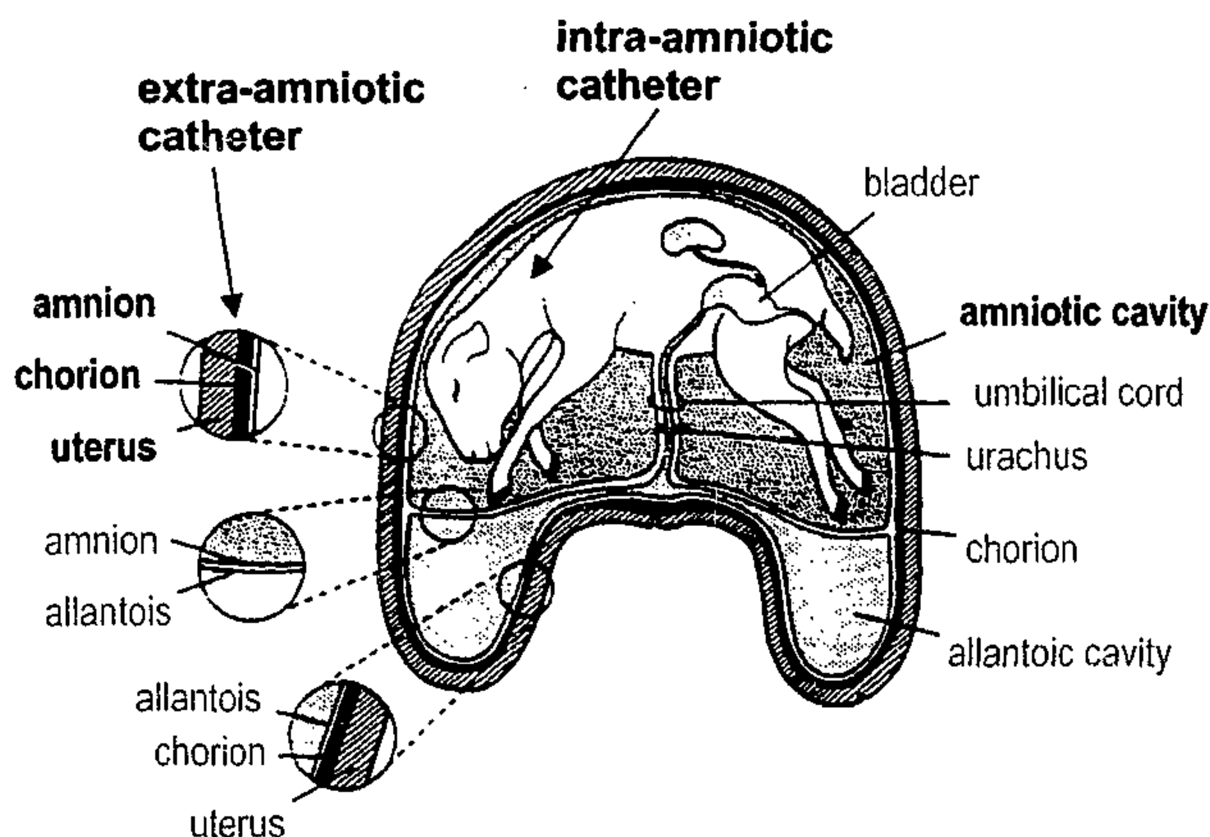

Figure 6.1 Schematic diagram showing the placement of the intra- and extra-amniotic cathethers, adapted from Gilbert (1993). The intra-amniotic fluid catheter was sutured to the nape of the fetal neck, while the extra-amniotic catheter was placed between the chorio-amnion and endometrial tissues and secured to the uterine wall.

6.2.2 Experimental design

Lipopolysaccharide (LPS), derived from *Escheridhia coli*, serotype: 0127:B8 (Sigma Chemical Company, St Louis, Missouri, U.S.A) was used as the inflammatory agent, diluted in sterile saline (Baxter Healthcare Pty. Ltd., Australia). Control values were taken from each animal at -12, -0.5 and -0.25 h prior to LPS treatment, allowing each animal to be used as its own control. Results for -12, -0.5 and -0.25 h prior to LPS treatment were thus combined to give pre-treatment values for each animal.

(a) Maternal systemic LPS treatment

On 130 ± 1 days of gestation, ewes were administered LPS as a bolus injection via the maternal JV catheter (2 μg/kg, maternal body weight, in 5 ml saline; n = 5). Ewes and their fetuses were monitored prior, during and up to 48 h after maternal LPS treatment.

(b) Intra-amniotic LPS administration

On 125 ± 1 days of gestation ewes were administered LPS as a bolus injection via the intra-amniotic catheter attached to the fetus (400 μg/kg maternal body weight, a total of ~20 mg, in 10 ml saline; n = 4). Ewes and their fetuses were monitored prior, during and up to 48 h after intra-amniotic LPS treatment.

(c) Extra-amniotic LPS administration

On 132 ± 2 days of gestation ewes received a constant infusion of LPS via the extra-amniotic catheter at three doses (0.1, 1.0 and 10 μg/kg/d maternal body weight, in saline 0.5 ml/h; n = 4). Ewes and their fetuses were monitored before, and during the extra-amniotic infusion for 72 h.

6.2.3 Sampling regime

Blood samples were obtained from the fetal CA, maternal CA and amniotic fluid in all ewes prior to, during and after the administration of LPS at regular intervals. At the completion of each experiment, ewes and their fetuses were killed by barbiturate overdose (Lethabarb; Arnolds of Reading Pty. Ltd., Australia), administered via the maternal JV catheter (refer to Section 2.4). Maternal and fetal blood respiratory gases (PaO_2 mmHg, $PaCO_2$ mmHg, O_2 sat), haemoglobin (Hb) and pH were measured as described in Section 2.2.3.

The intra- and inter-assay co-efficients of variation for the PGE_2 RIA were 18% and 21%, with a mean sensitivity of 0.26 ± 0.08 nmol/L. The intra- and inter-assay co-efficients of variation for the PGFM RIA were 4% and 15%, respectively, with a mean sensitivity of 0.30 ± 0.08 nmol/L. Cortisol was measured in fetal and maternal plasma after extraction with dichloromethane as described previously in Section 2.5.4. The intra- and inter-assay co-efficients of variation for this assay were 10% and 15%, respectively, while the mean sensitivity of the assay was 1.75 ± 0.58 nmol/L. IL-6 concentrations in fetal and maternal plasma and amniotic fluid samples were measured by ELISA (refer to Section 2.6). The intra- and inter-assay co-efficients of variation were 7% and 24%, respectively. The mean sensitivity of the assay was 0.39 ± 0.17 ng/ml.

6.2.4 Uterine electromyographic (EMG) activity

Uterine EMG activity was recorded continuously before, during and after administration of LPS using a ML135 dual bio-amplifier connected to a ML795 PowerLab/16sp data recording system (ADInstruments Pty. Ltd., Castle Hill, New South Wales, Australia). Uterine EMG activity was analysed using Chart v3.4.11 (AD Instruments Pty. Ltd., Australia). For each animal the number of discrete bursts of uterine activity occurring within each 2 h period was measured, defined previously in Section 3.2.4 (Grigsby *et al.*, 2000).

6.2.5 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Data were first tested for homogeneity of variance, with heterogeneous data rendered homogeneous by square root or logarithmic transformation. All results were grouped into 2, 6 or 12 h time periods after LPS treatment, and the data averaged for collective overall analysis. Basal values taken at -12, -0.5 and -0.25 h prior to LPS treatment were averaged for analysis and used as pre-treatment values. Differences were identified by comparison with mean basal values, prior to LPS treatment. Differences in uterine activity were identified by comparison with basal uterine activity, 12 h prior to LPS treatment. Statistical analysis was performed by identifying differences between means using a one-way repeated measures analysis of variance (ANOVA), time being the variable factor (Statistical Packages for Social Sciences, SPSS Inc., U.S.A.). Least significant difference (LSD) tests were used to identify significant differences between pairs of mean values. Significance is reported at the 5% level ($P < 0.05$).

6.3 RESULTS

6.3.1 Outcome of animals

Premature delivery did not occur in any of the animals studied, despite uterine activity significantly increasing after LPS administration into the maternal circulation and directly into the amniotic fluid. Fetal or maternal death did not result with any of the doses of LPS used or the experimental conditions employed in this study.

Fetal blood gases were monitored at regular intervals prior to and after the administration of LPS into the maternal circulation, directly into the amniotic fluid, or into the extra-amniotic compartment, and are presented in Table 6.1-6.3. Within 2 h after maternal systemic LPS administration, fetal O_2 sat was significantly decreased, reaching a minimum value of 35.75 ± 4.31%, at 6-12 h after LPS administration (Table 6.1). Correspondingly, fetal $PaCO_2$ was slightly elevated and fetal pH was significantly decreased. Fetal PaO_2 also decreased at this time, although it was not significantly different from pre-treatment PaO_2 values ($P = 0.064$). Fetal O_2 sat and fetal pH returned to normal levels by 48 h after maternal systemic LPS administration, while fetal $PaCO_2$ levels remained slightly below basal levels. There were no significant changes observed in the maternal O_2 sat after systemic LPS administration, however, there was a significant reduction in maternal $PaCO_2$ levels from 30.56 ± 0.66 to 25.85 ± 0.94 mmHg, 2-4 h after maternal systemic LPS administration. $PaCO_2$ remained significantly reduced for the subsequent 8 h. A significant increase in maternal pH (7.55 ± 0.02) was observed 2-4 h after maternal systemic LPS administration compared to pre-treatment values (7.48 ± 0.01). These ewes displayed an increase in body temperature (39.26 ± 0.04 to 41.05 ± 0.25 °C), 3.5 h after maternal LPS treatment (Figure 6.1). These changes were also associated with mild respiratory distress (tachypnea, nasal flaring and grunting) in all animals, lasting for approximately 8 h after maternal systemic LPS treatment.

Table 6.1 Fetal *(top panel)* and maternal *(bottom panel)* arterial blood gas parameters from ewes treated with LPS via the maternal circulation (2.0 μg/kg, maternal body weight; n = 5). Asterisks (*) indicate values significantly different from basal values, prior to LPS administration at time 0.

Time (h) relative to the onset of LPS administration	*PaO_2 (mmHg)*	*$PaCO_2$ (mmHg)*	*pH*	*Hb (g/dL)*	*O_2 sat*
			Fetal arterial blood gases		
0	19.80±0.58	41.33±1.93	7.39±0.01	9.75±0.19	66.64±1.55
0-2	18.42±0.85	41.58±0.70	7.41±0.01	9.90±0.24	62.27±2.38
2-4	17.35±0.97	38.45±0.49	7.42±0.01	9.97±0.39	57.45±4.20*
4-6	17.10±1.04	39.90±0.91	7.42±0.01	9.80±0.32	57.43±3.56*
6-12	13.98±1.22	44.00±1.08	7.22±0.11*	9.39±0.55	35.75±4.31*
12-24	18.80±2.96	37.60±2.48*	7.40±0.02	8.52±0.78*	52.96±6.94*
24-48	17.93±0.71	37.20±2.40*	7.41±0.01	8.37±0.62*	62.64±2.07
			Maternal arterial blood gases		
0	111.13±2.18	30.56±0.66	7.48±0.01	9.55±0.47	98.77±0.89
0-2	95.60±4.81*	28.65±0.94*	7.47±0.01	10.79±0.56*	99.14±1.14
2-4	103.08±5.53*	25.85±0.94*	7.55±0.02*	10.57±0.52*	100.11±0.84
4-6	104.55±0.79*	28.45±1.91*	7.52±0.02*	10.91±0.52*	100.42±0.69
6-12	95.68±2.99*	33.27±1.19*	7.51±0.01	10.03±0.54*	99.56±0.90
12-24	95.60±4.45*	32.20±0.58	7.50±0.02	9.32±0.27	99.74±0.74
24-48	103.63±2.67	29.83±0.69	7.51±0.01*	9.29±0.39	100.26±0.80

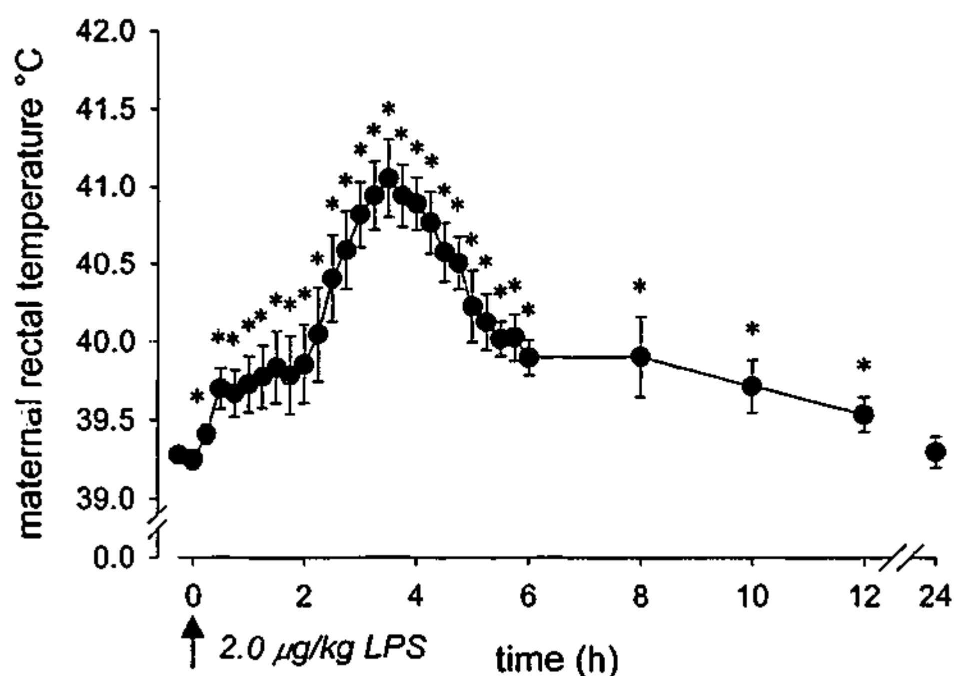

Figure 6.1 Maternal rectal temperature from ewes treated with LPS via the maternal circulation (2 µg/kg/d, maternal body weight; n = 5). Asterisks (*) indicate values significantly different from basal values, prior to LPS administration at time 0.

Direct administration of LPS into the amniotic fluid resulted in a small, yet significant decrease in fetal O_2 saturation, 6-12 h after treatment (Table 6.2).

A small but significant reduction in O_2 sat was observed in fetuses of ewes that received LPS into the extra-amniotic compartment, at the dose of 10 µg/kg/d (Table 6.3). There were no significant changes in the other fetal blood gases measured prior to, during or after the administration of LPS into the extra-amniotic compartment. There were also no significant changes in maternal blood gas parameters measured after intra-amniotic LPS administration or into the extra-amniotic compartment.

Table 6.2 Fetal *(top panel)* and maternal *(bottom panel)* arterial blood gas parameters from ewes treated with LPS via the intra-amniotic fluid (400 μg/kg, a total of ~20 mg; n = 4). Asterisks (*) indicate values significantly different from basal values, prior to LPS administration at time 0.

Time (h) relative to the onset of LPS administration	*PaO_2 (mmHg)*	*$PaCO_2$ (mmHg)*	*pH*	*Hb (g/dL)*	*O_2 sat*
			Fetal arterial blood gases		
0	19.91±1.60	46.75±2.35	7.37±0.00	9.1±0.51	74.46±1.31
0-6	22.88±0.66*	49.93±2.58*	7.38±0.01	9.0±0.35	73.14±1.90
6-12	19.00±0.82	50.50±2.10*	7.37±0.01	9.0±0.44	64.35±3.06*
12-24	19.63±0.69	51.38±2.36*	7.36±0.01	9.0±0.28	63.44±2.79*
24-48	19.50±0.65	49.75±2.06*	7.37±0.00	8.8±0.31	63.70±2.21*
			Maternal arterial blood gases		
0	86.13±2.79	33.38±1.98	7.47±0.01	8.20±0.31	96.64±0.98
0-6	97.38±7.87	34.50±1.69	7.48±0.01	8.20±0.35	95.59±1.10
6-12	94.38±8.89	34.25±1.18	7.51±0.01	7.90±0.34	96.50±1.26
12-24	97.50±6.70	33.00±0.91	7.48±0.01	7.80±0.46	96.88±1.28
24-48	95.75±6.57	32.50±0.96	7.48±0.01	7.60±0.49	97.40±1.30

Table 6.3 Fetal *(top panel)* and maternal *(bottom panel)* arterial blood gas parameters from ewes treated with LPS via the extra-amniotic compartment (0.1, 1.0 or 10 μg/kg, maternal body weight; n = 4). Asterisks (*) indicate values significantly different from basal values, prior to LPS administration at time 0.

Time (h) relative to the onset of LPS administration	*PaO_2 (mmHg)*	*$PaCO_2$ (mmHg)*	*pH*	*Hb (g/dL)*	*O_2 sat*
			Fetal arterial blood gases		
0	45.78±1.96	41.23±1.13	7.35±0.00	8.5±0.38	71.63±4.01
0-12	42.38±6.76	43.25±0.63	7.35±0.01	8.2±0.34	66.84±3.68*
12-24	46.13±4.71	42.50±0.96	7.36±0.01	8.1±0.40	69.35±4.49
24-36	46.13±2.82	43.41±0.65	7.35±0.01	8.2±0.33	64.34±5.34*
36-48	33.13±5.66	44.25±0.25	7.36±0.01	8.1±0.37	65.28±4.39
48-60	36.75±2.39	43.00±0.41	7.36±0.00	7.9±0.32	62.98±5.67*
60-72	34.00±6.75	44.25±1.03	7.35±0.01	8.2±0.30	63.93±5.79*
			Maternal arterial blood gases		
0	118.3±14.34	28.0±0.58	7.44±0.00	8.4±0.19	97.02±0.82
0-12	122.8±10.55	27.6±0.74	7.44±0.01	8.1±0.07	97.30±0.28
12-24	114.7±16.67	27.3±0.67	7.41±0.01	8.6±0.28	96.84±0.24
24-36	98.3±3.17	29.8±0.63	7.44±0.01	7.8±0.02	96.12±0.16
36-48	98.3±9.94	30.2±1.48	7.44±0.01	7.9±0.25	95.75±0.32
48-60	110.5±2.57	29.4±2.39	7.44±0.02	8.1±0.39	95.36±0.74
60-72	108.0±7.00	30.3±2.40	7.44±0.02	8.4±0.46	94.73±0.97

6.3.2 Uterine EMG activity

Uterine EMG activity in animals treated with LPS via the maternal circulation significantly increased within the first 2 h of treatment, (Figure 6.2 A). The significant increase in uterine EMG activity was consistently maintained for more than 30 h, before returning to basal levels, by 48 h after maternal systemic LPS treatment. Uterine EMG activity from animals that received LPS directly into the amniotic fluid is represented in Figure 6.2 (B). There was a small, but significant increase in uterine EMG activity observed in these animals, commencing 2 h after intra-amniotic LPS administration. Uterine EMG activity then returned to basal levels, 8 h after intra-amniotic LPS treatment and remained at these levels for the following 48 h. The extra-amniotic infusion of LPS had no observable effect on uterine EMG activity at any of the three doses of LPS administered (0.1, 1.0 or 10 μg/kg/d, maternal body weight), as shown in Figure 6.2 (C). Uterine activity in these animals was maintained at basal levels (< 5 bursts/2 h) for the entire study period.

6.3.3 Prostaglandin concentrations

PGE_2 and PGFM concentrations in the fetal and maternal circulations and amniotic fluid, before and after LPS administration into the maternal circulation or directly into the amniotic fluid are presented in Figure 6.3-6.5. There was a significant increase in fetal plasma PGE_2 concentrations observed by 4-6 h after maternal systemic LPS administration and this increase was maintained for up to 48 h after maternal systemic LPS treatment (Figure 6.3 A). There were no changes in maternal plasma PGE_2 or amniotic fluid PGE_2 concentrations during the entire experimental period (Figure 6.3 A).

There was a significant increase in PGFM concentrations in the fetal circulation within 4-6 h after maternal systemic LPS administration with concentrations continuing to rise until 12-24 h after treatment (Figure 6.3 B). A slight decrease was noted over the following 24 h, however, fetal PGFM concentrations remained significantly elevated above pre-treatment PGFM concentrations for the duration of the study. There was a small, but significant increase in PGFM concentrations in the maternal circulation 2-4 h after maternal systemic LPS administration, with a further increase at 12-24 h after treatment, (Figure 6.3 B).

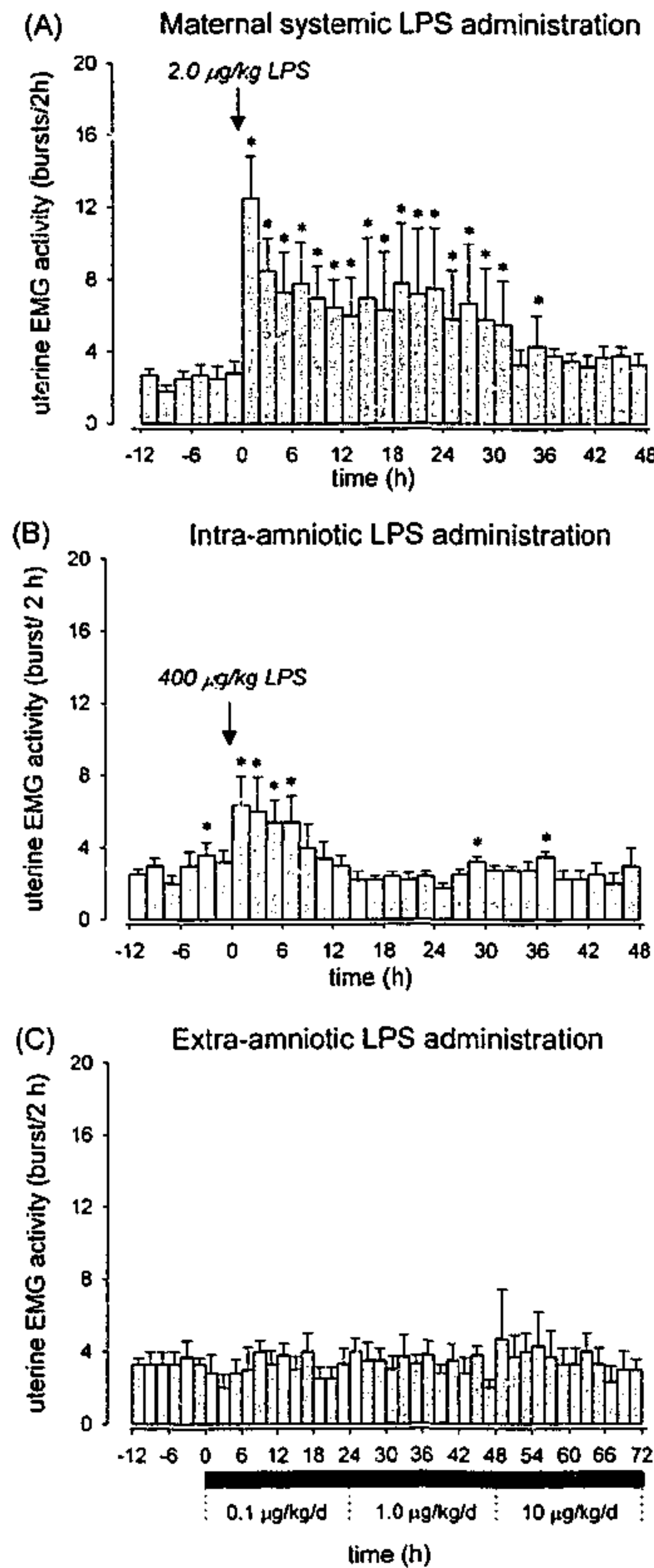

Figure 6.2 Uterine electromyographic (EMG) activity, measured as the frequency of discrete uterine bursts, from 12 h prior to LPS administration (time 0), either via the maternal circulation A (2 μg/kg maternal body weight, 5 ml saline, bolus injection; n = 4), the intra-amniotic fluid B (400 μg/kg, total ~20 mg, 10ml saline, bolus injection; n = 4), or the extra-amniotic compartment C (0.1, 1.0 and 10.0 μg/kg/d maternal body weight, continuous infusion, 0.5 ml/h; n = 4). Asterisks (*) indicate values significantly different from basal uterine EMG activity, prior to LPS administration at time -12 h.

There was also a significant increase in amniotic fluid PGFM concentrations observed from 6-12 h after maternal systemic LPS treatment, which rose further over the following 48 h.

Plasma PGE_2 concentrations in the maternal and fetal circulations remained unchanged after LPS administration directly into the amniotic fluid (Figure 6.4 A). However, the amniotic fluid PGE_2 concentrations increased within 2-4 h after intra-amniotic LPS administration, and continued to rise over the subsequent 48 h (Figure 6.4 A). PGFM concentrations in the fetal circulation increased moderately from 24-48 h after intra-amniotic LPS administration (Figure 6.4 B). A small increase in amniotic fluid PGFM concentrations was also noted, 24-48 h after intra-amniotic LPS administration. Maternal plasma PGFM concentrations were not affected by intra-amniotic LPS administration for the duration of the study.

There were no significant changes observed in fetal and maternal plasma or amniotic fluid PGE_2 concentrations during the infusion of LPS (0.1, 1.0 or 10 μg/kg/d) in to the extra-amniotic compartment, (Figure 6.5 A). In these animals the mean PGE_2 concentrations observed in the fetal and maternal circulations were 2.37 ± 0.43 and 8.87 ± 1.39 nmol/L, respectively. The amniotic fluid PGE_2 concentrations observed in these animals during the LPS infusion into the extra-amniotic compartment were 14.40 ± 5.80 nmol/L. There was a small, increase in PGFM concentrations observed in the fetal plasma samples during the infusion of 1.0 μg/kg/d (1.15 ± 0.48 nmol/L, $P < 0.05$, n = 4) and 10 μg/kg/d (2.12 ± 0.50 nmol/L, $P < 0.05$, n = 4) of LPS into the extra-amniotic compartment compared to basal concentrations (0.71 ± 0.50 nmol/L). PGFM concentrations in the maternal circulation and amniotic fluid remained unchanged during extra-amniotic LPS infusion (Figure 6.5 B).

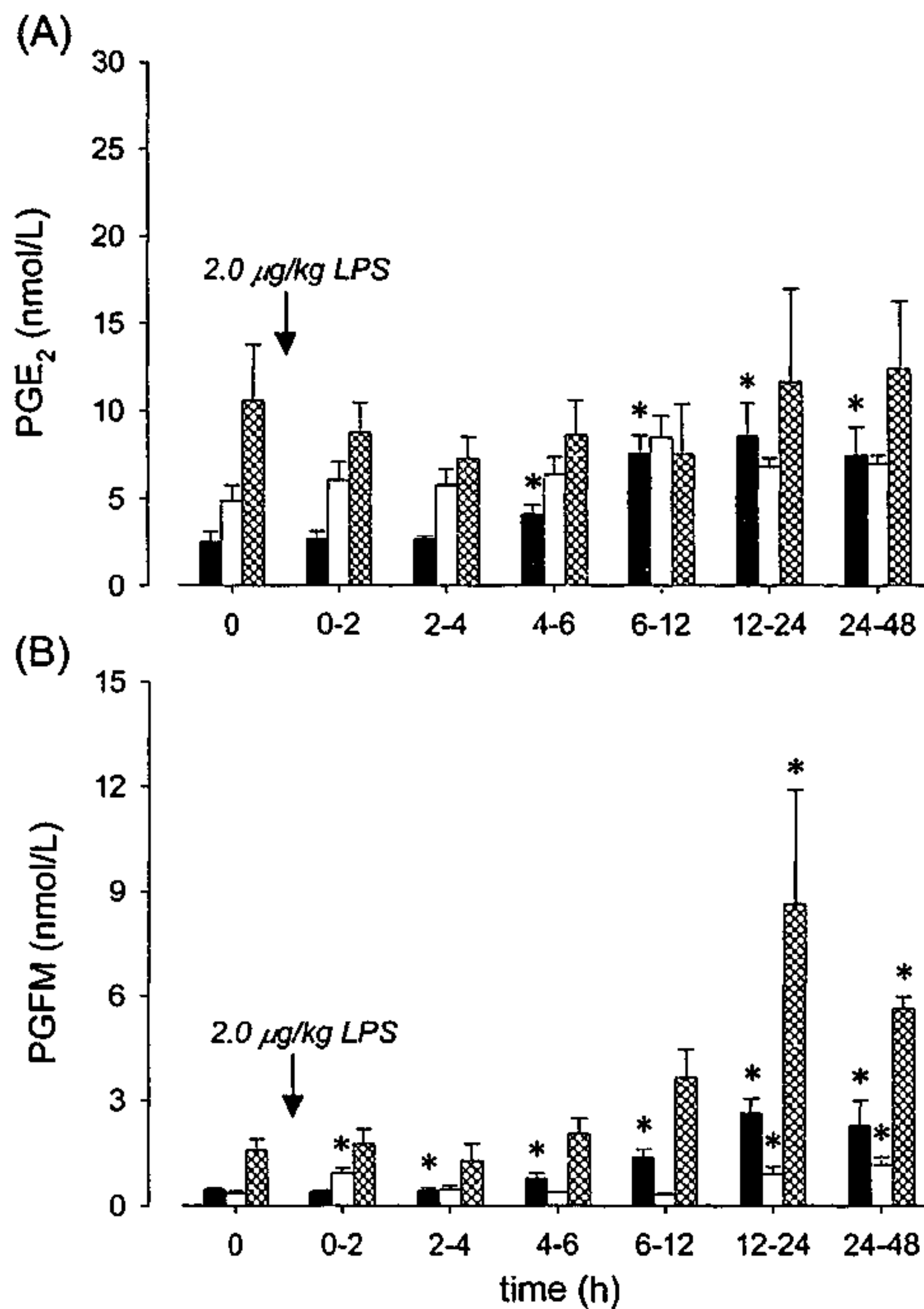

Figure 6.3 PGE_2 (A) and PGFM (B) concentrations prior to and after maternal systemic LPS (2.0 μg/kg, maternal body weight) administration (time 0), in the fetal (*solid bars*; n = 5) and maternal (*open bars*; n = 5) circulations, or amniotic fluid (*hatched bars*; n = 3). Asterisks (*) indicate values significantly different from prostaglandin concentrations, prior to LPS administration at time 0.

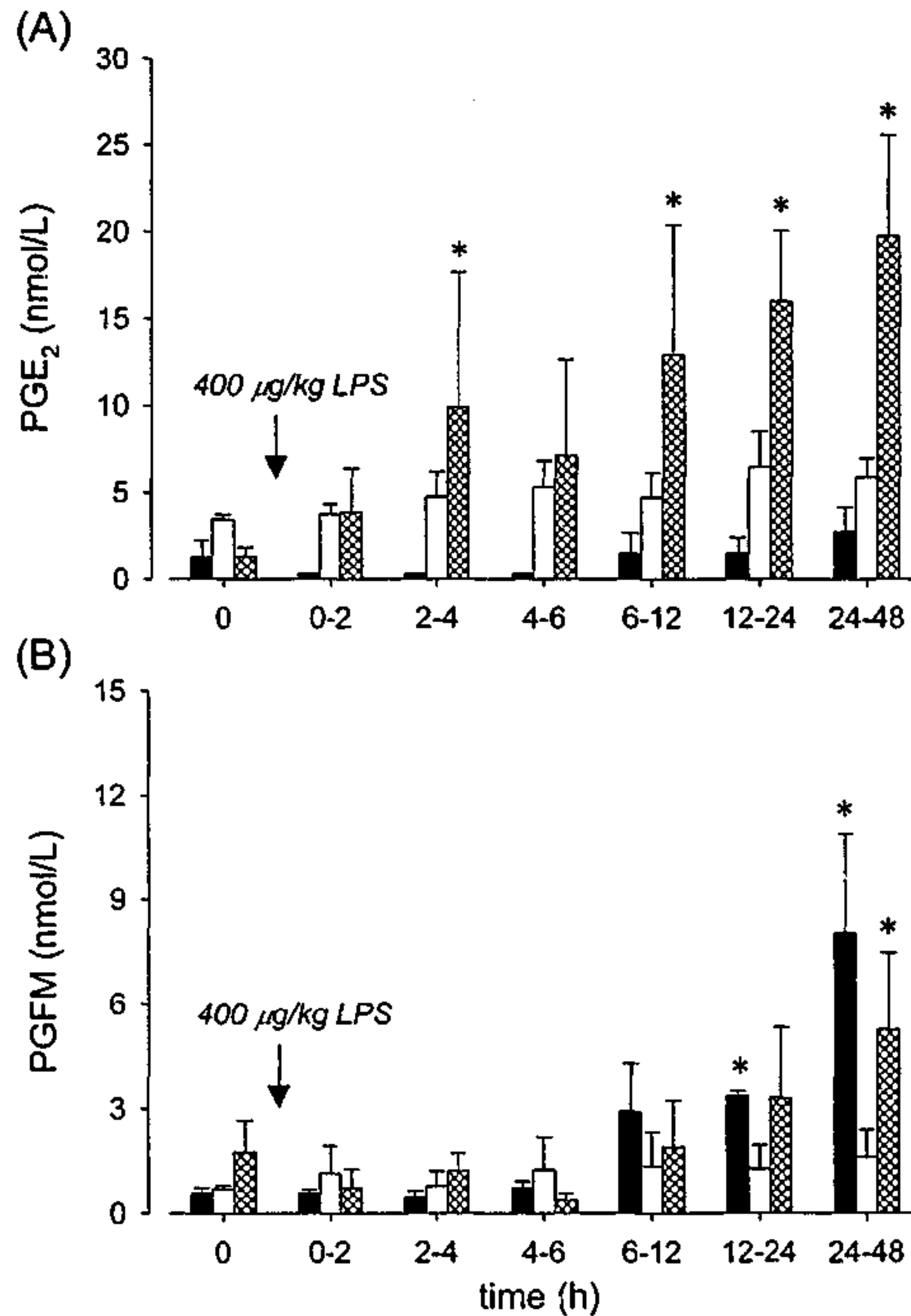

Figure 6.4 PGE$_2$ (A) and PGFM (B) concentrations prior to and after intra-amniotic LPS (400 µg/kg, maternal body weight, total of ~20 mg) administration (time 0), in the fetal (*solid bars*; n = 4) and maternal (*open bars*; n = 4) circulations, or amniotic fluid (*hatched bars*; n = 4). Note panel B, amniotic fluid PGFM concentrations were measured in three animals only. Asterisks (*) indicate values significantly different from prostaglandin concentrations, prior to LPS administration at time 0.

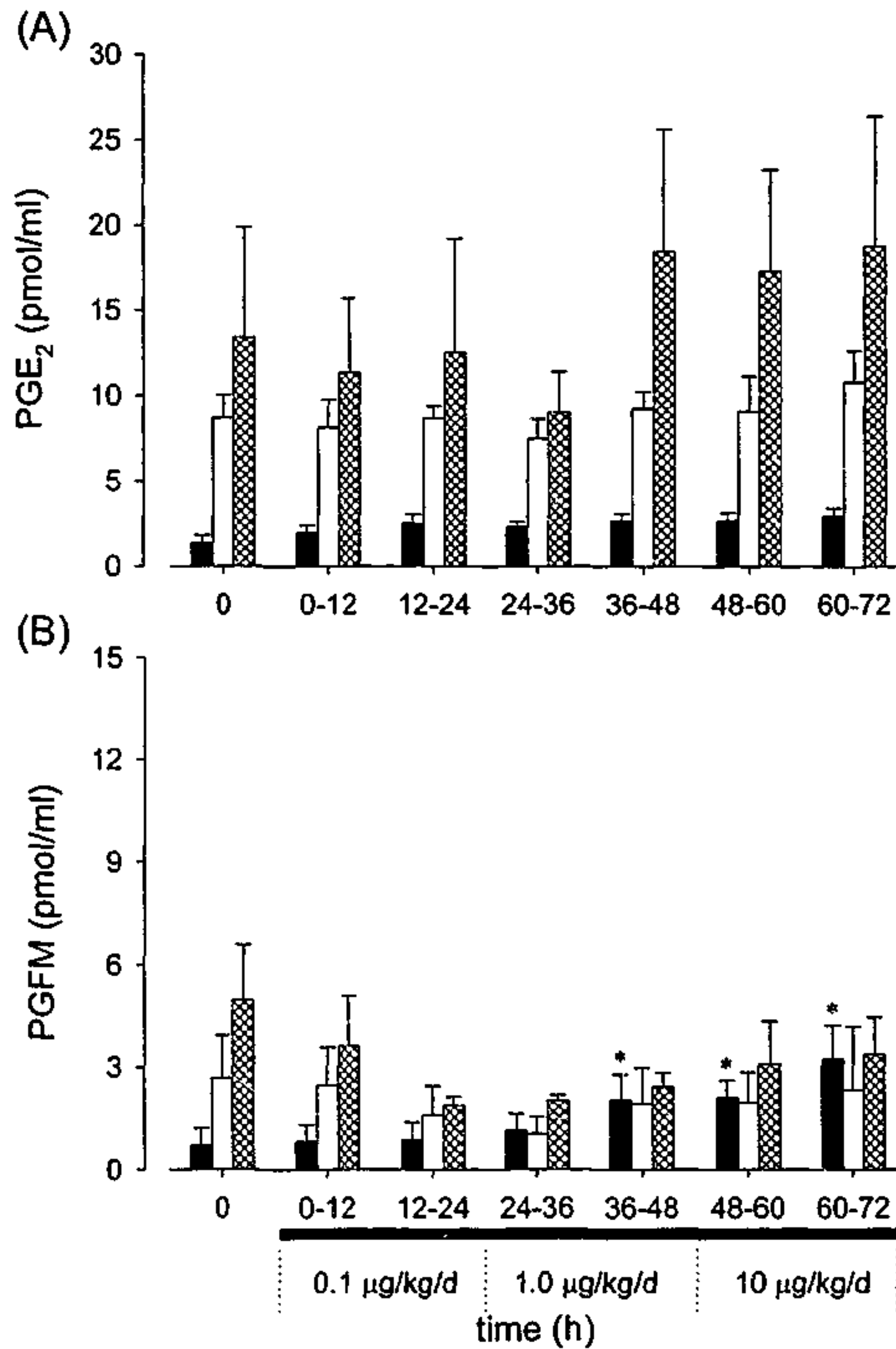

Figure 6.5 PGE_2 (A) and PGFM (B) concentrations prior to and after extra-amniotic LPS (0.1, 1.0 or 10 µg/kg/d, maternal body weight) administration (time 0), in the fetal (*solid bars*; n = 4) and maternal (*open bars*; n = 4) circulations, or amniotic fluid (*hatched bars*; n = 4). Black bar represents the continuous infusion of LPS in to the extra-amniotic compartment. Asterisks (*) indicate values significantly different from prostaglandin concentrations, prior to LPS administration at time 0.

6.3.4 Cortisol concentrations

Maternal systemic LPS administration resulted in a dramatic increase in cortisol concentrations in the maternal circulation within 2 h, and peaked at 4 h after treatment (Figure 6.6). Importantly, there was also a significant rise in cortisol concentrations in the fetal circulation within 2 h after maternal systemic LPS administration. These concentrations continued to rise over the subsequent 24 h.

There was a significant rise in cortisol concentrations in the fetal circulation 4-6 h after intra-amniotic LPS administration, (Figure 6.7), however there were no significant changes in maternal cortisol concentrations. There were also no significant changes observed in fetal and maternal plasma cortisol concentrations during the continuous infusion of LPS (0.1, 1.0 and 10 μg/kg/d) to the extra-amniotic compartment (Figure 6.8).

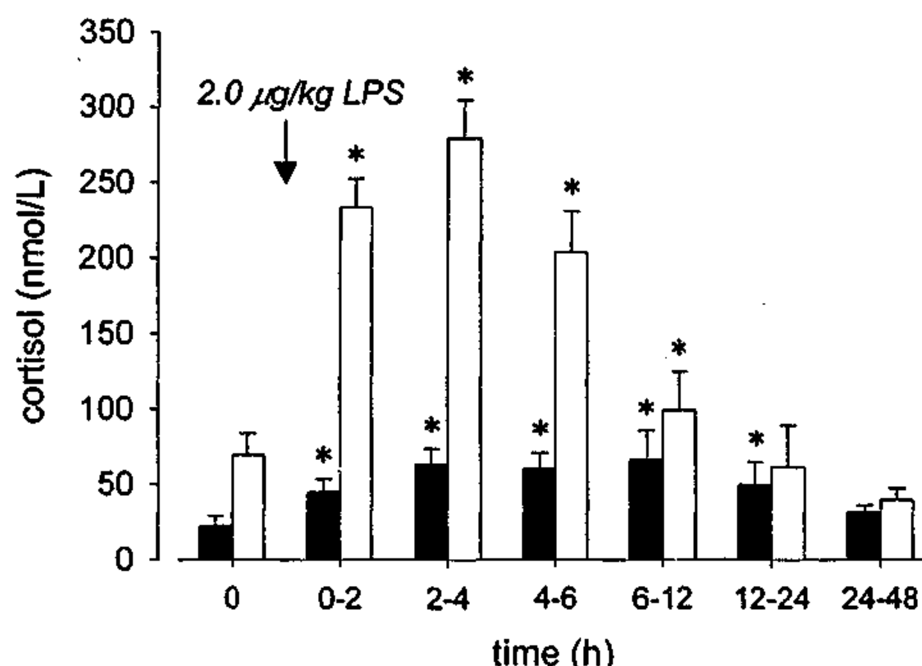

Figure 6.6 Fetal *(solid bars)* and maternal *(open bars)* plasma cortisol concentrations prior to and after maternal systemic LPS (2.0 μg/kg, maternal body weight; n = 5) administration (time 0). Asterisks (*) indicate values significantly different from cortisol concentrations, prior to LPS administration at time 0.

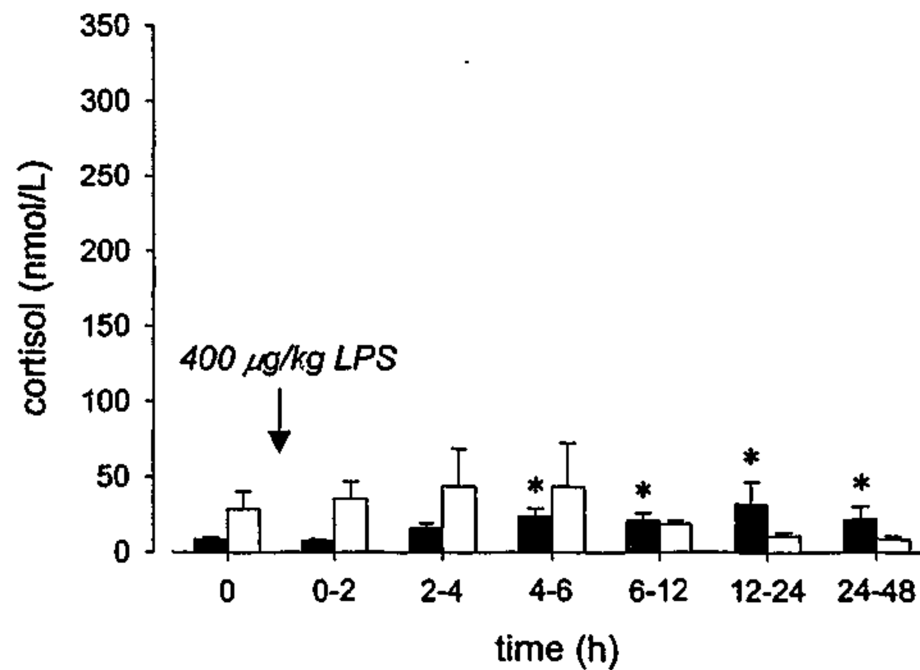

Figure 6.7 Fetal *(solid bars)* and maternal *(open bars)* plasma cortisol concentrations prior to and after intra-amniotic LPS (400 μg/kg, maternal body weight, total ~20 mg; n = 4) administration (time 0). Asterisks (*) indicate values significantly different from cortisol concentrations, prior to LPS administration at time 0.

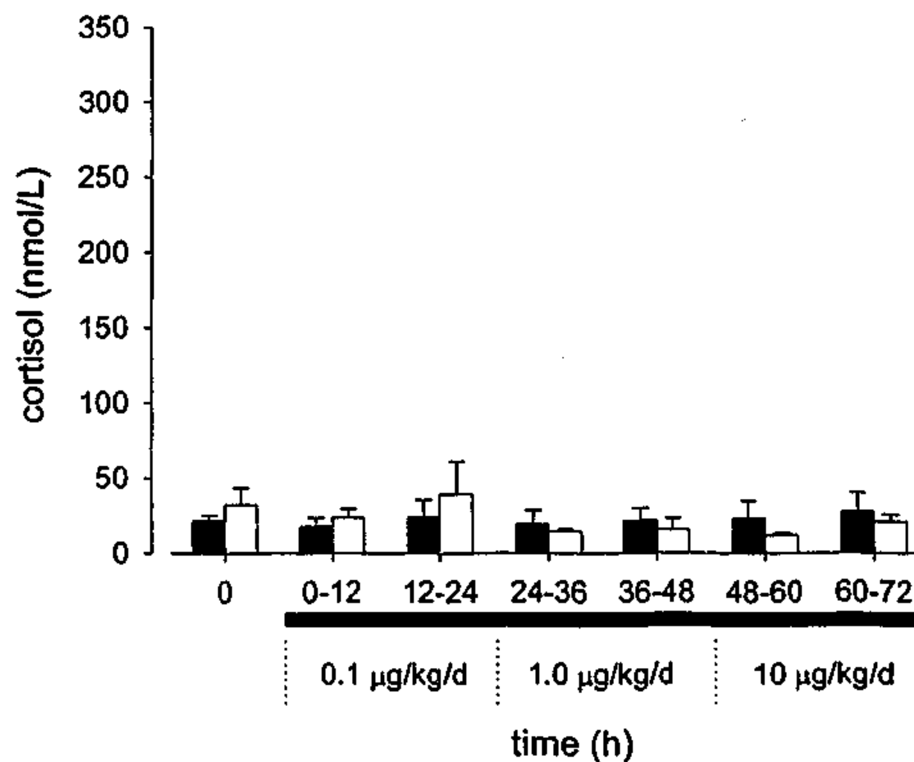

Figure 6.8 Fetal *(solid bars)* and maternal *(open bars)* plasma cortisol concentrations prior to and after extra-amniotic LPS (0.1, 1.0 or 10 μg/kg, maternal body weight; n = 4) administration (time 0). Black bar represents the continuous infusion of LPS in to the extra-amniotic compartment.

6.3.5 IL-6 concentrations

IL-6 concentrations dramatically increased in the maternal circulation by 3 h after systemic LPS treatment, and remained significantly above basal IL-6 concentrations for up to 24 h after maternal systemic LPS treatment (Figure 6.9). There was a trend towards higher IL-6 concentrations in the fetal circulation 12 h after maternal systemic exposure to LPS, however, these values were not significantly different from pre-treatment concentrations. There were no significant differences found with IL-6 concentrations in the fetal, maternal or amniotic fluid after intra-amniotic LPS administration (Figure 6.10). There were also no significant changes in IL-6 concentrations in the fetal and maternal circulations, or in amniotic fluid, during LPS administration into the extra-amniotic compartment (Figure 6.11).

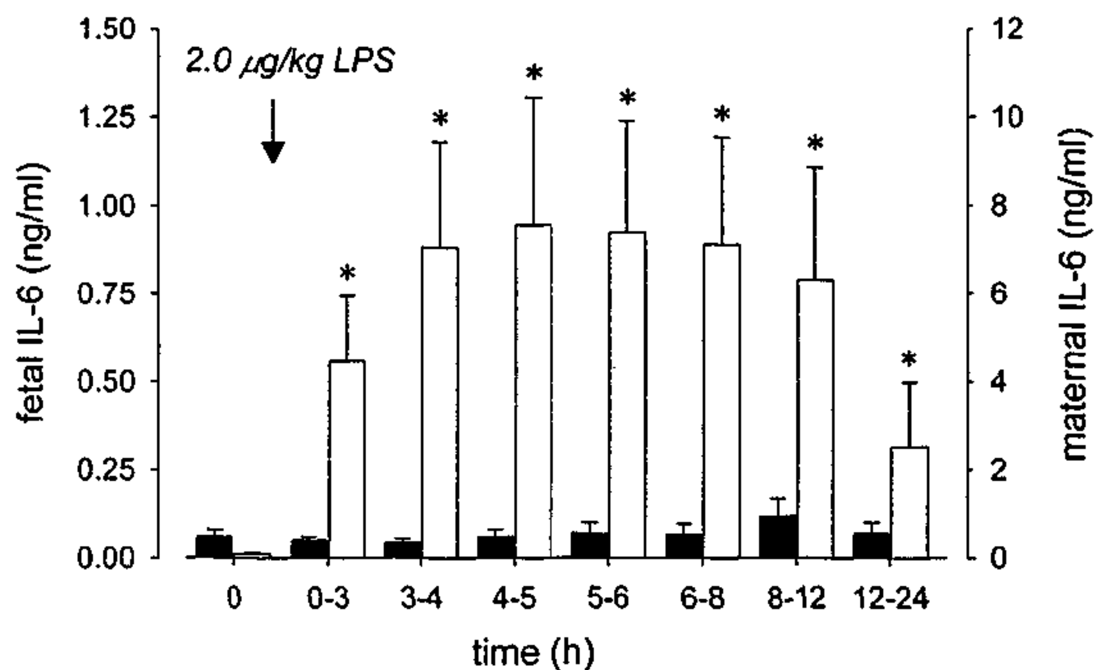

Figure 6.9 Fetal *(solid bars)* and maternal *(open bars)* plasma IL-6 concentrations prior to and after maternal systemic LPS (2.0 μg/kg, maternal body weight; n = 5) administration (time 0). Maternal values correspond to the right hand y-axis, while fetal values correspond to the left hand y-axis. Asterisks (*) indicate values significantly different from IL-6 concentrations, prior to LPS administration at time 0.

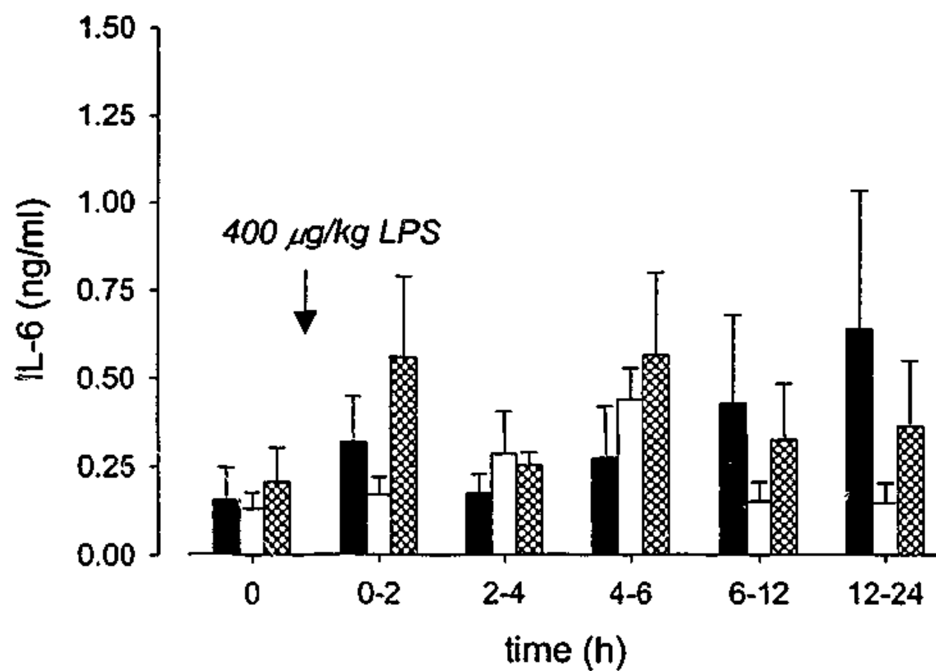

Figure 6.10 Fetal *(solid bars)* and maternal *(open bars)* plasma and amniotic fluid *(hatched bars)* IL-6 concentrations prior to and after intra-amniotic LPS (400 μg/kg, maternal body weight, total of ~20 mg; n = 4) administration (time 0).

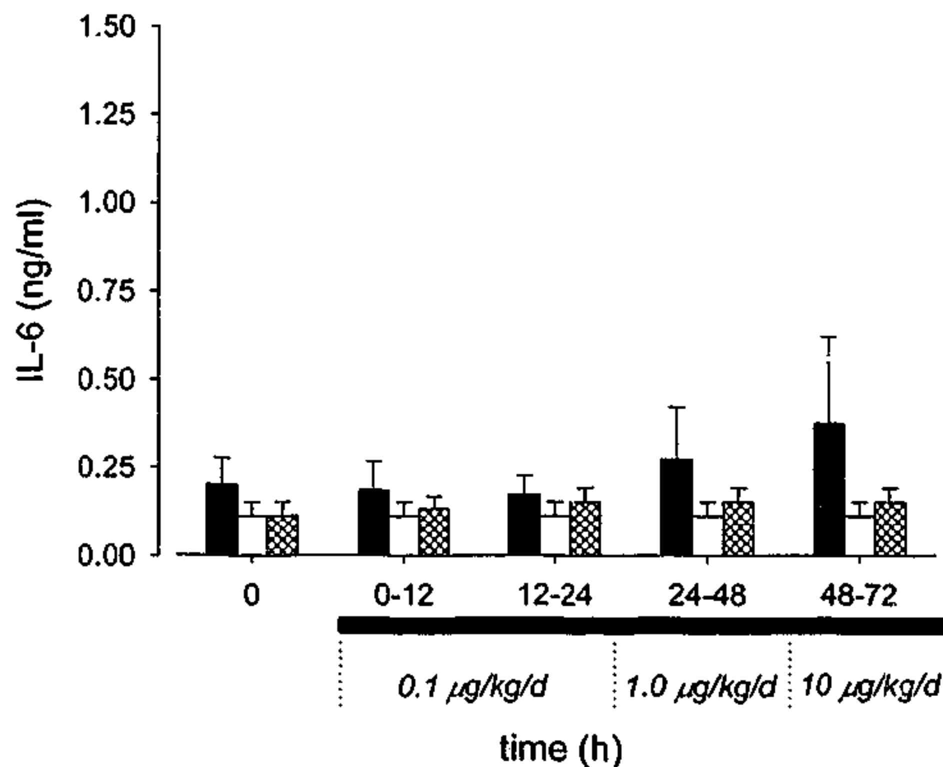

Figure 6.11 Fetal *(solid bars)* and maternal *(open bars)* plasma and amniotic fluid *(hatched bars)* IL-6 concentrations prior to and after extra-amniotic LPS (0.1, 1.0 or 10 μg/kg, maternal body weight; n = 3) administration (time 0). Black bar represents the continuous infusion of LPS in to the extra-amniotic compartment.

6.4 DISCUSSION

The association of intra-uterine infection and premature labour in women is well documented (Goldenberg *et al.*, 2000; Romero *et al.*, 2001; Goncalves *et al.*, 2002). The aim of this study was to investigate the effect of an inflammatory challenge during pregnancy in ewes with chronically catheterised fetuses so that the fetal responses during this inflammatory process could be examined. An inflammatory state was induced by LPS administration to the maternal systemic, intra- or extra-amniotic compartments to elucidate the mechanisms that may aid or adversely affect fetal well-being. Previous studies have used the administration of bacteria or bacterial products in animals to examine the mechanism of premature labour associated with infection, however, placental damage with consequent fetal death were also seen, and fetal monitoring was not possible with these models (Dombroski *et al.*, 1990; Kaga *et al*, 1996; Fidel *et al.*, 1998). The greatest advantage of using a sheep model in comparison with other laboratory species, is the ability to gain access to the fetal compartment and it's uterine environment for the insertion of physiological monitoring and sampling devices. Neither fetal or maternal death occurred in the present study, and although an increase in uterine activity was observed after LPS administration into the maternal circulation, delivery did not occur during this time. Interestingly, LPS administration directly into the amniotic fluid, or between the chorio-amnion and endometrial tissues also did not induce premature delivery in this study.

The principal finding of this study was that exposure of the maternal side of the placenta to LPS resulted in a marked rise in fetal PGE_2 and cortisol concentrations. Importantly, these increases were of a sustained nature, persisting for up to 48 h after maternal LPS treatment. There were also significant alterations in the fetal blood gases, consistent with previous observations (Schlafer *et al.*, 1994). Fetuses became hypoxemic, hypercapnic and acidemic at 6-12 h after maternal systemic LPS treatment. Ewes treated with systemic administration of LPS displayed an increase in body temperature, 3.5 h after LPS administration, a significant decrease in maternal arterial PaO_2 and, consistent with the observed maternal hyperventilation, ewes were mildly hypocapnic and alkalotic. These changes were also associated with clinical signs of mild respiratory distress (tachypnea, nasal flaring and grunting), which lasted for approximately 8 h. These effects are in agreement with previous observations of the pulmonary effects after systemic LPS administration in the ewe (Cefalo *et al.*, 1980). Thus, while ewes showed the usual signs of a mild inflammatory response, the present findings indicate that this maternal

response may have marked effects on fetal prostaglandin and cortisol concentrations as well as fetal well-being.

The rise in maternal cortisol and IL-6 concentrations are consistent with the induction of a maternal immune response following LPS administration (Fidel *et al.*, 1994; Schlafer *et al.*, 1994). The mechanisms that initiate these fetal changes after maternal systemic LPS exposure remain unclear. Maternal systemic LPS administration may stimulate the endometruim to secrete cytokines that then act on the placenta to increase prostaglandin production. Alternatively, it is possible that the fetus is affected by the placental passage of monocyte derived cytokines produced in the maternal compartment (Fidel *et al*, 1994). These cytokines would then be expected to enhance prostaglandin production by stimulating prostaglandin synthase type-2 (PGHS-2) within the placental trophoblast (McLaren *et al.*, 1996). This could account for the elevated prostaglandin concentrations in the fetal and maternal plasma, however this would not explain the different prostaglandin profiles in the two circulations and the amniotic fluid. The finding that, in the fetus, both PGE_2 and PGFM were elevated, whereas in the maternal circulation and amniotic fluid only PGFM rose, suggests that stimulation of PGHS-2 within fetal fibroblasts or cells on the fetal side of the placenta may cause the secretion of predominantly PGE_2 (Gyomorey *et al.*, 2000). The moderate rise in maternal PGFM in the plasma likely results from higher production within the uterine tissue. This rise would account for the increase in uterine activity that was observed following maternal LPS treatment. Furthermore, maternal LPS administration could also interfere with placental progesterone secretion causing prostaglandins to increase in the placenta and particularly in the endometrium. The finding that the animals did not deliver following maternal systemic or intra-amniotic LPS administration, despite increases in uterine activity, are consistent with the potent suppressive influence of progesterone produced by the placenta in this species, and which may not have been reduced sufficiently to cause premature labour. In contrast, intra-uterine infection has been associated with a fall in luteal progesterone production and plasma concentrations prior to the onset of parturition in the rabbit (Fidel *et al.*, 1998), and results in the onset of premature delivery in many other species (Dombroski *et al.*, 1990; Gravett *et al.*, 1994b; Kaga *et al.*, 1996).

The acute administration of *E. coli* endotoxin (0.2-0.5 mg/kg) to pregnant sheep has been shown to cause a significant fall in maternal arterial blood pressure as well as utero-placental blood flow, and therefore a reduction in oxygen delivery to the fetus (Bech-Jansen *et al.*, 1972). The induction of a mild maternal immune response following

systemic LPS administration may be sufficient to cause a reduction in maternal arterial blood pressure and utero-placental blood flow, and thus, may account for the fetal blood gas responses observed in the study. The increase in fetal cortisol concentrations after maternal LPS treatment, may have resulted from the passage of cortisol across the placenta, however, in late gestation the fetus is thought to be protected from the placental transfer of maternal cortisol by the abundant metabolising capacity within the placental barrier (Sun *et al.*, 1997). Alternatively, factors induced by LPS and released into the fetal circulation by the placenta may stimulate the fetal adrenals. The latter possibility is supported by the finding that maternal cortisol concentrations were not affected by injection of LPS into the amniotic fluid, but fetal concentrations were significantly increased by this treatment. Indeed, intra-uterine infections have been shown to increase fetal hypothalamic and placental production of corticotropin-releasing hormone, causing an increase in fetal corticotrpoin secretion, which in turn increases fetal adrenal production of cortisol (Gravett *et al.*, 2000). In addition, previous studies have shown a correlation between fetal oxygen saturation and hypothalamic-pituitary-adrenal activation, where fetal cortisol concentrations only increase when the decrease in oxygenation is close to that associated with the onset of metabolic acidosis (Carmichael *et al.*, 1997). The reduction in fetal oxygenation and pH observed in this study were in the ranges previously reported to increase cortisol concentrations (Carmichael *et al.*, 1997), thus, the increase in fetal cortisol concentrations observed in this study may have resulted from the fetal hypoxia associated with decreased utero-placental blood flow caused by maternal systemic LPS treatment. Moreover, the fetal cortisol concentrations in this physiological response were clearly not high enough or sustained long enough to initiate parturition in these ewes (Bassett & Thorburn, 1969; Schwartz & Rose, 1998). However, the finding that fetal cortisol concentrations are elevated during both maternal and intra-amniotic LPS treatment, may have significant clinical implications. Recent evidence from animal experiments suggests that repeated doses of antenatal corticosteroids to fetuses at risk of premature labour may have beneficial effects for fetal lung function, but may also have adverse effects on fetal brain function and fetal growth (Aghajafari *et al.*, 2002). Therefore elevation of fetal cortisol concentrations in response to maternal or intra-amniotic infection, coupled with subsequent antenatal corticosteroid treatment, may be detrimental to the fetus.

Previous studies by Kallapur *et al.* (2001), showed that an intra-amniotic LPS injection (20 mg), resulted in chorioamnionitis, an elevation in pro-inflammatory cytokine expression in the amnion/chorion and lung inflammation. We therefore used this model of intra-amniotic LPS administration to further investigate the fetal and maternal responses to endotoxin within the intra-uterine environment. In the current study, the presence of LPS in the amniotic fluid induced responses that were mainly restricted to the amniotic and fetal compartments. Intra-amniotic administration of LPS, 400 times that which would be sufficient to kill the fetus if given by intra-muscular injection (Kallapur *et al.*, 2001), only caused a moderate increase in uterine activity, amniotic fluid PGE_2 and fetal cortisol concentrations. These observations suggest that a mild fetal inflammatory response occurs following entry of endotoxins into amniotic fluid, although the route of entry of LPS and/or pro-inflammatory agents into the fetus remains to be determined. The fetus may swallow or aspirate the amniotic fluid containing pro-inflammatory agents, and once in the circulation, could lead to the fetal response and possibly sepsis (Romero & Mazor, 1988). A number of studies have suggested that the fetus is very sensitive to infectious challenges. The fetal sheep is much less sensitive to LPS compared to the adult, with doses of LPS > 1 μg/kg often being fatal (Schlafer *et al.*, 1994), while doses of 1-100 μg/kg are used in studies with adult animals (Fidel *et al.*, 1994; Kaga *et al.*, 1996). Our finding that fetal responses were moderate, despite the relatively high dose of LPS administered, may indicate a potential role for the amniotic fluid in protecting the fetus from endotoxin exposure during pregnancy. Preliminary studies investigating the effect of a continuous infusion of a lower dose of LPS into the amniotic fluid (1 mg/h for 24 h, data not shown), to mimic that of a continuous inflammatory challenge resulted in no change in prostaglandin concentrations, uterine activity or blood gas parameters, suggesting that the infusion of LPS into the amniotic fluid is not as effective in producing the inflammatory responses as seen after bolus intra-amniotic LPS administration. The factors responsible for the increased prostaglandin concentrations in the amniotic fluid after intra-amniotic LPS administration probably involve direct stimulation of the amnion and chorion by pro-inflammatory cytokines (Gravett *et al.*, 1994a). Alternatively, LPS administration may decrease the metabolism of prostaglandins by decreasing prostaglandin dehydrogenase expression and activity within uterine tissues (Hahn *et al.*, 1998).

To assess the accessibility of endotoxins produced in the fetal membranes to the amniotic fluid and fetus, a space between the chorio-amnion and the endometrial tissues

was created to allow LPS to enter directly between the fetal membranes and maternal endometrium. Extra-amniotic placement of catheters, similar to that used in the present study, have been successfully used without destroying the integrity of the fluid sacs, and are a useful delivery route to test the effects of substances on myometrial activity (Robinson *et al.*, 1978; Thorburn *et al.*, 1982). Extra-amniotic administration of LPS at the doses of 0.1, 1.0 or 10 μg/kg/d resulted in no overt fetal or maternal inflammatory responses. In this group of animals we chose to administer LPS as a continuous infusion, as prolonged exposure to bacterial products at sublethal doses have been suggested to be effective in triggering the onset of premature labour (Kaga *et al.*, 1996), and a more appropriate way to investigate fetal inflammatory responses during pregnancy. The small infusion volume was chosen as we did not wish to disrupt the chorio-amnion membranes. The finding that there were no changes observed in the uterine EMG activity or maternal prostaglandins in these ewes, suggests any inflammatory process induced may have been too limited in area to have created a systemic response. Interestingly, extra-amniotic infusion of $PGF_{2\alpha}$ to late gestation sheep using the same procedure as that used in this study, increases fetal and maternal plasma PGFM concentrations, but does not alter myometrial activity, suggesting that the ovine myometrium, when under the influence of progesterone, is relatively insensitive to prostaglandins (Thorburn *et al.*, 1982). Although an increase in fetal PGFM concentrations were observed during the infusion of 1.0 μg/kg/d and 10 μg/kg/d of LPS into the extra-amniotic compartment, this was of insufficient magnitude to increase myometrial activity under the influence of progesterone at this time. The lack of effect of LPS within the extra-amniotic compartment may also be explained by the differences in the placentation of sheep, compared to humans. In the sheep, placentation is less invasive than in humans (Steven, 1975), with the uterine epithelium remaining intact and is only in loose contact with the chorio-amnion. This difference may explain why infectious agents associated with the fetal membranes are more likely to transmit to the decidua and subsequently result in premature labour.

The present findings show that maternal LPS treatment caused marked changes in fetal blood gas parameters and sustained increase in fetal prostaglandins and cortisol concentrations. The presence of LPS in the amniotic fluid induces responses that are restricted to the amniotic and fetal compartments, with no overt responses detected in the maternal circulation. The attenuated fetal responses to intra-amniotic LPS administration, despite infusions of LPS at doses two hundred times those used in the

adult, may indicate a role of the amniotic fluid in protecting the fetus from endotoxin exposure during pregnancy.

Chapter 7

General Discussion

Normal labour results from physiological activation of a common terminal pathway involving the production and release of prostaglandins. This pathway underlies the major physiological changes that occur leading to enhanced uterine contractility and the onset of parturition in many species (Karim, 1972; Liggins *et al.*, 1973). Premature labour is a pathological condition caused by multiple aetiologies that can activate several components of this pathway (Ivanisevic *et al.*, 2001). The objective of treatments for delaying premature labour is to reduce perinatal morbidity and mortality associated with very premature babies. By increasing the length of pregnancy, neonatal survival rates are dramatically improved. Although our ability to care for these premature neonates has vastly improved, the rate of premature labour remains unchanged, partly due to our inability to selectively inhibit the labour associated uterine and cervical changes.

Labour is a process involving multiple factors that work in concert amplifying the cascaded of events leading to the delivery of the fetus. Increased prostaglandin concentrations (Challis *et al.*, 1976) and oxytocin receptors concentrations (Fuchs *et al*, 1982a) are involved in normal and premature labour, but drugs that inhibit the synthesis and/or action of prostaglandins and oxytocin, have been extensively used for the inhibition of premature labour, with variable success (Sawdy *et al.*, 1997; Moutquin *et al*, 2000; Group, 2001b). The studies presented here are the first to describe the use of such drugs in combination for the inhibition of premature labour (Grigsby *et al.*, 2000). The two drugs that were used in the present studies were nimesulide, a selective PGHS-2 inhibitor, and the oxytocin receptor antagonist, atosiban. The inclusion of a second drug that inhibits the action of oxytocin, markedly improved the effectiveness of prostaglandin inhibition for delaying premature labour. In addition, these findings also indicate that the combined treatment with these compounds was effective in blocking the interplay between $PGF_{2\alpha}$ and oxytocin before labour onset as well as in suppressing this positive mechanism after premature labour is initiated. Furthermore, fetal well-being appeared not to be compromised during treatment with nimesulide and

atosiban, adding to the potential advantages of this drug regime over previously available treatments. These findings strongly support the use of selective inhibitors of PGHS-2 in combination with oxytocin receptor antagonists as a potential approach for delaying premature delivery. The use of drugs such as atosiban to supplement PGHS-2 inhibition, may therefore enable sufficiently low doses of prostaglandin synthase inhibitors to be used to minimise problems associated with fetal distress. These studies have identified a new strategy for blocking premature birth with the possibility of future clinical application for improving the survival and health of prematurely delivered babies.

These studies have also highlighted the importance of oxytocin in the process of parturition. The role of oxytocin in ovine and human parturition, for many years has been controversial, more conclusive evidence came with the demonstration that oxytocin receptors increase gradually throughout pregnancy and dramatically increase with the onset of labour (Soloff, 1979; Fuchs *et al.*, 1982a; Wathes *et al.*, 1996). The present observations suggest that oxytocin is, in fact, an important factor in the generation of adequate uterine activity during labour, switching the uterine activity from contractures to contractions during parturition, a notion previously reported by Nathanielsz *et al.* (1995). Furthermore, the involvement of the oxytocin-induced prostaglandin release during the final stages of parturition was also demonstrated. Circulating prostaglandin concentrations in ewes treated with nimesulide alone were increased at the time of delivery or elective *post mortem*. This rise was modest, and the level achieved was no greater than that observed prior to nimesulide treatment. These findings suggest that the rise in prostaglandin concentrations in ewes treated with nimesulide alone may have been due to oxytocin-induced PGHS activity, which was not totally inhibited at the dose of nimesulide used. An increase in oxytocin together with the small increase in prostaglandin concentrations, reflecting an increase in production in gestational tissues, may have facilitated cervical dilation. This may have initiated further oxytocin release during treatment with nimesulide alone, allowing adequate uterine activity for fetal expulsion. These studies have demonstrated the ability of nimesulide together with atosiban treatment to block the interplay between prostaglandins and oxytocin before the onset of labour, and also in suppressing this positive feed-back mechanism after premature labour is initiated.

Placental PGHS-2 expression in nimesulide alone and nimesulide and atosiban-treated ewes were not different from that in control ewes that were not in labour. These

findings support and extend the novel suggestion that this enzyme may be positively regulated by one of its products, a concept which has been suggested previously by Tsai and Wiltbank (1997) and Wu *et al.* (1998). These observations are important in the clinical context as they indicate that due to the lack of up-regulation of the PGHS-2 mRNA at this time, a rebound production of prostaglandins is unlikely to occur after the cessation of tocolytic treatment. This is further supported by the observation that ewes failed to show a dramatic increase in prostaglandin concentrations or uterine activity after the cessation of nimesulide and atosiban infusions for at least 6 h, despite continued fetal glucocorticoid treatment for the induction of premature labour (refer to Chapter 5). This was surprising considering the relatively short half-lives of nimesulide and atosiban. Whether this ongoing suppression of prostaglandin synthesis is a consequence of continued glucocorticoid exposure creating a high oestrogenic environment or loss of the stimulus for PGHS-2 expression requires further investigation. However, the present findings indicate that a possible rebound rise in prostaglandin production and uterine activity following the cessation of tocolytic treatment with selective PGHS-2 inhibitors may not be a problem in the clinical application of this treatment.

Any tocolytic agent carries the risk of adversely effecting the mother and fetus, and there is always the added risk, when combining two drugs, that the chance of any detrimental side effects is significantly increased. However, serious adverse side effects were not observed in either of the nimesulide and atosiban studies. There were no significant differences in fetal blood gas parameters between groups except for values taken immediately before delivery or elective *post mortem*. At this time there was a significant decrease in fetal oxygen saturation in ewes that had been treated with nimesulide alone. This may have resulted from the prolonged period of elevated uterine activity seen in these animals. Examination of the ductus arteriosus of each fetus at *post mortem*, indicated patency was maintained in all fetuses from ewes that had been treated with nimesulide and atosiban. The finding that nimesulide does not adversely affects fetal survival supports the proposal that a selective PGHS-2 inhibitor may suppress placental PHGS-2 activity, but may have little or no effect on PHGS-1 activity in the premature ductus (Coceani *et al.*, 2001). In addition, the blockade of glucocorticoid-induced premature labour requires higher inhibitor doses than may be needed with less potent stimuli. Consequently, lower doses of inhibitors with less impact on the fetus may be needed, for example, in women where the rise in intra-uterine PGHS-2 mRNA

expression at premature labour is relatively small and of similar magnitude to the rise seen at normal labour onset (Mijovic *et al.*, 1998). Thus, by combining selective PGHS-2 inhibitors with atosiban, it may be possible to titrate the amount of PGHS inhibitor needed to delay labour adequately, thus further reducing the potential detrimental effects on the fetus.

An increase in glucocorticoids associated with premature labour in sheep has been suggested to be responsible for placental separation in this species (Jack *et al.*, 1975). Despite dexamethasone administration initially stimulating prostaglandin production, no such detachment was seen in animals completing the nimesulide and atosiban treatment, in both inhibitor studies, indicating that placental separation may not be due to a direct effect of glucocorticoids but may also require a rise in prostaglandin production. If glucocorticoids play a major role in placental separation, independent of prostaglandin concentrations, a greater reduction in fetal oxygen might be observed in the nimesulide or nimesulide and atosiban-treated animals, due to prolonged placental degradation and reduced placental blood flow. This was however, not the case, further indicating that placental malfunction did not occur in these animals. Furthermore, the effectiveness of nimesulide and atosiban in inhibiting uterine activity also likely contributes to maintaining normal placental function. These finding indicate that inhibition of oxytocin-induced uterine activity further delays premature labour and reduces the potential adverse effects on fetal blood gas parameters.

Recently, a range of new drugs with greater selectivity for PGHS-2, for example SC-56835 and celecoxib, have become available that may allow for further specific suppression of PGHS-2 activity. Although these selective PGHS-2 inhibitors are now available for the treatment of inflammatory conditions, few studies have investigated the effect of these compounds in pregnancy. Whether these new inhibitors may be able to completely block the activity of PGHS-2 and residual uterine activity observed in the present studies, and thus have greater efficacy in delaying premature labour remains to be determined.

Recent studies have shown the expression of PGHS-2 mRNA in the ductus arteriosus and that the new PGHS-2 inhibitors such as celecoxib, can produce significant constriction in the fetal lamb ductus *in vivo* (Takahashi *et al.*, 2000). Evidence also now suggests that PGHS-2 may be vital for certain aspects of fetal organ development and physiological function since PGHS-2 mRNA expression increases in human fetal kidney, lung and intestine in the third trimester (Olson *et al.*, 2001). The effect of

prostaglandin inhibition and oxytocin receptor blockade on fetal renal function was not directly investigated in the current studies, however, recently nimesulide has been shown to caused oligohydramnious (Holmes & Stone, 2000). A similar side effect has previously been associated with indomethacin treatment (Novy *et al.*, 1974; Itskovitz *et al.*, 1980). Together, these findings suggest that while short term exposure to highly selective PGHS-2 inhibitors may not be detrimental to the fetus, longer term treatment may have serious adverse effects on fetal development.

The initial activation of the myometrium involves a number of processes, leading to the increased appearance of myometrial oxytocin receptors (Fuchs *et al.*, 1982a; Wathes *et al.*, 1999), and prostaglandin receptors (EP_{1-4}, FP) (Brodt-Eppley & Myatt, 1998; Ma *et al.*, 1999; Smith *et al.*, 2001a). Further stimulation of the myometrium results from increased concentrations of $PGF_{2\alpha}$, PGE_2 and oxytocin, that enhance contractile activity of the activated myometrium. A significant increase in contractile myometrial prostaglandin $F_{2\alpha}$ receptor (FP) mRNA expression occurs with the onset of labour in many species including human, sheep and baboon (Brodt-Eppley & Myatt, 1999; Ma *et al.*, 1999; Smith *et al.*, 2001a). For these reasons, the FP receptor is gaining much attention as a potential new target for tocolytic treatment. Indeed, newly designed FP receptor antagonists have been shown to delay premature labour in rats (Quiniou *et al*, 2001). These preliminary studies indicate FP receptor antagonists may be an alternative treatment for the prevention of premature labour. The combination of an FP receptor antagonist with atosiban may prove beneficial for the safe prevention of premature labour in the future.

Whether it be the inhibition of prostaglandins or prostaglandin receptors, the concept of a combined tocolytic treatment, as demonstrated in these studies, is now being trialed in clinical practice. The BORN clinical trial (Birth Outcomes with Rofecoxib and Nifedipine), aims to evaluate the use of a combination of the highly specific PGHS-2 inhibitor, rofecoxib, and nifedipine, compared to nifedipine treatment alone and will hopefully lead to improved neonatal outcomes.

Another major problem that adversely affects neonatal outcome and often leads to premature labour is exposure to infection during late pregnancy. The mechanisms by which the fetus and mother respond to an inflammatory challenge during pregnancy was investigated by examining the effect of the administration of LPS, in an attempt to elucidate the mechanisms that may aid or adversely affect fetal well-being. The major finding of this study was that exposure of the maternal side of the placenta to LPS

resulted in a marked rise in fetal prostaglandin and cortisol concentrations. Importantly, these increases were of a sustained nature, persisting for up to 48 h after LPS treatment. There were also significant alterations in the fetal blood gases, consistent with previous observations (Schlafer *et al.*, 1994).

The finding that fetal cortisol concentrations were elevated during both maternal and intra-amniotic LPS treatment, may have significant clinical implications. Recent evidence from animal experiments suggests that repeated doses of antenatal corticosteroid administration to women at risk of premature labour may have beneficial effects for fetal lung function, but may also have adverse effects on fetal brain function and fetal growth (Aghajafari *et al.*, 2002). Therefore elevation of fetal cortisol concentrations in response to maternal or intra-amniotic infection, coupled with subsequent antenatal corticosteroid treatment, may have major detrimental effects on the fetus.

Interestingly, the presence of LPS in the amniotic fluid, at much larger doses than that normally tolerated systemically, induced responses that were mainly restricted to the amniotic and fetal compartments, with no overt responses detected in the maternal circulation. A number of studies suggest the fetus is very sensitive to infectious challenges. The fetal sheep is much less tolerant of LPS compared to the adult, with doses of LPS > 1 μg/kg often being fatal (Schlafer *et al.*, 1994), whereas doses of 1-100 μg/kg are used in studies with adult animals (Fidel *et al.*, 1994; Kaga *et al.*, 1996). This suggests the attenuated fetal responses are not due to the inability of the fetus to response to an inflammatory challenge, but that the fetus is highly sensitive. These observations have lead to the suggestion that the amniotic fluid appears to act to suppress fetal responses and may protect the fetus from endotoxin exposure during pregnancy. This protection could either be from the direct actions of LPS or protection from the response to LPS that is observed in the amniotic fluid via an induction of cytokines.

The presence of modulators capable of down regulating cytokine production within the amniotic fluid may be a necessary component of immune regulatory mechanisms working during pregnancy (Gravett *et al.*, 1994a). Under conditions of reduced uterine placental blood flow, the placenta and fetal membranes produce considerable amounts of activin A. This concomitantly increases concentrations of activin A in the amniotic fluid and fetal plasma, consequently, activin A has been suggested as a marker of fetal distress in late gestation (Jenkin *et al.*, 2001). Activin A has also been shown to display anti-inflammatory properties, by blocking the effect of IL-1β and IL-6 (Brosh *et al*,

1995; Ohguchi *et al.*, 1998; Yu *et al.*, 1998). Activin A concentrations were measured in samples obtained from the fetal and maternal plasma and amniotic fluid of ewes that were administered LPS directly into the amniotic fluid (Assays performed by Dr. D. Phillips and Ms A. O'Connor, data not presented in this thesis). Activin A concentrations were higher in the amniotic fluid than those observed in the fetal and maternal circulation. In addition, activin A concentrations were significantly elevated in the amniotic fluid at 6-12 h after intra-amniotic LPS administration. Thus activin A may be a candidate involved in protecting the fetus from LPS present in the amniotic fluid and from inflammatory responses during pregnancy.

Another naturally occurring modulator capable of down regulating cytokine production, is the interleukin-1 receptor antagonist protein (IL-1ra) (Gravett *et al.*, 1994a), which has been shown to increase in the amniotic fluid from non-human primates after intra-uterine infection (Gravett *et al.*, 1994a). IL-1ra competitively inhibits the biological responses induced by IL-1 such as IL-6, collagenase, and PGE_2 production (McIntyre *et al.*, 1991). Large amounts of IL-1ra within the amniotic fluid can inhibit the induction of the cytokine cascade and prostaglandin production thereby preventing the development of overt inflammation (Fukuda *et al.*, 2002). Whether or not these inflammatory modulators are involved in suppressing the fetal response to LPS in the present animal model remains to be fully elucidated.

Romero and Mazor (1988) suggested that in the human, an intra-uterine infection results from an ascending infection originating from the extra-amniotic cavity, which then proceeds through the fetal membranes resulting in an intra-amniotic infection and subsequent premature labour. Many studies have investigated this hypothesis by administering bacteria or bacterial products to pregnant animals as a model of human premature labour (Gravett *et al.*, 1994b; Fidel *et al.*, 1998 ; Kaga *et al*, 1996 ; Schlafer *et al.*, 1994). Many of these studies have also been accompanied by fetal death (Fidel *et al*, 1994; Kaga *et al.*, 1996; Schlafer *et al.*, 1994). Interestingly, LPS administration directly into the amniotic fluid, or between the chorio-amnion and endometrial tissues did not induce premature delivery in sheep, as the Romero model would predict, nor did it casue fetal death. These observations thus indicate that this is an appropriate model for examining the fetal responses to a maternal inflammatory challenge during pregnancy, as opposed to other studies which have used higher doses of LPS that have not only resulted in premature labour, but also in fetal death. This study may therefore aid in our understanding of the ambiguous responses to inflammation during pregnancy and, in

turn, why some women develop intra-uterine infections and deliver prematurely and why others do not. With respect to the latter situation, in this study no overt responses were detected in the maternal circulation after intra-amniotic LPS administration. Similarly, this has been shown to occur in women, where intra-uterine infections can occur quiet early in pregnancy and remain undetected for several months (Gravett *et al*, 2000), thus exposing the fetus to the effects of infection for a considerable period. As demonstrated in this study, the fetus is however, affected by, and does respond, to this type of inflammatory challenge. Although in this model, the fetal responses are somewhat dampened compared to the fetal responses observed after maternal systemic LPS administration, the consequences of a sustained and continual infection on fetal well-being may be considerable, and should be investigated further. Understanding the fetal inflammatory responses to infection is essential for the development of better approaches in the detection and treatment of intra-uterine infections during pregnancy.

In summary, premature labour is a complex and multi-factorial event, thus the treatment for premature labour should be aimed at inhibiting the synthesis and action of two of the separate but related major uterine stimulants involved in this process. Prostaglandins and oxytocin are excellent candidates due to the essential role of these stimulants in the positive feed-forward system in the processes of parturition. These studies have clearly demonstrated the effectiveness and advantages of a combined treatment of PGHS-2 inhibition and oxytocin receptor antagonism for delaying premature labour. In addition, these studies have provided insight into the fetal responses to inflammatory challenges during pregnancy and may provide an indication as to when it may be appropriate to delay cases of premature labour especially when associated with infection. The model used in these studies may also aid in our interpretation of the ambiguous responses to inflammation during pregnancy.

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