The expression of prostanoid receptor genes in uterine and fetal tissues.

Studies in the maternal and fetal baboon and the fetal and neonatal lamb

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Abstract.

1. The aim of this project was to determine whether advancing gestational age and parturition were associated with alteration in the relative level of expression of genes encoding prostanoid receptors in key uterine and fetal tissues. I also sought to determine whether advancing gestational age and parturition were associated with alteration in the expression of genes encoding lipoxygenase (LOX) enzymes in key intra-uterine tissues.

2. Caesarean hysterectomy was performed on 15 pregnant baboons in the last third of pregnancy. Samples of myometrium (from multiple uterine sites), cervix, decidua and chorion were obtained. In addition, the ductus arteriosus was obtained from nine fetal baboons, 28 fetal lambs and 4 neonatal lambs. Expression of genes was studied using Northern blot analysis and in situ hybridization. Expression of genes was quantified by Northern analysis as a ratio of the signal for the gene of interest to each of three housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH], beta-actin and cyclophilin). Statistical comparison of the effects of gestational age and labour was performed using linear regression, Student's t-test, repeated measures analysis of variance and analysis of covariance, as appropriate.

3 Initial studies of animals not in labour using cDNA probes demonstrated transcripts of similar size to the human genes for prostanoid EP_2, EP_3, EP_4, and FP receptor mRNA using Northern blot in myometrium. Myometrium from the lower uterine segment (LUS) had greater expression of EP_2 receptor mRNA and less expression of EP_3 mRNA compared with the fundus and corpus. However, similar levels of EP_4 and FP receptor

mRNA were observed comparing the fundus and LUS. Expression of EP_2, EP_3 and EP_4 receptor mRNA were also detected in cervix, decidua and chorion. EP_2 mRNA was most abundant in cervix, EP_3 was most abundant in myometrium and EP_4 mRNA was most abundant in decidua. The variation in myometrial expression of genes encoding EP receptor sub-types paralleled the contractile responses of paired samples (reported elsewhere).

4 When expression of prostanoid receptor genes was studied in myometrium obtained from animals both in labour and not in labour and the techniques employed were optimized (principally the use of riboprobes), transcripts of similar size to the human genes were detected for prostanoid EP_1, EP_2, EP_3, EP_4, IP, FP and TP receptor mRNA using Northern blot. There were no gestational age related changes in expression of these genes. Expression of EP_1, EP_3 and IP receptor mRNA was significantly higher in myometrium from the fundus (compared with lower segment) whereas EP_2 gene expression was significantly lower in the fundus. Labor was associated with a reduction in the regional variation of both EP_2 and IP receptor gene expression, but not EP_1 and EP_3 expression. Labor was also associated with an overall lower level of expression of EP_2 receptor mRNA.

5 When expression of prostanoid receptor genes was studied in cervix obtained from animals both in labour and not in labour, clear signals which were similar in estimated size to the human genes were detected by Northern analysis for EP_1, EP_2, EP_3, EP_4, FP, IP and TP receptors. Expression of the gene encoding the prostanoid EP_1 receptor increased with advancing gestational age prior to labor. Expression of the EP_2, FP and TP

receptor genes was much lower in animals that were delivered during spontaneous labor than in animals which were not in labor.

6 When expression of prostanoid receptor genes was studied in decidua and chorion obtained from animals both in labour and not in labour, expression of the genes encoding the EP_1 and FP receptor in decidua and the EP_4 receptor in chorion was lower with advancing gestational age. Expression of the EP_2 receptor gene was lower in labour in decidua, whereas expression of the IP receptor gene was higher in labour in both decidua (2-fold) and chorion (4-fold).

7 Expression of both the EP_3 and EP_4 receptor genes was detected in lamb ductus arteriosus and the level of expression of both genes in the fetal ductus was unaffected by maternal administration of corticosteroids. Expression of the EP_4 receptor gene was lower in the ductus obtained from term lambs compared with preterm lambs, and was lower still in neonatal lambs, whereas no variation was observed in EP_3 receptor gene expression with respect to gestational age and birth. Expression of the EP_4 receptor gene was also confirmed in fetal baboon ductus arteriosus and maternal administration of corticosteroid did not reduce EP_4 receptor gene expression in the baboon.

8 Signals of similar size to human 5-LOX and platelet 12-LOX genes were detected in myometrium, cervix, decidua and chorion. Expression of 5-LOX mRNA decreased with advancing gestational age in the cervix. In decidua, expression of 5-LOX mRNA was higher in tissues from animals in labor, whereas in chorion, 5-LOX expression was lower

in tissues from animals in labor. Expression of the platelet 12-LOX gene decreased with advancing gestational age in chorion and was lower in labor in cervix.

9 Cellular localization of the EP_3 receptor was possible in myometrium and of the EP_4 receptor gene in ductus arteriosus. In the latter case, expression of the EP_4 receptor gene was strikingly specific for the ductus arteriosus compared with neighbour vessels. Expression of the EP_3 receptor gene was evident in myometrial smooth muscles cells but not in the smooth muscle cells of muscular arteries supplying the uterus.

10 The following general conclusions are drawn from these studies:

(a) The myometrial expression of prostanoid receptor genes varies within the uterus and the reduced contractile response of LUS to PGE_2 is paralleled by greater inhibitory EP_2 receptor expression and less contractile EP_1 and EP_3 receptor expression, a similar pattern to the cervix.

(b) Regional variation in the expression of prostanoid receptor genes persists in labour and suggests a role for regional variation in myometrial sensitivity to prostaglandins in the polarity of uterine contraction

(c) The pattern of expression of prostanoid receptor genes varies with advancing gestational age in cervix, chorion and decidua. Variation in the sensitivity of these tissues to the effects of PGs may have a role in the preparation for parturition in the baboon.

(d) The pattern of expression of prostanoid receptor genes varies in association with labour in myometrium, cervix, chorion and decidua. In particular, decreased EP_2 receptor gene expression was noted in myometrium, cervix and decidua. This implies that

variation in the sensitivity of these tissues to PGs in general, and to PGE_2 in particular, might promote parturition in the baboon.

(e) The preparation of the fetus for birth also involved alterations in the relative expression of genes encoding contractile and inhibitory EP receptor sub-types. Specifically, contraction of the ductus arteriosus at birth may be stimulated by loss of inhibitory effects of PGE_2 through the EP_4 receptor in the presence of maintained contractile effects of PGE_2 through the EP_3 receptor.

(f) Variation in expression of LOX genes may play a role in the onset of parturition in the baboon. However, it is not apparent whether the role of LOX is principally to generate biologically active products, or to divert free cellular arachidonic acid to relatively inert products rather than PGs.

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Abbreviations

AA	arachidonic acid
ACTH	adrenocorticotropic hormone
ATP	adenosine triphosphate
BSA	bovine serum albumin
c	complementary
COX	cyclo-oxygenase
CRH	corticotrophin releasing hormone
CTP	cytosine triphosphate
d	deoxy
DHEAS	dihydroepiandrosterone
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMG	electromyogram
GTP	guanosine triphosphate
HPAA	hypothalamo-pituitary adrenal axis
HPETE	hydroperoxyeicosatetraenoic acid
LOX	lipoxygenase
LT	leukotriene
LUS	lower uterine segment
MLC	myosin light chain
MLCK	myosin light chain kinase
MOPS	3-(*N*-morpholino)propanesulfonic acid

NSAID	non-steroidal anti-inflammatory drug
PG	prostaglandin
PGDH	prostaglandin dehydrogenase
PL	phospholipase
PPH	post partum haemorrhage
PPROM	preterm, premature rupture of the membranes
PTHrP	parathyroid hormone related polypeptide
RNA	ribonucleic acid
TEA	triethanolamine
SDS	sodium dodecyl sulphate
SSC (1x)	0.15M NaCl and 0.015M sodium citrate, pH 7.0
TTP	thymidine triphosphate
Tx	thromboxane
UTP	uracil triphosphate
UV	ultraviolet

Presentations arising from this work.

Smith GCS, Baguma-Nibasheka M, Wu WX, Nathanielsz PW. The reduced contraction of baboon lower uterine segment to prostaglandin E_2 is paralleled by increased EP_2 and decreased EP_3 receptor mRNA compared with fundus. Society for Gynecologic Investigation, 45th Annual Meeting, Atlanta, GA, USA, March 1998, abstract published J. Soc Gyn Invest 1998;5:64A.

Smith GCS, Wu Wx, Nathanielsz PW. Prostaglandin EP receptor sub-types have characteristic patterns of expression in baboon myometrium, cervix, decidua and chorion. Society for Gynecologic Investigation, 45th Annual Meeting, Atlanta, GA, USA, March 1998, abstract published J. Soc Gyn Invest 1998;5:190A.

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Smith, GCS, Wu, WX, Nathanielsz, PW. Effects of gestational age and labor on expression of prostanoid receptor genes in baboon uterus. *Biology of Reproduction* (accepted pending revision).

Smith, GCS, Wu, WX, Nathanielsz, PW. Expression of prostanoid receptor genes in baboon chorion and decidua during pregnancy and parturition. *Journal of Endocrinology* (accepted pending revision).

There are three other first author papers submitted for publication on the work described in this thesis.

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Declaration.

This thesis is entirely my own work. The research it describes necessarily involved the contributions of a large group of people and the planning of experiments was outlined in association with Prof Nathanielsz. However, all the laboratory work described in this thesis was performed by myself. The analysis of data and writing up of these experiments was done by myself. Both Prof Nathanielsz and Prof Cameron made constructive comments on the final draft of this thesis and both Prof Nathanielsz and Dr Wen Wu made comments on the individual papers which make up this body of work.

Chapter 1. Prostaglandins and parturition: a review.

One of the key biological features of mammalian species is viviparity. By necessity, the successful completion of the process is essential for propagation of the species. Although parturition is common to all mammals, the physiological properties of the process are as diverse as mammalian species, which is to say different in many respects but with certain common features. Mammalian reproduction in characterized by (1) in vivo fertilization, (2) implantation of the conceptus and formation of the placenta, (3) development of the embryo, (4) growth of the fetus in an enlarging, essentially quiescent uterus, (5) expulsion of live born young, and (6) post-partum contraction of the uterus followed by uterine involution.

A detailed review of any aspect of mammalian parturition would require a volume many times larger than the whole of this thesis and the aims of this chapter are, first, to give an overview of the process of mammalian parturition accompanied with reference to more detailed discussion and, secondly, to discuss in greater detail the role of PGs in the control of parturition.

1.1 General aspects of mammalian parturition

One of the key differences in the control of parturition comparing mammalian species is the role and source of steroid hormones in the control of the process, namely, oestrogen, progesterone and corticosteroids [1]. These hormones have key roles in all species, but the source of these hormones and their effects on the process of labour significantly differ. Much of the research on the control of parturition has been in littering animals, specifically the rat. The reason for its widespread use is largely one of easy maintenance

in a laboratory setting. More recently the mouse has been a focus for study as it is readily utilized for studies of null mutations of its genome. A number of these so-called "knock out" experiments have demonstrated that mice which are null mutant for certain genes have delayed parturition, including the FP receptor gene [2], cyclo-oxygenase-1 (COX-1) [3] and steroid 5α reductase type I [4].

While studies in these animals can yield important information about the physiological control of parturition in these species, some caution has to be applied when extrapolating these results to the human and the attendant clinical problems which are the stimulus to much of this research. There are important species differences in the role and source of key steroid hormones in the process of labour (see section 1.5.2 for detailed discussion). The key factor regulating parturition in rodents is involution of the corpus luteum (luteolysis). The corpus luteum is the source of progesterone in these animals and high concentrations of progesterone maintain uterine quiescence. Falling concentrations of progesterone in these animals, following luteolysis, promote labour. In sheep, progesterone has the same role but the source is the placenta. In humans and non-human primates, progesterone is also formed in the placenta after the first trimester, but there is no change in circulating concentrations of progesterone preceding labour. Many of the cellular, autocrine and paracrine mediators are common to rodents and higher species. The specific differences are discussed below and have been reviewed elsewhere [1]. Prostaglandins, which are the focus of the present study, are thought to have a key role in the process of parturition in all mammalian species studied so far [5].

1.2. Uterine activity during pregnancy

The activity of the myometrium in pregnancy is frequently misunderstood. The state is caricatured as the uterus being quiescent during the antepartum period and then active throughout labour. The reality is that the uterine smooth muscle is active throughout pregnancy but that the patterns of activity are complex and change with its differing physiological roles.

1.2.1 Ante-partum uterine activity

For obvious reasons, most invasive studies of ante-partum uterine activity have been performed in animal species. These studies have revealed that in the ante-partum period the uterus exhibits low amplitude, low frequency activity which has been characterized as "contractures" [6]. This activity is associated with smooth muscle depolarisation, but only induces very small increases in intra-uterine pressure and does not result in dilatation or effacement of the cervix. This activity does, however, alter the environment of the fetus, such as fetal arterial oxygen tension, and the frequency of contractures can influence maturation of fetal cardiovascular control systems [7].

1.2.2 Intra-partum uterine activity

Uterine activity during labour is characterized by high frequency, high amplitude activity which are termed "contractions". In the human a typical pattern of intra-partum activity in the second stage of labour would be four contractions every 10 minutes, with a peak pressure of up to 60-70mmHg, and a total duration of 60 seconds with the pressure curve being bell-shaped [8]. From this it is evident that the uterus is only actively generating tension for 2 minutes out of every 10. For another 2 minutes it is actively relaxing and for

the remaining 6 minutes out of every 10, the intra-uterine pressure is at basal levels. Therefore, considering the uterus as merely moving from a purely passive state before labour into a purely active state during labour is not merely an unfortunate simplification, but is quite wrong.

Another physiological feature of intra-partum uterine activity is that it is associated with polarity, i.e. it is directional, forcing the presenting part of the fetus towards the cervix. This suggests that the contractile role of the myometrium during labour is likely to vary according to the site of that myometrium within the uterus. The fundus and corpus of the uterus would be expected to exert more force than the lower (cervical) pole of the uterus. This is another area that has been relatively neglected and is one of the major themes of this thesis.

1.2.3 Post-partum uterine activity.

In many considerations of the physiological roles of uterine contraction, post-partum uterine activity is neglected or even completely ignored. Yet in the absence of modern healthcare (i.e. the evolutionary environment which shaped human physiology), primary post-partum haemorrhage (primary PPH, defined as blood loss of greater than 500ml within 24 hours of delivery) is one of the major causes of maternal mortality [9]. Given that failure of post-partum contraction of the uterus is the major cause of primary PPH, it is clear that development of systems to allow effective post-partum contraction of the uterus were central to the evolution of placental reproduction.

1.3. Prostaglandin pharmacology

Prostaglandins are oxygenated derivatives of arachidonic acid (AA), a fatty acid formed from membrane phospholipids by phospholipases. Prostaglandins are generally synthesized de novo in the tissue which releases them in contrast to other bioactive endogenous agents (such as the monoamines) which are commonly pre-formed and stored in intra-cellular vesicles [10]. Prostaglandins exert biological effects by acting through a family of seven transmembrane domain, G-protein coupled receptors [11]. These receptors are coupled to a variety of second messenger systems and exert diverse biological effects [12]. Furthermore, different receptor types and/or sub-types commonly exert opposing effects on the same tissue [13]. Broadly speaking, PGs act at sites local to their site of synthesis. Some PGs, such as TxA_2 and PGI_2 have very short half lives and degrade before they exert systemic effects [14]. PGE_2 and $PGF_{2\alpha}$ are more stable, but are rapidly broken down in the lungs by 15 (OH) PG dehydrogenase (PGDH) [10]. An important exception is the fetus, where circulating PGE_2 is in the nanomolar range due to high levels of PGE_2 synthesis from the placenta and low levels of catabolism due to the relatively restricted pulmonary blood flow of fetal life [15]. All aspects of PG pharmacology have been previously reviewed in detail [12-14;16]. But an overview of the important aspects is presented below as the pharmacology of PGs is one of the central themes of this thesis.

1.3.1 Biosynthesis of prostaglandins

The main steps in the biosynthesis of prostaglandins (PGs) are: (1) formation of AA from membrane phospholipids, either by phospholipase A_2 (PLA_2) or phospholipase C (PLC), (2) the formation of the cyclic endoperoxides, PGG_2 and PGH_2 from AA by the enzyme

COX, and (3) the modification of PGH_2 to the main native PGs by modifying enzymes, such as PGE isomerase, thromboxane (Tx) synthase and PGI_2-synthase [14]. The PG pathway is only one of several pathways of AA metabolism which results in biologically active products [10]. Others include the epoxide pathway (cytochrome P450) [17], the isoprostane pathway (non-enzymatic generation of isoprostanes such as 8-epi- $PGF_{2\alpha}$) [18] and the lipoxygenase (LOX) pathway [19].

1.3.1.1 Phospholipase enzymes

Arachidonic acid is formed enzymatically from membrane phospholipids, principally phosphatidyl inositol and phosphatidyl choline. The enzyme PLC forms inositol triphosphate and diacylglycerol from phosphatidyl inositol in response to activation of a range of G-protein coupled receptors and inositol triphosphate acts on heparin sensitive sites to promote intracellular calcium release [20]. Diacylglycerol is metabolised by lipases to form AA and glycerol. Therefore, AA may be available within the cell following stimulation with a range of agonists. It is postulated that AA may itself act as a second messenger [21]. The other main pathway involved in AA release is PLA_2 and this is thought generally to be the more important pathway in PG biosynthesis, and it forms AA from phosphatidyl choline. A larger molecular weight cytosolic form ($cPLA_2$) and a number of smaller secretory forms ($sPLA_2$) have been identified, and these forms differ in their calcium concentration dependence [22].

1.3.1.2 Cyclo-oxygenase enzymes

The COX enzyme forms PGH_2 from one molecule of AA and two molecules of oxygen. The same enzyme catalyses two haeme requiring steps, first, an oxygenase activity

forming PGG_2 and, secondly, peroxidase activity forming PGH_2 from PGG_2. Two isoforms of the enzyme have been identified, COX-1 and COX-2 (see [23]). The expression of COX-1 tends to be fairly constant between cells and in a given tissue in different physiological states, and it is frequently referred to as the constitutive isoform. However, dynamic up regulation of COX-1 has been described in the uterine artery in association with the menstrual cycle and pregnancy [24]. Expression of COX-2 varies markedly between different tissues and expression in a given tissue may be very significantly increased under physiological and pathological situations, such as labour [25] and infection [23]. COX-2 is frequently referred to as the inducible form of the enzyme, although it does play a constitutive role in the physiology of the kidney, brain and endothelium [26].

The COX enzymes are inhibited by the non-steroidal anti-inflammatory drugs (NSAIDs). Aspirin was the first NSAID discovered, but this category of drugs includes an extensive range of widely prescribed medicines, including indomethacin, ibuprofen, and diclofenac. These drugs are very widely employed as analgesics, anti-pyretics, anti-inflammatories, and anti-spasmodics [27]. An increasingly large number of drugs are available which exhibit selective activity at the two COX isoforms [27].

Null mutant mice ('knock-outs') have been described for both isoforms. Mice lacking COX-1 exhibit reduced indomethacin-induced gastric ulceration and platelet aggregation [28]. Furthermore, the majority of pups from matings of homozygous males and females die and this appears to be due to delayed parturition[3]. Mice lacking the COX-2 gene exhibit a severe nephropathy and are susceptible to peritonitis [29]. These animals are also infertile, probably due to failure of ovulation and implantation [30].

1.3.1.3 Metabolism of PGH_2

The main naturally occurring PGs which are thought to be of importance physiologically are PGI_2, PGE_2, PGD_2, $PGF_{2\alpha}$ and TxA_2. Each of these is formed from PGH_2 by one (or more) enzymes. Since different PGs often have different effects on a given tissue, the relative activity of these enzymes may have an important role in determining the functional response of a tissue to an increase in free AA. However, a detailed review of these pathways is outwith the scope of this thesis and is available elsewhere [14]. Key enzymes include PGE synthase, PGI synthase and Tx synthase. Some PGs, for instance PGE_2, can be formed by degradation of PGH_2. Furthermore, both PGG_2 and PGH_2 can themselves stimulate the receptor for TxA_2[11].

1.3.2 Prostaglandin signal transduction

In the 1970s, there was general confusion about the possible existence of receptors for PGs. Such classifications as existed were very complex, [31] and some authors postulated that PGs did not act through conventional receptors but rather exerted direct effects on the cell membrane. The work of Dr Robert Coleman at Glaxo Group Research was pivotal in determining the existence of specific receptors for PGs and for understanding their physiological roles.

1.3.2.1 Prostanoid receptor pharmacology

Coleman characterised the relative potency of the main naturally occurring PGs in a wide range of tissues. These studies suggested that there were receptors for each of the main naturally occurring PGs which were at least 10-fold selective for the given PG, and he named them after the native PG: EP receptors for PGE_2, IP receptors for PGI_2, DP

receptors for PGD_2, FP receptors for $PGF_{2\alpha}$ and TP receptors for TxA_2 [13]. In addition, using a range of synthetic agonists (and a small number of receptor antagonists), it became apparent that there were sub-types of EP receptor. To date, four distinct sub-types have been identified and these are numbered 1-4 [32]. Broadly speaking, the IP, DP, EP_2 and EP_4 receptors inhibit smooth muscle contractility and the FP, TP, EP_1 and EP_3 receptors stimulate smooth muscle contractility [12]. In addition, activation of these receptors influences many physiological processes, including platelet aggregation, renal function, thermoregulation, and immune function[12].

Highly selective agonists exist for some of these receptors such as the IP receptor (e.g. cicaprost [33]), the DP receptor (e.g. BW245C [34]), the TP receptor (U46619 [35]) and the FP receptor (fluprostenol [36]). Selective agonists have been developed with activity at the EP_2 receptor (butaprost [31]and AH13205 [37]) and the EP_3 receptor (GR63799X [38]), but not for the EP_1 or EP_4 receptors. However, even among these "highly selective" agonists, BW245C and GR63799X are agonists of the EP_4 receptor in the nanomolar range[39]. Selective antagonists exist for some of the receptors. Many antagonists have been described for the TP receptor (e.g. GR32191B [40]). BW868C is a selective DP receptor antagonist [41]. AH6809 was originally described as an EP_1 receptor antagonist [42] but was also demonstrated to exhibit DP blocking activity. [43] Both AH23848B and AH13205 antagonize the EP_4 receptor [39].

The "classical" prostanoid receptors described above are of the G-protein coupled class with seven transmembrane domains (see below). More recently it has been demonstrated that PGs can activate intra-nuclear receptors which mediate effects by altering gene transcription [44;45]. The relative contribution of trans-membrane and intra-nuclear receptors in mediating the biological effects of PGs remains to be determined.

1.3.2.2 Prostanoid receptor genetics and second messenger coupling

The functional classification of Coleman has been validated, almost perfectly, by molecular biology [11;46]. Genes encoding all eight of the receptor types and sub-types identified pharmacologically have been cloned and sequenced in both mice and humans [11;12]. When recombinant receptors are expressed in cell systems, they exhibit very similar binding profiles to the native receptors [47] and these studies have also served to underline the lack of truly specific agonists and antagonists. The characteristics of these receptors, the findings in available knock outs, and the chromosomal location of murine prostanoid receptor genes is summarized in Table 1.1.

Table 1.1 Characteristics of murine prostanoid receptor genes.

Gene	Phenotype of knock out	Second messenger	Chromosomal Location
DP	Failure of mast cell derived asthma[48]	AC (positive) [49]	14
EP_1	Not described	IP3 [50]	8
EP_2	Impaired ovulation, hypertension [51;52]	AC (positive) [53]	3
EP_3	Impaired febrile response, duodenal mucosal dysfunction[54]	AC (negative) [55]	
EP_4	Neonatal death due to patent ductus arteriosus [56;57]	AC (positive) [58]	
FP	Failure of luteolysis/parturition [59]	IP3 [60]	3
IP	Thrombophilia, impaired inflammatory responses, hypoalgesia [61]	AC (positive) [62]	7
TP	Bleeding diatheses [63]	IP3 positive [64]	10

AC denotes adenylate cyclase IP3 denotes inositol triphosphate.

All the receptor types and sub-types which typically inhibit smooth muscle contractility have been found to be positively coupled to adenylate cyclase, i.e. the IP, DP, EP_2 and EP_4 receptors (Table 1.1). Homology studies have suggested that these receptors form a family, probably formed from mutation of a single ancestral gene [12]. Of the receptors which typically cause smooth muscle contraction, the EP_1, FP and TP receptors are usually positively coupled to the phosphatidyl inositol cascade, whereas the EP_3 receptor is usually negatively coupled to adenylate cyclase (Table 1.1).

A number of the currently identified receptor genes form multiple receptor isoforms due to alternative messenger ribonucleic acid (mRNA) splicing. The best characterized of these is the EP_3 receptor and there are at least eight alternatively spliced isoforms of the human EP_3 receptor [12]. The isoforms differ in the COOH terminal of the protein, which is the intracellular component of the receptor involved in G-protein coupling. The ligand binding site of the isoforms is the same. As stated above, the EP_3 receptor is, characteristically, negatively coupled to adenylate cyclase. However, transfection of different alternatively spliced receptor isoforms into cells demonstrates that different isoforms may be positively coupled to adenylate cyclase, or to the phosphatidyl inositol cascade [12;65]. It has also been demonstrated that agonist-induced desensitisation appears to vary directly with the length of the intracellular component [66]. Multiple bands have been demonstrated on Northern blot for the TP [67] and IP [68] receptors, and pharmacological studies have suggested isoforms of these receptors [69;70].

1.3.3 Metabolism of prostaglandins

Circulating PGs generally undergo rapidly pulmonary degradation: 95% of infused PGE_2 is inactivated in a single passage through the lungs [10]. In the fetus, pulmonary blood flow

is less than 10% of the combined ventricular output and this is one factor which results in elevated circulating concentrations of PGE_2 in fetal life [15]. The initial step of inactivation of some prostanoids (e.g. PGI_2 and TxA_2) is spontaneous hydrolysis, as they are very unstable. For PGE_2 and $PGF_{2\alpha}$, the initial step of inactivation is oxidation of the 15-OH to ketone by the enzyme 15-OH-PGDH and further oxidative steps of E series PGs yield metabolites which are chiefly excreted in the urine [10]. PGDH activity is thought to be a major local determinant of local concentrations of PGE_2 and $PGF_{2\alpha}$ in intra-uterine tissues [71].

1.4 Prostaglandins and parturition

Prostaglandins are thought to have a key role in the initiation of parturition in many species, including the human and non-human primate [5]. Exogenous PGs can induce delivery of the conceptus at any gestational age in human clinical situations although much lower doses of PGs are required if the administration of PG is preceded by a progesterone receptor antagonist such as mifepristone [72]. COX inhibitors prolong pregnancy [73;74] and can reverse the altered levels of expression of key genes in myometrium in established labour [75]. PGs are frequently referred to as a final common pathway in the process of parturition and have been implied in both term labour and preterm labour arising from diverse aetiologies [5].

1.4.1 Synthesis of prostaglandins in labour

Increased PG biosynthesis has been described in a number of tissues in association with both term and preterm labour. Labour at term is associated with increased circulating fetal [76] and maternal [77] concentrations of PGs as well as increased concentrations of PGs

in amniotic fluid [78;79]. The causal role of these findings in the initiation of labour are supported by the fact that these rises appear to precede the onset of labour. Analysis of key intra-uterine tissues also demonstrates increased expression of both PLA_2 and COX-2, but not COX-1 [80]. Increased expression of these enzymes in the baboon is observed in the amnion, myometrium, decidua and cervix and in some of these tissues the increase precedes the onset of labour [81]. Studies of amniotic fluid PGs also suggest variation within the cavity of the uterus, with higher concentrations of PGs in the forewaters (i.e. the liquor in the lower pole of the uterus) [82]. Consistent with this, myometrial expression of COX-2 appears to be greater in samples obtained from the lower pole of the uterus than the upper pole in the baboon, and myometrial COX-2 increases in the lower pole in late gestation, whereas it does not increase in samples from the fundus [81].

It is postulated that infection may account for a significant proportion of preterm births [83]. Clear links between infection, inflammation, and PG synthesis have been demonstrated in humans. Amniotic fluid concentrations of PGs appear to be elevated in preterm labour associated with infection compared with both idiopathic preterm labour and with preterm women not in labour [71]. There is an extensive literature on the effects of cytokines on expression of key enzymes determining the concentrations of PGs, such as PLA_2, COX-2 and PGDH in cells from key intra-uterine tissues, in particular the amnion [84]. The close link between inflammation and parturition is underlined by the observation that physiological term labour is associated with an infiltrate of inflammatory cells, particularly neutrophils and macrophages, and the process of parturition has much in common with an inflammatory process [85].

1.4.2 Effects of prostaglandins on key intra-uterine tissues

Prostaglandins have important effects on key uterine tissues involved in parturition. A number of naturally occurring PGs and synthetic derivatives have effects on myometrial contractility. Using a range of these agents, the prostanoid receptors present in myometrial strips from the lower uterine segment (LUS) obtained from pregnant women at the time of Caesarean section were characterized. These studies demonstrated that activation of DP, IP and EP_2 receptors inhibited myometrial contractility, whereas FP, TP and EP_3 receptor activation stimulated myometrial contractility [86]. The study predated the identification of the EP_4 receptor, and characterization of this receptor is limited by the lack of any selective agonist [11]. These studies also suggested that the bulk of the contractile effect of PGE_2 on myometrium was mediated by the EP_3 receptor, but there was also possibly an effect mediated through the EP_1 receptor. Studies on cultured human myometrial cells indicate that EP_1, EP_3 and FP receptor activation all appear to stimulate protein kinase C and calcium influx [87;88]. Interestingly, the intrinsic sensitivity of the EP_3 receptor for PGE_2 is generally 5 to 10-fold greater than the EP_2 receptor [47]. Consistent with this, low concentrations of PGE_2 have been shown to stimulate myometrium, whereas higher concentrations relax myometrium [86].

An important species difference exists between primates and non-primates: both the human and baboon exhibit inhibitory responses to selective EP_2 agonists [86;89], whereas ovine myometrium does not [90]. Expression of the gene encoding the EP_2 receptor decreases in association with advancing gestational age in the human [91] and in association with labour in the rat [92;93]. Conversely, expression of the gene encoding the excitatory FP receptor is increased in labour in both the rat and human [91-94].

The factors which regulate prostanoid receptor expression in the uterus in humans and non-human primates have not been studied. Studies in the pregnant rat have demonstrated that myometrial expression of the EP_2 receptor gene is unaffected by either exogenous oestradiol or by blocking the effects of endogenous oestradiol using the oestrogen receptor antagonist ICI 164384 [93]. Expression of the FP receptor gene in myometrium was increased by exogenous oestrogen or a progesterone receptor antagonist and was reduced by exogenous progesterone or an oestrogen receptor antagonist [93]. Unlike the oxytocin receptor gene, expression of the FP receptor gene was unaffected by stretch [94].

PGs also have important effects on the cervix and these have been exploited clinically, where administration of exogenous synthetic PGE_2 is widespread for the pre-treatment of the cervix prior to induction of labour [95]. The effects of PGs on the cervix are thought to be due both to associated uterine contraction and to direct effects of PGs on the cervix such as decreasing cervical collagen content [96]. The receptors mediating the direct effect of PGE_2 on the cervix have not yet been identified.

Both the decidua and fetal membranes are sources of PGs [5]. It is assumed that the effects of PGs released from these tissues are paracrine, specifically, acting on myometrium [97]. However, there are minimal data on possible effects of PGs on the fetal membranes or decidua. This is partly due to the difficulty in characterizing the effects of hormones on these tissues.

1.4.3 Metabolism of prostaglandins in labour

It is clear that enzymes which are important in the biosynthesis of PGs will have an important role in determining local concentration of PGs in key intra-uterine tissues in association with labour. In the case of PGI_2 and TxA_2 the compounds have very short half

lives and biosynthesis is likely to be the major determinant of local concentrations. However, in the case of PGE_2 and $PGF_{2\alpha}$, which are thought to have key roles in parturition [5], metabolism of PGs is also postulated to be important [71]. As discussed above, the enzyme PGDH catalyses the first step in inactivation of these compounds. Prior to the onset of labour, this enzyme is expressed in high levels in the chorion [71]. Since the chorion lies between the amnion and myometrium and the amnion is seen as the major source of PGs for stimulating myometrial contractility, it has been postulated that chorionic PGDH may have a key role in regulating parturition. Consistent with this it has been shown that both spontaneous term labour and preterm labour in the presence of genital tract infection are associated with decreased expression of PGDH in chorion (see ref [71] for review).

1.4.4 Prostaglandins and fetal cardiovascular adaptation at birth

Preparation for parturition clearly involves preparation of the fetus for life ex utero as well as preparation of maternal tissues to expel the fetus. One of the most acute needs of the neonate is to establish gaseous exchange through the lungs following loss of the umbilico-placental circulation. There are clearly many aspects to this adaptation. One of the best understood roles for PGs in this context is in the control of the ductus arteriosus, which is a shunt blood vessel of fetal life, extending between the main pulmonary artery and the descending aorta. In fetal life, the ductus diverts approximately 90% of the right ventricular output away from the lungs to the aorta and, ultimately, to the placenta [98]. Following birth, the pressure gradient across the ductus reverses and flow is from the aorta to the pulmonary artery. Closure of the ductus occurs in the first 24-48 hours of fetal life [99].

Like the uterus, PGs have a key role in mediating contraction of the smooth muscle at the time of birth. In fetal life, high local and circulating concentrations of PGE_2 inhibit ductal smooth muscle contractility [99] and following birth, circulating concentrations of PGE_2 fall promoting contraction of the vessel [15]. The ductus has high intrinsic contractile tone: administration of a PG synthesis inhibitor in pregnancy results in contraction of the ductus [100]. Ductal tone may be related to an intrinsically high sensitivity of the contractile proteins to calcium whereas PGE_2, acting through adenylate cyclase, inhibits intracellular calcium sensitivity in the vessel [101]. PGE_2 relaxes the ductus through the EP_4 receptor [39] but can also contract the vessel through the EP_3 receptor [102]. Mice lacking the EP_4 receptor die at birth due to patent ductus arteriosus [57;103]. It is as yet unclear why absence of a gene encoding an inhibitory receptor should result in failure of closure of the vessel in the neonatal mouse.

Although the pattern of control of the ductus arteriosus by PGE_2 (PGE_2 inhibits its contractility) is the opposite of myometrium (PGE_2 stimulates contractility), these two smooth muscles exhibit parallels. First, they both express mixed populations of stimulatory and inhibitory prostanoid receptor types and EP receptor sub-types. Secondly, current models for the control of both smooth muscles tend to focus on factors determining the concentration of PGs to which these vessels are exposed. In neither case is it yet clear whether variation in the relative expression of prostanoid receptor types and sub-types might act have a role in the physiological regulation of contractility.

1.5 Non-prostanoid control of labour

The PG system is only one of many that have been demonstrated to have a role in parturition. The process of preterm labour, in particular, may be the result of diverse

pathophysiological processes, and the relative importance of PGs may differ according to the aetiology. Furthermore, many physiological processes have levels of redundancy such that elimination of a single system may have relatively little effect on a process to which it is known to contribute. Thus the phenotypes of many 'knock out' mice are surprisingly normal. A number of other systems involved in the control of the uterus during parturition are discussed below.

1.5.1 Oxytocin

Oxytocin is a nine amino acid peptide which contracts myometrium through the phosphatidyl inositol pathway [104] and may also stimulate uterine activity by increasing COX-2 expression [105]. It was first localized in the posterior pituitary, although has been more recently identified as being produced in the decidua suggesting autocrine and/or paracrine roles in addition to its well described endocrine effects [106]. In fact, the possible role of oxytocin has been the subject of some debate and some authors maintain its effects on the uterus were entirely confined to the stimulation of postpartum contraction of the uterus and involution since oxytocin is released from the posterior pituitary in association with breast feeding, but circulating levels of oxytocin are not detectably elevated prior to the onset of labour [107]. Consistent with this view, mice which are null mutant for the oxytocin gene have normal labour and delivery, although the neonates die due to failure of lactation in the mother [108]. However, as well as the normal caveats which apply when interpreting these data, species differences in the control of parturition undermine extrapolation of this observation to the human. In mice which are null mutant for COX-1 and oxytocin, parturition occurs normally whereas mice null mutant for COX-1 alone have delayed parturition: this is explained by the opposing luteotrophic effect of

oxytocin and the luteolytic effect of COX-1 mediated increases in $PGF_{2\alpha}$ [3]. The luteotrophic effect of oxytocin complicates interpretation of the loss of myometrial effects of oxytocin in the knock out model.

Synthetic oxytocin has been widely used for many years in the induction of labour and management of postpartum haemorrhage. More recently, selective blockers of the oxytocin receptor have been developed as uterine tocolytics [109]. These agents have resolved some of the debate about the role of oxytocin in late gestation and in labour. Studies on non-human primates have shown that the oxytocin receptor antagonist atosiban inhibits the switch from low amplitude, low frequency contractures, to the high amplitude, high frequency contractions of labour at term [6] and a randomised controlled clinical trial demonstrated that the antagonist atosiban had similar tocolytic efficacy to ritodrine [110]. The discrepancy between the effectiveness of atosiban in inhibiting labour and the lack of any rise in circulating concentrations of oxytocin during labour may be explained by the drug blocking the autocrine and paracrine effects of decidual oxytocin [106;111;112] or by an increase in the number or affinity of oxytocin receptors in key tissues during labour [113]. In the rat, increased expression of oxytocin receptor gene in labour is mediated both by progesterone withdrawal [114;115] and mechanical stretch of the uterus [115].

1.5.2 Steroid hormones

The role of steroid hormones differs markedly between species. The key event triggering the onset of parturition in rodents is involution of the corpus luteum (luteolysis) which is mediated by $PGF_{2\alpha}$ acting through the prostanoid FP receptor [2]. Loss of luteal progesterone results in parturition. Extrapolation of findings in rodent species to the human and non-human primate is limited by the fact that luteolysis has no role in

parturition in these higher species [1]. In some ruminants, such as the cow, labour is also due to $PGF_{2\alpha}$ mediated luteolysis resulting in progesterone withdrawal [116]. However, in sheep, parturition is not related to luteolysis. It was observed that administration of corticosteroids to pregnant ewes induced labour and delivery and catheterisation of fetal lambs demonstrated that there was a rise in cortisol in fetal blood preceding labour [117]. The hypothesis that activation of the fetal hypothalamo-pituitary-adrenal axis (HPAA) initiates parturition was supported by the observation that labour can be delayed by interruption of the HPAA at the level of the adrenal, hypothalamus or para-ventricular nucleus [118]. Corticosteroids appear to act by inducing placental expression of 17α-hydroxylase which converts progesterone to oestradiol [119]. The role of this process in inducing labour is demonstrated by the observations that labour in the sheep is preceded by a sharp rise in maternal circulating concentrations of oestrogen [120] and exogenous oestradiol induces labour in the sheep [121], whereas administration of exogenous progesterone delays labour [122]. Oestradiol stimulates expression of a cassette of genes in the uterus which promote contraction [123].

Although sheep and primates share the properties that luteolysis occurs early in gestation and progesterone is produced by the placenta [124], there are important differences. For instance, there is no fall in progesterone preceding the onset of labour in primates [125;126]. Further, administration of exogenous progesterone does not prevent or delay labour [127]. There is evidence that supports a role for progesterone maintenance of uterine quiescence, however, as administration of a progesterone receptor antagonist induces uterine contraction, although cervical dilatation is apparently less efficient than in normal labour [128]. This has given rise to speculation that there may be a progesterone withdrawal at the level of its signal transduction system (see below). Furthermore, there is no abrupt

rise in oestradiol in primates as observed in sheep prior to labour, although there is a steady rise in maternal circulating concentrations of oestrogens with advancing gestational age in primates [125]. Administration of exogenous oestradiol does not induce labour in rhesus monkeys [129]. However, as in the sheep, a key role has been postulated for the fetal HPAA.

The primate (human and non-human) adrenal has a unique fetal zone which produces very large quantities of androgenic precursors of oestradiol [130]. It is hypothesized that stimulation of the fetal adrenal by adrenocorticotrophic hormone (ACTH) may stimulate the formation of precursors which are metabolised to oestrogens which in turn promote parturition. The hypothesis that androgens from the fetal adrenal might stimulate labour was tested by administering exogenous androstenedione to pregnant rhesus monkeys and this was associated with preterm labour [131]. This would appear to act through the formation of oestradiol as co-administration of an aromatase inhibitor (which blocks the conversion of androstenedione to oestradiol) blocked the effect of androstenedione [132]. This seems inconsistent with the observation that exogenous oestradiol on its own did not cause labour [129]. The difference may be due to a necessity for the formation of oestradiol at the site of action. Alternatively, it may be related to effects of androstenedione on androgen receptors which are expressed in key intra-uterine tissues [133].

The control of parturition by steroids in the human appears, as one would expect, to be most similar to non-human primates compared with other species. Progesterone has an important maintenance function for pregnancy. In the first trimester, the corpus luteum is the major source of progesterone but luteolysis occurs early in pregnancy and the placenta is the major source of progesterone in the second and third trimesters [134]. Removal of the corpus luteum after the first trimester does not terminate human

pregnancy. There is no fall in progesterone preceding labour in women [135], but administration of progesterone receptor antagonists can induce delivery in all three trimesters, although the successful termination of pregnancy is more commonly successful when a PG agonist is administered after the progesterone antagonist [72]. However, exogenous synthetic progesterone does not inhibit labour in humans [136].

The effect of progesterone in other species, the effect of progesterone blockade in both the human and non-human primate and the lack of effect of exogenous progesterone have led to theories that allow a role for progesterone withdrawal in the absence of changes in serum concentrations. Possible explanations include altered levels of 17-β,20α-hydroxysteroid dehydrogenase resulting in local increases in the ratio of oestrogen: progesterone [137], functional antagonism of the progesterone receptor by transforming growth factor-β [138], increased expression of heat shock proteins [139], and altered expression of progesterone receptor sub-types [140]. The relative contribution of these multiple pathways affecting the response of the uterus to progesterone in the physiological control of parturition remains to be determined.

There is a steady rise in circulating oestrogens with advancing gestation in women [141]. Oestradiol and oestrone are formed in approximately similar quantities from maternal and fetal precursor dihydroepiandrosterone sulphate (DHEAS) whereas oestriol is formed principally from fetal DHEAS from the adrenal [141]. Release of fetal ACTH may be stimulated by the fetal response to pathological stimuli, such as hypoxia [142]. Alternatively, conversion of maternal cortisol to cortisone by the enzyme 11β steroid dehydrogenase may reduce suppression of the fetal HPAA by maternally-derived corticosteroid [143]. Furthermore, corticotrophin releasing hormone (CRH) from the placenta may stimulate ACTH and thus the endocrine changes leading to parturition [144].

As in the sheep, corticosteroids have important effects on the developing fetus to promote lung maturation and other developmental changes to promote adaptation at birth [145]. These effects are very widely exploited clinically and the use of corticosteroids to accelerate fetal lung maturity at the time of threatened preterm delivery is one of a relatively small numbers of pharmacological interventions in perinatology which reduced the number of perinatal deaths [145]. However, unlike the sheep, betamethasone and dexamethasone do not induce labour: the human placenta lacks 17-α hydroxylase [1]. There are mechanisms, however, by which corticosteroids may influence the process of parturition. First, transplacental passage of corticosteroid suppresses the fetal HPAA and reduces circulating levels of oestrogens and this prolongs gestation [146]. However, corticosteroids stimulate release of CRH from the placenta [147], which would tend to promote parturition (see below). Secondly, corticosteroids inhibit expression of PGDH in placental and chorion cells which will tend to promote parturition [148]. These data suggest that the cortisol released from the fetal adrenal, although lacking the pivotal role of this hormone in the sheep, may influence the process of labour in the human and non-human primate.

It is currently assumed that, like the primate, the onset of human parturition is related to activation of the fetal HPAA and the combined effects of fetal cortisol and a rise in oestrogen mediated by increased precursors from the fetal adrenal. If this is the case, it suggests that, as in the rhesus monkey, local production of oestrogen is critical, since administration of exogenous synthetic oestrogens to pregnant women does not induce labour [149] However, the adverse effects of diethylstilboestrol on the genital tract of the developing female fetus [150] has led to considerable caution in evaluating the effects of

oestrogen receptor agonists to pregnant women and, interestingly, topical oestradiol appears to be an effective agent in priming the cervix [151;152].

1.5.3 Corticotrophin releasing hormone

Corticotrophin releasing hormone is a 41 amino acid peptide hormone, first identified in the hypothalamus, where it is released into the portal circulation to stimulate release of ACTH. Shortly after its identification in the hypothalamus, CRH was demonstrated in the human placenta [153]. In humans and some – but not all – non-human primates, CRH rises in the third trimester [153]. Release of CRH is stimulated by oestrogen, corticosteroids and PGE_2 and inhibited by progesterone [154;155]. Although CRH does not appear to exert a direct effect of myometrial contractility [156] expression of the CRH-R1 receptor is increased in association with labour [157]. The precise role of CRH in the direct control of myometrial contractility remains to be determined. However, placentally-derived CRH may stimulate myometrial contractility indirectly by activating the fetal HPAA [155]. Serum concentrations of CRH between 16-20 weeks gestation appear to predict the onset of labour at term [155]. Furthermore, the relationship between placental CRH and inflammation and also with fetal stress suggest that the hormone may have a role in the aetiology of preterm labour as well [144].

1.5.4 Non-prostanoid eicosanoids

PGs are only one of a number of pathways of locally active agents formed from AA. Other enzymatic pathways are the LOX pathway and the epoxide pathway (cytochrome P450). I could find no data on the possible role of the epoxide pathway in the control of labour. There are a limited number of studies on the LOX pathway. LOX enzymes

generate hydroperoxyeicosatetraenoic acid (HPETE) compounds from AA. There are three main types of enzyme, 5-LOX, 12-LOX and 15-LOX which generate 5HPETE, 12HPETE and 15HPETE compounds, respectively. Of these enzymes, it has been shown that labour in the human is associated with increased expression of 5-LOX gene and protein [158]. Consistent with this, 5-HETE has been shown to increase the contractility of isolated strips of human myometrium whereas 12-HETE was without effect [159].

There is another pathway for AA, namely, the non-enzymatic formation of isoprostanes, such as 8-epi- $PGF_{2\alpha}$. These agents act both on the TP receptor [160] and on a novel receptor [161] and have been shown to have a number of physiological roles in other organs. 8-epi- $PGF_{2\alpha}$ has been shown to have a moderate stimulatory effect on isolated strips of human myometrium [162] but the role of this agent, if any, in the control of parturition is unknown.

1.5.5 Other stimulatory factors

A range of other hormones have been shown to mediate contraction of the uterus. Specifically, monoamines, such as 5-hydroxytryptamine, noradrenaline and histamine contract isolated strips of myometrium [163-165]. The role of these agents in the control of labour remains obscure, but it is postulated that release of some of these agents by mast cells may promote uterine contraction during labour and may act synergistically with other systems, such as PGs [165].

1.5.6 Other inhibitory factors

A range of other inhibitory factors have been identified which may be involved in maintaining uterine quiescence in the antepartum period, for instance, relaxin,

parathyroid hormone related polypeptide (PTHrP) and nitric oxide. In these cases plausible explanations of how these agents may be involved in parturition can be elaborated. For instance, in the case of PTHrP, this agent profoundly inhibits the contractility of myometrial strips from the uterine corpus, lower segment and fundus of the baboon [166] and release of PTHrP from the amnion may be reduced in association with labour [167]. Despite such associations, information on the causative role of these agents in the regulation of parturition remains to be determined.

It cannot be assumed that because an agent has an inhibitory effect on myometrial contractility, that its effect is purely to inhibit the process of parturition. In the case of both relaxin and nitric oxide (NO), both agents may have effects which promote parturition in some key tissues and inhibit parturition in others. Relaxin inhibits myometrial contractility but has effects on the cervix which would tend to increase cervical compliance [168]. Although, synthetic human relaxin is not effective in priming the cervix prior to induction of labour [169], increased expression of the gene is implicated in preterm, premature rupture of the fetal membranes (PPROM) [170]. Similarly, NO donors inhibit the contractility of isolated strips of human myometrium [171] and were investigated as potential uterine tocolytics in the management of preterm labour [172]. However, increased activity of endogenous NO is implicated in cervical changes at term and in labour [173] and NO donors are effective agents for priming the human cervix prior to termination of pregnancy [174].

1.5.7 Changes in cellular function

The above principally deals with specific hormone signalling systems. However, general changes also take place in myometrial cellular function which would be expected to influence the response of the uterus to these agents.

1.5.7.1 Control of intracellular calcium concentration

Smooth muscle contraction is mediated by the interaction between the 20kDa myosin light chain (MLC_{20}) and actin which results in activation of myosin adenosine triphosphate (ATP)ase and contraction. The interaction between myosin and actin is regulated by phosphorylation of the serine 19 residue on MLC_{20} [175]. Phosphorylation and dephosphorylation of this residue is mediated by key regulatory enzymes, myosin light chain kinase (MLCK) and a smooth muscle myosin phosphatase (SMPP-1M), respectively [176]. Raised intracellular calcium concentration ($[Ca^{2+}]_i$) has a key role in initiating smooth muscle contraction, forming a complex with the regulatory protein calmodulin, and the calcium calmodulin complex stimulates contraction by activating MLCK [20].

Current models of the control of myometrial contractility invoke a key role for the control of intracellular calcium in the regulation of myometrial contractility [175]. $[Ca^{2+}]_i$ may be elevated either by influx of calcium from the extracellular space or by release of calcium from intracellular stores. In human myometrium, agonists induce an increase in $[Ca^{2+}]_i$ due both to influx of calcium from the extracellular space and release from intracellular stores [177]. Influx of calcium from the extracellular space is mediated by both voltage sensitive calcium channels and receptor operated channels. The mechanisms which

regulate $[Ca^{2+}]_i$ and the relationship between a given $[Ca^{2+}]_i$ and contraction have been reviewed at length [178].

1.5.7.2 Gap junctions

One of the features of intrapartum uterine contraction is that contraction is global, i.e. coordinated across the uterus [8]. A prerequisite for this is the ability of contraction to be propagated through the uterus. In the heart, communication of cells through gap junctions results in a functional syncytium. Unlike the heart, the normal state of the uterus is not that of a syncytium. However, there is a dramatic increase in the expression of gap junctions in myometrium in association with labour which allows excitation of uterine cells to be propagated and facilitates global, coordinated uterine contraction [179].

1.5.7.3 G-proteins

Many of the signal transduction systems which are thought to have keys roles in stimulating contraction during parturition are of the super-family of G-protein coupled receptors [180]. G-proteins are membrane associated proteins which bind guanosine triphosphate (GTP) and couple occupancy of the receptor by an agonist with activation or inhibition of a second messenger [176]. In the case of an inhibitory second messenger, such as adenylate cyclase, a stimulatory G-protein would mediate relaxation. In the case of a stimulatory second messenger, however (such as PLC), the same G-protein would promote contraction. Conversely, an inhibitory effect of a G-protein on an inhibitory second messenger would initiate contraction. The prostanoid EP_3 receptor typically mediates contraction of smooth muscle in this manner, namely, by a G-protein mediated decrease in adenylate cyclase activity [181]. Labour is associated with decreased myometrial

expression of G α s [182] which stimulates the inhibitory second messenger adenylate cyclase. The possible role of variation in G-protein expression in controlling human labour is outwith the scope of this thesis and has been reviewed in detail elsewhere [180].

1.5.7.4 Contractile proteins

There is much less information on the possible role of changes in calcium sensitivity (i.e. the relationship between $[Ca^{2+}]_i$ and contractility) in the physiological and pharmacological control of myometrial contractility. Studies of human myometrium have inferred possible effects of stimulatory agonists on calcium sensitivity from apparent dissociations of calcium concentration and force [183]. A single study of β-escin permeabilised smooth muscle demonstrated a greater intrinsic calcium sensitivity of human pregnant myometrium compared with non-pregnant [184] which may be related to a greater degree of contraction for a given level of activation of MLCK [185] There are, however, multiple mechanisms by which the sensitivity of the contractile proteins to a given $[Ca^{2+}]_i$ can be altered [178] and these have not been the subject of intense study in relation to myometrial activation in labour.

1.6. Clinical aspects of uterine contraction in pregnancy.

1.6.1 Preterm labour

Preterm labour is frequently referred to as if it is a distinct disease. However, it is a purely descriptive term, being labour and delivery before the start of the 37th completed week following the first day of the last menstrual period. Further, by implication, it is arbitrary as well as descriptive. Labour on the 258th day since the LMP is preterm, whereas labour on the 259th day is not. Preterm delivery may be the result of a diverse

range of clinical precipitating factors including, but not limited to, PPROM, fetal abnormality, polyhydramnios, multiple pregnancy, infection, intra-uterine growth restriction and uterine anomaly [145]. Many cases have no obvious precipitating cause and are referred to as idiopathic. Infection is one key antecedent of many cases of preterm labour, both with and without PPROM.

The ultimate goal of treating preterm labour is the stated aim of many basic studies of myometrial physiology and pharmacology. However, of the three principle clinical goals discussed, management of preterm labour is perhaps the one with the most ambiguous indication. Preterm labour is undoubtedly a major source of morbidity and mortality. Administration of steroids to pregnant women in preterm labour between 1-7 days prior to preterm delivery accelerates fetal lung maturation and reduces perinatal morbidity and mortality [145]. Tocolytic therapy concurrent with steroid administration is justified on the grounds that it might delay delivery to allow steroids to have their maximum effect. However, although tocolytic therapy does delay delivery, it has no effect on perinatal morbidity or mortality [186]. The lack of a clear beneficial effect of tocolysis may be related to the pathophysiology of preterm labour. For example a significant proportion of cases of preterm labour is thought to be secondary to intra-uterine infection and delaying delivery is potentially harmful in this context [83;145]. However, preterm labour is the end-point of diverse pathological processes. It is likely that inhibition of preterm uterine contraction confers a differing degree of benefit (or harm) depending on the aetiology.

1.6.2 Induction of labour

In contrast to the treatment of preterm delivery, induction of labour has been shown, in the context of post-dates pregnancy, to reduce both maternal morbidity (Caesarean

section)[187] and may also reduce perinatal mortality [188]. The apparent protective effect of induction of labour post-term on risk of Caesarean section is likely to be due to the increased risk of Caesarean section associated with advancing gestational age post-term [189] and the apparent reduction in perinatal mortality may be due to the increased risk of unexplained stillbirth post term [190]. However, at a given gestational age, a woman whose labour has been induced is more likely to require a Caesarean section than a woman in spontaneous labour [189]. The challenge is how to induce labour in a manner than does not increase the risk of operative delivery.

1.6.3 Post-partum haemorrhage

Failure of contraction of the uterus postpartum with consequent primary postpartum haemorrhage kills approximately 125,000 women in the world each year [9]. In this case there is no ambiguity in the purpose of treatment: it is to contract the uterus and maintain it in a contracted state. An effective, tonic constrictor of the pregnant human uterus that has long-term stability at tropical room temperatures would have considerable potential to reduce global maternal mortality. However, this aspect of the control of the uterus is relatively ignored in the context of myometrial pharmacology.

1.7 Aims of this work.

1.7.1 Variation in myometrial pharmacology according to uterine site

The vast majority of studies of myometrium from pregnant women use biopsies obtained from the lower segment of the uterus, obtained at the time of LUS Caesarean section. It is possible to obtain biopsies from the fundus and corpus of the uterus, but only a small number of studies use this technique. The alternative to this is to use samples obtained at

the time of Caesarean hysterectomy. However, this procedure is performed very infrequently in modern obstetric practice and, commonly, the indication for this radical procedure, e.g. severe atonic PPH, would mean that the results obtained from such specimens could not be extrapolated to the physiological situation. One aim of this project was, therefore, to address regional variation in gene expression in the primate uterus. To this end, the principle technique employed for obtaining tissues was total Caesarean hysterectomy.

1.7.2 Identification of tissue-specific expression of prostanoid receptors

Prostaglandins are known to be important in the control of myometrium and cervix in labour. Although the decidua and fetal membranes are also sites of PG synthesis, it is currently assumed that PGs from these sites have principally a paracrine role by influencing the myometrium or cervix. However, it is much harder to characterize decidual or chorionic function than myometrium function. The possibility that PGs produced in these tissues may have an autocrine effect has not been fully addressed. An aim of this project was to determine whether prostanoid receptor expression could be demonstrated in the fetal membranes and decidua.

Another aspect of this question related to the complex family of prostanoid receptor types and sub-types of EP receptor. Currently, native PGs are typically employed to induce labour. The use of PGE_2 to prime the cervix is dose limited by the effect of PGE_2 to stimulate myometrial contractility. I sought to determine whether there was evidence that prostanoid receptor expression differed in different intra-uterine tissues.

1.7.3 Identification of cell-specific expression of prostanoid receptors

Although analysis of total prostanoid receptor gene expression in a given tissue allows inferences to be drawn about the role of a given gene in the control of parturition, this approach ignores the cellular complexity of intra-uterine tissues. Even taking the simple example of smooth muscle in the uterine wall, there are two types of smooth muscle, namely, myometrial and vascular. There are also many other non-muscle cells types, such as fibroblasts and leukocytes (the latter infiltrate the uterine wall in association with labour). One aim of the project was to determine the cellular localization of prostanoid receptor genes.

1.7.4 The effect of labour on prostanoid receptor expression

A number of groups have made systematic study of expression of families of genes in labour in order to determine systems which are likely to have key roles in the control of parturition. These studies have led to the concept of a cassette of genes encoding myometrial contraction associated proteins which have a key role in labour. Despite the key role postulated for PGs in the control of labour, there has been no systematic study, to my knowledge, of the expression of prostanoid receptors in myometrium in any species. One aim of this work was, therefore, to describe the effect of labour on expression of all eight currently recognized G-protein coupled prostanoid receptors.

As discussed above, I also postulated that prostanoid receptors may have a role in the control of other, non-myometrial tissues, in the process of labour. I sought to infer the possible role, stimulatory or inhibitory, of prostanoid receptors in other tissues in labour, by determining the effect of labour on the level of expression of each of the currently recognized prostanoid receptor genes.

1.7.5 Prostanoid receptor expression in the fetus

Given that successful reproduction necessitates that delivery of the fetus by the maternal uterus has coincided with sufficient fetal development to allow independent survival of the newborn, it is clear that the processes required for fetal adaptation must mature in a parallel fashion with the preparation for labour in uterine tissues. Given that the fetal ductus arteriosus, like the uterus, is critically regulated by PGE_2, I sought to determine whether there were any parallels in developmental changes in prostanoid signal transduction in the ductus. I sought, therefore, to determine the effect of advancing gestational age and birth on the expression of prostanoid receptor genes in ductus arteriosus smooth muscle.

1.7.6 Comparison between prostanoid and related systems

A key role has been postulated for prostanoids in the control of parturition. However, as discussed above there are other pathways of AA metabolism which may generate eicosanoids with a role in the control of key intra-uterine tissues. While the expression of COX enzymes has been systematically studied in relation to labour in a number of species, there has been no systematic study of the expression of LOX genes in relation to labour in the human and non-human primate. A further aim of this work was, therefore, to determine whether expression of LOX genes changed in association with labour in key intra-uterine tissues in the baboon.

Chapter 2. Methods.

2.1 In vivo methods

All animal work was overseen by the Cornell Institutional Animal Care Committee and was under the terms of the current US animal legislation. Baboons were bred in one of two facilities in the USA (San Antonio and Chicago) and transferred to Cornell at approximately 80 days gestational age (dGA). Gestational age was assessed by a known date of mating and by early ultrasonography.

2.1.1 Animal housing conditions

Animals were housed in individual cages. At least 4 weeks prior to study, all animals were given a full medical examination to determine that they were in good health. At this time they were acclimated to a controlled light: dark cycle and animals which were destined for protocols involving chronic instrumentation were fitted to a jacket and tether restraint system [6]. A nutritionally balanced maintenance diet (Teklad 25% Monkey Diet (W) 8663, Harlan Inc., Madison, WI) and water were supplied to animals ad lib. Feed was supplemented daily with fresh fruits and/or vegetables and one chewable multivitamin tablet daily (Topco, Skokie, IL).

2.1.2 Surgery

All surgical procedures were carried out under general anaesthesia under conditions of strict asepsis.

2.1.2.1 Anaesthesia and pain relief

Anaesthesia was induced by ketamine given by intra-muscular injection. The animal was then transferred to an operating theatre and anaesthesia was maintained by inhalation using halothane, 1-2%, initially administered through a face mask and subsequently through an endotracheal tube. Post operatively, all animals received analgesia for three days (continuous intra-arterial infusion of Buprenorphine [Reckitt & Coleman, Ltd., Richmond, VA], $0.03mg.kg^{-1}.day^{-1}$) and prophylactic antibiotics for 5 days (Oxacillin, Bristol-Myers Squibb, Princeton, NJ) $100mg.kg^{-1}day^{-1}$) via continuous intravenous infusion. All catheters were continuously infused with heparinized saline (25 units of $heparin.ml^{-1}$ at $0.5ml.h^{-1}$ to the mother and at $0.125ml.h^{-1}$ to the fetus). Heparinized saline was also used as the vehicle for infusion of post-operative medications. When required with fetal preparations, the oxytocin antagonist Atosiban (RW Johnson Pharmaceutical Research Institute, Raritan, NJ), $0.188g.kg^{-1}.min^{-1}$ was given to alleviate uterine contraction activity.

2.1.2.2 Maternal femoral vein catheterisation

A 3cm incision was made in the long axis of the anatomical course of the femoral artery approximately 1cm below the inguinal ligament. The femoral vessels were exposed by blunt dissection and the distal end of each vessel was tied. Catheters were advanced into the vessel to 15cm, secured to surrounding connective tissue by interrupted 3/0 silk sutures, tunnelled subcutaneously to the exit point on the animal's dorsum and connected to the tether system. The skin incision was closed with 3/0 Vicryl.

2.1.2.3 Laparotomy.

The abdomen was opened through a mid-line incision. This was usually sub-umbilical, but occasionally extended above the umbilicus. The length of the incision varied with the procedure to be undertaken. Bipolar diathermy was applied to secure haemostasis. The rectus sheath was identified and cut in the midline. The linea alba was identified, divided in its vertical axis and the two halves of the rectus abdominis separated in this plane. The peritoneum was grasped with two small artery forceps, confirmed empty and opened with a blade. The peritoneal incision was extended with scissors. Repair was effected using a mass closure techniques employing 1 Vicryl in a continuous suture. The skin was closed using sub-cuticular 3/0 Vicryl.

2.1.2.4 Placement of uterine electromyogram (EMG) electrodes.

The abdomen was opened as described above, with an incision of no more than 4cm. The anterior surface of the uterus was identified. Five EMG electrodes were stitched into the anterior uterine wall. The wires entered a common sheath which was secured to the parietal peritoneum on the anterior abdominal wall by a suture. The electrodes were connected to the tether system as described for the femoral vascular catheters.

2.1.2.5 Chronic fetal preparations

A detailed description of the techniques involved here is outwith the scope of this thesis as fetal preparations are peripheral to the main focus of the work. In brief, the uterus was opened with a scalpel and the cut edge was grasped with Babcock forceps. A needle was passed through the exposed fetal membranes and amniotic fluid withdrawn and stored at 42C. The membranes were then excised and grasped with the Babock forceps along with

the myometrium. When the fetal skin was incised, its cut edge was also incorporated with the membranes and myometrium to prevent loss of amniotic fluid. Intra-uterine instrumentation varied between preparations. As a minimum it would include an intra-amniotic catheter and fetal carotid arterial catheter and variably included jugular venous catheter, carotid arterial flow probe, EMG electrodes (biceps and/or triceps), ECG electrodes, and tracheal catheter. The catheters, with or without leads, were connected to the tether system as described for the maternal instrumentation.

2.1.2.6 Caesarean section.

The abdomen was opened as described above. The uterine incision was transverse through the lower part of the uterine corpus. The full thickness of myometrium was opened with a blade to expose the fetal membranes and the cut edges of myometrium grasped with Babcock's forceps for haemostasis. At this stage, specimens of amniotic fluid were frequently removed. The membranes were then incised and the uterine incision extended with scissors. The fetus was delivered, and the cord clamped and divided. The fetal membranes were carefully stripped off and the placenta removed intact with gentle traction on the umbilical cord. The uterine cavity was explored manually and confirmed empty. The uterine incision was closed with a single layer of continuous locked 2/0 Dexon. Closure was otherwise as described above.

2.1.2.7 Caesarean hysterectomy.

The abdomen was opened as described above and the uterus was incised in the midline. After delivery of the fetus, placenta and membranes, the round ligaments were clamped and tied. The broad ligament was opened and the peritoneum was opened anteriorly. The

peritoneal incisions on either side were joined anteriorly and the bladder reflected inferiorly to below the level of the cervix. The peritoneum over the posterior aspect of the uterus was incised and the inferior cut edge was reflected inferiorly. In this way the rectum, if adherent to the Pouch of Douglas, was reflected inferior to the ultimate incision in the vagina. The infundibulo-pelvic ligament was then clamped, divided and tied (2/0 Dexon, lateral to the ovaries if oophorectomy was also being performed). Clamps were advanced lateral to the uterus to secure the uterine arteries. These pedicles were cut and tied (2/0 Dexon). The cardinal ligaments were then clamped, cut and tied (2/0 Dexon). The anterior vaginal wall at the level of the external cervical os was grasped and opened. The incision was extended laterally and the vascular angles were clamped. The uterus was freed by cutting the posterior wall of the vagina and the specimen was removed for immediate harvesting of tissues. The vaginal vault was closed with continuous locking 2/0 Dexon and the abdomen was closed as described above.

2.1.3 Acquisition of data from chronically instrumented animals

Maternal and fetal catheters and electrodes exited the animal's dorsum and entered the tether on its jacket. The tether exited the cage and was connected to transducers (for the catheters) and pre-amplifiers (for the electrodes). These signals were digitised, averaged over 6 second periods, and stored in an electronic form in a computer hard drive. They were also printed out on a dot matrix printer to provide a hard copy for each animal. A detailed discussion of the electronics of the system is outwith the scope of this thesis and has been described elsewhere [6].

2.2 In vitro methods

All reagents employed were molecular biology grade. Water was ultra filtered, deionized to 18.3 MΩ resistance and had less than 2 parts of organic impurity per billion. Glassware was carefully washed, rinsed with ultra filtered water and baked for at least 5 hours at 270C. As far as possible, disposable plastic ware was used which was sterile and individually wrapped. If it was not, it was autoclaved for 30 minutes. Items which were not suitable for either baking or autoclaving were treated with a commercial RNase inhibitor (RNase Zap, *Ambion*, Austin, TX, USA).

2.2.1 Northern blot.

Northern blot is a technique which allows the quantification of a specific ribonucleic acid (RNA). The process of Northern blot involves (1) extraction and quantitation of RNA, (2) electrophoresis of denatured samples in an agarose gel, (3) transfer of RNA from the gel to a solid medium where it is fixed (nitrocellulose or nylon membrane), (4) hybridisation of the blot with a labelled probe and (5) autoradiography of the probed blot.

2.2.1.1 Extraction of RNA.

Commercial kits were employed for extraction of RNA which were selective for polyadenylated RNA. For samples of less than 0.2g, Micro Fast track, version 2.2 was employed and for samples between 0.2 to 1.0g, Fast Track, version 2.0, was employed, both kits are made by *Invitrogen*, San Diego, CA. The overall process is similar for both kits and involves homogenisation and incubation of the sample in a buffer containing protein/RNase degrader, mixing of the sample with oligo dT cellulose to allow binding of polyadenylated RNA to the cellulose, washing of the cellulose pellet to remove protein,

deoxyribonucleic (DNA) and ribosomal RNA, and then elution of polyadenylated RNA from the pellet. The detailed protocol was as follows.

A sample of tissue stored at –80C was placed in a sterile plastic tube containing 200mM NaCl, 200mM Tris (pH 7.5), 1.5mM MgCl2, 2% sodium dodecyl sulphate (SDS) and RNase and protein degrader. The sample was then homogenised for 1 minute and incubated at 45C for 1 hour. The specimen was spun at 4000g for 5 minutes, the supernatant transferred to a clean labelled tube, 5M NaCl was added and mixed. DNA in the specimen was sheared by drawing the sample into a syringe through a 21G needle 4 times. The oligo dT cellulose pellet was added and the specimen mixed in a rocker for 1 hour. The suspension was then spun at 3000g for 5 minutes and the pellet washed three times in 500mM NaCl, 10mM Tris-Cl (pH 7.5). The pellet was then re-suspended in 250mM NaCl, 10mM Tris-Cl (pH7.5) and spun at 3000g for 5 minutes and this was also repeated 3 times. The cellulose pellet was re-suspended in the same buffer, transferred to a spin column and washed three times. The spin-column was transferred to a clean microfuge tube and the polyadenylated RNA was eluted by two washes with 200µl of 10mM Tris-Cl pH 7.5, heated to 65C. The RNA was precipitated with 0.15 volume of 2M sodium acetate and 2.5 volumes of 100% ethanol, placed in a –80C freezer until solid, thawed and centrifuged at 14,000g for 30 minutes at 4°C and the ethanol removed. The pellet of RNA was suspended in 10 mM Tris (pH 7.5) and the concentration and purity of RNA determined in a spectrophotometer using the ratio of the optical density at 260nM and 280nM with a ultraviolet (UV) light source. This procedure typically yielded RNA with a 260:280 ratio of 2.1-2.2.

2.2.1.2 Gel electrophoresis of RNA

The samples of the test RNA and tube containing a standard series of RNA size markers (Gibco) were concentrated by centrifugation in a Speed Vac for 10-20 minutes. Each pellet was then dissolved in 15 µl of 17.4% (vol/vol) formaldehyde, 50% (vol/vol) freshly de-ionised formamide, 20mM MOPS [3-(*N*-morpholino)propanesulfonic acid], 5mM sodium acetate, 0.025% bromophenol blue and 1mM ethylenediaminetetraacetic acid (EDTA), pH 7.0. Re-suspension was effected by vigorous pipetting and brief vortexing. Samples were concentrated at the bottom of the tube by a 5 second spin at 5000g. They were then heated for 5 min at 65C and chilled on ice for 3 minutes. The samples were loaded into wells of a 1.4% (wt/vol) agarose gel containing 0.66M formaldehyde, 20mM MOPS and 0.28µg per ml ethidium bromide in an electrophoresis tank containing 0.66M formaldehyde and 20mM MOPS. The voltage was generally set at 100-110V for large gels and 70-80 for small gels. After the electrophoresis was completed (typically 3-4 hours), the gel was photographed under UV light (see Figure 2.1).

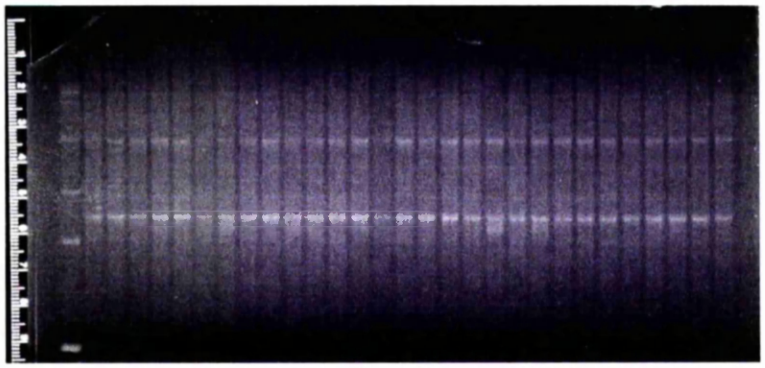


Figure 2.1 Photograph of RNA gel, stained with ethidium bromide, following electrophoresis and prior to Northern blot. 2μg of myometrial mRNA loaded in each lane, except first lane = 2.5 μg of RNA ladder.

2.2.1.3 Transfer onto membrane

The blot was washed 4 times for 5 minutes in water. The nylon membrane (Gene Screen plus, NEN, Dupont, DE, USA) was cut to the size of the gel, washed in water and then in 10X SSC (1X SSC is 0.15M NaCl and 0.015M sodium citrate, pH 7.0). The RNA was transferred onto the membrane by capillary blotting for 24 hours in 10X SSC. The gel was viewed under UV light to confirm that the RNA was transferred. The membrane was then washed briefly in 2X SCC and left over night. The process of drying was sufficient to fix the RNA onto the membrane. It was stored in a sealed bag at 4C until hybridisation.

2.2.1.4 cDNA probe synthesis

Probes were made from full length human complementary DNA (cDNA). The cDNAs were removed from the plasmid by restriction enzymes and separated in a low melting point agarose gel. The DNA corresponding to the probe was cut out of the gel and stored at –20C. Prior to use, the insert (in gel) was heated at 65C for 10 minutes. cDNAs were labelled with [α-^{32}P] deoxy-cytosinetriphosphate (dCTP) (3000Ci/mmol) using the random priming method to specific activities of approximately $1x10^9$ cpm/μg using a number of commercial kits, including NEG 013H (NEN, Dupont, Boston, MA, USA), Decaprime and StripEZ DNA (both Ambion) using a method described in detail elsewhere [191]. All kits used the same principle of denatured cDNA in an appropriate buffer with random decamers, deoxynucleoside mixture (dATP, dGTP and dTTP, [α-^{32}P] dCTP and large fragment DNA polymerase. Labelled and unlabelled DNA were then separated from unincorporated nucleotide using Sephadex G-50 chromatography. The probe was quantified in a scintillation counter and, prior to use, was boiled for 5 minutes and chilled on ice for 3 minutes. The advantage of the Decaprime kit over the NEG kit

was that it required less template DNA resulting in less unlabelled DNA in the final probe. The advantage of the StripEZ DNA kit was that one of the nucleotides was chemically modified allowing the probe to be removed with stripping solutions at 65C with minimal loss of blotted RNA.

2.2.1.5 cRNA probe synthesis

The prostanoid receptor cDNAs had been cloned into the pcDNA-3 vector (Invitrogen), which includes promoters for phage polymerases SP-6 and T-7, allowing synthesis of sense and anti-sense riboprobes. The plasmid was linearized by an appropriate restriction enzyme for the given promoter and type of probe. Probes were synthesized using a commercial kit (StripEZ RNA [Northern] or MaxiScript [in situ], both *Ambion*, TX, USA) and labelled with [α-^{32}P] uracil triphosphate (UTP) (800Ci/mmol) (NEN Life Science) for Northern analysis and with ^{35}S UTP (1250Ci/mmol) for in situ hybridization (see below). In both kits, the linearized plasmid is incubated in appropriate buffer (containing DTT and spermidine); unlabelled ATP, CTP and GTP; labelled UTP; the appropriate RNA polymerase and a ribonuclease inhibitor. The StripEZ RNA kit differed in that one of the unlabelled nucleotides was modified and this allowed the probe to be stripped using commercially supplied buffers at 65C with minimal loss of blotted RNA. Template DNA was removed by addition of 2U of RNase-free DNase and incubation for 15 minutes at 37C. Probes was separated from unincorporated nucleotide using a Sephadex G-50 spin column (Probe Quant G-50, Pharmacia Amersham) and quantified. Unlabelled competitor complementary RNA (cRNA) was synthesized using a commercial kit (MegaScript, Ambion) which was optimized for large scale synthesis of

RNA, allowing the unlabelled competitor to be added in greater than 100-fold excess to the labelled probe as a control when employing in situ hybridization (see below).

2.2.1.6 Source of cDNAs

The human EP_2 receptor cDNA was obtained from Dr D.F. Woodward of Allergen, Irvine, CA 92713, USA. The human prostanoid receptor EP_3, EP_4, IP and FP cDNAs were obtained from Dr Mark Abramovitz of Merck Frosst, PO Box 1005, Quebec H9R 4P8, Canada. The human TP receptor cDNA was obtained from Oxford Biomedical (MI, USA) and the mouse EP_1 receptor cDNA was obtained from Dr Y Sugimoto of Kyoto University, Japan. The plasmids (all TRIscript, Ambion) containing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin and cyclophilin cDNAs with RNA polymerase promoters were purchased from Ambion. The cDNAs for human 5-LOX, platelet 12-LOX, and 15-LOX and for murine leukocyte 12-LOX were all purchased from Oxford Biomedical.

2.2.1.7 Hybridisation and washing

Blots were pre-hybridised and hybridised in the same buffer except the latter contained the nucleic acid probe. In initial experiments using cDNA probes, hybridisation buffer of the following composition was employed: freshly de-ionised formamide (50% vol/vol), sodium phosphate (50mM), NaCl (0.8M), SDS, 2% wt/vol, herring sperm DNA (100μg /ml), yeast transfer RNA (20μg /ml) and 1X Denhardt's (1X = 1% solution of bovine serum albumin [BSA], Ficoll and polyvinylpyrrolidone). In later experiments, commercial hybridisation buffer was employed which was also based on 50% formamide (Northern Max or Ultrahyb, both Ambion).

When cDNA probes were employed, pre-hybridisation (>5 hours) and hybridisation (>18 hours) were carried out at 42C in sealed bags. Blots were washed sequentially in 2X SSC at 42C, 0.5X SSC and 0.1%SDS at 65°C (15 minutes), and 0.2X SSC and 0.1%SDS at room temperature (10 minutes). When cRNA probes were employed, pre-hybridisation (>1 hour) and hybridisation (>18 hours) were carried out at 65-68C in bottles in a hybridisation oven (Hybaid, UK). Membranes were washed at 65C: twice for five minutes in 2X SSC and 0.1% SDS and twice for one hour in 0.1X SSC and 0.1%SDS. In both cases probe was added to the pre-hybridisation buffer to a concentration of 1 million counts per minute per ml of buffer. Following washing, Kodak X-Omat film was exposed to the membrane with an intensifying screen at -80C. Following autoradiography, membranes were stripped according to the manufacturer's protocol. Densitometric analysis of autoradiographic signals was performed by scanning autoradiographs into Adobe Photoshop and then using Scan Analysis (Biosoft, Cambridge, UK) to quantify signals.

2.2.2 In situ hybridisation

Frozen sections (5-8 microns thick) cut onto commercially prepared poly-l-lysine coated slides (Sigma Chemical Company) were fixed in freshly prepared 4% paraformaldehyde in 0.1M phosphate buffer (5 minutes), washed twice in 0.1M phosphate buffer, immersed in triethanolamine (TEA)-HCl (3.71g TEA, 2ml 6M NaOH and 198ml of water), pH 8.0 and then TEA and acetic anhydride (0.25%) for 10 minutes. They were then washed in 2X SSC for 5 minutes and briefly in 70% ethanol and allowed to air dry. The specimens were incubated for 2 hours in a humidified container (55C) with 50-100µl of pre-hybridization buffer (50% freshly deionized formamide, 0.3M NaCl, 20mM Tris-HCl

(pH 8.0), 5mM EDTA, 10mM sodium phosphate buffer (pH 8.0), 1x Denhardt's solution, in RNase free water), and for least 16 hours in hybridization buffer (i.e. pre-hybridization buffer plus probe = 1x106 cpm per specimen, between 50-100μl). Control slides were hybridized with a sense riboprobe or an antisense probe in the presence of an excess of unlabelled competitor. Slides were then washed three times in 4X SSC and 4mM DTT; three times in NTE buffer (0.5M NaCl, 10mM Tris-HCl and 5mM EDTA, pH 8.0) at 37C (second NTE wash was for 30 minutes with 30μg/ml Ribonuclease A); 2X SSC and 1mM DTT; 0.1X SSC and 1mM DTT at 60C; and, finally 0.1SSC at room temperature. The slides were then dehydrated in a graded series of ethanol: 50%, 85% (both plus 0.3M ammonium acetate) and 100%. They were air dried overnight, dipped in emulsion (Kodak NTB2) and exposed for 1-4 weeks at 4C. Following developing and fixing they were counter stained with haematolxylin and eosin, mounted (Permount) and covered with a glass coverslip.

2.2.3 Cell *culture*

All procedures were carried out using strict aseptic technique. All manipulations of cells were performed in a laminar flow hood. All reagents used were tissue culture grade. If reagents were not already sterile (e.g. culture medium), they were filter sterilized. All reagents employed were obtained from the Sigma Chemical Company, unless otherwise stated. Myometrial cells were established in primary culture using the methodology described by Casey et al, [192] with certain modifications. Approximately a gram of myometrium was obtained at surgery and placed in Hank's balanced salt solution (buffered with HEPES [20mM] and sodium bicarbonate to pH 7.4) containing penicillin (1000U/ml), streptomycin (1mg/ml) and amphotericin (2.5μg/ml). The tissue was minced

into fragments of about $1mm^3$ using two sterile scalpel blades and transferred to Hank's balanced salt solution containing collagenase (type II, Gibco BRL) 2mg/ml, DNase (0.2mg/ml = 1400U/ml), penicillin (200U/ml) streptomycin (200μg/ml) and amphotericin 0.5μg/ml. The tissues was incubated in this solution (with agitation) for 12-16 hours. The suspension was then drawn into a 30ml syringe 4 times (to disperse the cells further) and filtered through 4 layers of sterile gauze cloth. The filtrate was centrifuged at 600g for 10 minutes and the pellet suspended Ham's F-12 Dulbecco's minimal essential medium containing 10% fetal bovine serum (v/v), penicillin (100U/ml), streptomycin (0.1mg/ml) and amphotericin (0.25μg/ml). The cells were then placed in a tissue culture flask and incubated at 37C in 95% air 5% CO_2. The culture medium was changed every 48-72 hours. Cell growth was assessed by visual inspection using a phase contrast microscope and confluence was reached in about 7 days. When confluent, the culture medium was removed and replaced with Hank's balanced salt solution (lacking calcium and magnesium) containing HEPES (25mM, pH7.2) and Trypsin-EDTA solution and incubated for 10 minutes at 37C until the cells detached. The cell suspension was added to two volumes of culture medium, centrifuged for 10 minutes at 600g and then re-suspended in culture medium and pelleted again. The cells were then re-suspended in culture medium and counted. The cells were then plated at a density of 100,000 per cm^2.

2.3 Statistics

Continuous variables were summarized by the mean and standard error of the mean (SEM). Comparison of two means was made by Student's t-test. Comparison of three or more means was made using analysis of variance (ANOVA). Comparison of paired data was performed using Student's paired t-test when comparing two groups and by repeated

measures ANOVA when comparing three or more groups. Multiple comparisons following ANOVA was performed using Tukey's method. Comparison of means adjusted for a continuous covariate was performed using analysis of covariance. Correlation was determined using both the Pearson correlation coefficient and by Spearman's rho. Statistical significance was assumed at the 5% level. Statistical analysis was performed using SPSS version 9.0 (SPSS Inc, Chicago IL, USA) and Stata version 6.0 (Stata Corporation, College Station, TX, USA).

Chapter 3. Regional and tissue variation in prostanoid receptor gene expression

To deliver the fetus, the uterus must exert force with a degree of polarity to direct the baby through the cervix and birth canal, which necessitates a relative relaxation of the LUS. Following delivery, however, the whole uterus must contract to prevent hemorrhage. Effective delivery of the fetus and contraction of the empty uterus post-partum are essential for neonatal and maternal survival. But the process by which differential, regional control of the uterus is effected is as yet unknown.

I sought to test the hypothesis that differential control of the upper and lower poles of the uterus would be reflected in regional variation in the response to PGs and that any such variations would in turn be paralleled by regional differences in the expression of prostanoid receptor mRNA. Furthermore, given the large family of receptor types and sub-types, there is clear potential for the use of selective agonists and antagonists, as appropriate, in the clinical situations described section 1.6. Therefore, I also sought to describe the pattern of expression of prostanoid receptors in key intra-uterine tissues.

3.1 Methods.

Caesarean section hysterectomy was performed on 8 animals at the following days gestational age (dGA): 121, 153, 159, 162, 162, 177, 177, 180 (term=180-185 days) and samples of myometrium were obtained from different regions of the uterus. The cervix was un-effaced and closed in all animals. Uterine electromyogram leads had been sited in 3 hysterectomy animals going to term (delivered at 177, 177 and 180dGA). Analysis of EMG traces of the 48 hours preceding surgery revealed no contraction activity. No drugs of any form had been administered to any of the animals in the 2 weeks preceding surgery. Samples were dissected immediately following removal of the uterus and flash frozen. The LUS was defined as the portion of the uterus superior to the internal os of the cervix. The fundus was defined as the portion of the uterus superior to the upper limit of the uterine cavity. Samples from the corpus were obtained from both the anterior and posterior wall. Upper and lower corpus were defined as lying above and below (respectively) the midline between the upper limit of LUS (which I took as 1cm superior to the internal os of the cervix) and the lower limit of fundus. The site of sampling are outlined schematically in Figure 3.1.

Northern blot was performed as described in Chapter 2. The composition of the pre-hybridisation buffer was freshly de-ionised formamide (50% vol/vol), sodium phosphate (50mM), NaCl (0.8M), sodium dodecyl sulphate (SDS, 2% wt/vol), herring sperm DNA (100μg /ml), yeast transfer RNA (20μg /ml) and 1X Denhardt's (1X = 1% solution of BSA, Ficoll and polyvinylpyrrolidone) and hybridisation buffer was the same with the probe added. Random primed DNA probes were used in all Northern blots described in this chapter.

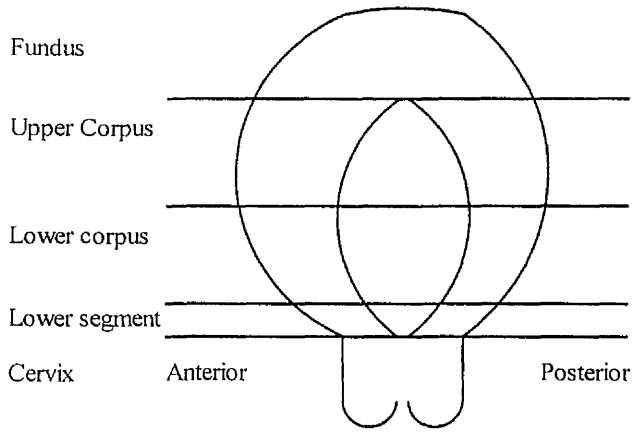


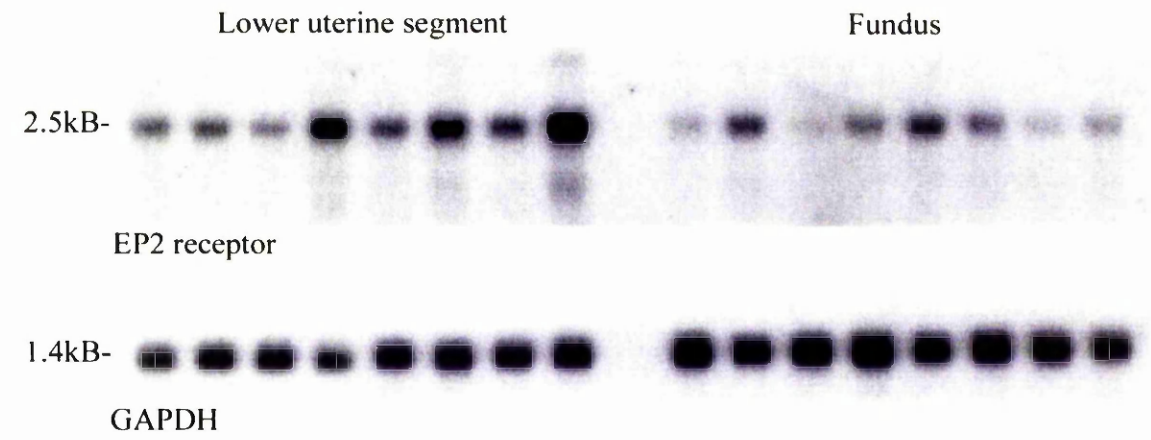
Figure 3.1 Schematic outline of site of sampling from myometrium. The figure represent the appearance of the uterus cut in a longitudinal section in the midline.

3.2 Results.

A preliminary screen for the eight currently recognized prostanoid receptor types and sub-types (EP_{1-4}, DP, IP, TP and FP) using Northern blot analysis and cDNA probes identified EP_2, EP_3, EP_4 and FP receptor mRNA in baboon myometrium. Samples of RNA from LUS and fundus from the 8 hysterectomies were then analysed for these receptors. Compared with fundus, LUS exhibited more EP_2 (Figures 3.2A and 3.3A), less EP_3 (Figures 3.2B and 3.3B) and similar levels of EP_4 and FP mRNA (Figures 3.3C and 3.3D). The corpus also had less EP_2 and more EP_3 mRNA compared with LUS (Figure 3.4). There was no correlation between the level of expression of any of these genes in either LUS or fundus with the gestational age of the animal at the time of Caesarean section.

Northern blot analyses of prostanoid receptor mRNA in myometrium (anterior corpus), cervix, decidua and chorion demonstrated EP_2, EP_3 and EP_4 receptor mRNA in all four tissues. FP receptor mRNA was only detected in myometrium and cervix (Figure 3.5). There was a characteristic distribution of EP receptor mRNA in the four tissues, with the EP_2 receptor being most abundant in cervix, EP_3 most abundant in myometrium and EP_4 most abundant in decidua (Figure 3.6). There was no difference in EP_4 or FP receptor mRNA comparing myometrium and cervix (Figure 3.6). EP_1, IP, DP and TP expression were not reliably detected by Northern blot in any of these tissues using cDNA probes, as were employed in these analyses.

A.



B.

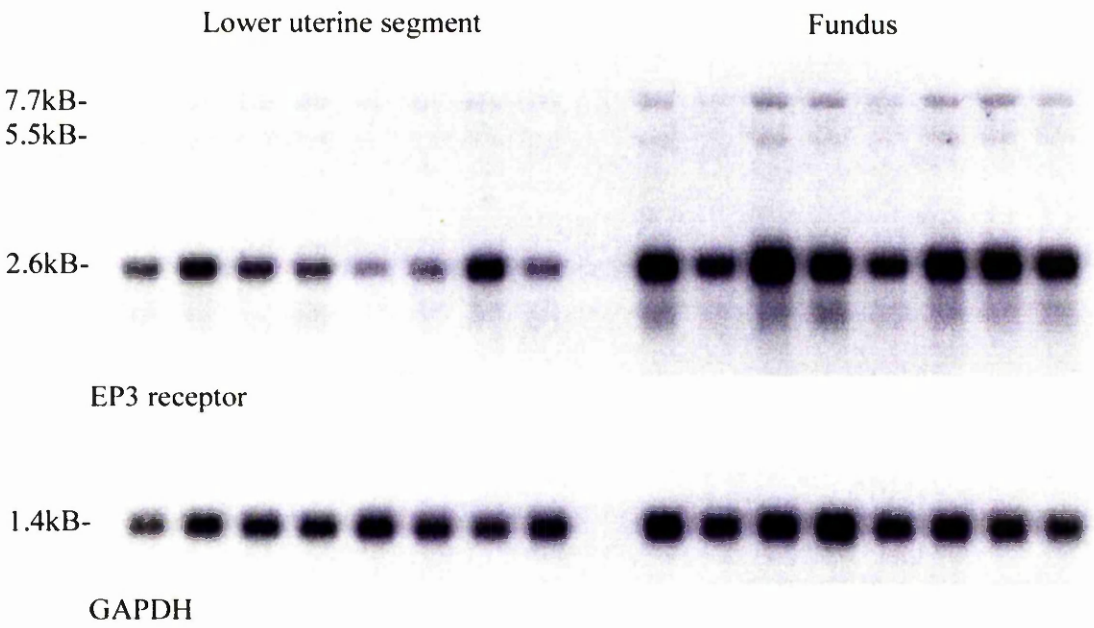


Figure 3.2A-B. Northern blot of EP_2 and EP_3 expression in lower uterine segment (LUS) and fundus from 8 pregnant baboons between 121-180dGA. Variation in loading was corrected by subsequent probing of the same blot for GAPDH. Each lane was loaded with 2μg polyadenylated RNA (EP_3) or 5μg (EP_2).

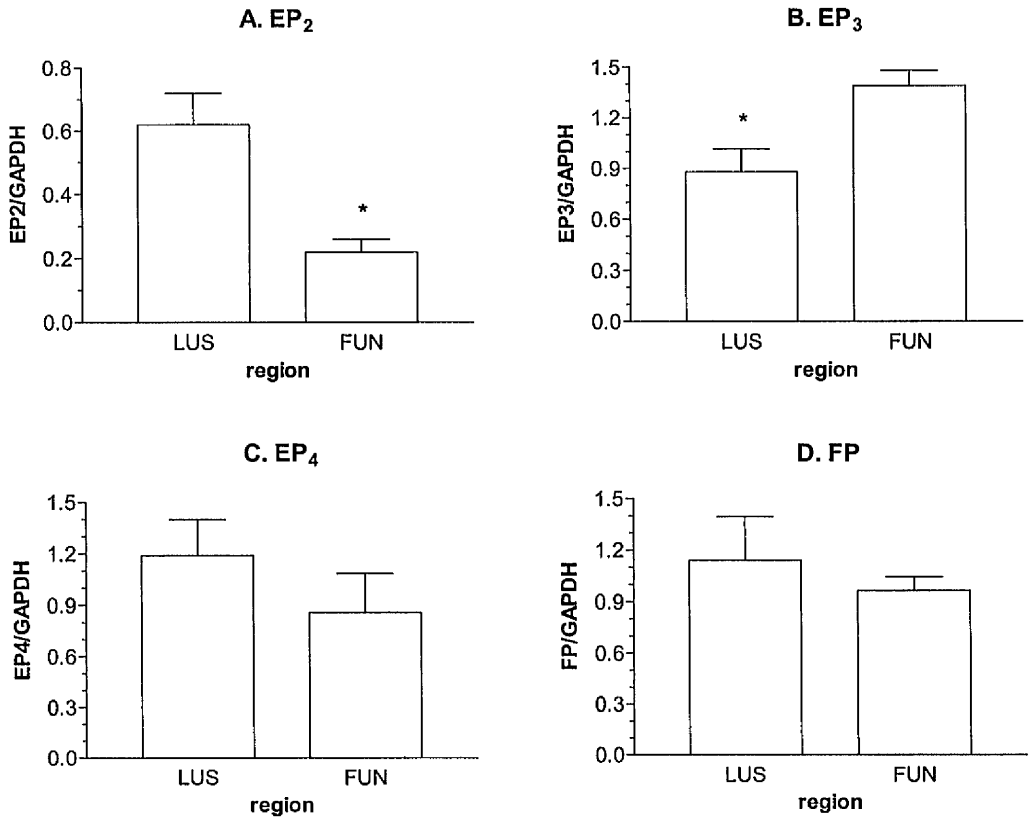


Figure 3.3. Densitometric quantitation of EP_2, EP_3, EP_4 and FP receptor mRNA relative to GAPDH comparing lower uterine segment (LUS) and fundus. Densitometry for EP_3 was performed on the signal of approximately 2.4kB (see Figure 2A). Statistical comparison of LUS vs. fundus (means ± SEM, n=8) by Student's t-test, *$p<0.01$.

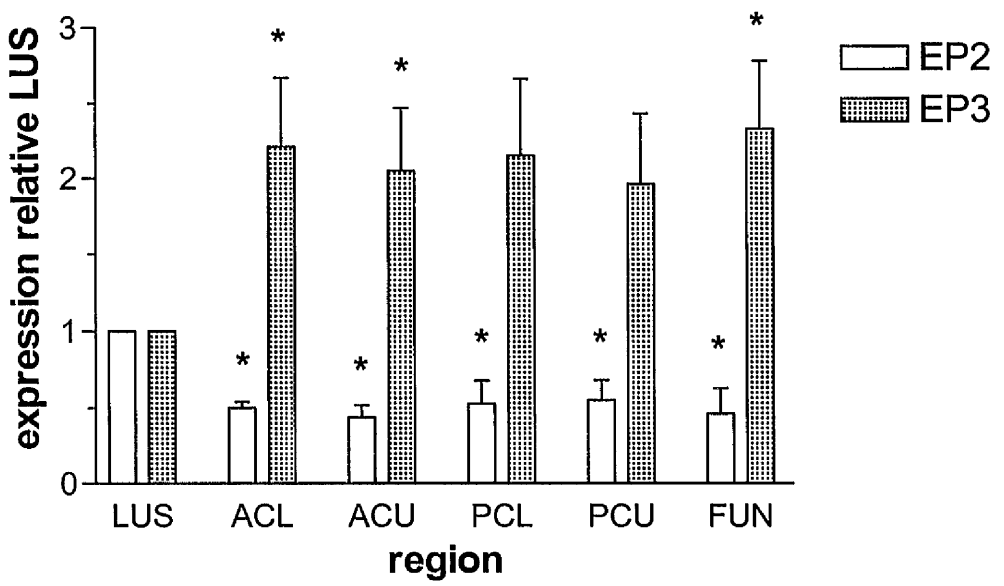


Figure 3.4. Densitometric quantitation of EP_2 and EP_3 mRNA in different regions of baboon uterus using lower uterine segment (LUS) as a reference. Each lane was loaded with 5μg of myometrial mRNA extracted from one of 6 regions of a single animal's uterus: LUS= lower uterine segment, ACL = anterior corpus lower, ACU = anterior corpus upper, PCL = posterior corpus lower, PCU = posterior corpus upper, FUN = fundus. Blots were probed for EP_2 or EP_3 and then for GAPDH. The signal for a given probe was expressed as a ratio to the signal for GAPDH. For a given blot, the signals in the other 5 regions were expressed as a ratio to the signal in LUS (means ± SEM, n=6-7). The data were then pooled and means and 95% CI were calculated for each region with the LUS as unity and a significant difference (*) with LUS was assumed where 95% CI excluded 1.0.

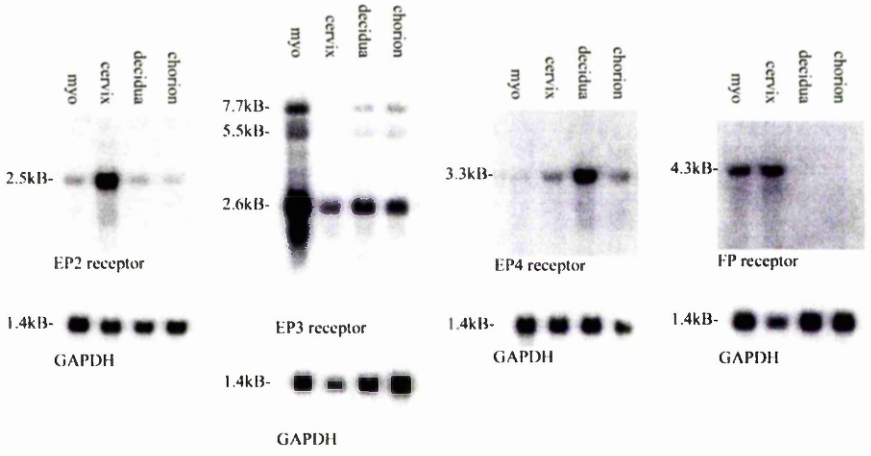


Figure 3.5A-D. Representative Northern blots of EP_2, EP_3, EP_4 and FP receptor mRNA in myometrium from anterior corpus (MYO), cervix (CX), decidua and chorion. Each lane was loaded with 10µg polyadenylated RNA.

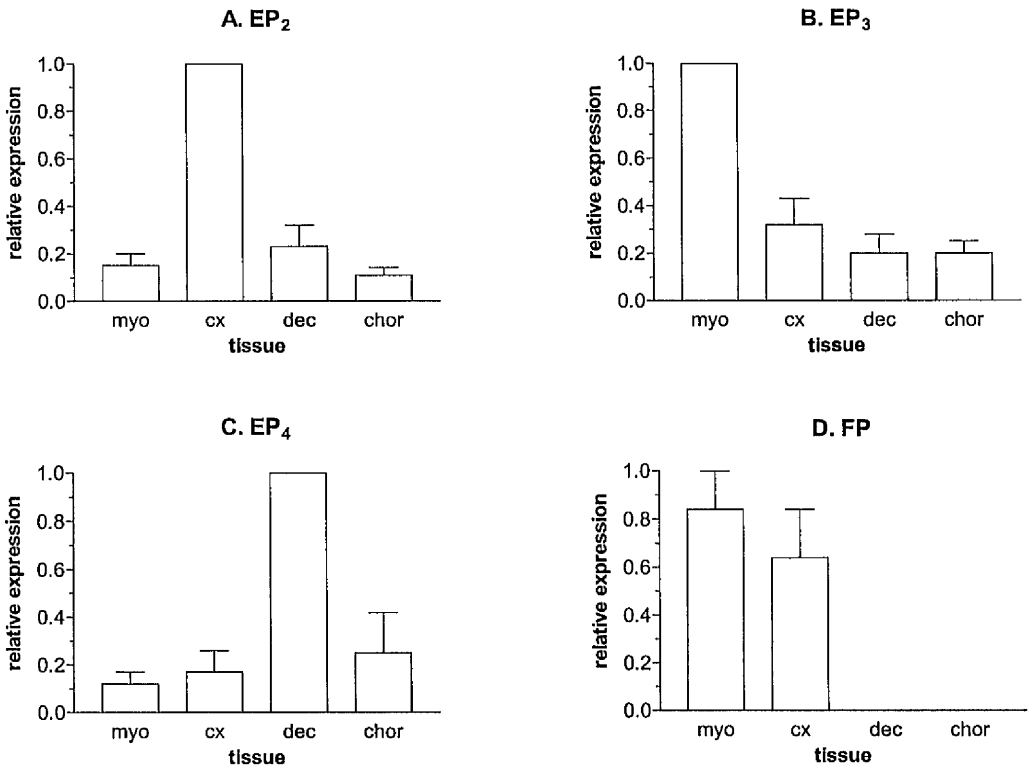


Figure 3.6A-D. Quantitation by densitometry of EP_2, EP_3, EP_4 and FP receptor mRNA in myometrium from anterior corpus (MYO), cervix (CX), decidua (DEC) and chorion (CHOR) as a ratio to GAPDH. In each blot (see Figure 3.5 for representative blots), the highest signal was expressed as unity and the others were expressed as a ratio to it. The ratios for 3-5 blots from different animals were then pooled and the 4 regions compared by ANOVA, plus Tukey's multiple comparisons. EP_2 mRNA expression was significantly greater in the cervix than in the other 3 tissues ($p<0.05$, $n=5$), EP_3 mRNA was significantly greater in MYO than in the other 3 tissues ($p<0.05$, $n=4$), EP_4 was significantly greater in decidua than in the other tissues ($p<0.05$, $n=3$). There was no significant difference in EP_4 or FP ($n=3$) expression comparing MYO and CX.

3.3 Discussion.

When considering the regulation of myometrial contractility, most attention is focused on factors controlling delivery of the fetus. However, effective post-partum contraction of the uterus is probably equally important from an evolutionary perspective. The processes of delivery of the fetus and post-partum contraction are quite different. Delivery of the fetus requires phasic contraction with a relative relaxation of the lower segment. Post-partum contraction requires tonic smooth muscle activation of the whole uterus. Therefore, a possible means by which differential regulation of uterine contraction (i.e. pre- and post-delivery of the fetus) might be studied is by addressing regional variation in the control of contractility.

One reason why these issues have not been fully addressed is the difficulty in obtaining specimens of healthy pregnant human myometrium from regions other than the LUS. There are some reports of analysis of Caesarean hysterectomy specimens and one report demonstrated regional variation in oxytocin receptors [193]. However, these reports are anecdotal and are necessarily limited by the opportunities to obtain healthy tissues. There are some studies comparing lower segment strips with biopsies of fundus also obtained at the time of lower segment Caesarean section [194]. However, these did not utilize full thickness strips of fundus which introduces a potential for bias in comparing LUS and fundus.

At the same time as samples were obtained for Northern analysis, paired samples were obtained for contractility studies. It was found that PGE_2 contracted myometrial strips from the fundus but not the lower segment [195]. (These findings are reported in a paper where I was first author, but I have not included them in this thesis. Although I directed these experiments and devised the experimental protocol, the actual practical work was

carried out by a colleague and, therefore, I have excluded these data from this thesis.) The LUS is formed during pregnancy from the cervix [196]. Given the fibrous nature of the cervix, it might be expected that the LUS would exhibit a generalized reduced contractility compared to the fundus. However, its response to $PGF_{2\alpha}$ was virtually identical to fundus [195]. These observations implied that the lack effect of PGE_2 on LUS was a manifestation of differences in prostanoid signal transduction.

As discussed in Chapter 1, four receptor sub-types have been identified pharmacologically which mediate the effects of PGE_2 [11]. EP_1 and EP_3 receptors generally stimulate and EP_2 and EP_4 receptors generally inhibit smooth muscle[11]. I tested the hypothesis that the reduced response of LUS to PGE_2 compared with fundus was secondary to higher expression of inhibitory EP receptors and/or lower expression of stimulatory EP receptors in LUS. I found that LUS had more inhibitory EP_2 receptor mRNA and less contractile EP_3 receptor mRNA than the fundus, but similar levels of EP_4 mRNA (Figure 3.3). This supported the hypothesis that varying receptor populations explain the different magnitude of the effects of PGE_2 on LUS and fundus. This hypothesis was lent further support as the levels of FP receptor mRNA were similar in LUS and fundus (Figure 3.3), also paralleling the response to native PGs [195]. Samples of myometrium from the corpus showed similar levels of EP_2 and EP_3 mRNA to the fundus (Figure 3.4).

Given that the LUS is formed from the cervix [196], I hypothesized that the cervix might exhibit similar quantitative differences in prostanoid receptor mRNA when compared with the corpus. EP_2, EP_3 and EP_4 receptor mRNA were expressed in myometrium, cervix, decidua and chorion (Figure 3.5). There was a characteristic pattern of expression of the sub-types with the EP_2 receptor mRNA most abundant in cervix, EP_3 in

myometrium and EP_4 in decidua. EP_4 and FP receptor expression were similar in myometrium and cervix (Figure 3.6). Therefore, the pattern of prostanoid receptor mRNA comparing the cervix and corpus paralleled the pattern comparing LUS and fundus. I conclude that different proportions of EP_2 and EP_3 receptors in LUS are likely to be due to its cervical origin.

The foregoing assumes that levels of mRNA parallel the amount of functional receptors in the respective tissues. This assumption is supported by the contractility studies [195]. It might be further supported by immunocytochemistry or Western blot to confirm translation of mRNA into protein. However, antibodies against the various EP receptor sub-types are not yet widely available. The size of transcripts observed in the present study was similar to previous reports of Northern blots from the human. As in previous studies in the human, multiple bands were present using the EP_3 probe [197], whereas discrete bands were observed using the EP_2 [198], EP_4 [199] and FP [200] probes. Furthermore, the estimated size of transcripts was similar to these reports of Northern analysis of human RNA. Such variation as was observed (less than 20%) may be related to variation in un-translated regions of the gene which has been previously been inferred by the discrepancy between the size of the full length cDNA and the size of transcripts in Northern analysis [201] as well as inevitable variation due to experimental error.

These pharmacological differences in LUS and fundus might explain the differential regional control of the uterus before and following delivery of the fetus. I postulate that high levels of PGE_2 from the fetal membranes during labour [202] stimulate EP_2 receptors in LUS during labour, which would favour selective relaxation of LUS to allow passage of the fetus. Following delivery of the membranes, PGE_2 levels will fall, which would favour stimulation of the EP_3 receptor, as its affinity for PGE_2 is more than an order of

magnitude greater than EP_2 receptor [47]. Furthermore, the activity of other uterotonic agents with a purely contractile effect on the LUS, such as $PGF_{2\alpha}$, would stimulate contraction without opposition by EP_2-mediated inhibition.

The complex family of prostanoid receptors in decidua and chorion were also of interest. Prostaglandins have been postulated to mediate the clinically evident relationship between infection and PPROM, but current models invoke an indirect action on the membranes, namely, by stimulating uterine activity which then promotes membrane rupture [97]. The presence of multiple EP receptor sub-types in chorion implies a direct effect of PGE_2 on the fetal membranes. The presence of EP_2, EP_3 and EP_4 receptors on decidua also suggests that PGE_2 acts directly on the decidua. It was interesting that the EP_4 receptor appeared to have much higher levels of expression in the decidua than in adjacent tissues. All these receptors are coupled to adenylate cyclase, EP_2 and EP_4 positively and EP_3 negatively [11]. Establishing the effects of cyclic AMP dependent protein kinases should shed light on the effects of PGE_2 on these tissues.

These data have very clear clinical significance. The high level of EP_2 expression in the cervix (Figure 3.5A) may be important for drug development to induce labour. PGE_2 is widely employed to prime the cervix [95]. The use of PGE_2 reduces the incidence of prolonged labour, Caesarean and instrumental vaginal delivery [203]. However, uterine hyper-stimulation (probably EP_3 receptor mediated [86]) is the dose-limiting side effect of vaginal PGE_2 [204]. PGE_2 stimulation of glycosaminoglycan synthesis by cervical fibroblasts is mediated by activating adenylate cyclase [205]. Given that the EP_2 receptor is coupled positively to adenylate cyclase [11] and EP_2 expression is high in the cervix (Figures 3.5 and 3.6), it is possible that the effect of PGE_2 on the cervix is mediated, at least in part, by the EP_2 receptor. Selective EP_2 agonists (which are currently available [13])

may, therefore, be as effective in priming the cervix as PGE_2, but would not be expected to cause uterine hyper-stimulation.

There is preliminary evidence to support the above from randomised controlled trials. There are two reports that misoprostol is more effective in priming the cervix than PGE_2 without increasing the risk of hyper-stimulation [206;207]. The affinity of PGE_2 for the EP_3 receptor is 14-fold greater than the EP_2 receptor [47]. However, the affinity of misoprostol for the EP_3 receptor is less than 4-fold greater than EP_2 [47]. If the EP_2 receptor mediates cervical priming, it would be predicted that the greater the relative affinity of a drug for the EP_2 receptor compared with the EP_3 receptor, the safer and more effective it will be in priming the cervix. However, this is somewhat at odds with the apparent efficacy of sulprostone, a selective EP_1 and EP_3 agonist, in priming the cervix in early pregnancy [208]. Sulprostone is a potent stimulator of myometrial contractility [86] and it is possible that selective agonists of different EP receptor sub-types might prime the cervix by different mechanisms of action. The possible role of EP_2 receptor activation in the control of cervical compliance during labour may be inferred by whether the gene is up-regulated or down-regulated in labour. This issue is addressed in a later chapter.

The finding that $PGF_{2\alpha}$ contracted both the LUS and fundus [195] and that expression of the FP receptor gene was similar in the two regions (Figure 3.3) provide a rational basis for the empirical usefulness of 15-methyl-$PGF_{2\alpha}$ in the treatment of PPH refractory to other uterotonic agents [209].

PG synthesis inhibitors are currently used in the treatment of pre-term labour but contract the fetal ductus arteriosus [210]. It has been proposed that selective antagonists of prostanoid receptors may be useful in the treatment of pre-term labour and have fewer effects on the fetus than COX inhibitors [39;99]. However, the complex pattern of multiple

EP receptor sub-types and FP receptor expression in key intra-uterine tissues controlling parturition (Figure 3.5) may limit the usefulness of a drug acting on a single receptor type or sub-type in the treatment of pre-term labour.

Chapter 4. The effect of labour and gestational age on expression of prostanoid receptor genes in myometrium

It is evident from the human clinical use of exogenous synthetic PGs to induce labour that the response to a standard dose of PGE_2 is highly variable between women, from virtually no effect to excessive stimulation of uterine contraction necessitating immediate delivery by Caesarean section [211]. This observation suggests that the sensitivity of the uterus to PGs might be a factor controlling myometrial contractility at term and in labour. In the preceding chapter it was established that variation in contractile responses to native PGs was paralleled by variation in the expression of prostanoid receptor types and sub-types. In the present chapter, I sought to test the hypothesis that variation in the expression of prostanoid receptors at term and in labour has a role stimulating myometrial contractility in primate parturition. I quantified the expression of prostanoid receptor genes in baboon myometrium obtained from 15 animals in the last third of pregnancy, five of which were in spontaneous labour. Given that I had also previously shown that expression of receptor genes varied comparing different regions of the uterus, I also determined (a) whether regional variation in prostanoid receptor expression persisted in labour and (b) the cellular localization of prostanoid receptor mRNA expression.

4.1 Methods

The general methods were as described in Chapter 2. The animals employed were as follows. Ten animals were not in labour and in the last 1/3 of pregnancy at the following days gestational age (dGA): 121, 128, 141, 153, 159, 162, 162, 177, 177, 180 (term=180-185 days). The cervix was un-effaced and closed in all of these animals. Uterine electromyogram leads had been sited in three of these animals going close to term (the animals delivered after 170 dGA, term is approximately 185dGA). Analysis of EMG traces of the 48 hours preceding surgery revealed that myometrial activity was solely in the contractures mode with no contraction activity. No drugs of any form had been administered to any of the animals in the two weeks preceding surgery. Caesarean hysterectomy was also performed on five animals in spontaneous labour. Of these, four had EMG electrodes sited. These animals had a baseline cervical examination at the time of their first EMG contraction activity and were re-examined when they had a sustained switch from contractures to contractions (>30 minutes). The gestational ages at hysterectomy were 164, 184, 191, 193. The cervical dilations for these animals were 6cm, 3cm, 3cm and 2cm, respectively (the cervix was closed in all four at the baseline examination). In a fifth animal without EMG electrodes, Caesarean section was performed at 172dGA and at the time of the procedure it was found that the cervix was 4cm dilated and fully effaced and a hysterectomy was performed. Samples of myometrium were obtained from the fundus and lower segment, flash frozen in liquid nitrogen and stored at –80C until use. The lower segment and fundus were defined described in Chapter 3.

Primary culture of myometrial cells was established from a sample of uterine fundus obtained from an animal in spontaneous labour at 193 days gestational age. In situ

hybridisation was performed on frozen sections of an animal at 177 days gestation, where hysterectomy was performed prior to the onset of labour, from the uterine fundus.

4.2 Results.

Clear signals of similar molecular weight to the cloned human prostanoid receptors genes were detected in myometrium for the EP_1, EP_2, EP_3, EP_4, FP, IP and TP receptors genes using riboprobes, both from the lower segment and uterine fundus and from animals in labour and not in labour (Figures 4.1 and 4.2). No clear signal above background could be detected using an antisense riboprobe for the human DP receptor. All blots were stripped and re-probed for each of three housekeeping genes, GAPDH, beta-actin and cyclophilin (Figure 4.3).

Densitometric analysis of these signals (expressed as a ratio to beta-actin) demonstrated that the level of expression of genes encoding the EP_4, FP and TP receptors were similar comparing samples obtained from the LUS and the fundus and comparing animals in labour and not in labour (Table 4.1).

The only signal that demonstrated a change in the overall level of expression in labour was EP_2 receptor mRNA, which was markedly decreased in myometrium obtained from animals in labour. Furthermore, expression of this gene was greater in the lower segment, but the magnitude of the difference between the lower segment and fundus diminished in association with labour (Figure 4.4 and Table 4.1). The expression of IP receptor mRNA also differed comparing lower segment and fundus and the magnitude of the difference also significantly differed comparing samples from animals in labour and not in labour (Figure 4.4 and Table 4.1). Expression of mRNA encoding the EP_1 and EP_3 receptors was also significantly different comparing samples from the lower segment and the fundus, but the magnitude of the differences comparing the two regions was not significantly affected by labour (Figure 4.4 and Table 4.1). There were no significant changes in the expression of prostanoid receptor genes related to gestational age among the animals

delivered prior to the onset of labour. All but one of the comparisons which were statistically significant with beta-actin were also significant when the signal was expressed as a ratio to GAPDH or cyclophilin (data not shown). The exception was comparison of the IP receptor signal between the lower segment and fundus. This was significant at P=0.02 and 0.0004 with beta-actin and cyclophilin, respectively, but the P value was 0.15 when related to GAPDH.

In situ hybridisation was attempted for the EP_2, EP_3, EP_4 and IP receptors. Clear signals above background were only detected for the EP_3 receptor (Figure 4.5A), which is most abundantly expressed in myometrium (Chapter 3). Hybridisation of the EP_3 riboprobe could be prevented by co-incubation with an excess of unlabelled probe (Figure 4.5B). The gene encoding the EP_3 receptor was expressed in myometrial cells (Figure 4.5C), but not in vascular smooth muscle cells of muscular arteries in the uterine wall (Figures 4.5A and 4.5D).

Expression of the genes encoding the prostanoid EP_2 and EP_3 receptors could not be detected in cultured baboon myometrial cells. Visual inspection of the cells demonstrated that they retained the characteristic spindle shape of smooth muscle cells in culture.

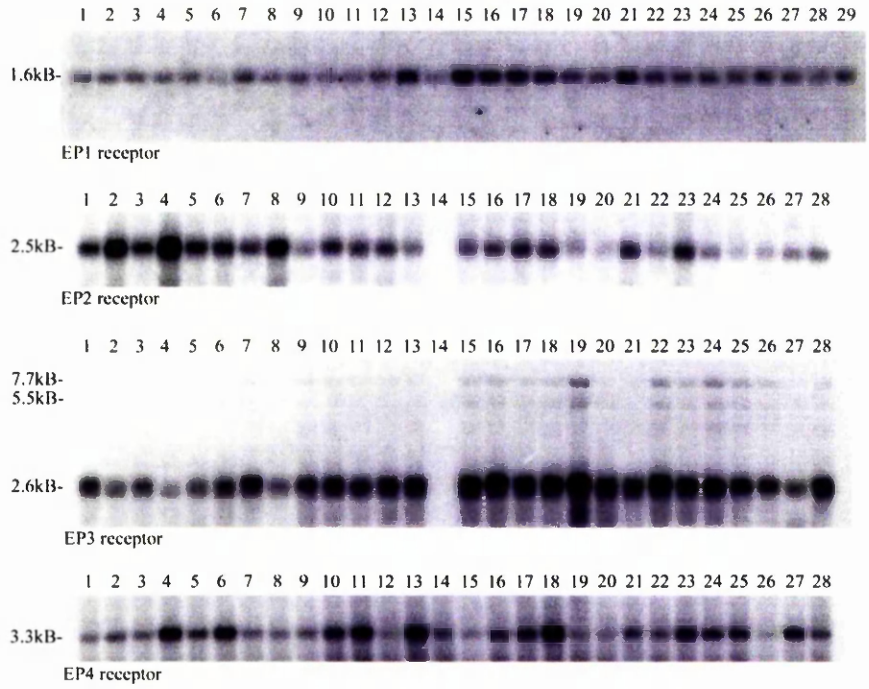


Figure 4.1. Northern blots of EP receptor genes in baboon myometrial mRNA from lower uterine segment (LUS) and fundus (FUN), both not in labour (NIL) and in labour (LAB). 2μg each lane loaded in each lane, probed for all four currently recognized EP receptor genes. Lanes as follows: **EP_1:** 1-9 = LUS, NIL; 10-14 = LUS, LAB; 15-24 = FUN, NIL; 25-29 = FUN, LAB. **EP_2 and EP_3:** 1-8 = LUS, NIL, 9-13 = LUS, LAB, 15-23 = FUN, NIL; 24-28 = FUN, LAB. **EP_4:**1-9 = LUS, NIL; 10-14 = LUS, LAB; 15-23 = FUN, NIL; 24-28 = FUN, LAB.

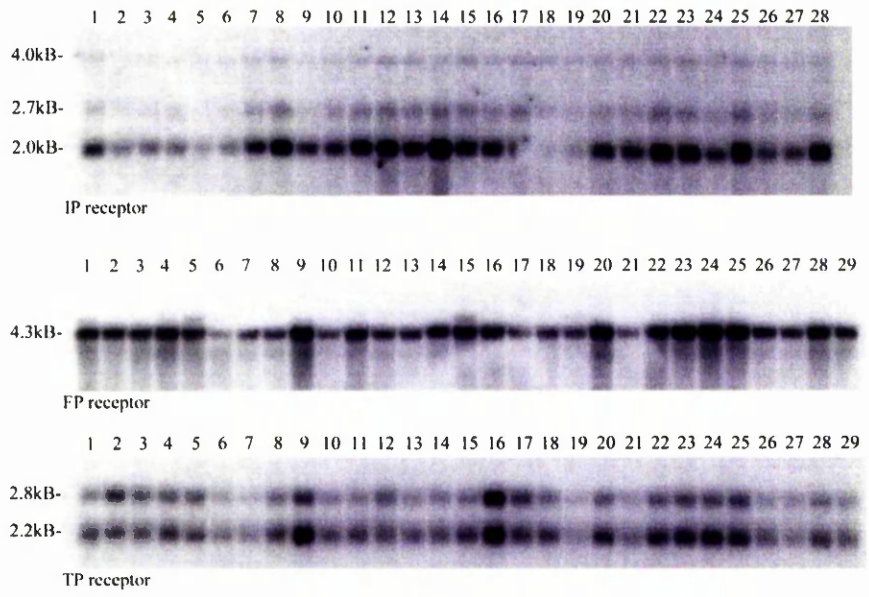


Figure 4.2. Northern blots of non-EP prostanoid receptor genes in baboon myometrial mRNA from lower uterine segment (LUS) and fundus (FUN), both not in labour (NIL) and in labour (LAB). 2μg each lane, probed for the IP, FP and TP receptor genes. Lanes as follows: **IP:** 1-9 = LUS, NIL; 10-14 = LUS, LAB; 15-23 = FUN, NIL; 24-28 = FUN, LAB. **FP and TP:** 1-9 = LUS, NIL; 10-14 = LUS, LAB; 15-24 = FUN, NIL; 25-29 = FUN, LAB.

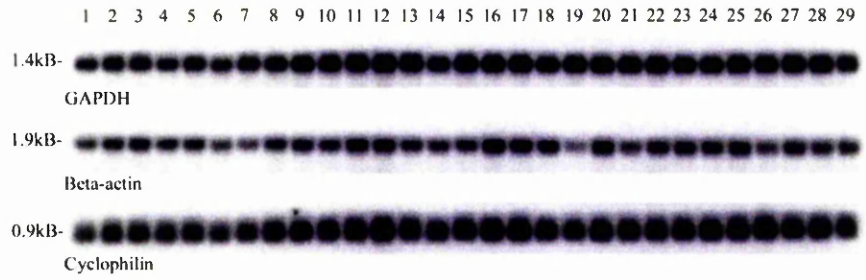


Figure 4.3. Northern blots of housekeeping genes in baboon myometrial mRNA from lower uterine segment (LUS) and fundus (FUN), both not in labour (NIL) and in labour (LAB). 2μg each lane consecutively probed and stripped for the GAPDH, beta-actin and cyclophilin genes. Lanes as follows: 1-9 = LUS, NIL; 10-14 = LUS, LAB; 15-24 = FUN, NIL; 25-29 = FUN, LAB.

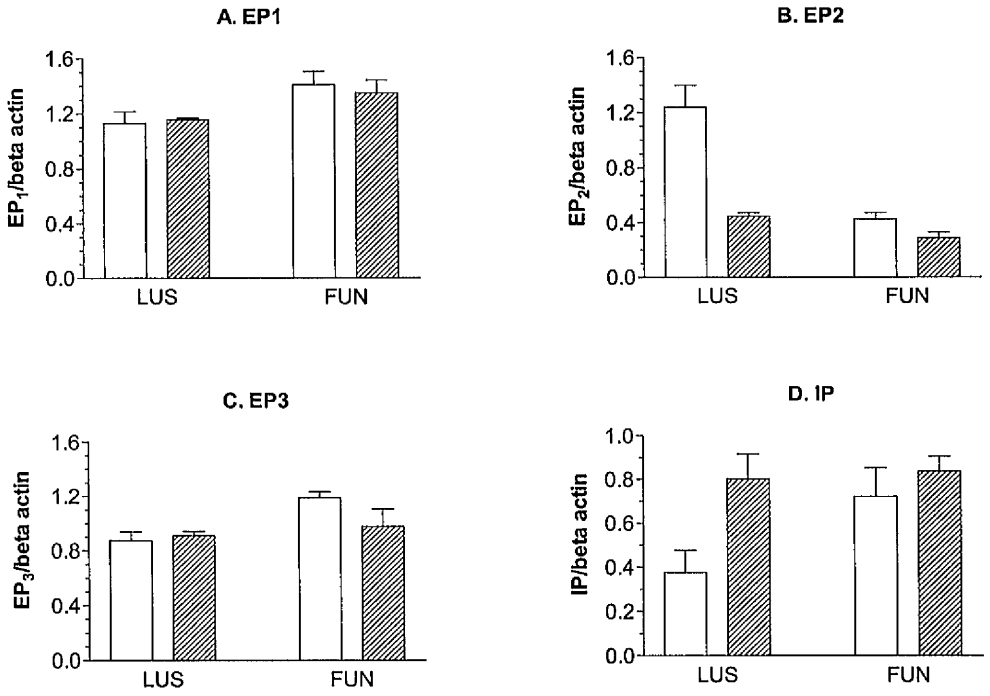


Figure 4.4. Densitometric quantitation of prostanoid receptor signals (as a ratio to beta-actin signal) for prostanoid receptor types in lower uterine segment (LUS) and fundus (FUN) from animals both not in labour (white bars) and in labour (shaded bars). A. EP_1 receptor **B.** EP_2 receptor **C.** EP_3 receptor and **D.** IP receptor. Significant differences between groups, compared with repeated measures ANOVA: effect of labour, EP_2 only; uterine site, EP_1, EP_2, EP_3 and IP; interaction between uterine site and labour, EP_2 and IP only. For details of statistical analysis, see table 4.1.

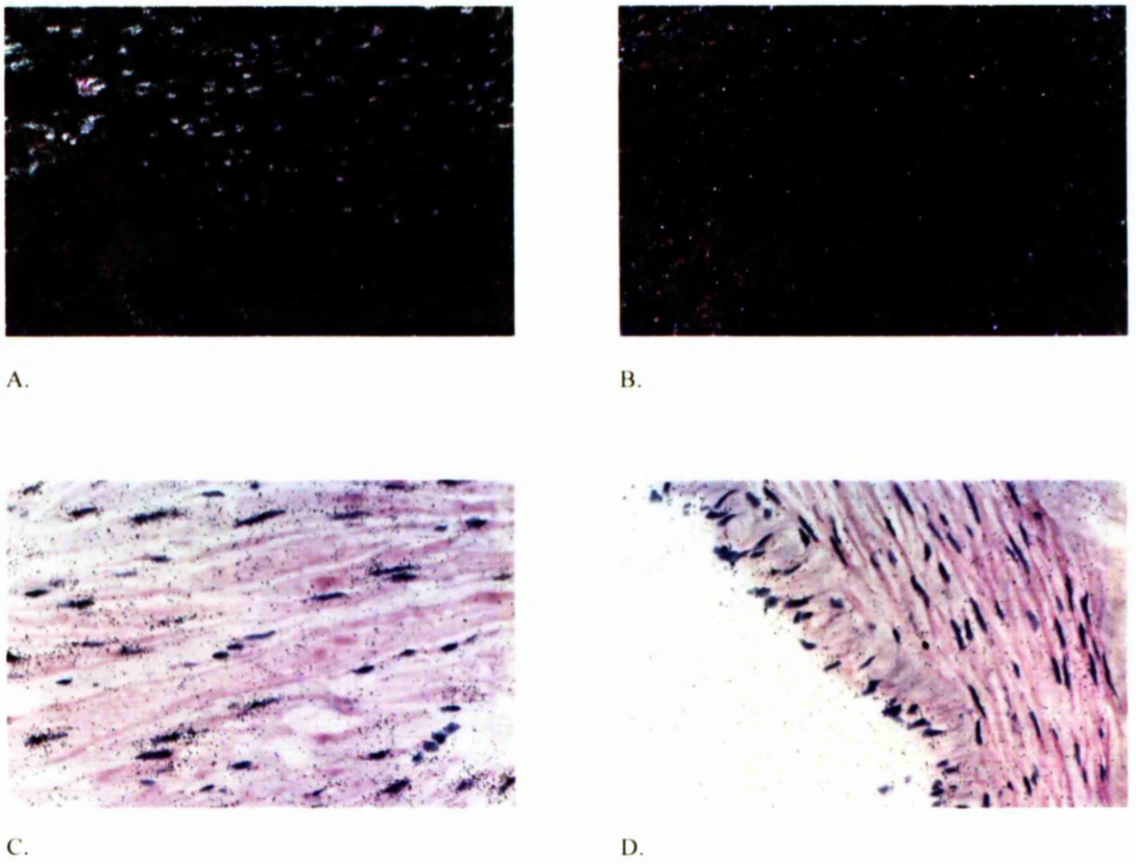


Figure 4.5. In situ hybridisation of EP_3 receptor riboprobe of section from uterine fundus, obtained from animal not in labour at 177 days gestation. A. Low power, dark field, probe without competitor. **B.** Low power, dark field, probe plus unlabelled competitor. **C.** Medium power of myometrial smooth muscle cells. **D.** Medium power vascular smooth muscle cells from muscular artery within uterine wall.

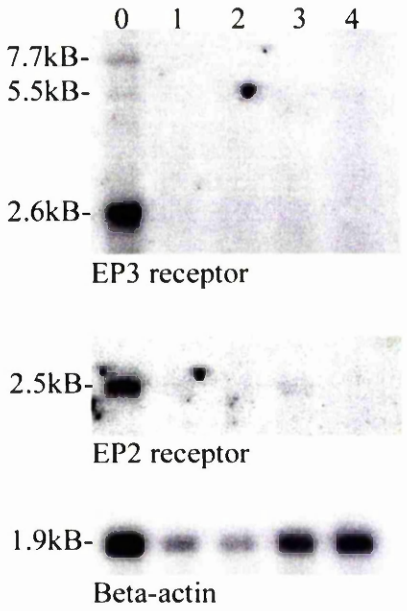


Figure 4.6 Expression of EP_2 and EP_3 receptor genes in fresh and cultured myometrium from uterine fundus of animal in term labour. Lane 0, flash frozen intact fundus, lanes 1-4 first four passages of primary culture of myometrium from same animal. 0.5 μg RNA loaded in each lane.

Table 4.1. Repeated measures analysis of variance of effect of anatomical site and labour on myometrial expression of prostanoid receptor genes, quantified densitometrically as a ratio to beta-actin.

Gene	Lower segment mean (SEM)		Fundus mean (SEM)		LAB vs. NIL	LUS vs. Fundus	Interaction
	NIL	LAB	NIL	LAB	P=	P=	P=
EP_1	1.13 (0.08)	1.16 (0.13)	1.40 (0.10)	1.35 (0.09)	0.94	0.02	0.57
EP_2	1.24 (0.16)	0.45 (0.03)	0.42 (0.05)	0.29 (0.04)	0.0003	0.002	0.02
EP_3	0.88 (0.06)	0.91 (0.03)	1.19 (0.04)	0.98 (0.13)	0.23	0.01	0.10
EP_4	0.82 (0.12)	0.69 (0.07)	0.63 (0.06)	0.66 (0.10)	0.67	0.17	0.28
FP	1.88 (0.18)	1.66 (0.20)	1.70 (0.19)	1.45 (0.12)	0.36	0.24	0.89
IP	0.38 (0.10)	0.80 (0.12)	0.72 (0.13)	0.84 (0.07)	0.12	0.02	0.05
TP	1.26 (0.12)	1.13 (0.07)	1.23 (0.08)	0.86 (0.08)	0.07	0.13	0.16

NIL = not in labour, LAB = labour, LUS = lower uterine segment. Not in labour n= 8-10, labour, n=5

4.3 Discussion.

There are a number of lines of evidence in a range of species that have indicated a key role for PGs in the control of parturition, and these were reviewed in Chapter 1. Much of the research on the potential role of PGs in the initiation and promotion of labour has focused on factors determining the concentration of endogenous PGs in intra-uterine tissues, in particular the relative balance of the expression and activity of PG synthetic enzymes (COX-1 and COX-2) [212] and PGDH, which converts PGs to inactive metabolites [148]. A number of other studies have looked at signal transduction aspects of myometrial smooth muscle which may affect the response of the uterus to PGs, such as expression of G-proteins [213] and genes encoding gap junctions [214]. However, there has been much less research on the role of variation in the expression of prostanoid receptor types and sub-types in the control of myometrial contractility in the human and non-human primate.

The most striking observation of the present study was that labour was associated with a much lower level of expression of the inhibitory prostanoid EP_2 receptor and this was observed both in the fundus and lower segment. Studies of isolated strips of human myometrium have demonstrated a profound inhibitory effect of EP_2 agonists on contractility and a profound excitatory effect of EP_3 receptor activation [86]. Reduced expression of EP_2 receptors would be anticipated to increase the stimulatory effect of a given concentration of PGE_2 on the myometrium acting through EP_1 and EP_3 receptors and, therefore, promote the process of parturition. I hypothesize, therefore, that reduced expression of the inhibitory EP_2 receptor in primate myometrium may have a key role in promoting myometrial contraction during labour. If confirmed in the human, this suggests that an EP_2 receptor antagonist might have a role in the induction of labour and that an EP_2 receptor agonist may have a role in the treatment or prophylaxis of preterm

labour, although the effect of such a drug may be diminished in active labour due to loss of EP_2 receptor expression.

I confirmed the previous observation (Chapter 3) of regional differences in EP_2 and EP_3 receptor gene expression comparing lower segment and fundus and that these differences were maintained in labour. Interestingly, the magnitude of the difference in EP_2 receptor gene expression between the lower segment and fundus was significantly diminished by labour, although a difference persisted.

By optimizing techniques, principally the use of riboprobes, I could also detect EP_1, TP and IP receptor gene expression using Northern analysis, whereas these genes could not be detected using the initial methodologies outlined in Chapter 3. These studies demonstrated regional variation in both the EP_1 and IP receptor genes. In the case of the EP_1 receptor gene, expression was greater in the fundus than the lower segment, but labour had no statistically significant effect on either the absolute level of expression or the difference between the two regions. In the case of the IP receptor gene, expression was lower in the lower segment before labour, but not in tissue obtained during labour. The higher level of expression of the excitatory EP_1 is consistent with the previous observation that strips of myometrium from the fundus exhibited a greater contractile response to PGE_2 than strips from the lower segment [195]. The greater level of expression of the inhibitory IP receptor in the lower segment during labour suggests that PGI_2 may inhibit myometrial contractility in the lower segment during labour, but this issue requires further study.

Relating the current observations to previous studies there are interesting parallels and differences. Reduced expression of EP_2 receptor mRNA in myometrium in association with labour (or advancing gestational age) has now been demonstrated in the baboon

(present study), rat [92] and human [91], but not in sheep [215]. This species difference in genetic expression is paralleled by functional differences in response of myometrial strips, since both human [86] and baboon myometrium [89] relax in response to EP_2 receptor agonists, whereas these drugs are without effect in ovine myometrium [90].

The lack of an effect of labour on expression of the FP receptor gene differs from studies using human LUS which demonstrated increased FP receptor mRNA in biopsies taken from women in labour at the time of Caesarean section, as well as animal studies in the rat [92] and sheep [215]. The contractile response to $PGF_{2\alpha}$ and expression of FP receptor mRNA is the same in myometrial strips from the lower segment and fundus of baboons [195]. I interpreted this observation as possibly indicating a major role for the FP receptor in post-partum contraction of the uterus, consistent with the efficacy of FP agonists in the management of refractory atonic PPH [209]. It is possible, therefore, that expression of FP receptor mRNA could increase in the final stages of labour, and that the time of sampling of myometrium with respect to the total duration of labour, may explain this difference.

The only receptor gene that could reliably be localized using in situ hybridisation was the EP_3 receptor, which is the prostanoid receptor most abundantly expressed in myometrium (Chapter 3). As expected from functional studies [86], this gene was noted to be highly expressed in myometrial cells (Figures 5A and 5C). Interestingly, the gene did not appear to be expressed in the smooth muscle of small uterine arteries (Figures 5A and 5D). This observation suggests that there may be differential control of myometrial and vascular smooth muscle in the uterus. The blood vessels supplying the uterine muscle would be expected to be dilated most when the uterus was most metabolically active, i.e. during labour. Therefore, uterine vascular smooth muscle should be in a relaxed state when myometrial smooth muscle is in its most active state. On the basis of the in situ

hybridisation findings, differential regulation of myometrial and vascular smooth muscle may be effected by differential expression of prostanoid receptor genes.

Further studies should attempt to confirm that variation in levels of gene expression in the present study are reflected in variation in the number of functional receptors. Furthermore, elucidation of the factors which control EP_2 receptor gene expression in primate myometrium may shed light on the control of primate myometrial contractility at the molecular level. The loss of expression of the prostanoid EP_2 and EP_3 receptor genes in cultured baboon myometrial cells was disappointing as it was hoped that the factors controlling expression of these genes might have been studied in this model.

Chapter 5. The effect of labour and gestational age on expression of prostanoid receptor genes in cervix

Prostaglandins (PGs) are known to have a key role in the control of cervical compliance in human pregnancy [95]. Appreciation of the physiological role of PGs led to clinical trials of exogenous synthetic PGE_2 as a means of improving cervical compliance prior to induction of labour. A large number of randomized controlled trials have demonstrated that PGE_2 is an effective agent in priming the cervix and that its use prior to induction of labour reduces the risk of failed induction and operative delivery [211]. The principal side effect of PGE_2 in this context is uterine hyper-stimulation. Although PGE_2 reduces the overall risk of Caesarean section, the risk is increased in the interval immediately following insertion of PG due to occasional induction of hypertonic uterine activity[211].

In Chapter 3, I demonstrated that the cervix exhibited much greater levels of expression of the adenylate cyclase coupled EP_2 receptor than myometrium, decidua or chorion [195]. I hypothesized that the EP_2 receptor may mediate the clinically important effects of PGE_2 on cervical compliance. Characteristically, expression of genes encoding receptors for hormones that have a stimulatory role in parturition is increased in the given tissue in association with labour [75]. This suggests that if any of the prostanoid receptors had a major role in mediating the changes in cervical compliance associated with labour, that there would be an increase in the expression of the gene encoding the receptor. Conversely, if any receptor mediated an inhibitory effect on these changes, it suggests that the expression of the gene may be decreased in labour. In the this chapter I report quantitative Northern analysis of all the currently recognized G-protein coupled prostanoid receptor genes in the cervix obtained from pregnant baboons by total

Caesarean hysterectomy, comparing animals in labour and not in labour at the time of surgery.

5.1 Methods.

The same animals described in Chapter 4 were employed and the general methods were as outlined in Chapter 2.

5.2 Results.

Clear signals of similar molecular weight to the human prostanoid EP_1, EP_2, EP_3, EP_4, FP, IP and TP receptors genes were detected using Northern analysis of cervical mRNA (Figures 5.1 and 5.2). Multiple transcripts were observed for the EP_3, IP and TP receptor genes. No clear signal above background could be detected using an antisense riboprobe for the human DP receptor.

There was a significant increase in the level of expression of the EP_1 receptor with advancing gestational age (Figure 5.3 and Table 5.1). There were no gestational age-related changes in the level of expression of any of the other receptor genes (Table 5.1).

There were marked and highly significantly lower levels of expression of the EP_2, FP and TP receptor genes in samples obtained from animals in labour compared with those not in labour (Figure 5.4 and Table 5.2). There was no significant difference in the levels of expression of the genes encoding the EP_1, EP_3, EP_4 or IP receptors comparing animals in labour and not in labour (Table 5.2).

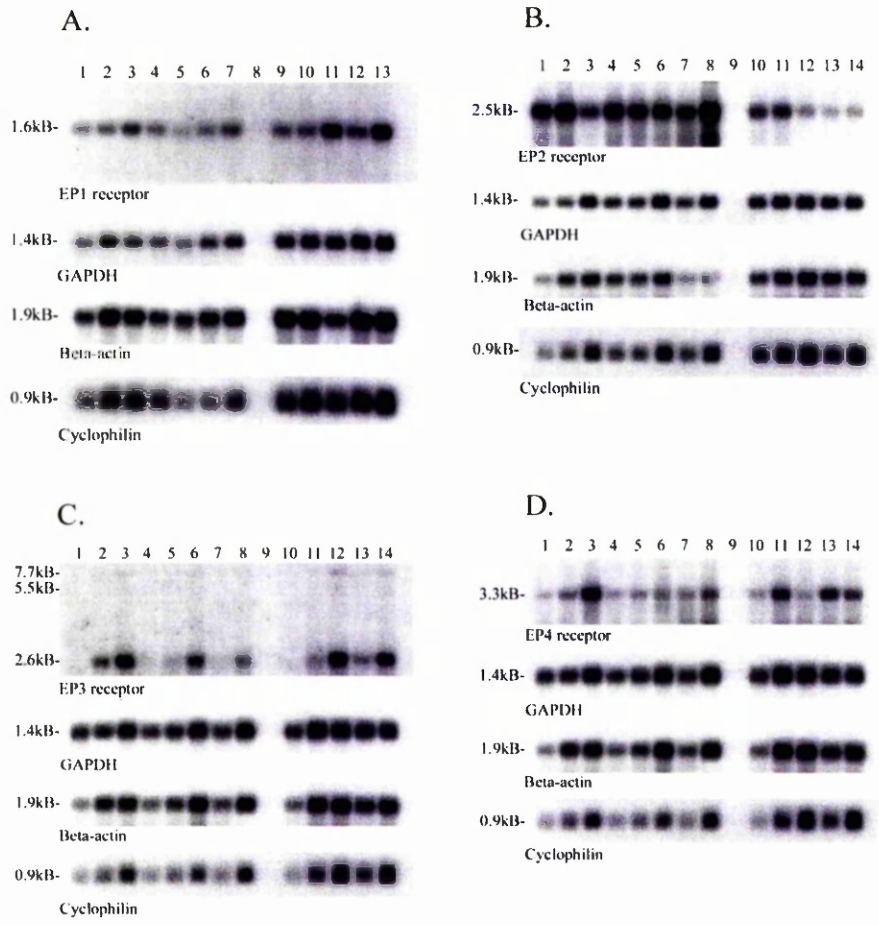


Figure 5.1. Northern blot of prostanoid EP receptor gene sub-types in baboon cervix. A. EP_1 receptor, B. EP_2 receptor, C. EP_3 receptor, D. EP_4 receptor. In each case the membrane was re-probed for each of the three housekeeping genes, GAPDH, beta actin and cyclophilin. The estimated molecular weight of transcripts is given in kilobases (kB) from interpolation of markers as described in Methods. The same quantity of mRNA (either 2 or 4 μg) was loaded in each lane in a given blot. A. Lanes 1-7 not in labour (NIL), lanes 9-13 in labour (LAB). B-D. Lanes 1-8 NIL, lanes 10-14 LAB.

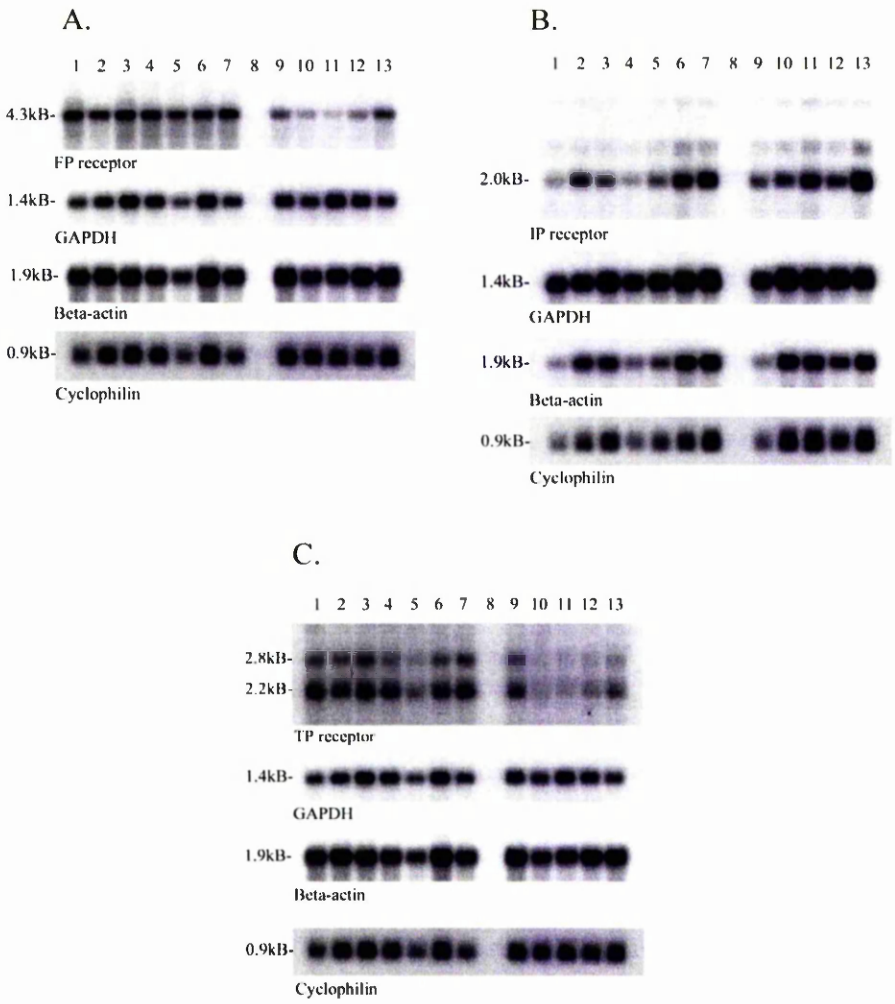


Figure 5.2. Northern blot of non-EP prostanoid receptor gene sub-types in baboon cervix. A. FP receptor, B. IP receptor, C. TP receptor. In each case the membrane was re-probed for each of the three housekeeping genes, GAPDH, beta actin and cyclophilin. The estimated molecular weight of transcripts is given in kilobases (kB) from interpolation of markers as described in Methods. The same quantity of mRNA (either 2 or 4 μg) was loaded in each lane in a given blot. All blots lanes 1-7 not in labour, lanes 9-13 in labour.

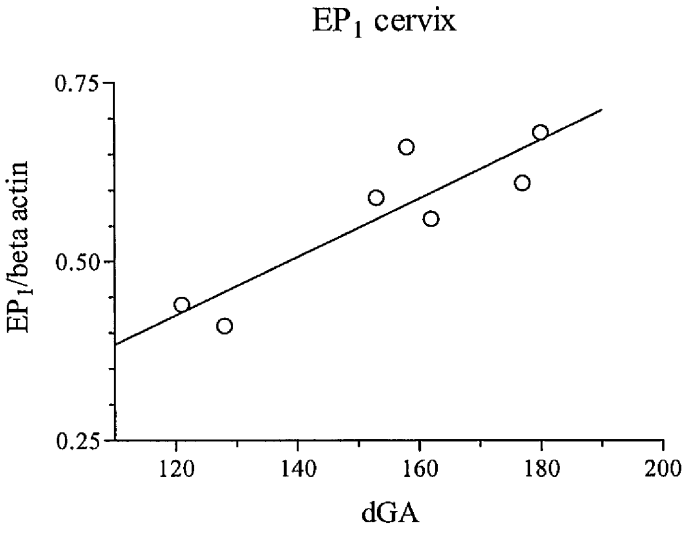


Figure 5.3. Expression of EP_1 receptor mRNA in cervix (n=7) with advancing days of gestational age (dGA). Simple linear regression of EP_1 receptor gene expression (relative to beta-actin, expressed as densitometric ratio) related to gestational age in animals not in labour (n=7). See Table 5.1 for statistical analysis.

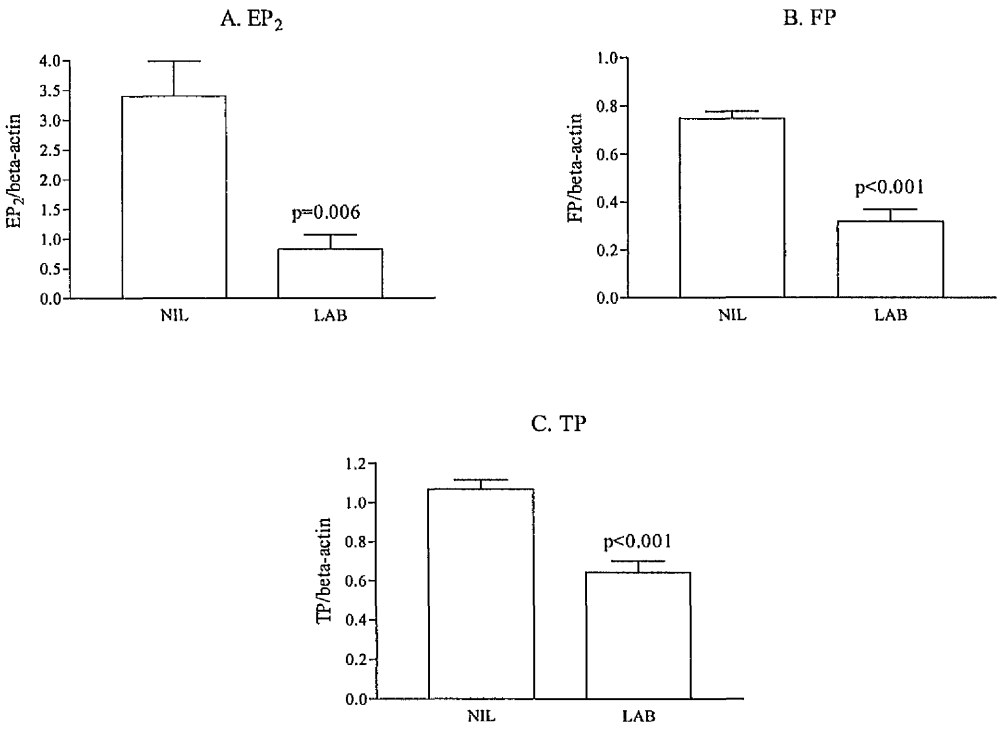


Figure 5.4. Expression of prostanoid receptor genes relative to beta-actin in cervix of animals in labour (LAB) and not in labour (NIL). A. EP_2 receptor gene (NIL n=8, LAB n=5), B. FP receptor gene (NIL n=7, LAB n=5), C TP receptor gene (NIL n=7, LAB n=5). See Table 5.2 for statistical analysis. Columns are means and bars are SEM.

Table 5.1. Relationship between gestational age and cervical prostanoid receptor gene expression as a ratio of three housekeeping genes.

	GAPDH		Beta-actin		Cyclophilin	
	p=	r^2	p=	r^2	p=	r^2
EP_1 (n=7)	0.04	0.61	0.007	0.79	0.01	0.76
EP_2 (n=8)	0.08		0.91		0.03	0.59
EP_3 (n=8)	0.69		0.94		0.80	
EP_4 (n=8)	0.56		0.69		0.91	
IP (n=7)	0.15		0.27		0.53	
FP (n=7)	0.22		0.91		0.32	
TP (n=7)	0.18		0.85		0.28	

The p value is given for simple linear regression, where the ratio of the gene of interest to the given housekeeping gene is the dependent variable and gestational age is the independent variable. Virtually identical results were obtained using Spearman's rho. None of the animals were in labour at the time of delivery. The relationship between EP_1 gene expression and gestational age was positive (Figure 5.3).

Table 5.2. Cervical prostanoid receptor gene expression as a ratio of three housekeeping genes comparing animals not in labour and in labour.

	GAPDH				Beta-actin				Cyclophilin			
	NIL (n=7-8)	Labour (n=5)	p=	95% CI difference	NIL (n=7-8)	Labour (n=5)	p=	95% CI difference	NIL (n=7-8)	Labour (n=5)	p=	95% CI difference
	mean (SEM)				mean (SEM)				mean (SEM)			
EP_1	1.11	1.20	0.49		0.56	0.74	0.12		0.70	0.69	0.91	
	(0.71)	(0.12)			(0.04)	(0.11)			(0.08)	(0.07)		
EP_2	2.75	0.80	0.002	0.92-2.98	3.40	0.83	0.006	0.89-4.25	2.69	0.74	0.002	1.16-2.71
	(0.35)	(0.18)			(0.57)	(0.25)			(0.35)	(0.20)		
EP_3	1.71	2.13	0.54		1.47	1.63	0.73		2.26	1.99	0.66	
	(0.37)	(0.64)			(0.26)	(0.42)			(0.40)	(0.40)		
EP_4	0.64	0.68	0.76		0.57	0.57	0.97		0.86	0.75	0.42	
	(0.08)	(0.10)			(0.06)	(0.08)			(0.08)	(0.13)		
IP	0.43	0.54	0.30		0.82	0.97	0.23		1.11	1.13	0.86	
	(0.06)	(0.09)			(0.06)	(0.11)			(0.09)	(0.11)		
FP	1.28	0.56	0.0008	0.38-1.26	0.75	0.32	<0.0001	0.30-0.56	1.02	0.42	0.0003	0.35-0.84
	(0.08)	(0.13)			(0.03)	(0.05)			(0.08)	(0.08)		
TP	1.83	1.08	0.004	0.30-1.21	1.07	0.64	0.0002	0.26-0.59	1.46	0.83	0.004	0.26-0.99
	(0.14)	(0.14)			(0.05)	(0.06)			(0.13)	(0.07)		

Means are compared using Student's t test. CI denotes confidence interval, NIL denotes not in labour, GAPDH denotes glyceraldehyde-3-phosphate dehydrogenase, SEM denotes standard error of the mean.

5.3 Discussion.

Administration of PGE_2 is well established as a method for preparing the cervix prior to induction of labour [211]. The identification of multiple sub-types of EP receptor [11;13] suggested that there may be a role for selective agonists of EP receptor sub-types in cervical priming. In Chapter 3, I compared the level of expression of EP and non-EP prostanoid receptors in the baboon and had found that there were high levels of expression of the adenylate cyclase-coupled EP_2 receptor in the cervix. I hypothesized that this may be the receptor which mediates the clinically important effects of PGE_2. A significant weakness in the hypothesis was the efficacy of sulprostone in priming the cervix [208] since the drug is virtually devoid of EP_2 receptor agonist activity [13].

In this chapter, I report that expression of the gene encoding the EP_2 receptor was actually lower in the cervix of animals that were in labour compared with animals not in labour (Table 5.2 and Figure 5.4A). This suggests that the EP_2 receptor does not mediate the effects of PGE_2 on the cervix which promote dilatation. Consequently, I now hypothesize that the EP_2 receptor might inhibit changes in the cervix which promote dilatation, that loss of EP_2 receptor expression may be a factor which promotes cervical dilatation, and that the effects of PGE_2 on the cervix which promote dilatation are mediated through a receptor other than the EP_2 receptor. The EP_1 receptor is a candidate since its level of expression increased with advancing gestational age (Figure 5.3). However, sulprostone, 16,16 dimethyl-PGE_1 and misoprostol are all effective agents for priming the cervix and all three drugs are agonists of the EP_3 receptor [13] whereas misoprostol is a weak EP_1 receptor agonist [11]. It seems most likely, therefore, that the EP_3 receptor mediates the effects of PGE_2 which promote dilatation of the cervix. I propose that a marked reduction in the expression of the inhibitory EP_2 receptor during labour

results in an uncovering of EP_3 receptor-mediated effects, promoting changes in cervical compliance and, consequently, cervical dilatation. This hypothesis is supported by the observation that the EP_2 and EP_3 receptors mediate opposing effects on adenylate cyclase [11].

A further unexpected finding in the present study was the lower level of expression of the genes encoding the FP and TP receptors in the cervix obtained from animals in labour (Figure 5.4B and C, Table 5.2). $PGF_{2\alpha}$ has been employed clinically as a means of inducing labour, but it requires an approximately 10-fold greater dose than PGE_2 for a given therapeutic effect and this is associated which a much greater level of systemic adverse effects [216]. There are very little data on the possible role of the TP receptor in the peripartum control of the cervix. But the current study suggests that a decrease in FP and TP receptor mediated effects may stimulate changes in the cervix which promote cervical dilatation.

Activation of the prostanoid EP_2 receptor usually stimulates adenylate cyclase [198], whereas both FP and TP receptor activation generally stimulate calcium influx [67;217]. In many tissues, including myometrium, activation of these receptors results in opposing effects [86]. It seems paradoxical, therefore, that receptors which mediate opposing effects might exhibit parallel changes in their level of expression in association with labour. The physiological significance of an increase or decrease in the overall level of expression of a given gene in the whole tissue depends on the cell type(s) that express the gene, the second messenger that the receptor is coupled to within the given cell type, the effect of activation of the given second messenger on the activity of the cell, and the role of that cell type in promoting cervical dilatation. I hypothesize that the parallel levels of expression of the EP_2, FP and TP receptor genes in labour might reflect changes in the

level of expression of these genes in different cells types with different roles in promoting cervical dilatation. I attempted to identify the cell types expressing prostanoid receptor genes in the cervix using in situ hybridization, but could not reliably detect signals above background due to diffuse low levels of expression.

The foregoing assumes that there is a correlation between expression of the gene encoding the receptor and the amount of functional receptor present. This assumption is supported by the data presented in Chapter 3 which demonstrated parallels between regional variation in the contractile response to PGs [195] and the expression of genes encoding prostanoid receptor genes in baboon myometrium. Ideally, however, the findings of our present study should be confirmed by other techniques such as radioligand binding or Western blot analysis. However, radioligand binding has limitations as a technique to characterize prostanoid receptor populations due to the lack of truly selective ligands [13], a problem underlined by studies using cells expressing recombinant prostanoid receptors which have demonstrated that many ligands which are supposed to be highly selective for a given receptor or receptor sub-type bind extensively to other receptors [47]. The use of Western blot analysis is limited by the fact that antibodies for all the receptor types and sub-types are not yet widely available. The size of bands observed were similar to those observed in myometrium (Chapter 4) which were themselves similar to previous studies using human tissues.

Assuming that the current findings can be extrapolated to the human, these data have implications for drug development. First, it has previously been demonstrated that the major effects of PGE_2 on human myometrium are mediated through the EP_3 receptor [86]. If, as seems likely on the basis of our studies in the baboon, the EP_3 receptor is also the major effector of PGE_2 in the cervix, it raises the possibility that an EP_3 receptor

antagonist might be an effective uterine tocolytic, which would also have an inhibitory effect on cervical compliance. Such a drug would have the advantage over COX inhibitors that it would not attenuate the inhibitory effects of EP_2 receptor activation [86]. It would also not be expected to contract the fetal ductus arteriosus, the dose limiting side effect of indomethacin [210], since this is mediated by loss of PGE_2 acting through the EP_4 receptor [99]. The present observations also suggest a possible role for an EP_2 receptor antagonist in the induction of labour. This is supported by the finding reported in Chapter 4 that expression of the EP_2 receptor gene is also reduced in myometrium in association with labour. Similarly, in human myometrium, decreased EP_2 receptor gene expression has been described with advancing gestational age [91]. Finally, the previous demonstration of a profound inhibitory effect of EP_2 receptor activation on myometrial contractility [86] and the current proposal that the same receptor might exert inhibitory effects on cervical dilatation suggest that an EP_2 agonist may have a role in the management of preterm labour.

Chapter 6. The effect of labour and gestational age on expression of prostanoid receptor genes in decidua and chorion

The fetal membranes and decidua are thought to have a key role in the control of parturition in the human and non-human primate. In analyses of cases of preterm labour, approximately 30-40% of births are associated with PPROM [145]. Furthermore, the membranes are thought to have an important paracrine role in the control of myometrial contractility, both in spontaneous term labour [218] and in preterm labour secondary to intra-uterine infection [97]. The decidua is also thought to have an important paracrine role in the control of the myometrium being both a source of PGs and other important regulators, such as oxytocin [111].

The amnion is thought to be a critical site of PG synthesis in relation to parturition in a number of species, including the human [218]. The chorion also expresses PG synthetic enzymes, although a key role has been postulated for the chorion by acting as a barrier for diffusion of PGs from the amnion to the myometrium due to high levels of expression of PG dehydrogenase (PGDH): expression of PGDH falls in association with labour [148;219], implicating a role for this barrier function in maintaining uterine quiescence.

Currently, most models for the role of PGs in the fetal membranes and decidua consider the membranes as sources of either PG biosynthesis or sites of PG catabolism [5]. Furthermore, any effects of PGs from the fetal membranes and decidua are thought to be paracrine, mediated through changes in myometrial contraction [97]. However, in Chapter 3, I demonstrated a complex pattern of expression of genes encoding prostanoid receptors in the chorion and decidua of pregnant baboons which suggests that PGs synthesized in the membranes and decidua may also have autocrine effects.

I hypothesize that PGs may have direct effects on decidua and chorion which in turn may have a role in the initiation of parturition in primates. Characteristically, genes encoding signal transduction proteins of systems which have a role in labour are expressed at higher levels in tissues (both myometrial and non-myometrial) from animals which are in labour compared with animals not in labour, and there are many examples of such phenomena [25;113;139;214]. As an initial test of the hypothesis that direct effect of PGs on decidua and chorion may have a role in the initiation of parturition, I sought to determine whether expression of prostanoid receptor genes varied according to gestational age and whether the animal was in labour or not in labour at the time the tissue was obtained.

6.1 Methods

The same animals described in Chapter 4 were employed and the general methods were as outlined in Chapter 2.

6.2 Results.

Clear signals of similar molecular weight to the cloned human prostanoid receptors genes were detected in decidua to probes for the EP_1, EP_2, EP_3, EP_4, FP, IP receptors (Figures 6.1 and 6.2). No clear signal above background could be detected using an antisense riboprobe for the human DP receptor. Clear signals of similar molecular weight to the cloned human prostanoid receptors genes were detected in chorion to probes for the EP_1, EP_2, EP_3, EP_4, and IP receptors (Figures 6.3 and 6.4). No clear signal above background could be detected using antisense riboprobes for the human DP or FP receptor genes. Weak signals could be detected in both tissues to a TP receptor probe (Figures 6.2 and 6.4). However, the signal to noise ratio for the TP receptor gene transcript was too low in both tissues for meaningful quantitative analysis.

There was a statistically significant negative correlation between the level of expression of the EP_1 and FP receptor genes in decidua and EP_4 expression in the chorion and the gestational age at the time of hysterectomy among animals which were not in labour (Figure 6.5). There were no other significant relationships between gene expression and gestational age in either tissue (Table 6.1). Results of similar statistical significance were obtained when correlation was tested using Spearman's rho (data not shown).

There was a significantly lower level of expression of the EP_2 receptor gene in decidua from animals in labour (Figure 6.6A) but a higher level of expression of the IP receptor gene in decidua (Figure 6.6B) and chorion (Figure 6.6C) from animals in labour. There were no other significant differences in the level of expression of the other prostanoid receptor genes in either tissue comparing samples from animals in labour and not in labour (Table 6.1).

For the signals which were observed to vary with gestational age (EP_1 and FP in decidua and EP_4 in chorion) a further comparison was made of tissues obtained from animals in labour with animals not in labour, where the comparison was adjusted for gestational age using analysis of covariance. In all three cases there was still no significant change in levels of expression associated with labour after adjusting for the effect of gestational age (EP_1 decidua, P= 0.65; FP decidua, P= 0.95; EP_4 chorion, P= 0.65).

All the above comparisons were made expressing the level of the given gene as a ratio to beta-actin. Significant results were also observed when levels were expressed relative to one of the other housekeeping genes, with one exception. There was a negative correlation of borderline statistical significance between gestational age and EP_1 expression in chorion relative to beta-actin (P=0.05). This association was not statistically significant when the EP_1 signal was related either to GAPDH (P=0.87) or cyclophilin (P=0.11).

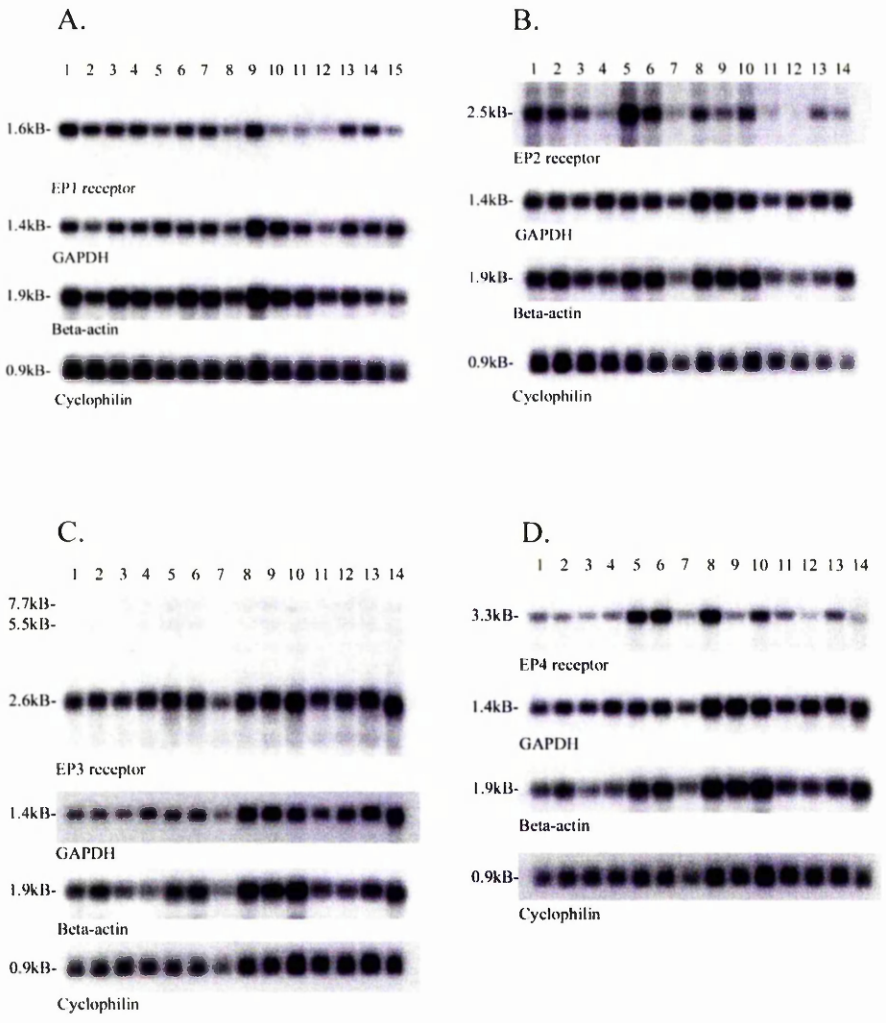


Figure 6.1. Northern blot of EP receptor mRNA in decidua. Either 2 or 4 μg each lane, same quantity loaded in each lane in given blot) probed for A. EP_1 receptor gene, B. EP_2 receptor gene, C. EP_3 receptor gene, D. EP_4 receptor gene. All membranes consecutively stripped and re-probed for three housekeeping genes. Estimate size of transcripts estimated in kilobases (kB).

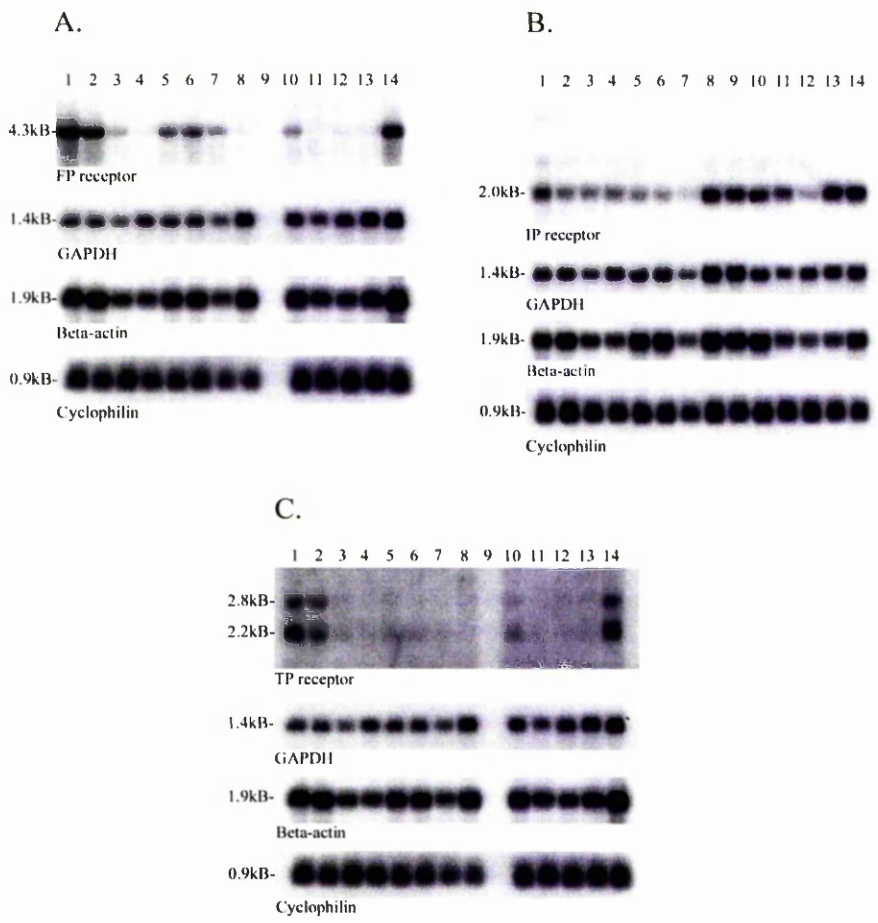


Figure 6.2. Northern blot of non-EP prostanoid receptor mRNA in decidua. Either 2 or 4μg each lane, same quantity loaded in each lane in given blot, probed for A. FP receptor gene, B. IP receptor gene, C. TP receptor gene. All membranes consecutively stripped and re-probed for three housekeeping genes. Estimate size of transcripts estimated in kilobases (kB).

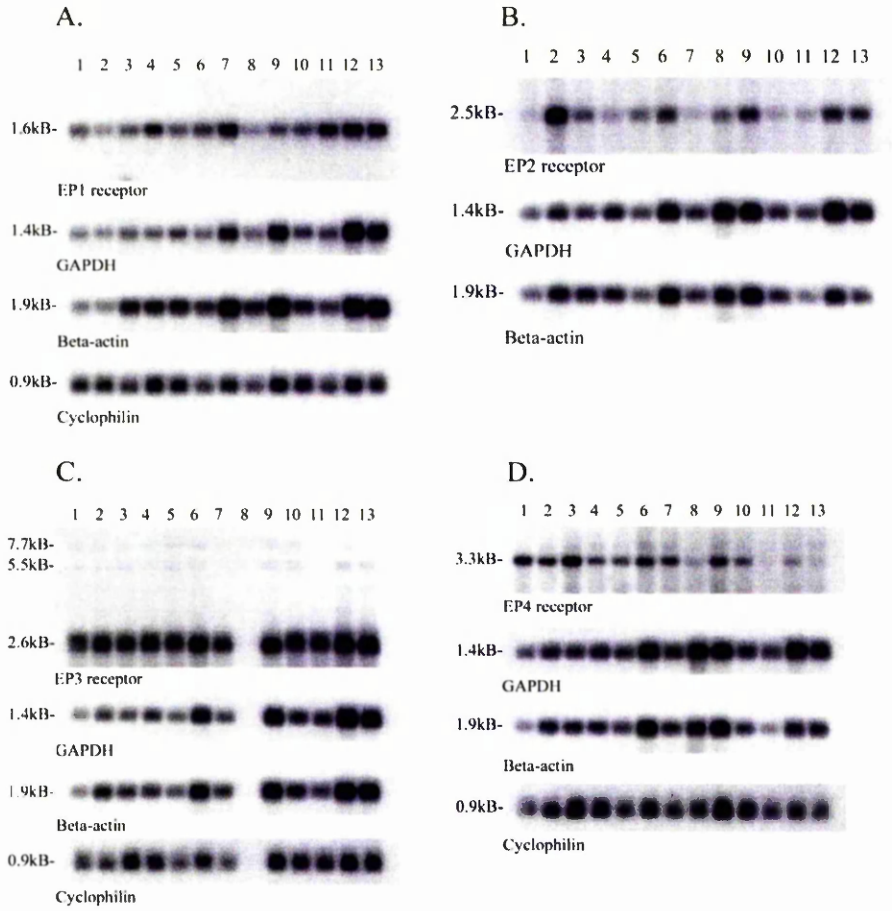


Figure 6.3. Northern blot of EP receptor mRNA in chorion. Either 2 or 4 μg each lane, same quantity loaded in each lane in given blot, probed for A. EP_1 receptor gene, B. EP_2 receptor gene, C. EP_3 receptor gene, D. EP_4 receptor gene. All membranes consecutively stripped and re-probed for three housekeeping genes. Estimate size of transcripts estimated in kilobases (kB).

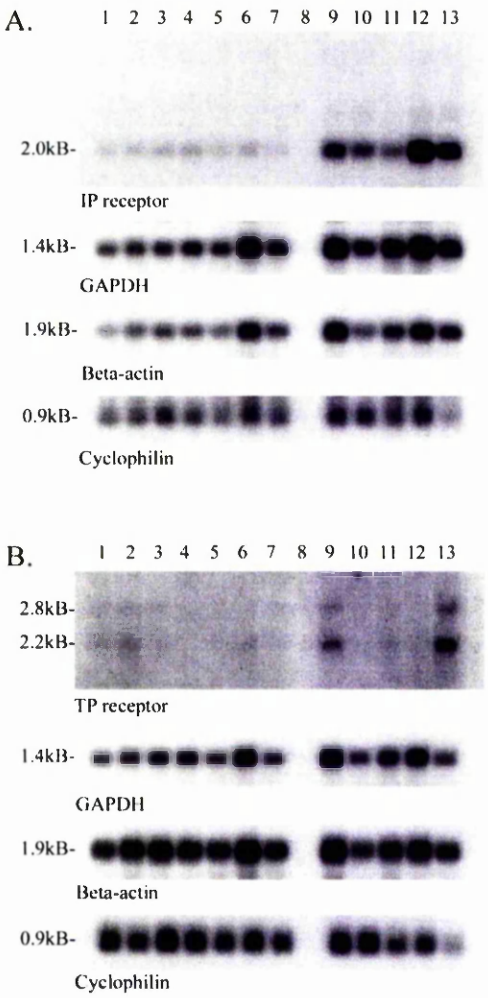


Figure 6.4. Northern blot of non-EP prostanoid receptor mRNA in chorion. Either 2 or 4 μg each lane, same quantity loaded in each lane in given blot, probed for A. IP receptor gene, and B. TP receptor gene. All membranes consecutively stripped and re-probed for three housekeeping genes. Estimate size of transcripts estimated in kilobases (kB).

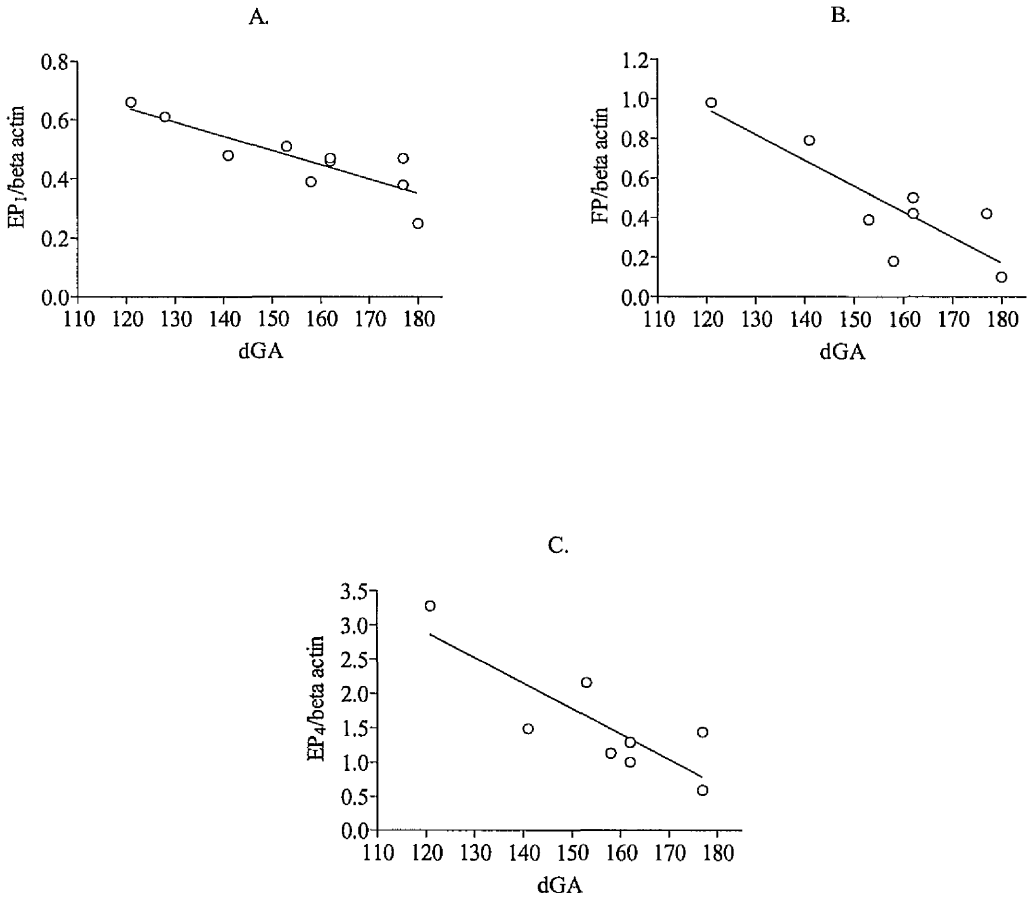


Figure 6.5. Prostanoid receptor gene expression related to gestational age among animals not in labour in decidua and chorion. A. EP_1 receptor gene in decidua (r^2 = 0.7, P=0.002), B. FP receptor gene in decidua (r^2 = 0.7, P=0.008), and C. EP_4 receptor gene in chorion (r^2 = 0.7, P=0.01). Signals quantified using densitometry and expressed as a ratio (arbitrary units) to signal for beta-actin.

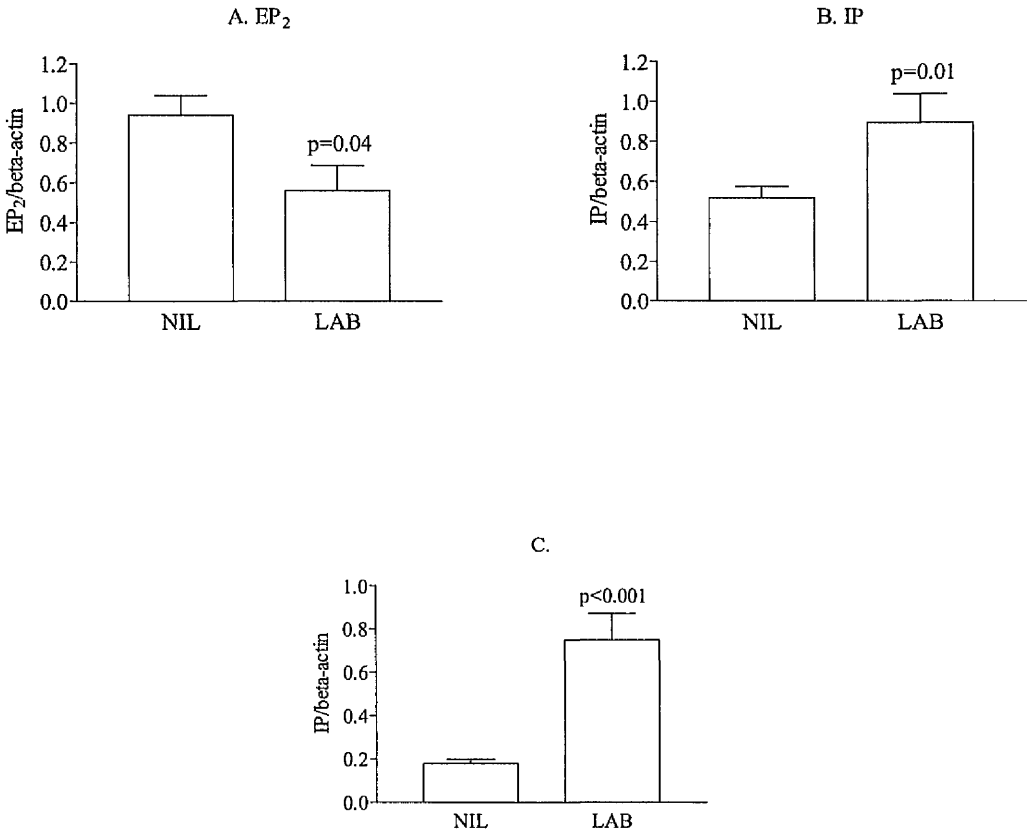


Figure 6.6. Variation in expression of prostanoid receptor genes (expressed as ratio to beta-actin) in relation to labour. A. EP_2 receptor gene in decidua, B. IP receptor gene in decidua, C. IP receptor gene in chorion. NIL = not in labour (n=8-10), LAB = labour (n=5). Statistical comparison by Student's t test. Columns are means, bars are SEM.

Table 6.1. Variation in expression of prostanoid receptor genes (relative to beta-actin) related to advancing gestational age and labour.

	Decidua				Chorion			
Gene	GA P (r^2) ¶	NIL mean (SEM)	LAB mean (SEM)	NIL vs. LAB P=	GA P (r^2) ¶	NIL mean (SEM)	LAB mean (SEM)	NIL vs. LAB P=
EP_1	0.002 (0.73)	0.47 (0.04)	0.37 (0.06)	0.17	0.05 (0.51)	0.85 (0.08)	0.89 (0.15)	0.79
EP_2	0.17	0.94 (0.10)	0.56 (0.13)	0.04	0.32	0.42 (0.09)	0.43 (0.08)	0.91
EP_3	0.99	1.25 (0.09)	1.36 (0.07)	0.42	0.08	2.21 (0.28)	1.45 (0.13)	0.06
EP_4	0.11	0.98 (0.09)	1.18 (0.23)	0.35	0.01 (0.68)	1.55 (0.29)	0.95 (0.14)	0.16
IP	0.94	0.52 (0.06)	0.89 (0.14)	0.01	0.08	0.18 (0.02)	0.75 (0.12)	0.0003
FP	0.008 (0.72)	0.47 (0.10)	0.26 (0.09)	0.19	_	_	_	_

Where GA denotes gestational age, NIL denotes not in labour, LAB denotes labour, and SEM denotes standard error of the mean. ¶Correlation co-efficient: gestational age independent variable, prostanoid receptor gene/beta-actin dependent variable, all animals not in labour at time of hysterectomy.

6.3 Discussion.

Studies on the control of parturition have naturally tended to focus on the myometrium, since uterine contraction is clearly central to the process of expulsion of the fetus. However, 30-40% of preterm births follow PPROM [145]. The relative ease of study of the effect of drugs and hormones on myometrium has probably increased the focus on this tissue. However, given the direct contribution of membrane integrity to the process of preterm delivery, and the secondary paracrine effects of choriodecidual activation on myometrial contractility [97], it is possible that drugs that directly target the membranes and decidua may be effective in the treatment or prophylaxis of preterm labour. Indeed, it is likely, that the apparent efficacy of indomethacin in delaying delivery may be due in a major part to its effects on PG synthesis in the fetal membranes and decidua.

Consequently, it is important to understand the regulation of function of the fetal membranes and decidua. In Chapter 3, I demonstrated that there is a complex pattern of expression of prostanoid receptor genes in baboon chorion and decidua. The genes expressed in chorion and decidua were also expressed in myometrium and cervix. Although receptors are often broadly described as inhibitory or stimulatory, these generalizations usually refer to the effects of receptor activation on smooth muscle. However, it does not follow that a receptor which usually mediates an inhibitory response on smooth muscle will always tend to inhibit non-smooth muscle tissues. A good example of this of the effect of beta-2 adrenoceptor agonists on different excitable tissues: these drugs typically inhibit myometrial and vascular smooth muscle contractility [220], whereas they increase myocardial contractility [221]. The factors determining the effect of an agonist on a tissue include the cell types in the tissue, the receptors expressed on the different cell types, the second messenger to which the receptor is coupled, the nature of the coupling between the receptor and second messenger, the effect of activation (or

inhibition) of the second messenger on the activity of the cell type, and the role of the given cell type in the regulation of the tissue. Clearly, it does not follow that an agonist which inhibits myometrial contractility will also inhibit the labour-promoting activities of all other intra-uterine tissues where the given signal transduction system is active. A good recent example of this are nitric oxide donors which inhibit myometrial contractility [171] but actually promote cervical dilatation [174].

The complex pattern of expression of prostanoid receptors in myometrium (Chapter 4) and the widespread clinical use of prostanoids and their synthesis inhibitors in the therapeutic control of myometrial contractility suggest possible roles for drugs with selective activity at prostanoid receptors in the clinical manipulation of the uterus in term and preterm labour. The demonstration that the genes encoding these receptors are expressed in the chorion and decidua suggested that these receptors might have a role in the control of these key tissues in labour. As stated above, it cannot be safely assumed that receptors which typically inhibit myometrium would also exert inhibitory effects on the chorion and decidua. In order to understand the effects of drugs with selective activity on prostanoid receptors in the control of labour, their effects on non-myometrial tissues also need to be resolved. One way of drawing inferences about the effect of a given system in the control of parturition is by studying the expression of its component parts in pregnancy and during labour. This approach has demonstrated (for example) increased expression in labour of genes encoding gap junctions in myometrium [179;214], COX-2 in amnion [25] and nitric oxide synthase in cervix [222]. There is evidence in each of these cases that the increased expression of the given gene is involved in the control of labour. Furthermore, the realization of the profound increase in COX-2 gene expression has led to the successful exploration of selective inhibitors of this enzyme in the management of preterm labour [223] and the recognition of the possible role of nitric oxide in the control of

the cervix led to successful trials of NO donors to prime the cervix clinically [174;224]. Therefore, as an initial investigation of the possible role of prostanoid receptors in the control of the primate chorion and decidua, I sought to determine whether expression of these genes varied in these tissues with advancing gestational age or in association with spontaneous term labour.

Four patterns of change in expression of prostanoid receptor genes in the decidua and chorion were observed. The first pattern observed was a decrease in expression of genes encoding receptors which are positively coupled to the phosphatidyl inositol cascade (EP_1 and FP) in decidua with advancing gestational age (Figure 6.5A and B). The parallel decrease in these receptors with advancing gestational age suggests that a prostanoid mediated response acting through the phosphatidyl inositol cascade might inhibit changes in the decidua which promote labour. However, activation of these receptors increases myometrial contractility [86] and, in the cervix, EP_1 receptor gene expression increases with advancing gestational age (Chapter 5). Therefore, these data suggest that EP_1 and FP receptor-mediated effects may promote parturition in some tissues and inhibit the process in others.

The second pattern that emerged was a decrease in the expression of a gene which encodes a receptor (EP_4) which is positively coupled to adenylate cyclase in chorion with advancing gestational age (Figure 6.5C). This suggests that an adenylate cyclase mediated effect may inhibit changes in chorion which promote parturition. This parallels myometrium, where adenylate cyclase coupled receptors inhibit myometrial contractility [86;220].

The third pattern was a lower level of expression of the gene encoding an adenylate cyclase coupled receptor (EP_2) in decidua obtained from animals in spontaneous labour (Figure 6.6A). This suggests that an adenylate cyclase-mediated effect may inhibit

parturition in decidua in cells expressing the EP_2 receptor. This parallels decreased EP_2 receptor expression in myometrium and cervix in labour (Chapters 4 and 5). I hypothesize that reduced expression of this inhibitory receptor may have a key role in promoting parturition in the primate. The absence of any change in expression of the EP_2 receptor in chorion suggests differential transcriptional control in maternal and fetal intra-uterine tissues.

The fourth pattern, observed in both the decidua and chorion, was an increase in the expression of a gene encoding an adenylate cyclase coupled receptor (IP) and this was particularly marked in the chorion (Figure 6.6B and C). This suggests that adenylate cyclase activation might promote parturition in cells expressing the IP receptor in these tissues. It was interesting to observe two adenylate cyclase coupled prostanoid receptors (EP_2 and IP) vary in opposite directions in decidua in association with labour. This underlines the potential for the same second messenger system to mediate different effects in different cells types and this presumably has its basis in differential effects of protein kinase A in different cell types. Activation of the IP receptor in myometrium inhibits myometrial contractility and this observation, like decreased EP_1 and FP expression in decidua with advancing gestational age, suggests dissociation of roles of receptors in choriodecidua compared with other key tissues. The present findings suggest that extreme caution should be applied in any studies seeking to exploit the IP receptor pathway as a means of uterine tocolysis, since any inhibitory, therapeutic effect on myometrium [36] may be undermined by effects on the choriodecidua.

Cellular localization of expression of these receptors may allow some inferences to be drawn regarding their physiological roles. I attempted in situ hybridisation for the EP_2, EP_3, EP_4 and IP receptor genes. However, reliable signals were not obtained due to low levels of expression and high levels of non-specific binding. Other techniques to localize

signal have certain limitations. Receptor autoradiography is limited by the extensive cross reactivity of available ligands [47], and immunocytochemistry is limited by the lack of well characterized and specific antibodies.

There are relatively few data on prostanoid receptors in the fetal membranes and decidua. A previous study failed to demonstrate variation in either EP_2 or EP_4 receptor gene expression in labour in ovine endometrium in association with labour [215]. However, species differences between the sheep and baboon have previously been demonstrated for EP_2 receptor mediated effects on myometrium [86;89;90]. Furthermore, myometrial EP_2 receptor gene expression decreases in both the baboon and human [91;225] in labour or at term, whereas it is unchanged in sheep [215]. These findings, taken together, suggest significant species difference between primates and sheep in terms of uterine prostanoid receptors and underline the importance of studies in non-human primates.

A number of studies have postulated a role for prostanoid receptors in the endometrium in association with implantation [226]. An important role for PGs in this context is supported by the failure of implantation and decidualisation in mice which are null mutant for the COX-2 gene [227], and this may be due to the absence of PGI_2 [45]. Other studies have demonstrated expression of the EP_1 receptor on amnion WISH cells. Expression of the EP_1 receptor in these tissues is stimulated by CRH [228], interleukin-1beta [229], interleukin-4 [230] and tumour necrosis factor-alpha [231]. Interestingly, the effect of CRH appeared to be mediated by PGE_2 [228], suggesting the possibility of a feedback loop. However, the data presented in this chapter are the first, to my knowledge, to support the concept that a changing pattern of prostanoid receptor expression may have a role in choriodecidual activation in labour.

Chapter 7. The effect of corticosteroids, gestational age and birth on expression of EP receptor genes in ductus arteriosus

The ductus arteriosus is a shunt blood vessel of fetal life. It diverts deoxygenated blood from the main pulmonary artery to the descending aorta. Following birth, the direction of flow across the ductus reverses and the vessel ultimately closes in the first 1-2 days of neonatal life.

Patency of the ductus in fetal life is an active state and is maintained by the tonic dilator effect of high circulating concentrations of PGE_2, which relaxes ductal smooth muscle through the adenylate cyclase coupled EP_4 receptor [39]. Cyclic adenosine monophosphate (cAMP) inhibits the intrinsically high sensitivity of ductal smooth muscle to the $[Ca^{2+}]_i$ [101]. Following birth, circulating concentrations of PGE_2 fall and loss of PGE_2-mediated activation of adenylate cyclase uncovers the vessel's intrinsically high sensitivity to $[Ca^{2+}]_i$ [99]. Furthermore, the physiological increase in arterial oxygen tension which follows birth depolarises ductal smooth muscle by closing oxygen sensitive delayed rectifier potassium channels resulting in intracellular calcium influx in ductal smooth muscle cells [232;233]. The central role of the prostanoid EP_4 receptor in the control of ductal patency has been underlined by the observed failure of normal closure of the vessel following birth in EP_4 receptor gene null mutant mice [103]. However, the paradox that the absence of a gene encoding an inhibitory receptor results in failure of ductal contraction following birth remains to be satisfactorily explained.

The sensitivity of the ductus arteriosus to the dilator effect of PGE_2 is decreased by antenatal exposure to corticosteroids [234], by advancing gestational age [235] and following birth [236]. However, these factors also affect the sensitivity of the vessel to a range of other vaso-active agents [see [99] for review], and it is not clear whether the altered response to PGE_2 represents a specific change in ductal PG signal transduction. Furthermore,

functional studies also suggest that the ductus expresses a contractile EP receptor, probably EP_3 [102]. It is not clear whether an alteration in the balance of contractile and dilator EP receptors has a physiological role in the ontogenic and perinatal control of the vessel.

The ductus arteriosus, like myometrium, is profoundly under the control of PGs and most current models of ductal control – like models of parturition - focus on the potential role of changes in PG concentration. In the preceding chapters, I demonstrated that advancing gestational age and labour are associated with changes in the relative expression of prostanoid receptor genes in key intra-uterine tissues. The aim of this part of my study was to determine whether this was also true of the fetal preparation for birth. Therefore, I sought to test the hypothesis that loss of EP_4 receptor gene expression might have a role in mediating ductal closure following birth. I also predicted that the ratio of EP_4 to EP_3 gene expression might change following birth, due to decreased EP_4 expression, increased EP_3 expression or both. I also sought to determine the effect of antenatal corticosteroids and advancing gestational age on the expression of these genes and to determine whether EP_4 receptor mRNA expression could be demonstrated in the non-human primate.

7.1 Methods.

The general methods described in Chapter 2 were employed. In these experiments, chronically instrumented fetal lamb preparations were used.

7.1.1 Obtaining fetal and neonatal lamb ductus arteriosus

Pregnant Rambouillet-Colombia ewes and baboons of known gestational age were anaesthetised (ketamine induction, then halothane), the fetus was delivered by Caesarean section, and exsanguinated. Neonatal Rambouillet-Colombia lambs were anaesthetized with halothane and exsanguinated.

7.1.2 Instrumented fetal lamb preparations

Rambouillet-Colombia ewes bred on a single occasion and of known gestational age were acclimated to the animal facilities for at least 5 days before surgery and kept in rooms with controlled light/dark cycles. Alfalfa cubes and water were provided ad libitum. Surgery was performed at 104±1 (mean ± SEM) days gestational age (dGA). Anaesthesia was induced with ketamine and maintained with halothane. Catheters were inserted into the ewe's carotid artery and jugular vein, into the fetal carotid and axillary arteries and jugular and pedal veins, and into the amniotic cavity. Electrodes were implanted bilaterally on the fetal parietal bone and above the orbit and on each side on the surface of the maternal uterine body and pregnant horn.

After surgery the ewe was returned to a metabolism cage and provided free access to food and water. Animals were allowed at least five days of post-operative recovery and all ewes received a daily antibiotics (ampicillin) and twice daily analgesics (phenylbutazone, until day three post-op).

Animals were randomly assigned to receive dexamethasone (n=4) or vehicle (n=4) at 110 dGA. Infusion of either vehicle or dexamethasone (2.2 μg/kg/min at 1 ml/h) was maintained for forty two hours. Immediately preceding and following the infusion, the fetuses were exposed to a continuous series of 2 minute infusions of phenylephrine (2.5, 5, 10, 20, and 40 μg/min) and, after a 60 minute recovery period and a set of fetal and maternal blood samples, a continuous series of 2 minute infusions of nitroprusside (1, 2, 4, 8, and 16 μg/min). Infusion of either phenylephrine or nitroprusside was stopped if the change in blood pressure exceeded 30% of baseline or if arrhythmia was noted on the pressure tracing. Ewes were euthanised by intravenous injection of pentobarbital sodium solution (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI).

7.1.3 Instrumented fetal baboons

Five pregnant baboons (Papio sp.) of known gestational age were employed as described in Chapter 2. At 118-134 days of gestational age (dGA, term = 185dGA) animals were instrumented under halothane (1-2% in 2 l/min O2) general anaesthesia. Maternal femoral artery and vein and fetal carotid artery catheters were placed and a 22G catheter was used for fetal carotid cannulation. Five electrodes (New England Wire Corp., Lisbon, New Hampshire) were implanted on the uterine body for electromyographic recording of uterine activity.

On the morning of the fifth day after surgery, animals were given an intra-muscular injection of 87.5μg/kg betamethasone (Celestone, betamethasone sodium phosphate injection, USP, Schering Corp., Kenilworth, NJ;) or vehicle. A total of 4 doses at 12 hour intervals were given and the fetus was delivered by Caesarean section 12 hours after the last dose. In addition, further baboon fetuses were delivered by planned Caesarean section as described in Chapter 2.

7.2 Results.

Northern analysis demonstrated clear signals for the EP_4 and EP_3 receptor genes from RNA extracted from fetal and neonatal lambs (Figure 7.1). The size of transcripts was similar to other reports of sheep prostanoid receptors in non-vascular tissue [215]. The blots were also probed with riboprobes for the EP_1, EP_2, FP and IP receptors, but none of these probes resulted in a signal that was clearly greater than background.

The level of expression of the EP_4 receptor gene in lambs at approximately 110dGA was not significantly different comparing un-instrumented lambs and instrumented lambs receiving vehicle or dexamethasone infusion (Table 7.1). However, EP_4 receptor gene expression was significantly lower in late term fetuses (145dGA) and lower still in neonates (Table 7.1, Figures 1 and 2). The pattern of EP_4 expression and statistical significance was similar when expression was normalized to either beta-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 7.1 and Figure 7.2). The level of expression of the 4.2 kB EP_3 receptor gene transcript was similar in all the groups studied (Figure 7.3A and Table 7.1). The ratio of the size of the EP_4 signal to the 4.2kB EP_3 signal was lower in late gestation and lower still in neonates (Table 7.1 and Figure 7.3B). The results were virtually identical for the 7.5 and 3.5 kB EP_3 transcripts (data not shown).

Samples were available from a number of fetal baboons, both controls and four steroid treated animals (Figure 7.4). In these animals there was no reduction in the level of EP_4 mRNA expression following treatment with betamethasone (Figure 7.5).

In situ hybridization of the baboon ductus confirmed that the gene was expressed in the smooth muscle cells of the vessel and demonstrated the specificity of the EP_4 signal for the ductus over the aorta (Figure 7.6A and C). The hybridization was specific as confirmed by the use of a sense probe (Figure 7.6B).

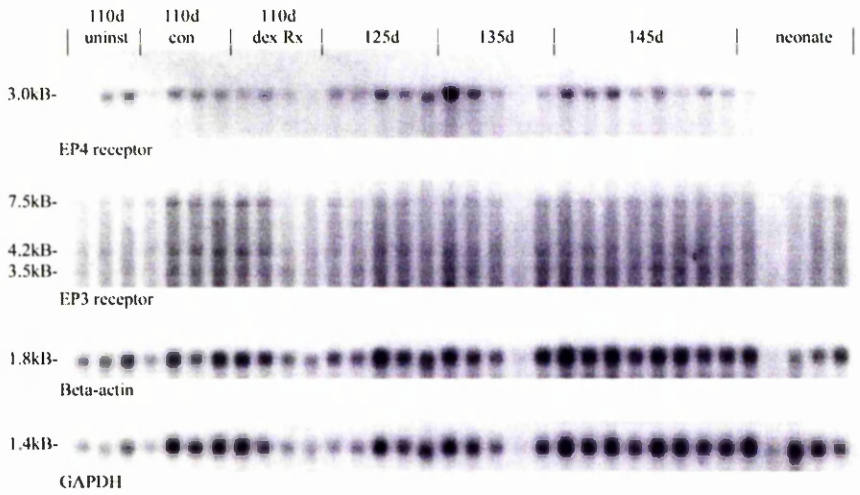


Figure 7.1. Northern blot of fetal and neonatal lamb mRNA, probed for EP_4, EP_3, beta-actin and GAPDH genes. One microgram of polyadenylated RNA was loaded in each lane. The estimated size of transcripts is expressed in kilobases (kB). Lanes 20 and 31 were not used in subsequent analysis due to the absence of a reliable signal to either GAPDH or beta-actin. Numbers equal gestational age at time of delivery, e.g. 145 = animals delivered at 145 days gestational age. "110d uninst" = un-instrumented animals at 110 days. "con" = instrumented animals at 110 days acting as controls for dexamethasone (received vehicle). "dex Rx" = instrumented animals treated with dexamethasone. All other animals un-instrumented.

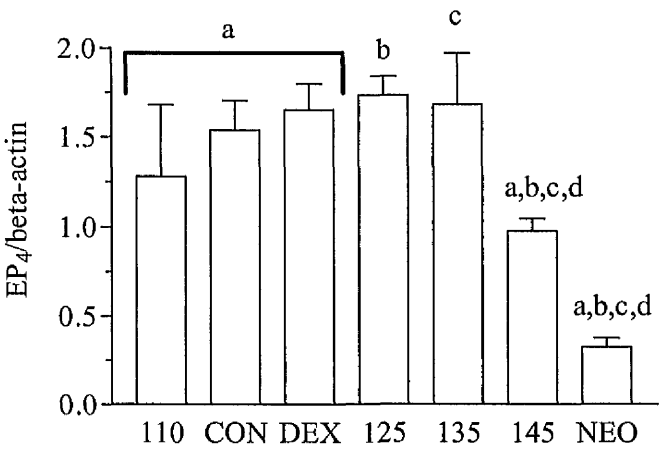


Figure 7.2. Densitometric quantitation of EP_4 signal in each of the seven groups relative to beta-actin. Key to X axis: un-instrumented at 110 days ('UN', n=3), instrumented and treated with dexamethasone vehicle at 110 days ('CON', n=4), instrumented and treated with intravenous dexamethasone at 110 days ('DEX', n=4), un-instrumented at 125 days ('125', n=5), un-instrumented at 135 days ('135', n=4), un-instrumented at 145 days ('145', n=8), un-instrumented neonates ('NEO', n=4). Groups with the same letter were statistically significantly different during post-hoc multiple comparisons (Tukey's method).

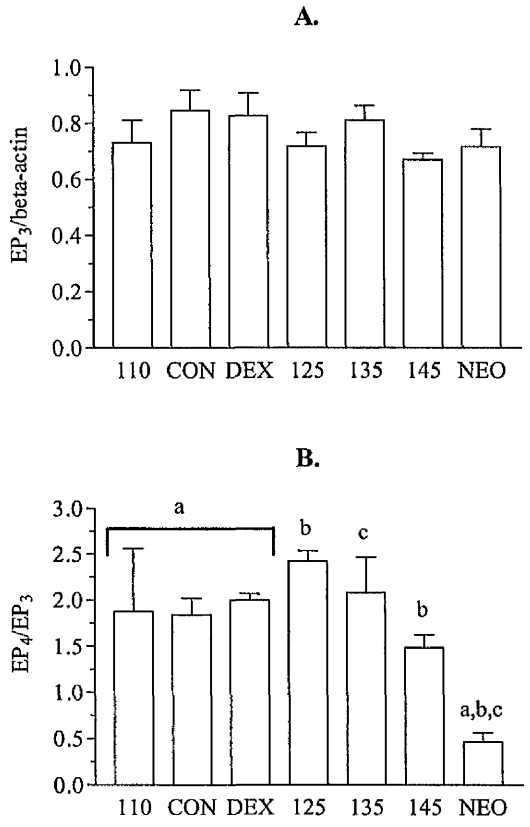


Figure 7.3. Densitometric quantitation of 4.2kB EP_3 transcript to A. ratio of EP_3 to beta-actin B. ratio of EP_4 to EP_3 in each of the seven groups. Key to X axis: un-instrumented at 110 days ('UN', n=3), instrumented and treated with dexamethasone vehicle at 110 days ('CON', n=4), instrumented and treated with intravenous dexamethasone at 110 days ('DEX', n=4), un-instrumented at 125 days ('125', n=5), un-instrumented at 135 days ('135', n=4), un-instrumented at 145 days ('145', n=8), un-instrumented neonates ('NEO', n=4). Groups with the same letter were statistically significantly different during post-hoc multiple comparisons (Tukey's method).

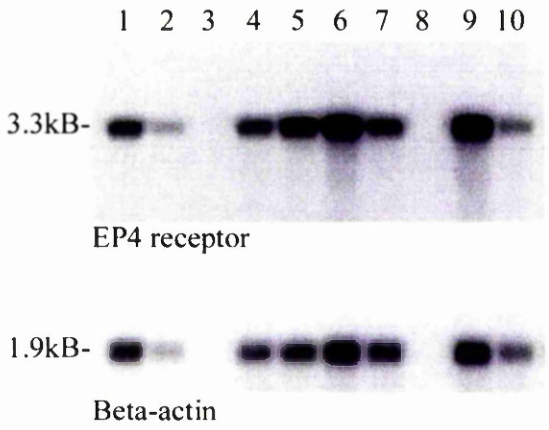


Figure 7.4. Northern blot of fetal baboon ductus arteriosus probed for EP_4 gene and beta-actin. 0.5μg of polyadenylated RNA was loaded in each lane, except lane two, where 0.35μg was loaded. The estimated size of transcripts is expressed in kilobases (kB). Lane 1=128dGA, un-instrumented; Lane 2=133dGA, instrumented, vehicle control for betamethasone; Lane 3=blank; Lanes 4-7, all instrumented and exposed to maternally administered betamethasone (dGA in order: 124, 127, 129, and 137); Lane 8=blank; Lane 9=un-instrumented 153dGA, Lane 10 = un-instrumented 158dGA.

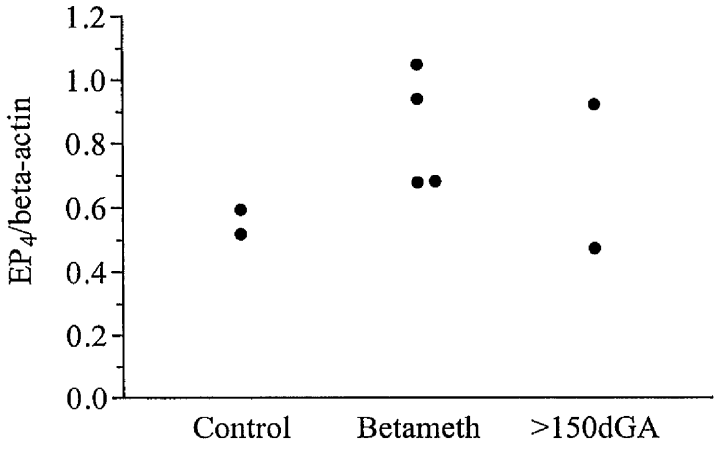


Figure 7.5. Densitometric quantitation of ratio of EP_4 signal to beta-actin in baboon ductus arteriosus. "Control" = 128 dGA, un-instrumented and 133 dGA, instrumented, vehicle control for betamethasone. "Betameth" = all instrumented and exposed to maternally administered betamethasone (dGA in order: 124, 127, 129, and 137). ">150dGA" = un-instrumented 153dGA and un-instrumented 158dGA. One way ANOVA of the three groups: P=0.35. Unpaired t-test of specific hypothesis that EP_4 expression was lower in the betamethasone treated animals compared with "Control": p=0.97.

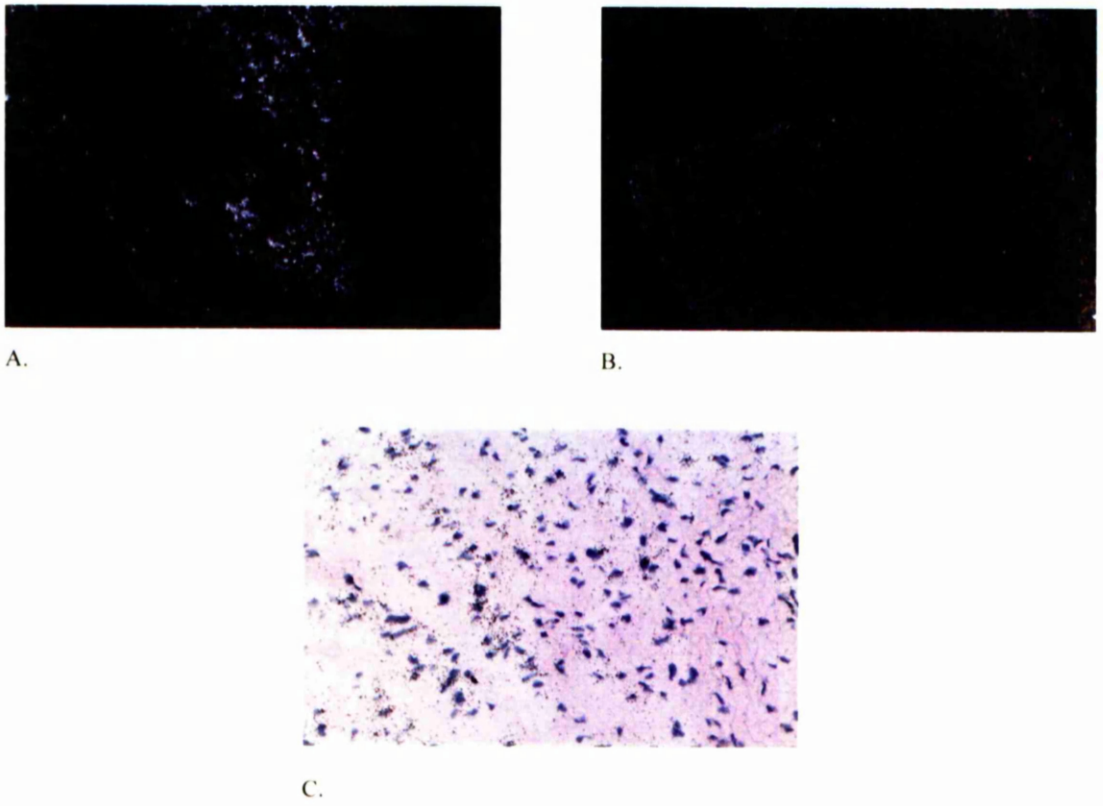


Figure 7.6. In situ hybridisation of EP_4 receptor gene expression in baboon ductus arteriosus and aorta. Sections from un-instrumented animal delivered by Caesarean section at 172 days gestational age, having received no antenatal drug treatment. A. Dark field, anti-sense probe, B. Dark field, sense probe, C. Haematoxylin and eosin section, anti-sense probe, EP_4 signal visualized as silver grains. A and B, low power: ductal tissue in middle of field. C, high power: ductal tissue on upper left of field and aorta on lower right.

Table 7.1. Three way analysis of variance: effect of fetal surgery, dexamethasone and gestational age and birth on expression of prostanoid receptor genes.

	Fetal surgery P=	Dexamethasone P=	Gestational age & birth P=
EP_4/beta-actin	0.35	0.65	<0.001
EP_4/GAPDH	0.36	0.31	<0.001
EP_3/beta-actin (4.2kB transcript)	0.22	0.60	0.36
EP_4/ EP_3 (4.2kB EP_3 transcript)	0.28	0.79	<0.001

GAPDH denotes glyceraldehydes-3-phosphate dehydrogenase, kB denotes kilobases.

7.3 Discussion

The main finding reported in this chapter is that expression of the gene encoding the prostanoid EP_4 receptor in the ductus arteriosus was lower in term fetal lambs compared with preterm lambs, and was lower still in neonatal lambs. However, there was no variation in expression of the gene encoding the contractile EP_3 receptor in the same animals. The main role of the EP_4 receptor in the control of the ductus had been thought to be to modulate variation in the local and circulating concentrations of PGE_2 [99]. The present data suggests that variation in the level of the EP_4 receptor gene may itself have a role in the control of the vessel. It seems likely that an altered balance of EP_4 and EP_3 mediated effects on the ductus, in the face of a given concentration of PGE_2, has a role in promoting contraction of the vessel in neonatal life.

The EP_4 receptor appears to be important in the control of the ductus with conservation in a number of species. It was first demonstrated in rabbits [39] and subsequently in mice [103]. In the present study I have now demonstrated expression of the gene encoding the receptor in the lamb and baboon ductus arteriosus (Figures 7.1 and 7.4). Given this high degree of species conservation, it seems likely that the EP_4 receptor will have a similar key role in the control of the human ductus. This is supported by the high sensitivity of the human ductus to PGE_2. In human infants with ductus dependent heart defects the vessel is dilated by exogenous synthetic PGE_2 in the sub-nanomolar range of circulating concentrations [237]. This is consistent with the known EC_{50} for the EP_4 receptor, which is more sensitive to PGE_2 than the other inhibitory EP receptor, the EP_2 receptor [13;32;198;238].

The current findings help explain the apparently paradoxical observation that the ductus is patent in utero in EP_4 null mutant mice, but fails to close following birth [103]. Taken at face value, this finding might be thought to suggest that the EP_4 receptor somehow mediates contraction of the vessel in neonatal life. This is implausible both from the

pharmacological perspective, as the receptor mediates relaxation of ductal smooth muscle [39], and on the basis of the current findings, since birth was associated with a decrease in expression of the EP_4 receptor gene (Figures 7.1 and 7.2). A major factor in the control of the ductus is the vessel's intrinsically very high level of endogenous contractile tone: acute withdrawal of PGE_2-mediated inhibition using a PG synthesis inhibitor (indomethacin) results in ductal closure and fetal heart failure [100]. The ductus of the EP_4 null mouse does not contract in response to indomethacin [103]. I hypothesize that the high level of EP_4 receptor-mediated inhibition of ductal tone is essential for the normal development of the vessel's intrinsic tone in utero. Since contraction of the vessel following birth is dependent on the vessel's intrinsic contractile tone, this model would explain the apparent paradoxical finding of patent ductus arteriosus in EP_4 null mutant mice [103]. This model would also explain the observation that human fetuses exposed to indomethacin in utero are more likely to have a patent ductus arteriosus in neonatal life than infants born at a similar gestational age that were not exposed to indomethacin [239].

The lack of effect of corticosteroids on the expression of the EP_4 and EP_3 receptor genes was of interest. It had previously been shown that treatment with corticosteroids decreased the sensitivity of the preterm lamb ductus to the effects of exogenous PGE_2 [234]. On the basis of the current data, it seems that this is unlikely to be due to variation in the relative balance of EP_4 and EP_3 gene expression. However, I cannot rule out altered translation of EP_4 and EP_3 mRNA following steroid treatment or an effect of corticosteroid on receptor mRNA transcription in late gestation and following birth. Furthermore, steroids may also affect the balance of EP_4 and EP_3 mediated effects by altering transcription and/or translation of other genes which might affect the relative balance of activation of the two receptors, such as genes encoding G-proteins and second messengers. However, it is of interest that there was a decrease in expression of the EP_4

receptor gene with advancing gestational age, but no apparent change following exposure of the preterm fetal lamb to corticosteroids (Figure 7.2). Many of the effects of advancing gestational age can be induced by antenatal exposure of the preterm fetus to corticosteroids and the use of corticosteroids is widespread in the management of threatened preterm delivery, as a means of advancing pulmonary maturity [240]. The present study demonstrates an effect of gestational age which was not induced by exogenous corticosteroids, despite the fact that corticosteroids have been shown to affect other aspects of ductal function in the preterm lamb [241]. This underlines the potential importance of endocrine systems other than the adrenocortical system in ontogenic development of the fetus. Thyroid hormones also have maturational effects on the preterm fetus [240] and been shown to alter contractility of the ductus [242].

It is possible, however, that the apparent lack of effect of corticosteroids is artefactual. It may represent a type II error due to lack of statistical power. However, using the standard deviations obtained from densitometry, I estimate that with an n of 4 in each group, the analysis had 71% power to detect the same magnitude of difference with steroid treatment as was observed comparing 110dGA and 145dGA fetuses. The apparent lack of effect of steroids may be methodological, e.g. the dose of corticosteroid was insufficiently high to induce altered gene expression. However, as in previous studies, the fetus was noted to be hypertensive during steroid treatment, both the lambs (unpublished data, Koenen, Nijland and Nathanielsz, 1999) and baboons [243] which suggests that the dose was not sub-therapeutic. Finally, the instrumented lambs were also exposed to both phenylephrine and sodium nitroprusside as part of another experiment. Administration of these drugs may have altered prostanoid receptor gene expression and masked an effect of dexamethasone. This seems unlikely, however, as there was no difference in EP_4 gene expression among 110dGA fetuses, comparing un-instrumented animals and

instrumented animals which were controls for dexamethasone and received phenylephrine and sodium nitroprusside (Table 7.1).

A study of the ductus arteriosus in piglets demonstrated several important species differences with respect to prostanoid receptors in the vessel [244]. These authors demonstrated the presence of EP_2, EP_3 and EP_4 receptors in the fetal piglet ductus, but that only EP_2 receptors were present in the neonatal piglet ductus. This is in contrast to the fetal rabbit ductus which does not express functional EP_2 receptors, as evidenced by the lack of response to the selective EP_2 agonist, AH13205 [39]. It is also suggests species differences in terms of the EP_4 receptor, which continued to be expressed at the level of mRNA in the neonatal ductus in the mouse [103] and in the present study (albeit at lower levels) in the lamb, and in terms of the EP_3 receptor since, in the present study, expression of EP_3 mRNA was observed in the ductus from neonatal lambs. Furthermore, since the EP_3 receptor mediates contraction of the ductus [102], it remains to be seen how loss of this receptor might be involved in ductal closure in the neonatal piglet.

Another aspect of prostanoid receptor pharmacology which appears to differ in the fetal piglet when compared with other species is the affinity of EP receptor sub-types. The neonatal piglet ductus expressed only EP_2 receptors, whereas the fetal ductus expressed approximately equivalent levels of EP_2, EP_3 and EP_4 receptors [244]. The EP_2 receptor has a five to tenfold lower affinity for PGE_2 than either the EP_3 or EP_4 receptor, which has been observed in functional studies in a number of species [37] and confirmed in recombinant mouse prostanoid receptors [47]. However, PGE_2 was bound with greater affinity in neonatal ductus than fetal ductus (K_d, 6.1nM versus 10.8nM, respectively), despite the apparent loss of receptors which bind PGE_2 with five to tenfold greater affinity than the EP_2 receptor in other species. Taken together, these observations suggest that the piglet ductus may be somewhat atypical in terms of prostanoid pharmacology.

Since the expression of EP receptor sub-types in the neonatal ductus is highly relevant for the development of drugs for the clinical manipulation of the human ductus, the expression of EP receptors in primate neonates should be an area of further study.

Chapter 8. The effect of labour and gestational age on expression of lipoxygenase genes in intra-uterine tissues

A large number of studies have underlined the key role of PGs in the control of parturition in a number of species [5]. PGs are formed from AA by one of two isoforms of the enzyme COX. Expression of the inducible form of this enzyme (COX-2) has been shown to increase both with advancing gestational age and labour [25;80;245] and these studies led directly to clinical studies of selective COX-2 inhibitors in the management of preterm labour [223].

The PG pathway is only one of several pathways of AA metabolism which results in biologically active products [10]. Others include the epoxide pathway (cytochrome P450) [17], the isoprostane pathway (non-enzymatic generation of isoprostanes such as 8-epi-$PGF_{2\alpha}$) [18] and the LOX pathway [19]. The last of these generates HPETE compounds which are metabolised to a range of eicosanoids, including the leukotrienes (LTs) and some of these compounds have been shown to have effects on myometrium [159].

Whereas the expression of COX genes has been systematically studied in a number of species, there are few studies systematically addressing variation in the expression of LOX genes with advancing gestation and in relation to labour. In this chapter I report expression of the three main recognized human LOX genes in myometrium (both fundus and lower segment), cervix, decidua and chorion.

8.1 Methods

The same animals described in Chapter 4 were employed and the general methods were as outlined in Chapter 2. All LOX probes were cDNA.

8.2 Results.

Clear signals of similar size to previous reports of Northern analysis of human RNA were detected using the 5-LOX and platelet-12-LOX probes in myometrium (Figure 8.1), cervix (Figure 8.2), decidua (Figure 8.3) and chorion (Figure 8.4). In the case of 5-LOX there was a single band of approximately 2.4kB. In the case of platelet-12-LOX there was a single band of 2.5kB in decidua, but in myometrium, cervix and chorion, there was also an additional band at 2.2kB. A clear 6.3kB band was observed using the human 15-LOX cDNA probe in myometrium and cervix (Figure 8.5). However, this differs markedly in size from previous reports of the 15-LOX gene in Northern analysis of human tissues [246] and it remains to be determined whether this signal truly represents the baboon 15-LOX gene. No signal in any tissues was observed using the murine leukocyte 12-LOX cDNA probe.

Expression of the 5-LOX gene (relative to beta-actin) decreased in the cervix with advancing gestational age, but did not vary significantly with gestational age in any of the other tissues (Table 8.1). Expression of 5-LOX was greater in decidua obtained from animals in labour than animals not in labour, whereas 5-LOX expression in chorion was lower in tissues obtained from animals in labour (Figure 8.6). There was no significant difference in 5-LOX expression comparing labour and not in labour in either cervix or myometrium, and myometrial expression was not significantly different comparing samples obtained from the fundus and lower segment (Table 8.1).

Expression of the 2.5 kB transcript of the platelet 12-LOX gene (relative to beta actin) decreased with advancing gestational age in chorion, but did not vary significantly with advancing gestational age in any of the other tissues (Table 8.1). Expression of the 2.5 kB transcript of the platelet 12-LOX gene in cervix was lower in tissues obtained from animals in labour (Figure 8.7A). There was no significant difference in the 2.5 kB

transcript of the platelet 12-LOX gene comparing labour and not in labour in myometrium, decidua or chorion and myometrial expression was not significantly different comparing the fundus and lower segment (Table 8.1).

Although the 2.2kB platelet 12-LOX transcript was also observed in myometrium and chorion, the signals were too weak for meaningful quantitative analysis. When related to beta-actin, there was no significant change in the level of expression of the 2.2kB transcript in association with either advancing gestational age or labour in the cervix (Table 8.1). The ratio of expression of the 2.5kB platelet 12-LOX gene transcript to the 2.2kB transcript was lower in cervical tissue obtained from animals in labour (Figure 8.7B) but there was no change in the ratio of the two transcripts in association with advancing gestational age in the cervix (Table 8.1).

The apparent 15-LOX signal showed no variation with either gestational age or labour in myometrium and cervix (Figure 8.5).

All cases where significant variation was observed when signals were related to beta-actin were also significant when related to at least one of the other two housekeeping genes (data not shown). Where signals were observed to change with gestational age prior to labour (namely, 5-LOX in the cervix and platelet 12-LOX in the chorion) adjusting the comparison between animals in labour and not in labour for the effect of gestational age (using analysis of covariance) did not alter the statistical significance.

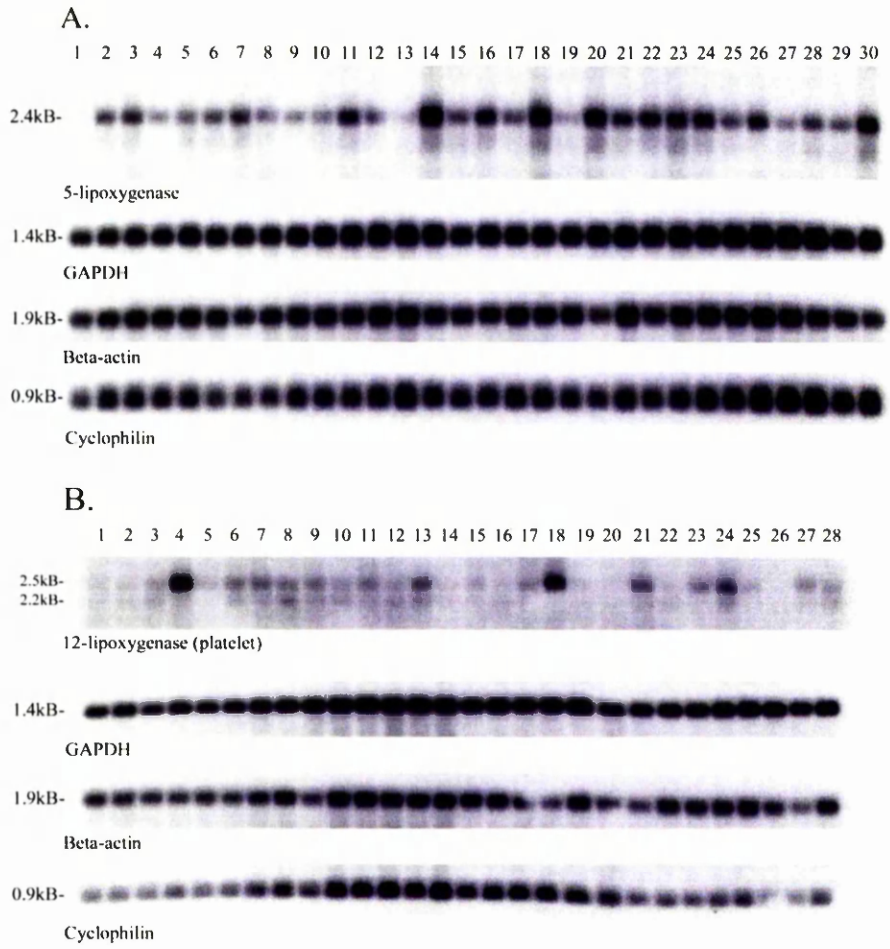


Figure 8.1. Northern blot of LOX expression in myometrium from lower uterine segment (LUS) and fundus (FUN), from animals not in labour (NIL) and in labour (LAB). A. 5-LOX, B. platelet 12-LOX. The estimated size of transcripts is given in kilobases (kB). 2µg of RNA was loaded in each lane. Lanes: A. 1-10 LUS, NIL; 11-15 LUS, LAB; 16-25 FUN, NIL; and 26-30 FUN, LAB. B. 1-9 LUS, NIL; 10-14 LUS, LAB; 15-23 FUN, NIL; 24-28 FUN, LAB.

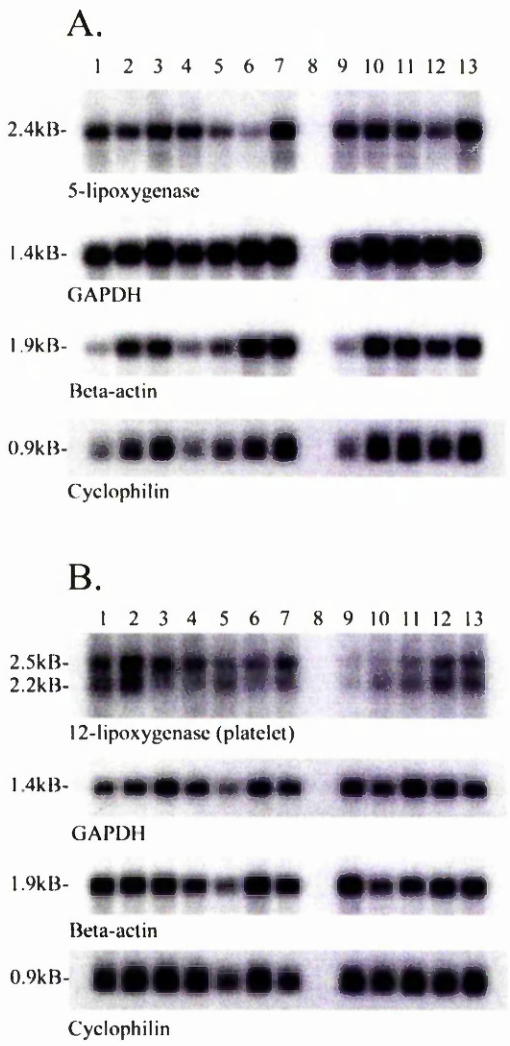


Figure 8.2. Northern blot of LOX expression in cervix from animals not in labour (NIL) and in labour (LAB). A. 5-LOX, B. platelet 12-LOX. The estimated size of transcripts is given in kilobases (kB). 4μg of RNA loaded in each lane in A and 2μg loaded in each lane in B. Both blots, lanes 1-7 NIL and lanes 9-13 LAB.

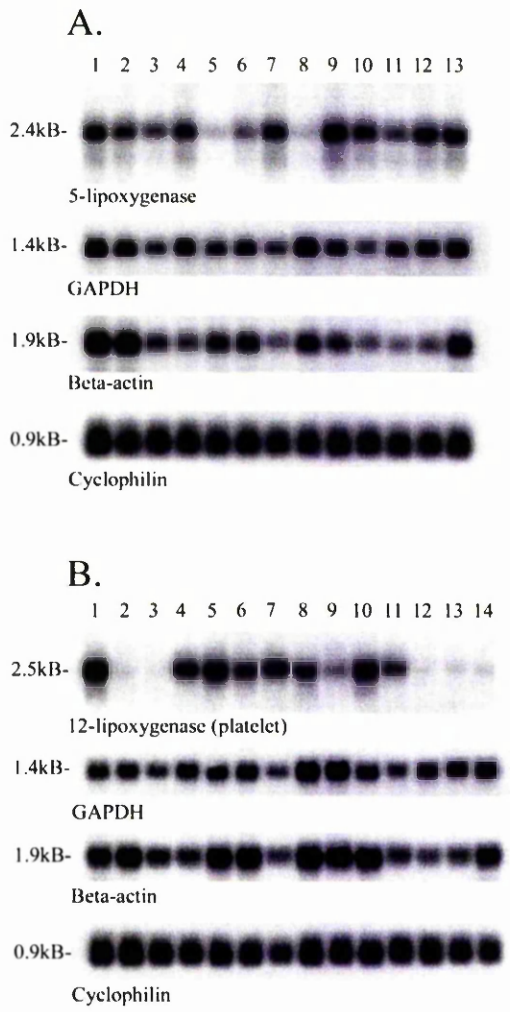


Figure 8.3. Northern blot of LOX expression in decidua from animals not in labour (NIL) and in labour (LAB). A. 5-LOX, B. platelet 12-LOX. The estimated size of transcripts is given in kilobases (kB). 5 μg of RNA loaded in each lane. Lanes: A. 1-8 NIL, 9-13 LAB; B. 1-9 NIL, 10-14 LAB.

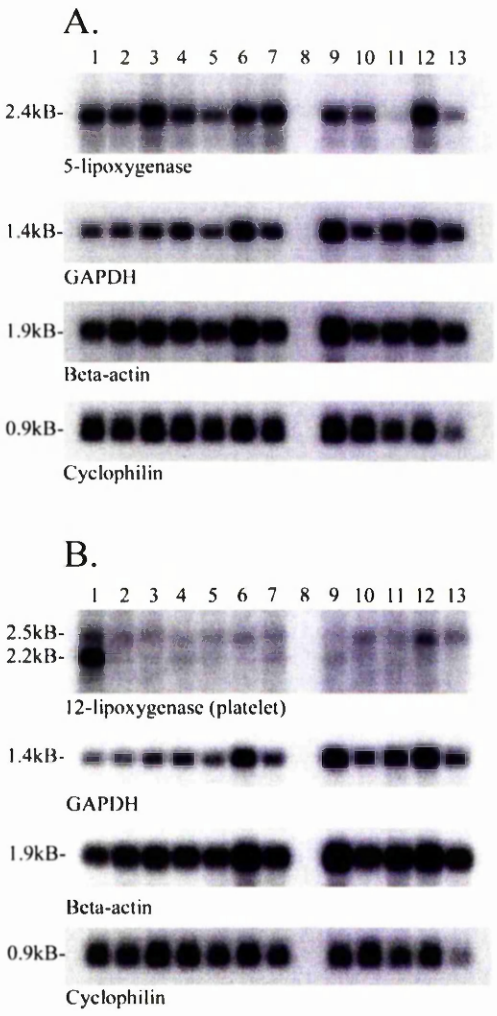


Figure 8.4. Northern blot of LOX expression in chorion from animals not in labour (NIL) and in labour (LAB). A. 5-LOX, B. platelet 12-LOX. The estimated size of transcripts is given in kilobases (kB). 5 μg of RNA loaded in each lane. Both blots, lanes 1-7 NIL and lanes 9-13 LAB.

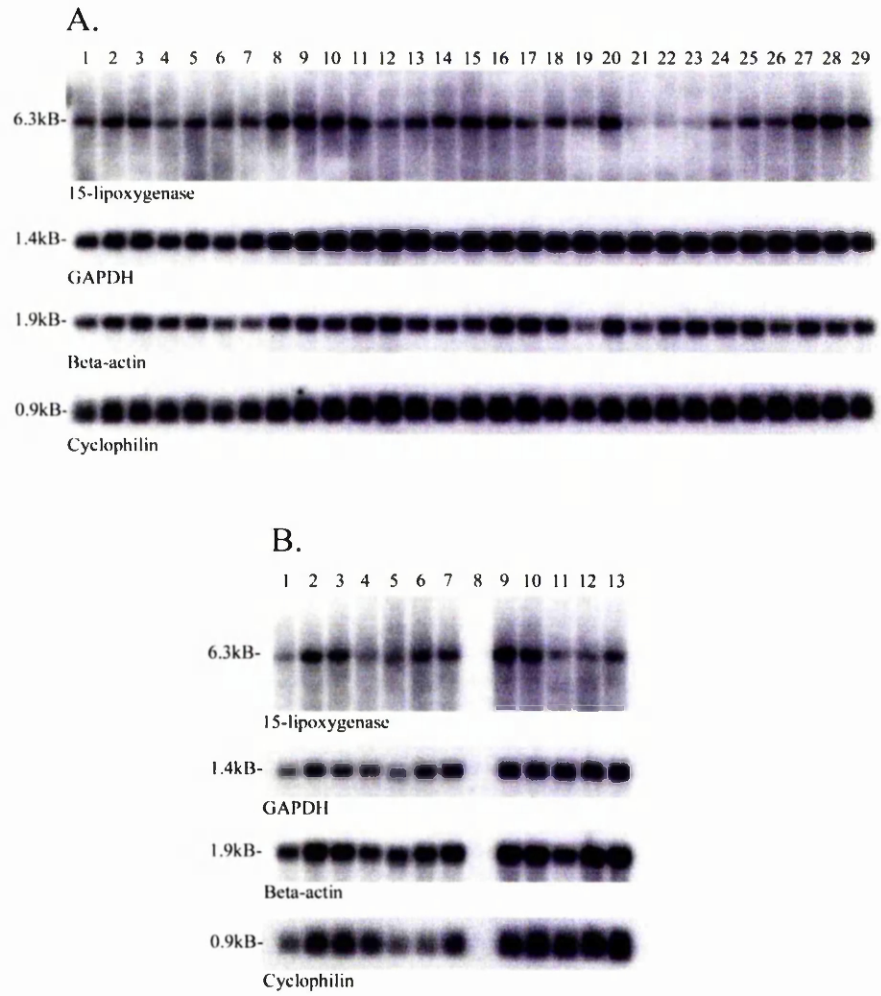


Figure 8.5. Northern blot of 15-LOX expression in A. Myometrium, B. Cervix. Samples of myometrium from both lower uterine segment (LUS) and fundus (FUN), and samples from both tissues obtained from animals not in labour (NIL) and in labour (LAB). The estimated size of transcripts is given in kilobases (kB). Lanes: A. 1-9 LUS, NIL; 10-14 LUS, LAB; 15-24 FUN, LAB; and 25-29 FUN, LAB. B. 1-7 NIL and 9-13 LAB. Statistical comparison (densitometry relative to beta-actin): A. FUN vs. LUS, p=0.19 (ANOVA), NIL vs. LAB, p=0.18 (ANOVA); B. NIL vs. LAB, p=0.38 (Student's t-test). Similar results obtained when signal quantified relative to GAPDH or cyclophilin.

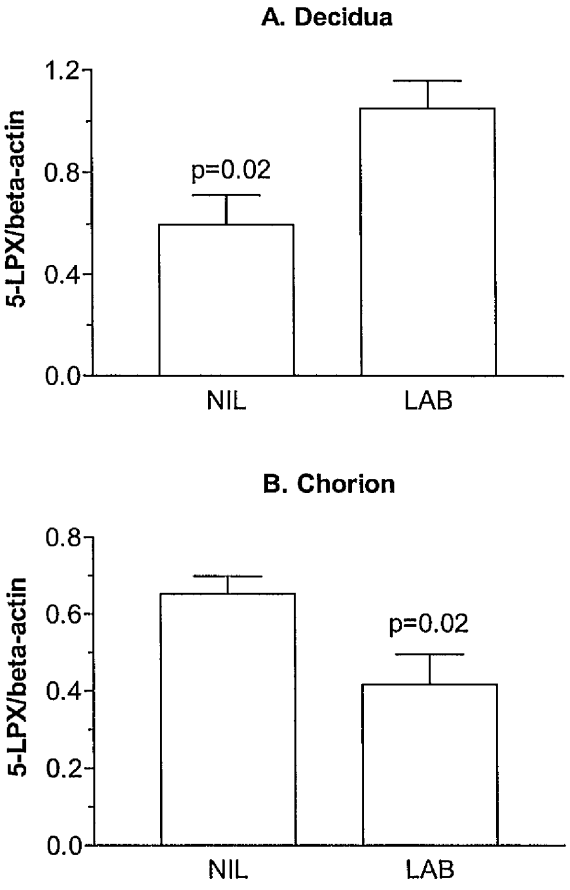


Figure 8.6. Densitometric analysis of 5-LOX expression (relative to beta-actin) decidua and chorion. A. Decidua and B. Chorion, comparing animals in labour (LAB, n=5) with animals not in labour (NIL, n=7-8). Columns are means, bars are SEM. Statistical comparison by Student's t-test.

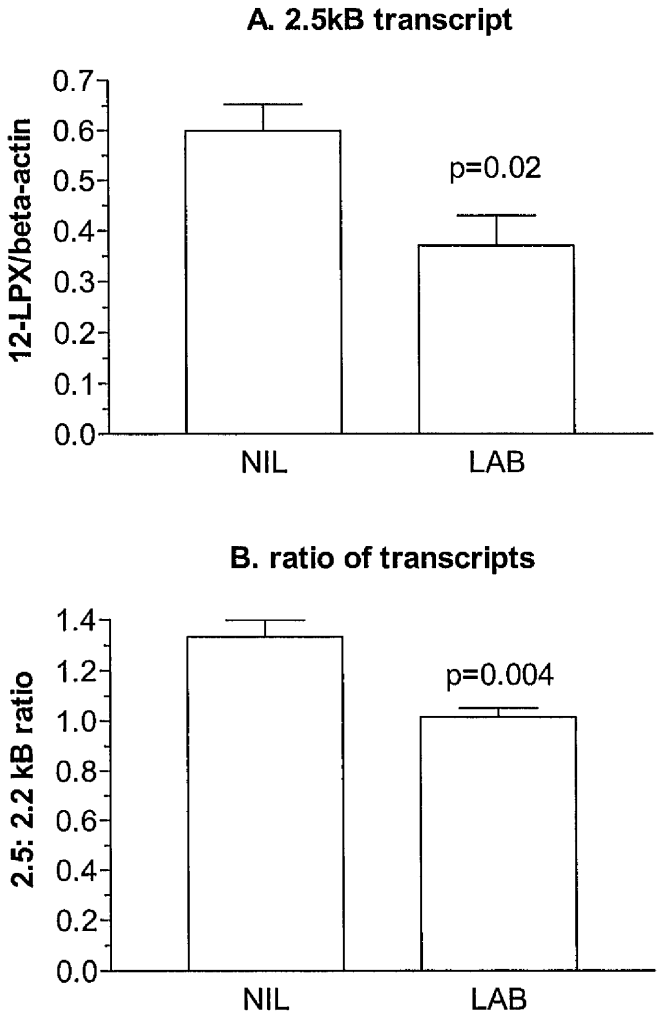


Figure 8.7. Densitometric analysis of 12-LOX expression in the cervix. A. 2.5kb transcript relative to beta-actin and B. 2.5kb transcript relative to 2.2kb transcript, comparing animals in labour (LAB, n=5) with animals not in labour (NIL, n=7). Columns are means, bars are SEM. Statistical comparison by Student's t-test.

Table 8.1. Expression of 5-LOX and platelet 12-LOX in relation to gestational age and labour in baboon intra-uterine tissues.

	5-lipoxygenase				Platelet 12-lipoxygenase¶			
	Means (SEM)		P =		Means (SEM)		P =	
	NIL (n=7-10)	LAB (n=5)	GA (NIL)	LAB	NIL (n=7-10)	LAB (n=5)	GA (NIL)	LAB
LUS	0.25 (0.03)	0.35 (0.10)	0.96		0.49 (0.14)	0.24 (0.04)	0.57	
Fundus	0.39 (0.05)	0.34 (0.09)	0.77	0.69	0.38 (0.13)	0.26 (0.07)	0.69	0.33
Fundus* labour				0.20				0.17
Cervix	1.23 (0.28)	1.11 (0.22)	0.02	0.75	2.5kB: 0.60 (0.05)	0.37 (0.05)	0.79	0.02
					2.2kB: 0.46 (0.05)	0.37 (0.06)	0.68	0.27
					ratio: 1.33 (0.07)	1.02 (0.03)	0.61	0.004
Decidua	0.59 (0.12)	1.05 (0.11)	0.72	0.02	0.90 (0.17)	0.62 (0.20)	0.95	0.32
Chorion	0.65 (0.05)	0.42 (0.08)	0.56	0.02	0.23 (0.04)	0.20 (0.02)	0.02	0.51

¶Unless stated otherwise, expression of platelet 12-LOX refers to the 2.5kB transcript.

GA denotes gestational age, NIL denotes not in labour, LAB denotes in labour, kB denotes kilobases, SEM denotes standard error of the mean.

8.3 Discussion.

A key role has been established for PGs in the control of labour in a number of species [5]. The elucidation of the physiological role of PGs in labour led directly to the clinical use of PG synthesis inhibitors in preterm labour, and these drugs are currently widely clinically employed [210]. The PG pathway is, however, just one of several pathways through which AA can be metabolised. Much less is understood about the role of these other pathways in the control of parturition. A wide range of drugs have been developed which both inhibit LOX enzymes and block receptors through which LOX products act [247;248] If LOX products have a role in parturition, there is the potential for these drugs to be employed in the clinical management of preterm labour. A key line of evidence in understanding the role and site of PG synthesis in the control of parturition was elucidation of quantitative changes in COX gene expression in association with labour [25;245] The aim of the present study was, therefore, the systematic study of LOX gene expression in key intra-uterine tissues with advancing gestational age and labour.

The three main LOX genes identified in the human are 5-LOX, platelet 12-LOX and 15-LOX [19]. In the mouse there is another isoform, namely, leukocyte 12-LOX. However, this enzyme also has 15-LOX activity and shares 65% amino acid homology with human 15-LOX [19]. It is thought that murine leukocyte 12-LOX is the homologue of 15-LOX in the human and, consistent with this hypothesis, I could not detect leukocyte 12-LOX expression in any of the baboon tissues. The signals observed in the present study using the 5-LOX and platelet 12-LOX probes were similar to previous reports of Northern analysis in human tissues [249;250] However, the transcript observed using the 15-LOX probe was approximately twice as large as previous descriptions of Northern blot of human tissue [246]. I have reported these findings with this probe for completeness (Figure

8.5), but suggest that the results be interpreted with some caution until more is known about the baboon 15-LOX gene.

The changes observed in expression of 5-LOX were tissue specific. Expression of the gene decreased with advancing gestational age in cervix and was lower in animals in labour in chorion. However, 5-LOX expression was greater in decidua obtained from animals in labour. Increased 5-LOX expression suggests that 5-HPETE or its metabolites might promote parturition. This could either be due to autocrine effects on the decidua or paracrine effects on myometrium. Consistent with this, 5-HETE has been shown to stimulate contractility in myometrial strips obtained from the LUS of pregnant women [159]. The decrease in 5-LOX expression in cervix and chorion may indicate that in these tissues, 5-HPETE or its metabolites may act to inhibit the process of parturition. Consistent with this hypothesis, LTB_4 has been shown to exert inhibitory effects on cervical tissue obtained from pregnant women at term [251]. An alternative possibility is that 5-HPETE and its metabolites are without significant effects in these tissues and that the main biological consequence of lower levels of 5-LOX expression may be to divert free AA to the PG pathway. These mechanisms may also act additively, since exogenous LTB_4 has been shown to inhibit PGE_2 release in human fetal membranes [252]. Therefore, decreased 5-LOX may stimulate PGE_2 release due to both an increase in the free AA pool and direct effects of LTs on PG biosynthesis.

The size of the platelet 12-LOX transcript observed in the baboon (2.5kB) was very similar in size to the human transcript (2.4kB) which supports the interpretation that this signal is likely to represent the platelet 12-LOX gene in the baboon [250]. However, in myometrium, cervix and chorion a second transcript of 2.2kB was also observed. The level of expression of the smaller transcript was lower than the 2.5kB transcript and quantitative analysis of the level of expression was only possible in cervix. Interestingly,

labour appeared to affect differentially expression of the two transcripts in the cervix (Figure 8.2, Table 8.1). The practical significance of these observed changes in relative expression is as yet unclear although isoforms of 12-LOX have previously been described [19]. However, the fact that changes were observed is consistent with a role for platelet 12-LOX, in some form, in the control of parturition in these tissues. As in the case of 5-LOX this could be by effects on PG synthesis or by effects of 12-HPETE (or its metabolites) on key intra-uterine tissues. It has been shown that 12-HETE had no effect on the contractility of isolated strips of human myometrium [159]. As in the case of 5-LOX, it may be that reduced platelet 12-LOX activity might be a factor in determining increased PG levels in labour, amplifying the effect of other known factors which increase PG synthesis in labour such as increased expression of PLA_2 and COX-2 [80].

Comparing the findings of this study with other studies, it has also been shown in the human that mRNA encoding the 5-LOX gene increases in the choriodecidua in association with labour and this was paralleled by increased 5-LOX protein [158]. This is consistent with the observation that amniotic fluid concentrations of both 5-HETE and LTC_4 are higher in women in samples obtained from women in labour compared with women not in labour [253;254]. The finding that 5-LOX and 12-LOX were the main LOX enzymes in intra-uterine tissues is consistent with biochemical studies of human intra-uterine tissues that have demonstrated that 5-HETE and 12-HETE are the main LOX products of AA in human intra-uterine tissues [255;256]. My hypothesis that decreased 12-HETE formation in labour may play a role in increasing free AA and thus promoting formation of PGs is supported by the observation that 12-HETE is quantitatively the major AA metabolite formed in key intra-uterine tissues [256] and that human labour is associated with an increased ratio of COX: LOX metabolites of AA [257].

There were no apparent differences in myometrial expression of LOX genes comparing the upper and lower segment of the uterus. This is in contrast to COX genes which have been shown to demonstrate differential expression according to uterine site in the baboon [81]. These observations also tend to support an interpretation of different physiological roles for COX and LOX products in the control of parturition.

These data do not strongly suggest, at present, a potential role for LOX inhibitors in the treatment of preterm labour. Indeed, the observations that both advancing gestational age and labour are associated with decreases in the expression of some LOX genes in key tissues suggests that pharmacological inhibition of LOX genes may actually promote parturition by diverting free AA to the PG pathway. However, increased expression of 5-LOX in decidua is consistent with a possible role for 5-LOX products (such as leukotrienes) in parturition. Multiple receptors exist for these compounds and drugs with selective antagonist activity have been described [247]. Unlike enzyme inhibitors, receptor antagonists would not be expected to increase the pool of free AA and the effect of available drugs on parturition in animals warrants study.

Chapter 9. Overall conclusions.

The specific aims outlined in Chapter 1 were largely achieved. The immediate significance of observations to a given area of study were discussed in each chapter. The purpose of this chapter is, briefly, to draw together these observations into such general conclusions as can be drawn from this work.

9.1 Variation in prostanoid receptor expression with advancing gestational age and labour

As outlined in Chapter 1, there has been a great deal of study of the regulation of the genes which control PG concentrations in key intra-uterine tissues during parturition. One of the key aims of this thesis was to determine whether variation in the level of expression of the receptors may also have a role in the control of primate parturition. This aim was achieved and I demonstrated variation in the level of expression of genes comparing animals in labour and animals not in labour in myometrium, cervix, decidua and chorion. Of particular significance was the observation that expression of the prostanoid EP_2 receptor gene was lower in labour in myometrium (both lower segment and fundus [Chapter 4]), cervix (Chapter 5) and decidua (Chapter 6). This gene encodes an adenylate cyclase coupled receptor which typically inhibits myometrial contractility. I speculate that this gene may also exert inhibitory effects in the cervix and decidua and that loss of EP_2 receptor mediated inhibition in these tissues may be a key molecular event in primate parturition. I also demonstrated that in a key, PG mediated fetal adaptive response at birth, namely, contraction of the ductus arteriosus, one could demonstrate altered expression of stimulatory and inhibitory prostanoid receptor genes. These data suggest that variation in the expression of prostanoid receptor genes may be a key feature of dynamic physiological control of smooth muscle by PGs.

9.2 Regional variation in myometrial function

Many studies in pregnant woman have examined the function of myometrium obtained at the time of lower uterine segment Caesarean section [86]. The assumption (often unstated) of these studies was that the function of LUS myometrium is the same as myometrium from the corpus and fundus. In this thesis I demonstrated that this assumption is unsound. Variation exists in the expression of prostanoid receptor genes in tissue obtained prior to the onset of labour (Chapter 3) and the pattern of variation is consistent with functional variation in the response to native PGs [195]. Furthermore, the nature of the differences between the LUS and fundus is affected by labour in terms of inhibitory but not excitatory receptor genes (Chapter 4). These observations underline the potential pitfalls of studying LUS samples in isolation from the rest of the uterus. The studies also demonstrated that there was no regional variation in expression of LOX genes (Chapter 8) in contrast to studies of COX2 expression in these baboon tissues [81].

9.3 Tissue specific expression of prostanoid receptors

In the initial screen of expression of receptor genes, it was apparent that there was variation in the level of expression of different prostanoid receptor types and sub-types comparing different intra-uterine tissues (Chapter 3). When levels of expression were then compared in samples obtained at the time of labour and from animals prior to the onset of labour, it was observed that certain genes changed in some tissues but not others. For instance, there was a lower level of expression of the prostanoid EP_2 receptor gene in maternal intra-uterine tissues (Chapters 4-6) but not chorion (Chapter 6). In contrast, IP receptor gene expression was increased in both decidua and chorion (Chapter 6), but did not significantly differ in relation to labour in myometrium (Chapter 4) or cervix

(Chapter 5). These observations suggest that different PGs may have differential effects on different intra-uterine tissues and, consequently, that drugs with selective activity at prostanoid receptor types and sub-types may allow more specific manipulation of intra-uterine tissues.

9.4 Cell specific expression of prostanoid receptors

A number of lines of study in this thesis demonstrated that even in a given tissue a given second messenger system could not be characterized as either inhibitory or stimulatory. This possibility was underlined by findings in the decidua. Labour was associated with lower levels of expression of the EP_2 receptor gene but higher levels of expression of the IP receptor gene (Chapter 6). Both receptors are coupled to adenylate cyclase [12]. This suggests that in cells which express the EP_2 receptor, cAMP dependent protein kinases have effects which inhibit the process of labour, whereas in cells expressing the IP receptor the same enzymes may have effects which promote parturition. The potential for these observations to have rational physiological explanations was apparent using in situ hybridization in myometrium which demonstrated differential expression of the prostanoid EP_3 receptor in myometrial and vascular smooth muscle (Chapter 4). This is likely to be due to the fact that uterine resistance vessels should be most widely dilated when uterine smooth muscle is most intensely activated.

9.5 The concept of stimulatory and inhibitory systems

The factors regulating myometrial contractility have been the natural focus of studies of the control of parturition. This reflects a key role for uterine contraction in the process of labour, but also probably also reflects the relative ease of study of myometrial function. Techniques have been available for more than 50 years that allow the study of the effects

of drugs on myometrial contractility. However, as discussed elsewhere in this thesis, other intra-uterine tissues have keys roles in the process of parturition, such as the fetal membranes and cervix. However, it is much less straightforward to characterize the effect of a drug on the process of cervical priming or the integrity of the fetal membranes than uterine contraction. This has led both to the natural focus on myometrium and a tendency to characterize a given system as either promoting or inhibiting the process of parturition on the basis of its effect on myometrial contractility. It is clear, however, that the simple fact of a drug having an inhibitory effect on myometrial contractility does not ensure that the same drug will inhibit the processes leading to expulsion of the fetus in the other key intra-uterine tissues. A recent, important clinical example of this is nitric oxide, which inhibits myometrial contractility [171], but which promotes the process of cervical ripening [174;222].

The current studies also demonstrated that receptors which would be anticipated to stimulate myometrial contractility were seen to have lower levels of mRNA expression in other tissues in association with labour. Both TP and FP receptor activation stimulate myometrial contractility [86], but expression of the genes encoding both receptors was lower in the cervix in association with labour (Chapter 5). Conversely, IP receptor activation inhibits myometrial contractility [86], but expression of the IP receptor gene was much higher in the chorion (and to a lesser extent in the decidua) obtained from animals in labour (Chapter 6). These observations suggest that key prostanoid receptor types might be stimulatory to the process of labour in some tissues but inhibit the process in others. These findings underline the necessity for caution when investigating a drug in the inhibition of labour on the basis of a negative effect on myometrial contractility.

9.6 Regulation of prostanoid receptor gene transcription

It is apparent from the studies reported in this thesis that there is variation in the expression of prostanoid receptor genes in association with advancing gestational age and labour which is manifested by altered levels of expression of the same receptor gene in some tissues but not others, or in a given tissue of some genes but not others. These findings suggest that factors modulate the control of expression of these genes in labour within key intra-uterine cells in a tissue specific manner. The global reduction in EP_2 receptor gene expression was taken to suggest a key role for this gene in the control of primate parturition. If true, the factors which control the onset of parturition may possibly be inferred by understanding the transcriptional regulation of this gene. There are very few data on the factors which control expression of any of the prostanoid receptors. For instance, one of the basic techniques in the study of factors which control gene transcription is DNA foot printing. A Medline search (September 2000) failed to yield any citations which used the MESH terms "DNA foot printing" and "receptors, prostaglandin". This area, particularly as it relates to parturition, would appear to be ripe for future research.

9.7 Technical considerations relating to prostanoid gene expression

Over the course of the three year project, I clearly modified the technical approach to the study of prostanoid receptor genes. The major change was the use of riboprobes instead of cDNA probes for Northern blot analysis. In my initial studies I could not detect EP_1, IP or TP receptor signals using cDNA probes (Chapter 3). I then found that using riboprobes, appropriate sized transcripts could be detected for these genes (Chapter 4). There are a number of advantages to the use of riboprobes over cDNA probes. First, cDNA probes are double stranded whereas riboprobes are single stranded. The two

complementary DNA molecules will tend to bind each other and therefore not be available to bind the target. In the case of riboprobes, this is not an issue. Secondly, the higher stability of RNA: RNA duplexes compared with DNA:RNA duplexes [258] means that higher temperatures can be used for both hybridisation and washing which results in a higher signal to noise ratio. Thirdly, the unlabelled template DNA can be removed from the riboprobe using RNase free DNase I. This can clearly not be done using a cDNA probe. Finally, as part of the random priming process, one employs random decamers to create stretches of double stranded DNA to allow the Klenow DNA polymerase to synthesize DNA. Consequently, only a proportion of the given dNTP (for instance dCTP) is radioactively labelled since dCTP will also be included in the random hexamers, which are unlabelled. In contrast every NTP (UTP in the present study) in a riboprobe is radioactively labelled unless 'cold' UTP is added with the intention of making the probe less active.

Another issue is that the present study focused entirely on the expression of genes. I did not use other techniques to confirm that the RNA was translated into protein and that the protein was formed into functional receptors. The close parallel between gene expression and functional responses when comparing uterine regions [195] (Chapter 3) supports my assumption that genes were translated. Ideally, one would employ protein analysis (such as immunocytochemistry or Western blot analysis) or receptor binding studies to confirm these assumptions. Unfortunately, antibodies are not widely available for the prostanoid receptor genes. Furthermore, such ligands as are available are not specific for a given prostanoid receptor type or sub-type [47].

9.8 Summary

Many genes encoding prostanoid receptor types and sub-types are expressed in key intra-uterine tissues and the level of expression varies systematically within the gravid baboon uterus in relation to anatomical site, gestational age and labour. Comparable changes were demonstrated in the fetal ductus arteriosus. The nature of this variation suggests that altered expression of prostanoid receptor genes may have an important role in the control of parturition and in the preparation of the fetus for birth. Drugs with selective activity at prostanoid receptor types and sub-types are likely to allow safer and more effective control of the human uterus in the clinical management of term and pre-term labour.

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