THE ROLE OF NITRIC OXIDE IN HUMAN PARTURITION

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Declaration

The contents of this thesis have not been submitted elsewhere for any other degree, diploma or professional qualification.

The thesis has been composed by myself, and I have been responsible for patient recruitment, tissue collection, clinical management and laboratory studies, unless otherwise acknowledged.

Andrew Thomson

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This work is dedicated to my wife, Lesley.

Abstract

Animal studies suggest that nitric oxide, a free radical derived from L-arginine by the action of nitric oxide synthase (NOS), is a fundamental mediator in the initiation of labour. The studies reported in this thesis have investigated the involvement of the L-arginine-nitric oxide system in the maintenance of myometrial quiescence and the initiation of parturition in human pregnancy. Further, the effects of nitric oxide donors on the pregnant human cervix have been determined.

The studies reported in chapter 2, were performed to determine whether a decrease in NOS enzyme activity and expression are involved in the final activation of spontaneous labour at term. Using immunocytochemistry, each of the three isoforms of NOS was localised in myometrium, placenta and fetal membranes collected before and during labour at term. NOS enzyme activity in each of these tissues was determined by measuring the conversion of radiolabelled L-arginine to L-citrulline. There were no differences in either the expression or enzyme activity of NOS within each tissue following the onset of labour indicating that a decrease in NOS enzyme activity is not involved in the final activation of labour at term in the human.

The enzymes superoxide dismutase (SOD) and xanthine oxidase (XO) are known to prolong and attenuate respectively, the biological activity of nitric oxide. Studies, presented in chapter 3, were therefore undertaken to determine whether changes in the uterine expression of these enzymes are associated with the onset of human parturition. Both SOD and XO were shown to be widely expressed within the pregnant uterus, although there was no difference in their expression in tissues collected before, compared with after the onset of labour. These data suggest that SOD and XO may be important in maintaining uterine quiescence in pregnancy, but that a change in the expression of these enzymes is not involved in the initiation of labour.

In chapter 4, the expression of the constitutive isoforms of NOS, eNOS and bNOS, was investigated in the non-pregnant uterus, the preterm pregnant uterus and the term pregnant uterus to determine whether these isoforms are up-regulated in the uterus during pregnancy. Using immunocytochemistry and Western blotting, eNOS and bNOS protein concentrations were greater in preterm, pregnant myometrium than non-pregnant myometrium, ($p<0.01$ and $p<0.05$, respectively). eNOS, but not bNOS, protein concentration was lower ($p<0.05$), in myometrial samples obtained at term compared with those obtained preterm. These data indicate that the constitutive isoforms of NOS are upregulated in human pregnancy and may play a role in maintenance of myometrial quiescence.

Concurrent histological studies on tissues obtained before and during spontaneous labour, reported in chapter 5, revealed that inflammatory cells infiltrate the myometrium during labour. Analysis of individual cell types using immunocytochemistry showed significantly more inflammatory cells, predominantly neutrophils and macrophages, in labouring myometrium compared to non-labouring biopsies, (Scheffe's test, $p = 0.0001$). The infiltrate was present in both lower and upper uterine segment myometrium, with significantly more inflammatory cells present in the lower uterine segment myometrium, (Scheffe's test, $p < 0.02$). These observations indicate that inflammatory cell infiltration is part of the physiological mechanisms that occur in the myometrium during labour.

The cervical ripening effects of nitric oxide donors, isosorbide mononitrate (IMN, 40 mg) and glyceryl trinitrate (GTN, 500 μg), were determined in a prospective, randomised trial involving women scheduled for surgical termination of pregnancy in the first trimester. During the operative procedure, the cervical resistance was measured objectively using a force sensing apparatus. The data, presented in chapter 6, showed that both IMN and GTN significantly reduced the cervical resistance, compared with no treatment ($p<0.006$ and $p<0.05$, respectively). A further randomised trial involving 66

primigravid women was performed to compare the side effect profile of IMN in comparison with the prostaglandin analogue gemeprost. Significantly more women remained asymptomatic following IMN (64%) than following gemeprost (14%). Pretreatment with gemeprost resulted in abdominal pain in 73% of women and vaginal bleeding in 32%, compared with 3% and 0% respectively following IMN. These data, reported in chapter 7, suggest that nitric oxide donors may have advantages over the prostaglandins for cervical ripening.

In summary, an up-regulation of NOS expression in myometrium during pregnancy suggests an involvement of the L-arginine-nitric oxide system in the maintenance of myometrial quiescence. A decrease in this system and in the expression of enzymes known to influence the bioavailability of nitric oxide, do not coincide with the onset of spontaneous labour at term, suggesting that a withdrawl of nitric oxide is not the final activating factor in the initiation of labour in women. This work has demonstrated that nitric oxide donors produce effective ripening of the human cervix in the first trimester of human pregnancy with fewer side effects than prostaglandins. Nitric oxide donors represent alternative cervical ripening agents, at least in the first trimester of human pregnancy.

Chapter 1

Literature Review

Introduction

Every moment dies a man,

Every moment one is born.

Alfred, Lord Tennyson (1809 - 1892)

The control and timing of delivery is crucial in the survival of any species, and in most, the interval between conception and parturition varies little. This is not the case in human pregnancy, where delivery can occur many weeks before or after the expected date. It is recognised in women that intervention by induction of labour in pregnancies prolonged beyond 41 weeks' gestation reduces the likelihood of perinatal mortality (Grant, 1994). However, the single biggest cause of perinatal and neonatal death in the developed world is preterm delivery. This is defined as delivery before 37 completed weeks of gestation (Hall *et al.*, 1997). The incidence of preterm delivery is between 5% and 10% of all births and has remained relatively stable over the last two decades; approximately 13 million infants are born preterm worldwide each year (Lumley 1993; Villar *et al.*, 1994). Severe neonatal morbidity, especially respiratory distress syndrome, intraventricular haemorrhage and necrotizing enterocolitis, is more common in preterm than in term infants (Robertson *et al.*, 1992), as is long-term impairment, such as cerebral palsy, broncho-pulmonary dysplasia, visual impairment and hearing loss (Knoches and Doyle, 1993).

The prevention of preterm labour remains one of the primary goals of obstetric research. An understanding of the mechanisms underlying preterm labour in women is fundamentally linked to an understanding of the mechanisms of parturition at term.

Indeed, they share a common terminal pathway of uterine contractility, cervical ripening and activation of the membranes. Romero *et al.*, (1997) propose that the fundamental difference between term and preterm labour is that the former results from physiological activation of the components of the common terminal pathway, while preterm labour results from disease processes activating one or more of the components of this pathway. At present, the factors maintaining myometrial quiescence during pregnancy, and those which stimulate the onset of uterine contractions and cervical ripening at term remain obscure. Until these factors are elucidated, it seems unlikely that effective strategies for the treatment of preterm labour will be found (Goldenberg and Rouse, 1998).

Part I.

The Factors Controlling the Onset of Parturition

Parturition is a physiological process - the same in the countess and in the cow
W.W. Chipman (1866 - 1950)

Despite major advances in molecular biology and the science of reproduction, the signals controlling the onset of human parturition remain elusive. The search for the triggering mechanism initiating human labour, which focused on oxytocin in the early 20th century, has since included prostaglandin production, growth factors, cytokines, endothelins, gap junction formation and, more recently, placental corticotropin-releasing hormone (Karalis *et al.*, 1996) and nitric oxide withdrawal (Izumi *et al.*, 1993). In non-primate mammals, the end of pregnancy is associated with a fall in maternal progesterone concentrations (Csapo, 1977), which together with increasing oestradiol concentrations, activates multiple pathways, including stimulation of oxytocin release, oxytocin receptors, and prostaglandin synthesis, which contribute to the initiation of labour (Casey and MacDonald, 1988; Liggins, 1994).

It would seem reasonable that the conceptus should trigger the onset of labour when maturity is adequate for extra-uterine survival, and this is certainly the case in sheep. In sheep, fetal cortisol triggers parturition by increasing the activity of placental enzymes, including 17α-hydroxylase and P450 C-17,20 lyase, that enable progesterone conversion to oestradiol (Flint *et al.*, 1975). The resultant increase in the ratio of oestradiol to progesterone stimulates myometrial oxytocin receptors, gap junction formation, prostaglandin production and maternal pituitary oxytocin release, events leading directly to labour and delivery (Schwartz, 1997). These same pathways are activated at term in human pregnancy when circulating levels of fetal plasma cortisol are also raised. However, the target for cortisol action in the ovine placenta, 17α-

hydroxylase, is absent in human placenta, and there is no consistent rise in maternal plasma concentrations of oestrogens or any consistent decline in progesterone concentrations before the onset of labour in man (Tulchinsky *et al.*, 1972; Flint 1979). Hence, if fetal cortisol is a factor controlling the onset of human parturition, its mode of action must be different from that in sheep.

The onset of human parturition is associated with an increase in the production of prostaglandins within the uterus. Prostaglandins are synthesised from arachidonic acid via the enzyme cyclo-oxygenase (COX), of which there are two isoforms. The type-1 gene (COX-1) encodes a constitutively expressed form of the enzyme, whilst the type-2 enzyme (COX-2) is inducible. With the onset of labour at term, there is an increase in COX-2 expression in the amnion and chorion-decidua (Slater *et al.*, 1995; Slater *et al.*, 1998) and an increase in the production of prostaglandins E_2 and $F_{2\alpha}$ by these tissues (Keirse, 1979; Mitchell, 1984; Casey and MacDonald, 1988). Prostaglandin production is stimulated by platelet-activating factor and various cytokines, including interleukin-1, interleukin-6 and tumour necrosis factor (Challis and Mitchell, 1994). Loss of chorionic prostaglandin dehydrogenase activity may be significant in initiating labour, particularly in preterm labour (Keirse, 1995), although *in vitro* studies have not found any change in prostaglandin metabolism with labour at term (Brennand *et al.*, 1998).

The myometrium is stimulated directly by both prostaglandins and oxytocin derived from the chorion-decidua (Fuchs *et al.*, 1984). Other myometrial stimulants, such as platelet-activating factor and endothelin, may also be involved in the initiation of myometrial contractions (Wolff *et al.*, 1996). Simultaneously, the connective tissue of the cervix undergoes complex biochemical changes that lead to softening and distensibility. Cervical ripening has been compared to an inflammatory reaction and is characterised by an accumulation of leukocytes in the cervical stroma (Junqueira *et al.*, 1980; Liggins, 1981; Bokstrom *et al.*, 1997).

The signal to this chain of events is unknown although the progesterone-withdrawal theory remains the leading hypothesis. Progesterone inhibits human myometrial contractions and decreases gap junction formation (Beck *et al.*, 1978; Ambrus and Rao, 1994). Labour is an inflammatory process (Kelly, 1996) and progesterone is recognised to be an anti-inflammatory agent (Sitteri *et al.*, 1977). Whilst exogenous progesterone does not postpone the onset of parturition in humans as it does in sheep, antiprogesterones activate many of the pathways involved in the onset of labour and induce uterine contractility and cervical ripening (Lælaidier *et al.*, 1994; Chwalisz, 1994). These observations propose a role for progesterone in the maintenance of pregnancy and suggest that a decline in progesterone sensitivity, or an uncoupling of progesterone action in late pregnancy, without an actual fall in the hormone's concentration, could be an important factor in the initiation of human labour. Possible uncoupling mechanisms include local metabolism of progesterone, progesterone inactivation by a specific binding protein (Westphal *et al.*, 1977), by an endogenous antiprogesterone (Casey and MacDonald, 1993; Wilson and Parsons, 1996), or by a change in the number or affinity of progesterone receptors (Khan-Dawood and Dawood, 1984).

A role for corticotropin-releasing hormone (CRH) in the initiation of human labour has been proposed. CRH is a peptide hormone which was originally recognised as a hypothalamic releasing factor, and is now known to be secreted by placental trophoblast into the maternal circulation (Keelan *et al.*, 1997). Maternal plasma CRH levels rise exponentially during pregnancy and elevated plasma CRH levels have been associated with preterm labour (Kurki *et al.*, 1991). The bioavailability of circulating CRH is known to be influenced by the CRH binding protein (CRH-BP) which binds to the hormone in an equimolar ratio and prevents its recognition at the CRH receptor. CRH-BP is present in the maternal circulation in concentrations that will block the bioactivity of CRH until the final three weeks before the onset of spontaneous labour (McLean *et*

al., 1995). At this time the continuing rise in plasma CRH concentrations is accompanied by an abrupt fall in CRH-BP concentrations in the maternal circulation and amniotic fluid (Florio *et al.*, 1997). CRH receptors are present in the myometrium (Hillhouse *et al.*, 1993) and fetal membranes (Petraglia *et al.*, 1990), and CRH stimulates the release of prostaglandins from human amnion and decidua *in vitro* (Jones and Challis, 1989) and has been reported to potentiate the action of oxytocin and prostglandin $F_{2\alpha}$ in stimulating myometrial contraction *in vitro* (Benedetto *et al.*, 1994) and *in vivo* (McLean *et al.*, 1994). CRH is present in the amniotic fluid and fetal circulation where it is capable of stimulating the fetal pituitary-adrenal axis to increase fetal adrenal glucocorticoid secretion, promoting fetal organ maturation. Further, CRH induces the synthesis of prostaglandins and glucocorticoids, which in turn stimulate further placental CRH secretion, creating positive feedback loops in the maternal, fetal and amniotic compartments, which may drive the onset of labour (Challis and Hooper, 1989).

Inflammatory mediators are recognised to play a crucial role in uterine contractility, cervical ripening and activation of the fetal membranes, (Kelly 1996). Inflammatory cells are known to infiltrate the cervical stroma, placenta, maternal decidua and the fetal membranes during parturition, and may play a role in spontaneous rupture of the membranes (Halgunset *et al.*, 1994, Rosenberg *et al.*, 1996). There are, however, no reports of inflammatory cells infiltrating the myometrium during parturition. In Chapter 5 of this thesis, the results of a study to determine whether inflammatory cells infiltrate the uterine myometrium, are reported.

Part II.

The Physiology of Uterine Contractility

The uterus is spontaneously active, and using electromyographic measurements, contractile activity can be detected in both pregnant and non-pregnant women (Morrison, 1996). Two different types of electromyographic activity have been described in the uterine myometrium of the pregnant rhesus monkey, referred to as contractures and contractions, and are believed to be present in most species (Nathanielsz *et al.*, 1992). Contractures represent low amplitude, long-acting uterine activity which commence early in pregnancy; in women these were first observed by Braxton Hicks in 1873, and are still known by his name. Contractions are high amplitude and of short duration, and are characterized by nocturnal or labour uterine activity (Nathanielsz *et al.*, 1992). The level of activity in the uterus throughout pregnancy is regarded as relatively low, compared with that measured both during labour and the immediate puerperium, when strong contractions occur to expel the fetus and placenta, and then maintain haemostasis.

Organisation of myometrial smooth muscle

The myometrium is composed of interlacing bundles of long, spindle-shaped smooth muscle fibres arranged in ill-defined layers. The myometrial smooth muscle cells are surrounded by extracellular material composed of collagen fibres, fibroblasts and bone marrow derived cells (Garfield and Yallampalli, 1994). During pregnancy, the myometrium grows dramatically due to hypertrophy and an increase in the number of smooth muscle cells by division, (Carsten, 1968). The myometrial cells communicate with one another through intercellular channels called gap junctions, which were first observed in myometrial cells in rats just prior to and during parturition (Garfield *et al.*, 1977) . Gap junctions consist of channels which connect the interiors of two cells.

The channels are composed of proteins, termed connexins, which span the plasma membranes to form a pore. They provide a low-resistance pathway between individual myometrial smooth muscle cells and allow the passage of inorganic ions and small molecules (Cole *et al.*, 1988). The number of gap junctions increases in the myometrium at the time of labour in most if not all species. They are under hormonal control, with progesterone inhibiting, and oestradiol stimulating their formation. In addition prostaglandins regulate gap junction formation and function (Garfield, 1994).

Unlike striated muscle, the actin and myosin filaments of myometrium are not organised into fibres and fibrils, but instead occur in random bundles throughout the myocyte (Somlyo, 1980; Garfield, 1984). However, like skeletal muscle, myometrial contractions occur from a sliding of actin and myosin filaments, without any change in the length of either filament (Huxley, 1971). A rise in intracellular calcium (Ca^{2+}) promotes the binding of calcium to calmodulin. Calcium-calmodulin activates myosin light chain kinase (MLCK) which itself phosphorylates myosin. The phosphorylated myosin filaments bind to actin, and contraction occurs with the hydrolysis of ATP.

Electrical activity

The myometrial smooth muscle cells are spontaneously active, such that isolated strips of pregnant or non-pregnant uterus will produce regular spontaneous contractions. These spontaneous contractions are preceeded by action potentials. Myometrial cells have a negative resting membrane potential of approximately -50 mV (Inoue *et al.*, 1990). Changes in the myometrial membrane potential occurring from ion flow across the membrane, are fundamental to the control of uterine activity, (Wray, 1993). When the magnitude of this potential is reduced beyond a certain threshold, an action potential may be stimulated. The basis of this myogenic mechanism is the spontaneous depolarisation of pacemaker cells within the myometrium. Unlike cardiac muscle, the

pacemaker cells in the myometrium are not anatomically fixed or defined and it is unclear why some cells or groups of cells should become pacemakers.

Calcium and uterine contractions

An increase in intracellular calcium is essential for uterine contractility. In the resting state, intracellular calcium is maintained at a concentration of about 10^{-7} M which increases to 10^{-6} M during contraction, (Thornton and Gillespie, 1992). The extracellular calcium concentration is 10^{-3} M. Intracellular Ca^{2+} can be increased by several mechanisms, and different agents are thought to operate via different routes. Voltage-dependent and receptor-operated calcium channels are two important mechanisms (Hurwitz, 1986). When the membrane is depolarised to an appropriate level, voltage-dependent calcium channels convert to an activated or open state, thus allowing a substantial calcium influx into the cell. Receptor-operated calcium channels are opened in response to activating ligands, such as hormones or neurotransmitters, which bind to specific receptors associated with the channel. Calcium may be released into the cytoplasm from stores within the cell. The sarcoplasmic reticulum represents the major intracellular store for calcium and in the myometrium it is well developed, though less well so than in skeletal and cardiac muscles (Garfield and Somlyo, 1985; Somlyo *et al.*, 1985). Inositol 1,4,5-trisphosphate (Carsten and Miller, 1985) or an increase in intracellular calcium itself, can stimulate the release of calcium from the sarcoplasmic reticulum.

Relaxation occurs with the dephosphorylation of myosin light chains by a phosphatase. Further, cell repolarisation results in a lowering of intracellular calcium by extrusion across the sarcolemma and uptake into the internal stores, thus activating the calcium-calmodulin MLCK complex.

Control of myometrial function

(i) Neuronal modulation

It has been recognised for more than a century that the uterus receives an extensive autonomic innervation (Krantz, 1959), yet the contribution of neuronal modulation of uterine activity has been less well characterised than the hormonal factors involved. Myometrial smooth muscle cells possess all four main types of adrenergic receptors, (α_1, α_2, β_1 and β_2), (Bottari *et al.*, 1985). It has been postulated that α_1-receptors mediate contraction and β_2-receptors are predominantly responsible for relaxation (Bulbring and Tomito, 1987). The α_2-receptors seem to have no contractile action (Hoffman *et al.*, 1981). Both β_1- and β_2-receptors are coupled to adenylate cyclase via an intermediate G-protein (G_s). Activation of adenylate cyclase results in an increase in cAMP which activates cAMP-dependent kinase. This in turn leads to a reduction in phosphorylation of myosin light chains and relaxation. Other mechanisms whereby cAMP may result in relaxation include calcium sequestration and efflux of calcium through the cell membrane (Diamond, 1990; Thornton and Gillespie, 1992). The α-adrenoceptor is coupled to adenylate cyclase by an inhibitory G-protein (G_i). This G_i may directly reduce adenylate cyclase activity or may prevent stimulation of the enzyme by G_s.

Cholinergic stimulation causes uterine contractions by increasing inositol 1,4,5-trisphosphate and hence elevating intracellular calcium, (Marc *et al.*, 1986). However, it is well established that contractions and expulsion of the fetus can proceed in the absence of neuronal activity (Reynolds, 1965) suggesting that the role of these nerves in the contractile activity of the uterus is of minor importance (Wray, 1993). Other neurotransmitters which are known to be present within the uterus include, vasoactive intestinal polypeptide (Alm *et al.*, 1977; Huang *et al.*, 1984), neuropeptide Y (Owman *et al.*, 1986), substance P (Alm *et al.*, 1978), calcitonon gene-related peptide (Gibbins

et al., 1985; Samuelson *et al.*, 1985), galanin, and gastrin-releasing peptide (Stjernquist *et al.*, 1986).

(ii) Hormonal modulation

Oestrogen and progesterone

Changes in hormonal conditions have profound effects on uterine activity. The steroid hormones oestrogen and progesterone have long been known to influence myometrial contractility, (Bozler, 1941; Fuchs, 1978). Human pregnancy is characterised by a state of markedly increased oestrogen levels, predominantly 17β-oestradiol and oestriol. Although oestrogens are known to stimulate prostaglandin production in the decidua, promote the formation of gap junctions and increase the synthesis of oxytocin receptors, their role in the initiation of parturition in the human remains uncertain, (Batra, 1994).

Progesterone is essential in the maintenance of pregnancy (Csapo, 1956), and has traditionally been perceived as having a "relaxing" effect on human myometrium. The proposed mechanisms for this have included diminished myometrial cell membrane permeability for calcium, increased adenosine monophosphate synthesis and an inhibition of decidual prostaglandin formation, (Egarter and Husslein, 1992). Furthermore, treatment with an anti-progesterone such as mifepristone, increases both uterine activity and myometrial sensitivity to prostaglandins (Swahn and Bygdeman, 1988). However, the role of progesterone in the onset of human parturition is unknown, since a fall in plasma progesterone is not seen in human pregnancy (Batra, 1985). In addition, administration of progesterone to strips of isolated human, pregnant myometrium *in vitro*, increases the frequency and strength of contractions, and enhances sensitivity to oxytocin (Fu *et al.*, 1993).

Oxytocin

Oxytocin is a powerful stimulator of uterine activity and can enhance the force, the frequency and the duration of uterine contractions. It is a nine amino acid peptide which is synthesised within the supraoptic and paraventricular nuclei of the hypothalamus as a large precursor molecule (Dawood and Khan-Dawood, 1985). This molecule is subsequently broken down to the active hormone and its neurophysin which are transported along neurones to the posterior pituitary gland. In 1909, Blair-Bell demonstrated the uterotonic action of posterior pituitary extracts at a Caesarean section. Since then, the pituitary extracts, and later purified oxytocin, have been used to arrest postpartum haemorrhage and to induce or augment labour. Whilst oxytocin has classically been described as being released from the posterior lobe of the pituitary gland, it is also expressed in amnion, chorion and decidua (Chibbar *et al.,* 1993).

Oxytocin stimulates uterine contraction by mechanisms involving activation of receptor-operated calcium channels and release of calcium from sarcoplasmic reticulum. Calcium-independent oxytocin contraction has been described (Matsuo *et al.*, 1989) and is probably due to a protein phosphorylating either a contractile or cytosolic protein, (Oishi *et al.*, 1991). The importance and role of oxytocin in the initiation of human parturition remains controversial (see Zeeman *et al.*, 1997 for review). There is a marked gestational difference in the pharmacological ability of oxytocin to cause uterine contractions, oxytocin being most effective in the third trimester. This is due to the presence of a higher concentration of oxytocin receptors at this time and is the basis of human uterine sensitivity to oxytocin throughout pregnancy (Theobald *et al.*, 1969; Fuchs *et al.*, 1984). Fetal oxytocin and local uterine and decidual sources of oxytocin can act on the myometrium in an endocrine and paracrine manner to initiate and maintain effective uterine contractions (Chard *et al.*, 1971). Furthermore oxytocin is now recognised to be released in discrete pulses of short duration and that pulse frequency and duration increase during spontaneous labour in women (Fuchs *et al.*, 1991).

Oxytocin antagonists are currently being investigated as tocolytic agents in the management of preterm labour, (Zeeman *et al.*, 1997).

Prostaglandins

The prostaglandins PGE_2 and $PGF_{2\alpha}$ increase uterine contractile activity in association with an rise in intracellular free calcium, which seems to be due primarily to an influx of extracellular calcium (Morrison and Smith, 1994). In contrast to oxytocin, prostaglandins do not increase inositol 1,4,5-trisphosphate production in human myometrium or release intracellular calcium stores in cultured myometrial cells (Thornton and Gillespie, 1992). There is strong evidence that prostaglandins play a central role in the initiation of human parturition, although the evidence for a regulatory role of eicosanoids is unavailable (see Schellenberg and Liggins, 1994 for review).

Endothelins

Endothelin (ET), a potent vasoconstrictor, is capable of contracting several non-vascular smooth muscles including non-pregnant and pregnant rat myometrium (Calixto and Rae, 1991) and human myometrium *in vitro* (Word *et al.*, 1990). The increase in smooth muscle force appears to be related to a dose-dependent rise in intracellular free calcium due to its release from stores and increased influx, producing increased myosin phosphorylation (Word *et al.*, 1990). ET_A endothelin receptors and a smaller proportion of ET_B endothelin receptors have been identified in human myometrium (Bacon *et al.*, 1995). Whilst ET_A is known to mediate myometrial contractility, ET_B receptors might be involved in myometrial relaxation (Wolff *et al.*, 1996).

(ii) Metabolic modulation

A variety of metabolic factors are thought to play a crucial role in the control of myometrial contractility. These include myometrial blood flow, uterine phosphorus metabolites, hypoxia, pH and stretch, and have been reviewed by Wray (1993) and Morrison (1996).

Part III.

The Physiology of Cervical Ripening

Throughout normal pregnancy, the cervix must remain closed and non-compliant to maintain the conceptus within the uterine cavity. With the onset of cervical ripening, it is converted into a compliant and easily dilating structure which allows the uterine contractions to effect the transport of the fetus through the birth canal. Our understanding of the physiological mechanisms involved in cervical ripening is far from complete. Interest in this process has mainly been concerned with the development of pharmacological agents to ripen the cervix in order to facilitate induction of labour. In contrast, research on preterm labour and therapeutic strategies aimed at preventing it have concentrated on abolishing the associated uterine contractions whilst largely ignoring the cervix, (Olah and Gee, 1992). This interest in tocolytic agents has arisen since the presence of uterine contractions is the most obvious manifestation of preterm labour. Yet it is clear that the process of cervical ripening is likely to begin prior to the onset of myometrial activity, as in normal labour at term, or at least concurrently. Hence a better understanding of the processes involved in cervical ripening may lead to new therapeutic strategies in the prevention of preterm delivery.

Structure of the cervix

The main formed element of the cervical stroma is extracellular connective tissue matrix. The extracellular matrix is made up of type I (66%) and type III (33%) collagen, (Kleissl *et al.*, 1978) with a small amount of type IV collagen in the basement membranes. The fibrils of collagen are bound together into dense bundles which confer on the cervix the rigidity which characterises its non-pregnant and early pregnant condition. A small amount of elastin is also present within the cervix. While the

collagen confers rigidity, elastin may be responsible for providing elasticity; this assists in closing the cervix after delivery and thereafter returning it to its non-pregnant shape.

The collagen is embedded in a ground substance consisting of large molecular weight proteoglycan complexes containing a variety of glycosaminoglycans (GAGs). Glycosaminoglycans are long chains of highly negatively charged repeating disaccharides containing one hexosamine (glucosamine or galactosamine) and one uronic acid (glucuronic or iduronic). There are several different GAGs, such as heparin and heparan sulphate and dermatan and chondroitin sulphate. These vary in their composition with regard to the exact combination of hexosamine and uronic acid residues, and each varies intrinsically with regard to chain length. In cervical tissue the most abundant GAGs are chondroitin and its epimer, dermatan sulphate, (von Maillot *et al.*, 1979; Uldbjerg *et al.*, 1983). As well as forming the ground substance of the tissue, proteoglycans invest collagen fibrils (Scott and Orford, 1981), with their protein cores attaching to the collagen. The relationship between the GAG side chains and the collagen fibrils is important in orientating the collagen and conferring on the cervix its mechanical strength (Lindahl and Hook, 1978). The binding affinity of GAGs to collagen increases with increasing chain length and charge density. Hyaluronic acid binds least strongly of the GAG molecules and will act to destabilise the collagen fibrils. GAGs such as dermatan sulphate, containing iduronic as opposed to glucuronic acid, bind strongly and promote tissue stability (Obrink, 1973). Changes in the proteoglycans/GAG composition can therefore alter collagen binding and facilitate collagen breakdown.

The major cellular component of cervical connective tissue is the fibroblast. These cells appear to be responsible for the synthesis of both collagen and ground substance. Whilst the bulk of the cervix consists of fibrous tissue, there is a varying amount of smooth muscle - usually about 10%, but it can vary between 2% and 40%. The functional role of this smooth muscle is controversial, although it is capable of both

spontaneous and drug induced contractions (Nixon, 1951, Hillier and Karim, 1970). It seems that the connective tissue in the cervix is more important than the muscle component. The muscle is unlikely to act as a sphincter mechanism, both on the basis of its low concentration and also its spatial arrangement within the cervix. It is proposed that it protects important blood vessels during labour and brings about prompt closure of the cervix following delivery (Calder, 1994).

The cervix during pregnancy

During pregnancy the cervix becomes metabolically more active. In the non-pregnant state, the cervix consists of around 80% water (Liggins, 1978) and this increases to around 86% in late pregnancy (Uldbjerg *et al.*, 1983). This water interacts with the matrix proteins and facilitates the function of elastin. Since GAGs are hydrophilic, these molecules may be important in controlling tissue hydration, with increased hydration destabilising the collagen fibrils and promoting ripening. There does not seem to be a change in cervical water content immediately before or after delivery in humans (Leppert, 1992). Whilst messenger RNA for tropoelastin, the precursor for elastin, is increased in pregnancy, the cervical elastin content does not appear to change throughout gestation (Leppert, 1992). The smooth muscle cells of the cervix become enlarged and prominent during pregnancy. Smooth muscle enlargement may play a role in cervical tissue rearrangement as collagen bundles are aligned in close approximation to the smooth muscle bundles.

The collagen content of the cervix, both type I and type III, undergoes marked changes in pregnancy. The spaces between the collagen bundles become dilated as early as 8-14 weeks' gestation. Although there is an increase in the total collagen content of the cervix at term, the collagen concentration is reduced by 30-50% compared to the non-pregnant cervix (Fosang *et al.*, 1984; Kokenyesi and Woessner, 1990; Jeffrey, 1991). The cervical collagen concentration measured biochemically also decreases (Danforth *et*

al., 1974; Uldbjerg *et al.*, 1983; Granstrom *et al.*, 1989). This arises because other components of the cervix, the water and non-collagen proteins are increasing in relatively greater amounts. In addition, the collagen fibrils are reduced in size (Danforth *et al.*, 1960). This decline in cervical collagen appears to be even more marked when studied histologically using stains specific for polymerised collagen. A much lower proportion of the collagen exists as intact fibres in the dilated cervix at term (Obrink, 1973). Several mechanisms have been proposed to explain these changes in collagen composition; essentially, these are increased enzymatic collagen degradation and/or alteration in the proteoglycan/GAG composition of the ground substance.

Collagen is amenable to breakdown by the action of lytic enzymes. These include collagenases, (MMP-1, MMP-8 and MMP-13) which are produced by fibroblasts and leukocytes, and leukocyte elastase produced by macrophages, neutrophils and eosinophils. Collagenase is secreted in a latent form, procollagenase, which is activated by cleavage of the proenzyme by plasmin or stromelysin (MMP-3) to the active form. The activated collagenase specifically breaks down the triple helix of the collagen fibril by hydrolysing peptide bonds (Wooley, 1984; Stricklin and Hibbs, 1988). Radiolabel studies suggest that the cells critically involved in collagen degradation during cervical dilatation are not the cervical fibroblasts, but rather neutrophils migrating from blood vessels. The neutrophils secrete both elastase and collagenase (MMP-8) (Osmers *et al.*, 1993). Elastase breaks down collagen by acting on the telopeptide non-helical domains. Elastase can degrade not only elastin and collagen, but also proteoglycans, and it may act synergistically with collagenase on collagen. As the cervical collagen content decreases through pregnancy, the leukocyte elastase and collagenase activities increase (Uldbjerg *et al.*, 1983). In addition, the amount of soluble collagen (reflecting partly degraded collagen) in the tissue increases in parallel with the increased enzyme activities (Ito *et al.*, 1979; Uldbjerg *et al.*, 1983). Mature collagen with many cross links may be broken down during pregnancy and replaced with new collagen which has fewer cross links and is more amenable to rapid breakdown at the time of parturition.

As the pregnancy advances, collagen is more easily extracted from cervical tissues, with the immature cross links in newly synthesized collagen contributing to this phenomenon.

In addition to increased collagen breakdown, there are changes in the cervical proteoglycan and GAG content as pregnancy advances. The total GAG content of the cervix increases substantially by term, indicating active synthesis although the concentrations of GAGs may remain relatively constant (Golichowski, 1980). Whilst some studies have shown an increase in the hyaluronic acid concentration in pregnancy, (von Maillot *et al.*, 1979; Golichowski, 1980), other studies have found no increase (Uldbjerg *et al.*, 1983; Fosang *et al.*, 1984; Uldbjerg and Malmstrom, 1991). Although this could reflect variation between species, these discrepancies could also be attributed to variations in the site of the cervical biopsies used in each study (Leppert, 1995). Studies employing more recently developed biochemical techniques have demonstrated that the concentration of hyaluronic acid increases almost 12-fold at 2-3 cm dilatation (Leppert, 1992). This GAG may help to "loosen" the collagenous network of the cervix whilst an increase in the hyaluronic acid available to bind water may be associated with an increase in tissue hydration, and tissue deformability. There may be a relative decrease in chondroitin and dermatan sulphate, compared to the non-pregnant cervix. It has been proposed that tissues become more rigid with increasing chondroitin sulphate concentration. Thus, a reduction in the chondroitin sulphate concentration might result in increased compliance and is likely to reduce the mechanical strength of collagen fibrils, and make them more amenable to breakdown by proteolytic enzymes.

The predominant proteoglycan in the non-pregnant, human cervix is dermatan sulphate proteoglycan II (decorin or DSPG II), a small proteoglycan with one dermatan sulphate side chain (Uldbjerg *et al.*, 1983). In addition, there are small amounts of two other proteoglycans, one with two dermatan sulphate side chains, biglycan (DSPG I) and a larger one with chondroitin /dermatan sulphate side chains called PGL (Norman *et al.*,

1991). It is proposed that the amount of decorin increases in the pregnant cervix (Leppert, 1995), until late pregnancy when the proteoglycan concentration decreases to about 50% of that in the non-pregnant cervix. After the onset of cervical ripening, the amount of decorin still dominates in the cervix, but there is an increase in the other proteoglycans (Norman *et al.*, 1993). Such an alteration in the proteoglycan and collagen composition of the ground substance might explain the altered biomechanical properties of the cervix (Greer, 1992). In the rat, a strong correlation exists between the cervical linear circumference and the decorin:collagen ratio (Kokenyesi and Woessner, 1990) supporting this contention.

The increase in total GAGs probably reflects an increased production by fibroblasts which become increasingly active as pregnancy advances (Junqueira *et al.*, 1980; Parry and Ellwood, 1981). Additionally, the increase could reflect breakdown of the proteoglycan complexes to provide free hyaluronic acid and proteoglycans. The proteases required for this could come from the activated fibroblasts, or the leukocytes which infiltrate the cervical connective tissue.

Cervical ripening

The phenomenon of cervical ripening is a prelude to the onset of labour and is most obvious during the last five or six weeks of pregnancy although it may have its origins even earlier. Clinically, ripening refers to the increased softening, distensibility, effacement and early dilatation which can be detected by pelvic examination. These changes are the result of profound alterations in the biomechanical properties of cervical tissue and include a reduction in collagen concentration, an increase in water content and a change in proteoglycan/GAG composition (Calder and Greer, 1992). One important change involved in cervical ripening is a rearrangement and realignment of collagen (Leppert, 1995). The cervical connective tissue at term shows widely scattered and dissociated fibrils of collagen and a marked increase in the ground substance when

compared to the non-pregnant or early pregnant cervix. The process of cervical ripening has been compared to an inflammatory reaction (Liggins, 1981) and infiltration of cervical tissue with inflammatory cells has been shown in experimental circumstances (Junquiera *et al.*, 1980; Rath *et al.*, 1988; Chwalisz, 1988).

The control of cervical ripening

Cervical ripening appears to be an active process in view of the changes which occur within the cervical connective tissue and cellular components. Furthermore, animal studies have shown that cervical ripening occurs even when the cervix is physically isolated from the uterus (Stys *et al.*, 1980; Ledger *et al.*, 1985). Those factors controlling cervical ripening, and which may be implicated in the pathophysiology of preterm labour, are incompletely understood.

Prostaglandins

Prostaglandins undoubtedly play a role in the control of cervical ripening in the human. The main prostaglandins produced by the cervix are PGE_2, PGI_2, and to a lesser extent $PGF_{2\alpha}$ and their production increases in association with cervical ripening (Ellwood *et al.*, 1980). Physiologically, PGE_2 is probably much more important than $PGF_{2\alpha}$, while the role of PGI_2 in cervical ripening is rather uncertain. Amniotic fluid concentrations of PGE_2 and $PGF_{2\alpha}$ correlate directly with the cervical score in women at term who are not in labour (Calder, 1980). In addition, receptors for PGE_2 and $PGF_{2\alpha}$ can be demonstrated in the cervix (Crankshaw *et al.*, 1979). These data suggest that prostaglandins have a physiological role in cervical ripening; there is no doubt that natural and synthetic prostaglandins are effective pharmacological agents for ripening the cervix at any stage in pregnancy (Calder, 1980; Calder and Greer, 1991). Prostaglandins might effect cervical ripening by inducing the breakdown of collagen. Alternatively, they could modify the binding of collagen and the hydration of tissue by altering the GAG/proteoglycan composition. Prostaglandin E_2 treatment will reduce

collagen concentration similar to the changes seen in physiological ripening (Ekman *et al.*, 1986; Uldbjerg *et al.*, 1981), but it is uncertain whether this is the result of collagenolysis. While some studies have shown an increase in collagenase activity following the administration of PGE_2, (Szalay *et al.*, 1989; Ding *et al.*, 1990), others have shown no such change (Ellwood *et al.*, 1981; Uldbjerg *et al.*, 1983; Rath *et al.*, 1987). While these conflicting results may, to some extent, reflect difficulties in assessing collagenase activity, Rath *et al.*, (1987) supported their findings by demonstrating an absence of collagen breakdown fragments on electrophoresis following prostaglandin therapy. In this study significant cervical ripening occurred in the treated group.

There is evidence that prostaglandins act by altering the ground substance in cervical tissue (Uldbjerg *et al.*, 1981; Uldbjerg *et al.*, 1983). An increase in hydration and hyaluronic acid concentration has been demonstrated in animal studies, after PGE_2 administration (Cabrol *et al.*, 1987). Prostaglandin E_2 can influence cervical fibroblast production of collagen and GAG. The production of these two substances is inversely related such that an increase in GAG production occurs when collagen synthesis is reduced (Norstrom, 1984; Norstrom *et al.*, 1985); Johnston *et al.*, (1993) have shown that PGE_2 administration in late pregnancy results in an increase in circulating levels of chondroitin sulphate, similar to those seen in spontaneous labour. Thus, PGE_2 mediated cervical ripening may be explained by alterations in GAG/proteoglycan content which will disperse and destabilise the collagen fibrils thereby increasing tissue compliance.

Oestrogens

Other naturally occurring agents also act to control cervical structural changes. Oestrogens, such as oestradiol have been used to bring about cervical ripening in the clinical situation (Gordon and Calder, 1977; Allen *et al.*, 1989; Magann *et al.*, 1995). The mechanism underlying the effects of oestradiol may be due in part to the induction

of prostaglandin synthesis within the tissues (Horton and Poyser, 1976). Oestradiol might also be responsible for the influx of protease-producing leukocytes which could induce ripening (Calder and Greer, 1992).

Progesterone & Antiprogesterones

Progesterone inhibits the effects of collagenase within the uterine corpus (Jeffrey *et al.*, 1971), and may have a similar role within the cervix. It has an inhibitory effect on cervical ripening and parturition in those animals where a decrease in progesterone at term results in ripening and labour. Such a decrease does not occur in the human but progesterone is a potent anti-inflammatory agent (Sitteri *et al.*, 1977), and could still be an important physiological inhibitor of the ripening process *in vivo* by inhibiting neutrophil influx and activation (Jeffrey and Koob, 1980). This is supported by the observation of the cervical softening effect of the antiprogesterones prior to termination of pregnancy (Gupta and Johnson, 1990; Radestad *et al.*, 1990), and which is associated with a neutrophil influx in animal models (Chwalisz, 1988). In addition, antiprogesterones might exert their effects through prostaglandins as they appear to stimulate prostaglandin synthesis and reduce catabolism *in vitro* (Kelly *et al.*, 1986; Kelly and Bukman, 1990). Morphological and biochemical studies in humans and animals, have shown evidence of collagenolysis in the cervix after treatment with antiprogesterones (Hegele-Hartung *et al.*, 1989; Radestad *et al.*, 1993). Other workers, however, have failed to show any change in cervical collagen content (Norman, 1992; Bokstrom and Norstrom, 1995).

Relaxin

There is good theoretical evidence that relaxin, a 6-kD dimeric peptide hormone, plays a role in the process of cervical ripening in the human. Relaxin increases collagenase activity (von Maillot *et al.*, 1977) perhaps via a mitogenic effect on fibroblasts which are known to exhibit relaxin receptors (McMurty *et al.*, 1980). Pharmacologically, porcine relaxin has been shown to cause cervical ripening in women (MacLennan *et al.*,

1980; Evans *et al.*, 1983). More recently, the efficacy of recombinant human relaxin, administered vaginally, has been investigated; while no adverse effects could be attributed to the preparation, recombinant human relaxin had no appreciable effects on cervical ripening (Bell *et al.*, 1993; Brennand *et al.*, 1997). While the specific role of relaxin during human pregnancy is unknown, increased relaxin concentrations in the maternal circulation are associated with preterm labour, perhaps by altering cervical connective tissue (Petersen *et al.*, 1992; Weiss *et al.*, 1993).

Inflammatory Mediators

Cervical ripening is considered to be a physiological inflammatory process, characterised by an accumulation of neutrophils and macrophages in the cervical stroma (Junquiera *et al.*, 1980; Liggins, 1981; Bokstrom et al., 1997). Interleukin-8 is an inflammatory cytokine which is capable of producing a selective neutrophil chemotaxis and activation (Baggiolini *et al.*, 1989). This cytokine can be produced by fibroblasts in the human cervix (Barclay *et al.*, 1993), and can induce cervical ripening in nonpregnant and pregnant rabbits (El Maradny *et al.*, 1994). Interleukin-8 may have a synergistic interaction with prostaglandin E_2 in promoting cervical ripening (Colditz, 1990). Other cytokines, including interleukin-1ß (El Maradny *et al.*, 1995) and tumour necrosis factor α (Chwalisz *et al.*, 1994a) have been shown to produce cervical ripening in animal studies. Recent work has indicated that the inflammatory mediator nitric oxide plays a crucial role in cervical ripening in animals (Buhimschi *et al.*, 1996; Chwalisz *et al.*, 1997). The role of nitric oxide in cervical ripening in women will be discussed later in this chapter, and also in chapters 6 and 7 of this thesis.

Apoptosis

Cervical ripening occurs spontaneously in a timely, species specific manner suggesting that apoptosis, or programmed cell death, may be involved (Leppert, 1995). Apoptosis is a phenomenon characterised by the shrinkage of cells, compaction of chromatin into uniformly dense bundles and clear halo nuclei. One study in pregnant rats showed that,

as gestation advanced, the numbers of dying smooth muscle cells in the cervix increased along with DNA degradation fragments and cervical softening, (Leppert and Yu, 1994).

As the processes involved in cervical ripening at term become clearer, then therapeutic strategies aimed at preventing this process occurring prematurely will become available. While the list of potential mediators of cervical ripening continues to grow, there are few reports of inhibitory substances for cervical maturation in vivo. Studies have shown that fetal urine and amniotic fluid contain large amounts of a substance called urinary trypsin inhibitor (UTI), (El Maradny *et al.*, 1994). UTI, which has been localised in myometrial and cervical cells during pregnancy (Kanayama, 1994) has an inhibitory effect on several enzymes including elastase, hyaluronidase and plasmin (Kosuzume *et al.*, 1983). It also inhibits many of the cytokines including interleukin-1 and interleukin-8, and one study in rabbits has shown that UTI suppresses premature cervical ripening (Kanayama *et al.*, 1995). Moreover, UTI has been reported to inhibit uterine contractions in patients in preterm labour (Kanayama *et al.*, 1992). Similarly, interleukin-10 or cytokine synthesis inhibitory factor is a potent natural attenuator of inflammatory cytokine reactions. These substances may have a therapeutic role in suppressing premature cervical ripening when an inflammatory aetiology is implicated (Keirse, 1995).

Part IV.

Nitric oxide

Nitric oxide has only recently been identified as a crucial biological mediator, involved in such diverse activities as smooth muscle relaxation, neurotransmission and host defence (Nathan, 1992). In 1987, Moncada and his colleagues showed that nitric oxide accounted for the biological activity of endothelium-derived relaxing factor and that L-arginine was its precursor (Palmer *et al.*, 1987, 1988). Only then did it become apparent that the therapeutic effects of glyceryl trinitrate, which has been employed in the management of angina pectoris for over one hundred years, are derived by the liberation of nitric oxide in the vascular endothelium (Feelisch, 1991).

Nitric oxide is a reactive gas with a very short physiological half life. It is synthesised from L-arginine by the enzyme nitric oxide synthase, of which three isoforms have been identified at present. These enzymes have common features with cytochrome P450 reductase and contain oxidative and reductive domains. The three isoforms of nitric oxide synthase (NOS) are known as endothelial NOS, (eNOS), neuronal or brain NOS, (bNOS) and macrophage or inducible NOS, (iNOS), and the genes for each of these enzymes has been localised to chromosome 7, 12 and 17 respectively. There is significant homology between the amino acid sequences of NOS from different species and between each of the different isoforms. Whilst the isoforms of NOS are named after the cell type in which they were first identified, it is now recognised that the three isoforms are widely distributed in a variety of tissues. bNOS was originally purified and cloned from neuronal tissues but is now known to have an important level of expression in skeletal muscle. iNOS was originally purified and cloned from an immunoactivated macrophage cell line, and has since been identified in neutrophils, mast cells, endothelial cells, vascular smooth muscle, cardiac muscle and hepatocytes. eNOS, the last of the three mammalian NOS isoforms to be isolated, was originally

purified and cloned from vascular endothelium, but has since been identified in cardiac myocytes, blood platelets, brain and elsewhere (Michel and Feron, 1997).

For all three NOS isoforms, nitric oxide synthesis depends upon the enzyme's binding of the calcium regulatory protein, calmodulin. For eNOS and bNOS, increases in intracellular calcium concentrations are required for their binding calmodulin, and consequently for their becoming fully activated. By contrast, iNOS appears able to bind calmodulin with extremely high affinity, even at the low intracellular concentrations characteristic of resting cells. Thus, in contrast to iNOS, the activity of eNOS and bNOS may be closely regulated by changes in intracellular calcium. eNOS and bNOS are constitutively expressed by cells although their expression can be induced or up-regulated. When cells containing constitutive NOS, that is eNOS and bNOS, (cNOS) are stimulated by for example, acetylcholine, bradykinin, glutamate, thrombin, ADP, physical pressure and shear stress, activation leads to an increase in cytosolic calcium, which activates the cNOS to produce nitric oxide. In general, expression of iNOS occurs after activation of cells with stimuli, including bacterial endotoxin or exotoxin, and inflammatory mediators, including the cytokines tumour necrosis factor and interleukin-1. However, iNOS may also function as a "constitutive" enzyme under physiological conditions in some cells (Guo *et al.*, 1995). iNOS produces larger amounts of nitric oxide than the other NOS isoforms (Anggard 1994).

The initial step in nitric oxide biosynthesis is hydroxylation of the nitrogen in the guanidino group of L-arginine (figure 1.1). This step incorporates molecular oxygen into nitric oxide and citrulline and requires cofactors including reduced pyridine nucleotides, reduced biopteridines and calmodulin. Whilst levels of L-arginine are normally sufficient for continuous secretory nitric oxide biosynthesis, under certain conditions the local cellular L-arginine levels may be insufficient (Drexler *et al.*, 1991). Furthermore, the limited availability of cofactors *in vivo* can attenuate the production of nitric oxide (Werner-Felmayer *et al.*, 1993; Sladek and Roberts, 1996).

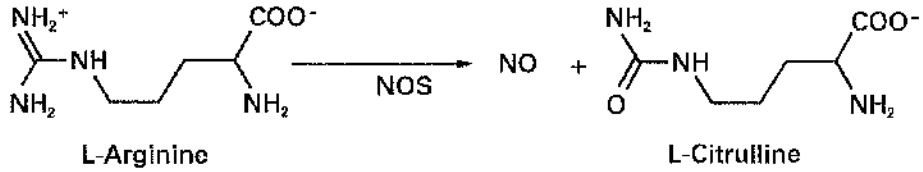

Figure 1.1 The generation of nitric oxide. Nitric oxide is derived from L-arginine by the action of nitric oxide synthase (NOS).

Nitric oxide is a free radical having an unpaired electron, and has a very short physiological half life of a few seconds since it readily combines with other free radicals (Beckman and Crow, 1993). Nitric oxide is rapidly converted in biological systems to nitrate and nitrite, a reaction that is catalysed by transition metals including iron. Haemoglobin inactivates nitric oxide by binding it to form nitrosohaemoglobin and by catalysing the degradation of nitric oxide to nitrate and nitrite, resulting in the formation of methaemoglobin. The very short half life of nitric oxide and its reactivity mean that it is most likely to act as a local messenger transferring messages within and between individual cells.

Other biological mediators can interact with nitric oxide to alter its effects. The biological actions of nitric oxide are abrogated via direct reaction with superoxide anion (McCall *et al.*, 1989; Huie and Padmaja, 1993). In the presence of superoxide anions, nitric oxide reacts to form peroxynitrite which in turn is oxidised to nitrate. Superoxide dismutase, which converts superoxide to hydrogen peroxide and oxygen (Klug-Roth *et al.*, 1973) prolongs the biological half-life of nitric oxide by removing superoxide. In contrast, xanthine and xanthine oxidase synthesise superoxide anion thereby attenuating the activity of nitric oxide (Miyamoyo *et al.*, 1996).

Nitrovasodilators, such as glyceryl trinitrate and sodium nitroprusside (SNP) are now known to produce their clinical effects by releasing nitric oxide. These drugs, known as nitric oxide donors, release nitric oxide either spontaneously, (eg SNP) or after metabolic conversion, (eg glyceryl trinitrate). Endogenous nitric oxide levels can be raised by administration of the nitric oxide substrate L-arginine. Analogues of L-arginine, such as N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA), attenuate the effects of NOS by competing with L-arginine for the active site of the NOS (Furchgott *et al.*, 1990). In experimental systems, nitric oxide may be inhibited by the addition of oxyhaemoglobin, or the effects of nitric oxide on the second messenger system, guanylate cyclase, may be blocked by methylene-blue.

Nitric oxide synthesised by constitutive isoforms of NOS, (eNOS and bNOS) exerts its effects through the stimulation of soluble guanylate cyclase and subsequent formation of cyclic GMP (Ignarro, 1992). Cyclic GMP activates protein kinases and leads in turn to the dephosphorylation of myosin light chains and muscle relaxation. Alternative mechanisms by which nitric oxide could cause smooth muscle relaxation, independent of guanylate cyclase, include activation of calcium-dependent potassium channels (Bolotina *et al.*, 1994) and ADP ribosylation (Brune and Lapetina, 1990). Nitric oxide produced in larger quantities by the activation of iNOS also activates guanylate cyclase, but has additional toxic effects. These toxic effects are mediated via the release of free copper and iron ions within the victim cell, and the generation of O_2 and highly toxic hydroxyl radicals. These effects, in turn, result in massive oxidative injury.

Since the short half-life of nitric oxide makes direct measurement difficult in experimental situations, techniques have been developed to measure nitric oxide activity indirectly. These include the Greiss reaction which measures nitrite, the oxidation product of nitric oxide (Green *et al.*, 1982). The protein and mRNA for each of the three isoforms of NOS can be localised by immunocytochemistry and *in situ* hybridisation, respectively. Quantification of tissue NOS mRNA and protein can be

achieved by Northern and Western blots respectively. The total activity of NOS in a tissue can be quantified by measuring the conversion of radio-labelled L-arginine to L-citrulline, in the presence of added co-factors. Identification of NADPH diaphorase activity *in vitro* has been used to identify NOS activity within a tissue since, certainly in neuronal tissue, NADPH diaphorase activity co-localises with NOS (Hope *et al.*, 1991). However, it is now recognised that not all NADPH diaphorase activity is NOS, casting doubt on the validity of this technique (Tracey *et al.*, 1993).

Nitric oxide in Human Parturition

Nitric oxide was first identified in the human reproductive system by Ignarro *et al.*, (1990), who demonstrated that nitric oxide was generated in response to non-adrenergic/non-cholinergic neurotransmission-mediated penile erection. Since then, the L-arginine-nitric oxide system and each of the three isoforms of NOS, have been identified in the female reproductive system, suggesting that locally synthesised nitric oxide is directly involved in the physiology of reproduction.

Identification of the nitric oxide system in the uterus

Nitric oxide production in the uterus

Human and animal studies suggest that nitric oxide is generated within uterine tissues. The ability of the rat and rabbit uterus to produce large quantities of nitric oxide has been demonstrated using the Greiss reaction, (Yallampalli *et al.*, 1993; Sladek *et al.*, 1993; Yallampalli *et al.*, 1994a; Dong and Yallampalli 1996). Pregnant and non-pregnant human myometrium can also generate nitrate/nitrite in culture (Buhimschi et al. 1995b). NOS activity in the rat and rabbit uterus has been detected using the radiolabelled arginine to citrulline conversion assay, (Natuzzi *et al.*, 1993; Sladek *et al.*, 1993; Yallampalli *et al.*, 1994a). This technique has also provided evidence of NOS activity within the pregnant guinea-pig uterus (Weiner *et al.*, 1994a) as well as in

pregnant human myometrium (Ramsay *et al.*, 1996). In these studies, both calcium-insensitive (iNOS) and calcium-sensitive (eNOS and/or bNOS) activity were identified. Rabbit (Sladek *et al.*, 1993) and human (Sladek *et al.*, 1994) decidua contained only calcium-insensitive NOS activity, which in the rabbit was 6-fold greater than in the myometrium.

In human pregnancy NOS activity was identified in myometrium (Buhimschi *et al.*, 1995b; Ramsay *et al.*, 1996), placental villous trophoblast (Gude *et al.*, 1994; Morris *et al.*, 1995; Ramsay *et al.*, 1996; Di Iulio *et al.*, 1996), and amnion (Di Iulio *et al.*, 1996). In contrast to Sladek *et al.*, (1994), Di Iulio *et al.*, (1996) detected no NOS activity within the choriodecidua in tissue collected before and during labour.

Localisation of uterine NOS

NADPH diaphorase staining has been used to localise NOS within the pregnant and non-pregnant uterus of various species and essentially identical patterns of staining have been found, (myometrial and vascular smooth muscle nerve bundles, vascular endothelium, endometrial epithelium and leukocytes). The density of nerve fibres was greater in the cervix than in the myometrium of the body of the uterus. This technique has been used in rats (Schmidt *et al.*, 1992; Shew *et al.*, 1993; Yallampalli *et al.*, 1994a; Suburo *et al.*, 1995; Papka *et al.*, 1995; Papka *et al.*, 1996), mice (Grozdanovic *et al.*, 1994; Huang *et al.*, 1995), cows and pigs (Majewski *et al.*, 1995), and humans (Telfer *et al.*, 1995; Yoshida *et al.*, 1995; Ekerhovd *et al.*, 1998).

Since NADPH diaphorase activity may not be specific for NOS, other techniques including immunocytochemistry and *in situ* hybridization have been employed to localise each of the three isoforms of NOS in uterine tissues. Using immunocytochemsitry, bNOS was localised to the endometrial epithelium (Schmidt *et al.*, 1992), perivascular neurones (Grozdanovic *et al.*, 1994; Majewski *et al.*, 1995), and myometrial neurones (Grozdanovic *et al.*, 1994; Majewski *et al.*, 1995; Papka *et*

al., 1995). eNOS protein has been immunolocalised to vascular endothelium, endometrial glandular epithelium and endometrial stromal cells but was not consistently detected in myometrial smooth muscle cells (Telfer *et al.*, 1995; Telfer *et al.*, 1997). eNOS mRNA was detected in endometrial glandular epithelium, stroma, myometrium and myometrial blood vessels by *in situ* hybridization (Telfer *et al.*, 1995). In mouse uterus, iNOS was identified using immunocytochemistry, in stromal macrophages and myometrial mast cells (Huang *et al.*, 1995) and in the human non-pregnant uterus, iNOS was immunolocalised to glandular epithelial cells, and, in some specimens, the vascular smooth muscle of the myometrium and endometrium (Telfer *et al.*, 1997). Tschugguel *et al.*, (1998) found that expression of iNOS in the glandular epithelial cells was strong in secretory phase endometrium but weak or absent in proliferative endometrium. They suggest that the increased production of nitric oxide may be relevant to the process of menstruation.

Using Western blotting, Gangula *et al.*, (1997) demonstrated that rat myometrial cells in culture express eNOS constitutively and can be stimulated by cytokines to express iNOS. However, it is unclear whether human myometrial smooth muscle cells contain NOS (Sladek *et al.*, 1997). Other human muscles, including cardiac myocytes (Brady *et al.*, 1992) and vascular smooth muscle (Nunokawa *et al.*, 1993) can be stimulated with endotoxin and/or cytokines to express iNOS, and human skeletal muscle contains bNOS (Nakane *et al.*, 1993; Kobzik *et al.*, 1994). Bansal *et al.*, (1997) have demonstrated, using immunocytochemistry and Western blotting, that human myocytes express iNOS protein and Myatt *et al.*, (1998) observed eNOS immunostaining in myocytes in human placental bed biopsies.

The expression of each of the three isoforms of NOS has been described in human placenta. eNOS localised to the endothelium of the resistance vasculature and the syncytiotrophoblast of term placentae and the endothelium of the umbilical artery and vein (Myatt *et al.*, 1993a; Buttery *et al.*, 1994). This distribution was also seen for

mRNA encoding eNOS using *in situ* hybridization (Conrad *et al.*, 1993). Similarly, in tissue collected in the first trimester of pregnancy, after spontaneous miscarriage, NOS was identified in syncytiotrophoblast, but not cytotrophoblast, using NADPH diaphorase activity and immunocytochemistry with an antibody raised against eNOS (Eis *et al.*, 1995). In placentae from pregnancies complicated by pre-eclampsia, eNOS immunostaining was also identified in small terminal villous vessels (Ghabour *et al.*, 1995; Myatt *et al.*, 1997a). Using immunocytochemistry, bNOS has been localised to the extravascular contractile system cells, the media and endothelium of villous vessels and the syncytiotrophoblast (Graf *et al.*, 1994).

Approximately 5-6 per cent of the NOS activity within the placenta is independent of calcium/calmodulin stimulation, and therefore characteristic of iNOS (Myatt *et al.*, 1993b; Conrad *et al.*, 1993). Immunolocalisation studies have shown that iNOS is present within cells in the villous stroma which have been identified as placental macrophages or Hofbauer cells (Myatt *et al.*, 1997b). In some, but not all placental tissue, iNOS staining was also identified in syncytiotrophoblast and vascular endothelium (Myatt *et al.*, 1997b). Schonfelder *et al.*, (1996), using reverse-transcriptase-polymerase chain reaction, Western blotting and immunocytochemistry, detected no iNOS mRNA and protein in normal term placentae but identified iNOS in endothelial cells and syncytiotrophoblast in placentae from pregnancies complicated by gestational diabetes.

Within fetal membranes collected from uncomplicated pregnancies at term, Dennes *et al.*, (1997) have identified eNOS and iNOS mRNA in amnion and chorio-decidua. The expression of bNOS mRNA was not determined. Using immunocytochemistry, Eis *et al.*, (1997) have localised iNOS within fibroblasts of the amniotic and chorionic mesenchyme, and in decidual macrophages.

Relaxation of myometrium by nitric oxide

In vitro studies

Whilst the smooth muscle relaxant effects of nitric oxide were described originally in blood vessels (Palmer *et al.*, 1987), numerous studies over the last few years have demonstrated that nitric oxide is also a powerful myometrial relaxant. Nitric oxide gas produced complete inhibition of spontaneous contractions of rat myometrium removed late in gestation prior to the onset of labour (Yallampalli *et al.*, 1993). There are no data showing an effect of nitric oxide gas on the contractions of myometrium obtained either from pregnant or non-pregnant women.

More commonly nitric oxide donors have been used to investigate the effects of nitric oxide on myometrial smooth muscle. With few exceptions in animal studies, when nitric oxide donors have been applied to myometrium *in vitro*, reductions in contraction amplitude and/or frequency have been observed (for reviews, see Norman 1996 and Sladek *et al.*, 1997). Similarly in human studies, nitric oxide donors produced inhibition of spontaneous and oxytocin-induced activity when amplitude or force of contractions was measured (Buhimschi *et al.*, 1995; Lee and Chang, 1995; Norman *et al.*, 1997). The effects of nitric oxide releasing agents on the frequency of myometrial contractions seem more complex (for review, see Norman, 1996).

Like agents which act by liberating nitric oxide, the precursor of nitric oxide, L-arginine inhibited spontaneous and carbachol-induced, but not KCl-induced, myometrial contractions (Izumi *et al.*, 1993; Yallampalli *et al.*, 1993, 1994a). This inhibitory effect of L-arginine on spontaneous and induced uterine contractility was itself abolished by the L-arginine analogue, L-NAME and by inhibitors of guanylate cyclase, including methylene blue (Yallampalli *et al.*, 1993, 1994a). L-NAME had a small stimulatory effect on rat myometrial contractility when administered alone *in vitro* (Yallampalli *et*

al., 1993a,b). In studies of pregnant and non-pregnant, human myometrium, L-arginine inhibited spontaneous and oxytocin-induced myometrial contractility (Izumi *et al.*, 1993; Lee and Chang, 1995) with a greater effect on frequency than amplitude. In both these studies, the inhibitory effect of L-arginine was reversed by the L-arginine analogues, L-NAME and N-nitro-L-arginine. Furthermore, when administered alone, the analogues L-NAME and L-NMMA have been reported to stimulate contractile activity suggesting that in human as in animal myometrium, endogenous nitric oxide production may maintain some inhibition of contractile activity *in vivo* (Buhimschi *et al.*, 1995b; Lee and Chang, 1995). However, in contrast to these findings, Jones and Poston, (1997) found that neither L-arginine nor L-NAME led to any change in spontaneous activity in myometrial strips obtained from women at term or preterm.

Myometrial relaxation in response to cGMP has been tested by using the cell-permeable nonhydrolyzable cGMP analogue, 8-bromo-cGMP. 8-bromo-cGMP *in vitro* produced relaxation in nonpregnant rat myometrium (Diamond, 1983; Osa *et al.*, 1994), and human myometrium, though not when the latter was precontracted with endothelin (Word *et al.*, 1991).

Growing evidence suggests that the effect of nitric oxide on myometrial contractions is dependent on whether the tissue is collected before or after the onset of labour. The same dose of L-arginine was found to completely inhibit spontaneous contractions of rat myometrium for longer when the tissue was obtained before the onset of labour, compared with tissue collected during labour (Yallampalli *et al.*, 1993). Furthermore, L-arginine (3mM) completely inhibited carbachol-induced contractions when myometrium was collected at mid-gestation, but had no effect when myometrium was removed during delivery (Izumi *et al.*, 1993). Similarly, one concentration of 8-bromo-cGMP had a profound inhibitory effect on contractions of rat myometrium removed at mid-gestation, but concentrations of 1000 times higher were required to produce a similar inhibitory effect when myometrium was removed from labouring animals (Izumi

et al., 1993). Buhimschi *et al.*, (1995b) have demonstrated a reduction in sensitivity to the tocolytic effect of nitric oxide in human myometrium collected following the onset of labour.

The presence of placental tissue may be necessary for nitric oxide donors to achieve their maximum effects on the myometrium. Buhimschi *et al.*, (1997) have shown that the spontaneously releasing nitric oxide donor, diethylenetriamine-nitric oxide has contrasting effects *in vivo* and *in vitro*. *In vitro* studies of rat myometrial contractility showed decreased inhibitory responses to diethylenetriamine-nitric oxide in tissues collected during spontaneous labour compared with tissues collected before the onset of labour. In contrast, studies *in vivo* showed that the nitric oxide donor can decrease uterine contractility even more effectively during delivery, suggesting that the feto-placental unit increases the availability of nitric oxide *in vivo*. Segal *et al.*, (1998) have recently demonstrated that the addition of placental tissue to the organ bath markedly increases the sensitivity of rat myometrium to the relaxant effects of glyceryl trinitrate.

In vivo studies

It has long been recognised that in the clinical arena, nitric oxide donors relax the human uterus. As early as 1882, Barnes reported the use of amyl nitrite to facilitate manual removal of a retained placenta. More recently, intravenous glyceryl trinitrate (GTN) has been reported in uncontrolled case reports to allow correction of uterine inversion (Altabef *et al.*, 1992; Bayhi *et al.*, 1992; Dayan and Schwalbe, 1996), to facilitate intrapartum external cephalic version (Belfort, 1993), and to allow internal podalic version of a second twin (Wessen *et al.*, 1995). In a prospective observational study, intravenous GTN produced relief of intrapartum fetal distress related to uterine hyperactivity (Mercier *et al.*, 1997). This agent has also been used to facilitate fetal delivery during Caesarean section (Mayer and Weeks, 1992), although a recent

randomised trial found that administration of GTN leads to no clinically relevant effect on fetal extraction (David *et al.*, 1998).

Two uncontrolled, observational studies have investigated the effects of GTN patches in women with a diagnosis of preterm labour (Lees *et al.*, 1994; Rowlands *et al.*, 1996). Although these studies concluded that GTN can arrest preterm labour, further controlled trials and side effect data are required before these agents are widely adopted as tocolytic agents, (Norman, 1996; Sladek *et al.*, 1997). Uterine activity can only be quantified accurately with the use of an intrauterine pressure transducer and this technique has only rarely been employed in clinical studies. Inhaled amyl nitrite suppressed oxytocin-induced contractions during human labour at term, but not spontaneous contractions (Kumar *et al.*, 1965). Administration of the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP), suppressed preterm hysterotomy-induced contractions in rhesus monkeys (Jennings *et al.*, 1993), and intravenous GTN suppressed spontaneous term labour in sheep (Heymann *et al.*, 1993). In contrast, Norman *et al.*, (1995) found that intravenous GTN had no effect on intrauterine pressure or uterine contractions in women undergoing second trimester therapeutic termination of pregnancy, in whom uterine activity had been induced using mifepristone.

The role of nitric oxide in parturition

Animal studies suggest that a nitric oxide 'withdrawal' may be involved in the timing of the onset of parturition. Several studies have demonstrated an up-regulation of uterine nitric oxide production during pregnancy, and down-regulation during term and preterm labour (Natuzzi *et al.*, 1993; Sladek *et al.*, 1993; Yallampalli *et al.*, 1993; Yallampalli *et al.*, 1994a). Hence, there is a consensus on a decrease in uterine NOS activity at the end of pregnancy, although the precise timing of the decrease has not been completely elucidated. NOS activity is high during rabbit pregnancy and progressively decreases,

(by 80%), in decidua during the last 4 days of gestation (Sladek *et al.*, 1993). Other studies, comparing 16 (Natuzzi *et al.*, 1993) and 18 days' gestation (Yallampalli *et al.*, 1993) with labouring rats (22 days), have reported that NOS activity decreases with advancing gestation in whole rat uterus, decidua and myometrium. However, these studies leave a 4- to 6-day window in which the rat uterine NOS activity decrease takes place. Examining this window, Sladek and Roberts, (1996) found that NOS activity decreased between days 15 and 21 of gestation and did not decrease further at term (day 22). These workers propose that the decrease in pregnant rat uterine NOS activity coincides with the preparation of the uterus for parturition rather than the final activation of labour. Although uterine NOS activity is increased during rat pregnancy, the isoforms responsible are not known (Yallampalli *et al.*, 1998). Data from gene knockout animals suggests that an absence of any single isoform of NOS has no effect on the normal gestational period (Huang *et al.*, 1993; Huang *et al.*, 1995; Wei *et al.*, 1995).

Changes in NOS activity appear to be less important during pregnancy in the guinea-pig (Weiner *et al.*, 1994a). In this species, there was no significant difference in NOS activity, measured by the arginine to citrulline conversion assay, between pregnant and non-pregnant animals. Despite this, there was a 200-fold increase in cGMP content from mid- to late-pregnancy, suggesting that factors other than nitric oxide are responsible for the changes in cGMP.

In human pregnancy, the L-arginine-nitric oxide system has been identified within myometrium (Buhimschi *et al.*, 1995), placenta (Myatt *et al.*, 1993a), and fetal membranes (Di Iulio *et al.*, 1996). It may be possible for nitric oxide derived from each of these tissues to regulate myometrial contractions. However, because of its short half life of a few seconds and its reactivity, it seems likely that nitric oxide produced within the myometrium itself, or tissues in close apposition to the myometrium, will influence myometrial contractility. Several studies have attempted to determine whether a change

in NOS activity occurs at the onset of human parturition (Gude *et al.*, 1994; Di Iulio *et al.*, 1996; Ramsay *et al.*, 1996). Examining tissues collected at Caesarean section, ('before' labour and 'during' labour), and assessing NOS activity using the arginine to citrulline conversion assay, these studies concluded that there was no significant fall in NOS activity during human parturition. Indeed, Ramsay *et al.*, (1996) found a slight increase in NOS activity in myometrium collected during labour compared with that collected before labour. One possible explanation for this apparent increase in NOS activity is given in chapter 5 of this thesis, in which data are presented showing that inflammatory cells, which are a rich source of NOS (Riesco *et al.*, 1993; Kolb *et al.*, 1994), infiltrate the myometrium during labour.

Chapter 2 of this thesis, describes a study to determine the activity and expression of each of the 3 isoforms of NOS in uterine tissue collected from women before and during spontaneous labour at term. Since other biological mediators, including superoxide dismutase and xanthine oxidase, can interact with nitric oxide to alter its effects, changes in the expression of these molecules might be involved in the onset of parturition. In chapter 3, the results of a study to investigate the expression of superoxide dismutase and xanthine oxidase during normal pregnancy and parturition are reported.

Bansal *et al.*, (1997) recently reported that myometrial iNOS expression, assessed by immunohistochemistry and Western blotting, was greater in the early third trimester (26-34 weeks gestation) than either the late third trimester (37-41 weeks gestation) or in the non-pregnant state. These data suggest that an increase in myometrial iNOS expression might contribute to the maintenance of uterine quiescence during pregnancy. In chapter 4, the results of a study to investigate the uterine expression of each of the constitutive isoforms of NOS (eNOS and bNOS) in non-pregnant women and pregnant women delivered preterm and term, are reported.

Factors controlling the up-regulation in uterine NOS expression during pregnancy remain uncertain. In animals both oestrogen and progesterone have been proposed as regulators of NOS *in vivo*. Oestrogen upregulates guinea pig constitutive NOS expression (Weiner *et al.*, 1994b). In sheep uterine artery, oestrogen enhanced endothelial NOS activity, and in rat hypothalmus, oestrogen increased bNOS expression (Veille *et al.*, 1996; Cecatelli *et al.*, 1996). In studies of women treated with a GnRH analogue together with either oestrogen replacement or placebo, fasting plasma nitrate concentrations were higher in the estrogen treatment group, suggesting that oestrogen stimulated nitric oxide production (Ramsay *et al.*, 1995). In contrast, Yallampalli *et al.*, (1994b) have demonstrated that treatment with either oestradiol alone or oestradiol and progesterone, resulted in lower nitrite/cGMP content in rat uterus, compared with animals treated with vehicle alone.

Nitric oxide and cervical ripening

The results of recent animal studies on nitric oxide production and NOS expression in the cervix indicate that nitric oxide may play a role in cervical ripening. The nitric oxide generating system is present in the rat cervix and in contrast to the body of the uterus, it is down-regulated during pregnancy, but up-regulated during term and preterm labour (Buhimschi *et al.*, 1996). Treatment of pregnant guinea-pigs with the NOS inhibitor L-NAME induced preterm labour but delayed physiological cervical ripening resulting in prolonged deliveries (Chwalisz *et al.*, 1994b). Furthermore, L-NAME treatment of pregnant rats significantly prolonged the duration of labour, suggestive of cervical dystocia, whilst a decrease in cervical extensibilty was observed after *in vitro* incubation with L-NAME (Buhimschi *et al.*, 1996). Chwalisz *et al.*, (1997) demonstrated that the local application of the nitric oxide donor sodium nitroprusside, produced effective ripening of the guinea-pig cervix, assessed by both force resistance measurements and morphological evaluation. It seems that, at least in animal pregnancy, nitric oxide represents a final metabolic pathway of cervical ripening. Acting in concert with

prostaglandins, particularly PGE_2, nitric oxide might induce local vasodilatation and increase vascular permeability and leukocyte infiltration, and perhaps also activate matrix metalloproteinases and other mechanisms responsible for the extracellular matrix remodelling (Chwalisz *et al.*, 1997).

There are no studies investigating the L-arginine nitric oxide system in the pregnant, human cervix. The effects of the nitric oxide donor GTN, administered sublingually, on the non-pregnant cervix have been subjectively assessed. Shaker *et al.*, (1993) found that GTN had no significant effects on the ease of embryo transfer after in-vitro fertilization. Yadava, (1990) reported that GTN facilitated the transcervical introduction of intra-uterine contraceptive devices. Chapter 6, describes the results of a study to determine the effects of the nitric oxide donors glyceryl trinitrate and isosorbide mononitrate on the first trimester human cervix. In chapter 7, the results of a randomised controlled trial to determine the side effect profile of isosorbide mononitrate in comparison with the prostaglandin analogue gemeprost, when used to ripen the first trimester human cervix, is reported and the clinical effectiveness of the nitric oxide donor for this indication, investigated.

In summary, the work in this thesis aims to determine whether changes in the activity and expression of the L-arginine nitric oxide system within the uterus, are involved in the maintainence of human pregnancy and the onset of parturition.

Chapter 2

Nitric oxide synthase activity and localisation do not change in uterus and placenta during human parturition

Introduction

Animal studies have suggested a role for nitric oxide in the onset of parturition. NOS activity is up-regulated during pregnancy and inhibits uterine contractility until term. At the onset of parturition, NOS activity decreases, thus freeing the myometrium from the relaxant effect of nitric oxide and stimulating uterine contractions (Yallampalli *et al.*, 1993a; Natuzzi *et al.*, 1993; Sladek *et al.*, 1993; Yallampalli *et al.*, 1994). In human pregnancy, the L-arginine-nitric oxide system is present within myometrium (Buhimschi *et al.*, 1995), placenta (Myatt *et al.*, 1993), and fetal membranes (Di Iulio *et al.*, 1996). It may be possible that nitric oxide derived from each of these tissues regulates myometrial contractions. However, its short half life of a few seconds, and its reactivity mean that nitric oxide is most likely to act as a local messenger molecule transferring messages within and between individual cells (Vallance and Collier, 1994). It seems likely therefore that only the myometrium itself, or tissues in close apposition to the myometrium will influence myometrial contractility. Several studies have attempted to determine whether a change in NOS activity occurs at the onset of parturition (Gude *et al.*, 1994; Ramsay *et al.*, 1996; Di Iulio *et al.*, 1996). These studies have failed to assess all of the cell types within the pregnant uterus and have relied solely upon in vitro analysis of NOS activity.

Since nitric oxide is produced within the pregnant human uterus, we hypothesise that in humans, as in animals, NOS activity is high during pregnancy and decreases at the onset of parturition. Such a mechanism would contribute to the control of the onset of labour. The aim of this study was to determine whether the expression of NOS

isoforms is altered in myometrium, placenta, decidua and fetal membranes in women before and after the onset of labour at term, and to localise, using immunocytochemistry, each of the three NOS isoenzyme proteins. Furthermore, we assessed whether a reduction in NOS enzyme activity occurs in these tissues following the onset of spontaneous labour.

Materials & methods

Subjects and collection of tissue

Twenty-two women, undergoing Caesarean section at term, (>37 weeks' gestation), were recruited to the study. Eleven of these women had an elective Caesarean section performed prior to the onset of labour. A further 11 were delivered by emergency Caesarean section during active labour, (cervical dilatation greater than 4 cm and less than 9 cm). Women were excluded from the study if they had a multiple pregnancy, or evidence of active infection, or following induction of labour. At operation, a myometrial strip was obtained from the upper margin of the lower uterine segment incision. In addition full-thickness biopsies of placenta and fetal membranes with attached decidua capsularis were obtained within 10 minutes of delivery. The biopsies were divided; one half was fixed in 10% neutral buffered formalin (BDH, UK) and embedded in paraffin, and the other half was snap frozen in liquid nitrogen and subsequently stored at -70°C. Informed consent was obtained from each woman prior to recruitment and the study was approved by the Local Research Ethics Committee.

Immunocytochemistry for eNOS

Eight micrometer thick sections were cut from the paraffin embedded tissues and mounted on silane-coated slides, heated to 60°C for 35 min, deparaffinized in xylene and rehydrated in a graded alcohol series. The sections then underwent enzymatic

digestion in a 0.01% (w/v) solution of calcium chloride containing 0.01% (w/v) protease type XXIV (Sigma, Poole, UK), for 10 min at 37°C. The sections were pre-incubated with 3% (w/v) immunoglobulin-free bovine serum albumin (BSA, Sigma) in PBS (10 mM sodium phosphate, pH 7.5, 120 mM sodium chloride) for 20 min at room temperature. They were then incubated for one hour at room temperature with a monoclonal antibody raised against a 20.4 kDa protein fragment corresponding to amino acids 1030-1209 of human eNOS (Affiniti, Nottingham, UK) diluted 1/100 in 3% BSA. Next, sections were washed in 0.1% Triton-X 100, followed by two washes in PBS before incubation with biotinylated anti-mouse immunoglobulin. This antibody, from a Vectastain Elite ABC kit (Vector, Peterborough, UK) was first diluted in 3% BSA and 1.5% normal human serum. After washing as before, sections were placed in 3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were thoroughly washed again, then incubated for 30 min with avidin DH / biotinylated horseradish peroxidase H reagent (Vectastain Elite ABC kit) in PBS before final washing. Immunoreactive eNOS was localised using 1 mg/ml diaminobenzidine tetrahydrochloride (DAB), 0.02% H_2O_2 in 50mM Tris.Cl, pH7.6, and appeared as a brown end-product.

Negative controls included sections incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 *Aspergillus niger* glucose oxidase (Dako Ltd., High Wycombe, UK), an enzyme which is not expressed in mammalian cell systems. A section of human umbilical cord was used as a positive control. Immunostaining for eNOS was confined to the endothelium.

Immunocytochemistry for bNOS

Immunocytochemistry was performed on eight micrometer paraffin-embedded sections using a polyclonal antibody raised against amino acids 724-739 of rat brain NOS (Serotec, Oxford, UK). This antibody recognises human bNOS, (100% sequence

homology) with no reported cross-reactivity with iNOS or eNOS. It was used on membranes, decidua and placenta in a working dilution of 1/1750 and 1/2000 on myometrium, with a rabbit IgG Vectastain ABC kit (Vector) according to the manufacturer's instructions. The sections were pretreated by microwaving at full power for 4 x 5 min in citrate buffer (10mM, pH 6.0).

Negative controls included slides incubated without the primary antibody and slides incubated with non-immune rabbit serum (SAPU, Carluke, UK), in place of the primary antibody. In addition, liquid-phase absorption controls were performed by incubating the primary antibody with increasing concentrations of the bNOS immunogen (0.01-100 nmol/ml), (Serotec, Oxford, UK) for 60 min at 37°C prior to incubation with the sections. A section of rat brain was used as a positive control.

Immunocytochemistry for iNOS

Immunocytochemistry was performed using 3 different rabbit polyclonal iNOS antibodies (table 2.1).

Table 2.1 iNOS primary antibodies used for immunocytochemistry

Antibody	Immunogen	Dilution	Presentation	Source
PA3-030	a.a. 1131-1144 of murine iNOS	1/800-1000	affinity purified	Cambridge Bioscience
sc-649	a.a. 1135-1153 of human iNOS	1/100-600	affinity purified	Santa Cruz
NO53	C- terminal heptamer of human iNOS	1/20 000	neat antiserum	Merck Research*

* Donated by Dr R. A. Mumford, Merck Research Laboratories, USA

Sections of each tissue were stained as follows:

(i) the polyclonal antibody PA3-030 (Cambridge Biosciences, Cambridge, UK) was used on untreated paraffin sections at a dilution of 1/800 to 1/1000, and a rabbit IgG Vectastain ABC kit (Vector, UK).

(ii) the polyclonal iNOS antibody, sc-649, (Santa Cruz, Heidelberg, Germany) was employed on serial sections of each tissue. With this antibody, the sections required antigen retrieval by microwaving at full power for 4 x 5 min in one litre of citrate buffer (10mM, pH 6.0). The antibody was used in a dilution of 1/100 to 1/600, and immunocytochemistry was carried out using a rabbit IgG Vectastain ABC kit (Vector, UK) according to the manufacturer's instructions.

(iii) an iNOS antibody (NO53) raised against a synthetic peptide containing the extreme carboxy-terminal heptamer of human iNOS, was kindly gifted to us by Dr R. A. Mumford, (Merck Research Laboratories, Rahway, USA). Since this antibody has been validated on cryosections, five micrometer thick sections were cut from the frozen tissue and mounted on silane coated slides. The sections were fixed in acetone for 10 mins., and then pre-incubated with 25% (w/v) normal goat serum/pooled human serum (SAPU, Carluke, Scotland) for one hour at room temperature. The antibody was then applied at a dilution of 1/20,000 and incubated for 16 hours at 4° C. Thereafter, the procedure was carried out as previously described using a rabbit IgG Vectastain ABC kit (Vector, UK).

For each of these antibodies, control slides were incubated without the primary antibody and liquid-phase absorption controls were performed using the appropriate immunogen peptides (Cambridge Biosciences, Cambridge, UK, Thistle Peptide, Glasgow, UK, and Merck Research Laboratories, Rahway, USA, respectively). Furthermore, antibody PA3-030 was used to immunolocalise iNOS protein on sections

of endometrium and myometrium from a wild type mouse and an iNOS knock-out mouse (gifted by Professor E.Y. Liew). This test could not be applied to antibodies sc-649 and NO53 which do not recognise murine iNOS. A section of diseased human lung tissue was used as a positive control, (Nicholson *et al.* 1996).

Vimentin and Cytokeratin Immunocytochemistry

In order to identify decidua and trophoblast respectively, immunocytochemistry was undertaken on serial paraffin sections using antibodies directed against vimentin and cytokeratins, thereby facilitating the localization of the NOS staining. A polyclonal antibody directed against vimentin, isolated from calf lens (Euro-path, Cornwall, UK) was used in a dilution of 1/150 with a rabbit IgG Vectastain ABC kit (Vector, UK). Control slides were incubated without the primary antibody. In addition, immunocytochemistry was performed using a monoclonal antibody directed against human cytokeratins (numbers 5, 6, 8, 17 and 19), (Dako, High Wycombe, UK). The sections required enzymatic digestion with a 0.1% (w/v) trypsin (Sigma, UK) solution in Tris buffer (pH 7.6) containing 0.1% (w/v) calcium chloride, for 10 min at room temperature, and the procedure was performed using a mouse IgG Vectastain ABC kit (Vector, UK).

Nitric oxide synthase enzyme activity

Nitric oxide synthase enzyme activity was determined by conversion of [^{14}C] L-arginine to [^{14}C] L-citrulline using a commercial kit, (NOS detect assay, Alexis Corporation, Nottingham). Frozen tissue was homogenised in five volumes of cold 250mM Tris-HCl (pH 7.4), 10mM EDTA and 10mM EGTA. Homogenised tissue was centrifuged at 11000g for 5 min at 4°C, thereafter supernatants were collected and kept on ice for no longer than 15 min before assay. The supernatant was incubated for either 60 min (placenta) or 90 min (myometrium and membranes) at 37°C with buffer

containing co-factors required for NOS enzyme activity, (1mM NADPH, 0.05μCi [^{14}C] L-Arginine, 0.6μM tetrahydobiopterin, 0.2μM flavin adenine dinucleotide, 0.2μM flavin adenine mononucleotide and 0.6mM Ca Cl_2). After incubation, 0.4ml of 50mM HEPES buffer (pH 5.5), 5mM EDTA was added to each tube. This mixture was added to 100μl of resin and transferred to a spin cup. This allows elution of the negatively charged L-citrulline but retains the positively charged L-arginine. The eluate was collected after spinning for 30 seconds in a microcentrifuge at full speed and counted in a liquid scintillation counter. Samples were incubated both in the absence and presence of 1mM nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NOS. NOS activity was determined from the difference between [^{14}C] citrulline produced from control samples and samples containing 1mM L-NAME.

Protein content was determined in homogenate supernatants using the Lowry reaction (Lowry *et al.*, 1951), and NOS activity was expressed as fmol of product formed per min per mg protein. The lower limit of detection of the assay is 0.5 fmol/min/mg protein.

Analysis of results

The localisation of each of the 3 isoforms of NOS and intensity of staining were recorded by two investigators independently, who were blind to the timing of biopsy in relation to labour. Intensity and consistency of staining was scored on a scale of 0 to 6, (0 = staining absent, 1 = weak, variable staining, 2 = weak, consistent staining, 3 = moderate, variable staining, 4 = moderate, consistent staining, 5 = strong, variable staining and 6 = strong, consistent staining). To determine whether there was a difference in the intensity of staining between those tissues obtained before and during labour, contingency table analysis of the scores was performed, and a chi-square p value of <0.05 was taken as significant.

NOS enzyme activity (fmol/min/mg protein) in those tissues obtained before and after labour, was analysed using a Mann-Whitney U test. A value of $p<0.05$ was taken as significant.

Results

(i) Patient characteristics

The mean age (SD), of the 11 women delivered prior to the onset of labour was 30 (6), years and their mean (SD), gestation at delivery was 38.2 (0.6), weeks. The mean age (SD), of the women in spontaneous labour at term was 30 (5), years and their mean (SD), gestation at delivery was 40.1 (0.9), weeks. One of the non-labourers and 3 of the labouring women were smokers.

(ii) Immunocytochemistry

Serial sections of placenta and fetal membranes were incubated with antibodies directed against vimentin and cytokeratins. These antibodies localised decidua and trophoblast respectively and these sections were used to identify the sites of staining produced by each of the NOS antibodies.

eNOS

eNOS protein was detected in all of the tissues before and after labour. There was no significant difference in staining intensity and localisation for eNOS, between individual cell types before and during labour. There was however, a considerable variability in the intensity of staining between samples which was not related to whether the sample was obtained in or outwith labour. Within myometrial sections, the protein was localised to the myometrial smooth muscle cells (myocytes) and the endothelium of

blood vessels, (figure 2.1 a). Within the placenta, the eNOS staining was of greatest intensity in the syncytiotrophoblast, of moderate intensity in the endothelium of blood vessels and was weakly present in villous stroma, (figure 2.1 b). In the fetal membranes, staining was strongest in the amniotic epithelium, moderately intense in the trophoblast (the remains of the cytotrophoblastic cells of the trophoblastic shell), and absent in the chorionic connective tissue. eNOS protein was also localised within the stromal cells of the decidua, (figure 2.1 c). There was no staining in the negative control slides.

bNOS

There was no significant difference in staining intensity and localisation for bNOS, between individual cell types before and during labour. In myometrial sections, bNOS protein was localised within the myocytes and in the smooth muscle and endothelium of blood vessels, (figure 2.2 a). The protein was detected in the placenta, within the syncytiotrophoblast, the villous stroma and endothelial cells, (figure 2.2 b). The amnion, chorion and trophoblast of the fetal membranes were positive, and the protein was also localised within the maternal stromal cells of the decidua capsularis, (figure 2.2 c). Staining was absent when the primary antibody was omitted. In liquid phase absorption controls, the staining disappeared when 10 nmol/ml of the bNOS immunogen was added to the primary antibody prior to incubation with the sections.

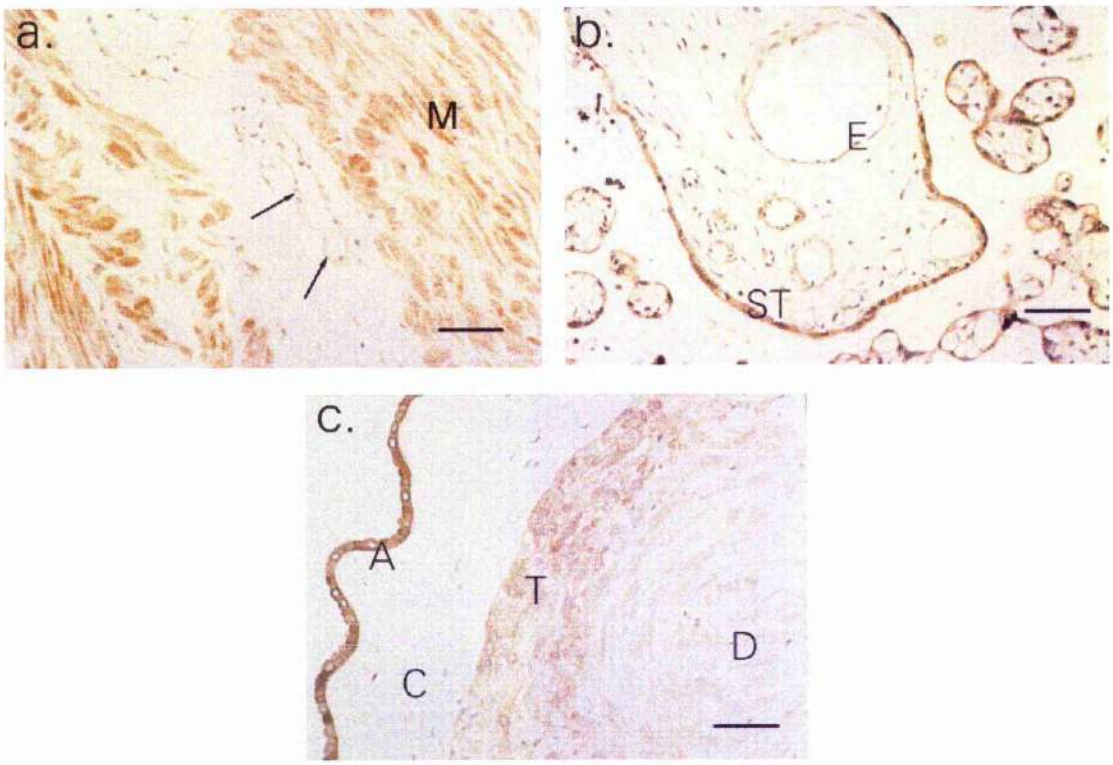

Figure 2.1 Localisation of endothelial NOS (eNOS) in **(a)** myometrium, **(b)** placenta and **(c)** fetal membranes with attached decidua. There was no significant difference in staining intensity and localisation for eNOS, between individual cell types before and during labour. **(a)** eNOS localised to the myometrial smooth muscle cells and the endothelium of blood vessels (arrows). **(b)** Within the placenta, the eNOS staining was of greatest intensity in the syncytiotrophoblast, of moderate intensity in the endothelium of blood vessels and was weakly present in villous stroma. **(c)** In the fetal membranes, staining was strongest in the amniotic epithelium, moderately intense in the trophoblast and absent in the chorionic connective tissue. eNOS protein was also localised within the stromal cells of the decidua. Nuclei were visualised with haematoxylin. M = myocytes, E = endothelium, ST = syncytiotrophoblast, A = amnion, C = chorion, T = extra-villous trophoblast and D = decidua. Bar = 50 μm.

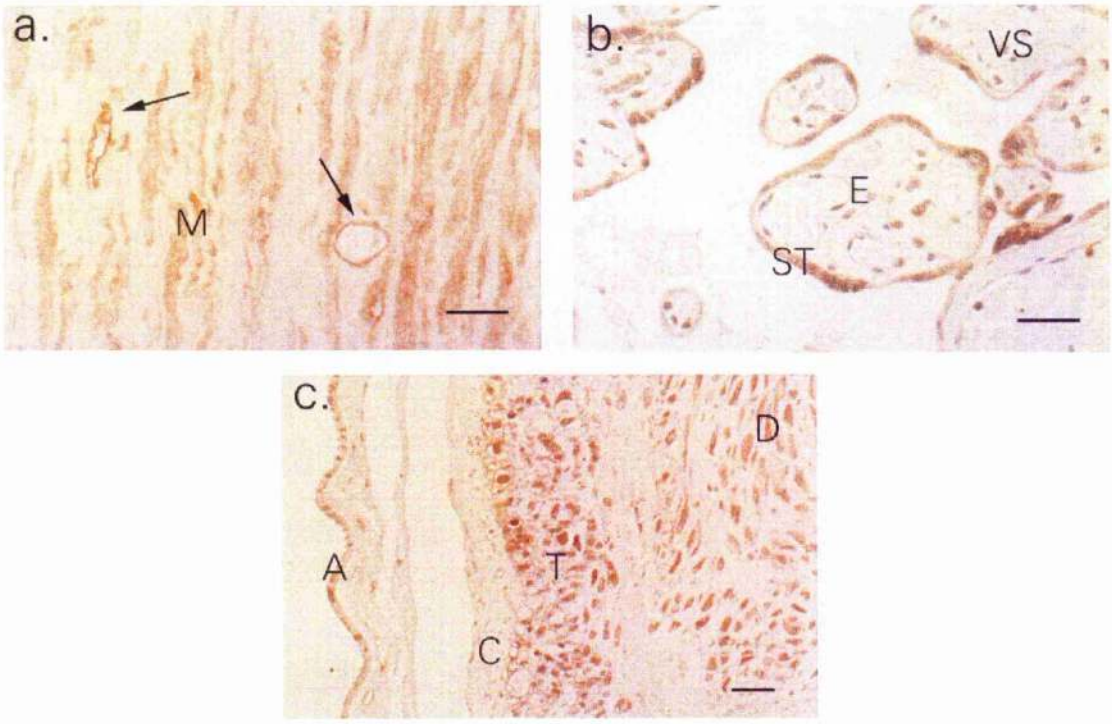

Figure 2.2 Expression of brain NOS (bNOS) in **(a)** myometrium, **(b)** placenta and **(c)** fetal membranes with attached decidua. There was no significant difference in staining intensity and localisation for bNOS, between individual cell types before and during labour. **(a)** bNOS localised to the myometrial smooth muscle cells and the endothelium of blood vessels (arrows). **(b)** Within the placenta, bNOS staining was expressed in the syncytiotrophoblast, the endothelium of blood vessels and the villous stroma. **(c)** In the fetal membranes, bNOS was expressed in the cells of the amniotic epithelium, the trophoblast and the fibroblasts of the chorion. bNOS protein was also localised within the stromal cells of the decidua. Nuclei were visualised with haematoxylin. The negative controls, (see Materials and methods) exhibited no reactivity. M = myocytes, E = endothelium, ST = syncytiotrophoblast, VS = villous stromal cells, A = amnion, C = chorion, T = extra-villous trophoblast and D = decidua. Bar = 50 μm.

iNOS

iNOS protein was localised in all the tissues sampled, and localisation was similar with the anti-murine iNOS, PA3-030 (Cambridge Bioscience), and anti-human iNOS, sc-649 (Santa Cruz) antibodies. However, there were some differences between the pattern of staining obtained with these antibodies and that obtained with antibody NO53 (Merck Research Laboratories).

(i). Cambridge Bioscience (PA3-030) and Santa Cruz (sc-649) antibodies.

There was no significant difference in staining intensity and localisation for iNOS, between individual cell types before and during labour. In myometrial sections, iNOS protein was localised with weak staining to the myocytes and vascular smooth muscle (figure 2.3 a). Within the placental sections, there was weak, variable staining for the protein in the syncitiotrophoblast, and more consistent staining within the vascular smooth muscle and stromal cells (figure 2.3 b). In the fetal membranes, staining for the iNOS protein was weak in the amniotic epithelial cells, and of moderate intensity in the chorionic connective tissue, the cells of the extravillous trophoblast and the stromal cells of the decidua (figure 2.3 c). Staining was absent when the primary antibody was omitted and, in liquid phase absorption controls, the staining disappeared when the primary antibody was incubated with the immunogen, prior to incubation with the sections. iNOS was localised to endometrial glandular epithelium and myometrial smooth muscle cells of the wild type mouse. There was no staining in the tissue sections from the iNOS knock-out mouse.

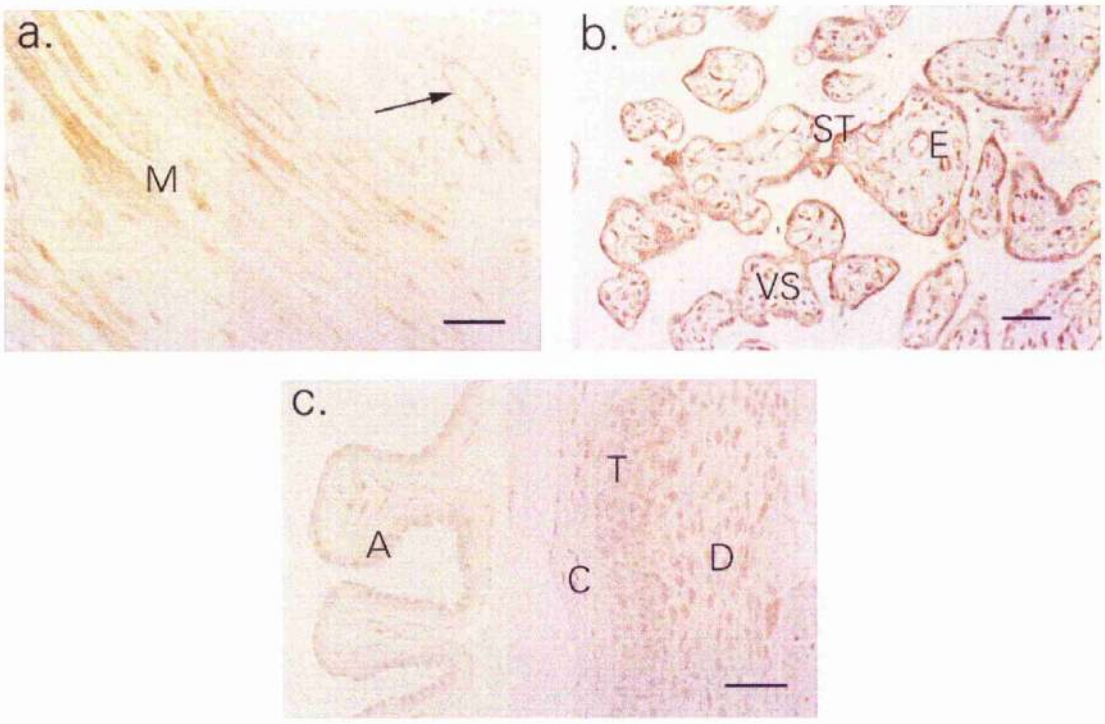

Figure 2.3 Localisation of inducible NOS (iNOS) in **(a)** myometrium, **(b)** placenta and **(c)** fetal membranes with attached decidua, using antibodies PA3-030 (Cambridge Bioscience) and sc-649 (Santa Cruz). There was no significant difference in staining intensity and localisation for iNOS, between individual cell types before and during labour. **(a)** iNOS localised to the myometrial smooth muscle cells and the smooth muscle of blood vessels (arrow) with weak intensity. **(b)** Within the placenta, iNOS staining was expressed in the syncytiotrophoblast, the endothelium of blood vessels and the villous stroma. **(c)** In the fetal membranes, iNOS was weakly expressed in the cells of the amniotic epithelium, and localised with moderate intensity in the trophoblast, the fibroblasts of the chorion and the stromal cells of the decidua. Nuclei were visualised with haematoxylin. The negative controls, (see Materials and methods) exhibited no reactivity. M = myocytes, E = endothelium, ST = syncytiotrophoblast, VS = villous stromal cells, A = amnion, C = chorion, T = extra-villous trophoblast and D = decidua. Bar = 50 μm.

(ii). Merck Research Laboratories, (antibody NO53).

Using antibody NO53, iNOS protein was localised in myometrial sections with weak staining in the myocytes and vascular smooth muscle (figure 2.4 a), a similar pattern to that obtained with PA3-030 and sc-649. Within the placenta, iNOS was localised in occasional and isolated cells within the villous stroma and in extravillous trophoblast. The villous trophoblast and endothelial cells were negative (figure 2.4 b). In the fetal membranes, staining was present in the amniotic epithelium and the extra-villous trophoblast, but in contrast to the results obtained with PA3-030 and sc-649, staining was absent in the cells of the chorion and decidua (figure 2.4 c). Control slides, including preabsorption with the immunogen peptide, showed no staining.

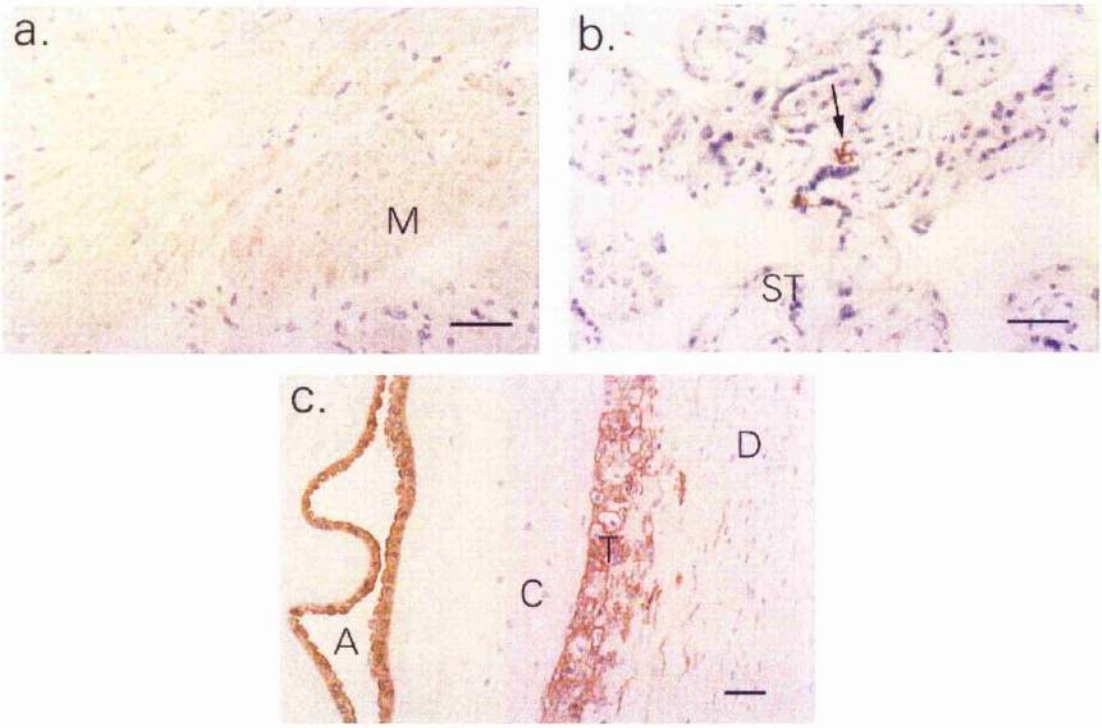

Figure 2.4 Expression of inducible NOS (iNOS) in **(a)** myometrium, **(b)** placenta and **(c)** fetal membranes with attached decidua, using antibody NO53 (Merck Research Laboratories). There was no significant difference in staining intensity and localisation for iNOS, between individual cell types before and during labour. **(a)** iNOS localised with weak intensity to the myometrial smooth muscle cells. **(b)** Within the placenta, iNOS was localised in occasional and isolated cells within the villous stroma (arrow). The villous trophoblast and endothelial cells were negative. **(c)** In the fetal membranes, iNOS was present in the amniotic epithelium and the extra-villous trophoblast, but in contrast to the results obtained with PA3-030 and sc-649, staining was absent in the cells of the chorion and decidua. Nuclei were visualised with haematoxylin. The negative controls, (see Materials and methods) exhibited no reactivity. M = myocytes, ST = syncytiotrophoblast, A = amnion, C = chorion, T = extra-villous trophoblast and D = decidua. Bar = 50 μm.

(ii) NOS enzyme activity

There was no significant difference in NOS enzyme activity in myometrium, placenta or fetal membranes, with attached decidua, before compared with during labour at term, ($p>0.05$), (figure 2.5). NOS enzyme activity was much greater in placenta than in the other tissues sampled. The median value of NOS enzyme activity in each tissue was: myometrium before labour = <1, myometrium during labour = <1, fetal membranes before labour = 5, fetal membranes during labour = 9, placenta before labour = 520, placenta during labour = 420 fmol/min/mg protein.

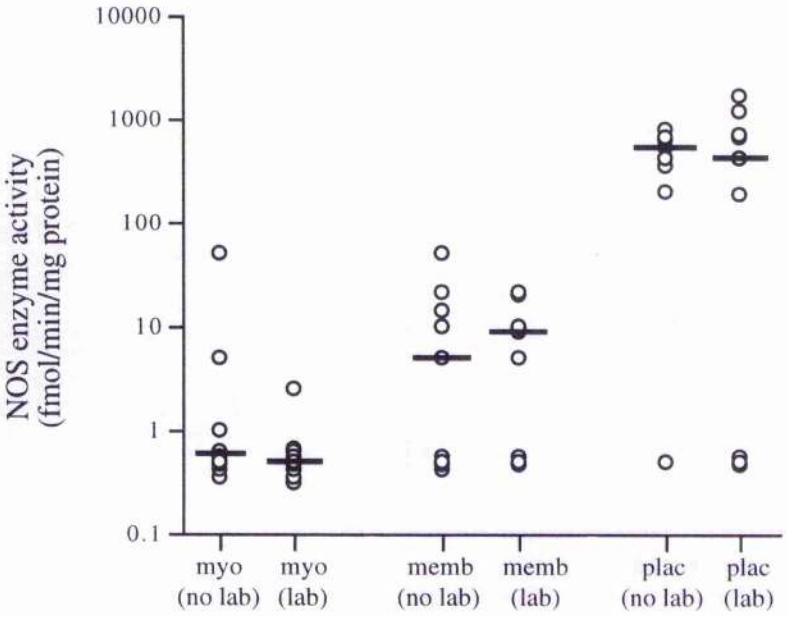

Figure 2.5 NOS enzyme activity (fmol/min/mg protein) in each of the tissues before, (no lab) and after, (lab) the onset of labour. Activity is plotted on a log 10 scale and n = 9 in each group. There is no significant difference ($p>0.05$, Mann Whitney U tests) in NOS enzyme activity in any of the tissues following the onset of labour. (myo = myometrium, memb = fetal membranes with attached decidua, and plac = placenta).

Discussion

We have localised each of the three isoforms of NOS in human myometrium, placenta, fetal membranes and decidua before and after the onset of parturition at term. Using a qualitative method to assess the intensity of staining, and a quantitative measure of enzyme activity, we have found no difference in either staining intensity and distribution, or enzyme activity of NOS in myometrium, placenta or membranes before and during labour at term. These results suggest that, in contrast to animal studies, a decrease in NOS enzyme activity may not be involved in the onset of parturition at term in the human.

Animal studies in the rat and rabbit have provided convincing evidence that nitric oxide has a physiological role in the maintenance of pregnancy and that a reduction in NOS enzyme activity is involved in the onset of parturition, (Yallampalli *et al.*, 1993a; Natuzzi *et al.*, 1993; Sladek *et al.*, 1993). Studies of human parturition have found no decrease in NOS enzyme activity, assessed as the conversion of L-arginine to L-citrulline, after the onset of spontaneous labour in myometrium (Ramsay *et al.*, 1996), placenta (Gude *et al.*, 1994; Di Iulio *et al.*, 1996) or fetal membranes (Di Iulio *et al.*, 1996). Indeed, Ramsay *et al.*, (1996) found an increase in calcium-independent NOS activity (iNOS activity) in myometrial samples obtained during labour compared with those obtained before the onset of labour.

We have failed to find a decrease in NOS activity following the onset of human parturition. In contrast to the rat, changes in nitric oxide appear to be less important during guinea-pig parturition (Weiner *et al.*, 1994). In this animal model, although a decrease in cyclic guanosine 3',5'-monophosphate levels, (cGMP, the second messenger system through which nitric oxide exerts its effects) can be demonstrated following the onset of labour, factors other than changes in NOS activity are

responsible. In human myometrium, a similar fall in cGMP has been demonstrated following the onset of labour (Buhimschi *et al.,* 1995), and again our data and others suggests that agents other than nitric oxide are involved. Whilst a decrease in NOS activity may be involved in the initiation of parturition in some animal species, nitric oxide may be less important in others, and caution should be exercised in extrapolating from one species to another.

One caveat to this conclusion relates to the method of measurement of NOS activity. All of the studies investigating NOS activity in human parturition, including our study, have assessed enzyme activity by measuring the conversion of radio-labelled L-arginine to L-citrulline. However, in vitro NOS enzyme activity may not reflect in vivo uterine NOS activity (Sladek and Roberts, 1996), where the limited availability of cofactors can attenuate the production of nitric oxide. Furthermore, availability of such co-factors may be regulated by cytokines in vivo (Werner-Felmayer *et al.,* 1993).

Whilst our results indicate that there is no reduction in either NOS activity or expression during human parturition, nitric oxide might still play a role in the onset of human labour. Lee and Chang, (1995) have demonstrated an increased sensitivity of human myometrium to the tocolytic effect of nitric oxide during pregnancy compared to non-pregnant myometrium, whilst Buhimschi *et al.,* (1995) have demonstrated a decreased sensitivity to the tocolytic effect of nitric oxide following the onset of labour. This change in sensitivity will cause an increase in myometrial contractions following the onset of parturition even in the presence of constant levels of nitric oxide. It is unclear at present whether this change in sensitivity is specific to nitric oxide or also includes other myometrial relaxants (Norman, 1996). Other biological mediators can interact with nitric oxide to alter its effects, for example the enzyme superoxide dismutase (SOD) prolongs the biological half-life of nitric oxide (Huie and Padmaja, 1993). SOD is known to be present within the endometrium of the non-pregnant uterus (Sugino *et al.,* 1996), and a reduction in SOD enzyme activity within uterine tissues at the onset of

labour would, in turn reduce the biological activity of nitric oxide, and stimulate uterine contractions. Such a hypothesis is addressed in chapter 3 of this thesis.

Immunocytochemical studies investigating the expression of NOS isoenzymes, and in particular iNOS, in human cells have aroused concern regarding the specificity of the antibodies employed (Nicholson *et al.,* 1996). Amin *et al.,* (1995), reported that a commercially available monoclonal antibody reactive with murine macrophage iNOS (Transduction Laboratories, Lexington, USA), binds to both human and rat bNOS.

In this study we observed differences in the localisation of iNOS protein when different antibodies were employed, suggesting either that these antibodies recognise different proteins or that there is non-specific binding. Although one of these antibodies, PA3-030, (Cambridge Biosciences), has recently been reported to bind specifically to mouse macrophage and human hepatocyte iNOS (Amin *et al.,* 1995), the specificity of antibody NO53 has been more rigorously characterised and validated (Nicholson *et al.,* 1996). Furthermore, the results obtained with antibody NO53, (Merck Research Laboratories) demonstrating an absence of iNOS staining in placental villous trophoblast, are in keeping with published data. No evidence of iNOS protein or gene expression can be demonstrated by in situ hybridisation, immunocytochemistry, (using both an IgG2a-monoclonal antibody and a polyclonal antibody), or polymerase chain reaction in human placentae under physiological conditions, (Conrad *et al.,* 1993; Garvey *et al.,* 1994; Schonfelder *et al.,* 1996). In contrast, we observed variable staining for the iNOS protein in the syncitiotrophoblast, and more consistent staining within the vascular smooth muscle and stromal cells with PA3-030 and sc-649.

The localisation of eNOS and bNOS enzymes within the placenta is consistent with previous morphological and immunocytochemical investigations, (Myatt *et al.,* 1993; Myatt *et al.,* 1997; Graf *et al.,* 1994). There have been no published data regarding the localisation of any of the NOS enzymes in fetal membranes, decidua or pregnant human

myometrium. However, our results reported here showing iNOS staining in pregnant human myometrium is in keeping with our previous results showing iNOS immunoreactivity in the myometrial smooth muscle cells of the non-pregnant uterus (Telfer *et al.,* 1997).

We and others (Ramsay *et al.,* 1996; Di Iulio *et al.,* 1996), have demonstrated that NOS enzyme activity is much greater in placenta than in the other tissues sampled. This finding is consistent with large amounts of constitutive NOS (bNOS and eNOS) within the trophoblast and endothelial cells of this tissue (Di Iulio *et al.,* 1996).

The study was designed to investigate NOS enzyme activity in human tissues obtained before and after the onset of labour at term and, extrapolating from animal studies, assumed that nitric oxide, a smooth muscle relaxant, maintains uterine quiescence until the onset of labour. Sladek and Roberts, (1996) recently found that spontaneous labour did not begin until more than 24 hours after the final decrease in uterine NOS activity in the gravid rat uterus at term. They concluded that the decrease coincides with the preparation of the uterus for parturition rather than the final activation of labour. Perhaps we have failed to demonstrate a decrease in NOS activity in the human uterus at term since the decrease occurs before 37 weeks' gestation, and this may be one of the factors governing the transition from Braxton Hicks contractions to the myometrial contractions of labour. This hypothesis is examined in chapter 4.

Chapter 3

The expression of superoxide dismutase and xanthine oxidase in myometrium, fetal membranes and placenta during normal pregnancy and parturition.

Introduction

A role for nitric oxide in the control of rat and rabbit parturition is supported by a variety of studies. Nitric oxide production within the uterus is high during animal pregnancy, but decreases at term thus initiating uterine activity at the onset of parturition (Sladek *et al.*, 1993; Natuzzi *et al.*, 1993; Yallampalli *et al.*, 1993). In contrast, we and others, have found no evidence that NOS activity is decreased following the onset of labour in human parturition either in the myometrium itself or in placenta or fetal membranes (Ramsay *et al.*, 1996; Di Iulio *et al.*, 1996; Thomson *et al.*, 1997 - chapter 2). However, a change in nitric oxide activity may control parturition by mechanisms other than nitric oxide production. The biological actions of nitric oxide are abrogated via direct reaction with superoxide anion (McCall *et al.*, 1989; Huie and Padmaja, 1993). Moreover superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide and oxygen (Klug-Roth *et al.*, 1973) prolongs the biological half-life of nitric oxide by removing superoxide. SOD exists as three isoenzymes, which are widely distributed. An intracellular copper/zinc containing enzyme is found predominantly in the cytoplasm and nucleus (Crapo *et al.*, 1992), a manganese containing SOD (Mn SOD) is found predominantly in mitochondria (Weisiger and Fridovich, 1973) and a third isoenzyme, extracellular SOD, is found in the extracellular matrix (Marklund, 1984). The importance of SOD in the augmentation of nitric oxide activity is illustrated by experiments showing that diethyldithiocarbamate (DETCA), an inhibitor of Cu/Zn SOD, diminishes nitric oxide-mediated relaxation of vascular smooth muscle (Rubanyi, 1988; Mugge *et al.*, 1991; Omar *et al.*, 1991).

Whilst SOD breaks down superoxide anion, xanthine oxidase synthesises superoxide. Xanthine oxidase appears to be one of the major superoxide producing enzymes in vessel walls (Jarasch *et al.*, 1986). Nitric oxide-induced relaxation can be potentiated in blood vessels by xanthine oxidase inhibitors (Miyamoyo *et al.*, 1996), suggesting that xanthine oxidase is an important regulator of nitric oxide activity.

We hypothesised that expression and activity of SOD and xanthine oxidase might change with the onset of labour in such a way that the biological activity of nitric oxide would be attenuated. The purpose of this study was to test this hypothesis by determining the activity and localisation of Cu/Zn, Mn SOD and xanthine oxidase in human myometrium, placenta and fetal membranes before and after the onset of labour.

Materials and methods

Tissue collection

Twenty-two women undergoing Caesarean section at term (>37 weeks' gestation) were recruited to the study. Eleven had an elective Caesarean section prior to the onset of labour. The other eleven were delivered by emergency Caesarean section during active labour, (cervical dilatation greater than 4 cm). Exclusion criteria and collection of tissue, (myometrium, placenta and fetal membranes with attached decidua) was as described in chapter 2. Each of the biopsies was divided; half was fixed in 10% neutral buffered formalin (BDH, UK) and embedded in paraffin. The other half was snap frozen in liquid nitrogen and stored at -70°C. Informed consent was obtained from each woman and the study was approved by the Local Research Ethics Committee.

Immunocytochemistry

Five micrometer sections of paraffin embedded tissues were cut and mounted on silane-coated slides, heated to 60°C for 30 min, deparaffinized in xylene and rehydrated in a graded alcohol series. Negative control slides were incubated without primary antibody. Two independent observers scored the intensity of staining in five different microscope fields. Intensity and consistency of staining was scored on a scale of 0 to 6, as described in chapter 2. Data were analysed using contingency tables and Chi squared p values were obtained.

Immunocytochemistry for Cu/Zn SOD

Immunocytochemistry was carried out using a monoclonal antibody against human Cu-Zn SOD (Sigma, Poole, UK) diluted 1/200, and an anti-mouse IgG peroxidase kit (Vectastain Elite ABC kit, Vector, Peterborough) as the detection system. Immunoreactive Cu-Zn SOD was localised using 1mg/ml diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.02% H_2O_2 in 50mM Tris HCl, pH 7.6. Following immunocytochemistry sections were washed in distilled water, conterstained with Harris hematoxylin and mounted in DPX (BDH, UK).

Immunocytochemistry for Mn SOD

Immunocytochemistry was performed using a polyclonal antibody raised against human Mn SOD (The Binding Site, Birmingham, UK) diluted 1/250. Sections were pre-treated with a 0.01% (w/v) solution of protease type XXIV (Sigma, Poole, UK) in 0.01% (w/v) calcium chloride for 10 min at 37°C then washed in water and phosphate-buffered saline (PBS), pH 7.5. Antibody binding was detected using an anti-sheep IgG peroxidase kit (Vectastain Elite ABC kit, Vector) and DAB as substrate.

Immunocytochemistry for xanthine oxidase

Immunocytochemistry was carried out on paraffin-embedded sections using a polyclonal antibody raised against purified xanthine oxidase from bovine buttermilk (Lorne Laboratories, Reading, UK). This antibody has been shown to cross-react with human xanthine oxidase. The antibody was diluted 1/1000 and antibody binding was detected on protease-treated sections using an anti-rabbit IgG peroxidase kit (Vectastain Elite ABC kit, Vector).

Isolation of mitochondria for SOD assay

Frozen tissue was homogenised in homogenisation medium (HM) containing 0.3M sucrose, 5mM [morpholino]propanesulfonic acid (MOPS) and 1mM EDTA and the resultant homogenate spun at 2500 g for 5min. An aliquot of the supernatant was collected for assay of total SOD activity and the remainder was spun at 10000 g for 10 min to separate mitochondrial and cytoplasmic cell fractions. The supernatant was collected (cytoplasmic fraction) for SOD assay and the pellet was resuspended in HM medium. Homogenization and low and high speed centrifugation was repeated and the pellet obtained was again resuspended in HM (mitochondrial fraction). The purity of this mitochondrial fraction was determined using transmission electron microscopy.

Determination of SOD activity

Frozen tissue was homogenised in HM and spun at 2500 g for 5 minutes to remove cell debris. SOD activity was determined using an assay kit from Calbiochem (Nottingham, UK). This kit makes use of a proprietary reagent that undergoes alkaline auto-oxidation which is accelerated by SOD (Nebot et al., 1993). Interference from haemoglobin was eliminated by precipitation prior to assay using ice-cold ethanol/chloroform 62.5/37.5 (v/v) followed by centrifugation at 3000 g for 5 min at 4°C. SOD activity was

determined from the Vs/Vc ratio of the auto-oxidation rates measured in the presence (Vs) and absence (Vc) of homogenised tissue. The data obtained was expressed as SOD activity units (U-525) per mg protein. One SOD activity unit is the activity that doubles the auto-oxidation background (Vs/Vc=2). Protein concentration was measured by the method of Lowry (Lowry, 1951). The detection limit of the SOD assay is 0.2U/ml. The intra-assay coefficient of variation was determined by calculating Vs/VC on 12 replicate samples of homogenate of placental tissue. The mean of the obtained data was 1.69, the SD was 0.09 and the CV was 5.2%. The inter-assay mean was 1.74, the SD was 0.09 and CV was 5.2%.

Results

Immunocytochemistry for Cu/Zn SOD in myometrium, placenta and fetal membranes

Cu/Zn SOD was identified in each of the tissues examined (figure 3.1 a-c). There was no difference in localisation or intensity of staining of Cu/Zn SOD in myometrium, placenta or membranes obtained from women delivered before or after the onset of labour. In the myometrium Cu/Zn SOD was localised to myocytes and endothelial cells of myometrial blood vessels (figure 3.1 a). In the placenta intense immunostaining was observed in the syncytiotrophoblast whilst endothelial cells of villous vessels and villous stromal cells exhibited moderate staining (figure 3.1 b). In the fetal membranes there was either a lack of staining or only very weak staining in the amnion, moderate staining in the chorion and extra-villous trophoblast and intense staining in decidua (figure 3.1 c). Specificity of antibody binding was confirmed by the presence of immunostaining for Cu/Zn SOD in epithelial cells in human endometrium, hepatocytes in human liver and epithelial cells in human kidney.

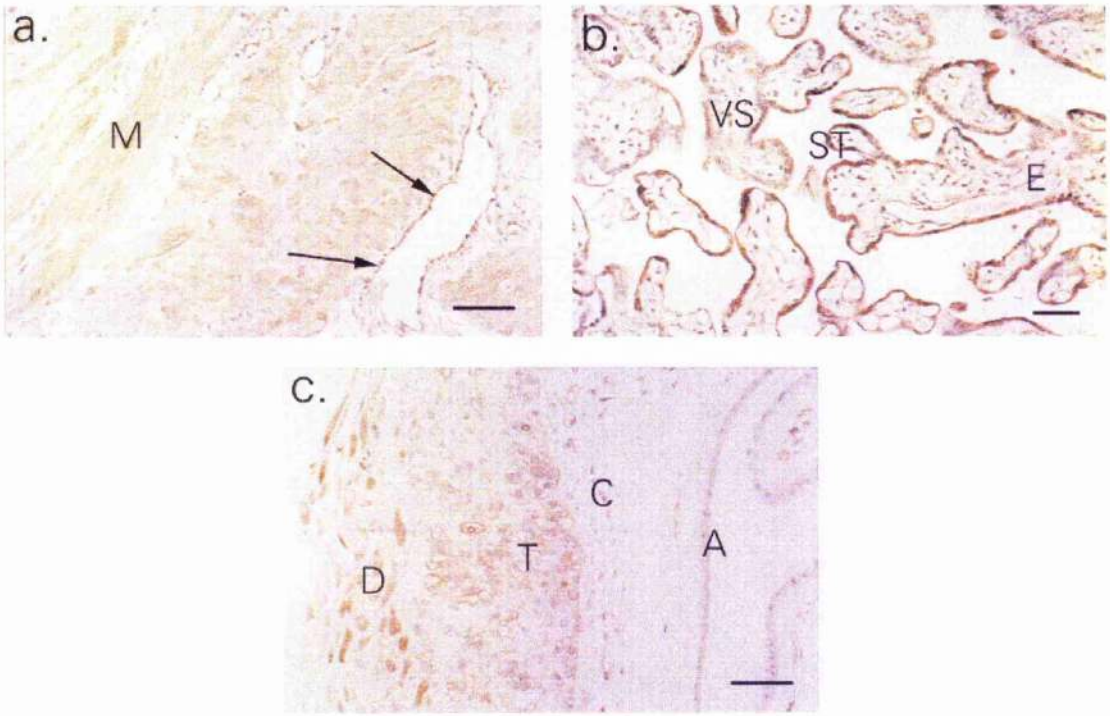

Figure 3.1 Localisation of Cu/Zn superoxide dismutase (Cu/Zn SOD) protein in myometrium, placenta and fetal membranes with attached decidua. **(a)** Cu/Zn SOD localised to myocytes and endothelium of blood vessels in myometrium (arrows). **(b)** In the placenta intense Cu/Zn SOD immunoreactivity was observed in the syncytiotrophoblast whilst endothelial cells of blood vessels and villous stromal cells exhibited moderate staining. **(c)** Faint staining for Cu/Zn SOD staining in amniotic epithelium and moderate staining in chorion and extravillous trophoblast was observed in fetal membranes. Attached decidua showed intense Cu/Zn SOD staining. M = myocytes, E = endothelium, VS = villous stromal cells, ST = syncytiotrophoblast, A = amnion, C = chorion, T = extra-villous trophoblast, D = decidua. Bar = 50 μm.

Immunocytochemistry for Mn SOD in myometrium, placenta and fetal membranes

The cellular distribution of Mn SOD cells in myometrium and placenta was similar to that of Cu/Zn SOD although the intensity of staining varied (figure 3.2 a-c). Mn SOD staining was localised to myocytes and endothelial cells in the myometrium (figure 3.2 a), although the staining was less intense than that observed with the Cu/Zn SOD antibody. In the placenta, intense staining of the synctiotrohoblast and moderate staining of villous vessel endothelial cells and villous stromal cells was observed (figure 3.2 b). In the fetal membranes, in contast to results with Cu/Zn SOD, intense staining of amnion and decidua, moderate staining of extravillous trophoblast and faint staining of chorion was observed (figure 3.2 c). There was no difference in the localisation of staining or in the intensity of staining between tissue obtained before or after the onset of labour. Specificity of antibody binding was confirmed by the presence of immunostaining for Mn SOD in epithelial cells in human endometrium, hepatocytes in human liver and epihtelial cells in human kidney and epithelial cells in human lung.

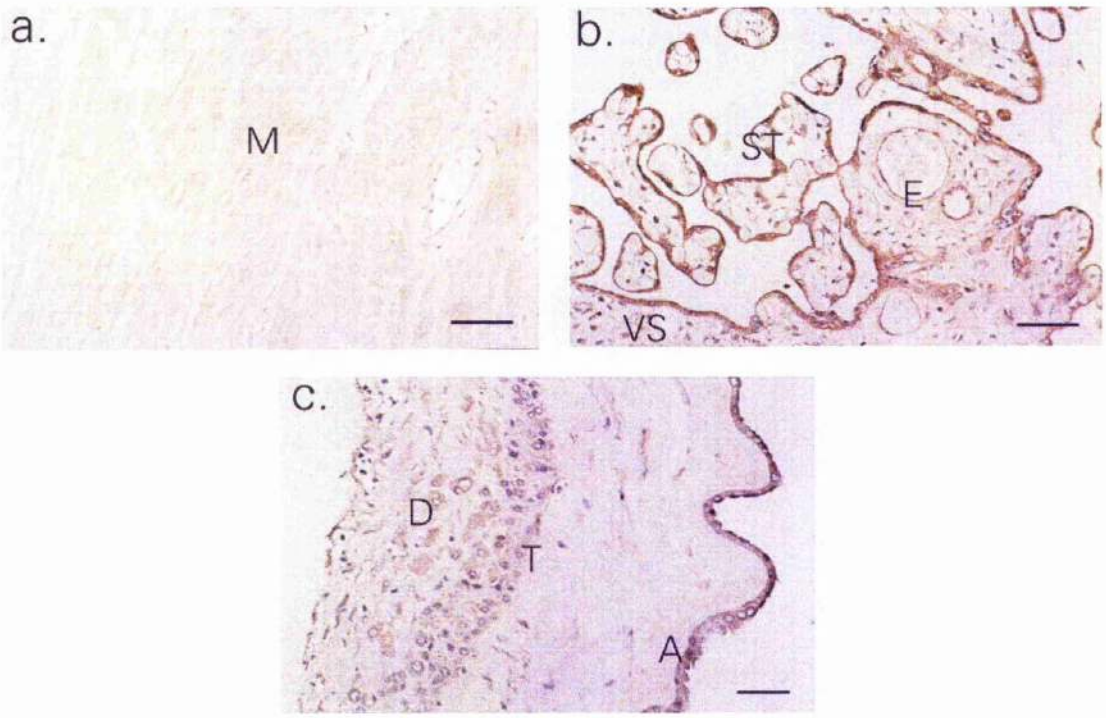

Figure 3.2 Localisation of Mn superoxide dismutase (Mn SOD) protein in myometrium, placenta and fetal membranes with attached decidua. **(a)** Faint Mn SOD staining was observed in myocytes and endothelium of blood vessels in myometrium. **(b)** Mn SOD was localised to syncytiotrophoblast, endothelium and stromal cells of placental villi. **(c)** Localisation of Mn SOD to amniotic epithelium, extravillous trophoblast and decidua. M = myocytes, E = endothelium, VS = villous stromal cell, ST = syncytiotrophoblast, A = amnion, T = extra-villous trophoblast, D = decidual cells. Bar = 50 μm.

SOD activity in myometrium, placenta and fetal membranes obtained prior to or during term labour

SOD activity in each of the tissues studied is shown in table 3.1. There was no significant difference in total or mitochondrial SOD activity between tissue obtained before and after labour. Electron microscopy confirmed that the isolation procedure for mitochondria resulted in a cell fraction consisting mainly of mitochondria.

Table 3.1 Total and mitochondrial SOD activity (mean ± SD, units/mg protein).

Tissue	Total SOD activity (n = 9)		Mitochondrial SOD activity (n = 8)	
	Not in labour	In labour	Not in labour	In labour
myometrium	7.7 ± 4.1	11.8 ± 7.0	ND	ND
placenta	30.6 ± 7.2	28.6 ± 14.5	ND	ND
membranes	10.1 ± 8.9	5.0 ± 3.0	5.7 ±5.8	3.7 ± 4.4

Not significant comparing tissues before and after labour using a Mann Whitney U test.
ND = not determined.

Immunocytochemistry for xanthine oxidase in myometrim, placenta and fetal membranes

Xanthine oxidase immunoreactivity was identified in each of the tissues examined. There was no difference in localisation or intensity of xanthine oxidase immunostaining in myometrium, placenta or membranes obtained from women delivered before or after the onset of labour (figure 3.3 a-c). In the myometrium, myocytes showed a variation of staining intensity for xanthine oxidase ranging from absent to moderate (figure 3.3

a). In myometrial vessel endothelial cells there was either a lack of staining or very weak staining. In the placenta weak to moderate immunostaining was observed in the syncytiotrophoblast and villous stromal cells whilst endothelial cells of villous vessels exhibited only weak staining (figure 3.3 b). In the fetal membranes there was a variation in staining intensity ranging from weak to strong in amnion, moderate to strong staining in the chorion and extra-villous trophoblast and intense staining in decidua (figure 3.3 c).

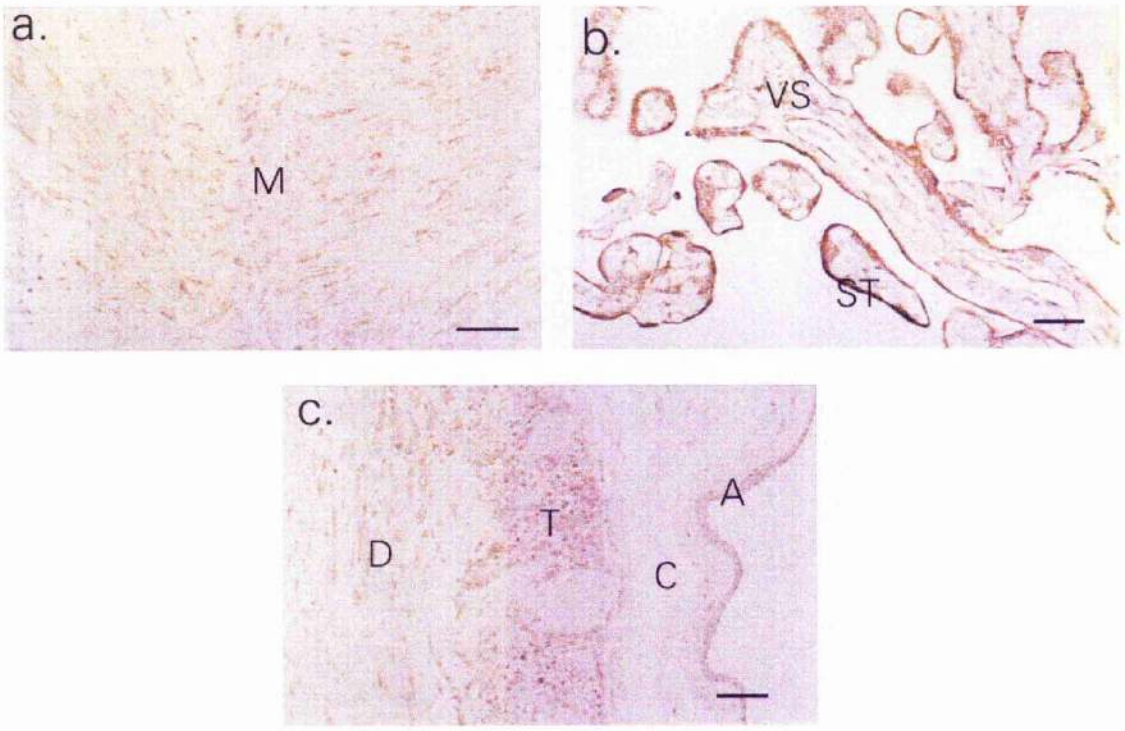

Figure 3.3 Immunostaining of myometrium, placenta and fetal membranes with attached decidua using a xanthine oxidase antibody. (**a**) Faint xanthine oxidase staining in myocytes in myometrium. (**b**) Moderate staining in syncytiotrophoblast and villous stromal cells of the placenta. (**c**) Xanthine oxidase staining was observed in amnion, chorion, extravillous trophoblast and decidua. M = myocytes, VS = villous stromal cell, ST = syncytiotrophoblast, A = amnion, C = chorion, T = extra-villous trophoblast, D = decidual cells. Bar = 50 μm.

Discussion

The results detailed in this study show that Cu/Zn, Mn SOD and xanthine oxidase are present in human myometrium, placenta and fetal membranes and decidua but that there is no difference in expression of these proteins as determined by immunocytochemistry between tissue obtained before or after labour. In addition we have shown that these tissues possess superoxide dismutase activity and that there is no difference in total SOD activity between tissue obtained before or after the onset of labour.

The enzyme superoxide dismutase is now implicated in an increasing number of clinical disorders. Mutations in the Cu/Zn SOD gene are responsible for familial amyotrophic lateral sclerosis, an autosomal dominant degenerative disorder affecting motor neurons in the cortex, brain stem and spinal cord (Rosen *et al.*, 1993). Affected individuals have a reduction of Cu/Zn SOD in their erythrocytes which may lead to oxyradical cell injury. In Down's syndrome, levels of Cu/Zn SOD are elevated by up to 50% (Garber *et al.*, 1979), probably due to the presence of the extra copy of chromosome 21, on which the Cu/Zn gene is located (Tan *et al.*, 1973). Evidence that CuZn SOD may be involved in the pathophysiology of Down's syndrome is suggested by experiments showing that overexpression of the CuZn SOD gene may impair neurotransmitter transport and alter neuromuscular junctions (Elroy-Stein *et al.*, 1988, Avraham *et al.*, 1991).

We hypothesised that the interaction between SOD and nitric oxide might be important in the regulation of the onset of human parturtion. Superoxide, which is a product of both oxidative metabolism and the inflammatory process, interferes with the biological activity of nitric oxide by converting it to peroxynitrite (Beckman and Croe, 1993). In contrast, superoxide dismutase converts superoxide to hydrogen peroxide and oxygen, and by removing superoxide anion prolongs the biological half life of nitric oxide. The precise mechanism by which the myometrium remains quiescent during pregnancy, and

is then converted to an actively contracting organ at the onset of parturition is unknown. Animal studies, and the work described in this thesis (chapter 4), have suggested a role for the smooth muscle relaxant nitric oxide. Uterine production of nitric oxide is high during rat and rabbit pregnancy, and declines at the onset of parturtion, initiating myometrial contractility (Sladek *et al.*, 1993; Natuzzi *et al.*, 1993; Yallampalli *et al.*, 1993). Although in human pregnancy nitric oxide is produced within the uterus, a decline in nitric oxide production at the onset of parturtion has not been demonstrated (Thomson *et al.*, 1997 - chapter 2). Both Cu/Zn SOD and Mn SOD may be important in promoting myometrial relaxation. It is believed that nitric oxide produced within the placenta, decidua, fetal membranes or myometrium may induce guanylate cyclase activity and thereby maintain myometrial quiescence during pregnancy. The biological activity of placental, decidual or myometrial nitric oxide will be potentiated by cytoplasmic Cu/Zn SOD in these tissues. In mitochondria, nitric oxide inhibits oxidative phosphorylation by binding to cytochrome oxidase and inhibiting electron transport (Bates *et al.*, 1995). This will in turn decrease ATP production, attenuating a large range of cellular processes including muscle contaction (Brown, 1992). If the biological activity of mitochondrial nitric oxide is increased, for example by Mn SOD, the effect of nitric oxide on inhibition of smooth muscle contraction should be enhanced. Changes in myometrial Mn SOD may therefore have profound effects on myometrial contractility.

Previous studies have also reported SOD and xanthine oxidase within the human reproductive tract. Cu/Zn SOD has been identified within the endometrium, and increases immediately prior to implantation (Narimoto *et al.*, 1991; Sugino *et al.*, 1996). Furthermore, decidual cells of early pregnancy show strong immunostaining for Cu/Zn and Mn SOD (Sugino *et al.*, 1996). These results agree with our own data showing intense immunostaining for Cu/ZnSOD and Mn SOD in decidual cells at term, and suggest that these enzymes may be expressed in the decidua throughout gestation. *In vitro* in the placenta, Mn SOD but not Cu/Zn SOD is induced in cultured human

trophoblast during differentiation (Church et al 1992). These results appear to be at variance with our own showing both Cu/Zn and Mn SOD expression in the placenta, but these differences may be explained by the fact that they were obtained *in vitro*. Xanthine oxidase activity and mRNA have previously been detected in placenta (Many *et al.*, 1996). Immunostaining for xanthine oxidase has previously been detected in villous trophoblast and non-villous trophoblast of the fetal membranes, but in contrast to our data was not identified within the villous stroma (Many *et al.*, 1996).

If SOD does not play a role in the initiation of parturition what is its function in the reproductive tissues within which it has been identified? After fertilization, embryonic and endometrial expression of both Cu/ZnSOD and Mn SOD has been observed (Harvey *et al.*, 1995; Sugino *et al.*, 1996). It has been suggested that such expression is important for successful implantation, by protecting the blastocyst from damage by superoxide radicals. The function of decidual SOD expression is not known, but expression of Mn SOD may protect the decidua from TNF-mediated cell damage. Decidual cells and predecidual cells produce tumour necrosis factor (TNF) (Tabibzadeh, 1991) and TNF production increases during labour (Opsjon *et al.*, 1993). TNF may damage cells via superoxide anion production (Zimmerman *et al.*, 1989), but TNF-resistant cells produce Mn SOD which protects them from the cytotoxicity of TNF (Wong and Goedell, 1988). Mn SOD is itself induced by TNF (Wong and Goeddel, 1988), and it may be that in decidua Mn SOD is expressed in order to protect decidual cells from high local concentrations of TNF.

In the placenta, SOD may be important in preventing lipid peroxidation. Lipid peroxidation involves oxidative or enzymatic conversion of unsaturated fatty acids to primary products known as lipid hydroperoxides and a variety of secondary metabolites (Carpenter, 1981; Taylor and Morris, 1983). Peroxidation of membrane-associated fatty acids and cholesterol alters membrane fluidity and may eventually induce widespread membrane damage (Kagan, 1988). Pre-eclampsia is associated with

elevated levels of lipid peroxidation products and it has been suggested that lipid peroxidation may play a role in aetiology of the disease (Hubel et al, 1989). If lipid peroxidation is not prevented during pregnancy, diseases such as pre-eclampsia may ensue. Moreover, placental and systemic SOD activity and mRNA is lower in pre-eclampsia compared with normal pregnancy (Wang and Walsh, 1996; Poranen *et al.*,, 1996), suggesting that SOD plays a role in the prevention of pre-eclampsia.

We have failed to show a decline in SOD activity following the onset of labour. Notwithstanding, the SOD present within the uterus may play a role in maintaining myometrial quiescence prior to the onset of parturtion. There is some evidence that the uterus is more sensitive to the tocolytic effects of nitric oxide *in vivo* during pregnancy compared with isolated myometrial strips *in vitro* (Lees *et al.*, 1994; Rowlands *et al.*, 1996; Norman *et al.*, 1997). *In vitro* studies have shown that the inhibitory effect of nitric oxide donors on myometrial contractility can be potentiated by the inclusion of placental tissue within the organ bath (Segal *et al.*, 1997). Taken together, these results suggest that the placenta contains an agent which enhances the effect of nitric oxide during pregnancy. This agent could be SOD.

In conclusion we have detected systems for synthesis and destruction of superoxide in the myometrium and the surrounding placenta, fetal membranes and decidua and have discussed ways in which alterations in the balance between nitric oxide and superoxide production and destruction may affect myometrial contractility. We have failed to demonstrate a change in superoxide production or destruction following the onset of labour and it seems unlikely that these processes are implicated in the initiation of labour. The widespread distribution of Cu/Zn and Mn SOD in reproductive tissues suggests that these enzymes may be important in other reproductive processes such as implantation, the prevention of pre-eclampsia and the maintenance of myometrial quiescence during pregnancy.

Chapter 4

The expression of constitutive nitric oxide synthase in the non-pregnant, preterm and term uterus: myometrial nitric oxide synthase expression is increased during pregnancy.

Introduction

We and others have demonstrated no change in nitric oxide synthesis in myometrium, placenta, fetal membranes or decidua, in tissues obtained immediately before and after the onset of labour at term (Gude *et al.*, 1994; Ramsay *et al.*, 1996; Di Iulio *et al.*, 1996; Thomson *et al.*, 1997a - chapter 2). Whilst nitric oxide withdrawl may not be the final "trigger" in the initiation of human labour, nitric oxide may still be responsible for the maintenance of uterine quiescence during pregnancy. Two strands of evidence support this hypothesis. Firstly, nitric oxide relaxes human myometrium both *in vitro* (Buhimschi *et al.*, 1995; Lee and Chang, 1995; Norman *et al.*, 1997) and *in vivo* (Lees *et al.*, 1994; Rowlands *et al.*, 1996), and secondly, products of the NO-cGMP pathway are present within both the non pregnant (Yallampalli *et al.*, 1994; Buhimschi *et al.*, 1995; Telfer *et al.*, 1997) and pregnant human myometrium (Buhimschi *et al.*, 1995; Thomson *et al.*, 1997a). Taken together, these data suggest that nitric oxide is produced in the myometrium during pregnancy, and could maintain uterine quiescence via a relaxant effect on the myometrium.

In support of a role for nitric oxide in the maintenance of pregnancy, Bansal *et al.* (1997) have reported that myometrial iNOS expression, assessed by immunohistochemistry and Western blotting, was greater in the early third trimester (26-34 weeks gestation) than either the late third trimester (37-41 weeks gestation) or in the non pregnant state. These data suggest that an increase in myometrial iNOS expression might contribute to the maintenance of uterine quiescence during pregnancy.

The role of the constitutive isoforms of NOS (eNOS and bNOS) has not been determined. We hypothesise that the constitutive isoforms of NOS might also be involved in the regulation of uterine activity during pregnancy. The aim of the studies described here was to test this hypothesis, by localising and quantifying eNOS and bNOS isoforms in non-pregnant, preterm pregnant and term pregnant myometrium. We also assessed NOS expression in other tissues within the pregnant uterus (decidua, placenta and membranes).

Materials and methods

Tissue collection

Three groups of women were recruited to the study:

(i) Thirteen non-pregnant, premenopausal women with regular menstrual cycles undergoing hysterectomy for benign disease.

(ii) Eleven pregnant women who were delivered preterm (25-34 weeks gestation) prior to the onset of labour.

(iii) Eleven pregnant women who were delivered at term (>37 weeks gestation) prior to the onset of labour.

Informed consent was obtained from each woman prior to recruitment and the study was approved by the local research ethics committee. Women were excluded from the study if they had a multiple pregnancy or evidence of active infection. The indications for delivery in the pregnant women delivered preterm were: pre-eclampsia (n=4), intrauterine growth restriction (n=2), HELLP syndrome (n=2), placenta praevia (n=2) and fetal distress of unknown aetiology (n=1).

In the non-pregnant uteri, myometrial biopsies were taken from the anterior wall of the lower uterine body immediately after hysterectomy. In the pregnant women myometrial biopsies were taken from the upper margin of the lower uterine segment incision during

Caesearean section. In all groups, myometrium was separated from surrounding structures by sharp dissection. Biopsies of placenta and fetal membranes with attached decidua capsularis were also obtained from pregnant women at the time of Caesarean section.

Once the biopsies were obtained they were divided in two; one half was snap frozen in liquid nitrogen and stored at -70°C for Western analysis. The other half was fixed in 10% neutral buffered formalin (BDH, UK) and embedded in paraffin for immunohistochemistry.

Western blotting

Western blotting was performed on randomly selected biopsies, six from non-pregnant women, five from preterm pregnant women and six from pregnant women at term. The indications for delivery in the preterm group were placenta praevia (n=2), pre-eclampsia (n=1), HELLP syndrome (n=1) and intrauterine growth restriction (n=1). Myometrial extracts were prepared by homogenising frozen tissue in 10mM Tris HCl (pH 7.4), 1% SDS containing protease inhibitors (BDH, UK). Cell debris was removed by centrifuging at 12 000 x g for 5 min. Protein content of the samples was determined by using the BCA protein reagent (Pierce, Chester, UK) and bovine serum albumin reference standards. Electrophoresis was performed using 75 µg aliquots of protein extract in 1 x loading buffer (Bio-Rad, Hemel Hempstead, UK) in 7.5% SDS-polyacrylamide gel. Resolved proteins were transferred onto Hybond-ECL membrane using electrophoresis (Amersham, Little Chalfont, UK). The membranes were allowed to air dry and then placed in blocking buffer (10% non-fat milk, 10 mM Tris, 100mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature.

The following primary antibodies were used: eNOS - monoclonal antibody raised against a 20.4 kDa protein fragment corresponding to amino acids 1030-1209 of human eNOS (Affiniti, Nottingham, UK) at a dilution of 1/1500; bNOS - a polyclonal

antibody raised against amino acids 724-739 of rat brain NOS (Serotec, Oxford, UK) at a dilution of 1/7500. Each primary antibody was diluted in 10mM Tris, 100mM NaCl, 0.1% Tween-20, pH7.5 (TBS-T). The blots were washed for 2 x 2 min, 1 x 15 min and 2 x 5 min in TBS-T then incubated with horseradish peroxidase-conjugated anti-mouse IgG (1/10 000) for eNOS, or anti-rabbit IgG (1/30 000) for bNOS in TBS-T for 1h. The membranes were washed as before then incubated with Amersham ECL detection reagent for 1 min. Thereafter, the membranes were exposed to autoradiographic film (Hyperfilm ECL, Amersham) and the intensity of specific immunoreactive bands was quantified using densitometric scanning. In each blot, one lane was loaded with protein from a positive control (human endothelial cells for eNOS and lysates of rat brain for bNOS). Density of immunostaining was corrected for transfer differences by comparison with intensity of positive control bands. Data were analysed statistically using a Mann Whitney U test. $P < 0.05$ was assumed to indicate statistical significance.

Immunocytochemistry

Immunohistochemistry for eNOS and bNOS was performed on each of the biopsies as previously described in chapter 2. Paraffin embedded sections of 8 µm diameter were pretreated with protease digestion (0.01% [w/v]) protease type XXIV in a 0.01% (w/v) solution of calcium chloride (Sigma, Poole, UK) for 10 min at 37°C prior to staining for eNOS or microwaving (4 x 5 min at full power in citrate buffer) prior to staining for bNOS. Antibodies were used as for Western blotting, at the following dilutions: eNOS 1/100 and bNOS 1/1750 for placenta and fetal membranes and 1/2000 for myometrium.

Negative controls for eNOS included sections incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 *Aspergillus niger* glucose oxidase (Dako Ltd., High Wycombe, UK), an enzyme which is not expressed in mammalian cell systems. A section of human umbilical cord was used as a positive control. Immunostaining for eNOS was confined to the umbilical cord

endothelium. Negative controls for bNOS included slides incubated without the primary antibody and slides incubated with non-immune rabbit serum (SAPU, Carluke, UK), in place of the primary antibody. In addition, liquid-phase absorption controls with the immunogen peptide were performed as described in chapter 2. A section of rat brain was used as a positive control.

The localisation of each of the two isoforms of NOS and intensity of staining were recorded by two investigators independently, who were blind to the source of the biopsy. Intensity and consistency of staining was scored on a scale of 0-6 as described in chapter 2. Contingency table analysis was performed to determine whether there was a difference in the intensity of staining between the three groups. A chi squared p value of <0.05 was assumed to indicate statistical sigificance.

Results

Western blotting

1. eNOS

The antibody against eNOS reacted with a band at 135kDa in the lysate of human aortic endothelial cells. Similar bands were detected in samples of human myometrium (figure 4.1). Densitometric analysis of bands indicated that eNOS protein levels were significantly higher in preterm myometrial biopsies compared with either term ($p < 0.05$) or non-pregnant myometrial biopsies ($p < 0.01$) (figure 4.2).

2. bNOS

The bNOS antibody reacted with a 160 kDa protein in the rat brain lysate corresponding to bNOS. Similar bands were detected in samples of human myometrium (figure 4.3). Densitometric analysis of bands indicated that bNOS protein levels were significantly higher preterm ($p < 0.05$) compared with non-pregnant, but that there were no significant differences in levels in term and preterm myometrium (figure 4.4).

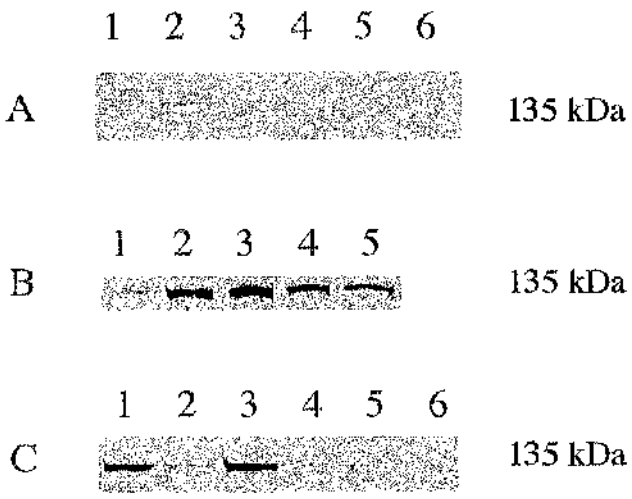

Figure 4.1 Western blots of non pregnant (A) (n=6), preterm (B) (n=5) and term (C) (n=6) myometrium using an antibody directed against eNOS. The putative eNOS band is seen at 135k Da.

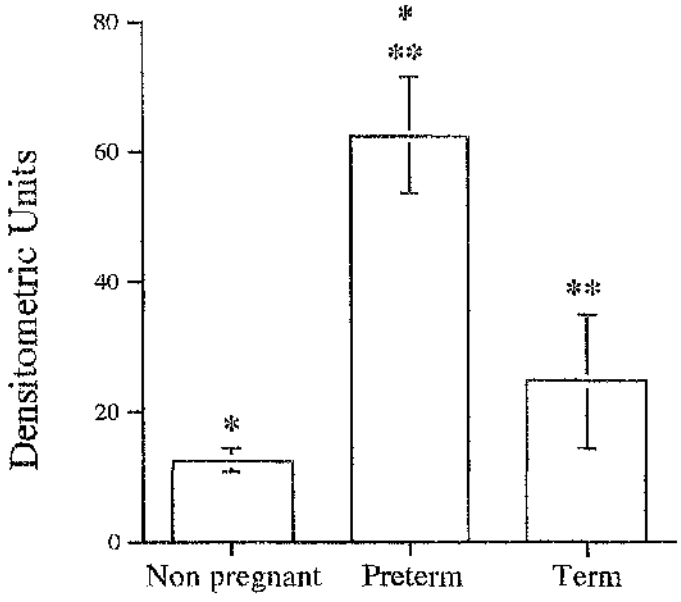

Figure 4.2 Graph showing mean (SE) relative optical density of bands of eNOS protein in non pregnant (n=6), preterm pregnant (n=5) and term pregnant (n=6) myometrium after Western blotting. Optical density was greater in the preterm pregnant than the non pregnant group (* $p < 0.01$); and in the preterm pregnant compared with the term pregnant group (** $p < 0.05$).

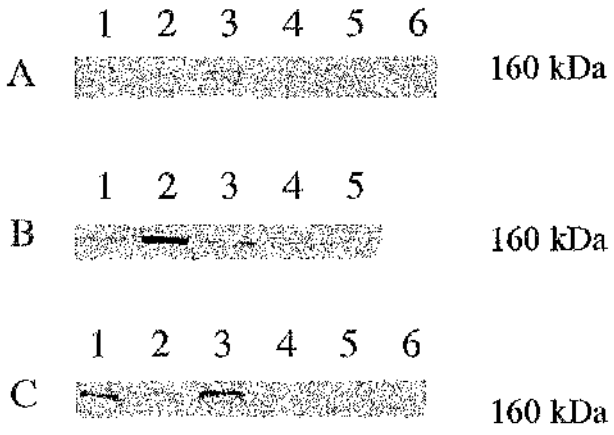

Figure 4.3 Western blots of non pregnant (A) (n=6), preterm (B) (n=5) and term (C) (n=6) myometrium using an antibody directed against bNOS. The putative bNOS band is identified at 160 kDa.

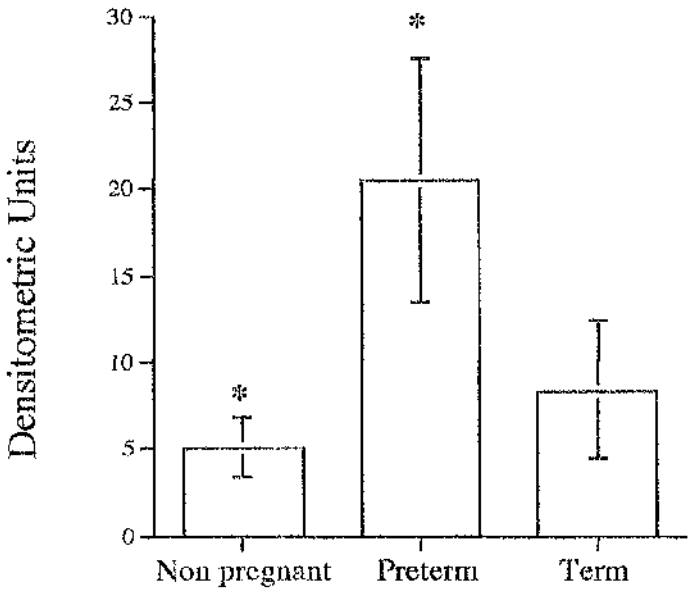

Figure 4.4 Graph showing mean (SE) relative optical density of bands of bNOS protein in non pregnant (n=6), preterm pregnant (n=5) and term pregnant (n=6) myometrium after Western blotting. Optical density was greater in the preterm pregnant than the non pregnant group (* $p < 0.05$).

Immunocytochemistry

eNOS

Within sections of myometrium, the eNOS protein was localised to myometrial smooth muscle cells (myocytes) and endothelial cells lining blood vessels. Intensity of staining within myocytes was greater in preterm myometrium compared to both non-pregnant ($p < 0.001$) and term myometrium ($p < 0.005$) (figure 4.5). Similar differences were seen in endothelial cells within the myometrium ($p < 0.05$ and $p < 0.01$ respectively). Myocytes in sections obtained at term had a significantly greater staining intensity to those in non pregnant samples ($p < 0.05$). No such differences were observed in endothelial cells.

Within the placenta, eNOS staining was of greatest intensity in the syncytiotrophoblast, of moderate intensity in endothelial cells and was weakly present in villous stromal cells. eNOS immunostaining was significantly more intense within syncytiotrophoblast and endothelial cells in preterm sections than those at term ($p < 0.02$) (figure 4.6). There was no difference in the staining intensity within stromal cells in the two groups. In fetal membranes, eNOS immunoreactivity was localised to amniotic epithelial cells, extravillous trophoblast (the remains of the cytotrophoblastic cells of the trophoblastic shell) and decidual stromal cells. Staining was absent from chorionic connective tissue. There was no difference in the intensity of staining in any of the above cell types when comparing preterm to term sections. There was no staining in the negative control slides.

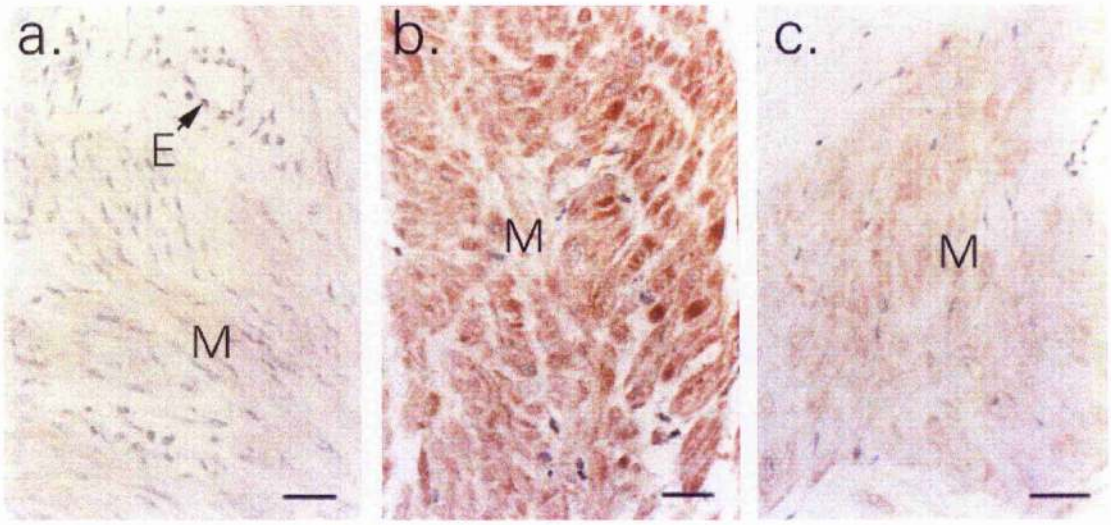

Figure 4.5 Immunohistochemical localisation of endothelial NOS (eNOS) in **(a)** non-pregnant, **(b)** preterm pregnant and **(c)** term pregnant myometrium. eNOS localised to the myometrial smooth muscle cells and the endothelium of blood vessels (arrow). Expression of eNOS was greater in preterm myometrium compared to both non-pregnant and term pregnant myometrium. The negative controls (see Methods) exhibited no reactivity. M = myocytes, E = endothelium. Bar = 50μm.

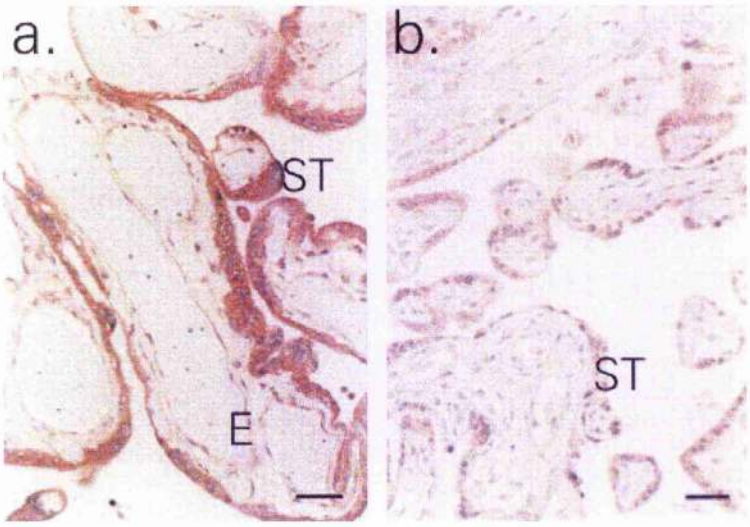

Figure 4.6 Expression of endothelial NOS (eNOS) in **(a)** preterm and **(b)** term placenta. Immunostaining for eNOS was more intense in syncytiotrophoblast and endothelial cells in preterm sections than those at term. The negative controls (see Methods) exhibited no reactivity. E = endothelium, ST = syncytiotrophoblast. Bar = 50μm.

bNOS

In myometrium, bNOS protein was detected within myocytes and endothelial cells (figure 4.7). The intensity of staining within myocytes was greater in preterm sections than either that of non-pregnant ($p < 0.001$) or term ($p < 0.05$) sections. In addition, bNOS staining in myocytes in sections obtained at term had a significantly greater staining intensity to those in non-pregnant samples ($p < 0.05$). Immunostaining intensity in myometrial vessel endothelial cells was greater in the preterm pregnant compared with the non pregnant group ($p < 0.05$). There were no other differences in the groups in endothelial cell staining.

bNOS protein was detected in syncytiotrophoblast cells, villous stromal cells and endothelial cells in placenta and within amniotic epithelial cells, chorion, trophoblast and decidual cells of the fetal membranes. There was no significant difference in the intensity of staining within any of these cell types when comparing the two groups of pregnant women. Staining was absent when the primary antibody was omitted. In liquid phase absorption controls, the staining disappeared when 10 nmol/ml of the bNOS immunogen was added to the primary antibody prior to incubation with the sections, indicating specificity of the primary antibody for bNOS.

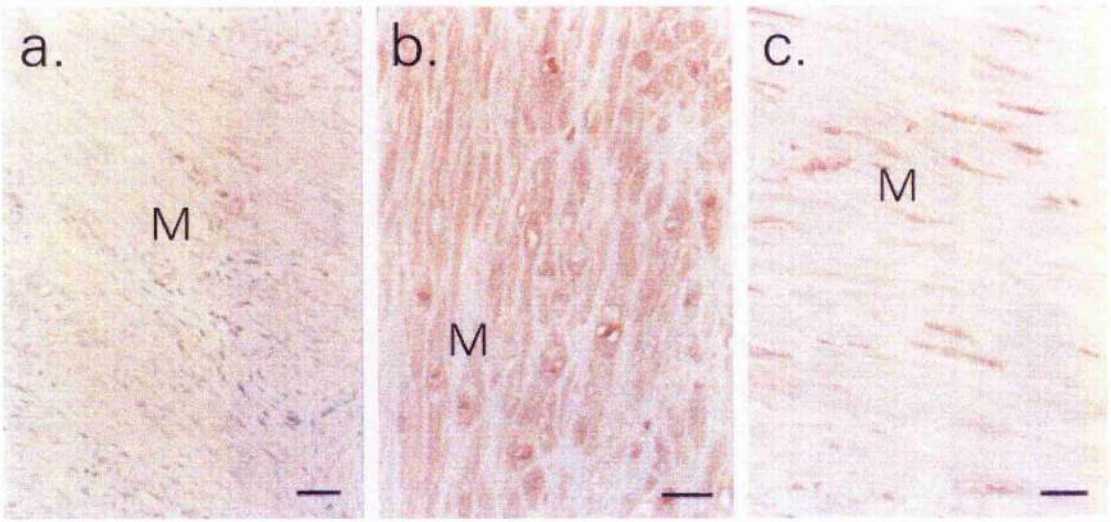

Figure 4.7 Immunohistochemical localisation of brain NOS (bNOS) in **(a)** non-pregnant, **(b)** preterm pregnant and **(c)** term pregnant myometrium. bNOS localised to the myometrial smooth muscle cells and the endothelium of blood vessels. Expression of bNOS was greater in preterm myometrium compared to both non-pregnant and term pregnant myometrium. The negative controls (see Methods) exhibited no reactivity. M = myocytes. Bar = 50μm.

Discussion

These studies demonstrate that the myometrial expression of constitutive NOS, (eNOS and bNOS), determined using Western blotting and immunohistochemistry, has been up-regulated by the early third trimester of pregnancy compared to the non-pregnant state. Furthermore, there is a decline in myometrial eNOS (assessed by immunohistochemistry and Western blotting), and in myometrial bNOS (assessed by immunohistochemistry) from the early third trimester to term. Taken together with the results of Bansal *et al.*, (1997) these data indicate that the myometrial expression of each of the three isoforms of NOS is increased during human pregnancy and declines during the third trimester. Since NO is a myometrial relaxant, we propose that such an increase in NOS will contribute to the quiescence of the myometrium during pregnancy.

The timing of parturition is a crucial event in the survival of any species, and it seems unlikely therefore to be controlled by a single biochemical pathway. In human pregnancy, numerous factors, including prostaglandin production, cytokines, gap-junction formation, corticotrophin releasing factor, G protein and thiol protein disulphide isomerase appear to play a role in the regulation of uterine contractility throughout gestation and at the onset of labour (Europe Finner *et al.*, 1994; McLean *et al.*, 1995; Hirst *et al.*, 1995; Morrison *et al.*, 1996; Schwartz, 1997). It seems clear that nitric oxide withdrawal does not play a part in the acute stimulus to labour at term since NOS expression decreases prior to the onset of labour. Furthermore there is no change in NOS activity or expression comparing uterine tissues taken immediately before and after the onset of labour (Thomson *et al.*, 1997a - chapter 2). However, it is recognised that myometrium from women in late gestation or in active labour, is less sensitive to tocolytic agents, and more sensitive to agents which stimulate uterine contractions than myometrium from women in mid-pregnancy (Soloff *et al.*, 1979; Yallampalli and Garfield, 1994; Kim *et al.*, 1995). A decline in myometrial NOS

activity prior to the end of the third trimester might be one of the mechanisms by which the uterus is sensitised to contractile agents, and thus prepared for the onset of labour.

We have shown that each of the isoforms of constitutive NOS is upregulated in the myometrium during pregnancy. Although NOS is increased during rat pregnancy, the isoforms responsible are not known. Data from gene knockout animals suggests that an absence of any single isoform of NOS has no effect on the normal gestational period (Huang *et al.*, 1993; Huang *et al.*, 1995; Wei *et al.*, 1995). Taken together, these data imply that each of the isoforms of NOS contributes to the maintanance of uterine quiescence during pregnancy, but that no single isoform is by itself crucial.

Factors controlling the observed up-regulation in myometrial constitutive NOS expression during parturition remain obscure. In animals both estrogen and progesterone have been proposed as regulators of NOS *in vivo*. Estrogen upregulates guinea pig constitutive NOS expression (Weiner *et al.*, 1994). In sheep uterine artery, estrogen enhanced endothelial NOS activity, and in rat hypothalmus, estrogen increased bNOS expression (Veille *et al.*, 1996; Cecatelli *et al.*, 1996). In studies of women treated with a GnRH analogue together with either estrogen replacement or placebo, fasting plasma nitrate concentrations were higher in the estrogen treatment group. This suggests that estrogen increased "total body" nitric oxide synthesis (Ramsay *et al.*, 1995). Since human pregnancy is associated with an increase in circulating estradiol concentration (Turnbull *et al.*, 1974), it is tempting to speculate that estrogen is responsible for the observed increase in myometrial constitutive NOS expression. Further studies are required to determine whether this hypothesis is correct.

A potential concern with our study is that the "preterm" group were made up of women with abnormal pregnancies. The majority of biopsies from women in the preterm Caesarean section group were collected from patients delivered either because of pre-eclampsia or intrauterine growth restriction. It is possible that the observed differences

between the groups reflect the indications for delivery rather than the differences in gestation. However, there is no evidence that eNOS expression in myocytes is altered by pre-eclampsia or intrauterine growth retardation. Indeed, in our study, when histological slides of preterm myometrium obtained from women with pre-eclampsia or intrauterine growth retardation were compared with preterm myometrium obtained from women delivered for other reasons, no differences were observed in eNOS or bNOS staining in myoctes. A formal statistical analysis was not performed because of the small size of the group. We believe therefore that the myometrial changes in eNOS and bNOS are dependent upon the stage of gestation rather than the indications for Caesarean section.

It is more likely that the observed differences in placental eNOS are due to the indications for delivery. We assessed the expression of eNOS and bNOS in placenta and fetal membranes, collected from women at term and preterm. Whilst the sites of localisation of eNOS were similar at each gestation, we found that the intensity of staining for eNOS (but not bNOS) was greater in preterm placental syncytiotrophoblast and preterm placental endothelium than in tissues obtained at term. We did not attempt to perform Western blotting on placenta to confirm this finding because of the multiple cell types present therein, each of which has variable expression of NOS (Thomson *et al.*, 1997a - chapter 2). Again, the majority of histochemically examined biopsies from women in the preterm Caesarean section group were collected from patients delivered either because of pre-eclampsia or intrauterine growth restriction. Each of these conditions is associated with an increase in placental eNOS expression (Myatt *et al.*, 1997). It has been postulated that the greater intensity of eNOS staining in the placenta in the preterm group is a compensatory mechanism attempting to increase feto-placental blood flow in this condition. We propose that differences we observed placental eNOS expression when term and preterm tissues were compared may reflect the indications for preterm Caesarean section in these patients, rather than a true gestational effect.

Endothelial NOS (but not bNOS) expression was also greater in myometrial vessels from preterm compared with term women. Previous studies have shown that the myometrial vessels in women with pre-eclampsia are different from those in normal pregnant women (Brocklesby *et al.,* 1998). The difference in eNOS expression between term and preterm myometrial vessels may be due to the high proportion of women with pre-eclampsia or intrauterine growth restriction in the preterm group. Further work is required to confirm this hypothesis.

We have demonstrated that the myometrial expression of each of the constitutive isoforms of NOS is increased during pregnancy, but declines during the third trimester. Bansal *et al.*, (1997) showed that preterm labour was associated with lower concentrations of myometrial iNOS. These data suggest that the timing of parturiton might be manipulated by the use of agents which will increase or decrease myometrial NOS expression. We suggest that the use of therapeutic agents which either liberate nitric oxide or alter myometrial NOS activity, should be targeted specifically to the myometrium. Later in this thesis (chapter 6), it is shown that nitric oxide donors induce cervical ripening in the first trimester of human pregnancy (Thomson *et al.*, 1997b). If such a mechanism also operates in late pregnancy, then the systemic administration of nitric oxide donors, whilst inducing myometrial relaxation, would stimulate cervical ripening and therefore potentially promote preterm delivery. If however, agents could be devised which would stimulate myometrial NOS expression specifically, these agents may be useful in the treatment of preterm labour, and reduce the substantial morbidity and mortality associated with this condition.

Chapter 5

Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process

Introduction

The studies reported in chapters 2 and 4 of this thesis focused on the smooth muscle relaxant effects of nitric oxide and investigated their involvement in the maintenance of myometrial quiescence and in the initiation of parturition in human pregnancy. However, nitric oxide is also a potent inflammatory mediator and leukocytes are recognised to be a rich source of nitric oxide (Michel and Feron, 1997). iNOS was originally purified and cloned from a macrophage cell line and has since been identified in neutrophils and mast cells (McCall *et al.*, 1989; Kolb *et al.*, 1994; Huang *et al.*, 1995). Similarly, the constitutive isoforms of NOS (eNOS and bNOS) have been identified within leukocytes (Riesco *et al.*, 1993).

Inflammatory cells play a crucial role in human parturition (Kelly, 1996). Cervical ripening, an early event in normal labour, has been compared to an inflammatory reaction and is characterised by an accumulation of leukocytes in the cervical stroma (Junqueira *et al.*, 1980; Liggins, 1981). The inflammatory infiltrate within the ripened cervix consists predominantly of neutrophils and macrophages and accumulates in the stroma before the onset of labour at term (Bokstrom *et al.*, 1997). These leukocytes contribute to cervical remodelling by releasing proteolytic enzymes such as collagenase and elastase (Rajabi *et al.*, 1988; Osmers *et al.*, 1992). Elsewhere in the pregnant uterus, inflammatory cells are known to infiltrate the placenta, maternal decidua and the fetal membranes during parturition, and may play a role in spontaneous rupture of the membranes (Halgunset *et al.*, 1994; Rosenberg *et al.*, 1996).

There are no data on whether inflammatory cells infiltrate myometrium during labour. However, indirect evidence suggests that inflammatory cells may infiltrate the myometrium at the time of parturition. Rechberger and Woessner, (1993) demonstrated that collagenase activity is increased in lower segment myometrium during labour and proposed that neutrophils may be responsible for much of the increase. Furthermore, Osmers *et al.*, (1995) demonstrated a significant increase in interleukin-8 concentration in lower segment myometrium during labour, and proposed that this increase could result in an influx of neutrophils. The presence of these leukocytes in myometrium has been examined prior to the onset of labour only. Butterworth *et al.*, (1991) found no neutrophils in the myometrium of both normal and pre-eclamptic women before the onset of labour.

The attachment and extravasation of circulating leukocytes is controlled by expression of cell surface adhesion molecules on both the circulating cells and the vascular endothelium (Akyama *et al.*, 1989; Bevilacqua, 1993). An increased expression of adhesion molecules on the endothelium occurs in inflammatory conditions and supports the recruitment and aggregation of leukocytes (Bevilacqua, 1993) The major endothelial adhesion molecules involved in leukocyte attachment and transendothelial migration include E-selectin, intercellular adhesion molecule-1 and 2 (ICAM-1 and 2), platelet endothelial cell adhesion molecule (PECAM) and vascular cell adhesion molecule-1 (VCAM-1). The purpose of the present study was to investigate inflammatory cell subpopulations in human myometrium during pregnancy and labour. Specifically, we aimed to determine whether myometrium in each of the lower and upper uterine segments, like cervical stroma, maternal decidua and the fetal membranes, is infiltrated by leukocytes during parturition. Furthermore, the expression and distribution of the cell adhesion molecules, E-selectin, ICAM-1 and 2, PECAM and VCAM-1, were assessed in myometrium during labour to investigate possible mechanisms of leukocyte accumulation.

Materials and Methods

Subjects and collection of tissue

Pregnant women delivered by lower uterine segment Caesarean section and non-pregnant women undergoing hysterectomy were recruited to the study. Informed consent was obtained from each woman prior to recruitment and the study was approved by the Local Research Ethics Committee.

Myometrial biopsies were collected from three groups of women:

(i). 18 pregnant women who were delivered before the onset of labour at term, (>37 weeks gestation).

(ii). 18 pregnant women who were delivered during active labour at term, (cervical dilatation greater than 4 cm and less than 9 cm). Women were excluded from the study if they had a multiple pregnancy, evidence of active infection, or following induction of labour.

(iii). 13 non-pregnant, pre-menopausal women with regular menstrual cycles undergoing hysterectomy for benign disease.

In groups (i) and (ii), a myometrial biopsy was obtained from the upper margin of the lower uterine segment incision during the Caesarean section. Additionally, in 7 of the women in group (i), and 5 of the women in group (ii), a myometrial biopsy was obtained from the upper uterine segment by dissecting a strip of myometrium from the inner aspect of the posterior uterine wall and inserting a haemostatic suture when required. In group (iii), myometrial biopsies were taken from the anterior wall of the lower uterine body, immediately following hysterectomy. Each of the 12 upper segment and the 13 non-pregnant myometrial biopsies was fixed in 10% neutral buffered formalin (BDH, UK) and embedded in paraffin. The 36 lower segment

myometrial biopsies were divided; one half was fixed in formalin and embedded in paraffin, and the other half was snap frozen in liquid nitrogen and stored at -70°C.

Identification of inflammatory cells

Inflammatory cells were detected using immunocytochemistry, with a panel of antibodies as shown in table 5.1. Serial sections were stained for naphthol AS-D chloroacetate esterase activity, an enzyme considered specific for cells of granulocytic lineage, (neutrophils, eosinophils and basophils).

Table 5.1 Primary antibodies used for leukocyte immunocytochemistry

Antigen	Cell Type	Clone	Pretreatment	Dilution	Supplier
CD 15	neutrophils	2.35.14	microwave	1/60	SAPU, UK
neutrophil elastase	neutrophils	NP 57	nil	1/150	Dako Ltd, UK
CD 68	macrophages	PG-M1	trypsin	1/300	Dako Ltd, UK
mast cell tryptase	mast cells	AA1	trypsin	1/300	Dako Ltd, UK
CD 3	T lymphocytes	polyclonal	microwave	1/50	Dako Ltd, UK
CD 20	B lymphocytes	L 26	microwave	1/50	Dako Ltd, UK

Immunocytochemistry

1. Neutrophils, macrophages, T-lymphocytes and B-lymphocytes

Inflammatory cells were identified in each of the biopsies obtained before and after the

onset of labour at term. Eight micrometer thick sections were cut from the paraffin embedded tissues and mounted on silane-coated slides, heated to 60°C for 35 mins, deparaffinised in xylene and rehydrated in a graded alcohol series. If required (table 5.1), the sections were then pretreated to retrieve the antigen by microwaving at full power for 4 x 5 mins in citrate buffer (10mM, pH 6.0), or by enzymatic digestion with a 0.1% (w/v) trypsin (Sigma, UK) solution in Tris buffer (pH 7.6) containing 0.1% (w/v) calcium chloride, for 10 mins at room temperature.

The sections were pre-incubated with 1.5% (w/v) horse serum in PBS (10 mM sodium phosphate, pH 7.5, 120 mM sodium chloride) for 30 mins at room temperature. They were then incubated for one hour with the primary antibody diluted in 1.5% horse serum. Next, sections were washed in PBS before incubation with biotinylated anti-mouse immunoglobulin. This antibody, from a Vectastain Elite ABC kit (Vector, Peterborough, UK) was first diluted in 1.5% horse serum and 1.5% normal human serum. After washing as before, sections were placed in 3% hydrogen peroxide in methanol for 10 mins at room temperature. The sections were thoroughly washed again, then incubated for 30 mins with avidin DH / biotinylated horseradish peroxidase H reagent (Vectastain Elite ABC kit) in PBS before final washing. The antigen was localised using 1 mg/ml diaminobenzidine tetrahydrochloride (DAB), 0.02% H_2O_2 in 50mM Tris.Cl, pH7.6, and appeared as a brown end-product.

Negative controls included sections incubated without the primary antibody and sections incubated with a mouse monoclonal antibody against IgG1 *Aspergillus niger* glucose oxidase (Dako Ltd., High Wycombe, UK), an enzyme which is not expressed in mammalian cell systems. Tonsillar tissue was used as positive controls for CD 3, CD 20 and CD 68.

2. Mast cells

Mast cells were localised in non-pregnant and pregnant myometrial biopsies. The paraffin embedded sections were prepared as before and digested in a trypsin solution to retrieve the antigen (table 5.1). The sections were then pre-incubated with 20% normal goat serum (SAPU, Carluke, UK) in PBS (10 mM sodium phosphate, pH 7.5, 120 mM sodium chloride) for 30 mins at room temperature. They were then incubated for 90 mins with a monoclonal antibody raised against mast cell tryptase (Dako Ltd., High Wycombe, UK) diluted 1/300 in 2% normal goat serum. The primary antibody was omitted from the negative control slides. Next, sections were washed in PBS before incubation with goat anti-mouse IgG alkaline phosphatase (Sigma, UK) diluted 1/200 in 2% normal goat serum and 5% normal human serum. The sections were thoroughly washed again and immunoreactive tryptase was localised using Fast Red Substrate (Sigma, UK). Finally, the sections were counter-stained with Harris haematoxylin.

3. Cell adhesion molecules

Five micrometer thick sections were cut from the frozen tissue and mounted on silane coated slides. The sections were fixed in acetone for 10 mins, washed in TBS, and placed in 0.5% hydrogen peroxide in methanol for 30 mins at room temperature. The sections were then washed as before and pre-incubated with 20% (w/v) normal goat serum (SAPU, Carluke, UK) in TBS for 30 mins at room temperature. The sections were then incubated for 16 hours at 4°C with the primary antibody diluted in 1.5% horse serum. Table 5.2 shows the characteristics of the primary antibodies. The primary antibody was omitted from the negative control slides. Next, sections were washed in TBS before incubation for one hour with biotinylated goat anti-mouse immunoglobulin (Dako Ltd., UK), diluted in 2% normal goat serum and 1.5% normal human serum. The sections were thoroughly washed again, then incubated for 30 mins with streptavidin horseradish peroxidase (Dako Ltd., UK) in TBS before final washing. The antigen was localised as previously described using DAB.

Table 5.2 Primary antibodies used for cell adhesion molecule immunocytochemistry

Antigen	Clone	Dilution	Supplier
ICAM-1	BBIG-I1	1/500	R&D Systems, Abingdon, UK
ICAM-2	CBR-IC2/2	1/200	Chemicon, Harrow, UK
PECAM	9G11	1/500	R&D Systems, Abingdon, UK
VCAM	BBIG-V1	1/500	R&D Systems, Abingdon, UK
E-selectin	BBIG-E4	1/250	R&D Systems, Abingdon, UK

Identification of granulocytes

Granulocytic cells were identified by staining for naphthol AS-D chloroacetate esterase activity. This activity was determined using a commercial kit (Sigma Diagnostics, UK). Briefly, 1ml of sodium nitrite solution (0.1 mol/L) was added to 1ml of Fast Red Violet LB Base Solution (15 mg/ml fast red violet LB base in 0.4 mol/L hydrochloric acid with stabiliser) and allowed to stand for 2 mins. This solution was then added to 40 ml prewarmed (37°C) deionised water, 5 ml of Trizmal 6.3 Buffer Concentrate (TRIZMA maleate, 1 mol/L with surfactant, pH 6.3) and 1 ml of Naphthol AS-D Chloroacetate Solution (naphthol AS-D chloroacetate 8 mg/ml and stabiliser). The slides were incubated in this solution for 15 mins at 37°C and then rinsed thoroughly in deionised water. Sites of activity showed violet granulation.

Quantification of inflammatory cells and statistical analysis

The inflammatory infiltrate was quantified (i) to determine whether inflammatory cells infiltrate the myometrium during labour at term, (ii) to compare the density of the

inflammatory cell infiltrate in upper and lower uterine segment myometrium and (iii) to compare mast cell density in non-pregnant and pregnant myometrium. Inflammatory cells were identified by either brown staining, (neutrophils, macrophages, T cells and B cells) or red staining, (mast cells). In each section of myometrium, the number of positive cells was counted in a high powered field, (x400 magnification within parts of a lined grid covering an area of 0.02 mm^2). Six different fields were counted by two observers who were blinded to the specimen details. Areas containing blood vessels were avoided so that leukocytes within vessels were not included. The average number (arithmetic mean) of positive cells recorded per field by each observer was calculated, and then a mean of these two values obtained. Statistical comparisons of the means were performed using 3 factor ANOVA with Scheffe's S as a *post hoc* test. Significant differences between groups were explored using Mann Whitney U tests. The sites of expression of each of the cell adhesion molecules were recorded by the observers who were blinded to the specimen details. Differences in the expression of these molecules before and after labour were analysed using the chi-squared test.

Results

Staining for naphthol AS-D chloroacetate esterase activity, which identifies inflammatory cells of granulocytic lineage, revealed that myometrial biopsies removed from labouring women (group ii) showed a marked inflammatory infiltrate in the muscle connective tissue in 17 of the 18 specimens (figure 5.1). In contrast, the myometrium in the non-labouring women (group i, n=18) did not exhibit an inflammatory infiltrate. The infiltrate was most dense at both the luminal (decidual) edge of the myometrium and also in and around blood vessels. Further, the infiltrate was most striking in the myometrium obtained from the lower uterine segment, but was also present in the myometrial biopsies obtained from the uterine fundus during labour.

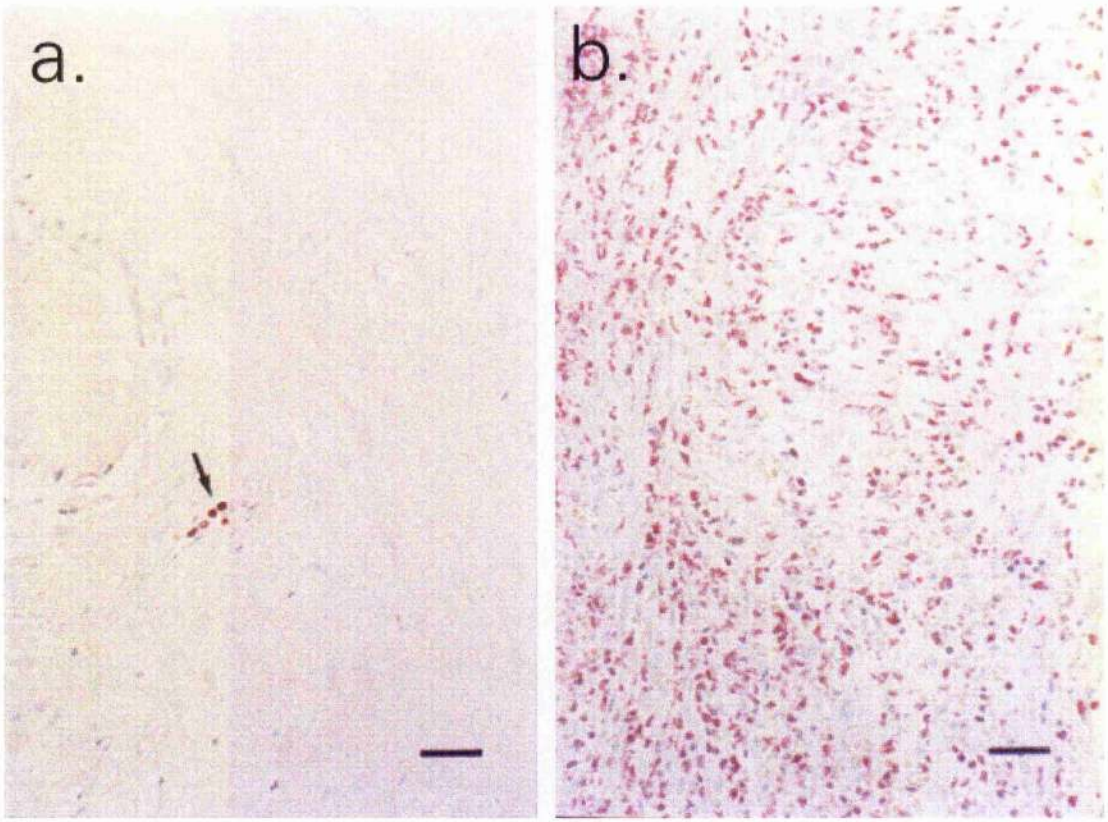

Figure 5.1 Identification of granulocytes in myometrium. The granulocytes are localised by staining for naphthol AS-D chloroacetate esterase activity and appear as violet granulation. **(a)** Granulocytes are sparse in myometrium collected from non-labouring women (arrow) and **(b)** are abundant in myometrium obtained during labour. Bar = 50 μm.

Analysis of individual cell types using immunocytochemistry showed significantly more inflammatory cells in labouring myometrium compared to non-labouring biopsies, (Scheffe's test, $p = 0.0001$), and significantly more inflammatory cells were present in the lower uterine segment compared to the upper uterine segment myometrium, (Scheffe's test, $p < 0.02$).

The leukocyte subpopulations were characterised as follows:

Neutrophils

Neutrophils were sparse in myometrium obtained before the onset of labour and abundant in biopsies obtained during labour, (figure 5.2). A significant increase in myometrial neutrophil density occurred following the onset of labour in both the lower, (table 5.3), and the upper uterine segments, (table 5.4). During labour, the neutrophil density was significantly greater in the lower than in the upper uterine segment, ($p<0.02$). Within the labouring biopsies, the elastase antigen was localised both within the neutrophil cytoplasm and extracellularly in the vicinity of the leukocytes, suggesting that a proportion of these cells had degranulated.

Table 5.3 Number of inflammatory cells in lower segment myometrium before and during labour at term, median (interquartile range) per high powered field.

Cell type	Before labour (n=18)	During labour (n=18)	Significance
Neutrophils	0.2 (0 - 0.3)	39.2 (17.7 - 92.3)	p<0.0005
Macrophages	3.8 (2 - 5.5)	27.5 (11.8 - 34.5)	p<0.0005
T-lymphocytes	0.7 (0.2 - 3.2)	10.6 (1.8 - 24.3)	p<0.005
B-lymphocytes	0 (0 - 0.3)	0.3 (0 - 0.7)	ns
Mast cells	0.2 (0 - 0.7)	0 (0 - 0.2)	ns

ns = not significant

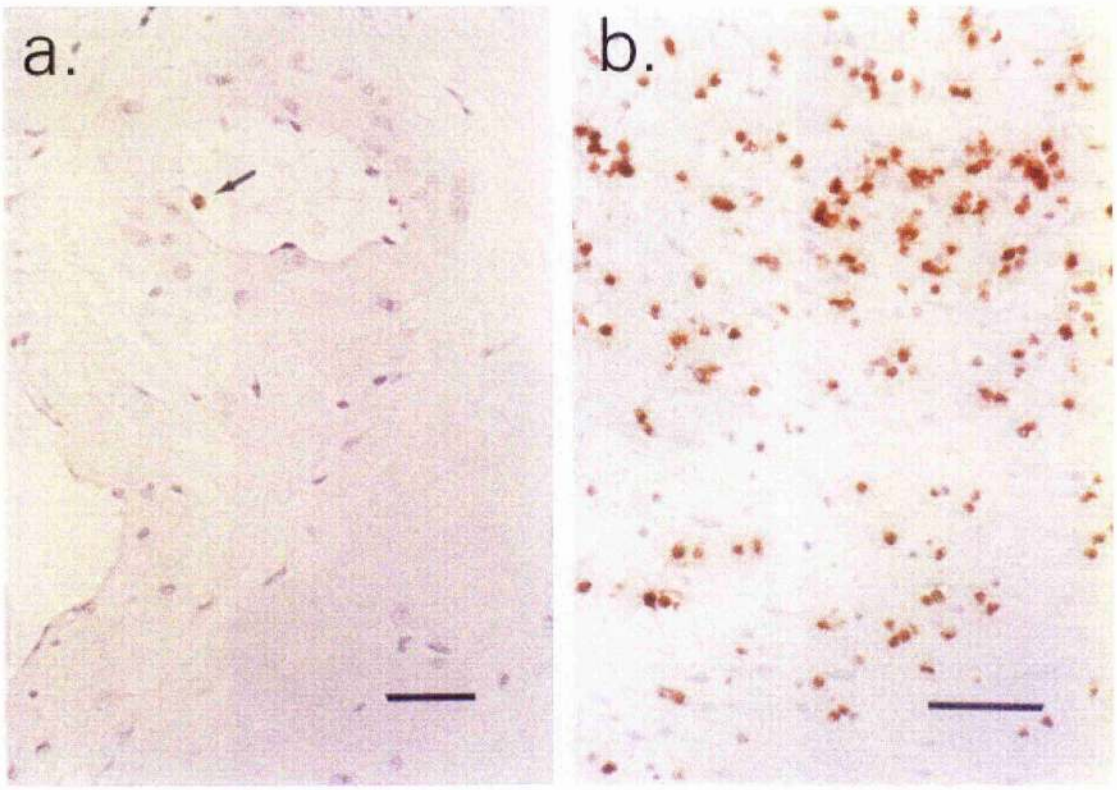

Figure 5.2 Identification of neutrophils in lower segment myometrium obtained **(a)** before and **(b)** during labour. The neutrophils are immunolocalised using an antibody directed against the enzyme human neutrophil elastase. Neutrophils are abundant in myometrium obtained during labour and sparse in myometrium obtained before the onset of labour (arrow). The negative controls, (see Materials and methods) exhibited no reactivity. Bar = 50 μm.

Table 5.4 Number of inflammatory cells in upper segment myometrium before and during labour at term, median (interquartile range) per high powered field.

Cell type	Before labour (n=7)	During labour (n=5)	Significance
Neutrophils	0.2 (0.2 - 0.3)	11.0 (2 - 22.5)	p<0.008
Macrophages	3.8 (2.3 - 6.2)	18.5 (17.5 - 20.0)	p<0.01
T-lymphocytes	3.3 (1.8 - 4.8)	4.0 (1.2 - 4.5)	ns
B-lymphocytes	0 (0 - 0.1)	0 (0 - 0.2)	ns
Mast cells	0 (0 - 0.5)	0.2 (0 - 0.5)	ns

ns = not significant

Macrophages

A population of immunoreactive macrophages was identified in myometrium before the onset of labour. The number of macrophages was significantly increased in both lower and upper uterine segment myometrium following the onset of labour, (figure 5.3, table 5.3 and table 5.4). Following the onset of labour, there was no significant difference in macrophage density between the upper and lower uterine segments.

Lymphocytes

There was a significant increase in the density of T-lymphocytes in lower segment myometrium following the onset of labour, (table 5.3). B-lymphocytes were sparse in lower segment myometrium and no significant change in their density occurred with the onset of labour. There was no significant change in either T or B-lymphocyte density following the onset of labour in upper segment myometrial biopsies, (table 5.4).

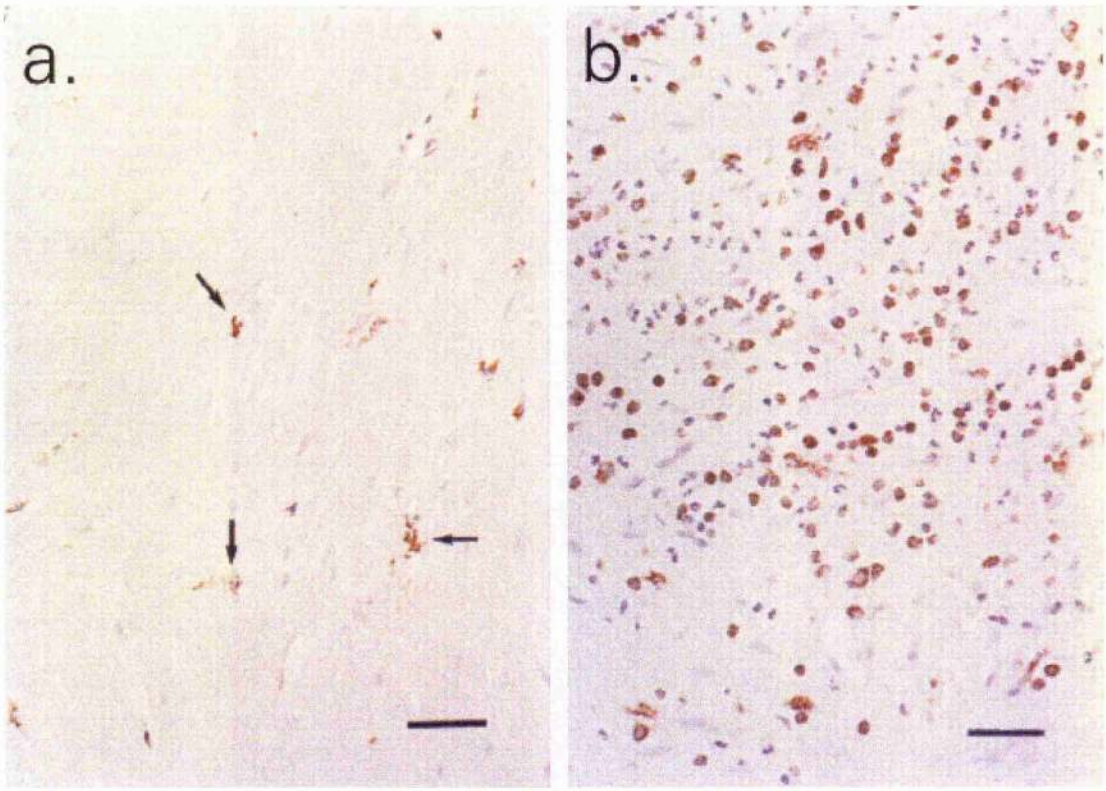

Figure 5.3 Identification of macrophages in lower segment myometrium obtained **(a)** before and **(b)** during labour. The macrophages are immunolocalised using an antibody directed against the antigen CD 68. Whilst a population of immunoreactive macrophages is identified before the onset of labour (arrows), significantly more macrophages are localised in myometrium following the onset of labour. The negative controls, (see Materials and methods) exhibited no reactivity. Bar = 50 μm.

Mast cells

There was no significant difference in mast cell density in myometrium before and after the onset of labour at term (table 5.3). Furthermore, mast cells were sparse in both upper and lower uterine myometrial biopsies (table 5.4), with no significant differences in mast cell density between the biopsy sites. We assessed mast cell density in non-pregnant myometrium (group iii), and found that this was significantly greater than in biopsies obtained from each of the pregnant groups, term not in labour (group i), and term in labour (group ii), (figure 5.4). These results are summarised in table 5.5.

Table 5.5 Presence of mast cells in human myometrium. Values are shown as median (interquartile range).

	Number of mast cells per high powered field.
Non-pregnant (n=13)	10.4 (6.3 - 14.8)*
Term, not in labour (n=17)	0.2 (0 - 0.7)
Term in labour (n=18)	0 (0 - 0.2)

*$p<0.0001$ compared with each pregnant group, (term not in labour and term in labour).

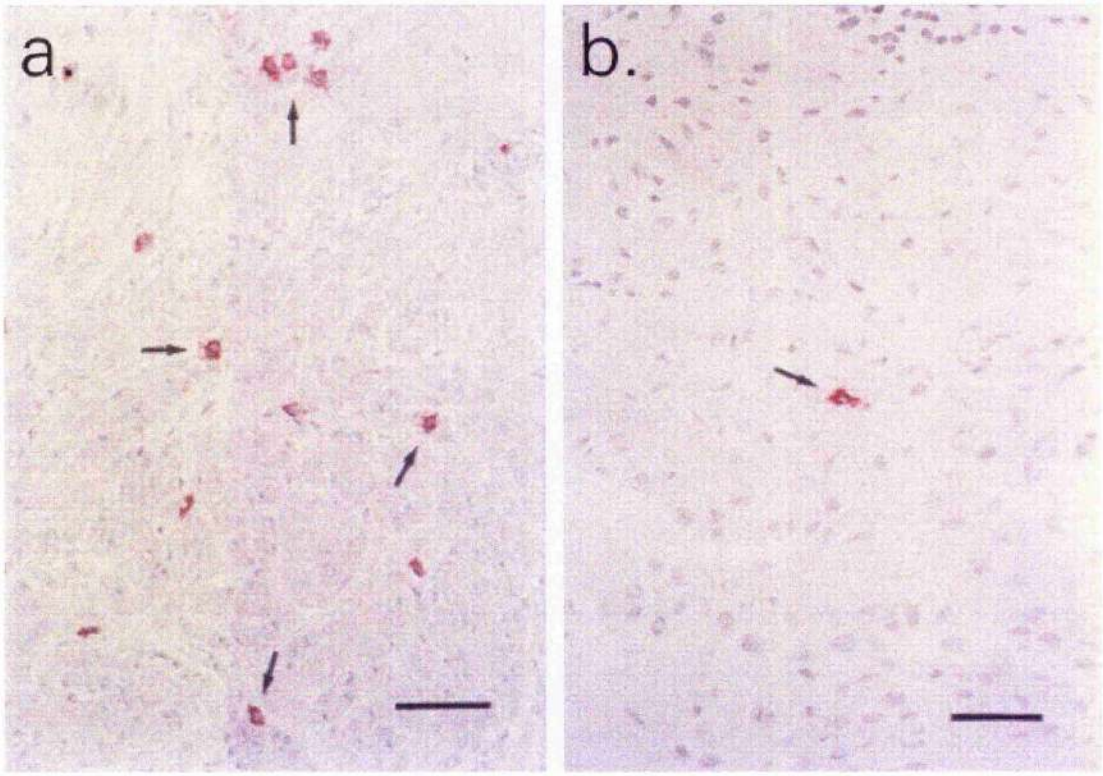

Figure 5.4 Identification of mast cells in **(a)** non-pregnant myometrium and **(b)** term, pregnant myometrium. The mast cells are immunolocalised (arrows) using an antibody directed against the enzyme, mast cell tryptase. There are significantly more mast cells in non-pregnant myometrium than in pregnant myometrium. The negative controls, (see Materials and methods) exhibited no reactivity. Bar = 50 μm.

Cell adhesion molecules

The cell adhesion molecules were localised as brown staining in serial cryosections of lower segment myometrium before, (n = 6) and after (n = 6) the onset of labour (figure 5.5). There were no changes in the immunolocalisation of ICAM-1, ICAM-2, PECAM or VCAM following the onset of labour. ICAM-1 immnostaining was of weak intensity and ICAM-2 staining was of moderate intensity in the myometrial vascular endothelium although not all vessels expressed these molecules. PECAM expression was strong and consistent in all of the vascular endothelium. In contrast VCAM expression was weak and absent in some vessels. E-selectin was not expressed in any of the tissues obtained before the onset of labour. However, 3 of the 6 biopsies obtained during labour showed immunostaining for E-selectin on the vascular endothelium (chi-square $p < 0.05$).

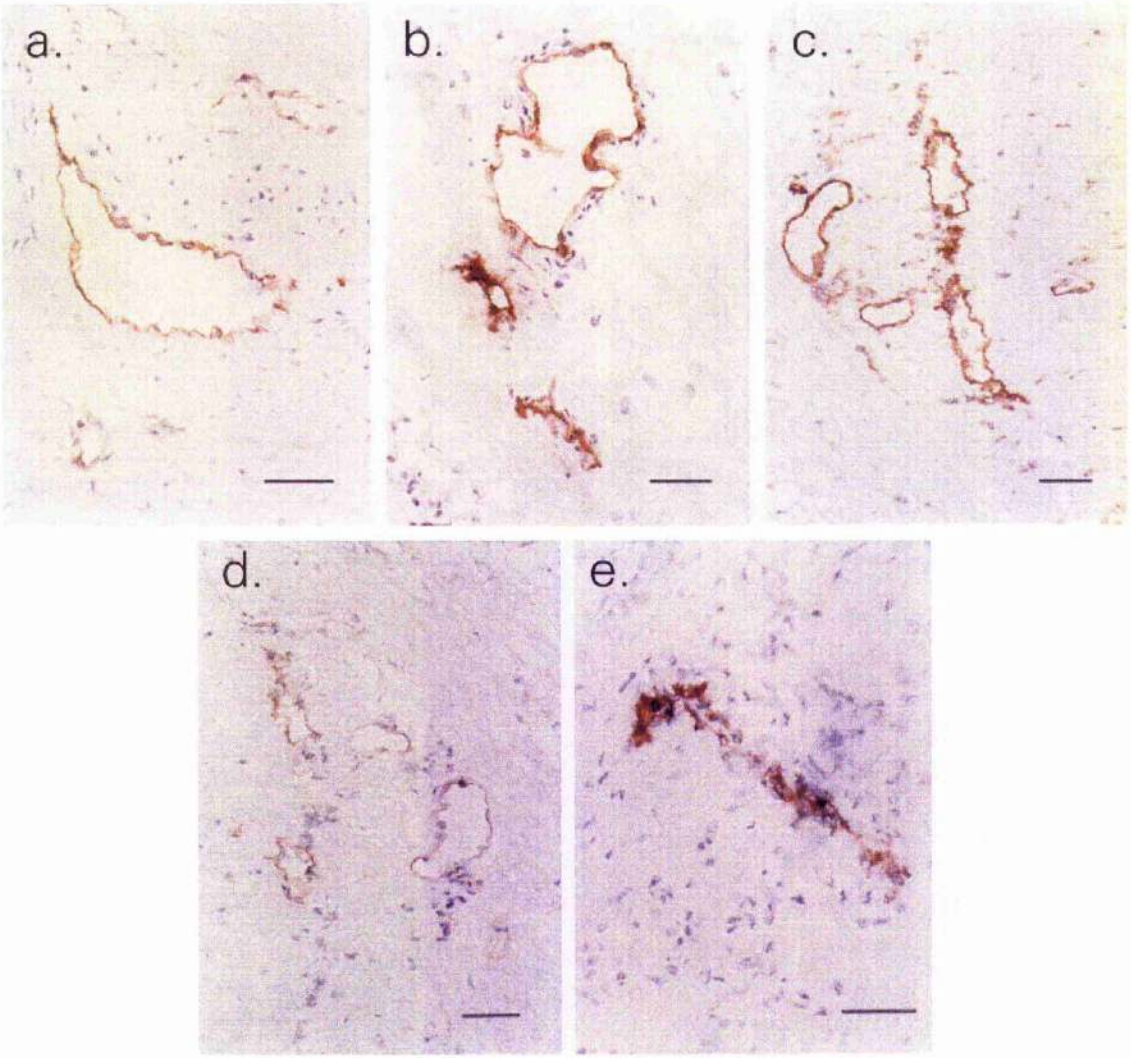

Figure 5.5 Localisation of cell adhesion molecules to the vascular endothelium in serial cryosections of myometrium. There are no changes in the expression of **(a)** ICAM-1, **(b)** ICAM-2, **(c)** PECAM or **(d)** VCAM in the vascular endothelium following the onset of labour. E-selectin is not expressed in any of the biopsies obtained before the onset of labour but is identified in 3 of the 6 biopsies obtained during labour. **(e)** Localisation of E-selectin in the capillary vascular endothelium in myometrium obtained during labour. The negative controls, (see Materials and methods) exhibited no reactivity. Bar = 50 μm.

Discussion

We have demonstrated that inflammatory cells, predominantly neutrophils and macrophages, infiltrate human myometrium during spontaneous labour at term. The inflammatory infiltrate is predominant in the lower uterine segment, but is also present in the myometrium of the uterine body. We also observed a significant increase in T-lymphocyte density in lower segment myometrium following the onset of labour. There was no change in B-lymphocyte and mast cell densities in myometrium during parturition.

In this study, we have examined both lower and upper uterine segment myometrium for the presence of inflammatory cells. Whether lower segment tissue reflects the state of the cervix or that of the uterine fundus, has aroused considerable interest over the last half century, (Calder, 1994). Some workers contest that lower uterine segment biopsies provide an alternative source of tissue that closely resembles cervix (Rajabi *et al.*, 1988). Because of the difficulties in obtaining cervical tissue for study, lower segment biopsies have been used in place of cervical tissue to investigate collagenase activity during cervical dilatation (Rechberger *et al.*, 1993). Our results indicate that lower uterine segment myometrium behaves quite differently from reported behaviour in cervix, and has similarities to upper segment myometrium. Bokstrom et al., (1997), demonstrated an abundance of neutrophils and macrophages in cervical biopsies in late pregnancy before the onset of labour with no significant increase in their densities during labour. In contrast, we have shown that neutrophils and macrophages are sparse in lower and upper segment myometrium before, and abundant during labour. Although the upper and lower uterine segments behave in a similar manner, they are not identical. During labour, the inflammatory infiltrate is more dense in lower segment myometrium than in upper segment myometrium. Consistent with this histological finding, functional studies have demonstrated that in normal labour the upper uterine

segment contracts more strongly than the lower, a situation which is reversed in abnormal labour, (Caldeyro-Barcia and Poseiro, 1960; Margono *et al.*, 1993).

Activated neutrophils and macrophages are a rich source of inflammatory mediators. These include plasminogen activators, eicosanoids, collagenase and elastase, and proinflammatory cytokines, including interleukin-1 and tumour necrosis factor-α, (Nathan, 1987; Osmers *et al.*, 1992; Casatella, 1995). Since these mediators have many diverse functions, the inflammatory infiltrate could have different roles in different regions of the uterus. Within the lower segment it could be involved in tissue remodelling and thereby facilitate cervical dilatation and passage of the fetus. In the upper segment, leukocyte products, including eicosanoids, interleukins and tumour necrosis factor-α, may stimulate uterine contractions directly, or indirectly by facilitating the production of uterotonic prostaglandins, (Casey *et al.*, 1990). Furthermore, inflammatory mediators may also initiate tissue remodelling in the uterine body. Granstrom *et al.*, (1989) demonstrated that the connective tissue of the uterine isthmus, (lower segment) and the uterine body undergo a biochemical ripening process similar to that found in the cervix with an increase in collagenolytic activity following the onset of labour. A breakdown of the connective tissue within the myometrium may facilitate the co-ordination of uterine contractions by allowing the formation of gap junctions, (Garfield and Hayashi, 1981).

Studies investigating the role of nitric oxide in the initiation of parturition have measured NOS enzyme activity using the arginine to citrulline conversion assay, in tissues collected before and during labour (Gude *et al.*, 1994; Di Iulio *et al.*, 1996; Ramsay *et al.*, 1996). These studies have concluded that there is no significant fall in NOS activity during human parturition at term. Conversely, Ramsay *et al.*, (1996), examining myometrium collected from the lower uterine segment during Caesarean section, found a slight increase in NOS activity, in tissue obtained during labour compared with that collected before labour. One possible explanation for this finding,

is that the increase in NOS enzyme activity originates from the inflammatory cell infiltrate since inflammatory cells are recognised to be a rich source of NOS (Riesco *et al.*, 1993; Kolb *et al.*, 1994).

We found no change in myometrial mast cell density before and after the onset of labour at term. The function of mast cells in the pregnant uterus remains unclear, although it has been proposed that myometrial mast cells regulate uterine contractility during labour, (Rudolph *et al.*, 1993). Mast cells produce mediators, (histamine and serotonin) and prostaglandins which can induce strong contractions in human myometrium *in vitro*, (Cruz *et al.*, 1989; Rudolph *et al.*, 1990; Rudolph *et al.*, 1993). Further, these cells are considered to play a pivotal role in wound healing, fibrosis and tissue remodelling (Galli, 1993), and might be involved in promoting collagen degradation and uterine involution in the post-natal period (Jeffrey *et al.*, 1991). We were surprised at the low density of mast cells in the pregnant myometrial biopsies since this is in contrast with the known distribution of mast cells in non-pregnant myometrium, (Mori *et al.*, 1997). Since mast cell mediators are capable of stimulating uterine contractions, the low density of mast cells in pregnant myometrium may be involved in the maintainence of myometrial quiescence as the uterus expands during pregnancy. Whilst we could demonstrate no change in myometrial mast cell density following the onset of labour, mast cell mediators might be capable of stimulating uterine contractions at term since the sensitivity of human myometrium to histamine and serotonin is upregulated at the end of pregnancy (Cruz *et al.*, 1989). This means that myometrial smooth muscle cells might be stimulated by mast cell mediators even when the mast cell density is reduced. In non-pregnant myometrium, the high density of mast cells has been proposed to have a role in implantation, (Brandon and Evans, 1983; Hore and Mehrotra, 1988), or in remodelling uterine smooth muscle and extracellular matrix during the menstrual cycle (Mori *et al.*, 1997).

The mechanisms involved in the accumulation, extravasation and degranulation of inflammatory cells in uterine tissues during parturition are poorly understood. Chemotactic cytokines, including interleukin-1, tumour necrosis factor-α and interleukin-8 seem to play a role (Osmers *et al.*, 1995, Barclay *et al.*, 1993, Chwalisz *et al.*, 1994), as well as other chemotactic agents, such as C5a (El Maradny *et al.*, 1995). E-selectin is involved in the infiltration of leukocytes to the maternal decidua and fetal membranes during labour (Rosenberg *et al.*, 1996).

Since the leukocytes in our myometrial biopsies were concentrated in and around blood vessels, we hypothesised that an up-regulation in the expression of vascular cell adhesion molecules in lower segment myometrium was involved in the accumulation of leukocytes in this tissue. ICAM-1, ICAM-2, PECAM and VCAM are members of the immunoglobulin superfamily, (Frenette and Wagner, 1996). ICAM-1 is important in the adhesion of monocytes, lymphocytes and neutrophils to activated endothelium, whilst VCAM binds to leukocyte integrins on many cells including eosinophils and activated T lymphocytes. E-selectin, a member of the Selectin family of adhesion molecules, is expressed by cytokine activated endothelial cells and has a major role in attracting neutrophils, monocytes, eosinophils and some lymphocytes, (Lasky 1992; Bevilacqua and Nelson, 1993).

The expression of cell adhesion molecules in the endometrium of the non-pregnant uterus is well described (Tawia *et al.*, 1993; Tabibzadeh *et al.*, 1994). A recent report has identified ICAM-1, VCAM, PECAM and E-selectin in pregnant human myometrium (Winkler *et al.*, 1998). We have shown that ICAM-1, ICAM-2, PECAM and VCAM are expressed on the vascular endothelium in myometrium obtained before the onset of labour at term and we propose that these molecules play a role in regulating leukocyte trafficking into this tissue. We found no change in the localisation and intensity of staining of ICAM-1, ICAM-2, PECAM and VCAM in the biopsies obtained during labour compared with those obtained before the onset of labour. In contrast, E-

selectin expression was absent in all of the biopsies collected before labour but was expressed in 3 of the 6 biopsies obtained during labour suggesting a role for this molecule in the recruitment of leukocytes in at least some of the tissues. These results are in broad agreement with Winkler *et al.*, (1998) who also found that E-selectin expression was up-regulated during labour. Since both we and Winkler *et al.*., (1998) have employed immunocytochemistry, further studies are required using quantitative techniques, to confirm these changes in cell adhesion molecule expression during parturition.

Factors responsible for the initiation of parturition remain obscure. We have demonstrated that leukocytes infiltrate both upper and lower uterine segment myometrium during spontaneous labour at term, and we propose that these cells play a fundamental role in normal parturition. A better understanding of the mechanisms involved in the initiation of labour both at term and preterm, would allow the development of novel strategies to prevent premature delivery. Our results suggest that strategies aimed at preventing the influx of inflammatory cells into the myometrium could be crucial in averting preterm delivery, and thus in reducing the excess perinatal mortality and morbidity associated with this condition.

Chapter 6

The biophysical effects of nitric oxide donors on the first trimester uterine cervix - a randomised controlled trial.

Introduction

The control of cervical ripening is crucial to the process of human parturition. Animal studies have shown that nitric oxide is a fundamental mediator of the ripening process. The nitric oxide generating system is present in the rat cervix and is up-regulated during labour both at term and preterm (Ali *et al.*, 1995; Buhimschi *et al.*, 1996). In pregnant guinea pigs, the local application of the nitric oxide donor, sodium nitroprusside effectively produces cervical ripening without inducing labour (Chwalisz *et al.*, 1997). Cervical ripening is an active process involving remodelling of the cervical tissue. This process can be induced pharmacologically in the first trimester to facilitate procedures such as surgical termination. Pre-operative cervical ripening reduces the morbidity of surgical termination, including haemorrhage, incomplete uterine evacuation, uterine perforation and cervical trauma (MacKenzie and Fry, 1981; Schultz *et al.*, 1983; Grimes *et al.*, 1984). The role of nitric oxide in human cervical ripening has not been investigated.

We hypothesize that nitric oxide donors can effect cervical ripening in humans. We therefore performed a randomised controlled trial to determine whether the nitric oxide donors isosorbide-5-mononitrate (IMN), and glyceryl trinitrate (GTN), can induce effective cervical ripening prior to surgical termination of pregnancy. In addition, we compared their ripening effects to those of the prostaglandin analogue gemeprost, the cervical priming agent currently used in our hospital.

Methods

A prospective, randomised controlled trial was performed involving 48 primigravid women referred for surgical termination of pregnancy by vacuum aspiration in the first trimester. The study was approved by the Local Research Ethics Committee and informed consent was obtained from each woman on the morning prior to operation. Forty-nine consecutive primigravid women undergoing termination of pregnancy were approached and one woman declined to participate in the trial. The remaining 48 women were randomly allocated to one of 4 treatment groups: IMN, GTN, gemeprost, or no treatment (vaginal examination only). Block randomisation was performed using sequentially numbered, sealed, opaque envelopes prepared using random number tables and 12 women were allocated to each treatment group. The envelopes were opened and the treatment allocated by one investigator after consent was obtained.

We calculated that by studying 48 women (12 in each group), the study had a power of 90% to show a difference of 20 Newtons between the treatment and no treatment groups at the 5% significance level assuming a standard deviation of 15. We considered this difference in force to be clinically significant.

Treatments in the 4 groups were either the nitric oxide donor IMN 40 mg (Ismo®, Boehringer Mannheim, Livingston, UK), the nitric oxide donor GTN 500 μg (non-proprietary supply), the prostaglandin analogue gemeprost 1 mg (16, 16-dimethyl-trans delta 2 PGE_1 methyl ester, Farillon, Essex, UK), or no treatment (vaginal examination only). Each medication was administered by one investigator to the posterior vaginal fornix 3 hours prior to surgery. Mean arterial blood pressure recordings were obtained before the medication was given and again when the patient was in the operating theatre.

General anaesthesia was induced using an intravenous injection of propofol, (Diprivan®, Zeneca Pharma, Cheshire, UK), and alfentanil, (Rapifen®, Janssen, Bucks., UK), and maintained with intermittent repeat doses as required. The cervical diameter before surgical dilatation was recorded and the force required to dilate the cervix to 8mm was measured with a force sensing apparatus coupled to cervical dilators, (figure 6.1) as previously described (Richardson *et al.*, 1989; Anthony *et al.*, 1982). All the procedures and recordings were performed by an experienced gynaecologist who was blind to the treatment given. Each of the treatments dissolved completely in the vagina, further ensuring that the operator was blinded to the treatment group. Suction evacuation of the uterus was then undertaken.

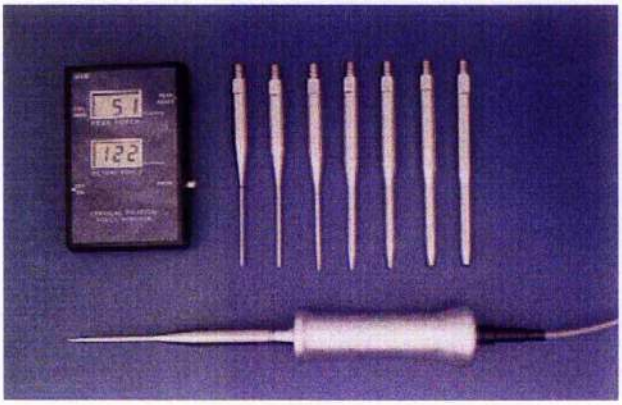

Figure 6.1 The force sensing apparatus was used to provide an objective assessment of the cervical resistance (Newtons).

One factor ANOVA was used to compare the patient characteristics (age, gestation and interval between treatment and surgery), and blood preassure data. The measurements for cervical diameter (mm) and forces required during cervical dilatation (Newtons) were analysed using

the Kruskal-Wallis test and significance determined using Mann-Whitney U tests. A value of $p<0.05$ was taken as significant. Each intervention group was compared to the no treatment group and each nitric oxide donor treatment group was compared to the gemeprost group.

Results

The mean age, gestation and the interval between pretreatment and surgical dilatation for each group are shown in table 6.1. Both IMN and gemeprost increased the cervical diameter before dilatation, and reduced the cumulative force required to dilate the cervix to 8 mm, compared to no treatment (table 6.2). Pretreatment with glyceryl trinitrate reduced the cumulative force required to dilate the cervix but had no effect on cervical diameter, (table 6.2). The force required to dilate the cervix in the gemeprost group was less than the IMN and GTN groups. No significant difference in the change in mean arterial blood pressure was detected among the groups after treatment.

Table 6.1 Clinical details of the study subjects.

Treatment	None (n=12)	Gemeprost (n=12)	IMN (n=12)	GTN (n=12)
Patient age in years	21.8 (4.1)	22.2 (5.1)	20.8 (3.4)	22.3 (5.7)
Length of gestation in weeks	8.6 (0.8)	9.2 (1.4)	9.4 (1.3)	9.2 (0.9)
Interval between pre-treatment & surgical dilatation in minutes	163 (29)	164 (19)	154 (39)	169 (24)

Data are given as mean (standard deviation). n= number of subjects in each group, IMN= isosorbide-5-mononitrate, GTN= glyceryl trinitrate.

Table 6.2 Results of the intra-operative recordings.

Treatment	None (n=12)	Gemeprost (n=12)	IMN (n=12)	GTN (n=12)
Cervical diameter before surgical dilatation in mm	4.5 (4-5)	6 (5-7) [a]	5 (5-6) [b]	5 (5-6)
Cumulative force to dilate cervix to 8mm in Newtons	68 (56-75)	18 [c] (6-27)	39 [d] (25-44)	54 [e] (27-58)

Data are given as the median (interquartile range). n = number of subjects in each group, IMN= isosorbide-5-mononitrate and GTN= glyceryl trinitrate.
The Kruskal-Wallis test was performed for cervical diameter, p=0.001, and for cumulative force, p=0.0002.
Cervical diameter greater than no treatment, (a) p<0.002, (b) p<0.006.
Cumulative force less than no treatment, (c) p=0.0002, (d) p<0.006, (e) p<0.05.

Discussion

This randomised trial is the first to demonstrate that a nitric oxide donor induces human cervical ripening. The effect of nitric oxide donors on human cervical ripening has not previously been investigated objectively. However, the effects of sublingual GTN on the non-pregnant cervix have been subjectively assessed. Shaker *et al.*, (1993) found that GTN had no significant effects on the ease of embryo transfer after in-vitro fertilization. Yadava (1990) reported that the nitric oxide donor GTN facilitated the transcervical introduction of intra-uterine contraceptive devices. A recent uncontrolled, observational study concluded that GTN patches suppress uterine contractions in preterm labour (Lees *et al.*, 1994) The effect of these patches on the cervix was not determined.

Cervical ripening is characterised by a decreased cervical collagen concentration, an increase in hydration and an alteration in the ground substance (Calder and Greer,

1992). The mediators of this process are incompletely defined. Liggins (1981) compared the process of cervical ripening to an inflammatory reaction and suggested that immunological mediators play a crucial role in the process. Nitric oxide is involved in the acute inflammatory response and amplifies the cytokine cascade stimulated during this response (Ianaro *et al.*, 1994). Potential mechanisms of action of nitric oxide in the ripening process are via interactions either with prostaglandin biosynthesis or with lytic enzymes such as matrix metalloproteinases. Nitric oxide is known to stimulate cyclooxygenase to increase the production of pro-inflammatory prostaglandins (Salvemini *et al.*, 1993). Furthermore, in the kidney, nitric oxide has been shown to stimulate matrix metalloproteinases which break down collagen (Trachtman *et al.*, 1996).

Whilst prostaglandins have an established therapeutic role in cervical ripening pre-operatively in the first trimester, they are associated with a number of adverse symptoms including abdominal pain, nausea, vomiting and diarrhoea (Henshaw and Templeton, 1991). Abdominal pain occurs in up to 74% of women receiving gemeprost prior to termination of pregnancy in the first trimester (Helm *et al.*, 1988) and arises from myometrial activity induced by the prostaglandin analogue. The ideal cervical ripening agent would induce cervical remodelling without stimulating uterine activity. Nitric oxide donors are such agents. Nitric oxide donors relax the myometrium whilst inducing cervical ripening (Norman, 1996). These agents could therefore be used as an alternative to prostaglandins to induce cervical ripening prior to intrauterine procedures in the first trimester thereby avoiding the side effects associated with prostaglandins. A formal assessment of adverse symptoms with each pretreatment was not performed in this study since a larger sample size would be required to address this issue. If nitric oxide donors are to be used in clinical practice, an assessment of their side effect profile in comparison with the prostaglandins is required. This issue is addressed in chapter 7.

This study has shown that nitric oxide donors can induce cervical ripening before first trimester surgical termination of pregnancy. We have shown that gemeprost (1 mg) is a more effective ripening agent than either IMN (40 mg) or GTN (500 μg). The efficacy of nitric oxide donors for this indication, may be improved by using a larger dose of the nitric oxide donor or by using an alternative preparation, such as a vaginal paste or gel, and further trials are required to test this hypothesis. If the efficacy of nitric oxide donors can be improved and an acceptable side effect profile demonstrated, this opens up the exciting possibility of a role for nitric oxide donors in cervical ripening prior to induction of labour at term. Before such randomised controlled trials are undertaken, the safety of nitric oxide donors in late pregnancy must be established.

Chapter 7

A randomised trial of nitric oxide donors for cervical ripening: an evaluation of the side effect profile of a nitric oxide donor in comparison with a prostaglandin.

Introduction

Cervical ripening before first trimester surgical termination of pregnancy facilitates the procedure whilst reducing the operative morbidity (MacKenzie and Fry, 1981; Schultz *et al.*, 1983; Grimes *et al.*, 1984). Pharmacological agents commonly used for this indication include laminaria in North America and prostaglandin analogues in the United Kingdom. Whilst prostaglandins produce more effective cervical ripening and are more convenient to administer than laminaria, (Helm *et al.*, 1988), they are associated with a number of adverse effects including abdominal pain, nausea, vomiting and diarrhoea Henshaw and Templeton, 1991). The ideal cervical ripening agent should be effective, easy to administer and have a low incidence of side effects.

The inflammatory mediator nitric oxide is a fundamental mediator of cervical ripening in animals (Buhimschi *et al.*, 1996; Ali *et al.*, 1995). The nitric oxide donor, sodium nitroprusside produces effective ripening when applied locally to the guinea pig cervix (Chwalisz *et al.*, 1997). In a randomised controlled trial, we have shown that the nitric oxide donors isosorbide-5-mononitrate and glyceryl trinitrate can induce effective cervical ripening compared to no treatment in primigravid women, when administered per vaginam in the first trimester of pregnancy (Thomson *et al.*, 1997b - chapter 6). Since these agents are smooth muscle relaxants (Norman, 1996), they should induce cervical ripening without causing abdominal pain due to myometrial contractions, the most commonly reported side effect in women receiving prostaglandins in early pregnancy (Helm *et al.*, 1988; Henshaw and Templeton, 1991; El-Refaey *et al.*, 1994).

We hypothesise that nitric oxide donors can effectively ripen the cervix before first trimester surgical termination of pregnancy with fewer side effects than prostaglandin analogues. We therefore performed a three-group randomised trial to compare the clinical effects of the nitric oxide donor, isosorbide mononitrate (IMN, 40 mg and 80 mg) and the prostaglandin analogue, gemeprost before first trimester surgical termination of pregnancy. In addition, we performed a concurrent observational study to determine whether the cervical ripening effects of IMN are clinically sufficient.

Methods

(a) Randomised study to compare the clinical effects of IMN (40 and 80 mg) and gemeprost.

Between January and November 1997, 67 primigravid women scheduled for surgical termination of pregnancy by vacuum aspiration in the first trimester were recruited to this randomised trial; one woman was excluded before randomisation when she was found to be multigravid (figure 7.1). The study was approved by the local research ethics committee and informed consent was obtained from each woman on the morning before the operation. Exclusion criteria from the study included: any previous pregnancy, previous cervical surgery, signs of threatened miscarriage or concurrent maternal disease. The gestation of each pregnancy was determined from both the menstrual history and clinical evaluation of uterine size. The women were randomly allocated to one of three treatment groups: the nitric oxide donor IMN 40 mg (Elantan® 40, Schwarz Pharma Ltd., Bucks., UK), IMN 80 mg, or the prostaglandin analogue gemeprost 1mg (16, 16-dimethyl-trans delta 2 PGE_1 methyl ester, Farillon, Essex, UK). Block randomisation was performed using sequentially numbered, sealed, opaque envelopes prepared using random number tables and 22 women were allocated to each treatment group. The envelopes were opened and the treatment allocated by one investigator after consent was obtained. Each medication was administered by the same

investigator to the posterior vaginal fornix three hours prior to surgery. Treatment allocation was concealed from patients until the end of the study period.

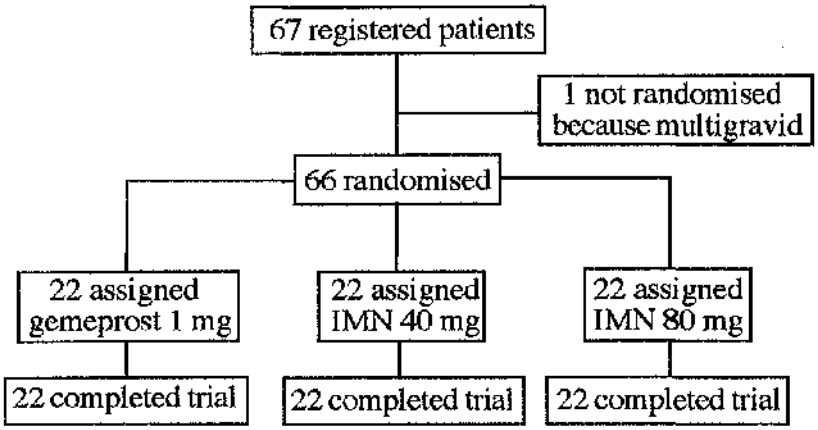

Figure 7.1 Flow chart showing the women's progress through the randomised study. IMN = isosorbide-5-mononitrate.

The primary outcome measures were the development of adverse symptoms for each active treatment group. These were assessed using a symptom questionnaire administered before the medication was given and again immediately prior to surgery. The same investigator administered the symptom questionnaire on each occasion. At these time points, peripheral arterial pulse rates and mean arterial blood pressure recordings were obtained using an automated electronic device, (Dinamap Plus®, Critikon, Florida, USA). For all women, general anaesthesia was induced using an intravenous injection of propofol, (Diprivan®, Zenecа Pharma, Cheshire, UK), and alfentanil, (Rapifen®, Janssen, Bucks., UK), and maintained with intermittent repeat doses as required. Secondary outcome measures were the cervical diameter before surgical dilatation and the force required to dilate the cervix to 8mm measured with a force sensing apparatus coupled to cervical dilators (figure 6.1), as previously

described in chapter 6. All the procedures and recordings were performed by two experienced gynaecologists who were blind to the treatment given. Each of the treatments dissolved completely in the vagina, further ensuring that the operators were blinded to the treatment group. Suction evacuation of the uterus was then undertaken and the total volume lost, including both blood and products of conception, was measured from a calibrated suction jar.

Using Altman's nomogram, (Altman, 1982) we calculated that by studying 66 women (22 in each active treatment group), the study had a power of 85%, at the 5% significance level, to show a reduction of two thirds in the incidence of abdominal pain between the active treatment groups. This calculation is based upon a 70% incidence of abdominal pain occurring in the gemeprost group (Helm *et al.*, 1988). We considered that such a reduction in the frequency of abdominal pain would be clinically significant.

(b) Observational study to determine whether the cervical ripening effects of IMN are clinically sufficient.

It is current practice to omit preoperative cervical ripening in parous women undergoing first trimester termination of pregnancy, (RCOG, 1997) since the force required to dilate the parous cervix is significantly less than that required for the primigravid cervix (Anthony *et al.*, 1982). We considered that for a cervical ripening agent to be considered clinically effective in primigravid women, it should reduce the cervical resistance to a level similar to that measured in parous women. We therefore recruited 22 consecutive parous women scheduled for surgical termination in the first trimester as a reference group for cervical resistance. The study was approved by the local research ethics committee and informed consent was obtained from each woman on the morning before operation. Each woman had had at least one normal vaginal delivery and exclusion criteria included previous cervical surgery and signs of threatened miscarriage. In these women, the primary outcome measures were the cervical diameter

before surgical dilatation, the cumulative force required to dilate the cervix and the intraoperative blood loss which were measured as described above.

Statistics

One factor ANOVA was used to compare the patient characteristics (age, gestation and interval between treatment and surgery), pulse and blood pressure data between the treatment groups. Data on intraoperative blood loss, cervical diameter and forces required during cervical dilatation were analysed using the Kruskal-Wallis test or the Mann-Whitney U test. The incidence of pre-operative side-effects was compared using Fisher's exact test.

Results

(a) Randomised study to compare the clinical effects of IMN (40 and 80 mg) and gemeprost.

The mean age, gestation and interval between pretreatment and surgical dilatation, in each active treatment group are shown in table 7.1.

Table 7.1 Clinical details of the women participating in the study. Data are given as mean (standard deviation). IMN = isosorbide-5-mononitrate.

	Gemeprost (n = 22)	IMN 40 mg (n = 22)	IMN 80 mg (n = 22)	Parous group (n = 22)
Age (years)	21.8 (4.1)	22.6 (6.6)	21.9 (6.2)	27.0 (4.9)
Gestation (weeks)	9.0 (1.2)	9.3 (1.1)	9.3 (1.0)	9.1 (1.0)
Interval between pre-treatment and surgical dilatation (min)	169 (24)	169 (37)	170 (42)	-

Sixty-eight per cent of women in the IMN 40 mg group and 59% in the IMN 80 mg group remained asymptomatic, significantly more than the 14% of women in the gemeprost group ($p<0.001$ and $p<0.005$ respectively). Significantly more women who received the prostaglandin analogue gemeprost experienced abdominal pain ($p<0.0001$) and vaginal bleeding ($p<0.01$) when compared with either of the IMN groups. More women in each of the IMN groups developed a headache ($p<0.05$) when compared with the gemeprost group. No significant difference was found between the IMN 40 mg and 80 mg groups in reported symptoms (table 7.2). No significant difference in the change in mean arterial blood pressure or pulse rate was detected among the groups after treatment.

Table 7.2 Incidence of new symptoms occurring after administration of the cervical ripening agent. IMN = isosorbide-5-mononitrate.

	Gemeprost (n = 22)	IMN 40 mg (n = 22)	IMN 80 mg (n = 22)
	n (%)	*n (%)*	*n (%)*
Asymptomatic	3 (14)	15 (68) [a]	13 (59) [a]
Abdominal pain	16 (73)	1 (5) [b]	0 (-) [b]
Diarrhoea	2 (9)	0 (-)	0 (-)
Dizziness	0 (-)	3 (14)	1 (5)
Headache	0 (-)	5 (23) [c]	7 (32) [d]
Nausea/vomiting	4 (18)	1 (5)	2 (9)
Palpitation	0 (-)	1 (5)	3 (14)
Vaginal bleeding	7 (32)	0 (-) [e]	0 (-) [e]

a. significantly greater than gemeprost group, $p<0.005$
b. significantly less than gemeprost group, $p<0.0001$
c. significantly greater than gemeprost group, $p<0.05$
d. significantly greater than gemeprost group, $p<0.01$
e. significantly less than gemeprost group, $p<0.01$

The efficacy of each agent to dilate the cervix and reduce the cervical resistance is shown in table 7.3. Pretreatment with gemeprost was more effective at ripening the cervix than either dose of IMN. Gemeprost (1 mg) increased the diameter of the cervical os before surgical dilatation compared with the 40 mg IMN group ($p<0.05$), and was associated with a lower cervical resistance than either dose of IMN ($p<0.02$). There were no significant differences in the cervical resistance between the two IMN groups. Prtreatment with gemeprost resulted in significantly less ($p<0.0005$) measured intraoperative blood loss compared with either dose of IMN (table 7.3).

Table 7.3 Results of the intra-operative recordings for both the randomised comparison and the observational study. Data are given as the median (interquartile range). IMN = isosorbide mononitrate.

	Gemeprost (n = 22)	IMN 40 mg (n = 22)	IMN 80 mg (n = 22)	Parous group (n = 22)
Cervical diameter before surgical dilatation (mm)	6 (5 - 7)*	5 (4 - 5)	5 (5 - 5)	5 (3 - 6)
Cumulative force to dilate cervix to 8 mm (N)	21 (7 - 38)**	40 (23 - 54)	36 (27 - 60)	34 (18 - 61)
Intra-operative blood loss (ml)	58 (45 - 90)***	143 (90 - 220)	160 (105 - 295)	135 (50 - 225)

(*) significantly greater than IMN 40 mg and parous groups, $p<0.03$

(**) significantly less than IMN 40 mg, IMN 80 mg and parous groups, $p<0.02$

(***) significantly less than IMN 40 mg, IMN 80 mg and parous groups, $p<0.0005$

(b) Observational study to determine whether the cervical ripening effects of IMN are clinically sufficient.

Pretreatment of primigravid women with IMN resulted in a cervical resistance similar to that in the parous group (table 7.3). There was no significant difference in the measured intraoperative blood loss between each IMN group and the parous group of women (table 7.3). Furthermore, no woman in any group required plasma volume expansion, blood transfusion or reoperation for vaginal bleeding and no woman in the study had a blood loss greater than 500 ml.

Discussion

This randomised study demonstrates that the nitric oxide donor IMN has fewer side effects than the prostaglandin analogue gemeprost when used to ripen the cervix before first trimester surgical termination of pregnancy. The nitric oxide donor is associated with a lower incidence of abdominal pain and pre-operative vaginal bleeding and a higher proportion of patients remaining asymptomatic. However, more women receiving the nitric oxide donor experienced a headache. The prostaglandin analogue produced a greater reduction in force and was associated with a lower intraoperative blood loss than either dose of the nitric oxide donor. The concurrent observational study demonstrated that pretreatment of primigravid women with IMN results in a cervical resistance similar to that of the parous group, whilst the intraoperative blood loss was similar in the IMN and parous groups.

The side effect profile of prostaglandin analogues administered in the first trimester of pregnancy is well characterised and includes nausea, vomiting, diarrhoea, abdominal pain and vaginal bleeding (Helm *et al.*, 1988; Henshaw and Templeton, 1991; Lawrie *et al.*, 1996). These effects are thought to arise because the prostaglandins stimulate contractions of uterine and gastrointestinal smooth muscle. Whilst it has been suggested that misoprostol, an orally active synthetic analogue of prostaglandin E_1, is

associated with fewer side effects than gemeprost, (El-Refaey *et al.*, 1994) a more recent study has reported a high incidence of vaginal bleeding (50%), abdominal pain (47%) and vomiting (22%) in women given misoprostol in the first trimester of pregnancy (Ngai *et al.*, 1996). It has been suggested that the antiprogestogen mifepristone represents an acceptable and effective cervical ripening agent (WHO, 1990; Henshaw and Templeton, 1991). Whilst this agent has the advantage of being effective after oral administration, it is also associated with side effects, (including nausea, vomiting, vaginal bleeding and abdominal pain) and requires a latent period of 36-48 hours for the drug to act (Ngai *et al.*, 1996).

The side effect profile of nitric oxide donors administered vaginally has not been described. Their recognised side effects when administered by other routes (oral, sublingual, transdermal and intravenous), include headache, dizziness, postural hypotension and tachycardia (Robertson and Robertson, 1995). Two studies have assessed the effects of nitroglycerin, a nitric oxide donor, administered either as transdermal patches or as an intravenous infusion (20 μg/min), during pregnancy (Lees *et al.*, 1994; Ramsay *et al.*, 1994). No significant changes in maternal heart rate or systemic blood pressure were detected, although headache was reported to Lees et al. (1994) by one third of the women. Other groups have found that larger doses of nitric oxide donors administered during pregnancy have significant adverse effects on the maternal cardiovascular system. Intravenous nitroglycerin, up to 5 μg/kg per minute (Cotton *et al.*, 1986; Grunewald *et al.*, 1995) and a single 5 mg dose of sublingual isosorbide dinitrate increased maternal pulse rate and decreased mean arterial blood pressure (Thaler *et al.*, 1996).

In our study, when IMN was administered locally to the cervix, 60 - 70 % of women remained asymptomatic and there were no significant changes in mean arterial blood pressure or pulse rate. This low incidence of side effects may reflect the first uterine pass effect whereby vaginal drug administration results in high uterine and low serum

concentrations (Bulletti *et al.*, 1997). We have previously shown that vaginal administration of IMN allows effective ripening of the uterine cervix (Thomson *et al.*, 1997b - chapter 6) and the study reported here indicates that such an approach is associated with minimal systemic side effects.

An accumulation of nitric oxide donor in the uterine smooth muscle would be expected to relax the myometrium (Norman, 1996) thereby minimizing preoperative abdominal pain and vaginal bleeding. One potential disadvantage of this effect is that myometrial relaxation, or lack of stimulation to contract during surgical evacuation might result in an increased intraoperative blood loss. Haemostasis in the placental bed depends not on the coagulation system, but rather myometrial contraction, the so-called living ligature, in the first instance. Prostaglandin analogues such as gemeprost, which cause myometrial contractions, are associated with a lower intraoperative blood loss when compared with placebo (Greer *et al.*, 1992). We found that the measured loss in each IMN group was not significantly greater than in a parous group of women receiving no treatment. Blood loss was lower in the gemeprost group, as would be expected with an agent provoking uterine contractility, than either the parous group or the groups receiving IMN. These data suggest that although the uterotonic effects of prostaglandins are associated with a reduction in measured blood loss, the myometrial relaxant effects of nitric oxide donors do not result in an increased blood loss compared with the parous control group. Moreover, no woman in any of the groups had a clinically significant intraoperative blood loss as assessed by the need for plasma volume expansion, blood transfusion or reoperation for blood loss.

The efficacy of the prostaglandin analogue gemeprost to ripen the cervix is significantly better than either dose of IMN. Our results suggest that the ability of IMN to ripen the cervix is not dose dependent, although we have assessed only two doses of the nitric oxide donor. Our previous work, which included a primigravid, control group given no treatment, has shown that IMN 40 mg and gemeprost 1 mg reduced the cervical

resistance by 40 % and 70 % respectively (Thomson *et al.*, 1997b - chapter 6). Whilst it is widely accepted that a reduction in the force required to dilate the cervix reduces both operative morbidity and long term effects of cervical damage (MacKenzie and Fry, 1981; Schultz *et al.*, 1983; Grimes *et al.*, 1984), there are no data on how much force is considered acceptable. In the absence of such data, we propose that the force required to dilate the parous cervix is a clinically acceptable target for ripening agents since it is current practice to omit preoperative cervical ripening in these women (RCOG, 1997). We have shown no significant differences in the cervical resistance of primigravid women pretreated with IMN and untreated parous women, suggesting that the cervical ripening effects of IMN are clinically sufficient. One caveat to this conclusion is that the comparison between the IMN groups and the parous group was made in an observational study since randomisation was not possible for the group of parous women. It seems essential that any cervical ripening agent used, should have minimal side effects. As we demonstrate in this chapter, the nitric oxide donor IMN appears to be such an agent.

This study has demonstrated that the nitric oxide donor IMN has fewer side effects than gemeprost when used to ripen the cervix in the first trimester of pregnancy. Later in pregnancy, cervical ripening is commonly employed to facilitate induction of labour. Whilst prostaglandins are the agents most commonly used to prepare the cervix at term, they are associated with excessive myometrial activity and an abnormal fetal heart rate pattern in 7% of women (Wing *et al.*, 1995). Nitric oxide donors relax the myometrium, and these agents may have advantages over the prostaglandins to ripen the cervix later in pregnancy (Norman *et al.*, 1998). However, the safety of nitric oxide donors in the third trimester must be established before their efficacy for this indication is determined.

Chapter 8.

Conclusions

The factors involved in the initiation of human parturition remain obscure. Studies in animals have shown that the L-arginine nitric oxide system is crucially involved in the maintenance of myometrial quiescence and the initiation of cervical ripening. The purpose of this work was to investigate the role of nitric oxide in human parturition. Data in chapter 2 shows that each of the three isoforms of NOS is widely expressed in the pregnant human uterus. However, there is no change in either the expression or enzyme activity of NOS in tissues, (myometrium, placenta or fetal membranes) obtained before and during the onset of spontaneous labour at term. These findings indicate that a decline in the synthesis of nitric oxide is not the final activating factor in the onset of human labour.

The biological effects of nitric oxide are prolonged by superoxide dismutase (SOD), and attenuated by xanthine oxidase. Data in chapter 3, show that Cu/Zn, Mn SOD and xanthine oxidase are present within pregnant uterine tissues. However, there is no change in the expression of these enzymes as determined by immunocytochemistry, between tissues obtained before or during labour. Our data also shows that these tissues possess SOD activity and that there is no difference in total SOD enzyme activity between tissues obtained before or after the onset of labour. It therefore seems unlikely that these enzymes are involved in the initiation of labour, although their wide distribution suggests that they may be important in the maintenance of pregnancy.

The data presented in chapter 4 show that the myometrial expression of the constitutive isoforms of NOS, cNOS and bNOS, is up-regulated during pregnancy and subsequently declines during the third trimester. This increase in the expression of the nitric oxide system may contribute to the relative quiescence of the myometrium during

pregnancy. Further, the decline in cNOS expression during the third trimester may be one of the factors governing the transition from Braxton Hicks contractions to the myometrial contractions of labour.

Inflammatory mediators play a crucial role in human parturition and inflammatory cells are known to infiltrate the cervix, placenta, decidua and fetal membranes during labour. Data in chapter 5 demonstrate that inflammatory cells, predominantly neutrophils and macrophages infiltrate the myometrium during spontaneous labour at term. Whilst the infiltrate is predominant in the lower uterine segment, it is also present in the myometrium of the uterine body. An increased expression of the cell adhesion molecule, E-selectin occurs on the vascular endothelium of myometrial biopsies obtained during labour, suggesting a role for this molecule in the accumulation of leukocytes. These results suggest that inflammatory cell infiltration is part of the physiological mechanisms that occur in the myometrium during parturition. Further understanding of this process may suggest new strategies aimed at preventing preterm delivery.

The results of a randomised controlled trial, presented in chapter 6, show that the nitric oxide donors IMN (40 mg) and GTN (500 μg), administered per vaginam produce significant ripening of the first trimester human cervix. The results of a larger randomised controlled trial, presented in chapter 7, demonstrate that IMN has fewer side effects than the prostaglandin analogue gemeprost when administered per vaginam in the first trimester of pregnancy. The nitric oxide donor is associated with a lower incidence of abdominal pain and pre-operative vaginal bleeding and a higher proportion of patients remaining asymptomatic. However, more women receiving the nitric oxide donor experienced a headache. The data from a concurrent observational study indicate that the cervical ripening effects of IMN (40 and 80 mg), though less than the effects of gemeprost (1 mg), are clinically sufficient.

In summary, it seems clear that nitric oxide withdrawal does not play a part in the acute stimulus to labour at term. An up-regulation of the L-arginine nitric oxide system in myometrium may be involved in the maintenance of uterine quiescence during pregnancy and further studies are required to determine whether a decline in uterine NOS expression occurs in preterm labour. The data presented here indicate that nitric oxide donors are an acceptable and effective class of cervical ripening agent and could replace prostaglandins for pre-operative cervical ripening in the first trimester of pregnancy. Since nitric oxide donors have been investigated as tocolytic agents in the management of preterm labour, and these agents induce significant ripening of the human cervix, at least in the first trimester of pregnancy, it would seem sensible that clinical studies investigating the tocolytic effects of nitric oxide donors should include an assessment of the cervical state.

References

Akyama, S.K., Yamada, S.S., Chen, W.T. and Yamada, K.M. (1989) Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly and cytoskeletal organization. *J. Cell Biol.*, **109**, 863-875.

Ali, M., Yallampalli, C., Chwalisz, K. and Garfield, R. (1995) Changes in mRNA levels of nitric oxide synthases in rat uterus and cervix during pregnancy. *J. Soc. Gynecol. Invest.*, **2**, 226.

Allen, J., Uldbjerg, N., Petersen, L.K. *et al.* (1989) Intracervical 17-ß oestradiol before induction of second trimester abortion with a prostaglandin E_1 analogue. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **32**, 123-127.

Alm, P., Alumets, J., Hakanson, R., Sundler, F. (1977) Peptidergic (vasoactive intestinal peptide) nerves in the genito-urinary tract. *Neurosci.*, **2**, 751-754.

Alm, P., Alumets, J.,Brodin, G., Hakanson, R., Sjoberg, N.O., Sundler, F. (1978) Peptidergic (substance P) nerves in the genito-urinary tract. *Neurosci.*, **3**, 419-425.

Altabef, K.M., Spencer, J.T., Zinberg, S. (1992) Intravenous nitroglycerin for uterine relaxation for an inverted uterus. *Am. J. Obstet. Gynecol.*, **166**, 1237-1238.

Altman, D.G. (1982) How large a sample? In: Statistics in Practice (eds Gore SM and Altman DG), London, British Medical Association, 6-8.

Ambrus, G. and Rao, ChV. (1994) Novel regulation of pregnant human myometrial smooth muscle cell gap junctions by human chorionic gonadotrophin. *Endocrinology*, **135**, 2772-2779.

Amin, A.R., Di Cesare, P.E., Vyas, P. *et al.* (1995) The expression and regulation of nitric oxide synthase in human osteoarthritis-affected chondrocytes: evidence for up-regulated neuronal nitric oxide synthase. *J. Exp. Med.*, **182**, 2097-2102.

Anggard, E. (1994) Nitric oxide: mediator, murderer, and medicine. *Lancet*; **343**, 1199-206.

Anthony, G.S., Fisher, J., Coutts, J.R.T., Calder, A.A. (1982) Forces required for surgical dilatation of the pregnant and non-pregnant human cervix. *Br. J. Obstet. Gynaecol.*, **89**, 913-16.

Avraham, K.B., Sugarman, H., Rotshenker S. *et al.* (1991) Down's syndrome: morophological remodelling and increased complexity in the neuromuscular junction of transgenic CuZn-superoxide dismutase mice. *J. Neurocytol.* , **20**: 208-215.

Bacon, C.R., Morrison, J.J., O'Reilly, G., Cameron, I.T., Davenport, A.P. (1995) ET_A and ET_B endothelin receptors in human myometrium characterised by the subtype selective ligands BQ123, BQ3020, FR139317 and PD151242. *J. Endocrinol.*, **144**, 127-134.

Baggiolini, M., Wakz, A., Kunkel, S.L. (1989) Neutrophil activating peptide-1/interleukin-8 a novel cytokine that activate neutrophils [review]. *J. Clin. Invest.*, **246**, 1045-1049.

Bansal, R.K., Goldsmith, P.C., He, Y., Zaloudek, C.J., Ecker, J.L. and Riemer, R.K. (1997) A decline in myometrial nitric oxide synthase expression is associated with labor and delivery. *J. Clin. Invest.* **99**, 2502-2508.

Barclay, C.G., Brennand, J.E., Kelly, R.W. and Calder, A.A. (1993) Interleukin-8 production by the human cervix. *Am. J. Obstet. Gynecol.* , **169**, 625-632.

Bates, T.E., Loesch, A., Burnstock, G. *et al.* (1996) Mitochondrial nitric oxide synthase: a ubiquitous regulator of oxidative phosphorylation? *Biochem. Biophys. Res. Comm.*, **218**, 40-44.

Batra, S. (1985) On the role of estradiol and progesterone in parturition: an updated proposal. *Acta Obstet. Gynecol.*, **64**, 671-672.

Batra, S. (1994) Hormonal control of myometrial function. In: Chard T, Grudzinskas JG, eds. The Uterus. Cambridge University Press. 173-192.

Bayhi, D.A., Sherwood, C.D., Campbell, C.E. (1992) Intravenous nitroglycerin for uterine inversion. *J. Clin. Anaesth.*, **4**, 487-488.

Beck, P., Adler, P., Szlachter, N., Steinetz, B.G., Weiss, G. (1978) Synergistic effect of human relaxin and progesterone concentration preceding normal labor. *Obstet. Gynecol.*, **51**, 686-690.

Beckman, J.S. and Crow, J.P. (1993) Pathological implications of nitric oxide, superoxide and peroxynitrite formation. *Biochem. Soc. Trans.*, **21**, 330-334.

Belfort, M.A. (1993) Intravenous nitroglycerin as a tocolytic agent for intrapartum external cephalic version. *S. Afr. Med. J.*, **83**, 656.

Bell, R.J., Permezel, M., MacLennan, A., Hughes, C., Healy, D., Brennecke, S. (1993) A randomized, double-blind, placebo-controlled trial of the safety of vaginal recombinant human relaxin for cervical ripening. *Obstet. Gynecol.*, **82**, 328-333.

Benedetto, C., Petraglia, F., Marozio, L. *et al.* (1994) Corticotropin-releasing hormone increases prostglandin $F_{2\alpha}$ activity on human myometrium. *Am. J. Obstet. Gynecol.*, **171**, 126-131.

Bevilacqua, M.P. (1993) Endothelial-leukocyte adhesion molecules. *Annu. Rev. Immunol.*, **11**, 767-804.

Bevilacqua, M.P. and Nelson, R.M. (1993) Selectins. *J. Clin. Invest.*, **91**, 379-387.

Bokstrom, H. and Norstrom, A. (1995) Effects of mifepristone and progesterone on collagen synthesis in the human uterine cervix. *Contraception*, **51**, 249-254.

Bokstrom, H., Brannstrom, M., Alexandersson, M. and Norstrom, A. (1997) Leukocyte subpopulations in human uterine cervical stroma at early and term pregnancy. *Hum. Reprod.*, **12**, 586-590.

Bolotina, V.M., Najibi, S., Palacino, J.J., Pagano, P.J., Cohen, R.A. (1994) Nitric oxied directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368**, 850-853.

Bottari, S.P., Vokaer, A., Kaivez, E., Lescrainier, J.P., Vauquelin, G. (1985) Regulation of α- and β-adrenergic receptor subclasses by gonadal steroids in human myometrium. *Acta. Physiol. Hung.*, **65**, 335-346.

Bozler, E. (1941) Influence of estrone on the electrical characteristics and the mobility of uterine muscle. *Endocrinology*, **29**, 225-227.

Brady, A.J., Poole-Wilson, P.A., Harding, S.E., Warren, J.B. (1992) Nitric oxide production within cardiac myocytes reduces their contractility in endotoxemia. *Am. J. Physiol.*, **263**, H1963-H1966.

Brandon, J.M. and Evans, J.E. (1983) Change in mast cells during the estrous cycle in the Syrian hamster. *Am. J. Anat.*, **167,** 241-247.

Brennand, J.E., Calder, A.A., Leitch, C.R., Greer, I.A., Chou, M.M., MacKenzie, I.Z. (1997) Recombinant human relaxin as a cervical ripening agent. *Br. J. Obstet. Gyneacol.*, **104**, 775-780.

Brennand, J., Leask, R., Kelly, R., Greer, I., Calder, A. (1998) The influence of amniotic fluid on prostaglandin synthesis and metabolism in human fetal membranes. *Acta Obstet. Gynecol. Scand.*, **77**, 142-150.

Brocklesby, J., Hayman, R., Warren, A., Johnson, I. and Baker, P. (1998) Pre-eclampsia - a role for vascular endothelial growth factor. *J. Soc. Gynecol. Invest.* **5** (suppl), 67A.

Brown, G.C. (1992) Control of respiration and ATP synthesis in mammalian mitochondrial cells. *Biochem. J.*, **284**, 1-13.

Brune, B. and Lapetina, E.G. (1990) Properties of a novel nitric oxide stimulated ADP ribosyltransferase. *Arch. Biochem. Biophys.*, **279**, 286-290.

Buhimschi, I., Chwalisz, K., Yallampalli, C., Garfield, R.E. (1995a) Nitric oxide may be involved in cervical ripening during pregnancy. *J. Soc. Gynecol. Invest.*, **2**, 225.

Buhimschi, I., Yallampalli, C., Dong, Y-L. and Garfield, R.E. (1995b) Involvement of a nitric oxide-cyclic guanosine monophosphate pathway in control of human

uterine contractility during pregnancy. *Am. J. Obstet. Gynecol.* **172,** 1577-1584.

Buhimschi, I., Ali, M., Jain, V., Chwalisz, K. and Garfield, R.E. (1996) Differential regulation of nitric oxide in the rat uterus and cervix during pregnancy in labour. *Hum. Reprod.*, **11,** 1755-1766.

Buhimschi, C., Buhimschi, I., Yallampalli, C., Chwalisz, K., Garfield, R.E. (1997) Contrasting effects of diethylenetriamine-nitric oxide, a spontaneously releasing nitric oxide donor, on pregnant rat uterine contractility in vitro versus in vivo. *Am. J. Obstet. Gynecol.* **177**, 690-701.

Bulbring, E. and Tomito, T. (1987) Catecholamine action on smooth muscle. *Pharmacol. Rev.*, **39**, 49-96.

Bulletti, C., De Ziegler, D., Flamigni, C. *et al.* (1997) Targeted drug delivery in gynaecology: the first uterine pass effect. *Hum. Reprod.*, 12, 1073-1079.

Butterworth, B.H., Greer, I.A., Liston, W.A. *et al.*, (1991) Immunocytochemical localization of neutrophil elastase in term placenta, decidua and myometrium in pregnancy-induced hypertension. *Br. J. Obstet. Gynaecol.*, **98,** 929-933.

Buttery, L.D.K., McCarthy, A., Springall, D.R., Sullivan, M.H.F., Elder, M.G., Michel, T., Polak, J.M. (1994) Endothelial nitric oxide synthase in the human placenta: regional distribution and proposed regulatory role at the feto-maternal interface. *Placenta*, **15**, 257-265.

Cabrol, D., Dubois, P., Sedbon, E. *et al.* (1987) Prostaglandin E_2-induced changes in the distribution of glycosaminoglycans in the isolated rat cervix. *Eur. J. Obstet. Gynecol.*, **26**, 359-365.

Calder, A.A. (1980) Pharmacological management of the unripe cervix in the human. In: Naftolin F, Stubblefield PG, eds. Dilatation of the uterine cervix. New York: Raven Press, 147-156.

Calder, A.A.. (1994) The cervix during pregnancy. In: Chard T, Grudzinskas JG, eds. The Uterus. Cambridge University Press. 288-307.

Calder, A.A. and Greer, I.A. (1991) Pharmacological modulation of cervical compliance in the first and second trimesters of pregnancy. *Semin. Perinatol.*, **15**, 162-172.

Calder, A.A. and Greer, I.A. (1992) Cervical physiology and induction of labour. In: Bonnar J, ed. Recent advances in obstetrics and gynaecology. Churchill Livingstone, **17**, 33-56.

Caldeyro-Barcia, R. and Poseiro, J.J. (1960) Physiology of the uterine contraction. *Clin. Obstet. Gynecol.*, **3**, 386-408.

Calixto, J.B. and Rae, G.A. (1991) Effects of endothelin, Bay K8644 and other oxytocics in non-pregnant and late pregnant rat isolated uterus. *Eur. J. Pharmacol.*, **192**, 109-116.

Carpenter, M. (1981) Antioxidant effects on the prostaglandin endoperoxidase synthetase product profile. *Fed. Proc.*, **40**, 189-194.

Carsten, M.E. (1968) Regulation of myometrial composition, growth and activity. In: The maternal organism, Vol 1 of the Biology of Gestation Series. New York, Academic Press. 355-425.

Carsten, M.E. and Miller, J.D. (1985) Calcium release by inositol trisphosphate from calcium-transporting microsomes derived from uterine sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.*, **130**, 1027-1031.

Casatella, M.N. (1995) The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today,* **16,** 21-26.

Casey, M.L. and MacDonald, P.C. (1988) Biomolecular processes in the onset of parturition: decidual activation. *Clin. Obstet. Gynecol.*, **31**, 533-552.

Casey, M.L. and MacDonald, P.C. (1993) Human parturition: distinction between the initiation of parturition and the onset of labor. *Semin. Reprod. Endocrinol.*, **11**, 272-284.

Casey, M.L., Cox, S.M., Word, A. and MacDonald, P.C. (1990) Cytokines and infection-induced preterm labour. *Reprod. Fertil. Dev.*, **2**, 499-510.

Ceccatelli, S., Grandison, L., Scott, R.E.M., Pfaff, D.W. and Kow, L-M. (1996) Estradiol regulation of nitric oxide synthase mRNAs in rat hypothalamus. *Neuroendocrinol* . **64**, 357-363.

Challis, J.R.G. and Hooper, S. (1989) Birth: Outcome of a positive cascade. *Bailliere's Clin. Endocrinol. Metab.*, **3**, 781-793.

Challis, J.R. and Mitchell, M.D. (1994) Basic mechanisms of pre-term labour. *Res. Clin. Forums*, **16**, 39-58.

Chard, T., Boyd, N.R.H., Edwards, C.R.W., Boyd, N. (1971) Release of oxytocin and vasopressin by human fetus during labour. *Nature (Lond.)*, **234**, 352-354.

Chibbar, R., Miller, F.D., Mitchell, B.F. (1993) Synthesis of oxytocin in amnion, chorion and decidua may influence the timing of human parturition. *J. Clin. Invest.*, **91**, 185-192.

Church, S.L., Farmer, D.R., Nelson, D.M. (1992) Induction of manganese superoxide dismutase in cultured human trophoblast during in vitro differentiation *Develop. Biol.*, **149**, 177-184.

Chwalisz, K. (1988) Cervical ripening and induction of labour with progesterone antagonists. Proceedings of the XI European Congress of Perinatal Medicine. Rome: CIC Edizioni Internaziolini, 60.

Chwalisz, K. (1994) The use of progesterone antagonists for cervical ripening as an adjunct to labour and delivery. *Hum. Reprod.*, **9**, 131-163.

Chwalisz, K., Benson, M., Scholz, P., Daum, J., Beier, H.M., Hegele-Hartung, C. (1994a) Cervical ripening with the cytokines interleukin 8, interleukin 1ß and tumour necrosis factor α in guinea pigs. *Hum. Reprod.*, **9**, 2173-2181.

Chwalisz, K., Ciesla, I., Garfield, R.E. (1994b) Inhibition of nitric oxide (NO) synthesis induces preterm parturition and pre-eclampsia-like conditions in guinea pigs. *Soc. Gynecol. Invest., 41st Annual Meeting*. A36.

Chwalisz, K., Shao-Qing, S., Garfield, R.E., Bier, H. M. (1997) Cervical ripening in guinea-pigs after a local application of nitric oxide. *Hum. Reprod.*, **12**, 2093-2101.

Colditz, I.G. (1990) Effects of exogenous prostaglandin E_2 and actinomycin-D on plasma leakage induced by neutrophil activating peptide-1/interleukin-8. *Imm. Cell. Biol.*, **68**, 397-403.

Cole, W.C., Garfield, R.E., Kirkaldy, J.S. (1988) Gap junctions and direct intercellular communication between rat uterine smooth muscle cells. *Am. J. Physiol.*, **249**, C20-31.

Conrad, K.P., Vill, M., McGuire, P.G. *et al.* (1993) Expression of nitric oxide synthase by syncytiotrophoblast in human placental villi. *FASEB J*, **7**, 1269-1276.

Cotton, D.B., Longmire, S., Jones, M.J., Dorman, K.F., Tesem, J., Joyce, T.H. (1986) Cardiovascular alterations in severe pregnancy-induced hypertension: effect of intravenous nitroglycerin coupled with blood volume expansion. *Am. J. Obstet. Gynecol.*, **154**, 1053-1059.

Crankshaw, D.J., Crankshaw, J., Brenda, L.A., Daniel, E.E. (1979) Receptors for E type prostaglandins in the plasma membrane of non-pregnant myometrium. *Arch. Biochem. Biophys.*, **198**, 459-465.

Crapo, J.D., Oury, T.D., Rabouillec, C. *et al.*, (1992) Copper zinc superoxide dismutase is primarily a cytosolic protein in human cells. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 10405-10409.

Cruz, M. A., Gonzalez, C., Acevedo, C. G. *et al.* (1989) Effects of histamine and 5-HT on the contractility of isolated pregnant and non-pregnant human myometrium. *Gynecol. Obstet. Invest.*, **28**, 1-4.

Csapo, A.L. (1956) Progesterone 'block'. *Am. J. Anat.*, **98**, 273-291.

Csapo, A.L. (1977) The "see-saw" theory of parturition. *Ciba Found. Symp.*, **47**, 159-210.

Danforth, D.N., Buckingham, J.C., Roddick, J.W. (1960) Connective tissue changes incident to cervical effacement. *Am. J. Obstet. Gynecol.*, **86**, 939-945.

Danforth, D.N., Veis, A., Breen, M. *et al.* (1974) The effect of pregnancy and labor on the human cervix: changes in collagen, glycoproteins and glycosaminoglycans. *Am. J. Obstet. Gynecol.*, **120**, 641-649.

David, M., Halle, H., Lichtenegger, W., Sinha, P., Zimmermann, T. (1998) Nitroglycerin to facilitate fetal extraction during cesarean delivery. *Obstet. Gynecol.*, **91**, 119-124.

Dawood, M.Y., Wang, C.F., Gupta, R., Fuchs, F. (1978) Fetal contribution to oxytocin in human labor. *Obstet. Gynecol.*, **52**, 205-209.

Dawood, M.Y. and Khan-Dawood, F.S. (1985) Oxytocin. In: Shearman, R.F., ed. Clinical reproductive endocrinology. New York: Churchill Livingstone. 233-249.

Dayan, S.S. and Schwalbe, S.S. (1996) The use of small dose intravenous nirtoglycerin in a case of uterine inversion. *Anest. Analg.*, **82**, 1091-1093.

Dennes, W.J., Slater, D.M., Bennett, P.R. (1997) Nitric oxide synthase mRNA expression in human fetal membranes: a possible role in parturition. *Biochem. Biophys. Res. Commun.*, **233**, 276-28.

Di Iulio, J.L., Gude, N.M., King, R.G. and Brennecke, S.P. (1996) Human placental and fetal membrane nitric oxide synthase activity before, during, and after labor at term. *Reprod. Fert. Dev.* ,**7**, 1505-1508.

Diamond, J. (1983) Lack of correlation between cyclic GMP elevation and relaxation of nonvascular smooth muscle by nitroglycerin, nitroprusside, hydroxylamine and sodium azide. *J. Pharmacol. Exp. Ther.*, **225**, 422-426.

Diamond, J. (1990) Beta-adrenoreceptors, cyclic AMP and cyclic GMP in control of uterine motility. In: Carsten, M.E. and Miller, J.D., eds. Uterine function: molecular and cellular aspects. New York: Plenum Press. 249-275.

Ding, J.Q., Granberg, S., Norstrom, A. (1990) Clinical effects and cervical tissue changes after treatment with 16, 16 dimethyl-trans delta 2 PGE_1 methylester. *Prostaglandins*, **39**, 281-285.

Dong, Y.L. and Yallampalli, C. (1996) Interaction between nitric oxide and prostaglandin E_2 pathways in pregnant rat uteri. *Am. J. Physiol.*, **270**, E471-476.

Drexler, H., Zeiher, A.M., Meinzer, K., Just, H. (1991) Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet*, **338**, 1546-1550.

Egarter, C.H. and Husslein, P. (1986) Biochemistry of myometrial contractility. *Clin. Obstet. Gynecol.*, **6**, 755-769.

Eis, A.L.W., Brockman, D.E., Pollock, J.S., Myatt, L. (1995) Immunohistochemical localisation of endothelial nitric oxide synthase in human villous and extra-villous trophoblast populations and expression during syncytiotrophoblast formation *in vitro*. *Placenta*, **16**, 113-126.

Eis, A.L.W., Brockman, D.E., Myatt, L. (1997) Immunolocalization of the inducible nitric oxide synthase isoform in human fetal membranes. *Am. J. Reprod. Immunol.*, **38**, 289-294.

Ekerhovd, E., Brannstrom, M., Delbro, D., Norstrom, A. (1998) Nitric oxide mediated inhibition of contractile activity in the human cervix. *Mol. Hum. Reprod.*, **4**, 915-920.

Ekman, G., Malmstrom, A., Uldbjerg, N. (1986) Cervical collagen: an important regulator of cervical function in term labour. *Obstet. Gynecol.*, **67**, 633-636.

El Maradny, E., Kanayama, N., Halim, A., Maehara, K., Sumimoto, K., Terao, T. (1994) Interleukin-8 induces cervical ripening in rabbits. *Am. J. Obstet. Gynecol.*, **171**, 77-83.

El Maradny, E., Kanayama, N., Halim, A., Maehara, K., Terao, T. (1994) Urinary trypsin inhibitor has a protective effect on the amnion. *Gynecol. Obstet. Invest.*, **38**, 169-172.

El Maradny, E., Kanayama, N., Halim, A., Maehara, K., Sumimoto, K., Terao, T. (1995) The effect of interleukin-1 in rabbit cervical ripening. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **60**, 75-80.

El-Refaey, H., Calder, L., Wheatley, D.N., Templeton, A. (1994) Cervical priming with prostaglandin E_1 analogues, misoprostol and gemeprost. *Lancet*, **343**, 1207-1209.

Ellwood, D.A., Mitchell, M.D., Anderson, A.B.M. (1980) The in vitro production of prostanoids by the human cervix during pregnancy: preliminary observations. *Br. J. Obstet. Gynaecol.*, **87**, 210-214.

Ellwood, D.A., Anderson, A.B.M., Mitchell, M.D. *et al.* (1981) Prostanoids, collagenase and cervical softening in sheep. In: Ellwood DA, Anderson ABM, eds. The cervix in pregnancy and labour: clinical and biochemical investigations. Edinburgh: Churchill Livingstone, 57-73.

Elroy-Stein, O., Bernstein, Y., Groner, Y. (1986) Overproduction of human Cu/Zn superoxide dismutase in transfected cells: extenuation of paraquat mediated cytotoxicity and enhancement of lipid peroxidation. *EMBO J .*, **5**, 615-622.

Europe-Finner, G.N., Phaneuf, S., Tolkovsky, A.M., Watson, S.P. and Bernal, A.L. (1994) Down-regulation of Ga_s in human myometrium in term and preterm labor: a mechanism for parturition. *J. Clin. Endocrinol. Metab.*, **79,** 1835-1839.

Evans, M.I., Dougan, M.B., Moawad, A.H., Evans, W.J., Bryant-Greenwood, G.D., Greenwood, F.C. (1983) Ripening of the human cervix with porcine ovarian relaxin. *Am. J. Obstet. Gynecol.*, **147**, 410-414.

Feelisch, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol., (suppl 3)*, **17**, S25-33.

Flint, A.P.F. (1979) Role of progesterone and estrogen in the control of the onset of labor in man: a continuing controversy. In: Keirse, M.J.N.C., Anderson, A.B.M., Bennebrock-Gravenhorst, J., eds. Human Parturition. New concepts and developments. The Netherlands, Leiden University Press, 85-100.

Flint, A.P.F., Anderson, A.B.M., Steele, P.A., Turnbull, A.C. (1975) The mechanism by which fetal cortisol controls the onset of parturition in the sheep. *Biochem. Soc. Trans.*, **3**, 1189-1194.

Florio, P., Woods, R.J., Genazzani, A.R., Lowry, P.J., Petragalia, F. (1997) Changes in amniotic immunoreactive corticotropin-releasing factor (CFR) and CRF-binding protein levels in pregnant women at term and during labor. *J. Clin. Endocrinol. Metab.*, **82**, 835-838.

Forstermann, U., Pollack, J.S., Schmidt, H.H.H.W. *et al.* (1991) Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc Natl Acad Sci USA*, **88**, 1786-1792.

Fosang, A.J., Handley, C.J., Santer, V., Lowther, D.A., Thorburn, G.D. (1984) Pregnancy-related changes in the connective tissue of the ovine cervix. *Biol. Reprod.*, **30**, 1223-1235.

Frenette, P.S. and Wagner, D.D. (1996) Adhesion molecules - Part 1. *N. Eng. J. Med.*, **334,** 1526-1529.

Fu, X., Masoumeh, R., Lofgren, M., Ulmsten, U., Backstrom, T. (1993) Anti-tachyphylactic effects of progesterone and oxytocin on term human myometrial contractile activity *in vitro*. *Obstet. Gynecol.*, **82**, 532-538.

Fuchs, A.R. (1978) Hormonal control of myometrial function during pregnancy and parturition. *Acta Endocrinol.*, **221**, 3-71.

Fuchs, A.R., Fuchs, F., Husslein, P., Soloff, M.S. (1984) Oxytocin receptors in the human uterus during pregnancy and parturition. *Am. J. Obstet. Gynecol.*, **150,** 734-741.

Fuchs, A.R., Romero, R., Keefe, D., Parra, M., Oyarzun, E., Behnke, E. (1991) Oxytocin secretion and human parturition: pulse frequency and duration increase during spontaneous labor in women. *Am. J. Obstet. Gynecol.*, **165**, 1515-1523.

Furchgott, R.F., Jothianandan, D., Freay, A.D. (1990) Endothelium-derived relaxing factor: some old and new findings. In: Moncada, S. and Higgs, E., eds. Nitric oxide from L-arginine: a bioregulatory system. Elsevier Science. 5-17.

Galli, S.J. (1993) New concepts about the mast cell. *N. Eng. J. Med.*, **328**, 257-265.

Gangula, P.R.R., Dong, Y.L., Yallampalli, C. (1996) Nitric oxide generation and expression of nitric oxide synthases by myometrial cells and their regulation by cytokines. *J. Soc. Gynecol. Invest.*, **3**, 348A.

Garber, P., Sinet, P.M., Jerome, H. *et al.* (1979) Copper/zinc superoxide dismutase activity in trisomy 21 by translocation. *Lancet,* **2**, 914-915.

Garfield, R.E. (1984) Myometrial ultrastructure and uterine contractility. In: Bottari, S., Thomas, J.P., Vokaer, A., eds. Uterine contractility. New York: Mason. 81-109.

Garfield, R.E. (1994) Role of cell-to-cell coupling in control of myometrial contractility and labor. In: Garfield, R.E., Tabb, T.N., eds. Control of uterine contractility. Boca Raton, CRC Press. 39-81.

Garfield, R.E., Sims, S.M., Daniel, E.E. (1977) Gap junctions: their presence and necessity in myometrium during parturition. *Science*, **198**, 958-960.

Garfield, R.E. and Hayashi, R.H. (1981) Appearance of gap junctions in the myometrium of women during labor. *Am. J. Obstet. Gynecol.*, **140**, 254-260.

Garfield, R.E. and Somlyo, A.P. (1985) Structure of smooth muscle. In: Calcium and contractility. Clifton, NJ: Humana. 1-36.

Garfield, R.E. and Yallampalli, C. (1994) Structure and function of uterine muscle. In: Chard, T., Grudzinskas, J.G., eds. The Uterus. Cambridge University Press. 54-93.

Garvey, E.P., Tuttle, J.V., Covington, K. *et al.* (1994) Purification and characterization of the constitutive nitric oxide synthase from human placenta. *Arch Biochem Biophys*, **311**, 235-241.

Ghabour, M.S., Eis, A.L.W., Brockman, D.E., Pollock, J.S., Myatt, L. (1995) Immunohistochemical characterization of placental nitric oxide synthase expression in pre-eclampsia. *Am. J. Obstet. Gynecol.*, **173**, 687-694.

Gibbins, I.L., Furness, J.B., Costa, M., MacIntyre, I., Hilliyard, C.J., Girgis, S. (1979) Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. *Neurosci. Lett.*, **57**, 125-130.

Goldenberg, R.L. and Rouse, D.J. (1998) Prevention of premature birth. *N. Eng. J. Med.*, **339**, 313-320.

Golichowski, A. (1980) Cervical stromal interstitial polysaccharide metabolism in pregnancy. In: Naftolin F, Stubblefield PG, eds. Dilatation of the uterine cervix: connective tissue biology and clinical management. New York: Raven Press, 99-112.

Gordon, A.J., Calder, A.A.. (1977) Oestradiol appllied locally to ripen the unfavourable cervix. *Lancet*, **ii**, 1319-1321.

Graf, R., Langer, J-U., Schonfelder, G. *et al.* (1994) The extravascular contractile system in the human placenta. *Anat. Embryol.*, **190**, 541-548.

Granstrom, L., Ekman, G., Ulmsten, U. and Malmstrom, A. (1989) Changes in connective tissue of corpus and cervix uteri during ripening and labour in term pregnancy. *Br. J. Obstet. Gynaecol.*, **96**, 1198-1202.

Grant, J.M. (1994) Induction of labour confers benefit in prolonged pregnancy. *Br. J. Obstet. Gynaecol.*, **101**, 99-102.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R. (1982) Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. *Anal. Biochem.*, **126**, 131-138.

Greer, I.A. (1992) Cervical ripening. In: Drife JO, Calder AA, eds. Prostaglandins and the uterus. London: Springer-Verlag, 191-209.

Greer, I.A., Miller, M., Calder, A.A. (1992) Gemeprost and cervical ripening: biophysical and histological effects. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **47**, 1-9.

Grimes, D.A., Schultz, K.F. and Cates, W.J. (1984) Prevention of uterine perforation during curettage abortion. *JAMA*, **251**, 2108-11.

Grozdanovic, Z., Mayer, B., Baumgarten, H.G., Bruning, G. (1994) Nitric oxide synthase-containing nerve fibres and neurons in the genital tract of the female mouse. *Cell Tissue Res.*, **275**, 355-360.

Grunewald, C., Kublickas, M., Carlstrom, K., Lunell, N.O., Nisell, H. (1995) Effects of nitroglycerine on the uterine and umbilical circulation in severe preeclampsia. *Obstet. Gynecol.*, **86**, 600-604.

Gude, N.M., Di Iulio, J., Brennecke, S.P. and King, R.G. (1994) Human placental villous nitric oxide synthase activity. *Pharmacol. Commun.*, **4**, 163-171.

Guo, F.H., De Raeve, H.R., Rice, T.W., Stuehr, D.J., Thunnissen, F.B., Erzurum, S.C. (1995) Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium in vivo. *Proc. Natl. Acad. Sci. USA*, **92**, 7809-7813.

Gupta, J.K. and Johnson, N. (1990) Effect of mifepristone on dilatation of the pregnant and non-pregnant cervix. *Lancet*, **i**, 1238- 1240.

Halgunset, J., Johnsen, H., Kjollesdal, A.M. *et al.* (1994) Cytokine levels in amniotic fluid and inflammatory changes in the placenta from normal deliveries at term. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **56**, 153-160.

Hall, M.H., Danielian, P., Lamont, R.F. (1997) Importance of preterm birth. In: Elder, M.G., Romero, R., Lamont, R.F. eds. Preterm Labor. New York, Churchill Livingstone, 1-28.

Harvey, M.B., Arecellana-Panlilio, M.Y., Zhang, X. *et al.* (1995) Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary bovine oviduct cultures employed for embryo co-culture. *Biol. Reprod.*, **53**, 532-540.

Hegele-Hartung, C., Chwalisz, K., Beier, H.M., Elger, W. (1989) Ripening of the uterine cervix of the guinea-pig after treatment with the progesterone antagonist onapristone (ZK98.299): an electron microscopic study. *Hum. Reprod.*, **4**, 369-377.

Helm, C.W., Davies, N. and Beard, R.J. (1988) A comparison of gemeprost (Cervagem) pessaries and Lamicel tents for cervical preparation for abortion by dilatation and suction. *Br. J. Obstet. Gynaecol.*, **95**, 911-15.

Henshaw, R.C. and Templeton, A.A. (1991) Pre-operative cervical preparation before first trimester vacuum aspiration: a randomized controlled comparison between gemeprost and mifepristone (RU486). *Br. J. Obstet. Gynaecol.*, **98**, 1025-30.

Heymann, M.A., Bootstaylor, B., Roman, C., Natuzzi, E.S., Parer, J.T., Harrison, M.R., Riemer, R.K. (1993) Glyceryl trinitrate stops active labor in sheep. In: Moncada, S., Feelish, M., Busse, R., Higgs, E.A., eds. The biology of nitric oxide. London: Portland Press. 201-203.

Hillhouse, E.W., Grammatopoulos, D., Milton, N.G.N., Quartero, H.W.P. (1993) The identification of a human myometrial corticotropin releasing hormone receptor that increases in affinity during pregnancy. *J. Clin. Endocrinol. Metab.*, **76**, 736-741.

Hillier, K. and Karim, S.M.M. (1970) The human isolated cervix: a study of its spontaneous motility and responsiveness to drugs. *Br. J. Pharmacol.*, **40**, 576-577.

Hirst, J.J., Teixeira, F.J., Zakar, T. and Olson, D.M. (1995) Prostaglandin endoperoxide-H synthase-1 and -2 messenger ribonucleic acid levels in human amnion with spontaneous labor onset. *J Clin. Endocrinol. Metab.*, **80,** 517-523.

Hoffman, B.B., Lavin, T.N., Lefkowitz, R.J., Ruffolo, R.R. (1981) Alpha adrenergic subtypes in rabbit uterus: mediation of myometrial contraction and regulation by estrogens. *J. Pharmacol. Exp. Ther.*, **219**, 290-295.

Hope, B.T., Michael, G.J., Knigge, M., Vincent, S.R. (1991) Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc. Natl. Acad. Sci. USA*, **88**, 2811-2814.

Hore, A. and Mehrotra, P. N. (1988) Presence of a blastocyst and mast cell depletion of the mouse uterus. *Acta. Anat.*, **132,** 6-8.

Horton, E.W., Poyser, N. (1976) Uterine luteolytic hormone: a physiological role for prostaglandin $F_{2\alpha}$. *Physiol. Rev.*, **56**, 595-651.

Huang, J., Roby, K.F., Pace, J.L., Russell, S.W., Hunt, J.S. (1995) Cellular localization and hormonal regulation of inducible nitric oxide synthase in cycling mouse uterus. *J. Leukoc. Biol.*, **57**, 2-35.

Huang, P.L., Dawson, T.M., Bredt, D.S., Snyder, S.H. and Fishman, M.C. (1993) Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* , **75**, 1273-1286.

Huang, P.L., Huang, Z., Mashimo, H., Bloch, K.D., Moskowitz, M.A., Bevan, J.A. and Fishman, M.C. (1995) Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* , **377**, 239-242.

Huang, W.M., Gu, J., Blank, M.A., Allen, J.M., Bloom, S.R., Polak, J.M. (1984) Peptide-immunoreactive nerves in the mammalian female genital tract. *Histochem. J.*, **16**, 1297-1310.

Hubel, C.A., Roberts, J.M., Taylor, R.W. *et al.*, (1989) Lipid peroxidation in pregnancy : New perspectives on preeclampsia. *Am. J. Obstet. Gynecol.*, **161**, 1025-1034.

Huie, R.E. and Padmaja, S. (1993) The reaction of nitric oxide with superoxide. *Free Rad. Res. Commun.*, **18**, 195-199.

Hurwitz, L. (1986) Pharmacology of calcium channels and smooth muscle. *Ann. Rev. Pharmacol. Toxicol.*, **26**, 225-258.

Huxley, H.E. (1971) The structural basis of muscular contraction. *Proc. R. Soc. Lond. [series B]*, **178**, 131-149.

Ianaro, A., O'Donnell, C.A., Di Rosa, M., Liew, F.Y. (1994) A nitric oxide synthase inhibitor reduces inflammation, down regulates inflammatory cytokines and enhances IL-10 production in carrageenin-induced mice. *Immunol.*, **82**, 370-75.

Ignarro, L.J. (1992) Haem-dependent activation of cytosolic guanylate cyclase by nitric oxide: a widespread signal transduction mechanism. *Biochem. Soc. Trans.*, **20**, 465-469.

Ignarro, L.J., Bush, P.H., Buga, G.M. *et al.* (1990) Nitric oxide and cyclic GMP formation can cause electrical field stimulation that can cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Comm.*, **170**, 843-850.

Inoue, Y., Nakao, K., Obabi, K., *et al.* (1990) Some electrical properties of human pregnant myometrium. *Am. J. Obstet. Gynecol.*, **162**, 1090-1098.

Ito, A., Kitamura, K., Mori, Y., Hirakawa, S. (1979) The change in solubility of type 1 collagen in human cervix in pregnany at term. *Biochem. Med.*, **21**, 262-270.

Izumi, H., Yallampalli, C. and Garfield, R.E. (1993) Gestational changes in L-arginine-induced relaxation of pregnant rat and human myometrial smooth muscle. *Am. J. Obstet. Gynecol.*, **169**, 1327-1337.

Jarasch, E-D., Bruder, G. and Heid, H.W. (1986) Significance of xanthine oxidase in capillary endothelial cells. *Acta Physiol. Scand. (Suppl).*, **548**, 39-46.

Jeffrey, J.J. (1991) Collagen and collagenase: pregnancy and parturition. *Semin. Perinatol.*, **15**, 118-12.

Jeffrey, J.J., Coffrey, R.J., Eizen, A.Z. (1971) Studies of uterine collagenase in tissue culture II. Effect of steroid hormones on enzyme production. *Biochim. et. Biophys. Acta.*, **252**, 143.

Jeffrey, J.J., Ehlich, L.S. and Roswit, W.T. (1991) Serotonin: an inducer of collagenase in myometrial smooth muscle cells. *J. Cell. Physiol.*, **146,** 399-406.

Jeffrey, J.J. and Koob, T.J. (1980) Endocrine control of collagen degradation in the uterus. In: Naftolin F, Stubblefield PG, eds. Dilatation of the uterine cervix. New York: Raven Press,135-145.

Jennings, R.W., MacGillivray, T.E., Harrison, M.R. (1993) Nitric oxide inhibits preterm labor in rhesus monkeys. *J. Mater. Fetal Med.*, **2**, 170-175.

Johnston, T.A., Hodson, S., Greer, I.A., Kelly, R.W., Calder, A.A.. (1993) Plasma glycosaminoglycan and prostaglandin concentrations before and after the onset of spontaneous labour. In: Prostaglandins in Reproduction (Proceedings of the Third European Congress), Edinburgh.

Jones, G.D. and Poston, L. (1997) The role of endogenous nitric oxide synthesis in contractility of term and preterm human myometrium. *Br. J. Obstet. Gynaecol.*, **104**, 241-245.

Jones, S.A. and Challis, J.R.G. (1989) Local stimulation of prostaglandin production by corticotropin releasing hormone in human fetal membranes and placenta. *Biochem. Biophys. Res. Commun.*, **159**, 192-194.

Junqueira L.C.U., Zugaib M., Montes G.S. *et al.* (1980) Morphological and histochemical evidence for the occurrence of collagenolysis and for the role of neutrophilic polymorphonuclear leukocytes during cervical dilatation. *Am. J. Obstet. Gynecol.*, **138**, 273-281.

Kagan, V.E. (1988) In : *Lipid peroxidation in Biomembranes*, Boca Raton, Florida: CRC Press, 13-146.

Kanayama, N. (1994) Intrauterine defensive mechanism of amniotic fluid and fetal membranes. *Acta. Obstet. Gynaecol. Jpn.*, **46**, 673-685.

Kanayama, N., Chinarong, L., Naruse, H. *et al.* (1992) The effect of granulocyte elastase inhibitor (ulinastatin) vaginal suppository on patients with imminent premature delivery. *Acta. Obstet. Gynaecol. Jpn.*, **44**, 477-482.

Kanayama, N., El Maradny, E., Halim, A., Maehara, K., Kajiwara, Y., Terao, T. (1995) Urinary trypsin inhibitor suppresses premature cervical ripening. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **60**, 181-186.

Karalis, K., Goodwin, G., Majzoub, J.A. (1996) Cortisol blockade of progesterone: a possible molecular mechanism involved in the initiation of human parturition. *Nature Med.*, **2**, 556-560.

Keelan, J.A., Myatt, L., Mitchell, M.D. (1997) Endocrinology and paracrinology of parturition. In: Elder, M.G., Romero, R., Lamont, R.F. eds. Preterm Labor. New York, Churchill Livingstone, 457-491.

Keirse, M.J.N.C. (1979) Endogenous prostaglandins in human parturition. In: Keirse, M.J.N.C., Anderson, A.B.M., Bennebroek-Gravenhorst, J., eds. Human Parturition. New concepts and developments. The Netherlands, Leiden University Press, 101-142.

Keirse, M.J.N.C. (1995) New perspectives for the effective treatment of preterm labor. *Am. J. Obstet. Gynecol.*, **173**, 618-628.

Kelly, R.W. (1996) Inflammatory mediators and parturition. *J. Reprod. Fertil.*, **106**, 89-96.

Kelly, R.W., Healy, D.L., Cameron, I.T. *et al.* (1986) The stimulation of prostaglandin production by two antiprogesterone steroids in human endometrial cells. *J. Clin. Endocrinol. Metab.*, **62**, 1116-1123.

Kelly, R.W. and Bukman, A. (1990) Antiprogestagenic inhibition of uterine prostaglandin inactivation: a permissive mechanism for uterine stimulation. *J. Steroid Biochem. Mol. Biol.*, **37**, 37-101.

Khan-Dawood, F.S. and Dawood, M.Y. (1984) Estrogen and progesterone receptor and hormone levels in human myometrium and placenta in term pregnancy. *Am. J. Obstet. Gynecol.*, **150**, 501-505.

Kim, B-K., Ozaki, H., Lee, S-M. and Karaki, H. (1995) Increased sensitivity of rat myometrium to the contractile effect of platelet activating factor before delivery. *Br. J. Pharmacol.*, **115**, 1211-1214.

Kleissl, H.P., van der Rest, M., Naftolin, F., Glorieux, F.H., De Leon, A. (1978) Collagen changes in human cervix at parturition. *Am. J. Obstet. Gynecol.*, **130**, 748-753.

Klug-Roth, D., Fridovich, I. and Rabani, J. (1973) Pulse radiolytic investigation of catalyzed disproportionation. Mechanism for bovine superoxide dismutase. *J. Am. Chem. Soc.*, **95**, 2786-2790.

Knoches, A.L. and Doyle, L.W. (1993) Long-term outcome of infants born preterm. *Baillieres Clin. Obstet. Gynaecol.*, **7**, 633-651.

Kobzik, L., Reid, M.B., Bredt, D.S., Stamler, J.S. (1994) Nitric oxide in skeletal muscle. *Nature*, **372**, 546-548.

Kokenyesi, R. and Woessner, J.R. (1990) Relationship between dilatation rate of the uterine cervix and a small dermatan sulfate proteoglycan. *Biol. Reprod.*, **42**, 87-89.

Kolb, J.P., Paul-Eugene, N., Damais, C., Yamaoka, K., Drapier, J.C., Dugas, B. (1994) Interleukin-4 stimulates cGMP production by IFN-γ-activated human monocytes: involvement of the nitric oxide synthase pathway. *J. Biol. Chem.*, **269**, 9811-9816.

Kosuzume, H., Ashida, Y., Takemura, M., Kato, K., Ohnishi, H. (1983) Mechanisms of enzyme inhibition by trypsin inhibitor in human urine. *Igaku. No. Ayumi.*, **125**, 187-190.

Krantz, K.E. (1959) Innervation of the human uterus. *Ann. NY Acad. Sci.*, **75**, 770-784.

Kumar, D., Zourlas, P.A., Barnes, A.C. (1965) In vivo effect of amyl nitrite on human pregnant uterine contractility. *Am. J. Obstet. Gynecol.*, **91**, 1066-1068.

Kurki, T., Laatikainen, T., Salminen-Lappalainen, K., Ylikorkala, O. (1991) Maternal plasma corticotrophin releasing hormone is elevated in preterm labour but unaffected by indomethacin or nylidrin. *Br. J. Obstet. Gynaecol.*, **98**, 685-691.

Lasky, L.A. (1992) Selectins: interpreters of cell-specific carbohydrate information during inflammation. *Science*, **258**, 964-996.

Lawrie, A., Penney, G., Templeton, A. (1996) A randomised comparison of oral and vaginal misoprostol for cervical priming before suction termination of pregnancy. *Br. J. Obstet. Gynaecol.*, **103**, 1117-1119.

Ledger, W.L., Webster, M., Harrison, L.P. *et al.* (1985) Increase in cervical extensibility during labor induced after isolation of the uterus in the pregnant sheep. *Am. J. Obstet. Gynecol.*, **151**, 397-402.

Lee, J.H. and Chang, K.C. (1995) Different sensitivity to nitric oxide of human pregnant and nonpregnant myometrial contractility. *Pharmacol. Commun.*, **5**, 147-154.

Lees, C., Campbell, S., Jauniaux, E. *et al.* (1994) Arrest of preterm labour and prolongation of gestation with glyceryl trinitrate, a nitric oxide donor. *Lancet*, **343**, 1325-1326.

Lelaidier, C., Baton, C., Benifla, J.L., Fernandez, H., Bourget, Ph., Frydman, R. (1994). Mifepristone for labour induction after previous Caesarean section. *Br. J. Obstst. Gynaecol.*, **101**, 501-503.

Leppert, P.C. (1992) Cervical softening, effacement and dilatation: A complex biochemical cascade. *J. Mat. Fet. Med.*, 1, 213-223.

Leppert, P.C. (1995) Anatomy and physiology of cervical ripening. *Clin. Obstet. Gynecol.*, **38**, 267-279.

Leppert, P.C., Yu, S.Y. (1994) Apoptosis in the cervix of pregnant rats in association with cervical softening. *Gynecol. Obstet. Invest.*, **37**, 150-154.

Liggins, G.C. (1978) Ripening of the cervix. *Semin. Perinatol.*, **2**, 261.

Liggins, G.C. (1981) Cervical ripening as an inflammatory reaction. In: Ellwood DA, Anderson ABM, eds. The cervix in pregnancy and labour: clinical and biochemical investigations. Edinburgh: Churchill Livingstone, 1-9.

Liggins, G.C. (1994) Mechanisms of the onset of labor: the New Zealand perspective. *Aust. N. Z. J. Obstet. Gynaecol.*, **34**, 338-342.

Lindahl, U. and Hook, M. (1978) Glycosaminoglycans and their binding to biological macromolecules. *Ann. Rev. Biochem.*, **47**, 385.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.

Lumley, J. (1993) The epidemiology of preterm birth. *Bailleres Clin. Obstet. Gynaecol.*, **7**, 477-498.

MacKenzie, I.Z. and Fry, A. (1981) Prostaglandin E_2 pessaries to facilitate first trimester aspiration termination. *Br. J. Obstet. Gynaecol.*, **88**, 1033-37.

Magann, E.F., Perry, K.G., Dockery, J.R., Bass, J.D., Chauhan, S.P., Morrison, J.C. (1995) Cervical ripening before medical induction of labor: A comparison of prostaglandin E_2, estradiol, and oxytocin. *Am. J. Obstet. Gynecol.*, **172**, 1702-1708.

Majewski, M., Sienkiewicz, W., Kaleczye, J., Mayer, B., Czaja, K., Lakomy, M. (1995) The distribution and co-localizaton of immunoreactivity to nitric oxide synthase, vasoactive intestinal peptide and substance P within nerve fibres supplying bovine and porcine female genital organs. *Cell Tissue Res.*, **281**, 445-464.

Many, A., Westerhausen-Larson, A., Kanbour-Shakir, A. *et al.* (1996) Xanthine oxidase/dehydrogenase is present in human placenta. *Placenta*, **17**, 361-365.

Marc, S., Leiber, D., Harbon, S. (1986) Carbachol and oxytocin stimulate the generation of inositol phosphates in the guinea pig myometrium. *FEBS Lett.*, **201**, 9-14.

Margono, F., Minkoff, H., and Chan, E. (1993) Intrauterine pressure wave characteristics of the upper and lower uterine segments in parturients with active-phase arrest. *Obstet. Gynecol.*, **81**, 481-485.

Marklund, S.L. (1984) Extracellular superoxide dismutase in human tissues and human cell lines. *J. Clin. Invest.*, **74**, 1398-1403.

Matsuo, K., Gokita, T., Karibe, H., Uchida, M.K. (1989) Calcium independent contraction of uterine smooth muscle. *Biochem. Biophys. Res. Commun.*, **155**, 722-727.

Mayer, D.C. and Weeks, S.K. (1992) Antepartum uterine relaxation with nitroglycerin at caesarean delivery. *Can. J. Anaesth.*, **39**, 166-169.

McCall, T.B., Boughton-Smith, N.K., Palmer, *et al.* (1989) Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem. J.*, **261**, 293-296.

McLean, M., Bisits, A., Davis, J., Woods, R., Lowry, P., Smith, R. (1995) A placental clock controlling the length of human pregnancy. *Nature Med.*, **1**, 460-463.

McLean, M., Thompson, D., Zhang, H-P., Brinsmead, M., Smith, R. (1994) Corticotropin releasing hormone and beta-endorphin in labour. *Eur. J. Endocrinol.*, **131**, 167-172.

MacLennan, A.H., Green, R.C., Bryant-Greenwood, G.D., Greenwood, F.C., Seamark, R.F. (1980) Ripening of the human cervix and induction of labour with purified porcine relaxin. *Lancet*, **i**, 220-223.

McMurty, J.P., Floerscheim, G.L., Bryant-Greenwood, G.D. (1980) Characterization of the binding of ^{125}I-labelled succinylated porcine relaxin in human and mouse fibroblasts. *J. Reprod. Fertil.*, **58**, 43-49.

Mercier, F.J., Dounas, M., Bouaziz, H., Lhuissier, C., Benhamou, D. (1997) Intravenous nitroglycerin to relieve intrapartum fetal distress related to uterine hperactivity: a prospective observational study. *Anesth. Analg.*, **84**, 1117-1120.

Michel, T. and Feron, O. (1997) Nitric oxide synthases: which, where, how and why? *J. Clin. Invest.*, **100**, 2146-2152.

Mitchell, M.D. (1984) The mechanisms of human parturition. *J. Develop. Physiol.*, **6**, 107-118.

Miyamoto Y, Akaike T., Yoshida M. *et al.* (1996) Potentiation of nitric oxide-mediated vasorelaxation by xanthine oxidase inhibitors. *Proceed. Soc. Exp. Med.*, **211**, 366-373.

Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) Nitric oxide: Physiology, Pathophysiology and Pharmacology. *Pharmacol Rev*, **43**, 109-142.

Mori, A., Zhai Y. L., Toki T. *et al.* (1997) Distribution and heterogeneity of mast cells in the human uterus. *Hum. Reprod.*, **12**, 368-372.

Morris, N.H., Sooranna, S.R., Learmont, J.G., Poston, L., Ramsey, B., Pearson, J.D., Steer, P.J. (1995) Nitric oxide synthase activities in placental tissue from normotensive, pre-eclamptic and growth retarded pregnancies. *Br. J. Obstet. Gynaecol.*, **102**, 711-714.

Morrison, J.J. (1996) Physiology and pharmacology of uterine contractility. In: The Yearbook of the RCOG, 1996. London, RCOG Press. 45-61.

Morrison, J.J., Charnock-Jones, D.S. and Smith, S.K. (1996) Messenger RNA encoding thiol protein disulphide isomerase in amnion, chorion and placenta in human term and preterm labour. *Br. J. Obstet. Gynaecol.*, **103**, 873-878.

Morrison, J.J. and Smith, S.K. (1994) Prostaglandins and uterine activity. In: Grudinskas, J.G. and Yovich, J.L., eds. Cambridge reviews in human reproduction: uterine physiology. Cambridge: Cambridge University Press. 230-251.

Mugge, A., Elwell, J.H., Peterson, T.E. *et al.* (1991) Release of intact endothelium-derived relaxing factor depends on endothelial superoxide dismutase activity. *Am. J. Physiol.*, **260**, C219-C225.

Myatt, L., Brockman, D.E., Eis, A.L.W., Pollock, J.S. (1993a) Immunohistochemical localization of nitric oxide synthase in the human placenta. *Placenta*, **14**, 487-495.

Myatt, L., Brockman, D.E., Langdon, G., Pollock, J.S. (1993b) Constitutive calcium-dependent isoform of nitric oxide synthase in the human placenta. *Placenta*, **14**, 373-383.

Myatt, L., Eis, A.L.W., Brockman, D.E., Greer, I.A. and Lyall, F. (1997a) Endothelial nitric oxide synthase in placental villous tissue from normal, pre-eclamptic and intrauterine growth restricted pregnancies. *Hum. Reprod.*, **12**, 167-172.

Myatt, L., Eis, A.L.W., Brockman, D.E., Kossenjans, W., Greer, I.A. and Lyall, F. (1997b) Inducible (type II) nitric oxide synthase in human placental villous tissue

of normotensive, pre-eclamptic and intrauterine growth-restricted pregnancies. *Placenta*, **18**, 261-268.

Myatt, L., Brockman, D.E., Sahay, R., Pijnenborg, R. Expression of endothelial nitric oxide synthase in placental bed biopsies of normotensive and preeclamptic pregnancies. *J. Soc. Gynecol. Invest.*, **5**, 132A.

Nakane, M., Schmidt, H.H.H.W., Pollock, J.S., Forstermann, U., Murad, F. (1993) Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS*, **316**, 175-180.

Narimoto, K., Noda,Y., Shiotani, M. *et al.* (1990) Immunohistochemical assessment of superoxide dismutase expression in human endometrium throughout the menstrual cycle. *Acta Histochem. Cytochem.*, **24**, 85-91.

Nathan, C.F. (1987) Secretory products of macrophages. *J. Clin. Invest.*, **79**, 319-320.

Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.*, **6**, 3051-3064.

Nathanielsz, P. and Honnebier, M. (1992) Myometrial function. In: Drife, J., Calder, A., eds. Prostaglandins and the uterus. London, Springer-Verlag. 161.

Natuzzi, E.S., Ursell, P.C., Harrison, M. *et al.* (1993) Nitric oxide synthase activity in the pregnant uterus decreases at parturition. *Biochem. Biophys. Res. Comm.*, **194**, 1-8.

Nebot, C., Moutet, M., Huet, P. *et al* (1993) Spectrophotometric assay of superoxide dismutase based on the activated autoxidation of a tetracyclic catechol. *Anal. Biochem.*, **214**, 442-451.

Ngai, S.W., Yeung, K.C.A., Lao, T., Ho, P.C. (1996) Oral misoprostol versus mifepristone for cervical dilatation before vacuum aspiration in first trimester nulliparous pregnancy: a double blind prospective randomised study. *Br. J. Obstet. Gynaecol.*, **103**, 1120-1123.

Nicholson, S., Bonecini-Almeida, M., Lapa e Silva, J.R. *et al.* (1996) Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J. Exp. Med.*, **183**, 2293-2302.

Nixon, W.C.W. (1951) Uterine activity, normal and abnormal. *Am. J. Obstet. Gynecol.*, **62**, 964-984.

Norman, J.E. (1992) Menstrual induction: methods and mechanisms of action. MD Thesis. Edinburgh.

Norman, J.E., Ward, L.M., Martin, W., Macklon, N.S., Cameron, A.D., MacLean, M.R., McGrath, J.C., Greer, I.A. and Cameron, I.T. (1995) Glyceryl trinitrate in the treatment of preterm labour - another placebo? *Endothelium*, **3**, S115.

Norman, J.E. (1996) Nitric oxide and the myometrium. *Pharmacol. Ther.*, **70**, 91-100.

Norman, J.E., Ward, L.M., Martin, W., Cameron, A.D., McGrath, J.C., Greer, I.A. and Cameron, I.T. (1997) Effects of cyclic GMP and the nitric oxide donors glyeryl trinitrate and sodium nitroprusside on contractions *in vitro* of isolated myometrial tissue from pregnant women.*J. Reprod. Fert.*, **110**, 249-254.

Norman, J.E., Thomson, A.J., Greer, I.A. (1998) Cervical ripening after nitric oxide. *Hum. Reprod.*, **13**, 251-252.

Norman, M., Ekman, G., Ulmsten, U., Barchan, K., Malmstrom, A. (1991) Proteoglycan metabolism in the connective tissue of pregnant and non-pregnant human cervix. An in vitro study. *Biochem. J.*, **275**, 515-520.

Norman, M., Ekman, G., Malmstrom, A. (1993) Changed proteoglycan metabolism in human cervix immediately after spontaneous vaginal delivery. *Obstet. Gynecol.*, **81**, 217-223.

Norstrom, A. (1984) The effects of prostaglandins on the biosynthesis of connective tissue constituents in the non-pregnant human cervix uteri. *Acta. Obstet. Gynecol. Scand.*, **63**, 169-173.

Norstrom, A., Bergman, I., Lindblom, B. *et al.* (1985) Effects of 9 deoxo- 16, 16 dimethyl-9-methylene PGE_2 on muscle contractile activity and collagen synthesis in the human cervix. *Prostaglandins*, **29**, 337-346.

Nunokawa, Y., Ishida, N., Tanaka, S. (1993) Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **191**, 89-94.

Obrink, B. (1973) A study of the interactions between monomeric tropocollagen and glycosaminoglycans. *Eur. J. Biochem.*, **33**, 387-400.

Office of Population Censuses and Surveys. (1993) *Mortality statistics. Perinatal and infant: social and biological factors.* DH3 **no 25,** 23-26.

Oishi, K., Takano-Ohmuro, H., Minakawa-Matsuo, N., Suga, O., Karibe, H., Kohama, K., Uchida, M.K. (1991) Oxytocin contracts rat uterine smooth muscle in calcium-free medium without any phosphorylation of myosin light chain. *Biochem. Biophys. Res. Commun.*, **176**, 122-128.

Olah, K.S. and Gee, H. (1992) The prevention of preterm delivery- can we afford to continue to ignore the cervix? *Br. J. Obstet. Gynaecol.*, **99**, 278-280.

Omar, H.A., Cherry, P.D., Mortelliti, M.P. *et al.* (1991) Inhibition of coronary artery superoxide dismutase attenuates endothelium-dependent and -independent nitrovasodilator relaxation. *Circ. Res.*, **69,** 601-608.

Opsjon, S.-L., Wathen, N.C., Tingulstad, S. *et al.* (1993) Tumor necrosis factor, interleukin-1 and interleukin-6 in normal human pregnancy. *Am. J. Obstet. Gynecol.*, **169**, 397-404.

Osa, T., Inoue, H., Todoroki, N., Cui, D. (1994) Effects of Mg and Mn ions on the inhibitory action of cyclic nucleotides in the longitudinal myometrium of rats. *Jpn. J. Physiol.*, **44**, 49-66.

Osmers, R., Rath, W., Adelmann-Grill, B.C. *et al.* (1992) Origin of cervical collagenase during parturition. *Am. J. Obstet. Gynecol.*, **166,** 1455-1460.

Osmers, R.G.W., Blaser, J., Kuhn, W., Tschesche, H. (1995) Interleukin-8 synthesis and the onset of labor. *Obstet. Gynecol.*, **86**, 223-229.

Owman, Ch., Stjernquist, M., Helm, G., Kannisto, P., Sjoberg, N.O., Sundler,F. (1986) Comparative histochemical distribution of nerve fibers storing noradrenaline and neuropeptide Y (NPY) in human ovary, Fallopian tube and uterus. *Med. Biol.*, **64**, 57-65.

Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524-526.

Palmer, R.M.J., Rees, D.D., Ashton, D.S., Moncada, S. (1988) L-arginine is the physiological precursor for the formation of nitric oxide in endothelium dependent relaxation. *Biochem. Biophys. Res. Comm.*, **153**, 1251-1256.

Papka, R.E., McNeill, D.L., Thompson, D., Schmidt, H.H.H.W. (1995) Nitric oxide nerves in the uterus are parasympathetic, sensory and contain neuropeptides. *Cell Tissue Res.*, **279**, 339-349.

Papka, R.E., Thompson, D., Schmidt, H.H.H.W. (1996) Identification of uterine-related sympathetic neurons in the rat inferior mesenteric ganglion: neurotransmitter content and afferent input. *J. Auton. Nerv. Syst.*, **59**, 51-59.

Parry, D.S. and Ellwood, D.A. (1981) Ultrastructural aspects of cervical softening in sheep. In: Ellwood DA, Anderson ABM, eds. The cervix in pregnancy and labour: clinical and biochemical investigations. Edinburgh: Churchill Livingstone, 74-84.

Petersen, L.K., Skajaa, K., Uldbjerg, N. (1992) Serum relaxin as a potential marker for preterm labour. *Br. J. Obstet. Gynaecol.*, **99**, 292-295.

Petraglia, F., Giardino, L., Coukos, G., Calza, L., Vale, W., Genazzani, A.R. (1990) Corticotropin-releasing factor and parturition: plasma and amniotic fluidlevels and placental binding sites. *Obstet. Gynecol.*, **75**, 784-789.

Poranen, A.K., Ekblad, U., Uotila, P. *et al.* (1996) Lipid-peroxidation and antioxidants in normal and preeclamptic pregnancies. *Placenta*, **17**, 401-405.

Radestad, A., Bygdeman, M., Green, K. (1990) Induced cervical ripening with mifepristone (RU486) and bioconversion of arachidonic acid in human pregnant uterine cervix in the first trimester. *Contraception*, **41**, 283-292.

Radestad, A., Thyberg, J., Christensen, N.J. (1993) Cervical ripening with mifepristone (RU486) in first trimester abortion. An electron microscope study. *Hum. Reprod.*, **8**, 1136-1142.

Rajabi, M.R., Dean, D.D., Beydoun, S.N. and Woessner J.R. (1988) Elevated tissue levels of collagenase during dilation of uterine cervix during human parturition. *Am. J. Obstet. Gynecol.*, **159**, 971-976.

Ramsay, B., De Belder, A., Campbell, S., Moncada, S., Martin, J.F. (1994) A nitric oxide donor improves uterine artery diastolic blood flow in normal early pregnancy and in women at high risk of pre-eclampsia. *Eur. J. Clin. Invest.*, **24**, 76-78.

Ramsay, B., Johnson,M.R., Leone, A.M. and Steer, P.J. (1995) The effect of exogenous oestrogen on nitric oxide production in women: a placebo controlled crossover study. *Br. J. Obstet. Gynaecol.* **102**, 417-419.

Ramsay, B., Sooranna, S.R. and Johnson, M.R. (1996) Nitric oxide synthase activities in human myometrium and villous trophoblast throughout pregnancy. *Obstet. Gynecol.*, **87**, 249-253.

Rath, W., Adelmann-Girill, B.C., Pieper, U. *et al.* (1987) The role of collagenases and proteases in prostaglandin induced cervical ripening. *Prostaglandins*, **34**, 119-127.

Rath, W., Osmers, B.C., Adelmann-Girill, B.C., Stuhlsatz, H.W., Severenyi, M., Kuhn, W. (1988) Biophysical and biochemical changes of cervical ripening. In: Egarter C, Husslein P, eds. Prostaglandins for cervical ripening and/or induction of labour. Vienna: Facultas, 32-41.

Rechberger, T. and Woessner, J.F. (1993) Collagenase, its inhibitors, and decorin in the lower uterine segment in pregnant women. *Am. J. Obstet. Gynecol.*, **168**, 1598-1603.

Reynolds, S.R.M. (1965) Physiology of the uterus. New York: Hoeber.

Richardson, W., Smith, D.C., Evans, A.L., Anthony, G.S. (1989) A novel cervical dilatation force measurement instrument. *J. Med. Eng. Technol.*, **13**, 220-221.

Riesco, A., Caramelo, C., Blum, G., Monton, M., Gallego, M.J., Casado, S., Farre, A.L. (1993) Nitric oxide-generating system as an autocrine mechanism in human polymorphonuclear leukocytes. *Biochem. J.*, **292**, 791-796.

Robertson, R.M. and Robertson, D. (1995) Drugs used in the treatment of cardiac ischaemia. In: Goodman and Gilman's The Pharmacological Basis of Therapeutics. (eds-in-chief Hardman JG and Limbird LE), McGraw-Hill, New York, 759-779.

Robertson, P.A., Sniderman, S.H., Laros, R.K. Jr., *et al.* (1992) Neonatal morbidity according to gestational age and birth weight from five tertiary care centers in the United States, 1983 through 1986. *Am. J. Obstet. Gynecol.*, **166**, 1629-1645.

Romero, R., Gomez, R., Mazor, M., Ghezzi, F., Yoon, B.H. (1997) The preterm labor syndrome. In: Elder, M.G., Romero, R., Lamont, R.F. eds. Preterm Labor. New York, Churchill Livingstone, 29-50.

Rosen, D.R., Siddique, T., Patterson D. *et al.*, (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, **362**, 59-62.

Rosenberg, S., Draper, L.D., Heine, R.P. *et al.* (1996) Polymorphonuclear leukocytes invade maternal decidua and fetal membranes via an e-selectin dependent pathway during human labour. *J. Soc. Gynecol. Invest.*, **3**, 553.

Rowlands, S., Trudinger, B., and Visva-Lingam, S. (1996) Treatment of preterm cervical dilatation with glyceryl trinitrate, a nitric oxide donor. *Aust. N.Z. J. Obstet. Gynaecol.*, **36**: 377-381.

Royal College of Obstetricians and Gynaecologists. (1997) Induced abortion. Guideline No. 11.

Rubanyi, G.N. (1988) Vascular effects of oxygen-derived free radicals. *Free Rad. Biol. Med.*, **4**, 107-120.

Rudolph, M. I., Reinicke, K., Cruz, M. A. *et al.* (1993) Distribution of mast cells and the effect of their mediators on contractility in human myometrium. *Br. J. Obstet. Gynaecol.*, **100**, 1125-1130.

Rudolph, M.I., Reinicke, K., Padilla, L. *et al.* (1990) The mast cells in pregnant uterus. *Euro. J. Pharmacol.*, **183**, 1765-1766.

Salvemini, D., Misko, T.P., Masferrer, J.L., Seibert, K., Currie, M.G., Needleman, P. (1993) Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci.*, **90**, 7240-44.

Samuelson, U.E., Dalsgaard, C.J., Lundberg, J.M., Hokfelt, T. (1985) Calcitonin gene-related peptide inhibits spontaneous contractions in human uterus and fallopian tube. *Neurosci. Lett.*, **62**, 225-230.

Schellenberg, J.C. and Liggins, G.C. (1994) Initiation of labour: uterine and cervical changes, endocrine changes. In: Chard T, Grudzinskas JG, eds. The Uterus. Cambridge University Press. 308-336.

Schmidt, H.H.H.W., Gagne, G.D., Nakane, M., Polock, J.S., Miller, M.F., Murad, F. (1992) Mapping of neuronal nitric oxide synthase in the rat suggests frequent co-localisation with NADPH diaphorase but not with soluble guanylate cyclase, and novel paraneural functions for nitrinergic signal transduction. *J. Histochem. Cytochem.*, **40**, 1439-1456.

Schonfelder, G., John, M., Hopp, H. *et al.* (1996) Expression of inducible nitric oxide synthase in placenta of women with gestational diabetes. *FASEB J.*, **10**, 777-784.

Schultz, K.F., Grimes, D.A. and Cates, W. (1983) Measures to prevent cervical injury during suction curettage abortion. *Lancet*, **i**, 1182-84.

Schwartz, L.B. (1997) Understanding human parturition. *Lancet*, **350**, 1792-1793.

Scott, J.E. and Orford, C.R. (1981) Dermatan sulphate rich proteoglycan associates with rat tail tendon collagen at the d band in the gap region. *Biochem. J.*, **197**, 213.

Segal, S., Csavoy, A.N., Datta, S. (1997) Placental tissue enhances uterine relaxation by nitroglycerin. *Anesth. Analg.*, **86**, 304-309.

Shaker, A.G., Fleming, R., Jamieson, M.E., Yates, R.W.S., Coutts, J.R.T. (1993) Assessments of embryo transfer after in-vitro fertilization: effects of glyceryl trinitrate. *Hum. Reprod.*, **8**, 1426-28.

Shew, R.L., Papka, R.E., McNeill, D.L., Yee, J.A. (1993) NADPH diaphorase-positive nerves and the role of nitric oxide in CGRP relaxation of uterine contraction. *Peptides*, **14**, 637-641.

Sitteri, P.K., Febres, F., Clemens, L.E. *et al.* (1977) Progesterone and maintenance of pregnancy: is progesterone nature's immunosuppressant? Ann. NY. Acad. Sci., **286**, 384-397.

Sladek, S.M. and Roberts, J.M. (1996) Nitric oxide synthase activity in the gravid rat uterus decreases a day before the onset of parturition. *Am. J. Obstet. Gynecol.*, **175**, 1661-1667.

Sladek, S.M., Regenstein, A.C., Lykins, D. and Roberts, J.M. (1993) Nitric oxide synthase activity in pregnant rabbit uterus decreases on the last day of pregnancy. *Am. J. Obstet. Gynecol.*, **169**, 1285-1291.

Sladek, S.M., Stranko, C.P., Roberts, J.M. (1994) Human decidual nitric oxide synthase activity decreases by the last week of gestation but not with labor. *Soc. Gynecol. Invest., 41st Annual Mtg., abstract*, 422.

Sladek, S.M., Magness, R.R., Conrad, K.P. (1997) Nitric oxide and pregnancy. *Am. J. Physiol.*, **272**, R441-R463.

Slater, D.M., Berger, L., Newton, R., Moore, G.E., Bennett, P.R. (1995) Changes in the expression of types 1 and 2 cyclo-oxygenase in human fetal membranes at term. *Am. J. Obstet. Gynecol.*, **172**, 77-82.

Slater, D., Allport, V., Bennett, P. (1998) Changes in the expression of the type-2 but not the type-1 cyclo-oxygenase enzyme in chorion-decidua with the onset of labour. *Br. J. Obstet. Gynaecol.*, **105**, 745-748.

Soloff, M.S., Alexandrova, M. and Fernstrom, M.J. (1979) Oxytocin receptors: triggers for parturition and lactation. *Science* , **204**, 1313-1315.

Somlyo, A.V. (1980) Ultrastructure of vascular smooth muscle. In: Bohr, D.F., Somlyo, A.P., Sparks, H.P., eds. Handbook of Physiology. The cardiovascular system. Vol. 2. Bethesda: American Physiological Society, 33-70.

Somlyo, A.P., Bond, M., Somlyo, A.V., Scarpa, A. (1985) Inositol trisphosphate induced calcium release and contraction in vascular smooth muscle. *Proc. Natl. Acad. Sci. USA*, **82**, 5231-5235.

Stjernquist, M., Ekblad, E., Owman, Ch., Sundler, F. (1986) Neuronal localization and motor effects of gastrin releasing peptide (GRP) in rat uterus. *Reg. Peptides*, **13**, 197-205.

Stricklin, G.P. and Hibbs, M.S. (1988) Biochemistry and physiology of mammalian collagenases. In: Nimni ME, ed. Collagen biochemistry, vol. 1. Boca Ranton: CRC Press, 187-205.

Stys, S.J., Clarke, K.E., Clewell, W.M. *et al.* (1980) Hormonal effects on cervical compliance in sheep. In: Naftolin F, Stubblefield PG, eds. Dilatation of the uterine cervix. New York: Raven Press, 147-156.

Suburo, A.M., Chaud, M., Franchi, A., Polak, J.M., Gimeno, M.A.F. (1995) Distribution of neuronal and non-neuronal NADPH diaphorases and nitric oxide synthases in rat uterine horns under different hormonal conditions. *Biol. Reprod.*, **52**, 631-637.

Sugino, N., Shinamura, K., Takiguchi, S. *et al.* (1996) Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and in early pregnancy. *Hum. Reprod.*, **11**, 1073-1078.

Swahn, M.L. and Bygdeman, M. (1988) The effect on the antiprogestin RU-486 on uterine contractility and sensitivity to prostaglandin and oxytocin. *Br. J. Obstet. Gynaecol.*, **85**, 126-134.

Szalay, S., Husslein, P., Grunberger, W. (1989) Local application of prostaglandin E_2 and its influence on collagenolytic activity of cervical tissue. *Sing. J. Obstet. Gynecol.*, **12**, 15.

Tabibzadeh, S. (1991) Ubiquitous expression of TNF /cachectin immunoreactivity in human endometrium *Am. J. Reprod. Immunol.*, **26**, 1-4.

Tabibzadeh, S., Kong, Q.F. and Babaknia, A. (1994) Expression of Adhesion Molecules in Human Endometrial Vasculature throughout the Menstrual Cycle. *J. Clin. Endocrinol. Metab.*, **79**, 1024-1032.

Tan, Y.H., Tischfield, J. and Ruddle, F.H. (1973) The linkage of genes for the human interferon induced antiviral protein and indophenol oxidase-B traits to chromosome G-21. *J.Exp.Med.*, **137**, 317-330.

Tawia S.A., Beaton L.A. and Rogers P.A.W. (1993) Immunolocalisation of the Cellular Adhesion Molecules, Intercellular Adhesion Molecule-1 (ICAM-1) and Platelet Endothelial Cell Adhesion Molecule (PECAM) in the Human Endometrium throughout the Menstrual Cycle. *Hum. Reprod.*, **8**, 175-181.

Taylor, G.W. and Morris, H.R. (1983) Lipoxygenase pathways. *Br. Med. Bull.*, **39**, 219-222.

Telfer, J.F., Lyall, F., Norman, J.E., Cameron, I.T. (1995) Identification of nitric oxide synthase in human uterus. *Hum. Reprod.*, **10**, 19-23.

Telfer, J.F., Irvine, G.A., Kohnen, G. *et al.* (1997a) Expression of endothelial and inducible nitric oxide synthase in non-pregnant and decidualized human endometrium. *Mol. Hum. Reprod.*, **3**, 69-75.

Telfer, J.F., Thomson A.J., Cameron I.T. *et al.* (1997b) Expression of superoxide dismutase and xanthine oxidase in myometrium, fetal membranes and placenta during normal human pregnancy and parturition. *Hum. Reprod.*, **12**, 2306-2312.

Thaler, I., Amit, A., Jakobi, P., Itskovitz-Eldor, J. (1996) The effect of isosorbide dinitrate on uterine artery and umbilical artery flow velocity waveforms in mid-pregnancy. *Obstet. Gynecol.*, **88**, 838-843.

Theobald, G.W., Robards, M.F., Suster, P.E.N. (1969) Changes in myometrial sensitivity to oxytocin in man during the last six weeks of pregnancy. *Br. J. Obstet. Gynaecol.*, **76**, 385-393.

Thomson, A.J., Telfer, J.F., Kohnen, G., Young, A., Cameron, I.T., Greer, I.A. and Norman, J.E. (1997a) Nitric oxide synthase activity and localisation do not change in uterus and placenta during human parturition. *Hum. Reprod.*, **12**, 2546-2552.

Thomson, A.J., Lunan, C.B., Cameron, A.D., Cameron, I.T., Greer, I.A. and Norman, J.E. (1997b) Nitric oxide donors induce ripening of the human uterine cervix: a randomised controlled trial. *Br. J. Obstet. Gynaecol.*, **104**, 1054-1057.

Thornton, S. and Gillespie, J.I. (1992) Biochemistry of uterine contractions. *Contemp. Rev. Obstet. Gynaecol.*, **4**, 121-126.

Tracey, W.R., Nakane, M., Pollock, J.S., Forstermann, U. (1993) Nitric oxide synthases in neuronal cells, macrophages, and endothelium are NADPH diaphorases, but represent only a fraction of cellular NADPH diaphorase activity. *Biochem. Biophys. Res. Commun.*, **195**, 1035-1040.

Trachtman, H., Futterweit, S., Garg, P., Reddy, K., Singhal, P.C. (1996) Nitric oxide stimulates the activity of a 72-kDa neutral matrix metalloproteinase in cultured rat mesangial cells. *Biochem. Biophys. Res. Commun.*, **218**, 704-8.

Tschugguel, W., Schneeberger, C., Unfried, G., Czerwenka, K., Weninger, W., Mildner, M., Bishop, J.R., Huber, J.C. (1998) Induction of inducible nitric oxide synthase expression in human secretory endometrium. *Hum. Reprod.*, **13**, 436-444.

Tulchinsky, D., Hobel, C.J., Yeager, E., Marshall, J.R. (1972) Plasma estrone, estradiol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. *Am. J. Obstet. Gynecol.*, **112**, 1095-1100.

Turnbull, A.C., Patten, P.T., Flint, A.P.F., Keirse, M.J.N.C., Jeremy, J.Y. and Anderson, A.B.M. (1974) Significant fall in progesterone and rise in oestradiol levels in human peripheral plasma before the onset of labour. *Lancet*, **i**, 101-104.

Uldbjerg, N., Ekman, G., Malmstrom, A. *et al.* (1981) Biochemical and morphological changes of human cervix after local application of prostaglandin E_2 in pregnancy. *Lancet,* **i**, 267-268.

Uldbjerg, N., Ekman, G., Malmstrom, A. (1983) Ripening of the human cervix related to changes in collagen, glycosaminoglycans and collagenolytic activity. *Am. J. Obstet. Gynecol.*, **147**, 662-666.

Uldbjerg, N., Ekman, G., Malmstrom, A., Ulmsten, U., Wingerup, L. (1983) Biochemical changes in human cervical connective tissue after local application of prostaglandin E_2. *Gynecol. Obstet. Invest.*, **15**, 291-299.

Uldbjerg, N., Malmstrom, A., Ekman, G., Sheehan, J., Ulmsten, U., Wingerup, L. (1983) Isolation and characterization of dermatan sulphate proteoglycan from human uterine cervix. *Biochem. J.*, **209**, 497-503.

Uldbjerg, N., Ulmsten, U., Ekman, G. (1983) The ripening of the human uterine cervix in terms of connective tissue biochemistry. *Clin. Obstet. Gynecol.*, **26**, 14-26.

Uldbjerg, N. and Malmstrom, A. (1991) The role of proteoglycans in cervical dilatation. *Semin. Perinatol.*, **15**, 127-132.

Vallance, P. and Collier J. (1994) Biology and clinical relevance of nitric oxide. *Br. Med. J.*, **309**, 453-457.

Veille, J-C., Li, P., Eisenach, J.C., Massermann, A.G. and Figueroa, J.P. (1996) Effects of estrogen on nitric oxide biosynthesis and vasorelaxant activity in sheep uterine and renal arteries in vitro. *Am. J. Obstet. Gynecol.*, **174**, 1043-1049.

Villar, J., Ezcurra, E.J., Gurtner de la Fuente, V., Campodonico, L. (1994) Pre-term delivery: the unmet need. *Res. Clin. Forims*, **16**, 9-38.

von Maillot, K., Stuhlsatz, H.W., Mohanaradhkrishan, V. *et al.* (1979) Changes in the glycosaminoglycan distribution pattern in the human uterine cervix during pregnancy and labour. *Am. J. Obstet. Gynecol.*, **135**, 503-506.

von Maillot, K., Weiss, M., Nagelschmidt, M. *et al.* (1977) Relaxin and cervical dilatation during partuition. *Archiv. Gynakol.*, **223**, 323-331.

Wang, Y. and Walsh, S.W. (1996) Antioxidant activities and mRNA expression of superoxide dismutase, catalase and glutathione peroxidase in normal and preeclamptic placentas. *J. Soc. Gynecol. Invest.*, **3**, 179-184.

Wei, X-Q., Charles, I.A., Smith, A., Ure, J., Feng, G-J., Huang, F-P., Xu, D., Muller, W., Moncada, S. and Liew, F.Y. (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* , **375**, 408-411.

Weiner, C.P., Knowles, R.G., Nelson, S.E. and Stegink, L.D. (1994a) Pregnancy increases guanosine 3',5'-monophosphate in the myometrium independent of nitric oxide synthesis. *Endocrinology*, **135**, 2473-2478.

Weiner, C.P., Lizasoain, I., Baylis, S.A., Knowles, R.G., Charles, I.G. and Moncada, S. (1994b) Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc. Natl. Acad. Sci. USA* , **91**, 5212-5216.

Weiss, G., Goldsmith, L.T., Sachdev, R., von Hagen, S., Lederer, K. (1993) Elevated first-trimester serum relaxin concentrations in pregnant women following ovarian stimulation predict prematurity risk and preterm delivery. *Obstet. Gynecol.*, **82**, 821-831.

Werner-Felmayer, G., Werner, E.R., Fuchs, D. *et al.* (1993) Pteridine biosynthesis in human endothelial cells. *J. Biol. Chem.*, **268**, 1842-1846.

Wesiger, R.A. and Fridovich, I. (1973) Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial localization. *J. Biol. Chem.*, **74**, 1398-1403.

Wessen, A., Elowsson, P., Axemo, P., Lindberg, B. (1995) The use of intravenous nitroglycerin for emergency cervico-uterine relaxation. *Acta Anaesth. Scand.*, **39**, 847-849.

Westphal, U., Stroupe, S.D., and Cheng, S.L. (1977) Progesterone binding to serum proteins. *Ann. NY Acad. Sci.*, **286**, 10-28.

Wilson, L.Jr. and Parsons, M. (1996) Endocrinology of human gestation. In: Adashi, E.Y., Rock, J.A., Rosenwaks, Z., eds. Reproductive endocrinology, surgery and technology. Philadelphia, Lippincott-Raven. 452-475.

Wing, D.A., Jones, M.M., Rahall, A. *et al.* (1995) A comparison of misoprostol and prostaglandin E_2 gel for preinduction cervical ripening and labor induction. *Am. J. Obstet. Gynecol.*, **172**, 1804-1810.

Winkler, M., Ruck, P., Horny, H.P. *et al.* (1998) Expression of cell adhesion molecules by endothelium in the human lower uterine segment during parturition at term. *Am. J. Obstet. Gynecol.*, **178**, 557-561.

Wolff, K., Faxen, M., Lunell, N.O., Nisell, H., Lindblom, B. (1996) Endothelin receptor type-A and receptor type-B gene-expression in human nonpregnant, term pregnant and pre-eclamptic uterus. *Am. J. Obstet. Gynecol.*, **175**, 1295-1300.

Wong, G.H.W. and Goeddel, D.V. (1988) Induction of manganous superoxide dismutase by tumour necrosis factor: possible protective mechanisms. *Science,* **242**, 941-944.

Wooley, D.E. (1984) Mammalian collagenases. In: Piez, K.A., Reddi, A.H., eds. Extracellular matrix biochemistry. New York: Elsevier,119-157.

Word, R.A., Kamm, K.E., Stull, J.T., Casey, M.L. (1990) Endothelin increases cytoplasmic calcium and myosin phosphorylation in human myometrium. *Am. J. Obstet. Gynecol.*, **162,** 1103-1108.

Word, R.A., Casey, M.L., Kamm, K.E., Stull, J.T. (1991) Effects of cGMP on calcium, myosin light chain phosphorylation and contraction in human myometrium. *Am. J. Physiol.*, **260**, C861-C867.

World Health Organisation. (1990) The use of mifepristone (RU486) for cervical preparation in first trimester termination by vacuum aspiration. *Br. J. Obstet. Gynaecol.*, **97**, 260-266.

Wray, S. (1993) Uterine contraction and physiological mechanisms of modulation. *Am. J. Physiol.*, **264**, C1-C18.

Yadava, R.P. (1990) Sublingual glyceryl trinitrate spray facilitates IUD insertion. *Br. J. Sexual. Med.*, **July**, 217.

Yallampalli, C., Garfield, R.E. and Byam-Smith, M. (1993) Nitric oxide inhibits uterine contractility during pregnancy but not during delivery. *Endocrinology*, **133**, 1899-1902.

Yallampalli, C. and Garfield, R.E. (1994) Uterine contractile responses to endothelin-1 and endothelin receptors are elevated during labor. *Biol. Reprod.*, **51**, 640-645.

Yallampalli, C., Izumi, H., Byam-Smith, M. and Garfield, R.E. (1994a) An L-arginine-nitric oxide-cyclic guanosine monophosphate system exists in the uterus and inhibits contractility during pregnancy. *Am. J. Obstet. Gynecol.*, **170**, 175-185.

Yallampalli, C., Byam-Smith, M., Nelson, S.O. and Garfield, R.E. (1994b) Steroid hormones modulate the production of nitric oxide and cGMP in the rat uterus. *Endocrinology*, **134**, 1971-1974.

Yallampalli, C., Dong, Y.L., Gangula, P.R., Fang, L. (1998) Role and regulation of nitric oxide in the uterus during pregnancy and parturition. *J. Soc. Gynecol. Invest.*, **5**, 58-67.

Yoshida, Y., Yoshida, K., Kimura, T., Toda, N. (1995) Distribution of NADPH diaphorase-reactive nerves in the human female genital organ. *Acta. Obstet. Gynecol. Scand.*, **74**, 171-176.

Zeeman, G.G., Khan-Dawood, F.S., Dawood, M.Y. (1997) Oxytocin and its receptor in pregnancy and parturition: current concepts and clinical implications. *Obstet. Gynecol.*, **89**, 873-883.

Zimmerman, R.J., Chan, A. and Leadon, S.A. (1989) Oxidative damage in murine tumour cells in vitro by recombinant human tumour necrosis factor. *Cancer Res.*, **49**, 1644-1648.

Appendix

Published Papers.

The following papers have been published based on the text of this thesis:

Original Articles.

1. Thomson AJ, Telfer JF, Kohnen G, Young A, Cameron IT, Greer IA, Norman JE. (1997) Nitric oxide synthase activity and localisation do not change in uterus and placenta during human parturition. Human Reproduction, **12**, 2546-2552.

2. Telfer JF, Thomson AJ, Cameron IT, Greer IA, Norman JE. (1997) Expression of superoxide dismutase and xanthine oxidase in myometrium, fetal membranes and placenta during human pregnancy. Human Reproduction, **12**, 2306-2312.

3. Thomson AJ, Lunan CB, Cameron AD, Cameron IT, Greer IA, Norman JE. (1997) Nitric oxide donors induce ripening of the human uterine cervix: a randomised controlled trial. British Journal of Obstetrics & Gynaecology, **104**, 1054-1057.

4. Thomson AJ, Lunan CB, Ledingham MA, Howat RCL, Cameron IT, Greer IA, Norman JE. (1998) A randomised trial of nitric oxide donors for cervical ripening: more acceptable than prostaglandins? The Lancet, **352**: 1093-1096.

5. Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CJR, Cameron IT, Greer IA, Norman, JE. (1999) Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. Human Reproduction, *in press.*

6. Norman JE, Thomson AJ, Telfer JF, Young A, Cameron IT, Greer IA. (1999) Myometrial constitutive nitric oxide synthase expression is increased during human pregnancy. Molecular Human Reproduction, *in press*.

Book Chapter and Review Article

1. Thomson AJ, Norman JE, Greer IA. (1997) The Cervix. In: Elder MG, Lamont RF, Romero R, eds. Preterm Labor London, Churchill Livingstone. 445-455.

2. Norman JE, Thomson AJ, Greer IA. (1998) Cervical ripening after nitric oxide. Human Reproduction, **13**, 251-252.