COMMITTEE CERTIFICATION OF APPROVED VERSION

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THE RELATIONSHIP BETWEEN NITRIC OXIDE SYNTHASE (NOS) AND CYCLOOXYGENASE (COX) IN THE CONTROL OF CERVICAL RIPENING AND PARTURITION

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SYNTHASE (NOS) AND CYCLOOXYGENASE (COX) IN THE CONTROL OF CERVICAL RIPENING AND PARTURITION

by

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This is dedicated in honor of my parents (Jackie G. Marx and Alan P. Marx), my brothers (Michael K. Marx and Louis D. Marx), and in loving memory of my grandparents (Beate Gureasko and Louis Gureasko; Anita Marx and Franck Marx).

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ABSTRACT

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The purpose of these studies is to examine if there is relationship between iNOS and COX-2 in the control of cervical ripening and parturition. Cervices were obtained from estrus and timed pregnant Sprague-Dawley rats (n = 4-10 per group) under normal conditions; or after treating with LPS (100µg i.p.), Onapristone (3mg/rat), progesterone (2.5 mg, twice daily), L-NAME (50mg/day), or SNP (0.3mg/rat). Collagen changes were measured and visualized with the picrosirius polarization method. Expression of iNOS and COX-2 mRNA was determined using RT-PCR. Immunohistochemistry (IHS) was performed for localization of the iNOS and COX-2 enzymes (significance: $P<0.05$). Picrosirius polarization showed a decrease in the organization and birefringence of the cervical collagen from the non-pregnant state through pregnancy and is supported by changes in the luminosity ($P<0.001$). The iNOS and COX-2 enzymes were mainly localized in the cervical muscle with labeling also in the vascular smooth muscle and epithelium. Under normal term pregnant conditions, iNOS mRNA levels decrease as COX-2 mRNA levels increased demonstrating an inverse correlation (Spearman $r = -0.497$; $P = 0.00295$). Onapristone stimulated preterm labor and/or birth causing a parallel increase in iNOS and COX-2 mRNA demonstrating a positive correlation (Spearman $r = 0.456$; $P = 0.03$). Progesterone prolonged pregnancy stimulating a decrease in the iNOS and COX-2 ($P=0.036$) mRNA. In comparing term to preterm laboring conditions, there is a significant increase in the iNOS mRNA ($P=0.004$) but not the COX-2 mRNA. LPS enhanced the iNOS mRNA ($P<0.001$) but had no effect on the COX-2 mRNA. L-NAME had no effect on the COX-2 or iNOS mRNA. SNP decreased the COX-2 and iNOS with the decrease in the iNOS being significant ($P=0.007$). In conclusion, under normal term pregnant conditions iNOS and COX-2 play an important role in regulating cervical ripening and parturition but the pathways appear to act independently of one another in regulating iNOS and COX-2 expression at the mRNA level. Under preterm laboring conditions, when NO is up regulated and/or over expressed, there may be an interaction between the NO and PG pathways in the control of cervical ripening and parturition.

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LIST OF ABBREVIATIONS

3,3'-diaminobenzidine (DAB)
5-hydroxytryptamine (5-HT)
17β-hydroxysteroid (17βHSD)
Adenosine 5'-triphosphate (ATP)
Adenylyl cyclase (AC)
Adrenocorticotropic hormone (ACTH)
Ammonium hydroxide (NH_4OH)
Aminoethyl carbazole (AEC)
Analysis of variance (ANOVA)
Brain nitric oxide synthase (bNOS)
Calcium (Ca^{2+})
Calcium chloride ($CaCl_2$)
Carbon dioxide (CO_2)
Celsius (C)
Connexon 43 (Cx43)
Corticotropin releasing hormone (CRH)
CREB-binding protein (CBP)
Cycle threshold (C_T)
Cyclic adenosine 5'-monophosphate (cAMP)
Cyclic guanidine 5'-monophosphate (cGMP)
Cyclooxygenase (COX)
Dehydroepiandrosterone (DHEA)
Deoxyribonucleic acid (DNA)
Deoxyribonucleic acid binding domain (DBD)
Endothelial nitric oxide synthase (eNOS)
Epidermal growth factor (EGF)
Estrogen receptor (ER)
Estrus (E)
Ethyl alcohol (ETOH)
Fibroblast growth factor (FGF)
Forskolin (FSK)
G protein coupled receptors (GPCR)
Glyceryl trinitrate (GTN)
Glycosaminoglycan (GAG)
Guanosine 5'-triphosphate (GTP)
Hydrochloric acid (HCL)
Immunohistochemistry (IHS)
Inducible nitric oxide synthase (iNOS)
Interleukin (IL)
Labor (L)
Ligand-binding domain (LBD)

Light-induced-fluorescence (LIF)
Lipopolysaccharide (LPS)
Low density lipoprotein (LDL)
Magnesium (Mg^{2+})
Matrix metalloprotease (MMP)
Messenger ribonucleic acid (mRNA)
Micro-liter (µl)
Milligram (mg)
Milliliter (ml)
Myosin light chain kinase (MLCK)
N-nitro-L-arginine methyl ester (L-NAME)
Nana-gram (ng)
Nana-Mole (nM)
Neuronal nitric oxide synthase (nNOS)
N^G-monomethyl-L-arginine (L-NMMA)
Nitric oxide (NO)
Nitric oxide synthase (NOS)
Non-labor (NL)
Non-pregnant (NP)
Nuclear factor (NF)
Nuclear factor-kappa B (NF-?B)
Oxytocin (OT)
Oxytocin receptor (OTR)
Phosphate buffer saline (PBS)
Phosphatidylinositol 4,5-bisphosphate (PIP_2)
Platelet-derived growth factor (PDGF)
Polymerase chain reaction (PCR)
Postpartum (PP)
Preterm birth (PTB)
Preterm labor (PTL)
Preterm premature rupturing of the membrane (PROM)
Progesterone antagonists (PA)
Progesterone receptor (PR)
Progesterone receptor modulator (PRM)
Progesterone response element (PRE)
Prostaglandin (PG)
Prostaglandin E_2 (PGE_2)
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)
Prostaglandin G_2 (PGG_2)
Prostaglandin H_2 (PGH_2)
Prostaglandin I_2 (PGI_2)
Protein kinase C (PKC)
Reverse transcription polymerase chain reaction (RT-PCR)
Ribonucleic acid (RNA)
Ribosomal ribonucleic acid (rRNA)

Sodium nitroprusside (SNP)
Sprague-Dawley (SD)
Standard error mean (SEM)
Steroid receptor co-activator-1 (SRC-1)
Subcutaneous (s.c.)
Tissue inhibitors of metalloprotease (TIMP)
Toll-like receptor 4 (TLR-4)
Transforming growth factor (TGF)
Tumor necrosis factor (TNF)
United States (US)

CHAPTER 1: INTRODUCTION: THE RELATIONSHIP BETWEEN NITRIC OXIDE SYNTHASE (NOS) AND CYCLOOXYGENASE (COX) IN THE CONTROL OF CERVICAL RIPENING AND PARTURITION

PARTURITION

Successful parturition requires uterine activity, such as uterine contractions, to coordinate with changes occurring in the cervix during pregnancy, such as the ripening of the cervix (Bernal, 2003). Labor is defined as uterine contractions that cause effacement and dilation of the cervix and is associated with changes in the steroid hormone levels, specifically progesterone and estrogen (Bedin et al., 1987 and Cunningham et al., 2001). Changes in steroid hormone levels reportedly result in an increase in prostaglandin (PG) levels, cervical softening and the onset of myometrial contractions. Parturition likely results from coordinated changes in the endocrine systems of both the mother and the fetus (Bassett and Thorburn, 1969; Liggins, 1974 and Anderson et al., 1975). The fetus in humans and other animals may play a significant role in uterine adaptation to pregnancy and to the onset of parturition (Honnebier and Swaab, 1973). How these factors interact and control parturition are still largely unknown.

Myometrial contractility

The uterine myometrium is mainly composed of elongated spindle-shaped smooth muscle cells having a single nucleus and organized in continuous multilayered sheets that allow slow but sustained contractions. Smooth muscle cells contain both actin and myosin-II filaments forming a loosely arranged contractile apparatus. The actin and myosin filaments are roughly aligned along the long axis of the cell being attached to the plasma membrane at disc-like junctions which connect it to the adjacent cells. Unlike striated muscle, smooth muscle has the advantage of permitting a much greater degree of shortening which can produce larger movements (Alberts et al., 1994 and Bernal, 2003).

At the end of pregnancy the myometrium becomes increasingly active with the frequency, magnitude, and strength of the contraction depending upon the electrical activity, the frequency of the action potentials and the total number of smooth muscle cells simultaneously activated (Marshall, 1962). Cell to cell propagation of the action potential throughout the myometrium is important for regulating uterine contractility (Cole and Garfield, 1989).

The progress of labor in pregnant animals is associated with a series of sequential hormonal changes leading to normal parturition (Nathanielsz, 1978a, b; Thorburn and Challis, 1979 and Challis and Mitchell, 1981). Late in gestation, around the time of progesterone withdrawal, there is an increase in estradiol and PGs. This is associated with gap junction formation which may coordinate an increase in uterine activity, upsetting the regulatory balance that maintained the pregnancy (Garfield et al., 1980a, b; Csapo et al., 1981; Challis and Mitchell, 1981 and Puri and Garfield, 1982). The size, number, and area of gap junctions found in the myometrium increase and are a possible mechanism for the propagation of electrical activity from cell to cell (Garfield et al., 1977; Garfield et al., 1978; Garfield et al., 1979a, b; Garfield and Hayashi 1981; Garfield et al 1982a,b and Puri and Garfield 1982).

The electrophysiological basis for myometrial contractions is not well understood but involves coordinating the electrical activity, the influx of calcium (Ca^{2+}) in the myometrial cell which is regulated by voltage-operated Ca^{2+} channels, and Ca^{2+}-activated potassium channels, and the development of tension. Ca^{2+} may also regulate contractions through setting the threshold for the activation of the cell membrane (Sanborn, 2000). Ca^{2+} activates the Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK) which is responsible for smooth muscle contractions (Word et al., 1994 and Alberts et al., 1994). MLCK is at rest when Ca^{2+} levels are low and when the Ca^{2+} levels increase MLCK is activated. There are two different regulatory light chains on each head of the myosin-II molecule and when activated MLCK catalyzes the phosphorylation of a particular site on one of the two myosin light chains. This allows for an interaction of the myosin head with the actin filament forming a complex capable of converting the

chemical energy of adenosine 5'-triphosphate (ATP) to the mechanical energy of a contraction (Sobieszek, 1994; Alberts et al., 1994 and Bernal, 2003).

The G protein coupled receptor (GPCR) is one of the most abundant receptors in the uterus and may play a role in modulating uterine activity during pregnancy by interacting with heterotrimeric (α, β, γ subunits) proteins. G proteins hydrolyze guanosine 5'-triphosphate (GTP) and/or activate or inhibit a number of effector enzymes or ion channels. Oxytocin (OT), prostanoid FP and TP, and endothelin-receptors are examples of G-protein coupled receptors that couple to the $G\alpha_q$ protein, stimulating contractions through the activation of the phospholipase C/Ca^{2+} pathway. This causes phosphatidylinositol 4,5-bisphosphate (PIP_2) in the cell membrane to hydrolyse into inositol 1,4,5,-trisphosphate and diacylglyserol. Inositol 1,4,5-trisphosphate stimulates the release of calcium from the sarcoplasmic reticulum which activates the protein kinase C and the MAP kinase cascade leading to the stimulation of uterine contractions. β_2 – adrenoceptors, muscarinic and some 5-hydroxytryptamine receptors, for example 5-HT_1, activate the stimulatory pathway by coupling to the $G\alpha_i$ G protein, which stimulate uterine contractions by inhibiting cyclic adenosine 5'-monophosphate (cAMP) production (Bernal, 2003).

Fetal adrenal cortisol is thought to induce cyclooxygenase-2 (COX-2) in the placental trophoblast causing an increase in prostaglandin E_2 (PGE_2) and activation of the P450 cascade leading to the onset of cervical ripening and parturition (Whittle et al., 2001). A different mechanism for initiating cervical ripening and parturition is luteolysis. It is found in goats, rabbits and small rodents and is associated with a decrease in progesterone. Luteolysis is mediated by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which is released from the endometrium, activating the FP prostanoid receptor in the corpus luteum (Sugimoto et al., 1997).

Estrogen may have an affect on uterine contraction and parturition by increasing the number of myometrial gap junctions or by a direct oxytocin effect on the myometrium (Pinto et al., 1964; Schiff, 1977; Garfield et al., 1980a and Allen et al., 1989). Antiprogestin treatment in rats and guinea pigs stimulated an increase in

myometrial activity due to up regulation of gap junctions (Garfield et al., 1988; Chwalisz et al., 1991a, b; Sakai et al., 1992 and Chwalisz and Garfield, 1997). The antiprogestin RU486 increased uterine reactivity to prostaglandins but not oxytocin, suggesting that progesterone does not control oxytocin receptor (OTR) expression in guinea pigs or humans (Swahn and Bygdeman, 1988 and Chwalisz and Garfield, 1997).

In rodents, OT is believed to play a role in uterine activation and labor and is associated with an increase in uterine OTR levels (Soloff et al., 1974; Soloff et al., 1977; Soloff et al., 1979 and Bernal, 2003). In the myometrium and decidua of women the OTRs are increased, peaking early in the labor process which may be regulated through estrogen in the myometrium (Soloff and Sweet, 1982 and Fuchs et al., 1982). In the myometrium OT inhibits myometrial (Ca^{2+} + Mg^{2+})-ATPase activity by associating with the OTR causing an increase in Ca^{2+} and uterine contractility. OT stimulates an increase in prostaglandins (PGs) in the decidua but not the myometrium suggesting that OT stimulates uterine activity by acting directly on the myometrium via the OTR and indirectly through decidua PG production (Fuchs et al., 1982). This suggests a possible mechanism for the regulation of uterine contractions through OT and its receptors.

COX expression and PG production plays an important role in regulating uterine contractions during pregnancy, increasing as pregnancy progresses and peaking at labor (Puri and Garfield, 1982 and Dong et al., 1996). $PGF_{2\alpha}$ levels increase beginning in the day 16 rat uterus, peaking in the day 22 uterus and in the uterine vein. When progesterone levels are low, PG levels increase suggesting that COX expression and PG production may play a role in regulating uterine contractions through progesterone (Puri and Garfield, 1982).

An increase in $PGF_{2\alpha}$ (derived from macrophages and decidual cells) may play a role in uterine activation (Norwitz et al., 1991). Pro-inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) are produced by macrophages in response to bacterial stimuli which may also play a role in uterine activation possibly through PGs. Decidual macrophages may play a role in initiating early labor through the release of inflammatory cytokines and PGs in the decidua and/or fetal membranes in

close proximity to the myometrium under conditions of intrauterine infection (for e.g., chorioamnioitis) (Norwitz et al., 1991).

Progesterone acts as a gene suppressor. It down regulates genes associated with parturition, including the gap junction protein connexon 43 (Cx43), calcium channels, and OTRs. Progesterone also acts to stimulate an increase in nitric oxide (NO) (Chwalisz and Garfield, 1994a, b and Chwalisz and Garfield, 1997). NO plays a role in regulating the relaxation of the smooth muscle within the uterus during pregnancy by binding to the iron-heme moiety of guanylate cyclase which activates it, resulting in smooth muscle relaxation (Chwalisz and Garfield, 1997). Other mechanisms that relax the uterus include GPCRs, such as β_2 –adrenoceptors, prostanoid EP2 and IP and 5-HT_7, which cause an increase in cAMP in the myometrium stimulating uterine relaxation by coupling to the $G\alpha_s$ G-protein (Bernal, 2003).

Cyclic nucleotides are associated with smooth muscle relaxation. β_2-adrenoceptor agonists are commonly used as tocolytic agents by increasing the cAMP production from ATP. This is catalyzed by an adenylyl cyclase (AC) enzyme which is found in clusters in the cell membrane around receptors coupled to the $G\alpha_s$ G-protein. Increased coupling of receptors to AC may be responsible for uterine quiescence during pregnancy. GTP-dependent AC activity in the myometrial membranes is higher during pregnancy than in non-pregnant or postpartum tissues. AC activity increases in PGE_2 challenged membranes. This results in an increase in cAMP and relaxation of the uterus during pregnancy suggesting that receptors coupled to AC via the $G\alpha_s$ G-protein are important in maintenance of pregnancy (Europe-Finner et al., 1994).

What is the relationship between myometrial contractility and cervical ripening? Is there a relationship? Under normal conditions uterine contractions and cervical ripening are subject to similar endocrine regulation. Most patients with prolonged pregnancy have had an insufficiently ripe cervix which suggests an altered relationship between uterine activity and the cervix (Harris et al., 1983 and Huszar and Walsh, 1989). Cervical effacement and dilation is one of the most accurate ways of predicting the onset of labor. The uterine smooth muscle is continuous with the cervical smooth muscle. As

contractions of the uterus gradually increase, there is a gradual increase in the effacement of the cervix. During labor, or just prior to labor, contractions of the uterus are frequent, becoming quick and rapid, which may be associated with cervical dilation, the final stage in the ripening process (Embrey and Siener, 1965).

A number of factors govern the relationship between uterine contraction and cervical ripening. During pregnancy high levels of circulating progesterone, is associated with a firm, closed cervix, few myometrial gap junctions, low Ca^{2+} levels in the muscle, and a quiescent myometrium. At term there is a rise in estrogen levels which increases uterine contractility, OTR and the formation of gap junctions. In rats, luteolysis is induced by $PGF_{2\alpha}$ causing a decrease in progesterone levels and an increase in myometrial estrogen, OTR, gap junction formation, thus triggering preterm labor. PGs in general appear to increase myometrial activity and stimulate cervical ripening. Therefore, oxytocin mediated decidual PGs likely play a role in regulating uterine and cervical function during pregnancy (Alexandrova and Soloff, 1980a, b and Huszar and Walsh, 1989).

Cervical ripening

Cervical ripening (softening, effacement and dilation of the cervix) must occur for labor to progress normally to term, but can lead to preterm labor if it appears earlier in gestation. Mechanisms regulating and/or initiating the ripening process are unknown but are thought to be similar during both term and preterm labor (Stys et al., 1978; Uldbjerg et al., 1983b, e; Calder and Greer, 1992 and Shi et al., 1999). Biochemical and structural changes that occur in the cervix must be coordinated (timing and sequencing) with changes that occur in the uterus during pregnancy (Stys et al., 1978; Uldbjerg et al., 1983b, e; Calder and Greer, 1992 and Shi et al., 1999). Whether or not the delivery is slow and/or difficult depends upon the ability of the cervix to dilate. The “resistance” of the cervix dictates the ease at which dilation occurs.

Cervical ripening is a multifactorial process. A combination of biochemical and physical processes leads to the alteration of the mechanical properties of the cervix during the ripening process. The shape of the cervix gradually changes. Cervical

connective tissues, collagen (hydroxyproline) and proteoglycan concentrations decrease, while the soluble and/or extractable collagen, proteolytic activity, and turnover of matrix molecules increase (Epperson et al., 1951; Danforth et al., 1974; Uldbjerg et al., 1983b, e; Granström et al., 1989; Leppert and Yu, 1994; Glassman et al., 1995; Yu et al., 1995 and Shi et al., 1999). There is an increase in the ground substance. The glycosaminoglycans (GAG) composition changes (Yu et al., 1995). There is also an increase in the disorganization of the collagen bundles and a decrease in the myofibrils of the smooth muscle cells (Leppert and Yu, 1994). Collagen fibrils dissociate from one another and become scattered. The intrafibrillar space enlarges due to changes in the fluid movement into and out of the cervix (Calder and Greer, 1992 and Leppert and Yu, 1994). The water content increases in association with an increase in hyaluronic acid (Kroc et al., 1959; Zarrow and Yochim, 1961; Williams et al., 1982 and Calder and Greer, 1992). The distribution and composition of proteoglycans in the cervix changes (Golichowski et al., 1980; Kokenyesi and Woessner, 1989 and Kokenyesi and Woessner, 1990). There is a change in the soluble proteins and the cervical mass becomes enlarged and expanded (Leppert and Yu, 1994 and Yu et al., 1995). There is also an invasion of a large number of neutrophils, leukocytes, macrophages, and mast cells into the cervix during the ripening process (Liggins, 1981).

Problems associated with cervical ripening and parturition

Ineffective cervical softening can lead to prolonged labor and increased need for instrumental deliveries (Calder et al., 1977; Uldbjerg et al., 1983b, e and Granström et al., 1991). On the other hand, early onset of cervical changes or early ripening may result in preterm labor (PTL) and preterm birth (PTB) (Calder et al., 1977; Uldbjerg et al., 1983b, e; Granström et al., 1991 and Almström et al., 1991). Abnormalities that include preterm labor, dystocia, ineffective ripening of the cervix and cervical incompetence cause considerable obstetrical problems that compromise both the child and the mother.

Preterm labor and preterm birth

PTL or PTB is defined by the World Health Organization as labor onset or birth occurring prior to 37 but greater than 20 completed weeks of gestation in humans

(Berkowitz and Papiernik, 1993; Lockwood, 1995 and Goldenberg, 2002). PTB is the single most important clinical health problem in the practice of obstetrics and gynecology. PTL and PTB affects 10 to 15% of all pregnant women and is the leading cause of infant mortality and morbidity (e.g. responsible for 75% of all infant mortalities and 50% of long-term neurological disorders) (Berkowitz and Papiernik, 1993; Creasy, 1993; Simpson, 1997 and Goldenberg and Rouse, 1998). Three factors that contribute to delivery prior to 34 weeks of gestation are preterm premature rupture of the fetal membranes, spontaneous preterm labor with intact fetal membranes, and pregnancy complications jeopardizing fetal and/or maternal health requiring delivery (Goldenberg et al., 2000). Health care costs for PTB are more than 14 billion dollars a year in the United States (US). This does not include the emotional and financial stress placed on parents of premature infants (Simpson, 1997). In spite of significant advances in obstetrical care in the last three decades there has been little improvement in the prevention or treatment of PTL and PTB (Iams, 1995).

Women at higher risk for preterm premature rupturing of the membranes (PROM) demonstrate one or more of the following characteristics including prior PROM, prior PTB, low body mass index, cervical shortening and a positive fibronectin screening. Other factors associated with preterm PROM include a lower socioeconomic status, cigarette smoking, sexually transmitted infections, uterine distention, cervical cerclage, amniocentesis and vaginal bleeding during pregnancy (Mercer, 2003). The infection of the amniotic membrane and the decidua causes the release of proteases, the production of phopholipase A_2 and the initiation of the PG cascade which weakens the membrane and leads to PROM (Regan et al., 1981; Naeye, 1982; McGregor et al., 1987; Lonky and Hayashi, 1988 and Grable and Heine, 2003). Biochemically, PROM has been associated with a decrease in the collagen content of the membrane, an increase in the amniotic fluid matrix metalloproteases (MMPs) (1, 8 and 9), and a decrease in the amniotic fluid tissue inhibitors of MMPs (Skinner et al., 1981; Vadillo-Ortega et al., 1996 and Maymon et al., 2000a, b, c). In many of PROM the cause is unknown.

Spontaneous PTL and PTB with intact fetal membranes is distinguished from those with preterm ruptured membranes. Mechanisms regulating normal labor and birth, such as the remodeling of the cervix allowing it to open to the width of the reproductive tract and the initiation of rhythmic contractions with increasing frequency and amplitude, are thought to be similar to those regulating PTL and PTB except that they are accelerated and occur at an earlier time point during pregnancy (Peltier, 2003). Common characteristics associated with PTL and preterm delivery are preterm cervical dilation, cervical incompetence, cervical effacement, fundal abnormalities of the uterus, fetal anomalies, multifetal pregnancy, severe maternal illness, maternal thermal injury, autoimmune diseases, pregnancy induced hypertension, and maternal systemic disorder. A common denominator has not been identified among the fetal and maternal conditions associated with the onset of PTL and PTB (Cunningham, 2001).

Dystocia

Dystocia is a clinical term that means a difficult labor or childbirth and is characterized by the abnormally slow progression or arrest of labor (Garfield, 1987). Dystocia not only subjects the fetus to danger such as neonatal asphyxia but similarly often leads to postpartum hemorrhages and infections in the mother (Chelmow et al., 1993). Difficult labors are common and are a consequence of four distinct abnormalities that occur individually and/or in combination (Cunningham, 2001). The first is when the uterine force is insufficient and/or incorrectly coordinated with the effacement and dilation of the cervix or when the effort of the voluntary muscles during the second stage of labor, after the cervix has dilated, is inadequate for fetal expulsion. This is one of the most common causes of prolonged labor, affecting about 3 to 8 percent of women and may be caused by a decrease in the function and/or the absence of gap junctions, an over stimulation of inhibitory nerves or an under stimulation of excitory nerves, inadequate hormone levels and/or their receptors and/or a combination of the three (O'Driscoll et al., 1984; Seitchik, 1987; Garfield, 1987 and Wray, 1993). The second is associated with the maternal bony pelvis and pelvic contractions or cephalopelvic disproportion, when the size of the maternal pelvis is smaller than the fetal head. The third is the presentation or

the development of the fetus. The final abnormality is when the soft tissues of the reproductive tract form an obstacle to fetal descent.

Dystocia is the most common indicator for primary caesarean delivery (Shiono et al., 1987). Factors leading to increased use of caesarean delivery for dystocia are controversial and include epidural analgesia, fear of litigation, and obstetrician convenience (Fraser et al., 1987; Thorp et al., 1993; Savage and Francome, 1994 and Lieberman et al., 1996).

The identification of abnormal labor and the institution of proper management techniques such as labor augmentation for dystocia requires the assessment of the uterine contractile power and/or the expulsive force (ACOG Technical Bulletin, 1995). Amniotomy (the intentional rupturing of the membranes, the chorion and the amnion, allowing the amniotic fluid to flow out), oxytocin therapy, or both, are utilized in the augmentation of labor associated with dystocia. The potential advantage of amniotomy is the correction of the hypo contractility with the need for oxytocin. However, treatments with oxytocin, associated with intact membranes, may reduce the risk of infectous morbidity (Keirse, 1989 and Rouse et al., 1994). The assessment of the passenger, the fetus, in terms of cephalic presentation and passage through the pelvis includes estimating the fetal weight, position and attitude in association with their roles in dystocia (ACOG Technical Bulletin, 1995).

Ineffective cervical ripening

By the last two weeks of pregnancy, ripening of the cervix is nearly complete. Ineffective cervical ripening occurs in about 5 percent of women. It indicates the potential for prolonged labor and is associated with a delay in the dilation of the cervix (Calder et al., 1977; Uldbjerg et al., 1983b, e and Ekman et al., 1986a, b, c). One explanation for an unripe cervix may be an over estimation of the maturity of the fetus. However, in most cases when the normal ripening of the cervix does not occur, it is simply a failure or delay in the normal ripening process in the presence or absence of uterine contractions. In women, PGE_2 is commonly used for the management of

ineffective cervical ripening and is safe in post term pregnancies (Ekman et al., 1986a, b, c).

Cervical incompetence

An incompetent cervix is unable to maintain a pregnancy to term due to a deficiency in the structure of the cervix and is associated with cervical dilation in the second or early third trimester of pregnancy in the absence of uterine contractions (Neuman et al., 1980; Calder, 1981 and Conrad and Ueland, 1983). Factors associated with cervical incompetence include prolapse, ballooning of the membranes into the vagina, rupturing of the membranes and early expulsion of the fetus (Calder, 1981). What causes cervical incompetence is unknown but it is thought to be associated with both etiological and biochemical factors. Etiological factors include trauma to the cervix both present and previous, especially during the ripening process (Cunningham, 2001). Biochemical factors include the early breakdown of the cross-links between the collagen fibers, a change in the length and/or orientation of the fibers and/or abnormalities in the synthesis of the collagen. Patients showing a higher extensibility of the cervix in situ early in the pregnancy process are at a higher risk for developing an incompetent cervix (Neuman et al., 1980 and Conrad and Ueland, 1983). A surgical procedure such as a cerclage or some type of purse string suture is utilized to strengthen the cervix in the treatment of cervical incompetence (Cunningham, 2001).

CERVIX

Anatomy

The cervix is a heterogeneous organ containing mostly fibrous connective tissue. Collagen is the predominant fibrous extracellular element of the cervix (up to 80% - 85% total protein), playing a major role in the mechanical properties of the tissue (Aspden, 1988; Leppert and Yu, 1994 and Yu et al., 1995). Elastic fibers are also embedded in a ground substance of proteoglycan complexes (non-fibrillar) containing glycosaminoglycans (GAGs) (Danforth, 1947; Danforth, 1983 and Granström et al., 1991). The most abundant of these GAGs are chondroitin and its epimer dermatin sulphate (Uldbjerg et al., 1983a, d). The cellular portion consists mainly of actively

contractile smooth muscle (15% - 20%) but little is known of its role in the remodeling cascade and the ripening process (Schwalm and Dubrauszky, 1966; Rorie and Newton, 1967 and Leppert and Yu, 1994). Blood vessels and epithelial cells are found in the cervix and in humans there are mucus filled crypts that extend deep into the stromal tissue. Other cells (1%) found in the cervix include fibroblasts, mast cells, macrophages, neutrophils and leukocytes. Other non-fibrillar components include hyaluronic acid and structural glycoproteins.

The distal portion of the cervix contains more connective tissue than the proximal part which is mainly composed of smooth muscle (Uldbjerg et al., 1983b, e). In stretch tests of the non-pregnant human cervix, it was found that the slope of the stress strain curve varied depending on location within the cervix indicating that the load bearing ability of the cervix is not uniform and is related to the distribution of tissues within the cervix (Conrad et al., 1980 and Conrad and Ueland, 1983).

The human cervix

The human cervix is a specialized portion of the uterus that lies below the isthmus and projects into the vagina. Based on its attachment to the vagina, the cervix is divided into a vaginal and supravaginal portion which is on the posterior surface covered by the peritoneum of the recto-uterine space. The upper boundary of the cervix is the internal os, the uterine portions of the cervix. The lower is the external os, the vaginal portion of the cervix (the portio vaginalis), which projects into the vagina and is bound by the fornices (Krantz, 1973 and Cunningham et al., 2001).

The cervical mucosa is a series of vertical and lateral folds with the lateral folds emanating from the vertical folds (Deaver, 1927; Lowrie, 1952; Smout and Jacoby, 1953; Schaeffer, 1953 and Fluhmann, 1961a, b). Mucous can become encrusted forming small cysts frequently found on the portio vaginalis and are called ovules of Naboth or Nabothian cysts. The anterior wall of the vagina is continuous with the upper portion of the cervix, without distinct separation (Krantz, 1951).

The cardinal ligament of Mackenrodt is the main means of support, fixing and suspending the cervix from the lateral pelvic wall (Krantz, 1973). It is a band of smooth

muscle and connective tissue that protrudes from the anterior and posterior margins of the supervaginal cervix and the uterus to the lateral pelvic wall (Fluhmann and Dickmann, 1958).

Blood vessels supplying blood to the cervix are the uterine artery and vaginal artery. The uterine arteries are the main source of blood for the cervix. These divide into two main branches as they travel through the cardinal ligament. Here they cross over the uterus to the margins of the cervix and/or laterally through parametrical tissue ascending along the borders of the uterus sending lateral branches to both sides of the cervix. The smaller of the two branches, cervicovaginal artery, supplies the lower portion of the cervix. The main branch is of considerable size and provides blood to the upper portion of the cervix. Other sources of blood to the cervix are the azygos arteries of the vagina and the ascending branches of the vaginal arteries. The terminal branches of the uterine artery anastomose with the azygos vaginal artery (Smout and Jacoby, 1953). The upper portion of the cervix is attached to the 2nd-4th sacral vertebrae by the uterosacral ligament containing blood vessels, lymphatics, smooth muscle, and nerves (Fluhmann, 1954).

The main venous drainage for the cervix parallels the arterial vessels. Also, there is a venous network around the cervix that communicates with a similar network around the neck of the bladder emptying into the internal ilias veins (Deaver, 1927; Krantz, 1951; Smout and Jacoby, 1953 and Krantz, 1973).

The lymphatics of the cervix are found superficially beneath the peritoneum in the mucosa and deeply in the fibrous collagen draining into a plexus of lymph channels for which there are three major channels (Henriksen, 1949). The first follows the direction of the uterine artery to the external iliac and obturator nodes arising from the paracervical plexus. The second also arises from the paracervical plexus and follows the uterine veins posteriorly to the iliac nodes. The third is the smallest and most variable of the lymphatic channels. These pass posteriorly along the uterosacral ligaments, lateral to the rectum and into the sacral nodes (Eichner et al., 1954). During gestation there is an enlargement of the lymphatic system.

The anatomy and histology of the rat cervix

Anatomically, the vaginal fornix is associated with the distal portion of the cervix and the uterus with the proximal portion. Figure 1.1 demonstrates the anatomy and histology of the rat cervix, which contains two cervical canals. The cervical canals are lined with epithelial tissue. Stratified squamous epithelium is associated with the distal portion of the cervix and columnar epithelium with the proximal portion of the cervix. The two types of epithelium meet near the external os to form the squamo-columnar junction. The rat uterus contains glandular tissue. The lack of glandular tissue within the cervix differentiates it from the uterus. Tissues within the rat cervix are organized into the mucosa (epithelium) and the sub mucosa, which constitutes the main body of the cervix.

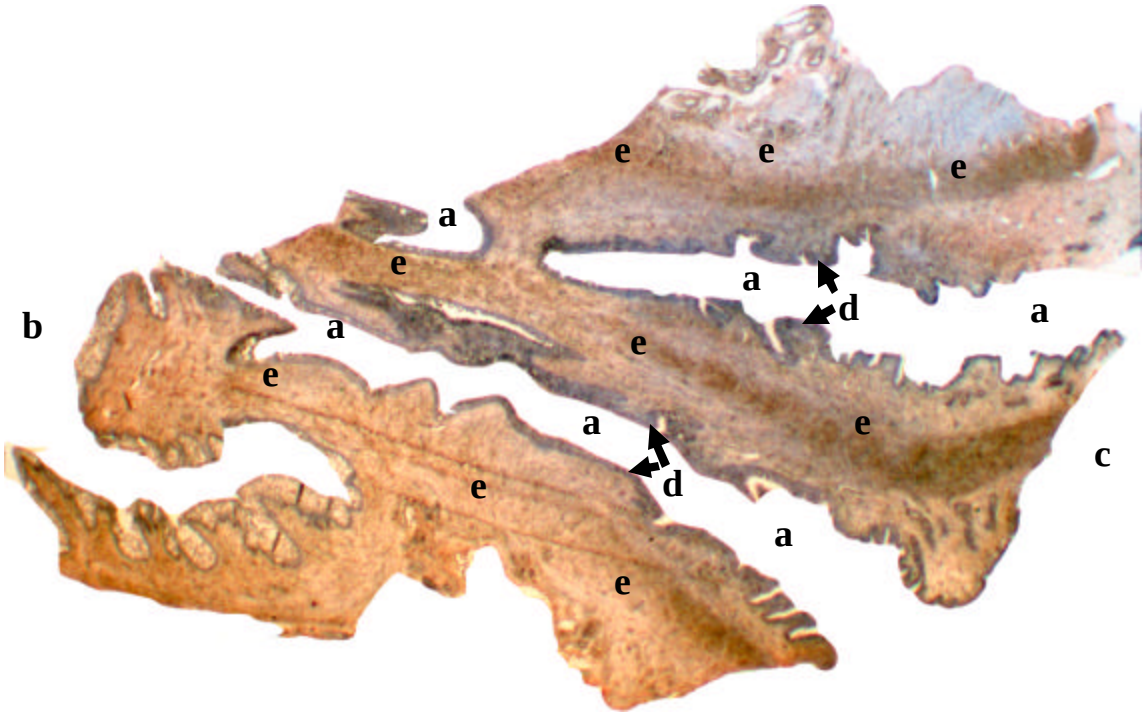

Figure 1.1: The photo is a longitudinal section of a gestational day 14 rat cervix and shows the general anatomy and histology of the rat cervix**.** (25X) **a)** cervical canals, **b)** fornix/distal portion, **c)** uterus/proximal portion, **d)** mucosa/epithelium, **e)** sub mucosa/cervical body

Collagen fibers within the rat cervix are well organized and densely packed mainly being found parallel to the smooth muscle layers in three distinct regions of the cervix (Leppert and Yu, 1994 and Yu et al., 1995). There are two small longitudinal layers of collagen with one located in the periphery and the other adjacent to the cervical canals. The circular collagen layer is a much larger layer located in the middle region of the cervix and arranged in multiple circular layers around the cylindrical cervix (Yu et al., 1995). During pregnancy the circular collagen in the middle layer demonstrates the greatest amount of change during the ripening process compared to the other layers, thus our studies will focus on changes in the circular collagen during the ripening process (Yu et al., 1995).

The rat cervix as a model for cervical ripening

The rat cervix is an established model for studying the ripening of the cervix and parturition and therefore was chosen for these studies. Our studies focus on cervical ripening and parturition in rat cervical tissues. The rat cervix is an established model for the study of cervical ripening and parturition. Rats have a short, well defined gestational period of 22 days, with day 1 being when sperm is observed in a vaginal smear. The different stages of cervical ripening have also been well characterized in rat cervical tissue through molecular (immunohistochemistry, RT-PCR, and Westerns) and functional studies (cervical resistance and extensibility studies) (Leppert, 1995; Buhimschi et al., 1996; Dong et al., 1996; Ali et al., 1997 and Shi et al., 1999).

Function

During pregnancy the cervix acts as a barrier allowing the fetus to be maintained in the uterus until term. Around mid-gestation in rats or at the end of the 2nd trimester/beginning of the 3rd trimester in humans the cervix will start to soften (Friedman, 1978 and Leppert, 1995 and Yu et al., 1995). Late in gestation when the cervix is already soft, effacement and dilation occurs allowing for the birth canal to open and the fetus to be born (Shi et al., 1999 and Calder and Greer, 1992). Initially, cervical function and/or changes occurring in the cervix during pregnancy were thought to be passive. It is now thought that these cervical changes during pregnancy are due to active

biochemical processes (Danforth et al., 1974; Ito et al., 1979; Conrad and Ueland, 1983; Uldbjerg et al., 1983b, e; Leppert, 1992 and Leppert and Yu, 1994).

Collagen synthesis and structure

Collagen synthesis occurs in fibroblasts starting with the pro-α chain which is synthesized on membrane bound ribosomes and injected into the lumen of the endoplasmic reticulum. The pro-α chain undergoes hydroxylation of selected proline and lysine residues forming hydroxyproline and hydroxylysine for which selected hydroxylysine residues are then glycosylated. The pro-α chain combines with two other pro-α chains to form a procollagen hydrogen bonded triple helix structure. The procollagen is placed in a secretory vesicle to be transported to the plasma membrane where it is secreted into the extracellular space contained within a large infolded area of the plasma membrane. The propeptides of the procollagen molecule are cleaved, with the collagen molecule then aggregating with other collagen molecules to form a collagen fibril. This then aggregates with other collagen fibrils to form a collagen fiber (Alberts et al., 1994).

The cervix is composed of three types of collagen fibers: type I (70%), type III (30%) and type IV (found in small amounts) (Winkler et al., 2003). Type I and type III collagen have a similar distribution within the cervix around the smooth muscle bundles. Type I (collagen fiber) forms thick fibers composed of closely packed thick fibrils and type III (reticular fiber) forms thin fibers composed of loosely packed thin fibrils. Type IV collagen is associated with the boundary lamina of individual smooth muscle fibers and the basement membrane of the blood vessels within the cervix.

Techniques utilized to measure and demonstrate cervical ripening

Changes in the physical characteristics of the cervix during pregnancy should be expressed as a change in the distensibility. A low distensibility indicating that the material in question is easily bent or stretched by a small force. Distensibility is commonly expressed as a length-tension curve. Other physical characteristics of the cervix that can be measured include tensile strength, breaking strength, and yield point.

Tensile strength refers to the amount of load that produces an irreversible change such as an increase in length due to stretching of the tissue. Breaking strength is the amount of load that causes the tissue to rupture. Yield point refers to the load at which the extension versus load curve becomes nonlinear. A linear extension is indicated when the resistance force to the extension is also linear (Conrad and Ueland., 1983).

Cervical ripening has been described as an inflammatory process (Liggins, 1981). Cervical ripening is not an acute process, but occurs gradually throughout gestation. Due to the structure and the nature of these changes, cervical ripening has been studied by measuring changes in cervical resistance, in light-induced-fluorescence (LIF) of the collagen (Shi et al., 1999) and in birefringence of the collagen.

Cervical resistance

Changes in the resistance to stretch of the rat cervix during gestation, as an indicator of ripening, was measured in our lab utilizing a device called the cervimeter (Shi et al., 1999). A stainless steel hook was inserted through each cervical canal of the isolated rat cervix. The lower hook secured the tissue in the organ bath and the upper hook is connected to a force transducer. The cervix is stretched incrementally producing a length-tension curve, whose slope indicates the extensibility of the cervix. The non-pregnant cervix is very rigid and the slope is steep. As pregnancy progresses to term, the tissue becomes more compliant meaning that there is a gradual decrease in the resistance of the cervix as it ripens. At post-partum there is a rapid increase in rigidity of the tissue comparable to the non-pregnant cervix. Changes in the resistance of the cervix are utilized to indicate and measure ripening and/or changes in the ripening process.

Cervical fluorescence

The collascope is an optical instrument designed to measure the auto-fluorescent properties of cross linked collagen. It is utilized in our lab to measure changes in the fluorescence of the rat cervical collagen during gestation as an indicator of ripening (Shi et al., 1999). The collascope measures pyridinoline which is a major crosslink in collagen that breaks down as the cervix ripens and pregnancy progresses to term. The intensity of the fluorescent signal at 390 nm is proportional to the amount of cross-linked collagen

within the cervix. Collagen fluorescence was high in the non-pregnant cervix, gradually decreased during pregnancy and increased again after delivery. As the cervix ripened there was a decrease in the fluorescence of the collagen in parallel to that of the resistance. Changes in the fluorescence of the collagen is therefore utilized as a valuable tool to indicate and measure ripening and/or changes in the ripening process during pregnancy.

Picrosirius red polarization method

Collagen is normally organized in an orderly fashion with a parallel orientation and exhibits a "normal" birefringence (Junqueira et al., 1982). Collagen, being rich in basic amino acids, strongly reacts with acidic dyes. Picrosirius red is a strongly acidic dye that reacts through an ionic interaction with the positively charged groups of the collagen, probably the lysine, hydroxylysine and histidine moieties. It enhances the normal birefringence of the collagen making it an effective stain that is selective for the fibrous connective tissue except for elastic tissue (Sweat et al., 1964; Junqueira et al., 1979; Junqueira et al., 1982 and Wolman and Kasten, 1986). Picrosirius red stain is routinely used on histological sections and for studying the distribution of the different types (types I, II and III) of collagen under normal and abnormal conditions making it an appropriate stain for studying the changes in the cervix during the ripening process (Junqueira et al., 1982).

NEURAL INNERVATIONS IN THE CONTROL OF THE UTERINE CERVIX AND PARTURITION

Innervation of the human uterine cervix originates from the pelvic autonomic system and includes the superior, middle and inferior hypogastric plexus consisting of several parallel nerves that descend into the pelvis terminating in the uterosacral ligament (Krantz, 1959 and Krantz, 1973). The inferior continues as the pelvic plexus which contains both sympathetic (inferior hypogastric plexus) and parasympathetic (nervi erigentes) segments (Krantz, 1959). Sensory fibers are mainly visceral and found in the nervi erigentes, however a significant number of sensory fibers are also found in the sympathetic component. The hypogastric ganglion is found at the base of the broad

ligament on both sides of the cervix parallel to the lateral pelvic wall. Postganglionic fibers form large branches that enter the uterus in the region of the internal os (Krantz, 1973).

The guinea pig uterine cervix is innervated by adrenergic nerves with 35% entering the uterus in the suspensory ligament and 65% from the hypogastric nerve at the cervical and tubal ends of the uterus. In the hypogastric nerves, 35% of the fibers are postganglionic from the paracervical sympathetic ganglia near the uterine wall at the level of the lower cervix (Owman and Sjoberg, 1966 and Thorbert, 1978). The uterine cervical tissue in the rat is innervated by both adrenergic and cholinergic nerves of the autonomic nervous system. Adrenergic nerves transverse the muscles layers of the rat uterus dividing into smaller branches which associate with the blood vessels but not the smooth muscle. There is no difference in the distribution of the adrenergic nerves between the cervical, miduterine, or ovarian regions of the uterus (Owman and Sjoberg, 1966; Bell, 1972; Thorbert, 1978 and Garfield, 1986). Adrenergic nerves in the rat probably control the blood flow to the cervix and the uterus, indirectly affecting cervical ripening and parturition (Garfield, 1986).

Cholinergic nerves innervate both the vascular tissue and the smooth muscle cells of the cervix originating from the Frankenhäuser's plexus on the lateral surface on each side of the cervix and from smaller ganglia immediately anterior to the cervix. The cholinergic nerves transverse through the mesometrium sending branches into the uterine wall with no observable difference in the innervation and distribution of the cholinergic nerves between the cervical, midhorn, and ovarian regions of the uterus (Garfield, 1986). The cholinergic nerves may play a role in regulating uterine contractility and cervical ripening due to their close approximation to the smooth muscle (Hollingsworth, 1974 and Garfield, 1986). The cholinergic nerves are also involved in blood flow due to their proximity to the blood vessels, but their exact role is unknown. Neurotransmitters may stimulate the release of various cytokines, hormones and enzymes such as the NOS and COX enzymes, MMPs and IL-8 in the control of cervical ripening and parturition (Garfield, 1986).

THE ROLE OF STEROID HORMONES IN THE CONTROL OF CERVICAL RIPENING AND PARTURITION

Steroid hormones such as estrogen and progesterone play an important role during pregnancy in the control of cervical ripening and parturition possibly through regulating NOS and COX expression (Dong et al., 1996; Allport et al., 2001 and Sato et al., 2001). Progesterone and/or estrogen may indirectly regulate COX-2 expression and PGE_2 production through NF-κB and inflammatory cytokines (Dong et al., 1996). Progesterone and 17β-estradiol have also been shown to directly suppress PGE_2 production in rabbit uterine cervical fibroblasts (Sato et al., 2001). NF-κB is associated with labor. It causes an increase in COX-2 expression in human amnion cells thus playing an indirect role, possibly through cytokines (Allport et al., 2001 and Slater et al., 1995). IL-1β decreases progesterone dependent transcription and increases NF-κB DNA binding and transcription which leads to an increase in COX-2 expression and PG production. Progesterone and/or progesterone receptors (PR) decrease NF-κB transcriptional activity suggesting that NF-κB may be a factor in functional progesterone withdrawal again leading to an increase in COX-2 expression and cervical ripening (Allport et al., 2001).

Estrogen receptors

Estrogen receptors (ER) have two isoforms, ERα and ERβ, that are derived from separate genes and share a common structural domain with other steroid receptors having a centrally located DNA binding domain, a C-terminal ligan binding/ligand dependent trans-activation domain (AF-2), and an N-terminal ligand independent trans-activation domain (AF-1). The selective action of estrogen and various estrogen agonists and antagonists are thought to be due to the differential expression of the ERα and ERβ receptors (Warner et al., 1999 and Gustafsson, 2000a, b). ERs are ligand activated macromolecules having a high affinity and low capacity for 17β-estradiol and other biologically active estrogens (Gustafsson, 2000a, b and Schreihofer et al., 2001). Once bound, ERs undergo a conformational change enhancing dimerization becoming a nuclear transcription factor that associates with the estrogen response element of specific

genes recruiting cofactors and thus initiating transcription and production of various proteins (McKenna et al., 1999 and Schreihofer et al., 2001). Cells responsive to estrogen normally produce additional estrogen receptors and progesterone receptors promoting the continued action of estrogen and responsiveness of the cell to progesterone (Cunningham, 2001).

ERs are also activated through intracellular signaling pathways in a ligand independent manner possible through increases in the intracellular cAMP levels. The cAMP is known to stimulate MAPK kinase activity which leads to the phosphorylation and activation of the ERα and ERβ and the initiation of transcription. (Aronica and Katzenellenbogen, 1993; Kato et al., 1995; El-Tanani and Green, 1997; Villalba et al., 1997; Moroo et al., 1998; Tremblay et al., 1997; Suzuki et al., 1999; Le Pechon-Vallee et al., 2000 and Schreihofer et al., 2001).

ERs are present in the human cervix with protein levels being down regulated in the term pregnant and in the postpartum cervix in association with a decrease in the collagen concentration and an increase in the collagen solubility, suggesting a role for estrogen and/or estrogen receptors in the remodeling of the cervix during pregnancy. ERs increased after antiprogestin, RU487 (Mifepristone), treatment suggesting that a decrease in progesterone and/or progesterone withdrawal may stimulate an increase in the ERs. PGs, which are normally increased in the term pregnant cervix and play an important role in initiating parturition, had no effect on the ER expression (Ekman-Ordeberg et al., 2003). Upon comparison of the expression patterns of the ERα and ERβ, it was found that both are localized in the epithelial and stromal tissues of the human cervix (Stygar et al., 2001and Ekman-Ordeberg et al., 2003). ERβ was also localized within the smooth muscle, endothelium and the granulocytes of vessels associated with the cervix (Stygar et al., 2001). The levels of ERβ increased while the ERα decreased in the term pregnant and postpartum cervix. Leukocytes identified in the term pregnant cervix stained positively for ERβ but not ERα which may regulate the release of various cytokines and MMPs during pregnancy (Stygar et al., 2001 and Ekman-Ordeberg et al., 2003).

Estrogen

Estrogen is produced in the placenta and ovarian follicles (granulosa cells). In non-human placentas the 17α-hydroxylase enzyme is found. It is essential for the synthesis of estrogen through the conversion of the C_{21}-steroids to C_{19}-steroids, the precursor for estrogen, which is utilized by animals metabolizing progesterone or pregnenonlone to estrogen (Ryan, 1959a, b and Liggins, 1983). The human placenta is deficient in the 17α-hydroxylase, yet large amounts of estrogen are still produced. Because the human placenta is unable to utilize acetate, cholesterol nor progesterone as a precursor for estrogen, it requires an external source of suitable substrate such as androgens which are converted by the aromatizing enzymes to estrogens (Liggins, 1983). There is a high capacity for the placenta to convert the C_{19}-steroids, which are found in the form of dehydroepiandrosterone, androstenedione, and testosterone, to estrone, 17β-estradiol or both. Androgens are an alternative to cortisol for progesterone/estrogen regulation by the fetus and are abundant in the placentas of primates. Androgens are mainly found in the form of dehydroepiandrosterone (DHEAs) which is secreted from the fetal zone of the adrenal cortex and the fetal adrenal glands of various species including primates and guinea pigs; however, the plasma C_{19}-steroids may also be a possible precursor for estrogen. Dehydroepiandrosterone sulfate was found in large amounts in the plasma and since it has a long half-life, it is uniquely qualified to be the principal circulating precursor for placental 17β-estradiol synthesis (Pulkkinen, 1961; Warren and Timberlake, 1962; Warren and Timberlake, 1963 and Warren and Timberlake, 1964).

Estrogen formation from androstenedione in the placenta is catalyzed by the aromatase enzyme mainly in the syncytiotrophoblast of the placenta, but also occurs in the granulosa cells of the ovaries and at a much lower level in the adipose tissues of the stromal cells, Sertoli and Leydig cells of the testis, hypothalamus, and in the liver of the fetus but not adults (Bonenfant et al., 2000). The type of estrogens being produced by the aromatase enzyme is dependent upon the substrate and the type of 17β-hydroxysteroid dehydrogenase (17βHSD) in that tissue. Aromatization of androstenedione in the ovaries

produces estrone which is converted in the granulosa cells to 17β-estradiol by the 17β-hydroxysteroid dehydrogenase type 1 enzyme. In contrast, in adipose tissue, estrone is not converted into 17β-estradiol but is released directly into the blood. Aromatization of testosterone in all tissues is directly converted to 17β-estradiol.

The synthesis of estrogens starts in the fetal adrenal glands with the secretion of DHEAs which is then converted in the fetal adrenal glands and/or the liver into 16α-hydroxydehydroepiandrosterone sulfate. The two types of estrogens that are secreted are 17β-estradiol and estriol. 17β-estradiol is produced from DHEAs and estriol is produced from 16α-hydroxyandrostenedione which is converted to 16α-hydroxyestrone, the substrate of estriol (Ryan, 1959a, b; MacDonald and Siiteri, 1965; Madden et al., 1976 and Madden et al., 1978). By the 30th week of pregnancy only a small fraction of the estrogen being produced and secreted by the placenta is derived from the dehydroepiandrosterone produced in the maternal adrenal glands (Siiteri and MacDonald, 1966). Near term, half of 17β-estrodiol is derived from the fetal and the other half from the maternal dehydroepiandrosterone sulfate (Siiteri and MacDonald, 1966). Regarding estriol in the placenta, 90 percent is derived from the fetal 16α-hydroxydehydroepiandrosterone and 10 percent from other sources. The fetal adrenal glands are an important source of placental estrogen precursors in human pregnancy with about 80 to 90 percent of the steroids being secreted into the maternal blood (Madden et al., 1976 and Madden et al., 1978).

Connective tissue remodeling during the ripening process occurs simultaneously with an increase in the serum levels of estrogen and/or the 17β-estradiol intensifying as pregnancy progresses to term. This is association with a down regulation of receptors within the cervical tissue and an increase in the COX-2 enzyme (Siiteri and McDonald, 1966; Turnbull et al., 1974 and Stjernholm et al., 1996). In vitro studies demonstrate that estrogen inhibits collagen synthesis in cervical tissue in early and term pregnancies in humans (Wiqvist and Linde, 1987a, b). Estrogen may therefore act to stimulate and/or regulate COX expression and PG production in the control of cervical ripening and

parturition (Hedin and Eriksson, 1997; Badawi and Archer, 1998; Xiao et al., 1998 and Sato et al., 2001).

The effect of estrogen in the human cervix during pregnancy is contraversal with few reports being available. Estradiol applied intravaginally during the first trimester had no effect on the resistance of the cervix, but pretreatment with 17β-estradiol in the second trimester caused a softening of the cervix improving the outcome of second trimester abortions induced by PGE_1 analogues (Anthony et al., 1984 and Allen et al., 1989). Intravenous infusion of 17β-estadiol in the second trimester caused a softening of the cervix in most of the women suggesting that local applications of estrogens may stimulate softening of the cervix (Pinto et al., 1964). PG induced labor did not improve with local applications of estrogen either in combination (simultaneous treatment with PGE_2 and estrogen) or when pretreated with estrogen 12 hours prior to PGE_2 induced ripening, suggesting that estrogens do not play a significant role in parturition (MacLennan et al., 1981).

Estrogen may have several possible actions including a direct effect on the cervix since softening can occur independently of contractions due to estrogens and is supported by estradiol's ability to cause a "loosening" and/or scattering of the cervical collagen in mice. DHEAs also increased the cervical collagenolytic activity (Leppi and Kinnison, 1971; Gordon and Calder, 1977; Quinn et al., 1981; Tromans et al., 1981 and Allen et al., 1989). Estrogen may effect MMP/TIMP production and/or expression. MMPs are colagenases that help break down the collagen as the cervix ripens and pregnancy progresses to term. MMPs will be discussed further later in chapter 1 (the role of matrix metalloproteinase's in the control of cervical ripening and parturition). Estradiol applied to cervical fibroblasts of rabbits in culture decreased MMP-1 and MMP-3 levels while increasing TIMP levels, which are inhibitors of MMPs. However, estradiol was not as effective as progesterone in regulating MMP and TIMPs (Sato et al., 1991). Circulating concentrations of 17β-estadiol are higher in women with successful labor induction by PGE_2 than in women where induction failed, suggesting the importance of estrogen and/or 17β-estadiol in the ripening processes and parturition (MacKenzie et al., 1979). In

support of this theory a deficiency in placental steroid sulfatase is associated with failure of the cervix to ripen and prolongation of pregnancy due to the lower levels of estrogen in the circulation (France, 1981).

Progesterone receptors

Progesterone receptors (PR) mediate the activity and/or actions of progesterone and their antagonists, depending on the amount or types of receptors present (Mulac-Jericevic et al., 2000). PR are ligand-activated transcription factors containing a highly conserved centrally located DNA-binding domain (DBD), a conserved C-terminal ligand-binding domain (LBD), a hinge region between the DBD and LBD, and a highly variable N-terminal domain (Giangrande and Mcdonnell, 1999 and Leonhardt and Edwards, 2002). The C terminus of the PR LBD is a 42-amino acid sequence required for progesterone to bind, determining how the PR will respond to a particular ligand (Vegeto et al., 1992). There are two isoforms, PR-A and PR-B, expressed from a single gene by alternating promoter usage (Kastner et al., 1990 and Mesiano et al., 2002). They are structurally similar although PR-B contains an N-terminal fragment (Kastner et al., 1990; Giangrande and McDonnel, 1999; Mesiano et al., 2002 and Leonhardt and Edwards, 2002). PR-B is a much stronger activator than PR-A, but PR-A can function as a ligand-dependent repressor of other steroid receptors including PR-B (Tung et al., 1993; Vegeto et al., 1993; Mcdonnell et al., 1994; Mcdonnell and Goldman, 1994; Mesiano et al., 2002; Giangrande et al., 2000 and Leonhardt and Edward, 2002). Progesterone causes a conformational change in the PR, activating the receptor from a non-deoxyribonucleic acid (DNA) -binding form to a DNA binding form. Once activated, the PR binds to the progesterone response element (PRE), a specific DNA sequence within the promoter region of the progesterone responsive gene. The agonist-bound PR activates transcription either directly with the general transcriptional machinery or indirectly with co-activators including steroid receptor co-activator-1 (SRC-1) and CREB-binding protein (CBP) (Wagner et al., 1998 and Liu et al., 2002).

PRs play a complex role in the reproductive process. Studies with knockout mice show that mice lacking both PRs (or PR-A only) are infertile (Lydon et al., 1995; Mulac-

Jericevic et al., 2000 and Mesiano et al., 2002). Mice lacking PR-B appeared to be fertile and have a normal pregnancy, suggesting the importance of PR-A in mice fertility. PR activity and/or actions in the human reproductive physiology is not well understood. One possible function is the differential regulation of the PRs creating a model of progesterone withdrawal (Mesiano et al., 2002). PRs are found in the human cervix, with the PRs being down regulated at term and in the postpartum cervical tissue at the protein level with a decrease in the collagen concentration and an increase in the collagen solubility suggesting a role for progesterone in the remodeling of the cervix during pregnancy through the PRs. There was no change in the mRNA expression suggesting post-transcriptional regulation of the PRs (Ekman-Ordeberg et al., 2003).

Progesterone

PR ligands can be pure agonists (progesterone), antagonists (also known as a type I antagonists, Onapristone) or both an agonist and an antagonist (mesoprogesterone) which is known as a progesterone receptor modulator (PRMs) or a type II antagonist (Spitz et al., 1996; Spitz and Chwalisz, 2000; Spitz and Coelingh Bennink, 2000 and Elger et al., 2000). The Type I antagonist prevents the PR from binding to the DNA, thus preventing the activation of transcription. The Type II antagonist, allow the PR to bind to the DNA where it may activate or inhibit transcription depending upon several factors that include the cell type, the promoter context, the co-activators and other signaling pathways (Gass et al., 1998). Progesterone antagonists (PA) and PRMs have numerous applications in female healthcare. For example, mifepristone, a type II antagonist, is used to terminate pregnancy and also as a contraceptive in emergencies. PA and PRMs are utilized in treating endometriosis and uterine myoma by inhibiting proliferation in the endometrium. As a contraceptive, they suppress follicular development, block the luteinizing surge and retard endometrial maturation.

Progesterone maintains the pregnancy and is initially produced in the corpus luteum of all species (Diczfalusy and Troen, 1961 and Liggins, 1983). Eventually the placenta will take over the function of the corpus luteum, at 7 weeks in humans and at 10 weeks in sheep. The corpus luteum in some species such as in rabbits and goats is

required for the continued secretion of progesterone throughout gestation. In animals dependent upon the corpus luteum, progesterone withdrawal is required for the effacement and dilation of the cervix and the initiation of parturition (Liggins, 1983).

Progesterone is synthesized in the granulosa cells of the corpus luteum from cholesterol taken up from the plasma in the form of low density lipoprotein (LDL) particles. It is converted in the mitochondria to pregnenolone (Tureck and Strauss, 1982; Cunningham et al., 2001 and Christenson and Devoto, 2003). The human placenta produces a significant amount of progesterone, but its capacity to synthesize cholesterol for conversion to progesterone is limited (Cunningham et al., 2001). The placenta must therefore rely on exogenous sources of cholesterol for progesterone formation with about 90 percent being derived from the maternal plasma (Hellig et al., 1970; Simpson et al., 1979a, b and Simpson et al., 1980).

The rate of progesterone synthesis is dependent upon the number of LDL receptors on the plasma membrane of the trophoblasts. LDL receptors are localized in coated pits on the microvillous membranes of the syncytium and are found as early as 4 weeks after conception with the affinity of these receptors remaining constant throughout human pregnancy (Cunningham et al., 2001).

Progesterone enters cells by diffusion and in responsive tissue becomes associated with high affinity PRs promoting gene transcription. Progesterone may play a role in regulating the actions of estrogen through several mechanisms such as by decreasing the synthesis of the ER, by increasing the rate of estradiol-17β inactivation through the increased activity of estradiol dehydrogenase or by increasing sulfurylation of estrogens (estrogen sulfotransferase), another means of estrogen inactivation (Tseng and Gurpide, 1974a, b; Tseng and Gurpide., 1975a, b and Tseng and Liu, 1981). Progesterone may also work through receptor independent, nongenomic processes.

Progesterone maintains uterine quiescence during pregnancy until term in all mammalian species studied to date. In sheep at term, a signal arises (corticotropin releasing hormone) from the brain of the fetus initiating the process of progesterone withdrawal and parturition. Corticotropin releasing hormone (CRH) is transported from

the fetal brain by the hypophyseal portal vessels to the fetal pituitary gland stimulating an increase in adrenocorticotropic hormones (ACTH) which causes the increased fetal adrenal secretion of cortisol (Myers et al., 1992a, b, c). In primates including humans, progesterone levels in the plasma increase throughout pregnancy and only decline after the delivery of the placenta (Challis and Lye, 1994). In humans fetal stress induces labor and a release of CRH from the hypothalamus. CRH may also be released from the trophoblast, amnion, chorion leave, and deciduas. CRH levels are increased in the third trimester during the last 5 to 6 weeks of the pregnancy and increase even further during labor (Goland et al., 1988; Petraglia et al., 1989 and Petraglia et al., 1990). CRH can increase the formation of PGs (Jones and Challis, 1989). Fetal stress may induce labor through the release of CRH stimulating an increase in PGs, uterine contractility and parturition. However, CRH does not appear to affect progesterone levels in humans in regards to parturition.

Progesterone plays an important role in the ripening of the cervix. Antiprogestins such as Onapristone (antiprogestin, ZK98299), a model for progesterone withdrawal and preterm labor, induces cervical ripening (Hegele-Hartung et al., 1989 and Chwalisz et al., 1991a, b). Ripening of the cervix with Onapristone is comparable to that found in term gestation and may act through inflammatory cells and/or fibroblasts (Junqueira et al., 1980; Hegele-Hartung et al., 1989; Chwalisz et al., 1991a, b; Buhimschi et al., 1996; Dong et al., 1996 and Ali et al., 1997). In guinea pigs, progesterone agonist R5020 (promegestone) blocked Onapristone induced ripening (Chwalisz, 1994; Chwalisz and Garfield, 1994a, b and Chwalisz and Garfield, 1997). This would strongly suggest that progesterone plays a role in the ripening of the cervix as pregnancy progresses to term.

Progesterone may act as an immunosuppressor in the cervix by inhibiting the actions and/or activities of PGs and chemotactic agents such as cytokines and leukotrienes during normal and antiprogestin induced labor in the control of cervical ripening. Progesterone decreased MMP-1 and MMP-3 in cervical fibroblasts of the rabbit while increasing tissue inhibitor of metalloprotease (TIMP) expression. MMPs are collagenases which play a role in degrading the collagen during the ripening process in

the cervix. MMPs are further defined later in chapter 1 (the role of MMPs in the control of cervical ripening and parturition). Progesterone had no effect on collagen synthesis by fibroblasts but inhibited RU486 stimulated IL-8 release in human choriodecidual cells in vitro (Sato et al., 1991; Ito et al., 1994 and Chwalisz and Garfield, 1997).

Progesterone is thought to regulate iNOS expression and NO production. PGs, steroids and cytokines such as IL-1β, IL-8, TNFα, transforming growth factor β (TGFβ) and relaxin may work together with NO in the ripening of the cervix and parturition (Ito et al., 1994). Onapristone induces an increase in iNOS expression and NO production in the rat cervix, changes similar to those found during term labor (Buhimschi et al., 1996 and Ali et al., 1997). Treatments of NO to the cervix in guinea pigs demonstrated similar characteristics that could not be distinguished from Onapristone induced cervical ripening (Buhimschi et al., 1996; Chwalisz et al., 1997; Ali et al., 1997 and Shi et al., 2000b) This strongly suggests that progesterone plays a role in regulatin iNOS expression and NO production during gestation.

Late in gestation, around the time of progesterone withdrawal in rats, there is an increase in the COX-2 expression (Dong et al., 1996). Progesterone decreases PG production and COX-2 activity and expression (Kelly et al., 1986; Sato et al., 2001 and Farina et al., 2004). Antiprogestins, RU486 (mifepristone) and ZK98734, increased PG production (Farina et al., 2004). This strongly suggests that progesterone plays a role in regulating COX-2 expression and PG production during gestation.

THE ROLE OF CYTOKINES AND INFLAMMATION IN THE CONTROL OF CERVICAL RIPENING AND PARTURITION

Reproductive events are associated with an inflammatory-like process, which is best suited for the required changes in the remodeling and rearrangement of the various reproductive tissues during pregnancy (Kelly, 1996). Cytokines (non-inflammatory and pro-inflammatory) are small polypeptides. Pro-inflammatory cytokines such as IL-1, 6, 8, TNF-α, and interferon gamma can act as local and systemic hormones in the inflammatory process (Dinarello, 1996a, b and Wilson et al., 1998). Non-inflammatory

cytokines can inhibit the actions of pro-inflammatory cytokines to induce tissue repair (Henderson and Poole, 1994 and Henderson, 1995).

Inflammation and the cervix

Cervical ripening is associated with cytokines and collagenases in an inflammatory-like process with the collagen becoming dispersed and disorganized (Schwalm and Dubrauszky, 1966). Neutrophils, which are attracted by chemokines such as IL-8, infiltrate the cervix during pregnancy and are the source of the collagenases (Osmers et al., 1991 and Osmers et al., 1992). The neutrophil degranulates in association with IL-8, releasing the collagenases such as MMP-8 (neutrophil collagenase) and 9 (gelatinase) initiating extracellular matrix reorganization of the cervical tissue (Lloyd and Oppenheim, 1992; Cassatella, 1995 and Osmers et al., 1995a, b). PGs are pro-inflammatory acting synergistically with IL-8 in attracting neutrophils into the cervix by altering the blood flow and the permeability of the vessel (Williams and Morley, 1973; Williams and Peck, 1977; Foster et al., 1989; Rampart et al., 1989 and Colditz, 1990).

Cytokines and the cervix

Local applications of IL-1β, IL-8 and TNFα stimulate ripening of the cervix in guinea pigs and are associated with an inflammatory-like response (Chwalisz et al., 1994 and El Maradny et al., 1994). Cytokines (IL-1β, IL-6, TNFα) stimulate the release of arachidonic acid, the precursor for PG and may play a role in regulating COX expression and PG production in the control of cervical ripening and parturition (Romero et al., 1989a, b; Mitchell et al., 1991b and Molnar et al., 1993). IL-8 is a chemotactic agent that preferentially recruits neutrophils, granulocytes and T-cells (Schroder, 1989; Larsen et al., 1989; Standiford et al., 1990a, b, c; Baggiolini and Clark-Lewis, 1992; Uchiyama et al., 1992 and Stadnyk, 1994). These are known sources of collagenases and other enzymes capable of digesting the extracellular matrix of the cervix which may play a role in the ripening of the cervix during pregnancy and parturition (Junqueira et al., 1980). IL-1 and TNF-α have been shown to stimulate IL-8 production in cultured fibroblast cells (Wuyts et al., 1998).

IL-6 increases during inflammation and promotes B and T-cell growth and differentiation, immunoglobulin production, maturation of megakaryocytes and stimulation of neutrophils (Mantovani, 1997). IL-6 acts on macrophages and endothelial cells to induce IL-8 production (Mantovani, 1997). IL-1 and TNF-α stimulate IL-6; however, IL-6 inhibits IL-1 and TNF-α (Aderka et al., 1989 and Schindler et al., 1990). 17β-estradiol has been shown to have an inhibitory effect on IL-6 (Jilka et al., 1992).

IL-6 and IL-8 are present in the amniotic fluid in normal pregnancies throughout most of gestation, but increase to high levels in association with intrauterine infection and preterm labor. IL-6 and IL-8 are produced by amnion mesenchymal cells and the amnion cells. IL-8 is also produced by the amnion epithelium cells (Keelan et al., 1997a, b and Maeda et al., 1997).

Interleukin-1 beta (IL-1β) is a primary cytokine that is produced rapidly in response to infection and immunological challenges promoting the synthesis of many other cytokines and mediators of inflammation. IL-1β is present in the amniotic fluid under both term and preterm laboring conditions suggesting that in infection and/or inflammation it may play a role in regulating parturition possible through IL-1β expression (Romero et al., 1987; Romero et al., 1990 and Cunningham, 2001). IL-1β stimulates an increase in IL-6 and IL-8 in the amniotic fluid (Cox et al., 1993).

Cytokines such as IL-1β are associated with an increase in NOS and COX expression, specifically iNOS and COX-2 (Corbett et al., 1993 and Swaisgood et al., 1997). Cytokines rapidly induce COX-2 expression suggesting an increase in PG production which takes part in various stages of the inflammatory cascade and increases vascular permeability, thus increasing leukocyte and/or monocyte infiltration into the cervix (Alberts et al., 1994; Armstrong et al., 1995; Kennard et al., 1995; Dong et al., 1996 and Swaisgood et al., 1997).

Lipopolysaccharide (LPS) and the cervix

LPS is commonly utilized for the study of cervical ripening and parturition and is a model for infection, inflammation and preterm labor under infectous conditions (Kaga

et al., 1996; Buhimschi et al., 1996 and Dong et al., 1996). LPS stimulates an increase in iNOS and COX-2 mRNA expression in mouse uterine tissues but not in placental tissues suggesting that the effects of LPS may be tissue specific (Liggins, 1981; Buhimschi et al., 1996; Ali et al., 1997 and Swaisgood et al., 1997).

THE ROLE OF MATRIX METALLOPROTEINASE'S (MMPS) IN THE CONTROL OF CERVICAL RIPENING AND PARTURITION

Proteolytic enzymes, such as MMP's and collagenase, degrade components of the extracellular matrix playing an important role in the control of cervical ripening and parturition (Mullins and Rohrlich, 1983). All MMPs are secreted from cells in a latent zymogen form and have optimal enzymatic activity at a neutral pH. MMP synthesis in normal connective tissue cells is negligible and regulated by cytokines, growth factors and several other agents. MMPs are all zinc proteinases containing zinc ions essential for activation and Ca^{2+} ions are also required for activation and stability. MMPs are all inhibited by TIMPs, present in tissues at low concentrations and difficult to extract possibly due to the binding MMPs to the matrix.

MMP-1 is produced by fibroblasts, macrophages and neutrophils as a set of zymogens, proMMP-1. One is 52 kDa and the other 56 kDa with the higher molecular weight species being glycosylated (Nagase et al., 1981; Nagase et al., 1983 and Campbell et al., 1987). MMP-1 is 57% identical to MMP-8 and when activated cleaves collagens types I, II and III generating a ¾ and ¼ fragment (Welgus et al., 1981a, b and Hasty et al., 1990). MMP-1 cleaves type III collagen at a faster rate than type I and digests collagens VII and X but not types IV and V (Welgus et al., 1981a, b; Schmid et al., 1986; Hasty et al., 1987 and Seltzer et al., 1989a, b). The α2-macroglobulin is the best substrate for MMP-1 (Enghild et al., 1989). Activation of proMMP-1 to MMP-1, which is 41 kDa with an NH_2-terminus of Phe^{81}, is dependent on the presence of a procollagenase activator MMP-3 (Vaes, 1972; Eeckhout and Vaes, 1977; Vater et al., 1983; Treadwell et al., 1986; Murphy et al., 1987; Ishibashi et al., 1987 and Ito and Nagase, 1988).

MMP-2 is secreted by rheumatoid synovial cells as proMMP-2, a 72 kDa gelatinase. When activated it is a type IV collagenase that readily digests gelatins known

as gelatinases and/or type IV collagen (Liotta et al., 1979; Murphy et al., 1985; Collier et al., 1988; Murphy et al., 1989a, b; Stetler-Stevenson et al., 1989 and Stygar et al., 2002).

MMP-3, also known as stromelysin, proteoglycanase and transin-1, is secreted by connective tissue cells as a 57 and 59 kDa zymogen. The 59 kDa proMMP-3 is the glycosylated form of the 57 kDa species and accounts for 20% of the proMMP-3. The primary structure of MMP-3 is similar to that of MMP-1 (54% identical) and is a type IV collagenase that digests gelatins (Nagase, 1991). MMP-3 degrades proteoglycans, fibronectin, laminin, collagen telopeptides and native collagens IV and IX (Galloway et al., 1983; Chin et al., 1985; Okada et al., 1989 and Okada and Nakanishi, 1989). MMP-3 is a collagenase activator that is critical for the activation of MMP-1 (Nagase, 1991). ProMMP-3 activation is attained by its interaction with a variety of proteinases (e.g. trypsin, chymotrypsin, plasmin, plasma, kallikrein, neutrophil elastase, cathepsin G, and thermolysin) to produce a first generation 45 kDa active form that is slowly converted to 28 kDa forms (Okada et al., 1989 and Okada and Nakanishi, 1989). Since proteinases have different substrate specificities, cleavage site for activation of proMMP-3 does not seem to be specific and MMP-3 cannot activate proMMP-3.

MMP-8 is a collagenase produced by neutrophils and has an amino acid sequence 57% identical to that of MMP-1 (Hasty et al., 1990). MMP-8 appears to cleave type I collagen at a much faster rate than type III collagen, but can cleave both (Hasty et al., 1987).

MMP-9 is produced by macrophages and is a 92 kDa gelatinase and a type IV and V collagenase with gelatinase activity that digests gelatins (Hibbs et al., 1985; Hibbs et al., 1987; Wilhelm et al., 1989 and Murphy et al., 1989a, b). MMP-9's structure is very similar to that of MMP-2, except that it has an additional 53 residue segment with some similarity in sequences with the $\alpha 2$ chain of collagen V. The difference in molecular weight between MMP-2 and MMP-9 is due to glycosylation of MMP-9.

MMPs play a role in the ripening process through the break down of the cross links between the collagen fibers and the continued break down of the collagen throughout pregnancy, which may be regulated by NOS and COX expression, and by NO

and PG production (Kelly, 1996 and Ishii et al., 2003). Collagenases have been shown to increase in the human serum and cervical tissues at term and during parturition (Uldbjerg et al., 1983b, c, e; Rajabi et al., 1988; Osmers et al., 1990 and Granstrom et al., 1992). Human cervical tissue at term contains free collagenase, collagenase bound by tissue inhibitor of metalloproteinase (TIMP) and collagenase complexed with alpha-2 macroglobulin (Kitamura et al., 1979 and Kitamura et al., 1980a,b). The MMP/TIMP ratio is important in regulating local matrix degradation. Serum TIMP-1 levels are lower in pregnant women compared to laboring women under both term and preterm laboring conditions, suggesting the importance of TIMPs in regulating MMP activity during the ripening process and parturition (Clark et al., 1994). MMP expression and secretion is controlled by various cytokines, growth factors, physical stress, as well as chemical agents acting through positive and negative feedback mechanisms (Nagase, 1991). The production of MMP-1 and MMP-3 have been shown to be stimulated by IL-1, TNF and a variety of growth factors (Mizel et al., 1981; Gowen et al., 1984 and Ito et al., 1991a, b). TNF-β, glucocorticoids and retinoic acid down-regulate both the MMP-1 and the MMP-3 gene. Progesterone treatment leads to a reduced secretion of endometrial MMP-1, MMP-3, and MMP-9, whereas progesterone withdrawal has been shown to increase MMP-1, MMP-3, and MMP-9 production from human endometrial tissue without altering TIMP-1 and TIMP-2 production (Marbaix et al., 1992; Salamonsen et al., 1997 and Lockwood et al., 1998). In the human cervix collagenase activity has been reported to be increased after PGE_2 application (Ekman et al., 1983c and Rath et al., 1993).

THE ROLE OF NO IN THE CONTROL OF CERVICAL RIPENING AND PARTURITION

The mechanisms and mediators that control cervical ripening are still largely unknown even though nitric oxide (NO) has been shown to be involved in the ripening cascade (Buhimschi et al., 1996; Chwalisz et al., 1997; Ali et al., 1997; Thomson et al., 1997 and Ledingham et al., 2000).

NO, the NO pathway, and the NOS isoforms

NO is a free radical produced by the nitric oxide synthase (NOS) enzymes, which

oxidizes the amino acid L-arginine into NO and L-Citrulline in equal amounts. Figure 1.2 demonstrates the pathway for NO production. There are three NOS iso-forms: inducible (iNOS), endothelial (eNOS), and brain or neuronal (b or nNOS) (Nathan, 1992). eNOS and bNOS are constitutive isoforms that are calcium-calmodulin dependent and produce NO in small amounts. eNOS is associated with the regulation of blood flow and blood pressure and is found in endothelial tissues associated with the endothelial cell membranes. bNOS is associated with neurotransmission and is found in neural tissues (Nathan, 1992). iNOS, which is calcium-calmodulin independent, is the inducible isoform and is found throughout the body associated with macrophages, immune responses, inflammation, and apoptotic stimulation (Gilbert and Herschman, 1993a, b; Kitajima et al., 1994 and Vane et al., 1994). iNOS is found in the soluble portion of tissue homogenates and produces large amounts of NO.

Animal and clinical studies looking at iNOS and NO in cervical ripening

NO is produced from both endogenous cervical tissues and from migrating cells.

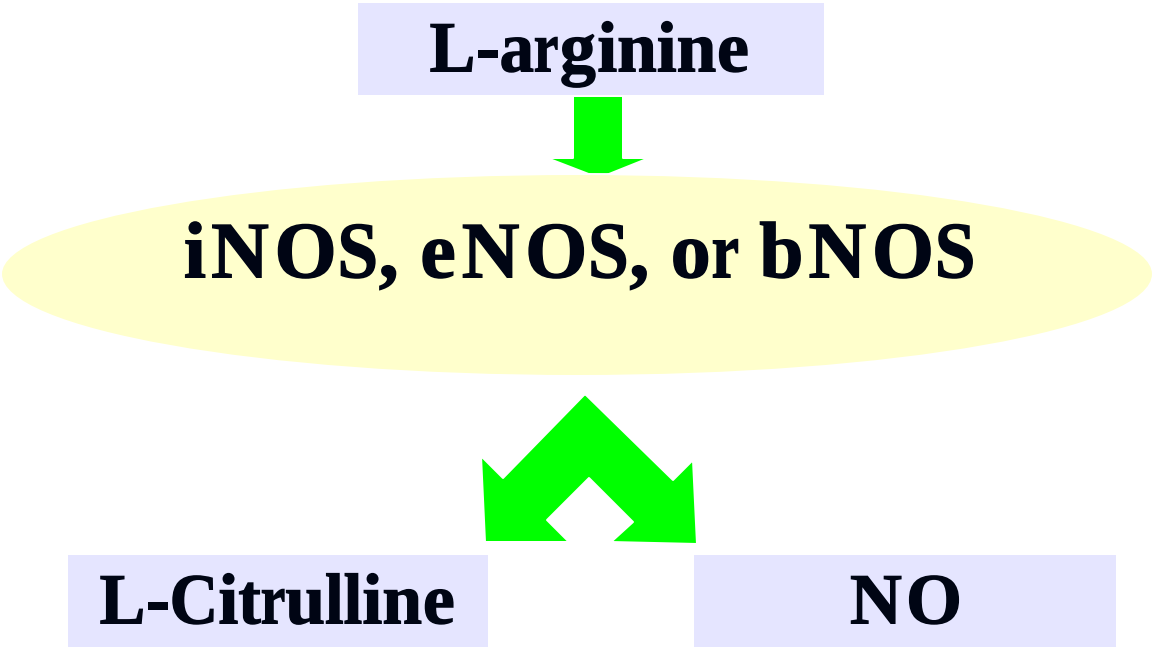

Figure 1.2: The pathway for the synthesis of nitric oxide

Cells within the cervix produce NO and contain all three NOS isoforms (Buhimschi et al., 1996). Leukocytes and macrophages have been shown to increase as pregnancy progresses to term (Mackler et al., 1999 and Yellon et al., 2003). Macrophages and leukocytes are known sources for NO production and iNOS expression (Buhimschi et al., 1996 and Ali et al., 1997). It is not known which plays a more important role in the ripening of the cervix, endogenous or migratory scourse of NO. iNOS increases significantly during labor (Buhimschi et al., 1996 and Ali et al., 1997). During most of gestation, NO production is maintained at non-pregnant levels in association with low levels of the iNOS enzyme. During labor, NO levels increase in association with an increase in the iNOS enzyme (Buhimschi et al., 1996 and Ali et al., 1997). Intracervical applications of NO to animals and humans produce characteristics similar to ripening. Endogenous NO decreases cervical resistance (Chwalisz et al., 1997; Thomson et al., 1997; Thomson et al., 1998 and Shi et al., 2000b). Sodium nitroprusside (SNP), a NO donating compound, induces a dissociation of collagen fibers and fiber bundles (Chwalisz et al., 1997). N-nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor, prolongs the time to delivery and decreases cervical extensibility (Buhimschi et al., 1996 and Shi et al., 2000a, b). Clinical studies have shown that NO donors, isosorbide-mononitrate and glyceryl-trinitrate (GTN), stimulate cervical ripening in humans and may have less severe effects than PGs (Thomson et al., 1997; Thomson et al., 1998 and Norman et al., 1998).

Function of NO in the cervix during the ripening process and parturition

NO may act on MMPs to ripen the cervix possibly being regulated through progesterone (Murrell et al., 1995; Drapier and Bouton, 1996; Tamura et al., 1996 and Upchurch et al., 2001). Progesterone in the endometrium inhibits MMPs, and antiprogestins enhance the effects of MMPs (Rodgers et al., 1994). Onapristone stimulates an increase in cervical iNOS suggesting that progesterone withdrawal may allow increased iNOS expression and NO production (Buhimschi et al., 1996 and Ali et al., 1997).

NO plays a role in regulating MMP production and activation (Murrell et al., 1995; Tamura et al., 1996; Drapier and Bouton, 1996; Sasaki et al., 1998; Yoshida et al., 2001 and Upchurch et al., 2001). Tamarua (1996) showed NO stimulates an increase in MMP-2 and MMP-9 in cultured rabbit articular chondrocyte cells. Sasaki (1998) showed an increase in MMP-1, MMP-9 and MMP-3 in that order in cultured rabbit articular chondrocyte cells. Upchurch (2001) showed NO to inhibit MMP-9 epxression in cultured rat aortic smooth muscle cells. Yoshida (2001) showed NO to increase MMP-1 production in cultured human uterine cervical fibroblast cells. NO plays a role in the transcription of MMP-1, MMP-3, MMP-10, and MMP-13 (Ito et al., 1998 and Ishii et al., 2003). Ishii (2003) showed NO upregulated MMP-1 promoter activity in human melanoma cell line C32TG. Deletion and mutational analysis identified and AP-1 binding site required for NO regulation of MMP-1. It was also shown that NO may also regulate MMP-1 transcription via the MAPK (ERK and p38) pathways (Ishii et al., 2003). MMP-3 is required for the activation of MMP-1. MMP-1 and MMP-3 are thought to play a role in the break down of the cross links between the collagen fibers in the softening of the cervix during the ripening process. High levels of NO are thought to inhibit expression of MMP-8 and MMP-9, but low levels increase expression of MMP-8 and MMP-9. Late in gestation iNOS and NO levels are low and are associated with an increase in MMP-8 and MMP-9. MMP-8 and MMP-9 are thought to be important for the continued break down and disorganization of the collagen late in gestation during the effacement and dilation stage of the ripening process (Winkler et al., 1999; Upchurch et al., 2001 and Marx Chapter 3 and 5 of the dissertation). The greater the degree of break down and disorganization of the collagen the easier it is for the cervix to efface and dilate.

Other processes and/or mechanisms that may involve NO in the ripening process include apoptosis, GAG remodeling, collagen remodeling and an increase in vascular permeability (Chwalisz and Garfield, 1998a, b). It has been reported that programmed cell death of cervical smooth muscle cells is associated with the ripening of the cervix possibly through a cascade that triggers the release of various cytokines and collagenase (Leppert and Yu, 1994 and Chwalisz and Garfield, 1998a, b).

NO is thought to play a role in apoptotic stimulation (Chwalisz and Garfield, 1998a, b). NO donors caused DNA fragmentation, a marker for apoptotic stimulation, in a time and concentration dependent manner. The inhibition of protein kinase C (PKC) allows an increase in NF-κB activity, which regulates apoptotic stimulation and COX-2 expression. NO may play a role in the ripening of the cervix through the inhibition of PKC and an increase in cAMP (Messmer et al., 1995 and Kim and Chun, 2003).

NO may play a role in GAG and collagen remodeling during the ripening process (Hickery and Bayliss, 1998). The mechanisms that regulate alteration of the GAG composition and the proteoglycan complexes during the ripening process are unknown. Late in gestation there is an increase in hyaluronic acid, which may inhibit iNOS expression and NO production (Takahashi et al., 2001 and Kobayashi et al., 2002). Eventually as pregnancy progresses to term the hyaluronic acid will break down into fragments. These hyaluronic acid fragments have been shown to stimulate iNOS expression and NO production through NF-κB (Mckee et al., 1997).

NO may take part in various stages of the inflammatory cascade during the ripening process (Liggins, 1981; Chwalisz et al., 1997 and Yellon et al., 2003). In rodents, activated neutrophils, lymphocytes, mast cells and macrophages can produce large amounts of NO (Hibbs et al., 1988; Buhimschi et al., 1996; Chwalisz et al., 1997 and Ali et al., 1997). Resident cells of inflamed tissues are capable of synthesizing large amounts of NO following stimulation by cytokines (Billiar et al., 1990 and Stadler et al., 1991). NO is thought to increase vasodilatation and vascular permeability allowing for the increased infiltration of inflammatory cells (Evans, 1995 and Lyons, 1996). The endothelial lining becomes leaky contributing to the formation of edema. It also becomes adhesive, facilitating the binding of neutrophils, monocytes, and lymphocytes to the endothelium as they migrate into the cervix (Osmers et al., 1992 and Kelly, 1996). These cells within the cervix communicate via paracrine and autocrine pathways to produce mediators which continue to degrade the cervix during effacement and dilation (Evans, 1995). NO may regulate the ripening process through the activation of the COX-2 enzyme at the protein level, thus increasing PG production leading to the continued

break down of the collagen during effacement and dilation of the cervix (Salvemini et al., 1993 and Corbett et al., 1993).

THE ROLE OF PGS IN THE CONTROL OF CERVICAL RIPENING AND PARTURITION

PGs, the PG pathway, and the COX isoforms

Endogenous release of PGs, which are locally acting agents produced by the cyclooxygenase (COX) enzymes, is thought to play a role in the ripening of the cervix (Calder and Greer, 1992 and Calder, 1994). COX is the rate-limiting enzyme in the biosynthesis of PGs and works by converting arachidonic acid into PGG_2 which is then converted into PGH_2 by the peroxidase enzymes (DeWitt, 1991). PGH_2 is converted into PGI_2, PGE_2, or thromboxane A_2 depending on which enzymes are present and in what quantities. Figure 1.3 demonstrates the pathway for PG production. COX has two isoforms COX-1 and COX-2. COX-1, the constitutive isoform, is found in most tissue and plays a house keeping function maintaining normal physiological levels of PGs. COX-2, the inducible isoform, is associated with an inflammatory like response (Flower, 1978a, b and Vane et al., 1994). COX-2 has a short half life, suggesting rapid regulation which could occur from transcription to post translation. It is found in endothelial cells, fibroblast cells, macrophages, mesangial cells and rat islets (Raz et al., 1988; Maier et al., 1990; Masferrer et al., 1992 and Salvemini et al., 1993). COX-2 can be induced by growth factors, cytokines, and other stimuli (Goppelt-Struebe, 1995).Animal and clinical studies looking at COX-2 and PGs in cervical ripening

Animal and clinical studies looking at COX-2 and PGs in cervical ripening

The cervix contains both COX isoforms and produces PGs (Calder et al., 1977; Calder and Greer, 1992 and Dong et al., 1996). During labor or just prior to labor, PG production in the cervix sharply increases and is associated with an increase in the COX-2 enzyme (Calder and Greer, 1992 and Dong et al., 1996). PGs, such as PGE_2, enhance cervical compliance and increase collagenase activity reducing collagen concentrations. High doses of PGE_2 induce a significant reduction in cervical resistance (Chwalisz et al., 1997). Intracervical application of PGE_2 in humans causes the cervix to soften and dilate

in the tenth week of pregnancy. In women with an “unfavorable” cervix at term, cervical ripening was pronounced after intracervical applications of PGE_2 (Wingerup et al., 1979a, b and Ekman et al., 1983a, b, c, d, e).

Clinically PGs are presently used to interrupt pregnancy during the second trimester and are used to stimulate cervical ripening at term (Szalay et al., 1981; Ekman et al., 1983a, b, c, d, e; Calder and Greer, 1992 and Calder, 1994). PG inhibitors may be used to prevent cervical ripening and preterm labor (Hearne and Nagey, 2000; Shi et al., 2000b and Shi et al., 2001). PG inhibitors have been used clinically to prevent spontaneous abortions and/or preterm labor (Calder, 1994).

Function of PGs in the cervix during the ripening process and parturition

PGs stimulate the production of glycosaminoglycans and proteoglycans in the human cervix and activate collagenases and proteases (MMPs) causing the dissociation and rearrangement of the collagen fibers. This reduces the tensile strength of the cervix (Norstrom, 1982 and Dong et al., 1996).

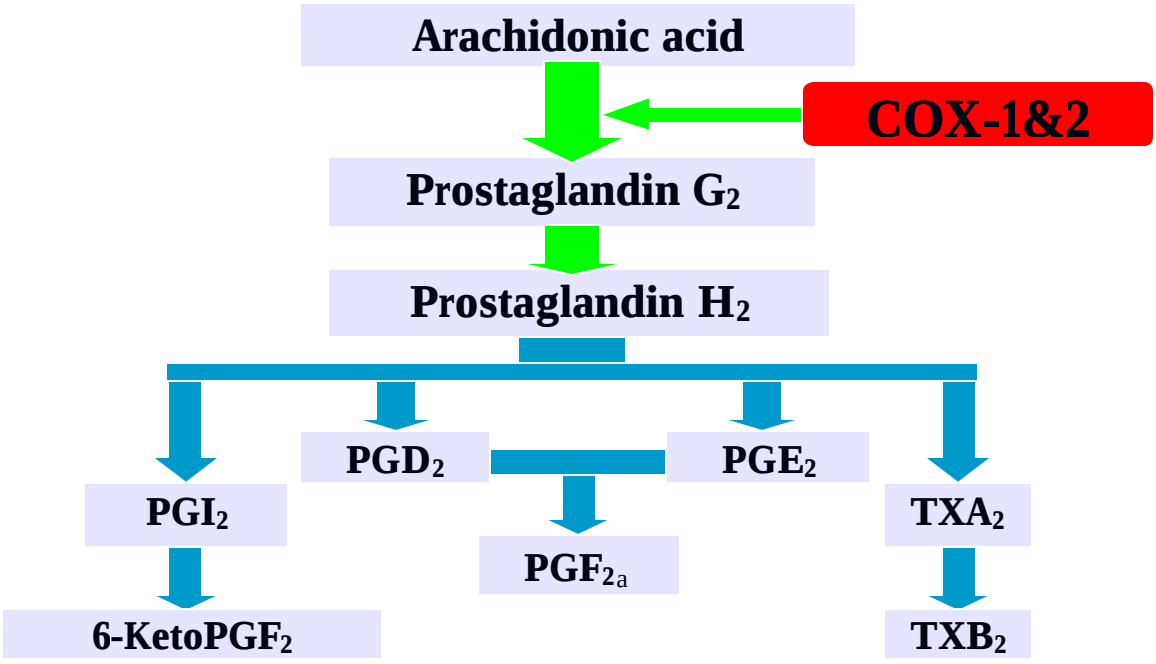

Figure 1.3: The pathway for the synthesis of prostaglandins

PGs, specifically PGE_2, are associated with an increase in MMPs such as MMP-8 and 9. In human cervical tissue PGE_2 stimulated IL-8 production. IL-8 is a chemotactic agent that preferencially recruites neutrophils into the cervix during the ripening process. Neutrophils are a source of MMP-8 and 9 (Williams and Morley, 1973; Williams and Peck, 1977; Foster et al., 1989; Rampart et al., 1989; Colditz, 1990; Lloyd and Oppenheim, 1992; Cassatella, 1995; Osmers et al., 1995a, b and Denison et al., 1999). PGs may act through leukocytes, smooth muscle cells and/or epithelial cells to stimulate an increase in MMPs and/or various cytokines. PGs may also act by altering the GAG composition and proteoglycan complexes to soften the cervix (Uldbjerg et al., 1981). GAGs are produced by fibroblasts, which is a cellular component of the cervical connective tissues (Calder and Greer, 1992). PGE_2 increases hydration and hyaluronic acid concentrations by acting on fibroblasts to induce hyaluronic acid synthetase (Murota et al., 1977 and Cabrol et al., 1987). Hyaluronic acid is hydrophillic leading to the increased water content of the cervical tissues during the ripening process which is characteristic of cervical softening. PGE_2 increases collagenolytic activity which may cause proteolytic breakdown of proteoglycan complexes and may cause an increase in the free hyaluronic acid (Szalay et al., 1981 and Kelly, 1996). Increases in GAG may cause collagen fibrils to disperse and become destabilized, allowing for an increase in the cervical compliance through the break down of the cross links between the collagen bundles (Calder and Greer, 1992).

Cervical effacement, associated with a gradual shortening of the cervix, occurs late in gestation prior to dilation. Cervical dilation is a rapid process that occurs during labor or just prior to labor. PGs are not known to play an active role in the effacement and dilation of the cervix; however it "softens" the cervix so that effacement and dilation can occur. PGs continue to alter the GAG composition and the proteoglycan complexes during effacement and dilation even though the cervix is soft (Uldbjerg et al., 1981; Conrad and Ueland, 1983 and Yu et al., 1995). Hyaluronic acid continues to increase within the cervix causing an influx of water into the cervix. The collagen fibrils continue to disperse and dissociate from one another. This maintains or increases the

disorganization causing further destabilization of the collagen within the cervix. What role this plays in the effacement and dilation of the cervix is unknown; however, it may determine how easily cervical effacement and dilation occurs. The greater the disorganization of the collagen within the cervix, the easier it will be for the cervix to efface and dilate.

PGs may take part in various stages of the inflammatory cascade. PGs increase vascular permeability (Armstrong et al., 1995). Late in gestation there is an infiltration of various leukocyte and/or monocyte like cells into the cervix (Osmers et al., 1992; Kelly, 1996 and Mackler et al., 1999). Increased infiltration of leukocytes and/or monocyte-like cells and increased vascular permeability are associated with an inflammatory like response.

COX-2 functions as an immediate early gene, which is rapidly induced by the actions of various hormones, cytokines and growth factors (Albert et al., 1994; Kennard et al., 1995; Dong et al., 1996; Swaisgood et al., 1997; Sato et al., 2001 and Kim et al., 2003). Little is actually known about the regulation of COX-2 in reproductive and/or cervical tissues and its potential interaction with NOS expression and NO production or its role in cervical ripening and parturition.

Estrogen and progesterone may play a role in regulating COX-2 expression and PG production (Gu et al., 1990; Romero et al., 1990; Badawi and Archer, 1998; Xiao et al., 1998 and Sato et al., 2001). ERs and PRs are present in the human cervix during pregnancy, but their involvement in regulating COX-2 expression and PG production is unknow (Ekman-Ordeberg et al., 2003). Their involvement could be determined utilizing antagonists that prevent estrogen and progesterone from binding to their recptor. COX-2 was shown to increase in the rat uterus at stages of the estrous cycle (proestrus and estrus) when estrodiol levels are elevated, suggesting that estrodiol may exert a stimulatory action on the expression of COX-2 (Dyal and Crankshaw, 1988; Dong et al., 1996 and Dong and Yallampalli, 1996). This suggests that estrogen or the lack of progesterone increases COX-2 expression and that PGs may act through NO, growth factors, and/or cytokines in the inflammatory cascade in the control of cervical ripening and parturition.

AN INTERACTION BETWEEN NOS AND COX EXPRESSION, NO AND PG PRODUCTION

It is possible that there is an interaction between NOS and COX expression (NO and PG production) in the control of cervical ripening and parturition. Progesterone and/or estrogen may be important in regulating the initial stages of a possible relationship between iNOS and COX-2 expression, and NO and PG production. Both iNOS and COX-2 have two NF-κB binding sites in their promoter region suggesting that NF-κB may regulate them (Appleby et al., 1994 and Zhang et al., 1998).

Proposed pathway for cervical ripening, NOS and COX expression and NO and PG production

Figure 1.4 demonstrates the proposed pathway for cervical ripening and the potential relationship between nitric oxide synthase (NOS) and cyclooxygenase (COX) expression in the ripening process. During term pregnancy, progesterone levels are thought to decrease while estrogen and/or estrodial levels increase. This leads to an increase in various cytokines specifically IL-1β, IL-8 and TNF-α, which are associated with chemotaxis, increased leukocyte infiltration and a dissociation of nuclear factor-kappa B (NF-?B) from I?B. Both iNOS and COX-2 have two NF-κB binding sites in their promoter region suggesting that NF-κB may play a role in NOS and COX regulation in the control of cervical ripening and parturition (Appleby et al., 1994 and Zhang et al., 1998). NF-?B is a nuclear transcription factor. I?B when bound to NF?B, prevents NF?B from entering the nucleus and stimulating transcription. IL-1β, IL-8 and TNF-α cause I?B to separate from NF-?B allowing NF-?B to be transported to and enter the nucleus. Once in the nucleus, NF?B can initiate the transcription of various deoxyribonucleic acids (DNAs) into ribonucleic acid (RNA). NF-?B stimulates an increase in NOS and COX expression NO and PG production. The increase in NO is thought to lead to a direct interaction with the COX enzyme. The COX enzyme at its activation site contains an iron group. NO is known to associate with various metals such as iron. NO, when it reaches a high enough concentration in the system, is thought to associate with the activation site of the COX enzyme. This causes an increase in PG production. This is then thought to lead

to a further increase in NOS and COX expression and NO and PG production. This leads to an increase in vascular permeability, matrix metalloproteinase activation and production, GAG and collagen remodeling and apoptosis. This leads to the degradation of the extracellular matrix, the ripening of the cervical, and eventually parturition (Chwalisz

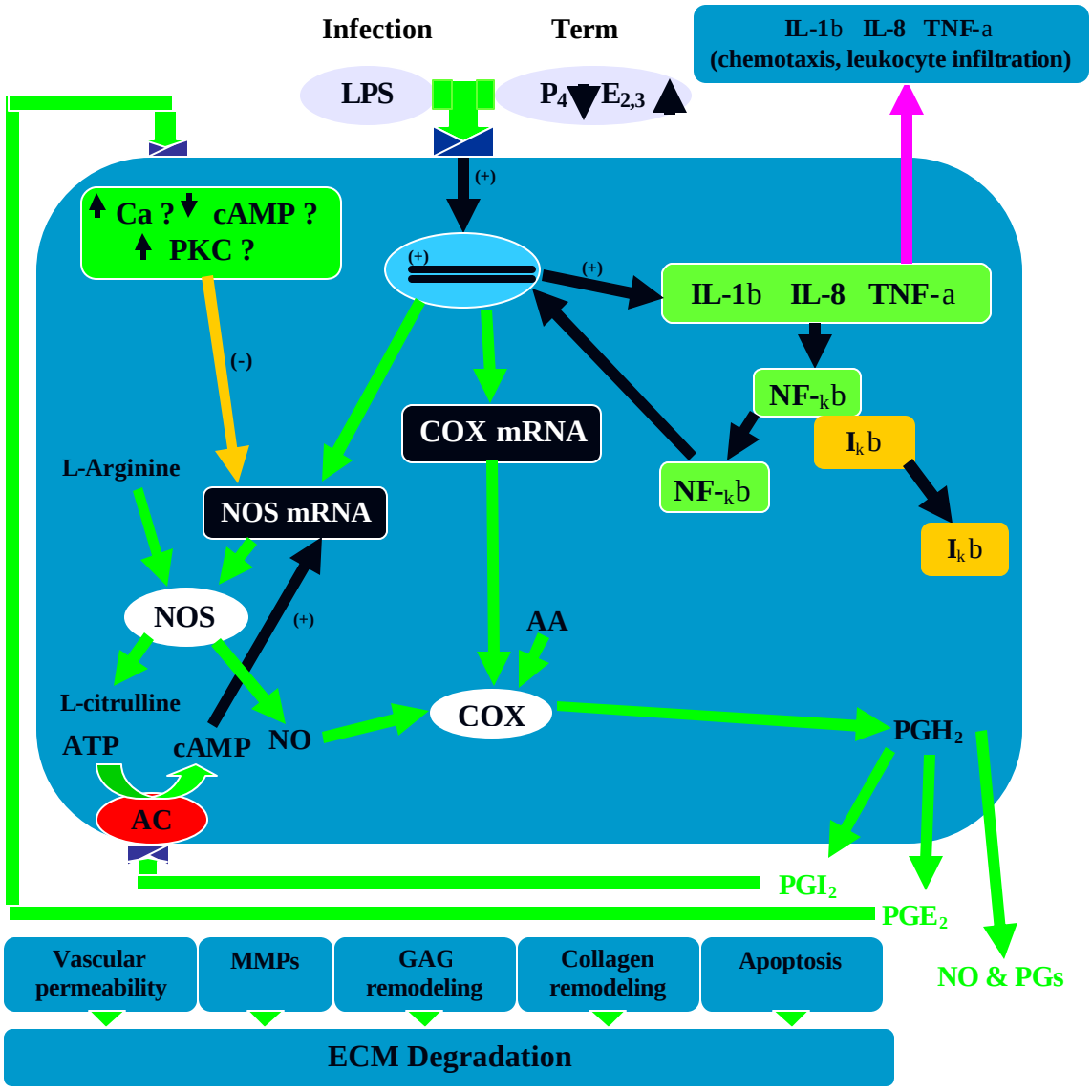

Figure 1.4: A pathway for cervical ripening and a potential relationship between NOS and COX expression (Tetsuka et al., 1994 and Chwalisz and Garfield, 1998)

and Garfield, 1998a, b). Initially while PG levels are still low in the system and depending on what prostanoid receptors are present in the system, PGs are thought to associate with a particular prostanoid receptor stimulating an increase in adenylate cyclase activity and cAMP. The increase in cAMP is thought to lead to a further increase in NOS and COX expression and NO and PG production. PG levels continue to increase in the system. When PG levels reach a high enough concentration, it is then thought to associate with a different prostanoid receptor stimulating an increase in Ca^{+2} and PKC and a decrease in cAMP. This leads to a decrease in NOS expression and NO production. Once NO levels reach a low enough concentration, it is then thought to stop associating with the activation site of the COX enzyme. This leads to a decrease in the PG production which leads to a further decrease in NOS and COX expression and NO and PG production. Once PGs decrease to a low enough concentration and depending on what factors are present in the system, the process will begin again.

Studies on NO, COX expression and PG production

The following studies examine the potential interaction between the NO pathway and the regulation of COX expression and PG production. L-arginine, the substrate for NO production, produces an increase in PGE_2 levels. Both N^G-Monomethyl-L-arginine (L-NMMA), a non specific NOS inhibitor, and aminoguanidine, a specific iNOS inhibitor, decrease PGE_2 levels after LPS stimulation in rat mesangial cells, whereas, D-arginine had no effect. Aminoguanidine abolished NO production and decreased PGE_2 production after IL-1β stimulation (Tetsuka et al., 1994). In RAW264.7 cells, PGE_2 levels were also decreased in the presence of L-NMMA, aminoguanidine and in the absence of L-arginine. In RAW264.7 cells with an inactivated L-arginine NO pathway, NO donor sodium nitroprusside (SNP) and Glyceryl trinitrate (GTN) increased PGE_2 production after arachidonic acid stimulation (Salvemini et al., 1993). Isosorbide 5-mononitrate, another NO donating compound, stimulated a significant increase in PGE_2 in human cervical specimens (Ekerhovd et al., 2002). Hemoglobin, which binds and inactivates NO, blocked the increase in PGE_2 production induced by L-arginine, NO, SNP, and GTN. The activation site of COX contains an iron-heme center that NO may

interact with to stimulate an increase in COX activity (Salvemini et al., 1993). These studies suggest a potential role for NO in the regulation of COX expression and/or activation and PG production.

Studies on PGs, NOS expression and NO production

PGs, depending on type and concentration, stimulate NOS expression and/or activation and NO production. PGs may influence NO activity through secondary messenger systems. Forskolin (FSK), which activates adenylate cyclase, increases nitrite and/or nitrate production, the by-product of NO metabolism, in IL-1β induced rat mesangial cells. PGI_2 activates adenylate cyclase, converting ATP to cAMP, which in turn may stimulate iNOS expression and NO production (Tetsuka et al., 1994). PGE_2 may down regulate iNOS expression through changes in Ca^{+2}, cAMP and/or the PKC pathways. Indomethacin enhances IL-1β induced nitrite production by inhibiting PGE_2 release, and increases IL-1β induced steady state iNOS mRNA levels and NO production. The effect of Indomethacin is reversed by exogenous PGE_2 (Tetsuka et al., 1994). However, other studies show that PGE_2 increases iNOS expression and NO production suggesting that the effect of PGs on NOS expression and NO production may be concentration dependent (Corbett et al., 1993; Salvemini et al., 1993; Franchi et al., 1994; Salvemini et al., 1994 and Murakami et al., 1997). Carba-prostacyclin, a stable analogue of PGI_2, increased iNOS mRNA levels and IL-1β induced NO production. The action of PGI_2 may be mediated through the activation of adenylate cyclase. In mesangial and vascular smooth muscle cells, agents that increase cAMP also enhanced the cytokine-induced iNOS induction (Tetsuka et al., 1994). It has been shown that IL-1β stabilizes COX-2 mRNA by phosphorylation of cytosolic factors that bind to the AUUUA-rich 3' end of COX-2. The iNOS mRNA has an AUUUA 3' motif in the untranslated region which is considered to be an mRNA instability determinate. PGs may have similar effects to that of IL-1β stabilizing iNOS (Srivastava et al., 1994 and Tetsuka et al., 1994). NOS expression and/or activation and NO production may be regulated through PG receptors such as the EP_1, EP_2, EP_3 and the alternatively spliced EP_3 isoforms. These receptors are

believed to utilize different signaling transduction pathways. EP_1 in mesangial and vascular smooth muscle cells is associated with the phospholipid pathway, PKC, and/or angiotensin II (Mene et al., 1987). The EP_2 receptor is associated with intracellular free Ca^{+2} and the Ca^{+2} inositol phospholipid pathway. The EP_3 receptor is associated with the PGE_2 attenuated catecholamine-induced increase in cAMP in cultured rat mesangial cells, and as a result can either up-regulate or down-regulate iNOS expression (Mene et al., 1987). The alternatively spliced and the normal EP_3 receptors differ at the C-terminal where it couples to G proteins thereby activating different secondary messenger systems (Namba et al., 1993). PGE_2 may down regulate IL-1β iNOS expression via the EP_2 receptor. Angiotensin II and/or PKC activity also decreases the iNOS expression induced by IL-1β in vascular smooth muscle (Nakayama et al., 1994). PGE_2 activates the Ca^{+2} inosital phospholipids pathway via the EP_1 receptors, which can inhibit iNOS expression (Kreisberg et al., 1984). These studies suggest a possible interaction between NOS and COX expression. Similar interactions may be involved in the control of cervical ripening and parturition.

QUESTIONS RAISED BY THE REVIEW ABOVE

The review above raises the following questions:

- What are the factors involved and/or associated with the changes taking place to the collagen during the ripening process and parturition?
- How do the nerves and the neural transmitters affect NO and PG production, the ripening process and parturition?
- What steroids and receptors are found in the cervix during the ripening process and parturition? How do these steroids and receptors change as pregnancy progresses to term and the cervix ripens?
- What role, if any, does estrogen and its receptors play in regulating NOS and COX expression, NO and PG production, cervical ripening and parturition?
- What are the effects of progesterone on NOS and COX expression, NO and PG production, cervical ripening and parturition during pregnancy?

- What role does infection and/or inflammation play in the ripening of the cervix and parturition and how does it effect NOS and COX expression, NO and PG production in the control of cervical ripening and parturition?
- What cytokines are present in the cervix and what role do they play during pregnancy in the ripening process and parturition?
- What affect does LPS have on NOS and COX expression, NO and PG production, cervical ripening and parturition? Can LPS be utilized as a model for studying infection and its affect on cervical ripening and parturition?
- What MMPs are present in the cervix and what role do they play during pregnancy in the control of cervical ripening and parturition?
- Do NO and PGs regulate expression and/or activation of MMPs during pregnancy?
- What role does NO play in the control of cervical ripening and parturition?
- What role do PGs play in the control of cervical ripening and parturition?
- Is there an interaction between the NO and PG pathways and, if so, is it direct or indirect? What role does this play in the control of cervical ripening and parturition?
- What are the expression patterns of NOS and COX? Where are they localized during the different stages of the ripening process and parturition?
- What are the expression patterns of NO and PGs during pregnancy?
- How does NO affect COX and NOS expression and PG and NO production during pregnancy?
- How do PGs affect NOS and COX expression and NO and PG production during pregnancy?

STUDY OBJECTIVES

Few studies have investigated the relationship, characterized the expression, or localized iNOS and COX-2 in the cervix during pregnancy and under various pathologic

conditions (Dong et al., 1996; Buhimschi et al., 1996; Ali et al., 1997; Ledingham et al., 1999; Ledingham et al., 2000 and Ekerhovd et al., 2002).

General objective

The general objective of this study is to determine if there is a relationship, either directly or indirectly, between iNOS and COX-2 in the control of cervical ripening and parturition. This will be accomplished through localizing and characterizing the expression patterns of iNOS and COX-2 in the rat cervix during term pregnancy and after various treatments during the different phases of ripening, with the use of reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHS).

Primary objectives

To visualize and measure changes in rat cervical tissue during the different stages of the ripening process

The first primary objective of the study is to determine if we can simultaneously visualize and measure the changes taking place in the organization of the collagen during the ripening processes using the picrosirius red method.

The picrosirius red polarization method

The picrosirius polarization method, which greatly enhances the natural birefringence of the collagen as much as 700% when observed under polarized light was used. Pictures were taken under polarized light using the OLY-200 digital camera and the brightness of the birefringence was analyzed using Photoshop 5.0 allowing us to measure the changes that occur. By simultaneously visualizing and measuring the changes in the cervix during the ripening process we can compare and directly visualize the changes in the collagen during the ripening process and parturition.

To localize and characterize the expression patterns of the inducible isoform of NOS and COX in rat cervical tissue during the different stages of the ripening process and parturition under normal conditions

A second objective of the study was to localize (IHS) and characterize (RT-PCR) the expression patterns of iNOS and COX-2 during the different stages of the ripening process and to define any relationship in the control of cervical ripening and parturition

during pregnancy. This was accomplished by utilizing immunohistochemical staining (IHS) to localize iNOS and COX-2 enzymes throughout gestation. RT-PCR was also utilized to determine the expression patterns of iNOS and COX-2 mRNA throughout gestation to see if there is any correlation between their expression.

To study the effect of progesterone on NOS and COX expression, cervical ripening and parturition in rat cervical tissues during pregnancy

A third objective of the study was to characterize the effect of progesterone, which inhibits cervical ripening, on iNOS and COX-2 expression in rat cervical tissues during pregnancy. These studies should show some relationship between progesterone, NOS and COX expression, cervical ripening and parturition.

Characterizing iNOS and COX-2 mRNA expression after the induction of preterm labor with the antiprogestin Onapristone (ZK98299) in rat cervical tissues

This was accomplished by characterizing and correlating the expression patterns of iNOS and COX-2 mRNA in rat cervical tissues utilizing RT-PCR after the induction of preterm labor with antiprogestin Onapristone. During normal pregnancy late in gestation, around day 18 in rats, progesterone withdrawal occurs. Progesterone withdrawal is thought to occur under both term and preterm labor conditions. Onapristone creates a model of progesterone withdrawal by blocking the progesterone receptors. A correlation between iNOS and COX-2 expression and the induction of early labor would suggest that progesterone plays a role in regulating NOS and COX expression in the control of cervical ripening and parturition.

Characterizing iNOS and COX-2 expression after the prolongation of pregnancy with progesterone in rat cervical tissue

This was accomplished by characterizing the expression patterns of iNOS and COX-2 mRNA utilizing RT-PCR in rat cervical tissues after the prolongation of pregnancy with progesterone. If iNOS and COX-2 are expressed at similar times and in similar amounts in association with the prolongation of pregnancy, this would suggest a possible relationship between iNOS and COX-2 expression.

Examining the expression patterns of iNOS and COX-2 mRNA in rat cervical tissues during both term and preterm parturition

This was accomplished by comparing iNOS and COX-2 mRNA expression in rat cervical tissues during both term and preterm labor utilizing RT-PCR. Depending upon the expression patterns of iNOS and COX-2 during term and preterm labor, this may suggest a relationship between iNOS and COX-2 in the control of parturition, possibly through progesterone.

To characterize NOS and COX expression after modeling infection and/or inflammation with LPS treatment in rat cervical tissues during the different stages of the ripening process and parturition

A fourth objective of the study was to characterize the expression patterns of iNOS and COX-2 after mimicking infection and/or inflammation with LPS in rat cervical tissues during the different stages of the ripening process and parturition. LPS is utilized as a model for infection, inflammation and preterm labor under infectous conditions allowing us to examine their effects. If iNOS and COX-2 are expressed at similar times and in similar amounts, this would suggest a possible relationship and/or communication between iNOS and COX-2 expression in the control of cervical ripening and parturition.

To study the effects of NO on COX and NOS expression in rat cervical tissue during pregnancy

A fifth objective of the study was to examine the relationship between NO and COX and NOS expression following the manipulation of NO levels in the control of cervical ripening and parturition. If a decrease (or an increase in NO) produces a change in COX and NOS expression this would suggest a relationship.

Characterizing the effects of L-NAME on COX-2 and iNOS mRNA expression in rat cervical tissues in the control of cervical ripening and parturition.

This was accomplished by characterizing the expression patterns of COX-2 and iNOS mRNA utilizing RT-PCR in rat cervical tissues after treating with L-NAME. L-NAME is a non-specific NOS inhibitor that prevents L-arginine, the substrate for NO, from being converted into NO creating a model in which NO is down regulated and/or under expressed. These studies may suggest that NO plays a role in regulating COX-2 and iNOS mRNA expression in the control of cervical ripening and parturition.

Characterizing the effects of sodium nitroprusside (SNP) on COX-2 and iNOS mRNA expression in rat cervical tissues in the control of cervical ripening and parturition

This was accomplished by characterizing the expression patterns of COX-2 and iNOS mRNA utilizing RT-PCR in rat cervical tissues after treating with SNP. SNP is an NO donating compound and is utilized as a model for the up regulation and/or over expression of NO. These studies may suggest that NO plays a role in regulating COX-2 and iNOS mRNA expression in the control of cervical ripening and parturition.

CHAPTER 2: VISUALIZING AND MEASURING CHANGES IN RAT CERVICAL CONNECTIVE TISSUE DURING THE DIFFERENT STAGES OF THE RIPENING PROCESS AND PARTURITION

INTRODUCTION

Changes occurring in the cervix during the ripening process (softening, effacement, and dilation) initially were thought to be passive; however, it is now known that the ripening process is an active process with the consistency of the cervix progressively changing (Danforth et al., 1974; Ito et al., 1979; Forman et al., 1982a, b; Conrad and Ueland, 1983; Uldbjerg et al., 1983b, c, e; Leppert, 1992 and Leppert and Yu, 1994). Changes in the physical characteristics of the cervix during pregnancy have been expressed as a change in the distensibility with a length tension curve. A low distensibility indicates that the cervix is easily distended or stretched by a given force. Other measurements that can be obtained from the cervix include tensile strength, breaking strength, and yield point. In our lab cervical ripening has been measured utilizing the cervimeter and the collascope confirming that the cervix gradually ripens as pregnancy progresses to term (Shi et al., 1999).

When studying the cervix, it is important to consider from which part the samples are obtained, due to the lack of uniformity within the cervix (Conrad et al., 1980 and Conrad and Ueland., 1983). The collagen fibers are found mainly interspersed in the smooth muscle layers of the cervix in three distinct regions with two small longitudinal layers, one being found in the periphery of the cervix and the other adjacent to the cervical canal (Leppert and Yu., 1994 and Yu et al., 1995). The third layer is a much larger circular layer located in the middle region of the cervix. It is the most active of the three layers with the greatest amount of change occurring during the ripening process (Yu et al., 1995). Our studies were conducted in the middle layer.

Collagen is normally found as a densely packed well organized structure with a characteristic birefringence. It is rich in basic amino acids which react strongly with

acidic dyes. Picrosirius red is an acid dye that is utilized to enhance the normal birefringence of collagen suggesting that changes in the collagen organization can be observed with the picrosirius red dye during the ripening process and potentially quantified by measuring the brightness of the birefringence (Sweat et al., 1964 and Wolman and Kasten, 1986).

Objective

The main objective was to visualize and quantify the changes in rat cervical collagen during the ripening process and parturition. Specific objective was to characterize the changes that occur to the collagen visually and quantitatively using the picrosirius polarization method in rat cervical tissue during the different stages of the ripening process and parturition.

MATERIALS AND METHODS

Animal treatment

Non-pregnant and timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were housed separately and allowed free access to food and water. Animals were maintained on a constant 12 hours light and dark cycle. Animals were killed by CO_2 inhalation according to the protocols approved by the Animal Care and Use Committee at the University of Texas Medical Branch.

Tissue preparation

Cervices were obtained on days 14 (mid-gestation), 18 (late-gestation) and 22 (term) of pregnancy and days 1 and 3 postpartum (PP). Non-pregnant (NP) rat cervices obtained at estrus were used as control. The tissue was fixed in 4% paraformaldehyde (in 0.1M phosphate buffer saline with $CaCl_2$) for 30 min to an hour, cleaned, cut into longitudinal sections, and stored overnight in 0.1M phosphate buffer saline with $CaCl_2$. The tissue was dehydrated and embedded in paraffin, sectioned at 5-6 µm, and placed on Superfrost/Plus microscope slides (Fisher Scientific, Fair Lawn, New Jersey). The charged slide surface retains the tissue sections. Slides were dried at 60°C for 30 to 45 minutes and stored at room temperature.

Visualizing and measuring the changes in rat cervical collagen utilizing the picrosirius polarization method

For picrosirius staining, sections were deparaffinized in xylene (EM Science, Gibbstown, New Jersey), rehydrated in a series of ethanol solutions (Fisher Scientific, Fair Lawn, New Jersey) and washed in running water for 10 minutes. Thorough washing of the sections prior to staining is essential as it allows the large Sirius red F3BA molecule to penetrate the connective tissue fibers.

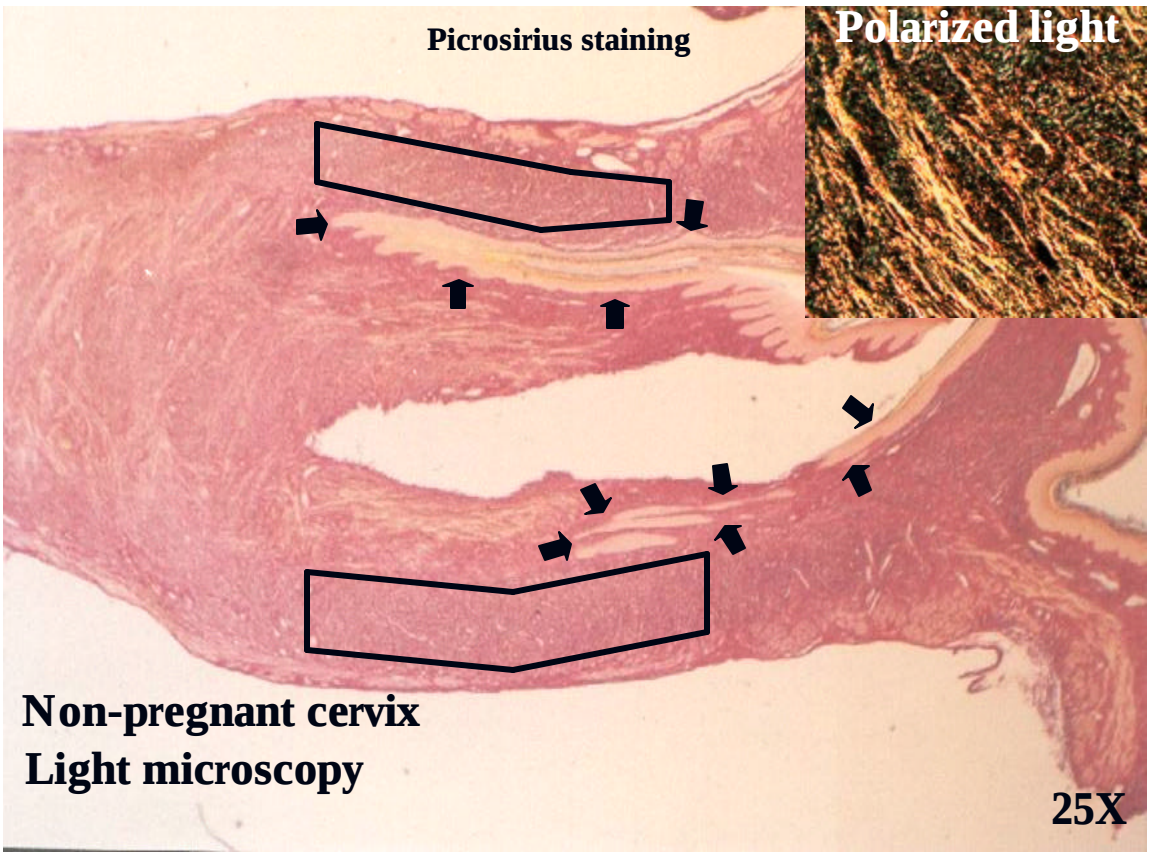

Figure 2.1: Picrosirius red staining under light microscopy (25X) and polarized light (100X). A digital image of the collagen stained with the picrosirius red. It gives a general representation of the location of where the samples were obtained from all the cervical tissues in this study. The figure is of the estrous cervix. The arrows indicate where the two cervical canals are located. The black boxes indicate the approximate region within the tissue from which the samples were obtained.

Sections are stained with picrosirius red F3BA solution (Direct Red 80, Aldrich, Milwaukee, WI) (0.1% Sirius (direct) red F3BA in saturated aqueous picric acid) for 30 minutes. The dye solution must stand for 24 hours before being used. Positive connective tissue staining, except for elastic connective tissues, is indicated with a deep red colorization. All other tissue structures stain bright yellow (Figure 2.1) (Sweat et al., 1964). Tissues were dehydrated, cleared, and mounted using Cytoseal 60.

Sections were viewed with an Olympus U-STP polarizing light microscope (Olympus America, Melville, NY; Figure 2.1; Sweat et al., 1964 and Junqueira et al., 1979). An OLY-200 digital camera (Olympus America, Melville, NY) was used with Adobe Premier 5.1 software (Adobe Systems Inc, San Jose, Ca) to obtain ten (100X) low-power-field images from the circular collagen of each cervix (Figure 2.1). The shutter speed (1/250 second) and light source were fixed so as to compare brightness of the collagen birefringence between groups. As the cross-links break down, the long strings of collagen are lost, reducing the ability of the collagen to reflect the polarized light. Images were analyzed for mean luminosity using the Adobe Photoshop 5.0 software (Image: histogram; Abode Systems Inc, San Jose, Ca).

Statistical analysis

Results are expressed as mean ± SEM. Statistical comparison between the groups was performed using a One-way ANOVA followed by the Student-Newman-Keuls test (significance: $P<0.05$; Sigma Stat – Jandel Scientific Software, San Raffael, CA, USA).

RESULTS

Visualizing and measuring the changes in the collagen (figure 2.2 and figure 2.3)

The changes in the organization of the collagen are visualized, based on changes in the birefringence of the collagen (figure 2.2). Luminosity is utilized to measure changes in the collagen based on the brightness of the birefringence (figure 2.3). There is a progressive decrease in the organization reflected by the loss of birefringent intensity of the collagen from the non-pregnant state (figure 2.2a), through pregnancy (figure 2.2b-d), which then appears to increase in the post-partum cervix (figure 2.2e and f). In samples

from the estrus (figure 2.2a) and day 14 (figure 2.2b) cervix the collagen appears to be dense and highly organized with a high birefringence (figure 2.3). On day 18 of gestation (figure 2.2c), term (figure 2.2d) and postpartum day 1 (figure 2.2e) cervix, the collagen appears to be dispersed and disorganized with a significantly low birefringence (figure 2.3). The birefringence (figure 2.3) and the organization of the collagen within the cervix appeared to return to estrus levels (figure 2.2a) in the postpartum day 3 cervix (figure 2.2f).

DISCUSSION

These studies characterize visually and quantitatively the changes taking place in the collagen of rat cervical tissue during the different stages of the ripening process and parturition. The data suggest that, as pregnancy progresses to term, there is a steady

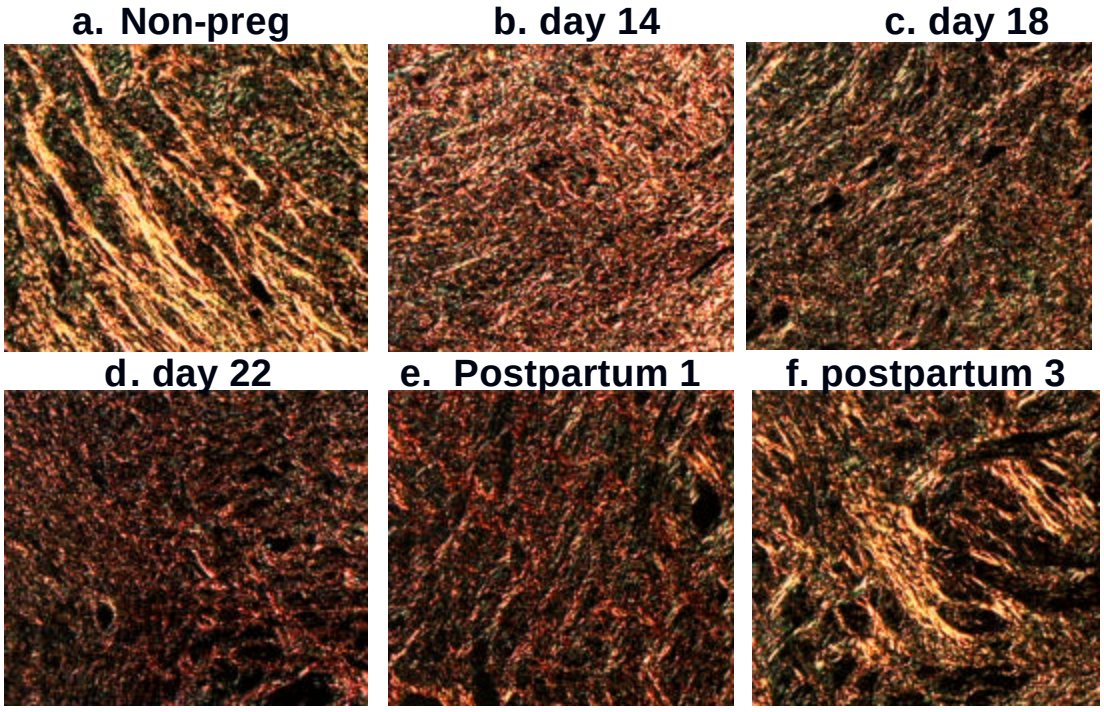

Figure 2.2: Changes observed were based on the organization and birefringence of the picrosirius red stained collagen within the cervix utilizing digital images (100X).

decrease in the birefringence and the organization of the collagen. Quantitatively, this is associated with a decrease in the luminosity. This indicates that as pregnancy progresses to term there is a progressive decrease in the cross-linked collagen and the long collagen fiber strands.

Our studies indicate that under polarized light, the picrosirius red F3BA dye can be utilized to visualize and measure changes in the collagen during the ripening process and parturition. These changes can be correlated with various treatments and/or changes in the expression of various enzymes that occur during pregnancy. In our studies there was a decrease in the organization and birefringence of the cervix as pregnancy progressed to term. This is comparable to previous studies showing a decrease in the

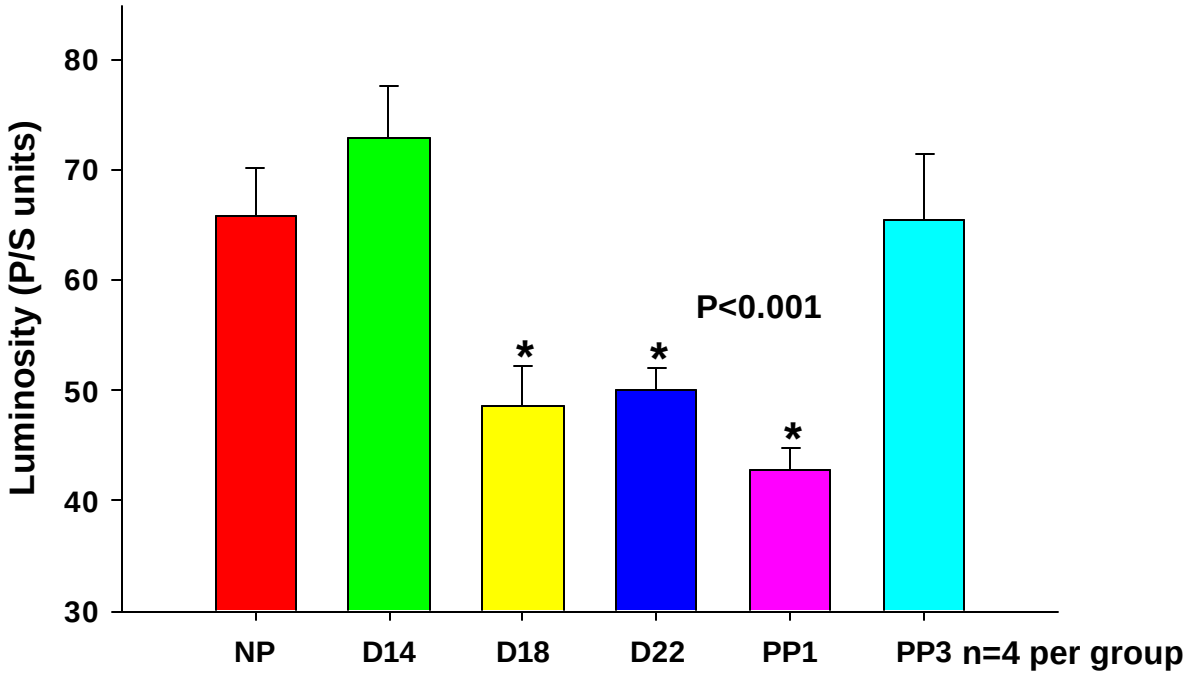

Figure 2.3: Changes in the cervical collagen during the ripening process measured by the picrosirius polarization method. The changes in the collagen are measured based on the luminosity of collagen as pregnancy progressed to term and the cervix ripens. * is an indication of significance. Non-pregnant estrus (NP), day 14 (D14), day 18 (D18), day 22 (D22), postpartum day 1(PP1), postpartum day 3 (PP3). 4 animals per group (n=4). P<0.001 is significant.

cervical resistance and light induced fluorescence as pregnancy progresses to term and the cervix ripens (Shi et al 1999). Tissue taken from the fibrous core of the human cervix demonstrated a decrease in the distensibility during pregnancy compared to the non-pregnant cervix (Conrad and Ueland 1983). This suggests that as pregnancy progresses to term the cervix becomes soft and pliable indicating the importance of the collagen and changes to the collagen in the ripening of the cervix and parturition (Danforth 1980; Conrad and Ueland 1983 and Shi et al 1999).

Our studies agree with others that conclude that the ripening of the cervix is a continual process that occurs throughout gestation preparing the cervix for delivery and a successful end to the pregnancy (Conrad and Ueland, 1983 and Shi et al., 1999). The human cervix is clinically soft several weeks prior to parturition but remains closed with a high tensile strength. The high tensile strength of the soft cervix may be due to the length and/or orientation of the collagen fibers (Yu et al., 1995). Our studies show that in the rigid cervix (estrus, day 14, postpartum 3) the collagen is oriented as bundles of long fibers suggesting a high tensile strength (Vidal et al., 1982; Leppert and Yu, 1994; Yu et al., 1995 and Shi et al., 1999). This is lost late in gestation when the cervix is already soft (day 18, 22, and postpartum 1 cervix). The long strands of collagen are gone, being replaced by aggregates of much shorter and thinner fibers and a birefringence that has decreased. With a change in the orientation of the collagen, the cervix is now soft and effacement and dilation can occur (Vidal et al., 1982; Leppert and Yu, 1994 and Yu et al., 1995). This suggests that the orientation and the length of the collagen fibers are important in maintaining the rigidity of the cervix and that it plays an important role in the ripening process during pregnancy.

Structural alteration of the collagen fibers in the pregnant cervix may be due to the expansion and/or swelling of the cervix caused possibly by an increase in the small molecular weight proteoglycans and water (Yu et al., 1995). Fluid mobility, along with softening, is important for the effacement and dilation of the cervix. An increase in the hydrostatic pressure may cause a further break down of the collagen tissue. The greater

the softening and/or break down of the collagen tissue, the easier it is for the cervix to efface and dilate (Conrad and Ueland, 1983).

Collagen and other extracellular matrix components may be digested in part by the enzymes secreted by the autophagosomes or lysosomes in association with smooth muscle and fibroblast cells (Yu et al., 1995). There are studies suggesting that some of the smooth muscle cells and fibroblast cells are in the process of dying and the number increases as pregnancy progresses to term, indicating that apoptosis may contribute to the softening process (Ito et al 1988; Rajabi et al 1988; Leppert and Yu 1994 and Yu et al 1995).

Inflammation is thought to play a role in the ripening process through the action of collagen degrading enzymes (Kitamura et al., 1980a; Rajabi et al., 1988 and Raynes et al., 1988). Women with pelvic infections are frequently experiencing preterm labor and cervical dilation (Romero et al., 1987 and Romero et al., 1991a, b). Softened or ripe cervical tissues are morphologically similar to those with inflammation of the cervix or cervicitis (Epperson et al., 1951). Mast cells, macrophages, neutrophils and leucocytes, which are associated with the inflammatory process, infiltrate into the cervix releasing collagenases and proteases that either directly or during phagocytosis degrade the collagen fibers (Liggins, 1981 and Hasty et al., 1990).

CONCLUSIONS

The picrosirius polarization staining method can be utilized to visualize and measure changes in the collagen during the ripening process and parturition. It may be used to correlate changes in the enzymes with structural changes that occur in the cervix. The organization and the birefringence of the collagen decrease as pregnancy progresses to term and the cervix ripens. This suggests a decrease in the cross-linked collagen and the long collagen fibers, and that the orientation of the collagen changes, becoming dispersed and disorganized as the cervix softens. Effacement and dilation will then occur as pregnancy progresses to term during the ripening process.

CHAPTER 3: LOCALIZATION AND EXPRESSION OF THE INDUCIBLE ISOFORMS OF NITRIC OXIDE SYNTHASE (INOS) AND CYCLOOXYGENASE-2 (COX-2) IN RAT CERVICAL TISSUES DURING THE RIPENING PROCESS AND PARTURITION

INTRODUCTION

Cervical ripening is a prerequisite for the normal progression of labor and requires the coordinated timing of biochemical and structural changes for a successful end to the pregnancy (Stys et al., 1978; Uldbjerg et al., 1983b, e; Calder and Greer, 1992; Buhimschi et al., 1996; Dong et al., 1996; Ali et al., 1997 and Shi et al., 1999). The mechanisms and mediators that control cervical ripening are still largely unknown even though nitric oxide (NO) and prostaglandins (PGs) have been shown to be involved in the ripening cascade (Szalay et al., 1981; Stys et al., 1981; Ekman et al., 1983a, b, c, e; Calder and Greer, 1992; Calder, 1994; Dong et al., 1996; Buhimschi et al., 1996; Ali et al., 1997; Chwalisz et al., 1997; Thomson et al., 1997 and Ledingham et al., 2000).

Treatment with NO donors such as isosorbide-mononitrate and glyceryl-trinitrate in humans stimulate the ripening process (Thomson et al., 1997; Thomson et al., 1998 and Norman et al., 1998). Inducible nitric oxide synthase (iNOS) is present in the cervix of many different species during pregnancy and increases during labor, suggesting a role for NO in the ripening process and parturition (Nathan, 1992; Gilbert and Herschman, 1993a, b; Kitajima et al., 1994; Vane et al., 1994; Buhimschi et al., 1996; Ali et al., 1997 and Ledingham et al., 2000). Local applications of NO in animals and humans produce characteristics associated with the ripening process (Chwalisz et al., 1997; Thomson et al., 1997; Thomson et al., 1998 and Shi et al., 2000b). N-nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor, prolongs pregnancy and decreases cervical extensibility (Buhimschi et al., 1996).

PGs are presently utilized to interrupt pregnancy in humans in the second trimester and also stimulate cervical ripening at term (Szalay et al., 1981; Theobald et al.,

1982; Ekman et al., 1983a, b, d, e; Calder and Greer, 1992 and Calder, 1994). Cyclooxygenase-2 (COX-2) is found in the cervix and sharply increases during labor or just prior to labor, suggesting a role for PGs in the ripening process and parturition (Calder et al., 1977; Szalay et al., 1981; Ekman et al 1983a, b, c, e; Calder and Greer, 1992; Calder, 1994; Dong et al., 1996 and Chwalisz et al., 1997). Local applications of PGs in animals and humans produce characteristics associated with the ripening process (Ekman et al., 1983a and Chwalisz et al., 1997). PG inhibitors, such as Indomethacin a non-specific COX inhibitor, prevent spontaneous abortions and/or preterm labor (Calder, 1994). PGs may play an important role in the control of cervical ripening and parturition.

Main Objective

The main objective was to localize and characterize the expression patterns of the inducible isoform of NOS and COX in rat cervical tissues during the different stages of the ripening process and parturition under normal term pregnant conditions.

Specific Objectives

The first objective was to localize the iNOS and COX-2 enzymes in rat cervical tissues utilizing immunohistochemical staining (IHS) at specific times during pregnancy and parturition. The studies may suggest a spatiotemporal relationship between the iNOS and COX-2 enzymes localization during cervical ripening and parturition.

The second objective was to characterize iNOS and COX-2 mRNA expression in rat cervical tissues during the different stages of normal pregnancy utilizing RT-PCR to quantitatively suggest an interaction between iNOS and COX-2 pathways in the control of cervical ripening and parturition.

MATERIALS AND METHODS

Animal treatment

Animal treatments are described in chapter 2.

Tissue preparation

Cervices were obtained on days 14, 18, 22 non-labor (NL) and labor (L – 1 to 3 pups expelled) of pregnancy and days 1 and 3 postpartum (PP). Non-pregnant (NP) rat cervices obtained at estrus were used as control.

Tissue embedding for immunohistochemistry (IHS)

The tissue was fixed in 4% paraformaldehyde in 0.1M phosphate buffer with $CaCl_2$ for 30 min to an hour, trimmed, cut into longitudinal sections, and stored overnight in 0.1M phosphate buffer saline with $CaCl_2$ at 4°C. The tissue was then dehydrated, embedded in paraffin, sectioned at 5-6 µm, and placed on Superfrost/Plus microscope slides (Fisher Scientific, Fair Lawn, New Jersey). Slides were dried at 60°C for 30 to 45 minutes and stored at room temperature until they were stained.

Preparation of RNA

Other cervical tissues were dissected, frozen in liquid nitrogen and stored at –70°C. Total RNA was extracted from the frozen tissue using the acid-guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). All samples were treated with Dnase I (Stratagene, La Jolla, California) to remove any contaminating genomic DNA. The amount of RNA was calculated using the A_{260} value.

Localization of iNOS and COX-2 in rat cervical tissue utilizing immunohistochemical staining

Standard IHS procedures were conducted. Sections were deparaffinized in xylene (EM Science, Gibbstown, New Jersey), and ethyl alcohol (reagent grade, Pharmco, Brookfield, Connecticut), and rehydrated in a graded series of ethanol solutions (95%, 70%, 50%) (Fisher Scientific, Fair Lawn, New Jersey). Sections were treated with 0.3% hydrogen peroxide to quench endogenous peroxidase activity and permeabilized with 0.1% Triton X-100 (Fisher Scientific, Fair Lawn, New Jersey) in phosphate buffered saline (PBS; pH 7.5). Nonspecific binding was blocked by incubating sections in PBS buffer (0.1 M) containing 0.025% Bovine Serum Albumin Type C (Sigma, St. Louis, Missouri) and 5 % normal horse serum (GIBCO Laboratories, Chagrin Falls, Ohio).

iNOS

Sections used to detect iNOS expression were incubated overnight at 4°C in rabbit polyclonal anti-iNOS antibody (Transduction Laboratories, Lexington, KY) at a 1:50 dilution. Sections were rinsed in PBS then incubated with a biotinylated secondary antibody (Zymed Laboratories, South San Francisco, CA). Aminoethyl carbazole (AEC - Zymed Laboratories, South San Francisco, CA) served as the chromagen to localize the iNOS enzyme.

Rat liver was used for the iNOS IHS positive control (Figure 3.1a). A red to

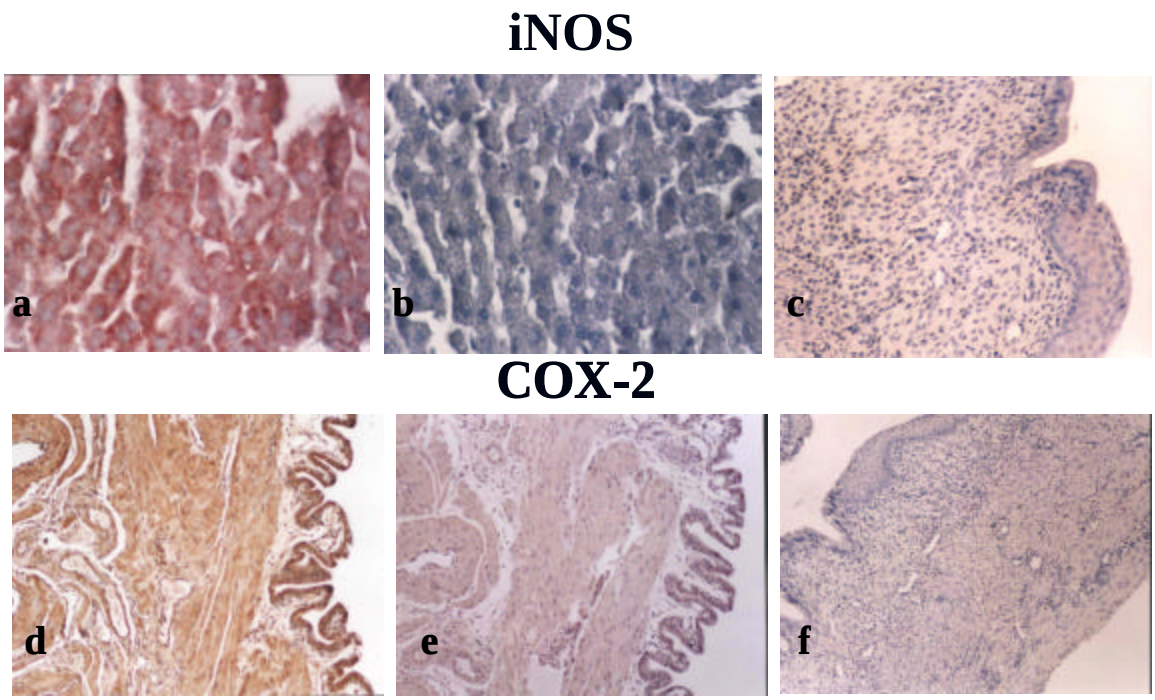

Figure 3.1: Control tissues for the localization of the iNOS (a-c) and COX-2 (d-f) enzymes utilizing immunohistochemical staining. iNOS: a.) The positive control for iNOS is the rat liver. 525X. b.) Negative control includes utilization of a non-reactive IgG on the rat liver (525X) and c.) staining of a longitudinal section of the estrus cervix omitting the primary antibody. 265X. COX-2: d.) The positive control for COX-2 is the day 22 laboring rat uterus. 525X. e.) Negative control includes utilization of a non-reactive IgG on the day 22 laboring rat uterus (525X) and f.) staining of a longitudinal section of the estrus cervix omitting the primary antibody. 265X.

brown reaction product is an indication of positive staining for the iNOS enzyme. Normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was substituted for the primary antibody (Figure 3.1b) or the primary antibody was omitted in the negative control sections (Figure 3.1c).

COX-2

Sections used to localize the COX-2 enzyme expression were incubated for 1 hour at 37^{o}C with a goat polyclonal anti-COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), at a 1:50 dilution. Sections were rinsed in PBS then incubated with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). Sections were then rinsed in PBS and incubated in an avidin-biotinylated peroxidase complex using the Vectastain Kit. 3,3'-Diaminobenzidine (DAB) served as a chromagen to localize the COX-2 enzyme.

Day 22 laboring rat uterus or kidney was used for COX-2 positive control sections (Figure 3.1d). A brown reaction product is an indication of positive staining for the COX-2 enzyme. Normal goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was substituted for the primary antibody (Figure 3.1e) or the primary antibody was omitted in the negative control sections (Figure 3.1f).

Sections of both iNOS and COX-2 preparations were lightly counterstained with Harris Modified Hematoxylin with acetic acid (Fisher Scientific, Fair Lawn, New Jersey). The excess hematoxylin was removed with an acid-alcohol dip (0.5 ml HCl to 49.5 ml 70 % ETOH) and then rinsed in ammonia water (2-3 drops of NH_4OH to 50 ml of distilled water). Tissues were dehydrated in graded alcohol, placed in xylene, and mounted with either GVA mounting solution (ZYMED Laboratories Inc. South San Francisco, CA) for iNOS or Cytoseal 60 (Fisher Scientific, Fair Lawn, NJ, USA) for COX-2.

Immunohistochemistry was performed for descriptive, but not quantitative purposes. Numerous sections were examined and representative tissues were photographed using a Nikon Microscope (Nikon Microphot – FXA EP1 – FL3, Japan).

Tissues from a minimum of 4 animals were examined at each time point with the distal portion of the cervix evaluated.

Measuring iNOS and COX-2 cervical mRNA levels with real time RT-PCR

Primers and probes (Table 3.1) specific for either iNOS or COX-2 were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) with the rat iNOS (Acc # L12562) and COX-2 (Acc # L20085) as reference sequences. The selected primers and probes served as queries in blast analyses to confirm specificity (reference identification number: iNOS – forward primer 988309628-20826-3898, reverse primer 988310400-814-12100, and probe 1049152419-02113-5929; COX-2 – forward primer 961013803-12918-21846, reverse primers 1049149992-02684-14705, and probe 1049153173-010389-12316).

RT-PCR assays were carried out using reagents provided in a TaqMan PCR Core Kit along with an RNase inhibitor and a Multiscribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). Final primer and probe sequences and concentrations for iNOS and COX-2 are listed in Table 1. The iNOS 18SrRNA concentration was 25nM each for the forward and reverse primers and probe. The COX-2 18SrRNA concentration was 50 nM each for the forward and reverse primers and probe. Each sample contained 100 ng of total RNA in a final volume of 50 µL and was reverse transcribed for 30 minutes at 48°C followed by AmpliTaq Gold activation at 95°C for 10 minutes. Real-time PCR amplification was measured by the ABI Prism 7700 Sequence detection System (Applied Biosystems, Foster City, CA) under the following conditions: denaturation at 95^{o}C for 15 seconds and annealing/extension at 60^{o}C for 1 minute for 40 cycles.

Tissues from 4 to 10 animals were analyzed in each experimental group. Relative RNA levels were calculated using the ΔC_T method described in Taqman Users Bulletin #2 (Applied Biosystems, Foster City, CA). The data was normalized against the internal 18SrRNA control (Applied Biosystems, Foster City, California). Results are expressed as the average % of cervical iNOS or COX-2 mRNA expression relative to the levels at estrus ± SEM.

Table 3.1: Primers and probe concentration and sequence information specific for iNOS and COX-2

Gene	**Gene Bank Accession #**	**Primer/probe concentrations**	Nucleotide position	Nucleotide sequence
INOS	L12562	Forward (50 nM)	1672 to 1692	5’-GTGCTAATGCGGAAGGTCATG-3’
		Reverse (300 nM)	1725 to 1748	5’-GCTTCCGACTTTCCTGTCTCAGTA-3’
		Probe (200 nM)	1697 to 1718	5’-CCCGCGTCAGAGCCACAGTCCT-3’
COX-2	L20085	Forward (900 nM)	1680 to 1701	5’-GTGTCCCTTTGCCTCTTTCAAT-3’
		Reverse (900 nM)	1732 to 1751	5’-GAGGCACTTGCGTTGATGGT-3’
		Probe (200 nM)	1686 to 1730	5’-AAGACCCGCAGCCTACCAAGACAGC-3’

Statistical analysis

Statistical comparisons between groups were performed using a 1-Way ANOVA followed by a Tukey test ($P<0.05$) and Spearmans correlation ($P<0.01$) (Sigma Stat – Jandel Scientific Software, San Raffael, CA, USA).

RESULTS

Localization of cervical iNOS and COX-2 during gestation

iNOS

Labeling for iNOS was evident in the cervical tissues at all time points examined. The iNOS enzyme was most prominent in the cervical smooth muscle (figure 3.2 and

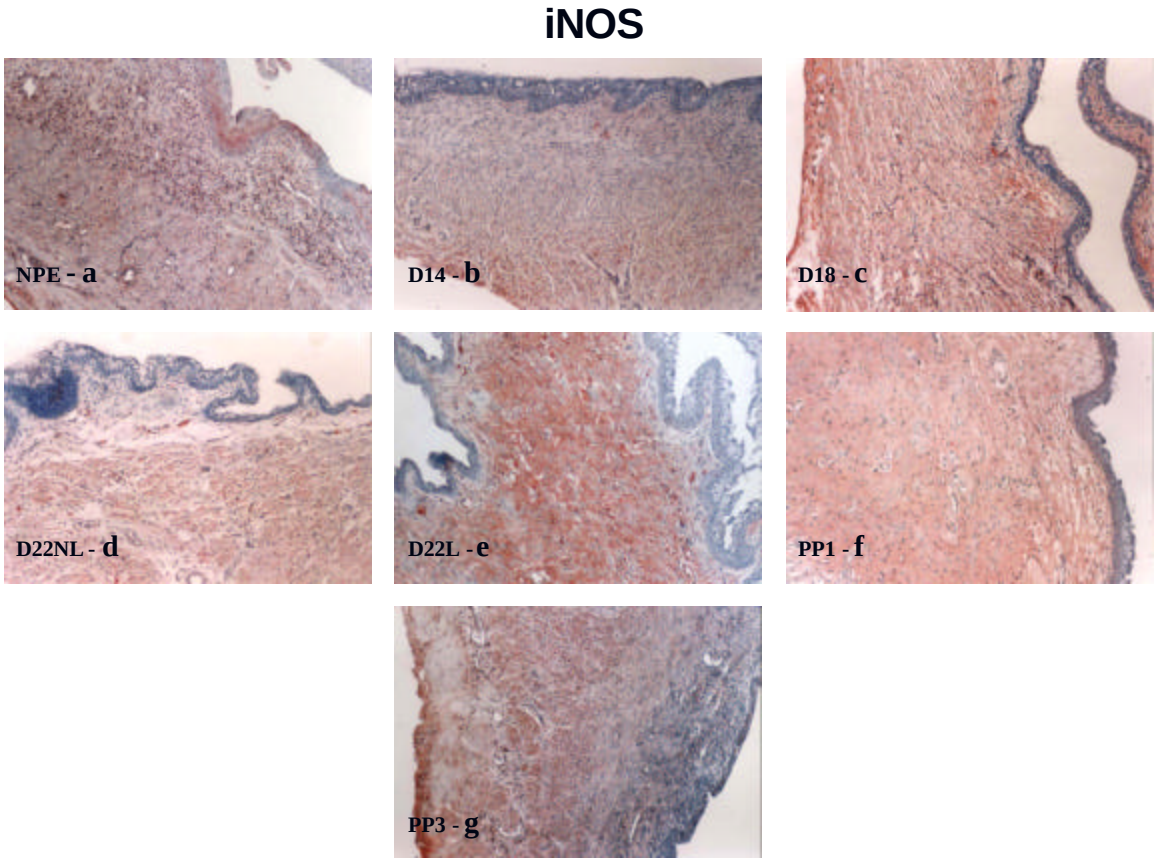

Figure 3.2: Localization sites for the iNOS enzyme in longitudinal sections of the rat cervical tissues during gestation utilizing immunohistochemical staining. a.) estrus, b.) day 14, c.) day 18, d.) day 22 non-labor, e.) day 22 labor, f.) postpartum day 1, and g.) postpartum day 3. 130X.

3.4b). Expression of iNOS was also localized in the vascular smooth muscle (figure 3.2 and 3.4a). There was also labeling of large leukocyte and/or monocyte-like cells (figure 3.4c). Epithelial labeling occurred in the postpartum day 1 cervix (figure 3.2f and 3.4e) and occasionally in the laboring cervix (figure 3.4d). iNOS in the estrous cervix, was present in the cervical smooth muscle, the vascular smooth muscle, the epithelium, and within a band of unknown cells located in the stroma, possibly fibroblasts (Figure 3.2a and 3.4f).

COX-2

Labeling for COX-2 was present in the cervical tissues at all time points

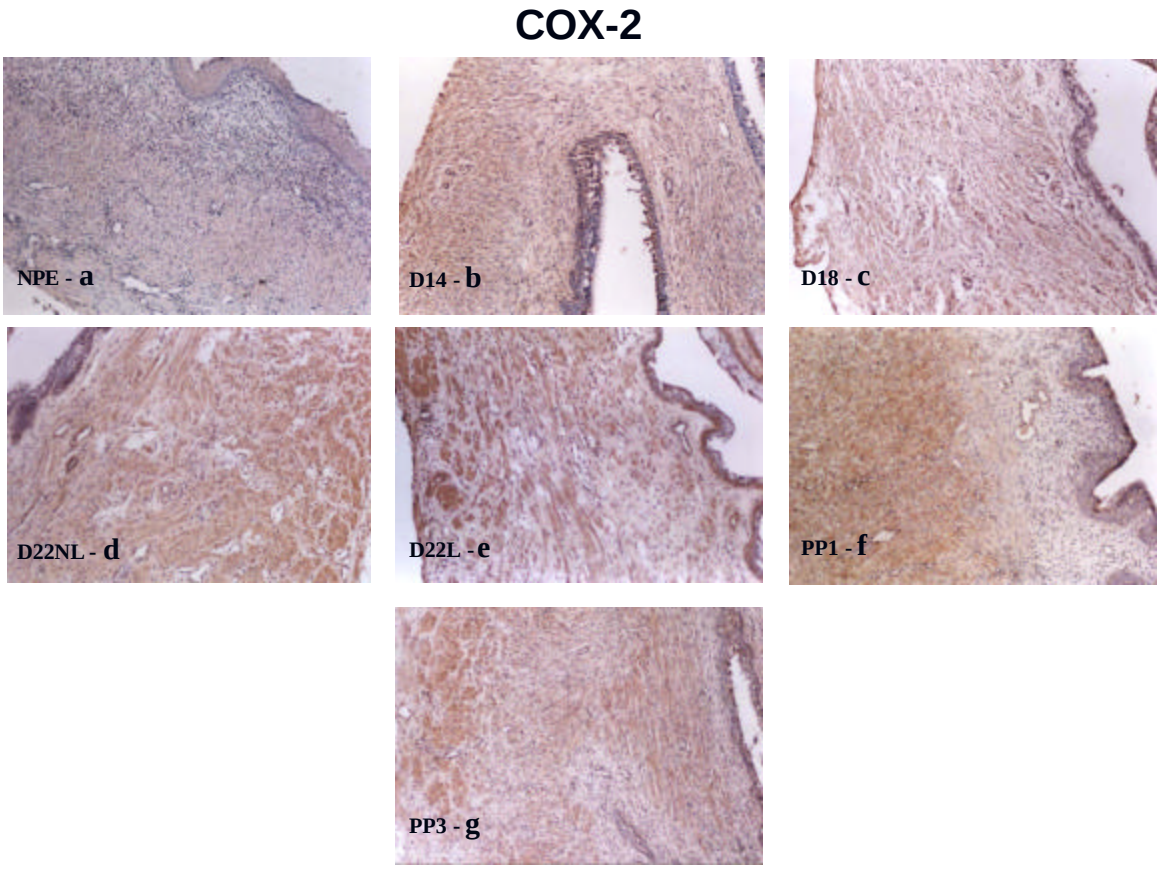

Figure 3.3: Localization sites for the COX-2 enzyme in longitudinal sections of the rat cervical tissues during gestation utilizing immunohistochemical staining. a.) estrus, b.) day 14, c.) day 18, d.) day 22 non-labor, e.) day 22 labor, f.) postpartum day 1, and g.) postpartum day 3. 130X.

examined. The COX-2 enzyme was mainly localized in the cervical smooth muscle (figure 3.3 and 3.4g). Expression of COX-2 was also localized in the vascular smooth muscle (figure 3.3 and 3.4i). Epithelial labeling occurred in the laboring cervix (figure 3.3e and 3.4h). The estrous cervix had label for the COX-2 enzyme in the cervical smooth muscle, the vascular smooth muscle and within the epithelium (Figure 3.3a).

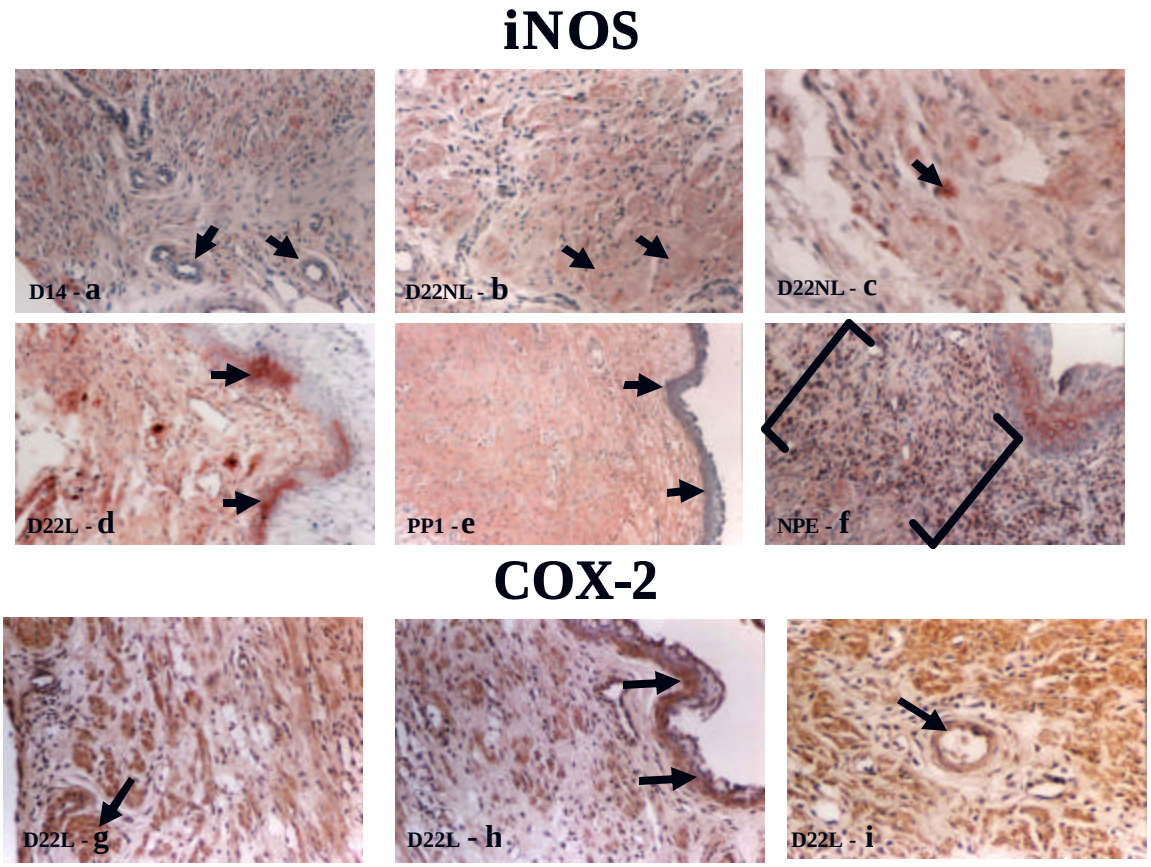

Figure 3.4: Representative localization sites at a higher magnification of specific areas demonstrating iNOS (a-f) and COX-2 (g-i) labeling in longitudinal sections of the rat cervical tissue utilizing immunohistochemical staining. iNOS: a.) day 14, vascular smooth muscle labeling; b.) day 22 non-labor, cervical smooth muscle labeling; c.) day 22 non-labor, labeling of a leukocyte/monocyte like cell; d.) day 22 labor, occasional epithelial labeling; e.) postpartum day 1, epithelial labeling; f.) estrus, a discrete unknown band of stromal cells. COX: g.) day 22 labor, cervical smooth muscle labeling; h.) day 22 labor, epithelial labeling; and i.) day 22 labor, vascular smooth muscle labeling. 265X.

Cervical iNOS and COX-2 mRNA expression during gestation

iNOS

The iNOS mRNA expression decreased as pregnancy progressed to term with the highest levels occurring in the non-pregnant and postpartum cervix and the lowest levels occurring at day 18 (figure 3.5) although the differences were not statistically significant.

COX-2

There was about a 50-fold increase in COX-2 mRNA expression in the day 22 laboring cervix compared to estrus, followed by a more than 19-fold decrease in the PP 3 cervix ($P<0.05$; figure 3.5).

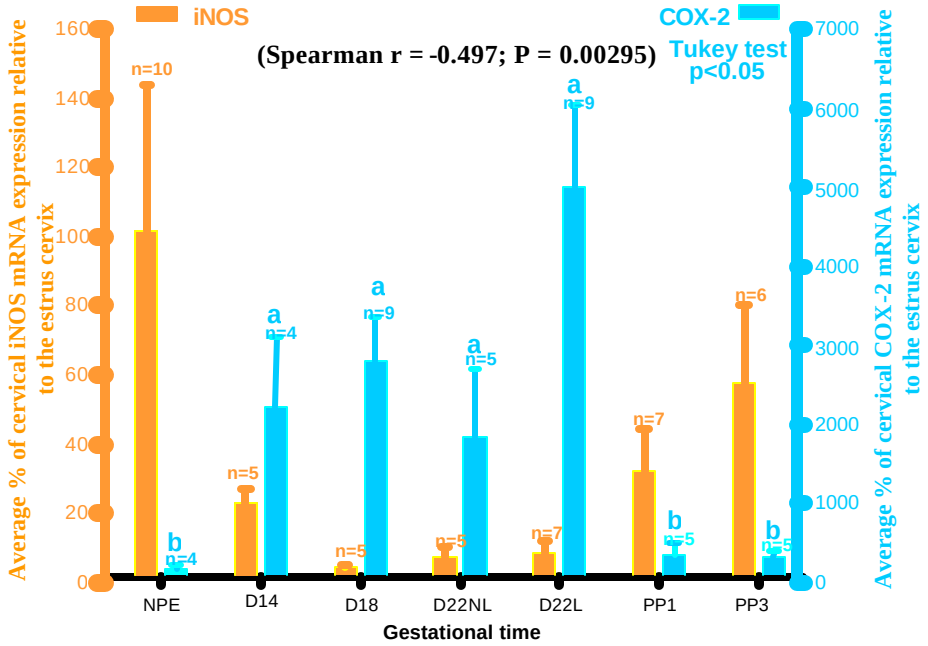

Figure 3.5: The expression patterns of iNOS and COX-2 mRNA in the rat cervix throughout gestation. The data is expressed as the average % of cervical iNOS or COX-2 mRNA relative to the estrus compared with gestational time. RNA levels were detected by real time analysis and calculated as described in the materials and methods section. Different letters are an indication of statistical significance.

iNOS and COX-2

There is a significant inverse relationship between iNOS and COX-2 mRNA expression (Spearman r = -0.497; P = 0.00295) (Figure 3.6).

DISCUSSION

These studies localize and characterize iNOS and COX-2 expression in rat cervical tissues during the different stages of the ripening process and parturition. The data suggests a possible relationship between iNOS and COX-2 expression in the control of cervical changes at the end of pregnancy. The iNOS and COX-2 enzymes were co-localized in the cervical smooth muscle, the vascular smooth muscle, and epithelium at similar times during gestation. The iNOS mRNA levels had a tendency to decrease while COX-2 mRNA levels increased as pregnancy progressed to term demonstrating an inverse relationship.

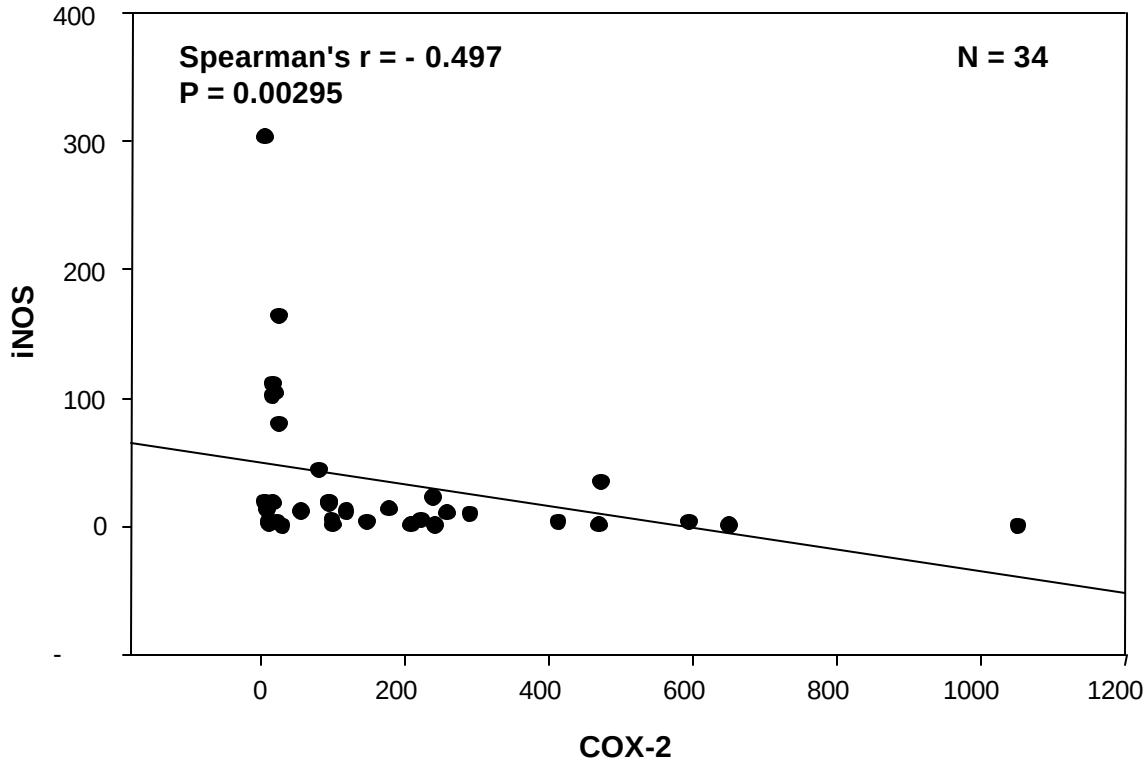

Figure 3.6: A significant inverse correlation was determined between iNOS and COX-2 mRNA expression under normal term pregnant conditions in the rat cervix.

It is thought that there may be a possible interaction between the NO and PG pathways at the protein and/or product level in the control of enzyme activity of iNOS and COX-2 during cervical ripening and parturition (Salvemini et al., 1993; Corbett et al., 1993; Tetsuka et al., 1994; Swaisgood et al., 1997; Ledingham et al., 1999 and Ekerhovd et al., 2002). Our data shows expression of the iNOS and COX-2 enzymes in similar tissues at similar times during gestation suggesting a possible interaction between the pathways. Buhimschi (et al., 1996) and Ledingham (et al., 2000) showed the iNOS enzyme to be present in the cervix during pregnancy. Dong (et al., 1996) showed the COX-2 enzyme present in the cervix during pregnancy. Salvemini (et al., 1993) and Ekerhovd (et al., 2002) showed a potential interaction between iNOS and COX-2 at the protein and product level. Our data supports these studies, indicating a possible interaction between the NO and PG pathways at the protein and/or product level.

At the mRNA level, iNOS had a tendency to decrease while COX-2 increased demonstrating an inverse relationship. Fittkow (et al., 2001) showed iNOS levels to be low in the guinea pig during pregnancy. Puri and Garfield (et al., 1982) showed an increase in the PG levels. Dong (et al., 1996) and Fittkow (et al., 2001) showed an increase in the COX-2 level. Our data confirms this. What is the importance of the inverse relationship between iNOS and COX-2? Why is it important for iNOS to decrease while COX-2 increases during pregnancy? As pregnancy progresses to term, there is thought to be an increase in MMP-8 and MMP-9 which may play a role in the continued break down of the collagen (Winkler et al., 1999). NO is thought to down regulate and/or inhibit MMP-8 and MMP-9 (Upchurch et al., 2001). The decrease in iNOS expression and/or NO production may be required to allow an increase in MMP-8 and MMP-9. The increase in COX-2 expression and/or PG production may be required for parturition and to allow an increase in IL-8. IL-8 is a chemotactic agent that attracts neutrophils which are known to express MMP-8 and MMP-9 (Chwalisz et al., 1994; El Maradny et al., 1994; Winkler et al., 1999; Elliott et al., 2001 and Watari et al., 2003).

The iNOS and COX-2 mRNA expression appears to be increased (iNOS: at estrus and in the postpartum tissues; COX-2: at labor) primarily at times during pregnancy

when epithelial staining occurred, suggesting that an increase in iNOS and COX-2 may be due to increased epithelial expression (Tschugguel et al., 1999).

The decrease in iNOS in my study was not significant which may be due to the variability within the physiological samples. Other studies report that iNOS and/or NO is increased rather than decreased during labor in the cervix (Buhimschi et al., 1996; Ali et al., 1997 and Ledingham et al., 2000). The difference in the data may be explained by the time points examined, since most of these studies examined the later stages of gestation. However, compared to the estrous and postpartum tissues, iNOS expression was low during pregnancy (Fittkow et al., 2001). The differences between our results and previous studies may be due to the differences in the techniques used, since our lab now utilizes a much more sensitive real time RT-PCR system. In addition, previous studies in rat cervical tissues utilized Sprague-Dawley rats from Harlan, while our studies used rats from Charles River. There may be subtle genetic variability in two outbred rat colonies of the same species (Shi et al., 2000c).

CONCLUSIONS

There maybe an interaction between the NO and PG pathways in the control of cervical ripening and parturition. Expression of iNOS and COX-2 in rat cervical tissues occurred throughout gestation and amounts varied with gestational age. The iNOS and COX-2 enzymes are co-localized in similar tissues at similar times during gestation. There was a tendency for iNOS mRNA levels to decrease while COX-2 mRNA levels increased as pregnancy progressed to term indicating an inverse relationship between iNOS and COX-2.

FUTURE DIRECTIONS

Our present study localizes and characterizes the expression patterns of iNOS and COX-2 expression during the ripening process and parturition at the protein (qualitative) and mRNA (quantitative) level. Quantitative data still needs to be collected and correlated for this study at the protein and product levels. In addition, the other isoforms for NOS and COX should also be taken into consideration, both quantitatively and

qualitatively, when studying the relationship and/or interactions between the NO and PG pathways in the control of cervical ripening and parturition under normal conditions. Other factors may be involved in the ripening process and parturition in association with NO and PG production, and should be considered both qualitatively and quantitatively. These include the prostinoid receptors (EP, FP, IP, DP, TP), Ca^{+2}, cAMP, PKC, steroid hormones (estrogen and progesterone), cytokines (IL-1, IL-8, TNF-α) and MMPs (1, 2, 3, 8, 9).

CHAPTER 4: THE EFFECT OF PROGESTERONE ON iNOS AND COX-2 EXPRESSION IN RAT CERVICAL TISSUES DURING THE RIPENING PROCESS AND PARTURITION

INTRODUCTION

Progesterone and progesterone antagonists act through progesterone receptors A and B (PR-A and PR-B), which are structurally similar except that the PR-B isoform contains an N-terminal fragment, which is absent in PR-A. There are two types of progesterone antagonists, types I and II. Type I is a pure progesterone antagonist that is thought to prevent the activation of transcription by preventing PR from binding to the DNA. Type II, depending on the cell type, promoter context and other signaling pathways, may act as an agonist and/or antagonist, for example mifepristone (RU486). Type II agents allow binding of the PR to the DNA. When acting as an antagonist, the type II agent appears to inhibit the activity of the receptor by failing to induce the appropriate conformational change required for enhanced transcription (Edwards et al 1995).

Onapristone, a type I progesterone antagonist, causes a ripening of the cervix, demonstrated in guinea pigs and humans, comparable to that of term gestation and is associated with an increase in inflammatory cells and/or fibroblasts (Elger et al., 1986; Frydman et al., 1988; Hegele-Hartung et al., 1989 and Chwalisz, 1994). A decrease in progesterone in the rat uterus and plasma is associated with an increase in prostaglandins (PGs) and parturition (Puri and Garfield, 1982). In the cervix, progesterone prolonged pregnancy in association with a decrease in iNOS expression (Buhimschi et al., 1996 and Ali et al., 1997). Onapristone stimulated preterm parturition and an increase in iNOS expression and nitric oxide (NO) production in rat cervical tissues (Buhimschi et al., 1996 and Ali et al., 1997). The above studies suggest that progesterone plays an important role in regulating iNOS and COX-2 expression and NO and PG production in the control of cervical ripening and parturition.

Main Objective

The main objective is to study the affect of progesterone on iNOS and COX-2 mRNA expression during cervical ripening and parturition.

Specific Objectives

Specific objective 1 is to characterize iNOS and COX-2 mRNA expression in rat cervical tissue after the induction of preterm labor with the antiprogestin Onapristone (ZK98299). Onapristone simulates and/or creates a progesterone-deficient environment and is utilized as a model for progesterone withdrawal and preterm labor. This allows us to study the effect of a decrease and/or a of lack progesterone on iNOS and COX-2 mRNA expression in regulating cervical ripening and parturition.

Specific objective 2 is to characterize iNOS and COX-2 mRNA expression in rat cervical tissues after the prolongation of pregnancy with progesterone, which simulates and/or creates an environment where progesterone withdrawal does not occur. This allows us to study the effect of elevated levels of progesterone on iNOS and COX-2 mRNA expression in the control of cervical ripening and parturition.

Specific objective 3 is to characterize and compare the changes in the levels of iNOS and COX-2 mRNA expression at term versus preterm parturition, to indirectly study the role of NO and PGs in parturition and their importance under term and preterm laboring conditions.

MATERIALS AND METHODS

Animal treatment

Animal treatments are described in chapter 2.

Tissue preparation

Term labor

Cervical tissues were obtained from SD rats at estrus (E) and at labor on day 22 of gestation.

Preterm labor

Cervices were obtained from timed pregnant SD rats at 6, 12, 24 and 28 hours after ZK98299 (3mg/rat subcutaneously) or vehicle control (200 µl of benzyl benzoate (1): Sesame oil (4) solution) (Sigma, St. Louis, Missouri) treatment on day 17 of gestation (n = 5-6 per group).

Prolongation of pregnancy

Timed-pregnant rats were chronically treated with either progesterone (2.5mg s.c. twice daily) or vehicle starting on day 18 of pregnancy. The animals were sacrificed 1 (control only, reference), 2, 3, 4 or 6 days after the initial treatment (n=6 per group).

Preparation of RNA

Preparation of the RNA was described in chapter 3.

Measuring iNOS and COX-2 cervical mRNA level with real time RT-PCR

Expression of iNOS and COX-2 mRNA levels were determined using semi-quantitative real-time RT-PCR (Taqman, PE Applied Biosystems) as described in chapter 3. The results are expressed as the average % of cervical iNOS or COX-2 mRNA expression relative to the 6 hour control cervix (ZK98299 study); the day 19 control cervix (progesterone study); or the estrus cervix (term), the 28 hour control cervix (preterm) and the cervix at estrus (term versus preterm labor study) $\pm$ SEM.

Statistical analysis

Preterm labor

Statistical comparisons between groups was performed using a 1-Way ANOVA followed by a Tukey test, and Spearmans correlation as appropriate (significance: $P<0.05$).

Prolongation of pregnancy

Data were analyzed using a 2-way ANOVA followed by a Tukey test for multiple comparisons as appropriate (significance: $P<0.05$).

Term versus preterm labor

Quantitative data were analyzed using a t-test or a Mann-Whitney Rank Sum Test when appropriate (Normal: iNOS P=0.022, COX-2 P=0.01; ZK98299: iNOS P=0.005, COX-2 P=0.004; Preterm versus Term: iNOS P=0.004, COX-2 P=0.874).

RESULTS

Cervical iNOS and COX-2 mRNA expression after the induction of preterm labor with the antiprogestin Onapristone (ZK98299)

Onapristone

Treatment with Onapristone stimulated preterm labor, reducing the duration of the pregnancy. The vehicle control rats did not deliver early, while the Onapristone treated

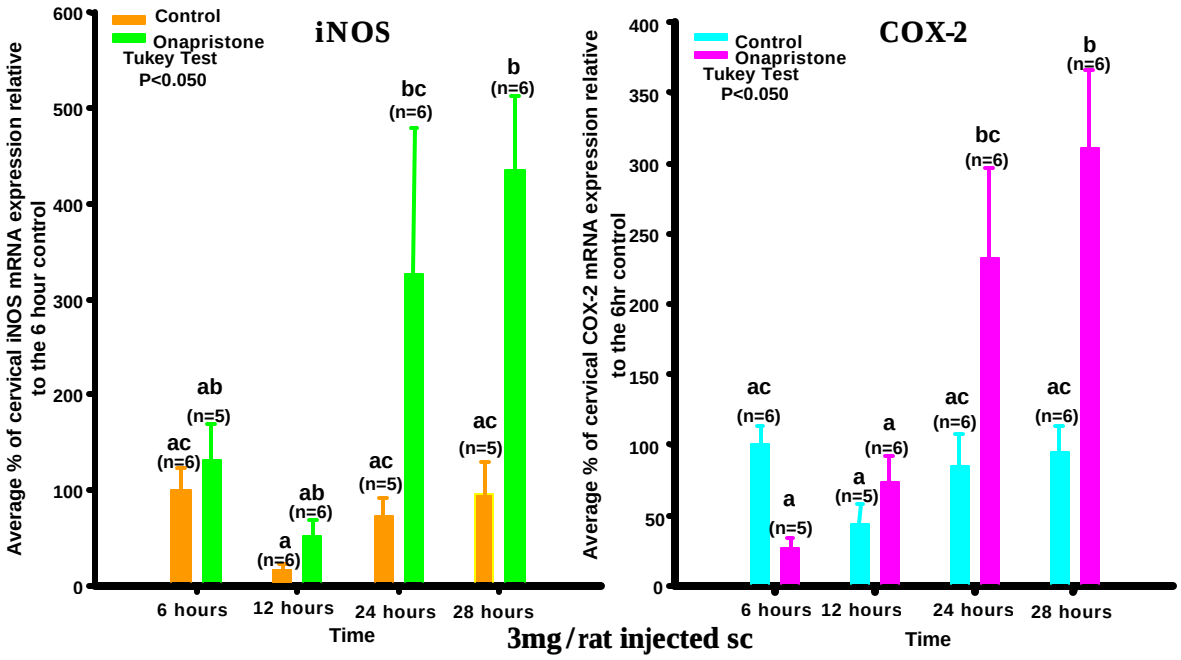

Figure 4.1: iNOS and COX-2 mRNA expression in rat cervical tissues treated on day 17 with ZK98299 (antiprogestin, Onapristone). The data is expressed as the average % of cervical iNOS or COX-2 mRNA relative to the 6 hour control cervix compared with gestational time. RNA levels were detected by real time analysis and calculated as described in the materials and methods section. Different letters are an indication of statistical significance.

rats were in labor and/or delivering preterm after 28 hours on the 18th day of gestation.

iNOS

Expression of the iNOS mRNA gradually increased 6, 12, 24 and 28 hours after Onapristone treatment in the day 17 rat cervix. iNOS mRNA levels peaked 28 hours after treatment with Onapristone (Figure 4.1). This peak was significantly different from all the control groups ($P<0.05$).

COX-2

Expression of the COX-2 mRNA gradually increased 6, 12, 24 and 28 hours after Onapristone treatment in the day 17 rat cervix. COX-2 mRNA levels peaked 28 hours

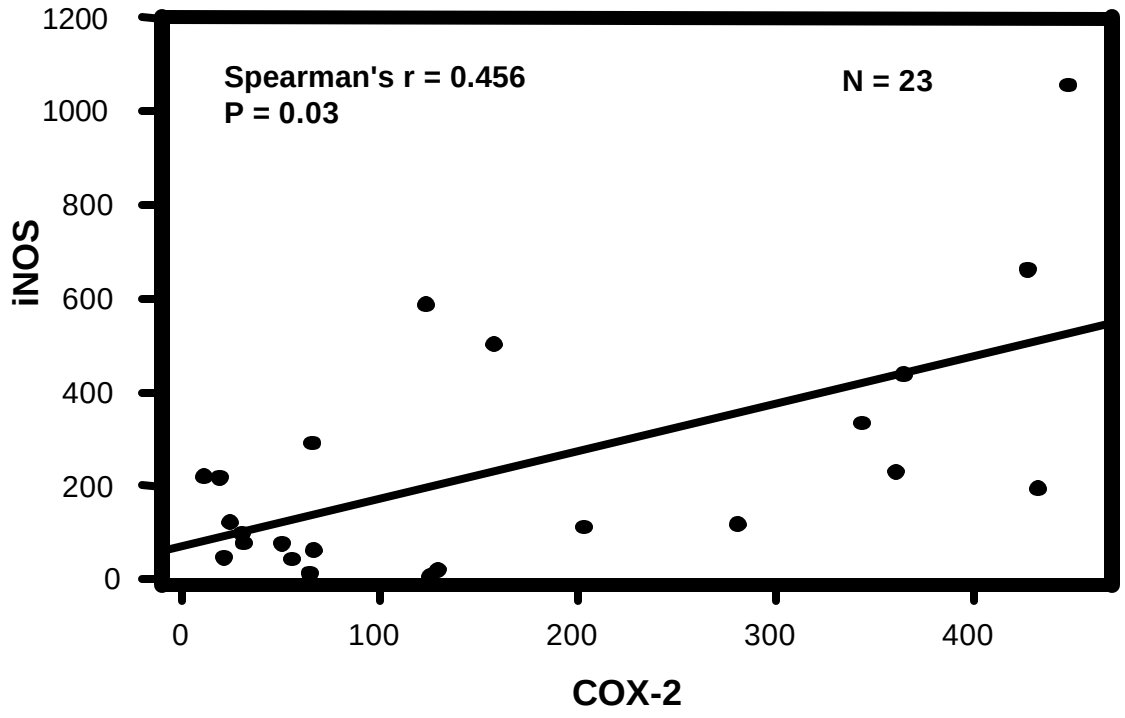

Figure 4.2: A significant statistical correlation between iNOS and COX-2 in Onapristone treated rat cervical tissues. A Spearman's correlation is utilized in this study, depicting the relationship between iNOS and COX-2 mRNA expression after Onapristone treatment in rat cervical tissues, demonstrating a significant positive correlation between iNOS and COX-2 mRNA expression after the induction of preterm labor with Onapristone. That as iNOS increases so does COX-2.

after treatment with ZK98299 (Figure 4.1). This peak was significantly different from all groups except for the 24 hour treated group ($P<0.05$).

iNOS and COX-2

A significant correlation between iNOS and COX-2 was demonstrated (Spearman r=0.456; P=0.03; N=23) (Figure 4.2). This does not mean they are related. It only suggests a possible relationship between iNOS and COX-2 which may be regulated initially through progesterone.

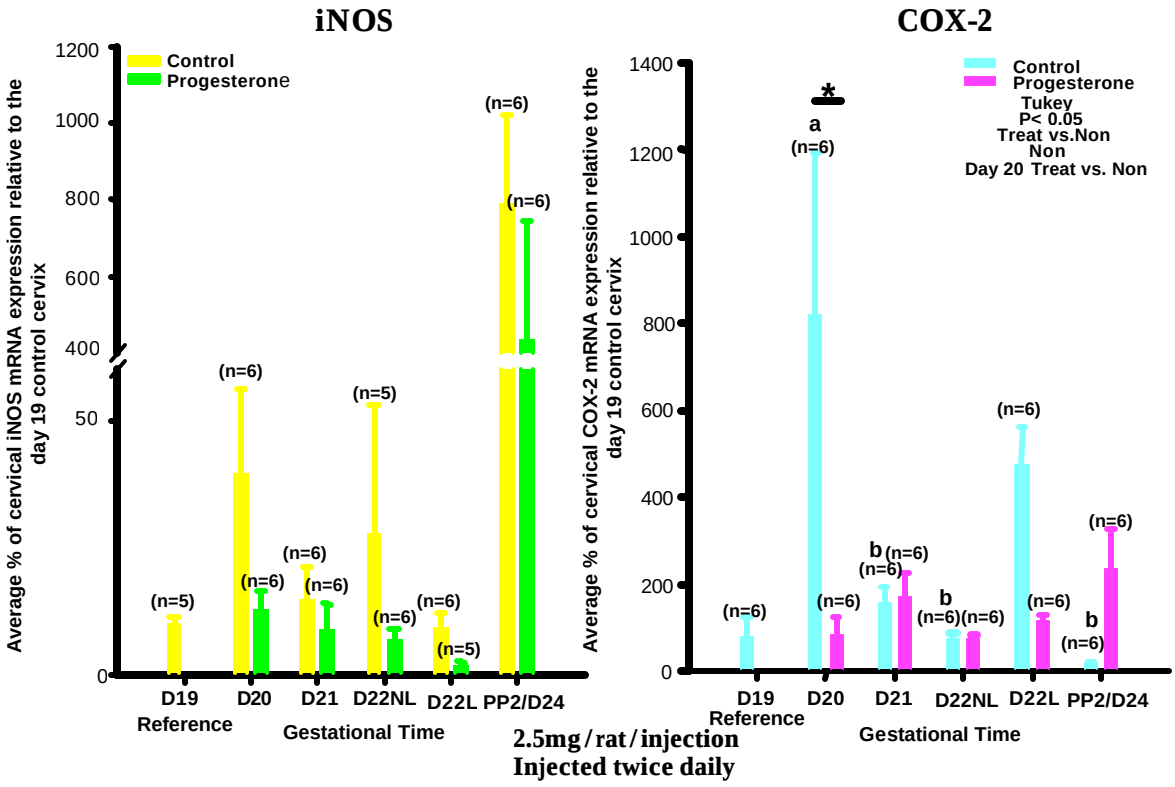

Figure 4.3: The expression of iNOS and COX-2 mRNA in rat cervical tissues after prolongation of pregnancy with progesterone. The data is expressed as the average % of cervical iNOS or COX-2 mRNA relative to the day 19 control cervix. Different letters are an indication of statistical significance. * indicates statistical significance between the control versus treated group at the particular day of gestatin being examined.

Cervical iNOS and COX-2 mRNA expression after the prolongation of pregnancy with progesterone

Progesterone

Treatment with progesterone inhibited term labor, prolonging the pregnancy. The vehicle control rats delivered at term (d22), while the progesterone treated rats remained pregnant even after 6 days of treatment.

iNOS (Figure 4.3)

In animals treated with progesterone, iNOS mRNA expression appeared to be reduced and maintained at a low and consistent level. However the decrease was not significant.

COX-2 (Figure 4.3)

In animals treated with progesterone, COX-2 mRNA expression was maintained at a low and consistent level demonstrating a significant ($P=0.036$) decrease in the treated versus the non-treated groups. COX-2 mRNA expression was significantly ($P<0.001$) decreased at day 20 in the treated versus the non-treated group. COX-2 significantly increased in the day 20 non-treated group compared to the day 21 ($P<0.001$), 22 non-labor ($P=0.001$) and postpartum day 2 ($P=0.005$) non treated rat cervix. The increase in COX-2 on day 20 of gestation in the cervix may be due to an increase in leukocyte infiltration and/or epithelial expression.

Cervical iNOS and COX-2 mRNA expression during both term and preterm laboring conditions

At term in the day 22 laboring rat cervix, iNOS mRNA significantly decreased ($P=0.022$), while COX-2 mRNA significantly increased ($P=0.011$) (Figure 4.4). The expression of iNOS ($P=0.005$) and COX-2 ($P=0.004$) mRNA was significantly increased during labor under preterm laboring conditions, 28 hours post treatment (Onapristone) compared to the 28 hour control cervix (Figure 4.4). During preterm versus term labor, iNOS mRNA was significantly increased ($P=0.004$); however, there was no difference in the COX-2 mRNA levels (Figure 4.4).

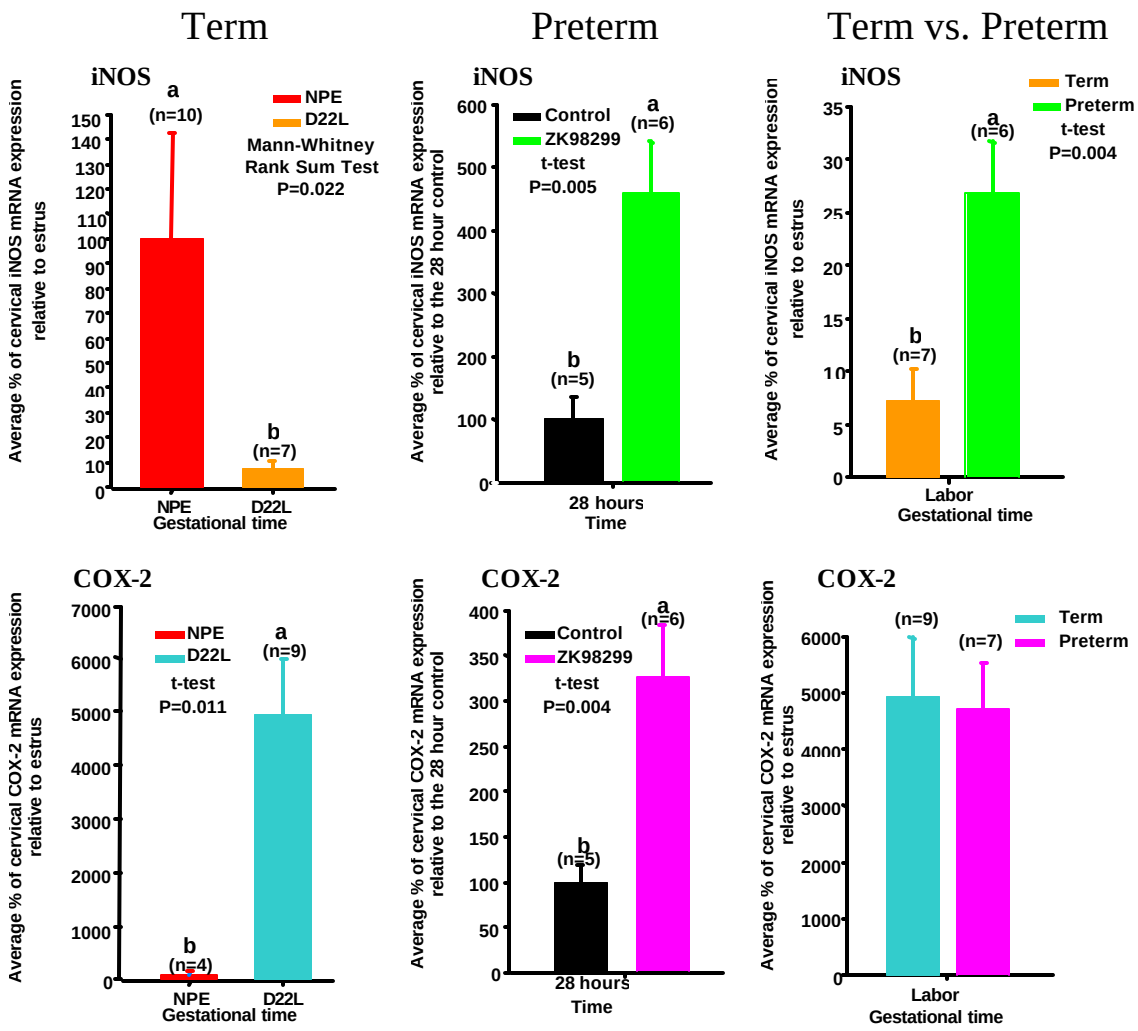

Figure 4.4: A comparison between iNOS and COX-2 mRNA expression during term and preterm laboring conditions. Under term pregnant conditions the data are depicted as the average % of cervical iNOS or COX-2 mRNA relative to the estrous control cervix. Under preterm laboring conditions the data is depicted as the average % of cervical iNOS or COX-2 mRNA 28 hours post treatment relative to the 28 hour control cervix. When comparing term versus preterm laboring conditions, the data is depicted as the average % of cervical iNOS or COX-2 mRNA relative to the estrus control cervix. Different letters are an indication of statistical significance.

DISCUSSION

These studies characterize the effect of progesterone on iNOS and COX-2 mRNA expression in rat cervical tissues during pregnancy. The data suggest that progesterone plays a role in regulating iNOS and COX-2 mRNA expression and parturition. Progesterone prolonged pregnancy and appeared to cause a decrease in iNOS and COX-2 expression. Onapristone stimulated preterm parturition and produced a parallel increase in iNOS and COX-2 mRNA expression demonstrating a positive correlation. At term under normal laboring conditions, iNOS mRNA decreased while COX-2 increased. Under preterm laboring conditions, both iNOS and COX-2 mRNA increased. When comparing term versus preterm laboring conditions, there was no change in the COX-2 mRNA expression but there was a significant increase in iNOS mRNA expression under preterm laboring conditions. This suggests that PGs are important for parturition but NO may be more important under preterm labor conditions possibly working through PGs.

Onapristone has been shown to ripen the cervix in many animals by preventing progesterone from binding to its receptor and creating a model comparable to that of progesterone withdrawal and preterm labor (Elger et al., 1986; Frydman et al., 1988; Hegele-Hurtung et al., 1989; Chwalisz et al., 1991a and Chwalisz et al., 1994). In our studies, Onapristone stimulated preterm parturition and produced a parallel increase in iNOS and COX-2 mRNA expression demonstrating a positive statistical correlation and suggesting a potential interaction between the NO and PG pathways under preterm laboring conditions (Nathanielsz, 1978a, b; Thorburn and Challis, 1979; Puri and Garfield, 1982; Yallampalli et al., 1996; Dong et al., 1996; Buhimschi et al., 1996; Ali et al., 1997 and Selles et al., 2002). A positive statistical correlation between iNOS and COX-2 does not indicate whether one affects the other. It does, however suggest that one may affect the other. There may be an interaction, a relationship under preterm laboring conditions between iNOS and COX-2 mRNA expression in the control of cervical ripening and parturition that may be regulated early on through progesterone.

Our studies and others suggest that cervical incompetence and/or preterm labor may be due to problems associated with progesterone and the regulation of iNOS and

COX-2 expression. Our studies indicate that Onapristone stimulates preterm labor and an increase in the iNOS and COX-2 epxression (Hegele-Hartung et al., 1989 and Ali et al., 1997). Hegele-Hartung (et al., 1989) showed that after treating with an antiprogestin, the collagen fibers become scattered and dissociate from one another. This was associated with stromal oedema and an increase in leukocyte infiltration into the cervix. Chwalisz (et al., 1997) showed a decrease in the cervical resistance after treating with antiprogestin, NO, or PGs. Ali (et al., 1997) showed an increase in iNOS expression after treating with an antiprogestin. These studies and ours suggest that progesterone plays a role in regulating the ripening of the cervix and parturition possibly acting through iNOS and COX-2 expression. This would also suggest that cervical incompetence and/or preterm labor may be due to deficiencies and/or abnormalities in the PR or a decrease in the progesterone.

Progesterone prolonged pregnancy and decreased iNOS and COX-2 mRNA expression maintaining them at a relatively low and consistent level. The decrease in iNOS was not significant possibly due to the iNOS levels already being low (see chapter 3). When increased and/or over expressed, progesterone prolonged pregnancy possibly due to a decrease in NO and PG production (Puri and Garfield, 1982; Dong and Yallampalli, 1996; Buhimschi et al., 1996 and Ali et al., 1997).

Our studies and others suggest that failure of the cervix to ripen and/or prolonged pregnancy may be due to problems associated with progesterone and the regulation of iNOS and COX-2 expression. Our studies indicate that the pregnancy was prolonged with progesterone and that there appeared to be a decrease in the iNOS and COX-2 epxression (Buhimschi et al., 1996). Buhimschi (et al. 1996) showed a decrease in NO production after treating with progesterone. This would suggest that progesterone plays a role in the ripening of the cervix and parturition possibly acting through an increase in NO and PG production. This would also suggest that the failure of the cervix to ripen and/or prolongation of the pregnancy may be due to an increase and/or over expression of progesterone and/or its receptor.

There is a peak in the COX-2 mRNA expression that occurs under both term and preterm laboring conditions suggesting that PGs may need to increase and/or reach a certain level for parturition to occur (Chwalisz et al., 1991a and Radestad and Bygdeman, 1993). Our data suggest that a shift in this peak to an early time point may stimulate preterm parturition or if the peak does not occur, pregnancy may be prolonged.

Presently fibronectin (a determination of the fibronectin protein in the vaginal fluid) and cervical changes are utilized in predicting preterm labor but they have been found to work mainly in high risk situations. Potentially this increase and/or peak in PGs may be utilized as a method along with fibronectin and changes in the cervix for predicting preterm labor in both high and low risk situations early in pregnancy.

CONCLUSIONS

Onapristone stimulates preterm labor and a parallel increase in iNOS and COX-2 mRNA expression demonstrating a positive correlation between iNOS and COX-2. Progesterone prolongs pregnancy maintaining iNOS and COX-2 mRNA expression at a low and consistent level. The data suggests a potential interaction between the NO and PG pathways under preterm laboring conditions in regulating parturition, and that progesterone plays a role in regulating iNOS and COX-2 mRNA expression. The COX-2 mRNA levels are increased under both term and preterm laboring conditions, while iNOS levels are only increased under preterm laboring conditions. This suggests that PGs play an important role in regulating parturition, but that NO may play a more important role in regulating parturition under preterm labor conditions. The failure of the cervix to ripen and/or prolonged pregnancy or cervical incompetence and/or preterm labor may be due to problems associated with progesterone and the regulation of iNOS and COX-2 expression.

FUTURE DIRECTIONS

The data in this chapter characterize the effects of progesterone on iNOS and COX-2 mRNA expression in the control of cervical ripening and parturition. Quantitative data still need to be collected and correlated for this study at the protein and product

levels. In addition, the other isoforms for NOS and COX should also be taken into consideration, both quantitatively and qualitatively. The data indicate that, Onapristone stimulates an increase in iNOS and COX-2 expression suggesting the importance of an increase in NO and PGs in the control of cervical ripening and parturition under preterm laboring conditions. Additional studies should be considered utilizing Onapristone with NOS and COX inhibitors, and NO donors and PGs to better understand the relationship between NO and PGs in the control of cervical ripening and parturition.

RU-486 is a type II agent that can act as an agonist or an antagonist. RU-486 and Onapristone create a model of preterm parturition by inhibiting transcription. Onapristone prevents transcription by preventing the receptor from binding to the DNA. RU-486 allows the receptor to bind to the DNA but transcription is inhibited by an improper conformational change of the receptor. RU-486 stimulates preterm parturition and is an effective model for studying progesterone and its effect on NOS and COX expression in the control of cervical ripening and parturition. Since RU-486 acts differently than Onapristone, studies that examine the effect of RU-486 on NOS and COX expression, cervical ripening and parturition should be considered.

Estrogen is thought to play a role in regulating NOS and COX expression, cervical ripening and parturition. Estrogen levels are known to increase as pregnancy progresses to term and is associated with a decrease in NO, and an increase in PGs and gap junction formation which is associated with cervical ripening and parturition. Estrogen has been shown to soften the cervix in humans. Studies examining the effect of estrogen on NOS and COX expression in the control of cervical ripening and parturition utilizing various estrogen agonists and antagonists should be considered.

Studies that localize NOS and COX expression and examine the changes in the collagen after estrogen and/or progesterone treatment should be considered. The picrosirius method should be utilized to study changes in the collagen after treating with estrogen and/or progesterone to suggest the importance of estrogen and/or progesterone in regulating the degradation of the collagen during the ripening process. Immunohistochemistry should be utilized to study if there are changes in the localization

of the NOS and COX enzymes in the epithelium after treating with estrogen and/or progesterone to suggest the importance of NOS and COX expression in the epithelium during pregnancy.

Clinical studies should be considered examining the importance of an increase COX-2 expression and PG production in humans and as a predictor of parturition under both term and preterm laboring conditions. Presently fibronectin and cervical changes are utilized as a predictor for preterm parturition usually only working consistently in high risk patients. Our data demonstrates (seen in chapter 3) an increase in COX-2 expression that peaked at labor and may be required for parturition to occur. This may be an indicator of preterm labor and impending parturition. With further studies, this may be utilized in humans either alone or along with fibronectin and/or cervical changes to predict and/or identify patients (at high and low risk) that will go into or are in preterm labor. Suggested studies would be to determine if there is a gradual increase in COX-2 expression and PG production that peaks at term in humans that can be identified and measured in the peripheral tissues and/or fluids (urine, blood and cervical mucus). Can this information be used with fibronectin and/or cervical changes to predict preterm labor in low and high risk patients?

CHAPTER 5: CHARACTERIZATION iNOS AND COX-2 EXPRESSION AFTER ADMINISTRATION OF LPS IN RAT CERVICAL TISSUES DURING THE DIFFERENT STAGES OF THE RIPENING PROCESS AND PARTURITION

INTRODUCTION

Inflammation is an immune response. It is a rapid response mechanism utilized for coping with tissue injury and is also associated with reproductive events. Cervical ripening and parturition is an inflammatory-like process (Liggins 1981 and Kelly 1996). Cytokines and collagenase are associated with the ripening process and parturition, playing a role in the inflammatory cascade (Sato et al., 1991; Osmers et al., 1992 and Osmers et al., 1995a,b). IL-1β, IL-8 and TNFα stimulate the ripening of the cervix (Cox et al., 1993; Chwalisz et al., 1994; El Maradny et al., 1994 and Cox et al., 1997). Cytokines may regulate NOS and COX expression in the control of cervical ripening and parturition (Romero et al., 1990; Corbett et al., 1993 and Swaisgood et al., 1997). PGs are associated with the inflammatory cascade and increase as the cervix ripens and pregnancy progresses to term (Romero et al., 1990; Dong et al., 1996 and Swaisgood et al., 1997).

LPS is commonly utilized to produce a model of inflammation and preterm labor under inflammatory conditions. LPS is thought to regulate the synthesis and stability of iNOS mRNA expression and NO production which in turn may control cervical ripening and parturition possibly through a direct interaction of NO with the COX-2 enzyme (Romero et al., 1990; Salvemini et al., 1993; Buhimschi et al., 1996 and Ekerhovd et al., 2002).

Main Objective

The main objective is to study the effect of infection and/or inflammation on iNOS and COX-2 mRNA expression in rat cervical tissues in the control of cervical ripening and parturition.

Specific Objective

The specific objective is to characterize iNOS and COX-2 mRNA expression in rat cervical tissue during the different stages of the ripening process and parturition after treatment with LPS. LPS is commonly utilized as a model of infection and/or inflammation allowing us the ability to study the function and/or effect of infection and/or inflammation on iNOS and COX-2 mRNA expression in the control of cervical ripening and parturition.

MATERIALS AND METHODS

Animal treatment

Animal treatments are described in chapter 2.

Tissue preparation

Infection and/or inflammation

Non-pregnant and timed-pregnant SD rats were acutely treated with either LPS (100µg i.p.) or vehicle (200 µl saline) then sacrificed 6 hours after treatment. Cervices were collected on days 14, 18, 22 non-labor, 22 labor, 22 postpartum, from days 1 and 3 postpartum and at estrus.

Preparation of RNA

Preparation of the RNA was described in chapter 3.

Measuring iNOS and COX-2 cervical mRNA level with real time RT-PCR

Expression of iNOS and COX-2 mRNA levels were determined using semi-quantitative real-time RT-PCR (Taqman, PE Applied Biosystems) as described in chapter 3. The results are expressed as the average % of cervical iNOS or COX-2 mRNA expression relative to the estrus control $\pm$ SEM.

Statistical analysis

Data was analyzed using a 2-way ANOVA followed by a Tukey test for multiple comparisons as appropriate (significance: $P<0.05$).

RESULTS

Cervical iNOS and COX-2 mRNA expression after stimulation with LPS

iNOS

When comparing all the LPS treated animals to all the control animals, the iNOS mRNA level was significantly increased in the LPS treated group (P<0.001) (Figure 5.1). When comparing the different groups in animals treated with LPS, iNOS mRNA expression increased with significance obtained at estrus (P<0.001), in the day 14 cervix (P<0.001) and in the day 22 postpartum cervix (P=0.032) compared to normal (control)

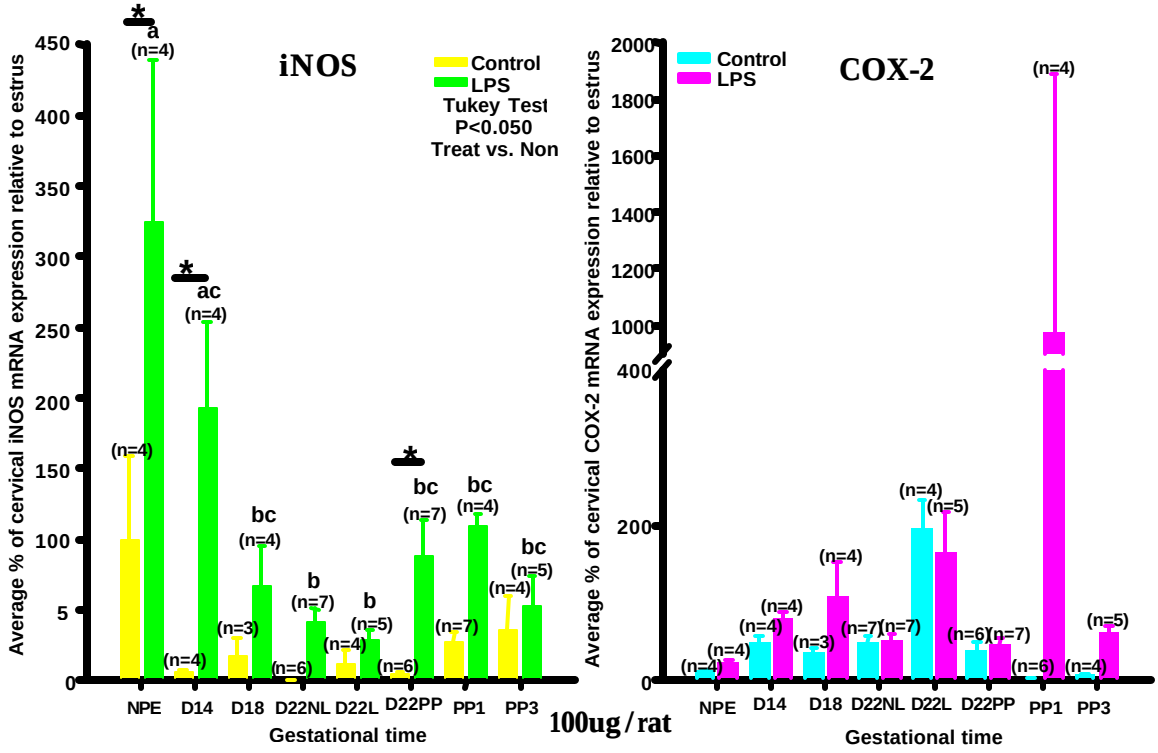

Figure 5.1: The iNOS and COX-2 mRNA expression 6 hours after LPS treatments in the rat cervix. The data is expressed as the average % of cervical iNOS or COX-2 mRNA relative to the estrous control cervix compared with gestational time. RNA levels were detected by real time analysis and calculated as described in the materials and methods section. Different letters are an indication of statistical significance. * indicates statistical significance between the control versus treated group at the particular day of gestatin being examined.

levels. However, expression levels decreased as pregnancy progressed to term with the lowest levels occurring in the laboring cervix. (Estrus versus: days 18, 22 non-labor, 22 labor, 22 postpartum and day 3 postpartum $P<0.001$; day 1 postpartum $P=0.001$; Day 14 versus: day 22 non-labor, $P= 0.022$ and labor, $P=0.021$). The pattern of expression for the LPS data as pregnancy progressed to term and the control data were comparable to the pattern of expression under normal term pregnant conditions, see chapter 3.

COX-2

Treatment with LPS had no effect on COX-2 mRNA expression in the cervix of non-pregnant and timed-pregnant rats (Figure 5.1). The data were comparable to the data previously collected under normal term pregnant conditions, see chapter 3. There appeared to be an increase in the COX-2 mRNA data in the postpartum day 1 cervix but this is due to one large outlier in the data skewing the result.

DISCUSSION

These studies characterize the effect of LPS on iNOS and COX-2 mRNA expression in rat cervical tissue during the different stages of the ripening process and parturition. The data suggests that endogenous LPS, plays a role in regulating iNOS mRNA expression but not COX-2 mRNA expression. LPS stimulated an increase in iNOS expression at each time point examined but the expression pattern decreased as pregnancy progressed to term. This suggests that LPS when given acutely may be a good model for studying NO during term gestation.

LPS is thought to stimulate an increase in NOS and COX expression, but the effect may be tissue and/or species specific (Silver et al., 1995; Buhimschi et al., 1996; Swaisgood et al., 1997; Gross et al., 2000 and Helmer et al., 2002). Swaisgood (et al., 1997) showed an increase in iNOS and COX-2 expression in the mouse uterus after treating with LPS but not in the placenta. In our studies, LPS stimulated an acute increase in iNOS expression but had no effect on COX-2 expression (Buhimschi et al., 1996). One reason for the lack of an observable increase in COX-2 expression may be because the increase in iNOS was for a very short period of time (acute) and not continuous

(chronic). Chapter 4 demonstrates a parallel and chronic increase in iNOS and COX-2 expression under preterm laboring conditions, with iNOS being significantly increased under preterm laboring conditions compared to term. This suggests that, for NO to stimulate an increase in COX expression and PG production there would have to be a continuous increase in NO. Another explanation is that NO may not have an effect on COX-2 expression at the mRNA level. It may occur at the protein or product level in the rat (Salvemini et al., 1993; Ledingham et al., 1999 and Ekerhovd et al., 2002).

LPS is normally utilized as a model for inflammation stimulating an increase in iNOS expression and NO production (Buhimschi et al., 1996 and Ali et al., 1997). LPS in our studies, when given acutely, appeared to elevate the normal levels of the iNOS mRNA expression at each time point examined, without changing the pattern of expression over time, which is that the iNOS mRNA significantly decreased as pregnancy progressed to term and the cervix ripened. This is comparable to the pattern of expression under normal term pregnant conditions. This would suggest that LPS when given acutely may not be a good model for studying the effects of inflammation, but may be a good model for studying the effects of NO under normal term pregnant conditions. LPS may better be utilized as a model of infection when given continuous, cronically, and/or in higher dosages.

Does the decrease in NO permit an increase in COX-2 expression? LPS increases the iNOS mRNA expression without increasing the COX-2 mRNA expression. However the pattern of expression for iNOS and COX-2 is comparable to the pattern under normal term pregnant conditions. The iNOS mRNA expression decreases, while the COX-2 mRNA increases as pregnancy progresses to term and the cervix ripens. This would suggest that under normal term pregnant conditions, there may not be an interaction between the NO and PG pathways in regulating iNOS and COX-2 mRNA expression. A decrease in NO does not appear to be responsible for an increase in the COX-2 expression.

LPS in mice stimulates preterm parturition and is associated with an increase in COX-2 expression (Kaga et al., 1996 and Gross et al., 2000). In our studies LPS did not

stimulate preterm parturition in the rat possibly because there was no shift in the peak of COX-2 expression at term which is demonstrated in chapter 4.

Cervical ripening and parturition is associated with an inflammatory-like response that occurs under both laboring and PTL conditions (Liggins, 1981). Human cervical smooth muscle cells contain the toll-like receptor 4 (TLR-4) and the cofactor membrane CD-14 which are the LPS receptors required for signal transduction. This is thought to stimulate the release of various MMPs, IL-8 and inflammatory cytokines leading to the degradation of the extracellular matrix, the ripening of the cervix and parturition (Watari et al., 2000). LPS is thought to play a role in regulating NOS and COX expression in the control of cervical ripening and parturition possibly through inflammatory cytokines such as IL-1β which stimulates an increase in iNOS and COX-2 expression. IL-1β also stimulates an increase in IL-8 promoter activity and expression (Masood et al., 2001 and Elliott et al., 2001).

CONCLUSIONS

LPS or infection may produce changes that are different from progesterone regulated NOS and COX expression. LPS, when given acutely in the rat, may not be a good model for studying the effects of infection, but may be a good model for studying the effects of NO under normal term pregnant conditions. Our data suggest that, under normal term pregnant conditions, iNOS mRNA decreases while COX-2 mRNA increases as pregnancy progresses to term and that there is no relationship between iNOS and COX-2 at the transcriptional (mRNA) level in the control of cervical ripening and parturition. However, this does not rule out the possibility of a relationship at the protein or product level.

FUTURE DIRECTIONS

The data in this chapter characterize the effect of LPS as a model of inflammation and/or infection on iNOS and COX-2 expression in the control of cervical ripening and parturition. Our present data showed that acute LPS treatments had no effect on COX-2 mRNA expression but stimulated an increase in iNOS mRNA expression. However the

pattern of iNOS mRNA expression decreased over time and was comparable to that of normal term pregnancy. Although there may not be a change in the expression of COX-2 at the mRNA level; it may occur at the protein and/or product level. Quantitative data still need to be collected and correlated for this study at the protein and product levels. In addition, the other isoforms for NOS and COX should also be taken into consideration. Other factors that may be involved in association with LPS stimulated NOS and COX expression NO and PG production are TLR-4, cofactor membrane CD-14, various cytokines (IL-1, TNF-α, IL-8) and MMPs (1, 2, 3, 8, 9).

A possible reason why acute LPS treatments had no effect on the COX-2 mRNA expression and parturition is that the exposure to the enhanced levels of the iNOS mRNA expression was for a very short period of time. Another possible reason is that the pattern of the iNOS mRNA expression over time decreased rather than increasing as pregnancy progressed to term (chapter 3). It is thought that NO must continuously (chronically) be increasing and/or elevated for an extended period of time for the COX-2 peak to shift. A continuous increase in NO and/or elevated levels of NO for an extended period of time would better represent a model of infection and/or inflammation. Studies examining the effect of LPS given chronically, continuously and/or for an extended period of time, on NOS and COX should be considered.

CHAPTER 6: THE EFFECT OF NITRIC OXIDE ON COX-2 AND iNOS EXPRESSION IN RAT CERVICAL TISSUE DURING PREGNANCY

INTRODUCTION

Nitric oxide is thought to play a role in the control of cervical ripening and parturition, possibly through a direct interaction with the COX-2 enzyme (Salvemini et al., 1993; Tetsuka et al., 1994; Ledingham et al., 1999 and Ekerhoved et al., 2002). COX-2 contains an iron-heme group at its activation site with which NO may interact directly (Salvemini et al., 1993). NO donors and inhibitors are commonly utilized to study the effect of NO in the regulation of iNOS and COX-2 expression (Salvemini et al., 1993; Tetsuka et al., 1994; Ledingham et al., 1999 and Ekerhoved et al., 2002).

L-arginine (the substrate for NO production) and NO donating compounds (SNP, sodium nitroprusside and GTN, glyceryl trinitrate) stimulate an increase in PGs (in rat mesangial cells, mouse macrophages and human fetal fibroblasts from foreskin) while N^G-Monomethyl-L-arginine (L-NMMA--non-specific NOS inhibitor) and aminoguanidine (specific iNOS inhibitor) decrease PGs (in rat mesangial cells and mouse macrophages) (Salvemini et al., 1993 and Tetsuka et al., 1994). Isosorbide 5-mononitrate (NO donating compound) stimulates an increase in PGE_2 in human cervical tissues suggesting that NO regulates PG production (Ekerhovd et al., 2002). Interleukin-1beta (IL-1β) stimulates an increase in iNOS expression that enhances COX-2 activity and PG production in rat mesangial cells (Tetsuka et al., 1994). Hemoglobin, which binds and inactivates NO, blocked the increase in PGE_2 production induced by L-arginine, NO, SNP, and GTN. These studies suggest a potential interaction in which endogenous NO enhances COX-2 activity and PG production which may play a role in regulating the ripening of the cervix and parturition.

Main Objective

The main objective is to study the effect of NO, through NO donors and NO inhibitors, on COX-2 and iNOS mRNA expression in pregnant rat cervical tissue.

Specific Objective

Specific objective 1 characterizes the effects of N^{ω}-nitro-L-arginine-methyl ester (L-NAME) on COX-2 and iNOS mRNA expression in rat cervical tissues. L-NAME is an analog of L-arginine and binds to the NOS enzymes non-specifically preventing the association of L-arginine, thus inhibiting the production of NO. L-NAME is commonly utilized to produce a model for the down regulation and/or inhibition of NO allowing the study of the effect of NO when down regulated and/or under expressed on COX-2 and iNOS mRNA expression in regulating cervical ripening and parturition.

Specific objective 2 characterizes the effects of the NO donor SNP on COX-2 and iNOS mRNA expression in rat cervical tissues. SNP is a NO donating compound that creates a model where NO is increased and/or over expressed allowing the study of the effects of NO when increased and/or up regulated on COX-2 and iNOS mRNA expression in regulating cervical ripening and parturition.

MATERIALS AND METHODS

Animal treatment

Animal treatments are described in chapter 2.

Tissue preparation

Down regulation and/or under expression of NO

Timed-pregnant Sprague-Dawley (SD) rats were chronically treated with either L-NAME (50µg/rat/day subcutaneously via osmotic mini-pumps) or vehicle (saline) starting on day 13 of pregnancy. Cervices were collected on days 15 and 22 labor of pregnancy and from day 1 postpartum. The days during gestation that were chosen for examination were based on information from previous studies. Gestational day 15 and postpartum day 1 cervix appear to have elevated levels of iNOS mRNA expression. The day 22 labor cervix demonstrated elevated levels of the COX-2 mRNA expression.

Up regulation and/or over expression of NO

Timed-pregnant SD rats were chronically treated with either SNP (0.3mg/rat/day intravaginally in 0.2ml of a 10% methylcellulose gel) or vehicle starting on day 12 of

pregnancy. Cervices were collected on days 14 and 16 of pregnancy. These days during gestation were chosen for examination, based on information obtained from previous studies. NO levels are relatively high but appear to start decreasing around mid gestation as pregnancy progresses to term. These days were chosen to prevent the decrease in NO around mid gestation and to see what happens to COX-2 and iNOS expression when NO levels remain elevated in the system.

Preparation of RNA

Preparation of the RNA was described in chapter 3.

Measuring iNOS and COX-2 cervical mRNA level with real time RT-PCR

Expression of iNOS and COX-2 mRNA levels were determined using semi-quantitative real-time RT-PCR (Taqman, PE Applied Biosystems), as described in chapter 3. The results are expressed as the average % of cervical iNOS or COX-2 mRNA expression relative to the day 15 control cervix (L-NAME study) or the day 14 control cervix (SNP study) $\pm$ SEM .

Statistical analysis

Data was analyzed using a 2-way ANOVA followed by a Tukey test for multiple comparisons as appropriate (significance: $P<0.05$).

RESULTS

COX-2 and iNOS mRNA expression after the down regulation and/or inhibition of NO with L-NAME

L-NAME had no significant ($P>0.05$) effect on COX-2 mRNA levels (Figure 6.1). The data was comparable to data previously collected under normal pregnant conditions in chapter 3.

The iNOS mRNA level in the L-NAME treated animals had a tendency to increase, but it was not significant different from control groups (Figure 6.1). Control data were comparable to data previously collected under normal pregnant conditions in chapter 3.

COX-2 and iNOS mRNA expression after the up regulation and/or over expression of NO with SNP

In animals treated with SNP, COX-2 mRNA expression appeared to be reduced but was not significant. There was a significant decrease in the iNOS mRNA level, when comparing all the SNP treated groups versus all the control groups (P=0.007) (Figure 6.2).

DISCUSSION

These studies characterize the effect of NO on COX-2 and iNOS mRNA expression in rat cervical tissue during the ripening process and parturition. The data suggests that endogenous NO when up regulated and/or over expressed may play a role in regulating COX-2 and iNOS mRNA expression.

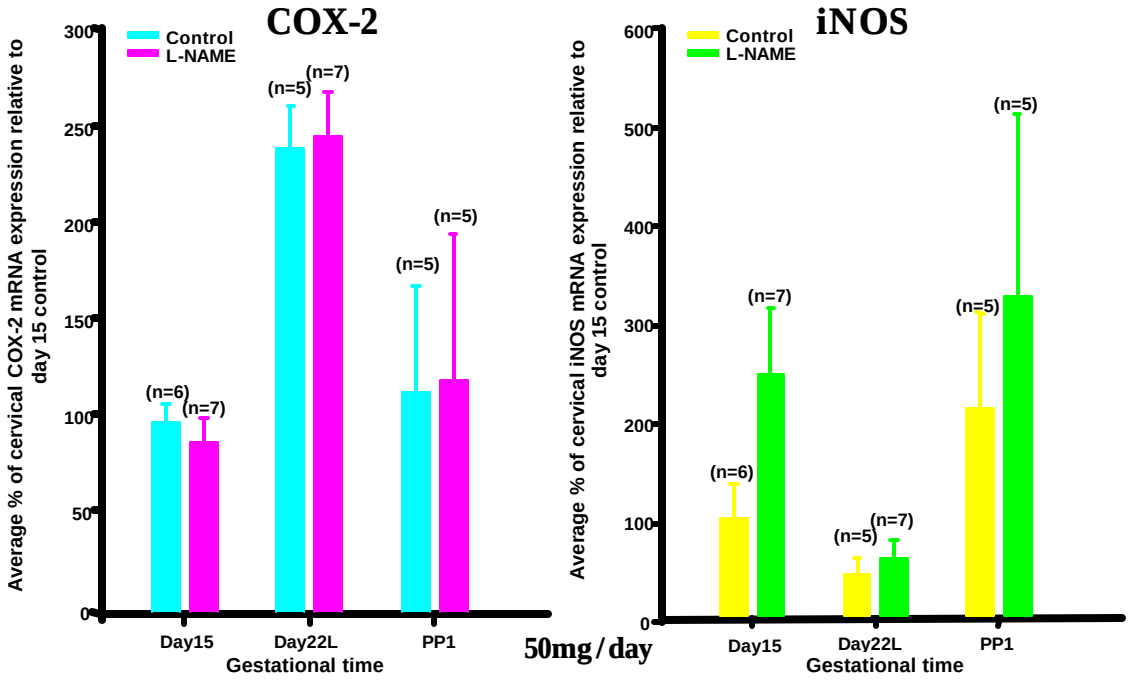

Figure 6.1: Examines the effect of NO on regulating COX-2 and iNOS mRNA expression in rat cervical tissue after down regulating and/or decreasing NO with L-NAME. The data is expressed as the average % of cervical iNOS or COX-2 mRNA relative to the day 15 control cervix compared with gestational time. RNA levels were detected by real time analysis and calculated as described in the materials and methods section.

L-NAME had no effect on COX-2 mRNA expression but there was a tendency for an increase in iNOS mRNA expression suggesting that there may not be a relationship between iNOS and COX-2 at the mRNA level under normal conditions. NO when down regulated and/or under expressed, may work through a feed back loop to regulate an increase in iNOS mRNA expression (Griscavage et al., 1993 and Mitchell et al., 1993). NO when down regulated and/or under expressed is thought to cause a decrease or have no effect on COX and NOS expression PG and NO production. NO inhibitors produce a decrease in NO, creating a model that is utilized to study the effects of NO when down regulated and/or under expressed. In our studies, L-NAME had no effect on COX-2 or

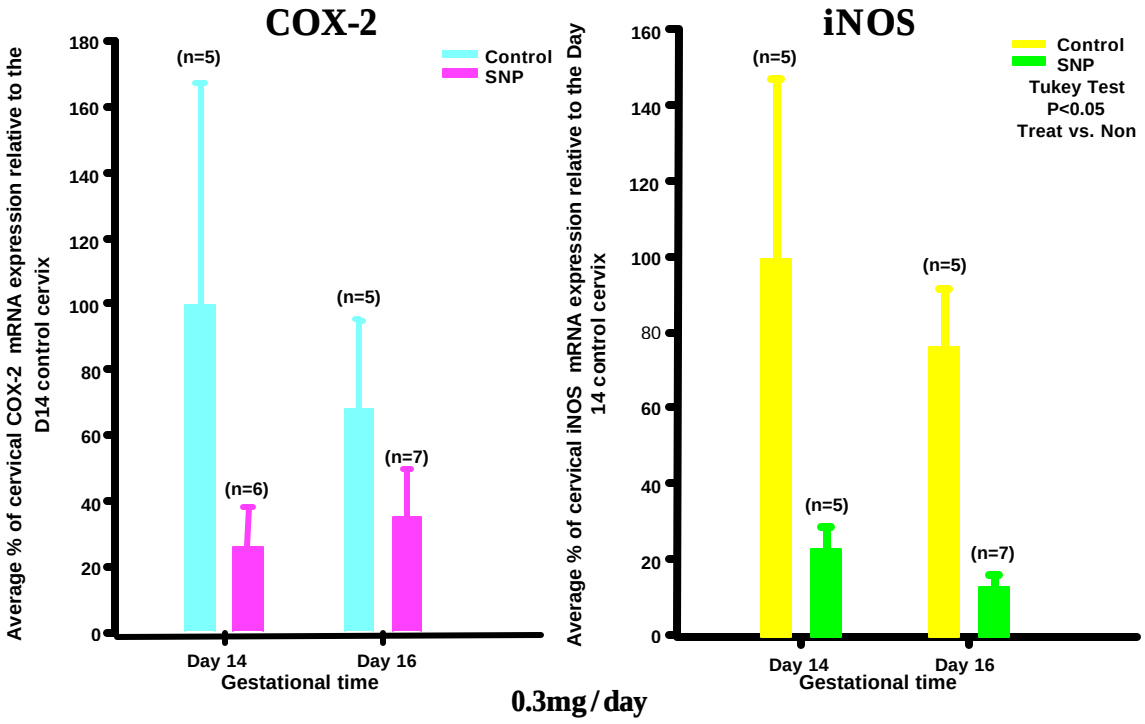

Figure 6.2: Examines the effect of NO on regulating COX-2 and iNOS mRNA expression in rat cervical tissue during pregnancy after the up regulation and/or over expression of NO with SNP. The data is expressed as the average % of cervical iNOS or COX-2 mRNA relative to the day 14 control cervix compared with gestational time. RNA levels were detected by real time analysis and calculated as described in the materials and methods section.

iNOS mRNA expression suggesting that there may not be an interaction between the PG and NO pathway in regulating COX-2 and iNOS mRNA expression under normal term pregnant conditions. SNP decreased iNOS mRNA expression. There was also a tendency for a decrease in the COX-2 mRNA expression. The decrease in COX-2, since not significant, may or may not be due to an increase in NO. There may or may not be an interaction between the PG and NO pathways in regulating iNOS and COX-2 expression. If the decrease in iNOS and COX-2 is due to the increase in NO levels, this would suggest a possible relationship between iNOS and COX-2 mRNA expression under pathological conditions when NO is up regulated and/or over expressed.

One reason for a decrease and not an increase in the iNOS and COX-2 mRNA expression after treating with SNP may be due to the amount of time the samples were exposed to the increased levels of NO (2 or 4 days). Another reason for the decrease, maybe be that in our studies the increase in NO is being maintained at a relatively constant level. There may need to be a continuous increase in the amount of NO for there to be an increase in COX and NOS expression, possibly leading to early parturition as indicated in chapter 4. Samples exposed to NO for a shorter period (3, 4, 6, 10, 12, 24, 28 hours) and/or with increasing amounts of NO may stimulate an increase in the COX-2 and iNOS mRNA expression (Ekerhovd et al., 2002). This also suggests that NO plays a role in regulating iNOS mRNA expression possibly through an increase in PGs (Inoue et al., 1993; Salvemini et al., 1993; Ledingham et al., 1999 and Ekerhovd et al., 2002).

CONCLUSIONS

L-NAME (a model for the down regulation and/or under expression of NO) had no effect on the COX-2 or the iNOS mRNA expression suggesting that NO inhibitors do not play a role in regulating COX-2 or iNOS at the transcription level. It also suggests that under normal term pregnant conditions or when NO is down regulated and/or under expressed there may not be an interaction between the PG and NO pathways. These pathways may act independently of each in the ripening of the cervix and parturition at the mRNA level. This does not rule out an interaction at the protein or product level.

SNP (a model for the up regulation and/or over expression between NO) appeared to cause a decrease in both COX-2 and iNOS mRNA expression. For COX-2 the decrease was not significant. Since there is a tendancy for both the COX-2 and iNOS to decrease, this would suggest that when NO is up regulated and/or over expressed that it may play a role in regulating COX-2 and iNOS mRNA expression. It also suggests that when NO is up regulated and/or over expressed such as in preterm laboring conditions, there may be an interaction between the PG and NO pathways in regulating the COX-2 and iNOS mRNA expression in the control of cervical ripening and parturition.

There may not be an interaction between the PG and NO pathways at the mRNA level under normal term pregnant conditions. However, there may be an interaction under pathological conditions (infection, inflammation and/or preterm laboring conditions) when NO is up regulated and/or over expressed in the control of cervical ripening and parturition.

FUTURE DIRECTIONS

The data in this chapter characterizes the effect of NO on COX-2 and iNOS mRNA expression in the control of cervical ripening and parturition. Our data showed that L-NAME had no effect on COX-2 mRNA expression, but may play a role in regulating iNOS mRNA expression. SNP played a role in regulating iNOS mRNA expression and possibly COX-2 mRNA expression. This indicates that during a normal term pregnancy, there may not be an interaction between the PG and NO pathways at the mRNA level in the control of cervical ripening and parturition. However there may be an interaction under pathological conditions when NO is up regulated and/or over expressed. Quantitative data still needs to be collected and correlated for this study at the protein and product levels. In addition, the other isoforms for NOS and COX should also be taken into consideration, both quantitatively and qualitatively. Other factors that may be involved in cervical ripening and parturition in association with a decrease or increase in NO production are various cytokines (IL-1, TNF-α, IL-8) and MMPs (1, 2, 3, 8, 9).

NO inhibitors produce a decrease in NO, creating a model that is utilized to study the effects of NO when down regulated and/or under expressed. In our studies, L-NAME

had no effect on COX or NOS expression. What is the role of NO when down regulated and/or under expressed with NO inhibitors in regulating the expression of the different COX and NOS isoforms? Studies examining the effect of NO when down regulated and/or under expressed by NO inhibitors (L-NMMA: non-specific NOS inhibitor, aminoguanidine and L-NIL: specific iNOS inhibitor), on COX and NOS expression in the control of cervical ripening and parturition should be considered.

L-arginine and NO donating compounds produce an increase in NO, creating a model that is utilized to study the effects of NO when up regulated and/or over expressed. In our studies, SNP (a NO donating compound) appeared to stimulate a decrease in COX and NOS expression instead of an increase. What is the role of NO when over expressed and/or up regulated to pharmacological levels with L-arginine or NO donating compounds in regulating the expression of the different COX and NOS isoforms? Studies examining the effect of NO when up-regulated and/or over expressed by L-arginine or NO donating compounds (DETA-NO: third generation NO donating compound, isosorbide mononitrate-IMN: NO donating compound used for studying NO in human tissues, glyceryl trinitrate-GTN: NO donating compound used in clinics and for clinical studies), on COX and NOS expression in the control of cervical ripening and parturition should be considered.

A deficiency in our studies is that it does not consider the effects of PGs on NOS and COX expression in the control of cervical ripening and parturition which should be examined in the future. At certain concentrations, PGs are thought to stimulate an increase in NOS and COX expression and NO and PG production. COX inhibitors should either down-regulate or have no effect on NOS and COX expression. If the PG levels decrease, NO levels are thought to increase in an attempt to compensate for the decrease in PGs. NO is thought to interact directly with the COX enzymes stimulating an increase in PG production. What effect do COX inhibitors (Indomethacin-Indo: non-specific COX inhibitor utilized to studying PGs and shown to prolong pregnancy in humans, ibuprofen: non-specific COX inhibitor utilized clinically, aspirin: non-specific COX inhibitor utilized clinically, Celebrex: specific COX-2 inhibitor utilized clinically and fairly new,

NS-398: specific COX-2 inhibitor, SC-S8236: specific COX-2 inhibitor, refecoxib: specific COX-2 inhibitor) have on the expression of the different NOS and COX isoforms? Studies examining the effect of PGs when down regulated and/or inhibited on NOS and COX expression in the control of cervical ripening and parturition should be considered.

PGs, when up regulated and/or over expressed, are initially thought to stimulate an increase in NOS and COX expression until a certain threshold concentration is reached. Once the threshold concentration is reached, iNOS expression and NO production are then thought to decrease. NO when it decreases to a low enough concentration, it will then stop associating with the activation site of the COX-2 enzyme. This will lead to a decrease in the PG production, and a further decrease in the NOS and COX expression. Arachidonate acid and PG agonists are utilized to produce an increase in PGs, creating a model that is utilized to study the effects of PGs when up regulated and/or over expressed. What is the role of PGs when over expressed and/or up regulated with Arachidonate acid or a PG agonist compound? Studies examining the effect of PGs when up regulated and/or over expressed by Arachidonic acid or PG agonists (PGE_2: used clinically to ripen the cervix and stimulate uterine contractions, sulprostone: PGE_2 analogue, $PGF_{2\alpha}$, iloprost: PGI_2 compound, U-46619: TXA_2) on NOS and COX expression in the control of cervical ripening and parturition should be considered.

Further studies that should be considered include the effect of the PG receptors on NOS and COX expression in the control of cervical ripening and parturition. (AH23848-selective EP4 receptor antagonist, ONO-AE3- 208---selective EP4 antagonist, ONO-AE1-329---selective EP4 agonist; sulprostone-selective EP3 agonist; AH6809-selective EP2 receptor antagonist; 17-phenyl-trinor-PGE_2---selective EP1 agonist; SQ29548-selective TP antagonist, U-46619-selective TP agonist; H89-selective cAMP dependent protein kinase inhibitor) AH6809 and AH23848 inhibit PGE_2 stimulated cAMP. PGE_2 and butaprost increases cAMP production. EP4 and EP2 are found in human cervical fibroblast cells. IL-1β has been shown to enhance the responsiveness of cervical fibroblast to PGE_2 by up regulating the EP4 receptor suggesting that PGE_2 may regulate

human cervical ripening via the EP4 receptor (Schmitz et al., 2001 and Schmitz et al., 2003). EP1 increased with advancing gestation age prior to labor in the pregnant baboon cervix. EP2 receptors decrease in laboring animals versus non-laboring animals along with the FP and TP receptors (Smith et al., 2001). This suggests that various PG receptor types and sub-types may play an important role in the control of cervical ripening and parturition. These studies would suggest a potential role for the PG receptors in the regulation of NOS and COX expression in the control of cervical ripening and parturition.

CHAPTER 7: SUMMARY

Under normal conditions, the iNOS and COX-2 enzymes are co-localized in similar tissues at similar times during gestation, suggesting a possible interaction between the iNOS and COX-2 pathway at the protein and/or product level. Expression of the iNOS mRNA decreases as the COX-2 mRNA increases, suggesting that during term pregnancy there is an inverse relationship between iNOS and COX-2 at the mRNA level. These two studies would indicate a possible relationship between iNOS and COX-2 in the control of cervical ripening and parturition under normal term pregnant conditions. Onapristone stimulates preterm labor and an increase in both iNOS and COX-2 mRNA expression, demonstrating a positive correlation. Progesterone inhibits term labor and decreases both iNOS and COX-2 mRNA expression. This suggests that under preterm laboring conditions there may be an interaction, there may be a relationship between the iNOS and COX-2 pathways in the control of cervical ripening and parturition that may be regulated early-on through progesterone. COX-2 expression and PG production is associated with both term and preterm laboring conditions indicating the importance of PGs in regulating parturition. Since NO levels are increased under preterm labor conditions compared to term laboring conditions, NO may play an important role in regulating parturition under preterm laboring conditions. Also the peak in PGs under preterm laboring conditions may be regulated through NO. PGs may need to reach a certain level and/or peak for parturition to occur and a shift in this peak may induce preterm labor. If PGs don't reach a high enough concentration, if the peak in PGs does not occur then this may cause the prolongation of the pregnancy. LPS stimulated an increase in iNOS mRNA expression, but had no effect on COX-2 mRNA expression. L-NAME had no effect on COX-2 or iNOS mRNA expression. These two studies would suggest that under normal term pregnant conditions or when NO is down regulated and/or under expressed, there may not be an interaction between the NO and PG pathways. These pathways may act independently of each other in the ripening of the cervix and

parturition at the mRNA level. This does not rule out an interaction at the protein or product level. SNP appeared to decrease both COX-2 and the iNOS mRNA expression. This suggests that when NO is up regulated and/or over expressed such as in preterm laboring conditions, there may be an interaction between the NO and PG pathways in regulating the iNOS and COX-2 mRNA expression in the control of cervical ripening and parturition.

The data suggests that under normal term pregnant conditions or when NO is down regulated and/or under expressed, there may not be an interaction between the NO and PG pathways. These pathways may act independently of each other in the control of cervical ripening and parturition at the mRNA level. This does not rule out an interaction at the protein or product level. Those studies have not been done and should be conducted in the future. Under preterm laboring conditions or when NO is up regulated and/or over expressed, there may be an interaction between the NO and PG pathway in regulating the iNOS and COX-2 mRNA expression in the control of cervical ripening and parturition. Further studies still need to be conducted to better understand the interaction, the relationship between NOS and COX expression and NO and PG production in the control of cervical ripening and parturition.

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VITA

Stephen G. Marx was born on May 18, 1970 to Alan and Jackie Marx. After attending Texas A&M University at Galveston for his Bachelor's degree in Marine Biology, Stephen matriculated at the University of Texas Medical Branch. While at graduate school, Stephen demonstrated himself to be a leader amoung his peers and in the surrounding communty. At the University of Texas Medical Branch, Stephen served as a member of the Student Government Association in the capacity of Treasurer for the student body (2003 to 2004) and as Senator for the Graduate School of Biomedical Science (2002 and 2003). In the community at large, Stephen served on the Board of Trustees (1995 to 2000) and as a Sunday school teacher (1997 to 2003) for Congregation B'nai Israel. Stephen as a graduate student, has received several honors. In 2001 Stephen was awarded the Who's Who Among Graduate Students and an NIH predoctoral fellowship (1 F31 HD08739 PI: MARX, STEPHEN G).

Stephen gained significant teaching and independent research experience at the University of Texas Medical Branch. Stephen was a Teaching Assistant for the Gross Anatomy lab for the Occupational Therapy and Physical Therapy students (1998 to 1999), the Microanatomy lab for first year Pathology and Cell Biology students (1999) and the Pre-Matriculation course (Molecules, Cells and Tissues) for the incoming medical students. Stephen has also given a lecture for the Microanatomy course on the Ovaries and Ovulation. Stephen studied the relationship between the nitric oxide and prostaglandin pathways in the control of cervical ripening and parturition obtaining experience in immunohistochemical staining, RT-PCR, Western blotting, other molecular techniques and isolated organ bath studies for the pharmacological study of smooth muscle.

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Ph.D. Cell Biology, University of Texas Medical Branch , Galveston, Texas, mentor: Dr. Robert E. Garfield, expected graduation May 2005

B.S. Marine Biology, Texas A&M University, Galveston, Texas, May 1996

Publications

A. Articles in Peer-Reviewed Journals:

Schlembach, D., Scalera, F., Fischer, T., Marx, S., Beinder, E. and Garfield, R. (2003). Neurokinin B peptide serum levels are higher in normotensive pregnant women than in preeclamptic pregnant women. *American Journal of Obstetrics Gynecology* **189**, 1418 – 22.

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