THE ATTENUATION OF PROGESTERONE EFFECTS ON THE UTERUS
AT THE TIME OF LABOUR

by

Grace Ellie Erb Tavares

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Institute of Medical Science
University of Toronto

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Abstract
THE ATTENUATION OF PROGESTERONE EFFECTS
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Doctor of Philosophy 1999
Grace Ellie Erb Tavares
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Progestosterone (P₄) is essential for the maintenance of pregnancy in part due to its inhibition of uterine contractility. In most species this inhibitory action is terminated by decreased P₄ production. In humans however, there is no evidence of any such decrease, suggesting that P₄ dominance over the myometrium must be overcome by a mechanism other than a decrease in maternal circulating levels of P₄. The actions of P₄ on the myometrium may be mediated in part through one of its 5α-reduced metabolites allopregnanolone (AP) which acts by agonistically binding to γ-aminobutyric acid receptors. As well, the signaling pathway through which P₄ acts includes its nuclear receptor A and B isoforms. The action of these receptors may be affected by heat-shock proteins (HSP) and steroid receptor coactivators. We therefore investigated whether labour was associated with: a decrease in the amount of AP; a change in the relative amounts of the A and B receptor isoforms; changes in HSP or steroid receptor coactivator expression. Samples from women who were not in labour at the time of delivery were compared to samples obtained from women who were in labour. AP levels were not found to significantly differ between serum samples obtained from women not in labour and women in labour at the time of delivery. No significant change in P₄ receptor isoforms was found in human myometrium at labour. HSP90α, and HSP90β, but not HSP70 increased in the human myometrium with term labour. SRC1 and TIF2, but not ARA70 increased in the human myometrium from preterm not in labour to term in labour samples. Therefore, functional withdrawal of P₄ in the human can not be explained by altering P₄ receptor levels, or by a decrease in its 5α-reduced metabolite AP, but may be due in part to increases in HSPs and steroid receptor coactivators within the myometrium. These changes might modify the transcriptional activity of the P₄R and/or estrogen receptor systems thereby modifying myometrial expression of various proteins associated with contractions, without there being a change in steroid hormone levels or a change in steroid receptor levels.
Acknowledgments

I would like to thank Dr. S. Lye for supervising me during the last half of my PhD and Dr. N. MacLusky for supervising me during the first half of my PhD. As well, I would like to thank Dr. J. Challis, and Dr. P. Walfish for being part of my supervisory committee, and Dr. T. Brown, Dr. D. Belsham, and Dr. B.F. Mitchell for being part of my examination committee.

I would like to thank members of Dr. Lye’s lab especially Dr. D. MacPhee, J. Mitchell, A. Orsino, Dr. R. Ou, as well as members of Dr. MacLusky’s lab, and Dr. Challis’ lab.

I would like to thank Dr. T. Brown, Dr. B. F. Mitchell and Dr. P. Walfish for cDNA probes used in the experiments presented in this thesis. Also, thank-you to Dr. R. Purdy for the antibody used for both allopregnanolone and dihydroprogesterone radioimmunoassays.

Thank-you to the research nurses: L. McWhirter, J. Zielonka, and C. Botsford who were instrumental in the collection of human myometrial samples.

Thank-you to those persons who’s suggestions contributed to methods used in this thesis, and those persons who read earlier versions of the thesis.

I would like to thank the Medical Research Council of Canada, the Genesis Research Foundation, and Interforest Ltd. for funding me through my PhD, as well as the MD/PhD program and the Institute of Medical Science for their support.

Finally, thanks to my parents for their love and support.
Contributions

The following persons contributed to particular samples presented in the indicated sections:

Section 4

- Ovariectomized non-pregnant rats treated with 17β-estradiol were prepared by Arawn Therrien (Dr. N.J. MacLusky's lab, Toronto General Hospital, Canada).

Section 5.2.1.2.2 (Progesterone treated pregnant rat myometrium) and Section 5.2.1.2.3 (Progesterone treated ovariectomized pregnant rat myometrium)

- Angela Orsino (Dr. S. J. Lye’s Lab, Samuel Lunenfeld Research Institute, Toronto, Canada) treated the animals, obtained myometrial tissues from these animals, extracted the RNA from these tissues, and prepared the RNA membranes.

Section 5.2.1.3 (Sheep myometrium)

- Members of Dr. J. R. Challis’ lab (University of Toronto, Canada) treated the animals and obtained myometrial tissues from these animals.

Section 3.1.2.2 (Allopregnanolone antibody preparation)

- Dr. R. H. Purdy (Department of Psychiatry, University of California, San Diego, School of Medicine, SD, USA) prepared the allopregnanolone antibody described in this section.

The following methods were developed for the data presented in this thesis:

- characterization of allopregnanolone antibody, and the measurement of allopregnanolone and 5α-dihydroprogesterone via high-pressure liquid chromatography followed by radioimmunoassay
- the identification of progesterone receptors via northern blot, PCR, western blot, and immunohistochemistry
- the identification of heat-shock proteins and steroid receptor coactivators via northern blots
- along with Arawn Therrien, the in situ hybridization technique which was later used to identify steroid receptor coactivator mRNA
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<td>AAG</td>
<td>α1-acid glycoprotein</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AF</td>
<td>activation function</td>
</tr>
<tr>
<td>AF1</td>
<td>activation function region 1</td>
</tr>
<tr>
<td>AF2</td>
<td>activation function region 2</td>
</tr>
<tr>
<td>AF3</td>
<td>activation function region 3</td>
</tr>
<tr>
<td>AIB1</td>
<td>amplified in breast cancer</td>
</tr>
<tr>
<td>AP</td>
<td>allopregnanolone (3α-hydroxy-5α-pregnane-20-one)</td>
</tr>
<tr>
<td>ARA70</td>
<td>androgen receptor coactivator 70</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBG</td>
<td>corticosteroid-binding globulin</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<tr>
<td>CID</td>
<td>CBP interaction domain</td>
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<tr>
<td>COPRA</td>
<td>cofactor of P4 receptor activation</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
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<tr>
<td>CRH</td>
<td>corticotrophin-releasing hormone</td>
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<td>cxn-43</td>
<td>connexin-43</td>
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<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
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<td>DHEAS</td>
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<td>DHP</td>
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<td>DHT</td>
<td>dehydrotestosterone</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>estradiol-17β</td>
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<td>estriol</td>
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<td>E/P₄ ratio</td>
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<td>estrogen receptor associated protein 140</td>
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<td>EthOH</td>
<td>ethanol</td>
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<td>F-SRC1</td>
<td>thyroid hormone receptor coactivator</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>histone acetyltransferase</td>
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<tr>
<td>HMG-1</td>
<td>chromatin high mobility group protein 1</td>
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<tr>
<td>hN-CoR</td>
<td>human thyroid/retinoic acid receptor corepressor</td>
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HSE  heat shock element
HSF  heat shock transcription factor
HSP  heat-shock proteins
IL-6  interleukin-6
MMTV mouse mammary tumor virus
mN-CoR  rat thyroid/retinoid acid receptor corepressor
mRNA  messenger ribonucleic acid
NCoA-1 / NCoA-2 nuclear receptor coactivator-1 / nuclear receptor coactivator-2
ND  nuclear domain
p/CIP  p300/CBP/co-integrator-associated protein
p160  proteins of 160 kilodaltons
P₄  progesterone (4-pregnen-3,20-dione)
PAPP-A  pregnancy associated plasma protein A
PBP  peroxisome proliferator-activated receptor binding protein
pCAF  p300/CBP associated factor
PCR  polymerase chain reaction
PGDH  15-prostaglandin dehydrogenase
PGE₂  prostaglandin E₂
PGF₂α  prostaglandin F₂α
pp1  one day post partum
PP12  placental protein 12
PPIase  peptidylprolyl isomerase
PRE  progesterone response element
RAC3  receptor-associated coactivator 3
RAR, RXR  retinoic acid receptors
RBF-1  receptor binding factor
RIP140  receptor interacting protein 140
RNAPII  RNA polymerase II
SHBG  sex hormone binding globulin
SMRT  silencing mediator of retinoic acid and thyroid hormone receptors
SP1  Schwangerschaft protein 1
SRC1  steroid receptor coactivator
TBP  TATA box binding protein
TBPS  t-butylbicyclophosphorothionate
TIF1  transcription intermediary factor 1
TIF2  transcription intermediary factor 2
TGF-β  transforming growth factor-β
TRIP1  thyroid receptor interacting protein 1
Trip230  thyroid receptor interacting protein 230
UG  uteroglobin
1. General Introduction

Progesterone (P\textsubscript{4}) is an important hormone during pregnancy in eutherian mammals. One of its major roles during this time is the maintenance of uterine quiescence. Therefore, at the end of gestation its effects must be overcome in some way, enabling the uterus to undergo the contractions necessary to deliver the fetus. In many species maternal circulating levels of P\textsubscript{4} decrease at labour. In humans however, maternal circulating levels of P\textsubscript{4} are maintained until the placenta is delivered. As well, the ratio of P\textsubscript{4} bound to serum proteins in the maternal circulation to that of free P\textsubscript{4} (and thus available for uptake into cells) does not change at labour. Thus, there must be some other mechanism(s) which results in the attenuation of P\textsubscript{4} effects.

One mechanism of steroid action involves the agonistic binding of 5α-reduced pregnane metabolites to GABA\textsubscript{A} (γ-aminobutyric acid A) receptors. Agonistic binding to GABA\textsubscript{A} receptors on smooth muscle cells inhibits smooth muscle contractions. Thus, the first study of this thesis examined whether 5α-reduced metabolites of P\textsubscript{4} are high during pregnancy thus inhibiting myometrial smooth muscle contraction, and whether their levels drop at the time of labour in women thus releasing the myometrium from this contractile inhibition.

P\textsubscript{4} exerts its intracellular action via steroid receptors, thus by significantly changing the levels of P\textsubscript{4} receptors the effects of P\textsubscript{4} could be attenuated. The total amount of P\textsubscript{4} receptors appears to decrease slightly with the onset of labour. Yet there are at least two isoforms of the P\textsubscript{4} receptor. These two isoforms differ with respect to their transcriptional activities. The second study presented in this thesis examines the relative levels of the two primary isoforms of the P\textsubscript{4} receptor, isoforms A and B, and compares their levels between myometrium collected from women not in labour and that collected from women in labour at the time of delivery. As well, samples from women who delivered preterm are compared to samples collected from women who delivered at term.

The actions of steroid hormone receptors are influenced by various intracellular proteins. One such family of proteins are known as heat-shock proteins (HSP). In general, HSP affect the three dimensional structure and folding of steroid receptors, as well as play an important role in chaperoning receptors within the cell. Another set of proteins important for steroid receptor action are known as steroid receptor coactivators. While steroid receptor coactivators do not affect basal
transcription of steroid receptors, when present they can significantly increase transcriptional activity. The third study presented in this thesis thus examines the levels of three major HSP and three major steroid receptor coactivators in the human myometrium (not in labour, in labour, preterm, and term). HSP levels are also examined in rat myometrium under various experimental conditions, as well as in sheep myometrium.
2. Literature review

2.1 General

2.1.1 Clinical problems associated with preterm delivery

Morbidity and mortality are closely related to prematurity in newborn infants; the more premature or underweight the newborn, the greater the risk of health problems (Knupel 1994; World Health Organization 1969). Improvements in obstetric management and the introduction of neonatal intensive care units have improved the outcome of preterm infants in recent decades, but the preterm birth incidence rate has remained relatively unchanged (Creasy 1989). In developed countries approximately 5-10% of all deliveries are preterm, and in developing countries the percentage is considerably higher (Challis and Lye 1986; World Health Organization 1969). In Canada approximately one third of infants born between 24 and 29 weeks do not survive (Health Canada 1998). Infants of low birth weight (500-1250 grams) are at significant risk for multiple severe disabilities such as cerebral palsy, mental retardation, and hearing loss (Robertson et al. 1992). Caregivers try to maintain a premature fetus within the uterus for as long as possible, but this is difficult since the metabolic and endocrine pathways responsible for parturition in humans remain uncertain, and safe and effective means of predicting and preventing preterm birth are unknown. Prevention of all preterm deliveries is an unrealistic goal due to the number of complications which lead to prematurity such as pre-eclampsia, premature rupture of the membranes, antepartum hemorrhage, hydramnios, cervical defects, and congenital abnormalities of the fetus (Challis and Olson 1988). However, idiopathic, or premature labour with no apparent complications, accounts for approximately 50% of all women who deliver spontaneously before term (World Health Organization 1969). Prevention of idiopathic preterm labour would thus have a considerable impact on perinatal morbidity and mortality (Bernal et al. 1993), and substantially decrease health care costs associated with caring for such premature infants. This requires an understanding of the processes involved in labour initiation.
2.1.2 Human uterine anatomy

During fetal life the uterus develops from the mullerian ducts, also known as the paramesonephric ducts, which form two tubes whose ends fuse (O'Rahilly 1989). Depending on the extent of the fusion, the uterus consists of either two horns (bipartite), as in rats, ewes, goats, and rabbits, or one cavity as in primates. Anatomically, the pregnant human uterus can be divided into three parts; the uterine body, the cervix, and the isthmus, which is the junction between the two other parts (Figure 2.1). The uterine wall is composed of three types of tissues: an inner mucosa, the endometrium; a surrounding muscle coat, the myometrium; and a thin external serous coat, the serosa (Figure 2.1). In animals with a bipartite uterus the myometrium consists of an outer longitudinal layer and an inner circular layer, which cross at right angles. In the pregnant human myometrium the organization of the muscle cells within the myometrium are similar but not as well defined anatomically as they are in animals with a bipartite uterus. The human myometrium is generally separated into either an outer longitudinal layer and an inner circular layer, or into three layers which are described as: 1) external layer with two sublayers (internal circular fibers and outer longitudinal fibers), 2) an intermediate layer that forms the most important part of the myometrium where fibers are diagonally interlaced, and 3) an internal layer containing circular fibers (Breuiller et al. 1987; Mitchell et al. 1990; Pinto et al. 1967c) (Figure 2.1). At the end of pregnancy as the uterus increases in size the external longitudinal layer tends to be localized to the uterine fundus and body, whereas the inner layer may be predominant in the isthmic region (Bengtsson 1982). The percentage of muscle fibers at term is 68.8% in the body, 28.8% at the top of the cervix, and only 6.4% at the bottom of the cervix (Schwalm and Dubrauszky 1966) (Figure 2.1).
Figure 2.1: Schematic diagram of human uterus. The major layers in the uterine wall from the most external are: serosa; outer/external/sub-serosal layer which is composed primarily of longitudinal muscle fibers; vascular/intermediate/plexiform layer which is composed of diagonally interlaced muscle fibers; inner layer which is composed of circular muscle fibers; and the most internal layer the endometrium/decidua (Breuiller et al. 1987; Devedeux et al. 1993). The body of the uterus contains a higher percentage of muscle fibers than does the cervix (Krantz 1973; Schwalm and Dubrauszky 1966). The most predominant layer in the uterine body is the outer longitudinal layer, and in the cervix it is the inner circular layer (Devedeux et al. 1993).
Muscle fiber content of tissue

**Body of uterus**
(primary layer = outer longitudinal)

**Isthmus**

**Cervix**
(primary layer = inner circular)

- Outer/external/sub-serosal layer (1st longitudinal muscle fibers)
- Vascular/intermediate/plexiform layer (diagonally interlaced muscle fibers)
- Inner layer (circular muscle fibers)

68.8%

28.8%

6.4%
2.1.3 Uterine contractions

The uterus can contract throughout pregnancy. During the first 30 weeks it exhibits very low amplitude contractions known as Alvarez waves, at a frequency of approximately one per minute (Caldeyro-Barcia and Poseiro 1960). From the twentieth week higher amplitude and lower frequency (one every 3 to 4 hours) contractions known as Braxton Hicks contractions, or contractures, appear. The frequency and strength of these contractions increase up to the end of pregnancy. At parturition a contractile wave is fully propagated through the whole uterus in a short time (about 20 seconds) (Wolfs and vanLeeuwen 1979). The intricate interlacing of smooth muscle bundles though the various layers of the human myometrium allows for such an isotropic contractile wave. Since myometrial cells are not organized into fibers, fibrils, and filaments as are skeletal muscles they require a functional link between the cells. Gap junctions perform such a role. Because of the intracellular organization of myometrial smooth muscle cells, this muscle can exert a pulling force in any direction (Stauffer et al. 1991).

2.1.4 Diurnal rhythm of uterine contractions

The majority of births occur during the late night or early morning hours in humans (Rodger and Baird 1987). Moore et al. (1994) examined 100 low risk pregnant women who delivered at term and recorded uterine contractions for 24 hours twice weekly from 20 to 40 weeks gestation. They found a strong diurnal rhythm present from 24 weeks onward, with 67% of contractions occurring at night. Contractions per hour increased with gestational age but rarely exceeded three per hour before term. Germain et al. (1993) measured uterine activity of women for 24 hours every 2 weeks from 26 weeks until delivery. They found that women had a nocturnal surge (4-7 am) in uterine activity 80 days prior to delivery. In women who delivered at term, this pattern was maintained through to delivery, while in patients who delivered preterm this pattern disappeared 24 hours prior to delivery. Germain et al. (1993) thus concluded that the diurnal rhythm is not a prerequisite for delivery but rather a characteristic of term labour. Several investigators have demonstrated marked circadian rhythmicity in uterine contractility of monkeys in
late pregnancy. There is increased activity during the night and relative quiescence during daylight hours. The frequency and duration of night time contractions increase during gestation until delivery occurs. In addition to a circadian pattern in myometrial contractions, Harbert (1977) found in rhesus monkeys a nocturnal increase in uterine blood flow, a decrease in blood pressure, and a decrease in intraamniotic pressure in the uterus during the last half of pregnancy. Taylor et al. (1983) examined the effects of the fetus on the circadian oscillation of myometrial contractions in the pregnant rhesus monkey. They found that fetal death abolished the circadian variation in contractile episodes, suggesting that the fetus plays a role in the regulation of these types of uterine activity in the Rhesus monkey. The increase in nocturnal contractions may be ultimately due to a diurnal decrease in those agents, such as progesterone, which inhibit labour, and/or to an increase in those agents, such as estrogen, which facilitate labour. This idea is supported by the results of the study conducted by Wilson et al. (1991). They examined circulating estradiol and P₄ levels baboons throughout the last trimester of pregnancy, and found a 3-5 hour time period at night in which the ratio of estrogen to P₄ (E/P₄ ratio) is significantly increased. This increase in E/P₄ ratio may be the trigger for the increase in nocturnal contractions leading up to labour in primates.

2.1.5 Different uterine layers and their contractile responses

The layers of the human myometrium appear to differ not only in their cellular orientation, but also with respect to distribution of adrenergic receptors which are capable of mediating a contractile response. There are at least two types of α-adrenergic receptors, α₁ and α₂, and at least two types of β-adrenergic receptors, β₁ and β₂ in the myometrium. Relaxation of myometrial cells occurs for the most part via β-adrenergic receptors and the resultant increase in cAMP. Contraction of myometrial cells is mediated by α-adrenergic receptors. Agonistic binding to α₁-adrenoceptors results in an increase in intracellular calcium through phosphatidylinositol turnover while the α₂-receptors are involved in adenylate cyclase inhibition. Breuiller et al. (1987) examined β-adrenergic receptors in human uterus and found at gestational week 35 the density of β-adrenergic receptors in the inner (circular) myometrial layer was about 50% higher than in the outer (longitudinal) myometrial layer. At term they found the densities of β-adrenergic receptors to be reduced and
exhibit the same values for both layers. Breuiller et al. (1988) examined \( \alpha_2 \)-adrenoceptors in inner and outer layers of the human uterus (Figure 2.1). They found the binding characteristics of both the outer and inner muscle layers to be the same, but the density of \( \alpha_2 \)-receptors in the inner layer was significantly higher than in the longitudinal layer (Breuiller et al. 1988). These results, along with those of other studies (Daels 1974; Pinto et al. 1967c), suggest that the inner layer of the myometrium is more responsive to contractile stimuli via \( \alpha \)-adrenergic receptors, while the outer layer is more responsive to relaxant stimulation via \( \beta \)-adrenergic receptors.

The layers of the human myometrium also differ in their sensitivity to various contractile and relaxing agents. Daels (1974) performed in vitro studies of spontaneous motility and the effect of adrenaline and oxytocin on sections of human myometrium taken from different parts of nonpregnant and pregnant uteri. The outer layer of the non-pregnant human uterus was stimulated by epinephrine, while the inner layer was unresponsive to this amine (Daels 1974). In the post-partum myometrium, epinephrine relaxed the outer segment and stimulated the inner (Daels 1974). Pinto et al. (1967c) examined strips of human myometrium obtained during classical cesarean sections. They divided the myometrium into three layers (external or superficial, medial and internal). They then recorded their activity in response to various agents. The inner layer contracted the greatest in response to oxytocin, while the external layer contracted the least. Estradiol reduced the latent period in response to oxytocin of the three layers, and increased the frequency or intensity of the contractions in all three layers. Spontaneous uterine activity was greatest in the internal layer. While such activity was occasionally measured in the middle layer, it rarely occurred in the external layer. Taken together, these results suggest that the inner (circular) muscle layer of the human uterus is primarily responsive to relaxation agents during gestation and primarily responsive to stimulatory agents during labour.

In animals with a bipartite uterus such as the rat the outer longitudinal and the inner circular muscles are more easily separated than in the human uterus (Kawarabayashi and Marshall 1981). While the functional roles of the circular and longitudinal muscles are not entirely clear, it has been suggested that contractions of the circular muscle during pregnancy prevent movement of the fetuses towards the cervix and that contractions of the longitudinal favor this movement (Nesheim 1972).
The electrophysiological and mechanical behaviour of the two myometrial layers in the rat differ (Anderson et al. 1981; Kawarabayashi and Osa 1976; Osa and Katase 1975), as do their responses to various drugs. Osa and Katase (1975) examined these muscle layers in pregnant rat uteri for 10-15 days by means of electrical and mechanical recordings. Application of stretch caused acceleration of spontaneous activity that was greater in extent in the inner circular muscle than in the outer longitudinal muscle. Stimulation of the longitudinal muscle caused membrane response in the circular muscle cells, and vice versa, suggesting electrical interference between longitudinal and circular muscle cells (Osa and Katase 1975). In the late term pregnant rat, α-adrenergic receptors which stimulate myometrial contraction are more abundant in the circular muscle layer, while β-adrenergic receptors which mediate a relaxation response are found primarily in the longitudinal muscle layer (Garfield et al. 1979; Kawarabayashi and Osa 1976). Anderson et al. (1981) examined the effects of two prostaglandin synthesis inhibitors, indomethacin and aspirin, on the longitudinal and circular muscle of the rat uterus. Microelectrode and tension records were obtained from isolated muscle strips from animals on days 17-21 (delivery day) of gestation. They found these drugs to delay parturition in rats by at least 24 hours. They also found the actions of these drugs were different in the two muscle layers and were unique for each drug.

2.1.6 Major physiological roles of progesterone in mammals

The steroid hormone progesterone (4-pregnen-3,20-dione, \( P_4 \)) is one of the major hormones of the female reproductive system and is essential for pregnancy. \( P_4 \) is produced by the corpus luteum, placenta and in minute amounts by the adrenal cortex. It has major physiological roles in the ovary, uterus, mammary glands, brain, bone, and immune system [see (Graham and Clarke 1997; Noci et al. 1994) for reviews]. In the ovary it helps control the final stages of folliculogenesis and regulates the release of mature oocytes, as well as transport of the ovum or zygote(s) through the oviduct (Mahesh et al. 1996). \( P_4 \) activates uterine proteases which lyse the zona pellucida (Renfree 1980). To prepare the uterus for implantation, \( P_4 \) stimulates cytodifferentiation and proliferation of estrogen-primed endometrial cells and initiates decidualization of the stromal cells around the spiral arteries. The sudden withdrawal of \( P_4 \) action at the end of the luteal phase leads to
a constriction of spiral arteries in the uterus, inducing menstruation in primates. In the presence of an embryo however, P₄ levels are maintained and decidualization continues [see (Lockwood et al. 1997) for a review]. After the placenta has developed and the fetus begins to grow, P₄ inhibits uterine myometrial contractions. P₄ is believed to do so by suppressing the excitability of uterine muscle by increasing the number of β₂-adrenergic receptors in myometrium (Putnam et al. 1991), and by suppressing the responsiveness of the uter to oxytocic stimuli [see (Challis and Lye 1994) for a review]. P₄ maintains cervical rigidity for most of pregnancy. Also, during pregnancy, P₄ is believed to suppresses many local inflammatory responses initiated by prostaglandins and cytokines. P₄ may play an important role in the maternal adaptation of the cardiovascular system during pregnancy. In mammary glands it is important for lobular-alveolar development in preparation for milk secretion, as well as for suppression of milk protein synthesis prior to parturition. In the brain of rodents P₄ mediates signals required for sexually responsive behaviour. P₄ also has a general metabolic effect by increasing basal and glucose-induced insulin production (Beck 1977). Finally, P₄ may also play a role in the maintenance of bone mass (Bowman and Miller 1996).

P₄ controls a number of endometrial proteins. In humans, P₄ stimulates endometrial secretion of various enzymes for carbohydrates, protein and prostaglandin metabolism, phosphatases, hydrolases, prolactin, and others. Intracellular components in the endometrium which are under P₄ control include P₄ receptors, estrogens, and estradiol metabolism enzymes, alphafucosidase, and type II cyclic AMP dependent protein kinase. In the myometrium P₄ inhibits estrogen receptors, P₄ receptors, type I cAMP dependent protein kinase and others [see (Savouret et al. 1988) for a review].

2.1.7 Progesterone synthesis and secretion

The ovary is the primary site of estrogen and P₄ synthesis and secretion in mammals. Circulating levels of these steroids fluctuate cyclically. Following ovulation, follicular granulosa cells mature and form the corpora lutea. It is this structure which is responsible for the production of estrogen and P₄ in the latter part of the cycle. If the ovum is not fertilized the corpus luteum will
soon regress. If fertilization occurs it will continue to grow and function. In humans, it does so for the first 2 to 3 months of pregnancy. After this time it slowly regresses as the placenta assumes the role of hormonal biosynthesis for the maintenance of pregnancy. This process is termed the ‘luteal-placental shift’. In other species, such as rodents, the corpora lutea remain the primary site of estrogen and P₄ production for the duration of gestation [see (Heap and Flint 1984) for a review].

2.1.8 Progesterone levels at term

P₄ is believed to play an essential role in maintaining uterine quiescence during pregnancy. In most mammals, labour is heralded by P₄ withdrawal, which is believed to be related to the activation of multiple pathways leading to parturition. In species such as the rabbit and rat, corpora luteal P₄ production is maintained throughout pregnancy and drops at the time of labour initiation (Figure 2.2). Ablation of the corpora lutea or ovaries leads to abortion. Similarly, normal parturition in such species can be prevented by exogenous P₄ administration (Bedford et al. 1972; Csapo 1961). As mentioned earlier, the placenta is the primary source of P₄ production after the sixth to ninth week of gestation in the human (Challis and Lye 1986). In non-primate animals such as sheep, cows, goats and rats, parturition is preceded by a precipitous decrease in P₄ and a rise in estradiol in the blood (Harris et al. 1993; Wilson et al. 1991). In humans there is no such fall in maternal plasma P₄ (Challis and Lye 1986) (Figure 2.2). In humans and other primates, despite a maintenance of placental P₄ production, activation of similar pathways may precede labour. Csapo (1971) collected serial blood samples from women with normal pregnancies during their last 7 weeks of gestation and spontaneous labour. They found plasma P₄ levels peaked at various times during the last 4 weeks of gestation in individual patients but subsequently decreased slightly with
Figure 2.2: Maternal circulating levels of progesterone (ng/ml) and estrogen (ng/ml) during pregnancy in four different species: humans, rats, sheep, and guinea-pigs. Figure adapted from Bedford et al. (1972).
the onset of clinical labour (Csapo et al. 1971). Most studies examining circulating levels as well as myometrial tissue levels of P₄ and estrogen however, have not found them to change significantly at term nor with preterm birth (Anderson et al. 1985; Bernard et al. 1988; Block et al. 1984; Cousins et al. 1977; Romero et al. 1988). Caldeyro-Barcia and Poseiro (1960) found that administration of large intramuscular doses of P₄ (400 mg per day for 4 days) to women at term did not abolish spontaneous contractility. Therefore, labour initiation in humans cannot be explained by a simple drop in maternal circulating levels, or myometrial tissue levels of P₄.

2.1.9 Circulating progesterone levels, bound verses free

In the serum of humans and most other mammals, P₄ and a number of other steroids may be bound to either albumin, corticosteroid-binding globulin (CBG) also known as transcortin, or α₁-acid glycoprotein (AAG) also known as orosomucoid (Pasqualini et al. 1985; Westphal et al. 1977). Steroids that are associated with these proteins are biologically inactive and can be activated by dissociation to the unbound hormone (Pasqualini et al. 1985). Binding of steroid hormones to these proteins: protects them from chemical or enzymatic attack, resulting in decreased metabolic clearance (Westphal et al. 1977); facilitates steroid transport (Westphal et al. 1977); and may be important for providing a steroid reservoir in order to have a rapidly available supply of the hormone should it be needed (Rosner 1990).

The effects of P₄ may be attenuated by decreasing the amount of it which is biologically active, that is, by increasing the amount that is associated with binding proteins in the serum thus decreasing the amount of available free hormone. Thus, it is conceivable that the effects of P₄ could be attenuated without a decrease in total circulating levels of P₄. The production rate and plasma concentration of P₄ increase significantly during pregnancy but only a small fraction of the native hormone, 5-7% of the total circulating P₄, is present in an unbound form (Buster et al. 1979). Batra et al. (1976) measured serum P₄ and estradiol-17β in women weekly from 23rd week of gestation until delivery. They found unbound/free P₄ increased with increasing gestational age; 6% of the total P₄ was unbound at week 24, and 13% was unbound at week 40. Both free and total P₄ decreased markedly very soon (2 hours) after delivery (Batra et al. 1976). They also measured
estradiol in the same women. At no time prior to delivery did they find a significant fall in P₄ (free or bound to plasma proteins) or an abrupt rise in estrogen (Batra et al. 1976). Tulchinsky and Okada (1975) measured bound and unbound P₄ in maternal serum and did not see a significant change in that bound or unbound between luteal phase, first trimester pregnancy, or term. Using radioimmunoassays, Willcox et al. (1985) examined concentrations of total and free (unbound) P₄, estradiol-17β in the plasma of pregnant women once daily from 10-17 days before delivery, during labour, and for 2-3 days postpartum. They examined samples from women who laboured spontaneously, as well as women whose labour was induced via fetal membrane rupture. The total concentrations of circulating P₄ and estrogen were not found to change significantly from 10-17 days preterm to delivery. This result was independent of whether total or free steroids were measured (Willcox et al. 1985). Overall then, the relative amount of free and serum protein bound P₄ in maternal plasma does not redistribute in favor of the physiologically inactive, bound form in women before labour (Willcox et al. 1985).

2.1.10 Estrogen/progesterone ratio

While P₄ maintains pregnancy, estrogen is important for stimulating a number of factors which ultimately lead to uterine contractions. Therefore, the ratio of these two hormones, E/P₄ ratio, may be more important than their absolute levels for the initiation of labour (Lye et al. 1993). A number of investigators have attempted to find such a change in E/P₄ ratio with the onset of labour in humans. Hartikainen-Sorri et al. (1981) measured P₄ and estradiol-17β weekly in plasma of pregnant women during the third trimester by radioimmunoassay. They found large individual variations in the levels of both hormones. The mean concentrations of both rose with advancing gestation but they did not find a change in the E/P₄ ratio. Moran et al. (1992) examined levels of estradiol-17β and P₄ in the saliva of women every 6 hours from 14 days pre-partum, to labour. They found a slight but non significant rise in the mean saliva E/P₄ ratio in the six women studied who laboured spontaneously, but no change in the 10 women whose labour was induced. The E/P₄ ratios were significantly higher in the spontaneous labour group than in the induced group. While there does not appear to be a significant change in E/P₄ ratio in maternal circulation, or in human
saliva, uterine contractions may be stimulated by local changes in the ratio of these two hormones which may not be reflected in the circulation. Romero et al. (1988) measured \( P_4 \), estradiol-17β and estriol in human amniotic fluid. They found a significant increase in estrogen levels, but no change in \( P_4 \) levels with the onset of labour. Thus, the \( E/P_4 \) ratio was significantly increased for women in active labour, compared to those women not in labour in amniotic fluid (Romero et al. 1988). Mazor et al. (1993) found similar results in human amniotic fluid. The ratio of \( E/P_4 \) has also been examined in human urine yet no differences in this ratio were found with the onset of labour (Anderson et al. 1985).

It is possible that changes in the \( E/P_4 \) ratio may be subtle and thus not detected by daily measurements of human serum, saliva, or urine. Wilson et al. (1991) examined circulating estradiol and \( P_4 \) levels in baboons throughout the last trimester of pregnancy, and found a significant circadian rhythm, with both surging nocturnally. During most of the third trimester the \( P_4 \) surge preceded the estradiol surge, but towards labour the timing of the estradiol surge changed such that it preceded that of \( P_4 \). Concurrent with this estrogen surge is an increase in myometrial contractions. As discussed in section 2.1.4., Wilson et al. (1991) proposed that this 3-5 hour time period in which the \( E/P_4 \) ratio is significantly increased initiates nocturnal uterine contractions by induction of biochemical alterations and generates other molecular messages that lead to labour and delivery in the baboon.

2.1.11 Progesterone inhibition of uterine contractions

As discussed earlier, \( P_4 \) inhibits uterine contractions by blocking excitation and conduction mechanisms within the myometrium (Mijovic and Olson 1996). During most of gestation the myometrium is unable to conduct action potentials, thus activity is not propagated but is confined to local areas of the uterus (Alvarez waves or Braxton Hicks contractions); in a \( P_4 \) dominated uterus only the segment actually stimulated contracts (Romanini 1994). In the absence of coordinated propagated activity there can be no significant development of intrauterine pressure. \( P_4 \) hyperpolarizes the cell membrane, decreasing action potential amplitude and frequency (Csapo 1961; Kao and Nishiyama 1964; Kuriyama and Csapo 1961; Kuriyama and Suzuki 1976; Marshall
Further, high levels of \( P_4 \) maintain low levels of intracellular calcium in myometrial smooth muscle, preventing a response to stimulators of myometrial contractility. \( P_4 \) also inhibits the formation of gap junctions (Csapo 1961). Pinto et al. (1967b) examined the effects of \( P_4 \) on the contractile activity of the three myometrial layers in the uterine body and of the lower segment in human pregnancy at term. \( P_4 \) diminished both the spontaneous uterine activity and the activity elicited by oxytocin in the three layers of both segments, especially those of the internal and medial layers.

Tissue concentrations of \( P_4 \) and estrogen have been examined in different layers of the myometrium. No differences however, have been found in the endogenous content of \( P_4 \), estradiol-17\( \beta \) or estrone between inner and outer myometrial layers when tissues adjacent to the site of placental attachment were examined (Ferre et al. 1978). Higher \( E/P_4 \) ratios were found at placental sites (Ferre et al. 1978).

2.1.12 Estrogen direct and indirect stimulation of uterine contractions

A change in the steroid environment from \( P_4 \) to estrogen dominance causes activation and stimulation of the myometrium and effects labour. Estrogens induce uterine hypertrophy, hypopolarize the cell membrane, increase action potential amplitude, the length of action potential bursts, and the number of action potentials per burst (Kao and Nishiyama 1964; Marshall 1959). Pinto et al. (1967a) administered 17\( \beta \)-estradiol (200 mg once daily, intravenously) to pregnant women at term and reported that estradiol treated women started labour significantly earlier than non-treated women (estrogen treated = 65 hours, control = 107 hours). Pinto et al. (1967c) also examined strips of human myometrum and found that treatment of these muscle strips with estradiol increased uterine tone and contraction intensity of all layers.

Estrogen can enhance uterine contractions indirectly by stimulating the formation of gap junctions (Chow and Lye 1994; Garfield et al. 1980; Lye et al. 1993). The most abundant myometrial gap junction protein has been identified as connexin-43 (cxn-43). In both pregnant and non-pregnant rats estrogen acts to increase the level of cxn-43 transcripts. \( P_4 \) can both block and reverse this action of estrogen (Chow and Lye 1994; Lye et al. 1993).
An increase in estrogen is believed to increase the number of oxytocin receptors in the uterus (Alexandrova and Soloff 1980). Estrogen also stimulates endocrine and paracrine production of the oxytocin peptide (Chibbar et al. 1995). Oxytocin synthesized in the posterior pituitary and in the fetal membranes, myometrium and placenta, leads to uterine contractions through a number of ways. It stimulates phosphatidylinositol hydrolysis in myometrial cells which results in increased inositol triphosphate generation, a second messenger step leading to an increase in intracellular calcium levels, and therefore myometrial contraction. This hydrolysis reaction also results in the release of arachidonic acid, a substrate for prostaglandins. Oxytocin also induces the production of endothelin-I in the decidua. This agent is as potent as oxytocin in inducing myometrial contraction by inducing calcium efflux into myometrial cells (Dawood et al. 1978, Fuchus 1986).

Estrogen also affects uterine contractions possibly by stimulating prostaglandin production, in particular, the prostaglandin isoforms PGE$_2$ and PGF$_{2\alpha}$. An increase in amnion, chorion and decidual production of prostaglandins, and rising levels of prostaglandins in amniotic fluid are thought to be important steps leading to increased uterine contractions, cervical change, and birth (Biswas and Craigo 1994; Collins et al. 1993; Green et al. 1974; Hollingsworth et al. 1994; Mitchell and Wong 1993; Williams and Collins 1976). PGF$_{2\alpha}$ and PGE$_2$ are thought to enhance calcium transport through plasma membranes of intracellular organelles within smooth muscle cells, ultimately resulting in cellular contraction. The rate-limiting step in metabolism of prostaglandins is the enzyme 15-prostaglandin dehydrogenase (PGDH). This enzyme is found in most intrauterine tissues, especially the chorion. P$_x$ stimulates PGDH, thus, during most of pregnancy PGDH levels are high, reducing the bioactivity of any prostaglandins formed. Local withdrawal of P$_x$ at term may reduce PGDH activity resulting in an increase in prostaglandin bioactivity [see (Challis and Lye 1994) for a review]. Prostaglandins in amniotic fluid may not in fact cross the fetal membranes in sufficient quantities to affect myometrial contractility (Collins et al. 1992). Therefore, prostaglandins in amniotic fluid are not believed to be a major stimulus of uterine contractions (Collins et al. 1993). Clinically, PGF$_{2\alpha}$ has been used in the form of vaginal suppositories to induce labour. PGE$_2$ cervical and vaginal gels are also used to prime or ripen the cervix prior to induction of labour. As well, parturition may be lengthened by the administration of...
cyclooxygenase inhibitors such as aspirin or indomethacin which prevents prostaglandin production and thereby suppress uterine activity (Challis and Lye 1986).

2.1.13 Role of the fetus in the initiation of labour

The ovine fetus plays a key role in the initiation of labour. The process begins in the fetal brain where hypothalamic paraventricular neurons are stimulated to secrete corticotrophin-releasing hormone (CRH). CRH regulates anterior pituitary corticotrope biosynthesis and release of adrenocorticotropin hormone (ACTH). ACTH levels begin to rise between 120-130 days of gestation (parturition 145-150 days). ACTH in turn stimulates the fetal adrenal P450 steroid hydroxylase enzymes necessary for de novo cortisol synthesis. Fetal plasma cortisol levels begin rising over the last 8 days of gestation. The increased levels of cortisol stimulate the enzyme 17α-hydroxylase which converts P₄ into 17α-hydroxyprogesterone, an estrogen precursor (Figure 2.3) [see Challis and Lye (1994) for a review]. As a result, P₄ production decreases while estrogen production increases (Challis et al. 1985). This change in the E/P₄ ratio promotes uterine activation, and increases the synthesis of uterotonic contractile agents resulting in myometrial contractions associated with labour.

It is unclear whether the human fetus plays an important role in the initiation of labour or not. Anencephaly and fetuses with hypoplastic adrenals do not have an increased incidence of prolonged pregnancy, although the precise timing of labour is disputed in these cases (Mijovic and Olson 1996). Administration of a synthetic glucocorticoid such as dexamethasone fails to induce labour in humans except in some patients already post-term (Pasqualini and Kincl 1985a). The human fetal adrenal gland is involved in parturition through the synthesis and secretion of steroids such as the C19 estrogen precursors, dehydroepiandrosterone (DHEA, Figure 2.3), and its sulfated form, DHEAS. After 6-9 weeks of gestation the placenta is the major site of steroid production in human pregnancy. Unlike the ovine placenta, the primate placenta can not synthesize estrogens de novo from cholesterol since it lacks the 17α-hydroxylase/C17-20 lysase enzymatic system necessary for this. However, the primate placenta is capable of converting C19 steroids into estrogen (Pepe and Albrecht 1995). In human and nonhuman primates maternal plasma
concentrations of unconjugated estrogens rise progressively throughout gestation reaching peak levels at term. This may be a result of the increase in DHEA and/or DHEAS synthesis.
Figure 2.3: Synthesis of the major sex steroids from cholesterol and the enzymes involved. C27, C21, C19, and C18 refer to the number of carbons which compose that group of steroids.
2.1.14 The contribution of fetal membranes to the initiation of labour

Local changes in estrogen and P₄ synthesis, not reflected in the maternal circulation, may significantly influence the progress of human parturition (Mitchell and Wong 1993). The fetal membranes are interposed between the fetal and the maternal compartments and are thus ideally positioned to regulate signals between fetus and mother. The amnion, chorion, and maternal decidua produce numerous hormones necessary for the maintenance of pregnancy, and because of the intimate anatomical relationship between these tissues and the myometrium, their metabolic activity may be an important determinant of myometrial contractility (Casey and MacDonald 1986; Liggins 1989; Mitchell and Powell 1984). Collins et al. (1993) found that human fetal membranes reversibly inhibit uterine contractions. They developed a dual chamber fetal membrane-uterine muscle model to study the effect of human fetal membranes on spontaneous uterine contractions in women. They found that amnionic, chorionic, and decidual tissues resulted in a 40% decrease in myometrial contraction strength and frequency, compared to basal conditions in which there were no membranes present. This inhibition was reversible after removal of the membranes.

The amnion, chorion, and decidual membranes may directly affect uterine contractions in part by their affects on prostaglandin production and metabolism as discussed in section 2.1.12. Also, there may be local regulation of P₄ within the chorion, amnion and decidua (Challis and Lye 1986; Lofgren and Backstrom 1994). Local changes in P₄ metabolism, especially in association with rising estrogen concentrations might provide a mechanism to effect a local P₄ withdrawal and promote uterine contractility at the time of labour (Challis and Lye 1986; Harris et al. 1993; Lofgren and Backstrom 1994; Martini et al. 1993). Both estrogen and P₄ are synthesized within human amnion, chorion, decidua, and placenta. Mitchell and Challis (1988) found an increase in the enzyme 17β-20α-hydroxysteroid dehydrogenase which converts P₄ into 20α-dihydroprogesterone in vitro, in the amnion, chorion, decidua and placenta, suggesting a local withdrawal of P₄ in association with human parturition.
2.1.15 The cervix as a monitor of labour progress

Changes in the cervix are an important marker of labour progress (Danforth and Ueland 1986). The cervix is essentially a fibrous connective-tissue structure; consisting primarily of tight bundles of collagen fibers with a few muscle cells irregularly scattered throughout (Danforth and Ueland 1986). The myometrium ends at the cervix and inserts into it much like a skeletal muscle inserts into an aponeurosis. The cervix remains thick, rigid, and closed until a few weeks before term. At this point the cervical canal maintains its non-pregnant length of 2-3 cm but begins to unfold and becomes softer. This change is referred to as effacement. As effacement continues, the cervix dilates slightly so that it becomes possible to introduce a finger into the cervical canal without resistance. When labour starts, the cervix is usually soft, about half of the canal has been taken up, and the external os is dilated approximately 2 cm. As labour contractions are established, further shortening occurs and the cervix becomes thinner but cannot yet be forcibly dilated. When labour is well established, effacement proceeds rapidly, and at 6-7 cm dilation the cervical thinning and softening are complete.

2.2 Progesterone receptors

2.2.1 Steroid hormone receptors -- general characteristics

The cellular actions of steroid hormones are mediated through intranuclear receptor proteins which function as ligand-modulated transcription factors. These hormone receptors belong to a large superfamily of nuclear proteins which includes receptors for glucocorticoids, mineralocorticoids, retinoic acid, androgen, estrogen, P₄, vitamin D, and thyroid hormone. The receptor proteins all have common structural domains responsible for properties such as ligand binding, nuclear localization, receptor phosphorylation, dimerization, deoxyribonucleic acid (DNA) binding, and transcription activation. (Goodman and Hodgen 1996; Kraus et al. 1993; MacLusky 1996) (Figure 2.4). The c-terminus contains the ligand binding domain, the central portion of the molecule contains a DNA binding domain, and the N-terminus is required for maximal
transactivation function (Edwards et al. 1993; MacLusky 1996) (Figure 2.4). The half life of $P_4$ receptors in a cell is approximately 2.5 hours (Mullick and Katzenellenbogen 1986).

2.2.2 Progesterone receptor isoforms

Hormone receptors for estrogen (Grandien et al. 1997), androgen (Wilson and McPhaul 1996), and $P_4$ exist in more than one form. The mammalian and avian $P_4$ receptors examined to date are expressed in two isoforms, with the exception of the rabbit in which only one isoform has been identified (Goodman and Hodgen 1996; Kastner et al. 1990a; Kastner et al. 1990b; Kraus et al. 1993). The two isoforms are referred to as the $P_4$ receptor A and B isoforms (Sartorius et al. 1993). These isoforms are either transcribed from two different promoters on the same gene (humans, rats, and mice $P_4$ receptors) (Kastner et al. 1990), or from alternate initiation of translation at two sites within the same mRNA (chicken $P_4$ receptor) (Conneely et al. 1989). The $P_4$ receptor A isoform is a truncated version of the $P_4$ receptor B; the human $P_4$ receptor A protein lacks the first 164 amino acids present at the amino terminus of the $P_4$ receptor B molecule which is a total of 933 amino acids in length (Chwalisz 1994; Fujimoto et al. 1995; Goodman and Hodgen 1996). The site of initiation of transcription lies between +1 and +15, and between +737 and +842 base pairs, for the human B and A $P_4$ receptor isoforms respectively (Fujimoto et al. 1995). Thus, the unique N-terminal region found in the B isoform is believed to ultimately result in the differences in activity between the two isoforms (Goodman and Hodgen 1996) (Figure 2.4).

Wei et al. (1996) described a third isoform of the $P_4$ receptor; the $P_4$ C isoform. Translation of this C isoform is initiated at a methionine 3' to the methionine start sites which generates the larger 94 kilodalton A and 116 kilodalton B human $P_4$ receptor isoforms. Because the C $P_4$ receptor isoform lacks the first zinc finger of the DNA binding domain, but contains sequences for dimerization, Wei et al. (1996) reasoned that it would be transcriptionally inactive and not bind DNA directly. Surprisingly, however, in the presence of A and/or B receptors, Wei et al. (1996) found that the C isoform can modulate transcriptional activity of these receptors. They argued that the C isoform is not a degradation product of the two larger $P_4$, receptor isoforms but a
distinct P₄ binding protein (Wei and Miner 1994). The truncated C P₄ receptor may help to explain some of the pleiotropic effects of P₄ (Wei et al. 1996).
Figure 2.4: Schematic illustration of the progesterone (P4) receptor mRNA and protein isoforms. The bold letters within the mRNA section are frequently used when describing steroid receptor regions. The A/B region is cell and promoter specific and contains at least one activation domain. The C region contains the zinc fingers necessary for DNA binding as well as the sequence necessary for receptor dimerization. The D region is referred to as the 'hinge' domain which links the steroid-binding and DNA-binding regions. The E region is the site of hormone binding, as well, it contains an activation domain. This region is ligand-dependent as well as cell and promoter-specific. The C and E regions are the most evolutionarily conserved. Sequences within the C, D and E regions are necessary for receptor binding to various proteins such as heat-shock proteins, coregulators, as well as other transcriptional regulators. Sequences within the F region are believed to be involved in transcriptional regulation. See MacLusky (1996) and Shibata et al. (1997) for reviews.

The P4 receptor is made up of 8 exons (Misrahi et al. 1987). The translation of the B isoform of the P4 receptor begins at nucleotide 744 (amino acid 1), the A isoform at nucleotide 1239 (amino acid 165), and the putative C isoform at approximately 2529 (amino acid 595) (Fujimoto et al. 1995; Wei et al. 1990; Wei and Miner 1994). The DNA binding domain begins at amino acid 556 (Takimoto et al. 1991) or 567 (Sheridan et al. 1989a). One activation domain (AF1) is located in the A/B region just upstream of the DNA binding domain (amino acids 456-546), a second activation domain (AF2) is located in the E region, and a third (AF3) has been described in the 164 amino acid region which is unique to the B isoform of the P4 receptor (Meyer et al. 1992; Sartorius et al. 1994b; Shemshedini et al. 1992). Basal serine phosphorylation sites are located at amino acids 81, 162, 190, and 400. Hormone dependent serine phosphorylation sites are located at amino acids 102, 294, 345 (Beck et al. 1996; Zhang et al. 1997). PR(C-20) refers to location of the peptide for which this antibody from SantaCruz Biotechnology, Inc., CA, USA, was directed against. The boxes on the mRNA band labeled PCR B and PCR A+B denote the location of cDNA regions amplified by polymerase chain reaction in Chapter 4.
Progesterone receptor

Exon 1

mRNA

Protein

5' Bases

Amino acids

Basal phosphorylation (Serine) sites

Hormone dependent phosphorylation (Serine) sites

AUG 1

AUG 2

AUG 3

81 162 190

102 294 345

545-564

PR(C-20) antibody

COOH

COOH

COOH

3'
2.2.3 Ligand binding

The first step in P4 receptor activation is binding of the specific ligand (hormone, agonist, or antagonist) at the hormone-binding domain (Goodman and Hodgen 1996). While ligand binding is no longer believed to be essential to all steroid receptor actions (Duval et al. 1983), it causes conformational changes in the receptor that permit the subsequent steps of receptor modification and DNA binding to affect transcription (Allan et al. 1992a; Allan et al. 1992b; Goodman and Hodgen 1996). Hormone binding also renders the entire ligand binding domain relatively resistant to digestion by proteases (Allan et al. 1992a). The conformational changes appear to differ in response to the particular ligand to which the receptor is bound (Allan et al. 1992a). For example, the ligand binding domain of agonist-occupied P4 receptors differs from that of antagonist occupied P4 receptors (Allan et al. 1992a). As hormone binding is the biological signal, the question arises whether hormone binding and steps associated with it, such as conformation changes in the receptor, are different between the P4 receptor A and B forms. Conformational changes related to ligand binding occur in the carboxy-terminal domain of steroid receptors (Allan et al. 1992a). The A and B isoforms of the P4 receptor differ only in their amino-terminal end, yet they display different conformations when bound to RU486, a P4 antagonist (Carbajo et al. 1996). The amino-terminal domain of the P4 receptor must therefore influence the carboxy-terminal ligand-binding domain either directly or indirectly (Carbajo et al. 1996).

2.2.4 Phosphorylation of the progesterone receptor

Phosphorylation is the metabolic process of introducing a phosphate group into an organic molecule. Phosphorylation plays an important role, as it does for many other proteins, in the regulation of steroid receptors (Kuiper and Brinkmann 1994; Sheridan et al. 1989a). In general, phosphorylation of steroid receptors occurs on serine or threonine residues, although tyrosine phosphorylation has been reported for the estrogen receptor (Migliacco et al. 1989), and for retinoic acid receptor-β (Rochette-Egly et al. 1992). Phosphorylation of P4 receptors occurs primarily on serine residues (Sheridan et al. 1989a; Sheridan et al. 1989b).
Steroid receptor phosphorylation has been shown to be a multi-step process. In the absence of hormone the nonactivated receptor is bound to heat-shock proteins. The details of P₄ receptor interaction with HSP will be discussed in detail in section 2.3. In their non-ligand bound state receptors appear to be constitutively phosphorylated (Beck et al. 1992). The role of this phosphorylation is not well understood. Hormone binding to P₄ receptors appears to unmask additional serine residues for phosphorylation and generally results in an increase in receptor phosphorylation. Phosphorylation of these newly accessible sites may enhance the dissociation of occupied receptor from certain accessory proteins. Phosphorylation following hormone binding may also enhance the transcriptional regulatory activity of the hormone-receptor complex (Goodman and Hodgen 1996; Kuiper and Brinkmann 1994; Migliacco et al. 1989; Rochette-Egly et al. 1992; Takimoto et al. 1992). This second phosphorylation step is extremely rapid, (within 10 minutes of hormone treatment). It is accompanied by receptor dimerization but precedes DNA binding (Guiochon-Mantel et al. 1989). The following, third round of phosphorylation is dependent on DNA binding (Beck et al. 1992; Goodman and Hodgen 1996; Takimoto et al. 1992). DNA binding, either directly, or by facilitating appropriate protein-protein contacts, may produce additional conformational changes in the receptors so that new serine residues become substrates for protein kinases (Takimoto et al. 1992).

As just discussed, phosphorylation of steroid receptors may be involved in the modulation of receptor activation, hormone binding, and DNA transcription. Treating cells with various activators of protein phosphorylation can result in steroid receptors within those cells inducing transcription of target genes in the absence of ligand. Ligand-independent trans-activation as such provides a mechanism for cross-talk between protein phosphorylation signal transduction pathways and nuclear steroid receptors (Edwards et al. 1993). It also permits steroid receptor mediated transcriptional regulation independent of steroid hormones in nonclassical target tissues or in certain pathological conditions (Edwards et al. 1993). Compounds such as okadaic acid, cyclic AMP enhancing agents, dopamine, epidermal growth factor, and insulin-like growth factor-I, have been shown to activate various receptors to a significant extent in the absence of hormone (Edwards et al. 1993). Such trans-activation has been shown for chicken P₄ receptors, mouse and human estrogen receptors, human vitamin D receptors, human thyroid hormone receptor-b, nuclear orphan

Phosphorylation is essential for, and in part regulates, $P_4$ receptor activity (Beck et al. 1992). Sheridan et al. (1989b) examined the $P_4$ receptor A isoform of approximately 94 kilodalton, and B isoform protein ‘triplet’ of approximately 114, 117, and 120 kilodaltons. All four of these proteins were phosphorylated and capable of binding DNA (Sheridan et al. 1989b). Sheridan et al. (1989b) synthesized a single A protein of 94 kilodaltons, and a single B protein of 114 kilodaltons. They found mature B ‘triplet’ forms 6-10 hours later via post-translational phosphorylation at sites not found in the A form. This step however, was not required for $P_4$ receptor activation to hormone binding states since receptors only 15 minutes following their formation were found capable of responding to progestins by undergoing transformation and nuclear binding accompanied by a rapid secondary phosphorylation common to both A and B isoforms (Sheridan et al. 1989b). Sheridan et al. (1989b) however, were unable to determine whether the immature B receptor was transcriptionally active.

The human $P_4$ receptor is phosphorylated on at least nine different serine residues (Zhang et al. 1997) (Figure 2.4). Three of these sites (Ser102, Ser294, and Ser345) are inducible by hormone agonist, while the other six (potentially more) sites are basally phosphorylated (Beck et al. 1996). Three phosphorylation sites are known to be unique to human $P_4$ receptor B isoform (Sheridan et al. 1989b). Of these three sites, one, the Ser102 site, is hormone inducible (Sheridan et al. 1989b; Zhang et al. 1997). These additional phosphorylation sites on the B isoform may contribute to the difference in transcriptional activities of the $P_4$ A and B isoforms (Lazmi et al. 1993).

If phosphorylation is so important for $P_4$ receptor function, antagonists to $P_4$ receptor actions may exert their effects in part by altering receptor phosphorylation. Beck et al. (1996) examined whether the two $P_4$ receptor antagonists RU486 and ZK98299 affected $P_4$ receptor phosphorylation. The biological effects of RU486 were not mediated by an alteration in the phosphorylation state of $P_4$ receptor. ZK98299 in contrast, inhibited phosphorylation of certain sites on the receptor. Beck et al. (1996) concluded that the antagonistic action of ZK98299 is
mediated, at least in part, by inhibiting P₄ receptor phosphorylation. RU486 appears to exert its inhibitory effects in another manner which will be discussed in section 2.2.17 of this review.

2.2.5 Dimerization of progesterone receptors

Following ligand binding and phosphorylation events a P₄ receptor dimer is formed (Goodman and Hodgen 1996). At least three dimeric species are produced: AA, BB homodimers and AB heterodimers (DeMarzo et al. 1991; El-Ashry et al. 1989; Sartorius et al. 1994a). P₄ receptor isoform C is also capable of dimerizing with the A and B isoforms, yet the functional consequence of these dimers has not been determined (Wei et al. 1990; Wei and Miner 1994). There is the potential for more dimeric species should more P₄ receptor isoforms be identified. Once formed the receptor dimers then bind to DNA at specific P₄ response elements (PRE) on the promoters of P₄ responsive genes, and regulate transcription (Sartorius et al. 1994a).

Dimerization is yet another step at which P₄ receptor activity may be regulated. Carbajo et al. (1996) examined the binding properties of the human A and B P₄ receptor isoforms. They measured these properties for different receptor combinations over a range of receptor concentrations to detect the effects of dimerization (Carbajo et al. 1996). Carbajo et al. (1996) found that hetero- and homodimers are not functionally equivalent, and that one isoform can influence the other. For example, human P₄ receptor A isoform, in a cell- and promoter-specific manner, repressed the transactivation activity of human P₄ receptor B (Carbajo et al. 1996). Antagonist-occupied B homodimers stimulated transcription, but AB heterodimers did not. This suggests that AB heterodimers have the transcriptional phenotype of A homodimers, such that when heterodimers are formed, the A receptors are dominant over B receptors (Carbajo et al. 1996; Sartorius et al. 1994a).

Dimerization of P₄ receptors is of particular interest with respect to hormonal antagonism (Goodman and Hodgen 1996). Since a dimer can be either a homodimer or a heterodimer, several permutations of agonist/antagonist-receptor-isoform combinations can be formed which provide an opportunity for complex, tissue-specific, even paradoxical effects (Goodman and Hodgen 1996). Meyer et al. (1990) showed that the antiprogestin RU486 can have weak agonist-like activities when
bound to B but not A receptors. Skafar (1993) found that RU486-bound receptor dimerizes at lower concentrations and forms a tighter dimer than does the P₄-bound receptor. They suggested that the difference in dimerization and the antagonistic biological activity of RU486 could both be related to the different conformational changes in the receptor produced by RU486 and P₄ agonists (Skafar 1993). DeMarzo et al. (1992) found P₄ receptors bound to RU486 have enhanced receptor dimerization correlated with higher DNA binding activity. Also, an RU486-bound P₄ receptor can dimerize with an agonist bound P₄ receptor. If it is a P₄ A receptor to which RU486 is bound the dimer it forms, with either a RU486 bound B P₄ receptor or an agonist bound P₄ receptor A or B, will bind to DNA but transcription will be impaired (DeMarzo et al. 1992).

2.2.6 Transcriptional activation regions

There are at least two regions within the P₄ receptor which are involved in transcriptional activation. These regions are termed transcriptional activation regions, or activation function (AF). It is not clear at present how these regions operate. We do know that they enhance transcription by interacting with various other proteins. The AF regions contain helical folds that form a well-conserved hydrophobic ligand-binding pocket (Henttu et al. 1997). Therefore, upon steroid binding the ‘pocket’ is formed which allows for the interaction of the receptor with other proteins (Henttu et al. 1997). These proteins, generally referred to as coregulators, will be discussed in detail in section 2.4 of this review. In P₄ receptors one of these functions is a 90-91 amino acid segment located in the A/B region just up-stream of the DNA-binding domain (AF1), and a second (AF2) is located within the E region/hormone-binding domain (Meyer et al. 1992; Sartorius et al. 1994b; Shemshedini et al. 1992). Sartorius et al. (1994b) described a third activation function domain (AF3) in the 164 amino acid region of the P₄ receptor which is unique to the B isoform (Figure 2.4) (Sartorius et al. 1994b). They suggested that the AF3, along with the unique phosphorylation sites found within the B isoform, may together contribute to the differences in transcriptional activation of the A and B isoforms (Sartorius et al. 1994b).
2.2.7 Activation of gene transcription

As a general mode of action, steroid hormones induce structural changes in the receptor, promoting its binding to specific hormone response elements (HRE) on target DNA and affecting rates of transcription (Beck et al. 1993a; Edwards et al. 1993; Goodman and Hodgen 1996). The precise structural modifications of receptors that are responsible for transformation and the molecular mechanisms by which receptor binding to HREs stimulates transcription initiation are not entirely understood (Beck et al. 1993a; Edwards et al. 1993; MacLusky 1996).

HREs are distinct for different hormones. They are around 15 bases long, and exhibit dyad symmetry (i.e., they are imperfect inverted repeats), which suggests that steroid receptors bind to their target DNA as dimers (Evans 1988). The P₄ response element (PRE) which bind the P₄ receptor dimers are enhancer sequences often within or contiguous with the 5'-promoter region of P₄-regulated genes (Goodman and Hodgen 1996). The PRE sequence is as follows (Liberman et al. 1993):

\[
5'\text{-GTTC\underline{A}AAACTGTTCT-3'}
\]
\[
3'\text{-CAATGTTTGACAAGA-5'}
\]

The underlined region of the PRE sequence denotes the spacer between the two halves of the palindromic recognition sequence (MacLusky 1996).

As mentioned earlier, P₄ receptor dimers bound to PRE undergo a second increase in phosphorylation. The phosphorylation state of the transcriptional activation domains of P₄ receptors is an important determinant of transcriptional control (Goodman and Hodgen 1996). Thus, binding of an activated P₄ receptor dimer at the PRE initiates a second nuclear molecular cascade that activates or accelerates gene transcription (Goodman and Hodgen 1996).

A PRE has been identified in the mouse mammary tumor virus (MMTV) (Archer et al. 1994; Savouret et al. 1988), in the chicken ovalbumin promoter (Savouret et al. 1988), and in uteroglobin (UG) (Savouret et al. 1994). MMTV has been frequently used as a model to study P₄ action. UG in the uterus is a preimplantation secretory protein that has immunosuppressive and...
anti-inflammatory properties through the inhibition of phospholipase-A₂ (Chilton and Hewetson 1998). The transcription of UG is stimulated by P₄ (Chilton and Hewetson 1998).

A number of P₄ target genes have been identified [see (Kester et al. 1997; Savouret et al. 1990) for reviews]. At least 20 proteins have been identified so far during gestation which are induced by P₄. Only a few appear interesting as markers of pregnancy evolution. Pregnancy associated plasma protein A (PAPP-A) is present in human trophoblast and plasma. P₄ induction of PAPP-A is inhibited by RU486. PAPP-A is involved in coagulation processes, as is placental protein 5, which has been identified as placental antithrombin III. Placental protein 12 (PP12) is a glycoprotein found in the trophoblast, decidua, amniotic fluid and maternal serum. Schwangerschaft Protein I (SPI) can be detected within 14 days of pregnancy [see (Savouret et al. 1990) for a review].

2.2.8 Effects of progesterone receptors on DNA bending/conformational changes caused by ligand binding

P₄ receptors when bound to the PRE of their target genes change the conformation of the DNA (Pendergast et al. 1996). Differential bending by the A and B P₄ receptor isoforms may be another mechanism by which they exert their differential effects. Pendergast et al. (1996) used circular permutation and phasing analyses to determine the ability of purified A and B forms of human P₄ receptors to bend target DNA. They also compared the effects of a P₄ agonist (R5020) and antagonist (RU486), and the influence of HMG-1 (chromatin high mobility group protein 1) on P₄ receptor-mediated DNA bending (Pendergast et al. 1996). Pendergast et al. (1996) found that P₄ receptor B was generally a stronger transcriptional activator than P₄ receptor A and mediated a greater degree of bending at the same PRE than did P₄ receptor A. Therefore, the same PRE bound to P₄ receptor A or B assumes different spatial conformations resulting in receptor contacting other proteins more efficiently (or contacting different proteins) to direct the assembly of a transcriptional unit that mediates distinct functional responses (Pendergast et al. 1996). HMG-1 may also be involved in transcriptional regulation by stabilizing the conformation of the transcription unit (Pendergast et al. 1996). DNA bending by P₄ receptors may be an important...
mechanism by which upstream activators contact other factors in the transcriptional apparatus (Pendergast et al. 1996).

2.2.9 Transcriptional regulation of progesterone receptors by estrogen and progesterone

Estrogen is known to 'prime' P₄ target tissues. For example, decidual cell formation in the mouse and rat uterus requires the prior administration of estrogen for P₄ to exert an optimal decidual reaction. The coordinated action of both hormones is also required for uterine implantation in many mammalian species (Heap and Flint 1984; Ing et al. 1993). Estrogen may be needed in such cases to control the relative amounts of P₄ receptors present. Estrogen is known to induce P₄ receptors at the level of mRNA as well as protein in most target tissues (Kraus and Katzenellenbogen 1993; Leavitt et al. 1977; Lydon et al. 1995; MacLusky and McEwen 1980; Parsons et al. 1980). With respect to the uterus, estrogen increases estrogen receptors as well as P₄ receptors in a number of species (Kraus and Katzenellenbogen 1993; Mayes et al. 1996; Spencer and Bazer 1995; Wathes and Hamon 1993; Zheng et al. 1996). Horwitz and McGuire (1978) examined the control of P₄ receptors by estrogen. They found in MCF-7 breast cancer cells treated 4 to 5 days with estradiol-17β (0.001 - 100 nM), basal P₄ receptor levels (0.3 - 0.7 pmol/mg DNA) increased 3- to 6-fold (Horwitz and McGuire 1978). This P₄ receptor response was dose-dependent; 0.1 nM estrogen, a physiological dose, was maximally effective (Horwitz and McGuire 1978). Induction of P₄ receptors by 0.1 nM estrogen was suppressed by the antiestrogen Tamoxifen at 10,000-fold molar excess (1 μM), but reversed by supraphysiological estrogen (10 nM) which reduces the molar excess of Tamoxifen to only 100-fold (Horwitz and McGuire 1978).

P₄ receptor A and B isoform mRNAs are transcribed by two distinct estrogen-responsive promoters. Those associated with the A isoform have been identified between +464 and +737 base pairs (Fujimoto et al. 1995; Kastner et al. 1990b; Kraus et al. 1994; Savouret et al. 1991b), while those associated with the B promoter have been identified between -711 to +31 base pairs (Fujimoto et al. 1995; Kastner et al. 1990b). The presence of these two distinct promoters suggests that the expression of the P₄ receptor isoforms may be differently regulated (Kastner et al. 1990a).
and further, that this differential regulation may be due to differential transcriptional stimulation by estrogen (Kastner et al. 1990b).

Estrogen has been found to affect the relative amounts of the two $P_4$ receptor isoforms. In T47D cells it appears to preferentially increase transcripts derived from the $P_4$ receptor B promoter, resulting in a decrease in the $P_4$ receptor A:B ratio (Graham et al. 1995). In the chicken, the A and B receptor isoforms are differentially expressed developmentally and differentially regulated by hormone treatment (Boyd-Leinen et al. 1982). Estrogen's effects on $P_4$ receptor isoforms in the chicken appear reversed to the situation found in mammals. Syvala et al. (1996; 1997), found estrogen upregulated $P_4$ receptor A in the chicken, and thus increase the A:B ratio in the chicken oviduct. In the chicken the A isoform, not the B, is capable of activating the ovalbumin gene promoter, and when these two isoforms are coexpressed $P_4$ receptor B acts as an inhibitor of $P_4$ receptor A mediated activation of ovalbumin promoter (Tora et al. 1988). $P_4$ receptor A stimulates while $P_4$ receptor B inhibits estrogen receptor mediated activation of the ovalbumin promoter (Tora et al. 1988). Boyd-Leinen (1982) described the seasonal changes in $P_4$ receptors in chickens. In the winter (non-laying season), the $P_4$ receptor B isoform was more abundant than the A isoform. In the summer during which the chickens laid their eggs the A and B isoforms were equal in abundance. These results would coincide with the effects of estrogen on oviduct of the chicken; estrogen and $P_4$ are important for the synthesis of various egg white proteins and their effects may be mediated in part by an increase of the A isoform of the $P_4$ receptor.

While estrogen increases, $P_4$ decreases the total level of $P_4$ receptor mRNA and protein (Graham et al. 1995; Horwitz and McGuire 1978; Kraus and Katzenellenbogen 1993; Mester and Baulieu 1977; Milgrom et al. 1973), as well as estrogen receptor mRNA and protein (Chaurchereau et al. 1992; Clarke 1990; Leavitt et al. 1987; Meyer et al. 1988; Miller et al. 1977). Leavitt et al. (1987) found $P_4$ blocks estrogen receptor chromatin binding sites, thus blocking transactivation of estrogen target genes. Cell and tissue specificity of $P_4$ regulation may be a result of estrogen and $P_4$ independently controlling the synthesis of transcripts arising from the $P_4$ and estrogen receptor promoter, along with estrogen altering the cellular $P_4$ receptor A:B ratio (Graham et al. 1995). The mechanisms of down-regulation of the $P_4$ receptor by its own ligand are not completely understood. It may involve $P_4$ receptor-mediated decreases in $P_4$ receptor gene transcription (Alexander et al.
1989; Read et al. 1988). Autoregulation of the P₄ receptor provides a self-limiting luteal phase in a number of species (Clarke 1990; Meyer et al. 1988; Miller et al. 1977; Spencer and Bazer 1995). In human endometrium P₄ and estrogen receptor levels are highest in late proliferative phase when circulating levels of estrogen are high and those of P₄ are low, and the receptor levels are lowest in late luteal phase when circulating estrogen levels are low and P₄ levels high (Bayard et al. 1978).

2.2.10 Transcriptional effects of progesterone receptors on other steroid receptors

As discussed earlier, while hormone and DNA binding properties of the P₄ receptor isoforms are similar, they display different transcriptional activities. As well, in mammals, while P₄ receptor B functions predominantly as an activator of P₄ responsive genes, P₄ receptor A functions as a modulator or repressor of P₄ receptor B activity (Vegeto et al. 1993). P₄ receptors can also affect the transcriptional activities of other steroid receptors within a cell. This effect differs between the P₄ receptor A and B isoforms. For example, the P₄ receptor A isoform, but not the B, has a potent dominant negative activity on glucocorticoid receptors, androgen receptors, as well as P₄ receptor B activity (McDonnell and Goldman 1994; Tung et al. 1993; Vegeto et al. 1993; Yen et al. 1997). The A isoform may thus serve as a means of cross-talk between these receptors in cells in which they are coexpressed (Wen et al. 1994). Thus, the coexpression of steroid hormone receptors may allow multiple ligands to regulate target gene expression (Yen et al. 1997).

Both isoforms of the P₄ receptor can act as potent ligand-dependent estrogen receptor activity repressors (Kraus et al. 1995). Kraus et al. (1995) found that P₄ receptor A isoform more strongly repressed estrogen receptor-mediated transcriptional activity than did P₄ receptor B. This suggests that the N-terminal extension found in P₄ receptor B isoform must attenuate the repressive activities of this isoform. As well, antagonist occupied P₄ receptor was a more effective estrogen receptor repressor than was agonist-occupied P₄ receptor (Kraus et al. 1995). Repression by antagonist-occupied P₄ receptor B was observed under all conditions, whereas repression by agonist-occupied P₄ receptor B was generally not observed (Kraus et al. 1995; Wen et al. 1994).

The exact mechanism by which P₄ receptors down-regulate estrogen receptor activity is not clear. Using in vitro DNA and ligand binding assays Wen et al. (1994) determined that P₄ receptor
A: does not heterodimerize with estrogen receptors; has no effect on the ability of estrogen receptors to interact with DNA; has no direct effect on estrogen receptor biochemistry; and has no effect on estrogen receptor cellular expression in transfected mammalian cells (Wen et al. 1994). P₄ receptors may induce estrogen degrading enzymes (e.g. 17β-hydroxysteroid dehydrogenase), or possibly both receptors are competing for cofactors (Clarke and Sutherland 1990; Onate et al. 1995). Kraus et al. (1995) suggest that P₄ receptors may interfere with the ability of estrogen receptors to interact productively with the transcriptional machinery, a process referred to as quenching or autosquelching (Wen et al. 1994). This interference of P₄ receptors with estrogen receptors may be the reason why human total P₄ receptor protein levels in the myometrium appear to decrease somewhat at labour (How et al. 1995) -- to remove any inhibitory effect on estrogen receptors. Knowing the effects of P₄ receptor A isoform on transcription of other steroid receptors, it makes sense that estrogen primarily stimulates the B isoform of the P₄ receptor (Kraus and Katzenellenbogen 1993), since if it stimulated the A isoform it would be inhibiting P₄ activity as well as the activity of a number of other steroid receptor systems, including estrogen receptor transcription.

Multiple steroid receptors within a cell may affect each other’s transcriptional abilities by potentially competing for limiting amounts of coregulatory proteins within the cell. Zang et al. (1996) found that ligand-occupied, transcriptionally active thyroid receptors (both A and B isoforms of this receptor) were capable of interfering with P₄ receptors, suppressing transactivation of PRE without binding DNA. It is possible that such interference is a result of limiting coactivator protein(s), which, once bound to the transcriptionally active receptor, are unavailable for binding to other liganded receptors.

2.2.11 Progesterone receptor expression

P₄ receptors have been identified in P₄ responsive tissues such as: uterus, ovary, testes, vagina, breast, brain, vascular endothelium, thymus, pancreatic islets, osteoblast-like cells, and lung [see (Graham and Clarke 1997) for a review]. They have been identified in a number of different species such as: human (Bayard et al. 1978; Horwitz and McGuire 1978; Illingworth et al. 1975;
Logeat et al. 1981); rat (Haukkamaa and Luukkanen 1975; Janne et al. 1976; VuHai et al. 1978); chicken (Boyd and Spelsberg 1979; Conneely et al. 1989; Jeltsch et al. 1990; Tora et al. 1988); baboon (Hild-Petito and Fazleabas 1997; Hild-Petito et al. 1992); rhesus monkey (Okuiiez et al. 1990); rabbit (Davies et al. 1974; Hegele-Hartung et al. 1992; Misrahi et al. 1987); guinea pig (Glasier and Hobkirk 1993); turtle (Giannoukos and Callanr 1995; Giannoukos and Callard 1996); cat (Li et al. 1992); alligator (Vonier et al. 1997); porcine (Geisert et al. 1994); sheep (Mayes et al. 1996; Spencer and Bazer 1995; Zheng et al. 1996); and elephant (Greyling et al. 1998; Meyer et al. 1997).

The levels of P4 receptor isoforms has been described at the message level using northern blot analysis. The presence of multiple bands has made the analysis of results from this method somewhat complex (Table 2.1). Wei et al. (Wei et al. 1990; Wei et al. 1988; Wei and Miner 1994) described P4 receptors in northern blot analyses of T47D cells. They analyzed the bands in detail and suggest that the 2.5 and 5.2 kilobase species must encode for the A species since they lack the sequences surrounding AUGB (codon 1), the translation initiation site for B-receptors, but they have the AUGA (codon 165), the translation initiation site for A receptors (Wei et al. 1990). The 3.2, 4.5, and 6.1 kilobase species, as well as bands I and II of the 11.4 kilobase species, have both AUGA and AUGB and are thus capable of encoding either the A or B isoforms. The 11.4 kilobase III, and IV bands have neither codons so are unlikely to code for either A or B receptors (Wei et al. 1990). Wei et al. (Wei et al. 1990; Wei and Miner 1994) suggested that since these two bands contain an initiator methionine at codon 595 in exon 2 they may code for the C form of the P4 receptor.

Protein analysis has been used to describe P4 receptors in a number of different species, in a variety of tissues. Table 2.2 summarizes the size and location of the P4 receptor isoform proteins designated as ‘A’ and ‘B’ which have been described to date. Sheridan et al. (1989b) described a triplet B receptor of 114, 117, and 120 kilodaltons, with the A form at 94 kilodaltons. They found the P4 receptors to be formed initially as the 114 and the 94 kilodaltons forms. The 117 and 120 kilodalton forms were found to be a result of post-translational modifications, which included phosphorylation (Sheridan et al. 1989b). The 94 and 114 kilodalton forms were also phosphorylated (Sheridan et al. 1989b).
Table 2.1: Identification of progesterone (P₄) receptor RNA bands using northern blot analyses. Species and tissue used for analysis are listed. As well, type of RNA used for analysis and probes used are identified.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Ribonucleic acid amount &amp; type examined</th>
<th>Probe size (kilobases)</th>
<th>Bands identified (kilobases)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baboon</td>
<td>-corpora lutea</td>
<td>20 μg, total</td>
<td>2.5</td>
<td>4.4, 3.1, 1.6, and 0.95</td>
<td>Hild-Petito et al. (1997)</td>
</tr>
<tr>
<td>Chicken</td>
<td>-immature oviduct</td>
<td>total</td>
<td>chicken P₄ receptor cDNA</td>
<td>8.0, 4.4, 3.9, 3.1 (3.9 increases with estrogen treatment)</td>
<td>Sylvála et al. (1997)</td>
</tr>
<tr>
<td>Chicken</td>
<td>-oviduct</td>
<td>poly(A)*</td>
<td>chicken P₄ receptor cDNA</td>
<td>8.2, 4.5, 4.1, 3.3</td>
<td>Jeltsch et al. (1990)</td>
</tr>
<tr>
<td>Human</td>
<td>-Ishikawa cells</td>
<td>poly(A)*</td>
<td>cDNA</td>
<td>12, 7, 6, 5, 4</td>
<td>Lessey et al. (1996)</td>
</tr>
<tr>
<td>Human</td>
<td>-leiomyoma and adjacent myometrium</td>
<td>10 μg, total</td>
<td>(human P₄ receptor cDNA)</td>
<td>3.8</td>
<td>Brandon et al. (1993)</td>
</tr>
<tr>
<td>Human</td>
<td>-rabbit uterus and -human uterus poly(A)*</td>
<td>40 μg, total</td>
<td>(rat P₄ receptor cDNA)</td>
<td>2.8</td>
<td>Misrahi et al. (1987)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>-MCF-7 and T47D breast cancer cells</td>
<td>poly(A)*</td>
<td>receptor cDNA</td>
<td>5.1, 4.3, 3.7, and 2.9, and a weaker band at 5.9</td>
<td>Wei et al. (1990; Wei et al. 1988; Wei and Miner 1994)</td>
</tr>
<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>7.5-20 μg, poly(A)*</td>
<td>oligo-nucleotide and cDNA fragment</td>
<td>11.4 complex (I,II,III,IV), 6.1, 5.2, 4.5, 3.2, and 2.5</td>
<td>Wei et al. (1990; Wei et al. 1988; Wei and Miner 1994)</td>
</tr>
<tr>
<td>Rat</td>
<td>-decidua</td>
<td>probes</td>
<td>cDNA</td>
<td>Ogle et al.</td>
<td></td>
</tr>
<tr>
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<td>----------</td>
<td>----------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-granulosa cells</td>
<td>0.9</td>
<td>11, 7.2, 6.8, 6.2, 4.4, 3.4.</td>
<td>Natraj and Richards</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mouse cDNA)</td>
<td>3.1</td>
<td>(1993)</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>-mammary tissue</td>
<td>10 µg, poly(A)*</td>
<td>8.7, 6.9, 4.2, 3.5, and 2.7</td>
<td>Shyamala et al.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(human P₄ receptor cDNA)</td>
<td>(6.9 and 8.7 kilobase forms accounting for 70-80% of the total mRNA).</td>
<td>(1990)</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>-uteri</td>
<td>poly(A)*</td>
<td>1.9</td>
<td>14.0, 10.3, 9.7, 6.8, 6.2, 5.5, 4.6, and 3.1</td>
<td>Kraus and Katzenellenbogen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(rat P₄ receptor cDNA)</td>
<td></td>
<td>(1993)</td>
<td></td>
</tr>
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</table>
Table 2.2: Identification of progesterone (P₄) receptor protein bands using western blot analyses. Species and tissue used for analysis are listed, as are the antibodies used for analyses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Antibody used</th>
<th>Size of A isoform (kilodaltons)</th>
<th>Size of B isoform (kilodaltons)</th>
<th>Relative abundance of A and B isoforms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>-oviduct (cytosol)</td>
<td>-C-262,</td>
<td>72</td>
<td>86</td>
<td></td>
<td>Weigel et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>-hPRa6, hPRa7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-breast cancer tissues</td>
<td>-hPRa6,</td>
<td>81-83</td>
<td>115-120</td>
<td></td>
<td>Graham et al. (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hPRa7</td>
<td>78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-endometrium (cycle)</td>
<td>-Clarke et al.</td>
<td>81</td>
<td>116 (triplet or doublet)</td>
<td>A&gt;B</td>
<td>Feil et al. (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1987)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-human breast carcinoma cell extracts</td>
<td>-IZB39,</td>
<td>95</td>
<td>120 (T47D cells only)</td>
<td></td>
<td>Press et al. (1988)</td>
</tr>
<tr>
<td></td>
<td>-T47D cells</td>
<td></td>
<td></td>
<td>(T47D cells only)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-Ishikawa cells and T47D cells</td>
<td>-hPRa1,</td>
<td>81</td>
<td>116 (3 bands visible)</td>
<td></td>
<td>Lessey et al. (1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and hPRa7</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Human</td>
<td>-leiomyoma</td>
<td>-ABS2</td>
<td>94</td>
<td>120</td>
<td>A&gt;B</td>
<td>Navarro et al. (1989)</td>
</tr>
<tr>
<td>Human</td>
<td>-leiomyoma and adjacent myometrium</td>
<td>-B39</td>
<td>90</td>
<td>120</td>
<td></td>
<td>Brandon et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>(Ben May Laboratories)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-leiomyoma and adjacent myometrium</td>
<td></td>
<td>94</td>
<td>120</td>
<td>1.6 x more A than B</td>
<td>Viville et al. (1997)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-P₄ receptors expressed in insect cells</td>
<td>-AB-52</td>
<td>94</td>
<td>120</td>
<td></td>
<td>Christensen et al. (1991)</td>
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<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>-(³H-R5020 for photo-affinity labeling)</td>
<td>82</td>
<td>118</td>
<td>Horwitz and Alexander (1983)</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Tissue/Cell Line</td>
<td>Antibody</td>
<td>MW (kDa)</td>
<td>Ratio</td>
<td>Comments</td>
<td></td>
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</tr>
<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>-C262</td>
<td>94</td>
<td>120</td>
<td>(Weigel et al. 1992)</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>-PR-6</td>
<td>94</td>
<td>116.5</td>
<td>A&gt;B</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>-AB-52</td>
<td>94</td>
<td>114</td>
<td>(SuIlivan et al. 1986)</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>B-30</td>
<td>94</td>
<td>117</td>
<td>(Estes et al. 1987)</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>AB-52</td>
<td>94</td>
<td>120</td>
<td>(Estes et al. 1987)</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>-C-262</td>
<td>94</td>
<td>120</td>
<td>(Weigel et al. 1992)</td>
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</tr>
<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>AB-52</td>
<td>94</td>
<td>120</td>
<td>(Wei and Miner 1994)</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>-C262</td>
<td>94</td>
<td>120</td>
<td>-C isoform at 60 kilodaltons</td>
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<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>-MCF-7</td>
<td>94</td>
<td>120</td>
<td>(Estes et al. 1987)</td>
<td></td>
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<tr>
<td>Human</td>
<td>-T47D breast cancer cells</td>
<td>-endometrium</td>
<td>94</td>
<td>120</td>
<td>(Wei et al. 1988)</td>
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<tr>
<td>Human</td>
<td>-uteri (not-pregnant)</td>
<td>-Mo482</td>
<td>79</td>
<td>110</td>
<td>(VuHai et al. 1989)</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>-uteri (pregnant)</td>
<td>-AT4.14</td>
<td>94</td>
<td>110</td>
<td>A&gt;B</td>
<td></td>
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<tr>
<td>Mouse</td>
<td>-mammary gland</td>
<td>-hPRA-4,5,7 and aPR6</td>
<td>83</td>
<td>115</td>
<td>Shyamala et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>-uteri, mammary glands, vagina from normal, ovariectomized and</td>
<td>-Clarke et al. (1987)</td>
<td>83</td>
<td>115</td>
<td>A&gt;B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3:1 uterus 2:1 vagina 3:1</td>
<td>Schneider et al. (1991)</td>
</tr>
<tr>
<td>Animal</td>
<td>Tissue</td>
<td>Conjugation</td>
<td>A1</td>
<td>B</td>
<td>C</td>
<td>Reference</td>
</tr>
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</tr>
<tr>
<td>Porcine</td>
<td>-endometrium (cytosol)</td>
<td>-C-262</td>
<td>90</td>
<td>117</td>
<td></td>
<td>Geisert et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>cycle(+) and pregnant(-)</td>
<td>(A+B)</td>
<td></td>
<td></td>
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<tr>
<td>Rat</td>
<td>-decidua</td>
<td>-h928</td>
<td></td>
<td></td>
<td></td>
<td>Ogle et al. (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(D. Edwards)</td>
<td>A1=90</td>
<td>B=110</td>
<td>C=60-64</td>
<td></td>
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<tr>
<td>Rat</td>
<td>-granulosa cells</td>
<td>-938</td>
<td></td>
<td></td>
<td>115</td>
<td>A&gt;B</td>
</tr>
<tr>
<td>Rat</td>
<td>-uteri (non-pregnant,</td>
<td>-KC146</td>
<td></td>
<td></td>
<td>110</td>
<td>Kraus and Katzenelenbogen (1993)</td>
</tr>
<tr>
<td></td>
<td>hormone treated)</td>
<td>(Zymed-B</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>only)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turtle</td>
<td>-liver (laying and non-</td>
<td>-anti-PR 22</td>
<td>88</td>
<td></td>
<td>125</td>
<td>Giannoukos and Callanr (1995)</td>
</tr>
<tr>
<td></td>
<td>laying)</td>
<td>(D.O.Toft)</td>
<td></td>
<td></td>
<td>-C isoform 64</td>
<td></td>
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</table>
2.2.12 Progesterone receptor distribution in the human myometrium

Progestin receptor distribution in the human myometrium. Immunological studies of P₄ receptors have not found the total amount of P₄ receptors in this tissue to change significantly during the menstrual cycle (Lessey et al. 1988; Snijders et al. 1992). Some investigators however, using steroid receptor binding techniques, have described highest levels of total and cytosolic P₄ receptor binding in the myometrium in the proliferative phase of the cycle, and lowest levels during the luteal phase (Giannopulos and Tulchinsky 1979; Illingworth et al. 1975; Janne et al. 1976).

Brandon et al. (1993) examined biopsy samples of tumor and adjacent normal myometrium from nine patients for P₄ receptor protein. Immunoassay, immunohistochemistry, and western blot analysis showed increased P₄ receptor protein in tumor tissue, with an equal increase in the A and B isoforms (Brandon et al. 1993). They concluded that P₄ receptor mRNA is over-expressed in uterine leiomyomas, suggesting amplified P₄-mediated signaling is important for the abnormal growth of these tumors (Brandon et al. 1993). Potgieter et al. (1995) examined total cytosolic P₄ receptors and estrogen receptors in leiomyoma and adjacent myometrium by steroid binding techniques. They found the levels of both receptor types to be greater, (although estrogen more so than P₄ receptors), in leiomyomas as compared to normal myometrium. They thus found a significant increase in the cytosolic estrogen receptor/progesterone receptor ratio in leiomyoma tissue.

P₄ receptors have been described in the uterus of pregnant women. Padyachi et al. (1990) used enzyme-linked immunoassays to examine nuclear and cytosolic P₄ receptors in human myometrium, and found levels during pregnancy to be undetectable. Khan-Dawood and Dawood (1984) found that total P₄ receptor binding was higher in the nucleus as compared to cytosol fractions in samples obtained from women not in labour at the time of term cesarean section. They did not look at P₄ receptor binding in labouring samples. Giannopulos et al. (1985) also found total P₄ receptor levels to be higher in the nucleus than in the cytosol of term human myometrial samples, while nuclear P₄ levels were higher in myometrium from women at term than in samples from cycling women.
Few studies have examined and compared $P_4$ receptors in non-labouring and labouring human myometrium at the protein level. Bernard et al. (1988), using enzyme-linked immunoassays, found a significantly higher level of cytosolic total $P_4$ receptors in myometrium collected from women in labour at the time of cesarean section, compared with samples collected from women who were not in labour. How et al. (1995) examined total $P_4$ receptors in human myometrium using immunohistochemistry and found levels to be lower in samples collected from women in labour as compared to those not in labour at the time of cesarean section. They found $P_4$ receptor immunoreactivity to be higher in samples collected from preterm as compared to samples collected from term deliveries. Using western blots, How et al. (1995) also examined $P_4$ receptor isoforms in the myometrium of four women who were not in labour at the time of cesarean section and found the A $P_4$ receptor isoform to be dominant. Geimonen et al. (1998) recently presented the only study in which $P_4$ receptors were found to be higher in pregnant than in non-pregnant human myometrium. For their immunoblot analysis they examined only a 112 kilodalton band, and with the same antibody (sc-539 from Santa Cruz) they examined $P_4$ receptors immunohistochemically. Methodological variations probably contribute to their contradictory findings.

2.2.13 Progesterone receptor distribution in human amnion, chorion and decidua

As discussed in section 2.1.14, the human amnion, chorion, and decidua are capable of synthesizing estrogen and $P_4$ (Mitchell and Challis 1988). Chibbar et al. (1995) examined estrogen and $P_4$ receptors in human chorio-decidual tissues obtained, at term immediately after spontaneous labour and vaginal delivery, or after elective repeat cesarean sections. RNAsese protection assays revealed that both estrogen receptors and $P_4$ receptor mRNA are expressed in amnion, chorion, and decidua and that estrogen receptor mRNA increases significantly at labour onset. $P_4$ receptor mRNA levels however, were not found to change significantly with labour in these tissues (Chibbar et al. 1995). $P_4$ receptors (combined A and B isoforms) have recently been detected in the placenta using reverse transcription and polymerase chain reaction (Rossmanith et al. 1997).
2.2.14 Progesterone receptor distribution in rat uteri

P₄ receptors have also been examined in rat uteri during pregnancy, primarily by radioligand binding to nuclear fractions (Chikusu et al. 1982; Hai et al. 1978; Myatt et al. 1994). Hai et al. (1978) found nuclear P₄ receptor concentration to be lowest at the beginning of pregnancy, with maximum levels found between day 9 and 15 of gestation. After this time they found a decrease in nuclear P₄ receptors with the lowest (almost non-detectable) nuclear levels found on day 22 (parturition on day 23). Myatt et al. (1994) also examined nuclear P₄ receptors and found the highest concentration between days 16 and 18 of gestation. Saito et al. (1985) examined total (both A and B isoforms together) nuclear and cytosolic P₄ receptors and estrogen receptors in rat myometrium, along with circulating P₄ and estrogen levels from days 15 of gestation to 12 hours post partum. They found P₄ receptor levels to be correlated with estrogen receptors, as well as with serum levels of P₄ and estrogen (Saito et al. 1985). Other investigators have also found a decrease in rat myometrial P₄ receptor concentration with labour (Davies and Ryan 1973; Rezapour et al. 1997). Levels of P₄ receptor A and B isoforms in the rat myometrium through labour have yet to be examined.

2.2.15 Progesterone receptors in the uteri of other species

P₄ receptors have been described in the uteri of a number of other species, in addition to the human and rat. Hild-Petito et al. (1992) examined the localization of estrogen receptors and P₄ receptors within specific uterine cell types during implantation and pregnancy in the baboon (Papio anubis). They found estrogen receptors and P₄ receptors were maintained in smooth muscle cells of myometrium during early and late pregnancy, with an overall decrease in the number of receptor-positive cells during pregnancy. Hild-Petito et al. (1992) did not find estrogen receptors and P₄ receptors in the developing or mature placenta, amnion or chorion in the baboon. Davies et al. (1974) described P₄ receptors in the myometrium of pregnant rabbits. Through binding assays they found levels of P₄ receptors decrease slightly 2/3 through pregnancy, then to increase as P₄ levels decrease (Davies et al. 1974). Glasier and Hobkirk (1993) investigated estrogen receptors and P₄ receptors nuclear binding in myometrium, endometrium, and chorion in the guinea pig.
between 32 days of gestation and delivery at 67-71 days. They found decreased P₄ binding in the myometrium from a high at 49-51 days to a low on the day of detectable pubic relaxation (Glasier and Hobkirk 1993). This decrease commenced at 60-63 days gestation (Glasier and Hobkirk 1993). A similar though less well defined change occurred in endometrium (Glasier and Hobkirk 1993). Glasier and Hobkirk (1993) concluded that there is a potential for decreased P₄ effects in myometrium at about one week before delivery and increased estrogen action in that tissue immediately before delivery in the guinea pig.

2.2.16 Progesterone receptor mutant mice

P₄ receptors are induced by estrogen in most target tissues. Thus, it is often difficult to differentiate the specific effects of P₄ from those of estrogen. Lydon et al. (1996; 1995) generated a novel mouse strain in which both forms of the P₄ receptor were ablated using gene targeting/embryonic stem cell techniques. This P₄ receptor null mouse has helped elucidate progesterone’s many important roles in female reproduction and development. They have also helped to distinguish between estrogen and P₄ effects. Both male and female embryos, homozygous for the P₄ receptor null mutation develop to adulthood at the normal Mendelian frequency, with no deviation in the sex ratio (Lydon et al. 1996). While developmental defects have yet to be detected in the adult male P₄ receptor null homozygous mouse, females have extensive reproductive abnormalities (Lydon et al. 1996). The reproductive phenotype consisted of an inability to ovulate, uterine hyperplasia and inflammation, severely limited mammary gland development and an impairment in the induction of a sexual behavioural response (Lydon et al. 1996). Lydon et al. (1995) found estrogen-primed ovariectomized female mice, homozygous for P₄ receptor mutation, were unable to exhibit a significant lordosis response in the presence of an experienced male when administered P₄. Conneely et al. (1998) have recently created two new mutant mouse lines that express only the P₄ receptor A or B isoforms. To date, only the P₄ receptor A isoform mutant has been examined. As with the P₄ receptor A and B mutant mice, the mice lacking only the P₄ receptor A isoform are infertile and have a severely impaired ability to ovulate.
With further investigation these $P_4$ receptor mutant mice will undoubtedly be an important tool whereby the differential effects of $P_4$ receptor isoforms A and B may be elucidated.

2.2.17 Progesterone receptor antagonists

Steroid hormone antagonists and agonists are important clinical drugs as well as useful tools to study normal receptor activation mechanisms (Beck et al. 1993a). There are a number of $P_4$ antagonists and agonists which have frequently been used in studies of $P_4$ receptors. The potencies of these agents are tissue and/or species specific. Some agents such as ZK98299 are considered type I antiprogestin since when bound to $P_4$ receptors they prevent $P_4$ receptor-DNA binding, while others such as RU486 and ZK112993 are type II antiprogestins since when bound to $P_4$ receptors the receptors are still capable of binding DNA (Beck et al. 1993b; Klein-Hitpass et al. 1991, Tung et al. 1993, Vegeto et al. 1993).

$P_4$ receptor agonists and antagonists bind to the receptors differently. Agonists require the presence of particular amino acids in the C-terminal end of the $P_4$ receptor which are not required for antagonist binding (Lanz and Rusconi 1994). Agonists and antagonists of $P_4$ receptors also cause different conformational changes in the receptor which can affect receptor interactions with cellular targets (Allan et al. 1992a; Allan et al. 1996; El-Ashry et al. 1989; Weigel et al. 1992).

RU486 is the drug most studied with respect to $P_4$ receptor antagonist action [see (Cadepond et al. 1997) for a review]. It is an antiglucocorticoid as well as an antiprogestosterone in humans. RU486 has been used for early pregnancy termination (Kovacs et al. 1984; Rodger and Baird 1987; Ulmann and Silvestre 1994; World Health Organization 1990). Prior to surgical abortion RU486 has been shown to increase cervical diameter and decrease cervical resistance to mechanical dilation (Chwalisz 1994). It has been proven more effective and better tolerated when followed by prostaglandin administration than surgical pregnancy termination techniques for pregnancies of less than 50 days of amenorrhea. Expulsion of a dead fetus has been shown to occur significantly earlier in patients treated with RU486 than in placebo controls (Cabrol et al. 1990). For therapeutic pregnancy termination in the second trimester RU486 treatment with a dose of prostaglandin results in a shorter time to expulsion and a lesser dose of prostaglandin than
treatment with prostaglandins alone. RU486 has also been used to induce labour in the third trimester (Chwalisz 1994; Frydman et al. 1992; Mahajan and London 1997; Ulmann and Silvestre 1994). The optimal therapeutic dose needed to do this has not been established. The effects of glucocorticoid antagonism on the fetus have not been determined.

The C-terminal end of the P4 receptor contains a repressor domain which inhibits the transcriptional activity of those receptors bound to RU486 (Xu et al. 1996). Xu et al. (1996) described a 12 amino acid domain present in the C-terminus of P4 receptors which is both necessary and sufficient for the repressor function of RU486; P4 receptor mutants lacking the C-terminus region become transcriptionally active in the presence of RU486. This 12 amino acid region in the C-terminus of the P4 receptor results in a major conformational difference between agonist- and antagonist-bound receptors (Xu et al. 1996). Xu et al. (1996) suggested that the 12 amino acid region within the C-terminus may be a region which associates with a corepressor protein, which in turn would inactivate P4 receptor activity when it is bound to RU486.

The transcriptional activities of P4 receptors A and B are different, and this difference is dependent upon the cell and promoter context (McDonnell and Goldman 1994; Meyer et al. 1992; Tora et al. 1988; Vegeto et al. 1993; Wen et al. 1994). Thus, the relative abundance of human P4 receptor forms A and B is a way in which P4 effects on transcription of P4-responsive genes may be regulated (Fujimoto et al. 1995; Kastner et al. 1990a; Kastner et al. 1990b). P4 receptor B functions predominantly as an activator of P-responsive genes, while P4 receptor A functions as a modulator or repressor of P4 receptor B activity (Wen et al. 1994). This suggests that high P4 receptor A expression may result in reduced P4 responsiveness, and that P4 receptor A may be a repressor, and P4 receptor B an activator of progestin action. Full length P4 receptor B is generally found to be a stronger transcriptional activator than P4 receptor A (Pendergast et al. 1996; Vegeto et al. 1993; Wen et al. 1994). Coexpression of P4 receptor A can also block transcription activation by antagonist-occupied P4 receptor B (Vegeto et al. 1993).

The P4 receptor A and B isoforms respond differently to P4 antagonists; antagonist-occupied P4 receptor B can act like it is bound to P4 and activate transcription, whereas antagonist-occupied P4 receptor A does not (Horwitz 1992; Rothchild 1996; Tung et al. 1993). P4 receptor isoform A always appears to transmit the blocking action of a P4 antagonist (Tung et al. 1993). P4
A receptors bound to an antagonist can also block action of antagonist bound to P₄ receptors (Tung et al. 1993). Thus, when both receptor forms are present, the P₄ receptor A phenotype appears dominant.

2.3 Heat-shock proteins

As described in section 2.2 steroids enter their target cells and bind to their specific receptors. The ligand/receptor combination then moves into the nucleus where the receptors bind to the response element in its target DNA. Steroid stimulation of transcription and translation thus follows. This process however, can be modified at a variety of steps. As mentioned, numerous phosphorylation events are necessary to render the appropriate receptor conformation. There are also numerous interactions of the receptor with other proteins which are needed in order to render the receptor ‘active’ and to transport the receptor to appropriate regions within the cell. To date, two major groups of proteins that are involved in this process have been described: heat-shock proteins (HSP), also known as chaperone proteins; and steroid receptor coactivators and corepressors, which are collectively referred to as coregulators. Information on these proteins is growing rapidly. Much of the information we have at present is based on in vitro work; comparatively few studies have been performed in vivo. Knowing that the presence and/or absence of these proteins in vitro can have profound affects on steroid receptor actions, then knowledge of their presence in vivo might help explain many steroid affects which do not appear to follow the traditional steroid-steroid receptor interaction paradigm. With respect to labour initiation in humans, P₄’s effects may be attenuated in part by altering the levels of such coregulatory proteins without a change in P₄ or P₄ receptor levels.

HSP were so named because their expression was found to increase in cells hours following an increase in their growth temperature. It was later discovered that HSP were expressed not only in response to elevations in temperature but in response to a large variety of cellular insults (Akerlund 1993; Blake et al. 1991; Copin et al. 1995; Holownia et al. 1995; Khanna et al. 1995; Kregel et al. 1995; Murdoch 1995; Pahlavani et al. 1995). During stressful conditions HSP can constitute up to 15% of total cellular protein content. Following stress HSP are believed to protect
cellular structures by preventing protein denaturation and aggregation, and by mediating protein folding (Schneider et al. 1996). Most of the cellular targets for HSP are inherently unstable signal transducers which act as cell-cycle and developmental regulators (Rutherford and Zuker 1994). Therefore, HSP may maintain or 'buffer' genetic variations within a cell, and thus, when HSP functions are compromised, for example by temperature, such variations may be expressed. Rutherford and Lindquist (1998) recently suggested this as a mechanism for promoting evolutionary change in Drosophila.

A number of HSP are constitutively expressed at low levels. These proteins do not themselves bind to ligand (hormone, agonist or antagonist), nor are they essential for progestin agonist/antagonist binding to the receptor molecule (Goodman and Hodgen 1996). HSP are also called 'chaperone proteins' since they are believed to play a role in the intracellular assembly and disassembly of oligomeric structures as well as to folding and unfolding of proteins during membrane translocation into various cellular compartments. For general reviews of HSP see (DeMaio 1995; Hendrick and Hartl 1993; Peetermans 1995; Perdrizet 1995; Pratt and Toft 1997; Wu 1995).

HSP can be grouped into three major size classes: 20-30, 68-73 and 80-90 kilodaltons, the second commonly referred to as HSP70 and the latter as HSP90. Members within each class are evolutionarily conserved. Their high degree of conservation suggests that the structural integrity of these proteins is essential for cellular function and survival. Homozygous HSP90 (HSP83) mutations in Drosophila are lethal (van der Straten et al. 1997)

2.3.1 Heat-shock protein 90

The mammalian HSP90 family consists of at least 2 isoforms which are encoded by separate genes, present on different chromosomes (Moore et al. 1989). In humans, the HSP90 family consists of HSP90α and HSP90β (Hickey et al. 1986; Hickey et al. 1989; Moore et al. 1989) which correspond to HSP86/89α and HSP84/89β in the mouse (Ullrich et al. 1986). The cDNA for the two human HSP90 isoforms differ slightly in size; HSP90α is 2.95 kilobases and HSP90β is 2.7 kilobases (Simon et al. 1987). There is a 99 base pair difference between HSP90α
and HSP90β (Hickey et al. 1989). Not all studies examining HSP90 have distinguished between these two isoforms. The mouse and human sequences are very similar. Hickey et al. (1989) determined the mouse HSP86/89α gene sequence to be 83% homologous with human HSP90α, and mouse HSP84/89β to be 97% identical to the human HSP90β. The two HSP90 isoforms have also been identified in the rat (Izumoto and Herbert 1993). HSP90 associates with unliganded, inactivated receptors, also known as aporeceptors. HSP90 is not found bound to activated (transformed) receptors (Sanchez et al. 1987). It appears to be capable of directly binding to several regions within the hormone binding domain (HBD) of P4 receptors since deletions of individual regions within the HBD do not completely abolish HSP90-P4 receptor binding (Schowalter et al. 1991). The stoichiometry of HSP90 with respect to the receptor is believed to be two HSP90 proteins for every steroid receptor (Chikusu et al. 1982; Smith et al. 1990; Wiech et al. 1992).

HSP90 has sites which bind not only to the HBD of steroid receptors, but to a number of other proteins as well (Bagchi et al. 1991; Renoir et al. 1994). While the full functional anatomy of HSP90 has yet to be determined, we do know that it is found in complex with p23, p59/p56 (Lebeau et al. 1992; LeBihan et al. 1993), p60 (Chen et al. 1996; Hickey et al. 1986; Smith et al. 1993), and immunophilins such as FKBP52 and CyP-40 [see (Pratt and Toft 1997) for a review]. These proteins are ubiquitous and evolutionarily conserved. Their roles in steroid receptor activation are not yet clearly defined. Immunophilins are proteins that bind immunosuppressive drugs and may be classified as either cyclophilins, which bind to cyclosporin A, or FKBPs which bind FK506 and rapamycin (Renoir et al. 1994). Immunophilins have peptidylprolyl isomerase (PPIase) activity suggesting they may play a role in protein folding in the cell. In the absence of receptor these proteins may be found in complex with each other. A complex commonly found contains HSP90, HSP70 and p60 (Smith et al. 1992). The binding of p23 to HSP90 requires adenosine triphosphate (ATP) while the binding of p60 and immunophilins apparently does not. ATP is also required for HSP90 and HSP70 binding to steroid receptors (Csermely and Kahn 1991; Smith et al. 1992).

HSP90 affects steroid receptors in a number of ways. In general, HSP90 appears to be involved in the transport, stabilization, regulation, and conformation of steroid receptors (Csermely
and Kahn 1991; Smith et al. 1992). HSP90 in some way determines the ability of the steroid receptor to assume and/or maintain a conformation which can be activated, possibly by affecting the bending or folding of the receptor protein. While HSP90 binding to steroid receptors facilitates ligand binding, it keeps the receptor in an inactive state, blocking the biological activity of the receptor until hormone is bound (Catelli et al. 1985; Picard et al. 1990; Wiech et al. 1992). Steroid binding causes dissociation of HSP90 and other proteins (Smith et al. 1992) which results in the appearance of other receptor activities (Picard et al. 1990). Binding of steroid receptors to HSP90 appears to permanently alter receptor structure. This was suggested by Picard et al. (1990) who found that aporeceptor molecules which have never been complexed with HSP90 differ qualitatively from those whose previous HSP90 interaction has been reversed.

HSP90 is predominantly found in the cytoplasm but some is also found in the nucleus (Akner et al. 1992; Lai et al. 1984). HSP90 binds actin and may thus transport proteins through the cytoplasm via its interactions with cytoskeletal actin (Ramachandran et al. 1988). It has been suggested that P₄ receptors and HSP90 can not exist in complex with each other in the nuclei (Tuohimaa et al. 1993), if so, then receptors bound to HSP90 may be in the nucleus in the absence of appropriate steroid agonist. It is not known what role HSP90 plays in such situations; whether HSP90 is performing a chaperone function, or whether HSP90 has just been brought into the nucleus along with other receptors.

Analysis of the HSP90 cDNA has revealed various important regions for steroid receptor binding. Sullivan and Toft (1993), through mutational studies of the chicken HSP90 cDNA, found that NH₂ deletions which resulted in the removal of amino acids 1-380 did not result in substantial loss of receptor binding activity. Deletions of the COOH-terminal half of HSP90 did result in partial or complete loss of this activity. They concluded that two regions of HSP90 cDNA, amino acid 381-441, and 601-677, are particularly important for receptor binding.

It is presently unclear whether HSP90 requires post-translational processing prior to its interaction with steroid receptors. Phosphorylation is presently the only post-translational step known to occur with HSP90. Two serine phosphorylation sites have been identified on human HSP90 (Sullivan and Toft 1993), which are in a highly charged region that is not essential for receptor binding. Csermely and Kahn (1991) suggested that dissociation of HSP90 from steroid
receptors and its autophosphorylation may be linked events. They also suggested that histones may play a role in the dissociation of HSP90 from steroid receptors which in turn may be linked with the autophosphorylation of HSP90. Sultan and Toft (1993) however, found that newly synthesized HSP90 can interact with P4 receptors under the same conditions needed to bind receptor to the endogenous HSP90 of reticulocyte lysate. Thus, they suggested that extensive post-translational processing of HSP90 is unnecessary.

2.3.2 Heat-shock protein 70

Another major class of HSP is HSP70. There are at least two forms of HSP70: HSP70 constitutively expressed [also known as HSP cognate (HSC)] and HSP70 protein which is heat-shock/stress inducible. In the rat mRNA transcripts of HSP70 are 2.3 kilobases for the constitutively expressed form and 2.8 and 3.1 kilobases for the inducible forms (Ehrenfried et al. 1996; Menoret et al. 1995; O'Malley et al. 1985). Wu et al. (1985) cloned human HSP70 mRNA transcript and found it to be 2.6 kilobases. The HSP70 protein in the human is 69.8 kilodaltons (Hunt and Morimoto 1985). HSP70 associates with aporeceptors and remains bound to the activated/transformed receptor (a receptor capable of binding ligand) following ligand binding and transport of the receptor from the cytoplasm to the nucleus. HSP70 appears to bind directly to the HBD of the receptor as does HSP90 (Schowalter et al. 1991). The stoichiometry of HSP70 with respect to the receptor is not well established, but is believed to be substoichiometric; Diehl and Schmidt (1993) found there to be one HSP70 molecule for every five glucocorticoid receptors.

HSP70 interacts with a number of proteins when bound to steroid receptors. DnaJ is considered a chaperone partner for HSP70 in mammals (Bohen et al. 1995). It is believed necessary for the assembly and maintenance of aporeceptors and for proper folding of the activated receptor after ligand binding i.e. for the folding of the receptor into its transcriptionally active conformation (Bohen et al. 1995). The binding of DnaJ is an ATP dependent process (Bohen et al. 1995). Prapapanich et al. (1996) found an association of p48 with HSP70 and, to a lesser extent, with HSP90 and p60. HSP70 is involved in the binding of HSP90 to receptor; HSP70 is an ATP-binding protein with weak ATPase activity, and it is thought to direct folding/unfolding reactions of
other proteins, such as HSP90, in an ATP-dependent manner (Smith et al. 1992). It has also been proposed that HSP70 might function in the assembly of protein oligomers. In these roles, HSP70 is thought to function as a molecular chaperone (Smith et al. 1992).

As with HSP90, we are only beginning to understand the role of HSP70 in steroid receptor activation. HSP70 is believed to maintain steroid receptors in an unfolded state which presumably facilitates their translocation across membranes (Bagchi et al. 1991). Very little is known about the mechanism that promotes the release of HSP70 and the proper folding of the protein after it emerges through the nuclear membrane (Bagchi et al. 1991). HSP70 is diffusely located in both the cytoplasm and the nucleus in unstressed cells. In stressed cells there is an overall increase in levels of HSP70 with a specific increase in the nucleoli (Welch and Feramisco 1984). The significance of this apparent nuclear distribution of HSP70 in cells is not clear (Welch and Feramisco 1984).

### 2.3.3 Heat-shock transcription factor

Transcriptional induction of heat-shock genes is mediated by the binding of a transcriptional activator, heat-shock transcription factor (HSF), to a short highly conserved DNA sequence known as the heat-shock element (HSE) present in the promoters of heat-shock genes (Morimoto et al. 1992; Rabindran et al. 1991; Sorger and Pelham 1987; Shen et al. 1997). At least two genes encode HSF in humans which are known as HSF1 and HSF2 (Schuetz et al. 1991). These two HSF appear to differ functionally; HSF1 mediates the response to stress while HSF2 is activated in the absence of physiological stress, perhaps during differentiation or development (Morimoto et al. 1992). Upon heat-shock, HSF are transformed from a monomer into a trimer. The HSF trimer then binds to HSE and stimulates transcription [see (Lis and Wu 1993; Morimoto et al. 1992; Wu 1995; or Morimoto et al. 1997) for reviews]. The mechanism by which binding of HSF to HSE leads to stimulation of transcription is unknown.

HSFs are regulated by steroids, in particular estrogen (Xiao and DeFranco 1997). Estrogen appears to positively regulate both HSF1 and HSF2 at both the mRNA and protein level (Yang et al. 1995). HSF1 and HSF2 have both been identified in the uterus of mice. Yang et al. (1995)
found levels of HSF1 and HSF2 decrease in uteri, vagina and mammary glands in mice following ovariectomy. In the ovariectomized rats estrogen treatment increases HSF levels, R5020 had a marginal effect on HSF, while testosterone had no effect (Yang et al. 1995). Phosphorylation of newly synthesized HSF appears necessary for HSF function (Ding et al. 1997). Ding et al. (1997) found activation of protein kinase C causes phosphorylation of HSF1 which leads to an increase in HSP70 production.

2.3.4 Steroid induction of heat-shock proteins

As discussed earlier, P, and in particular estradiol can stimulate HSF which in turn increases HSP transcription. Both estradiol and P, affect the levels of HSP found in uteri, a potential result of their effects on HSF in this tissue. Estradiol has been shown to increase HSP90 protein levels in the mouse and rat uterus (Olazabal et al. 1992; Ramachandran et al. 1988). Ramachandran et al. (1988) found that ovariectomy reduces mouse uterine concentration of HSP90, and that estradiol treatment for 24 hours results in an increase of HSP90 up to 4 times that of pre-ovariectomy levels. This result could not be repeated by treating ovariectomized rats with the synthetic estrogen diethylstilbestrol, the androgens dihydrotestosterone or testosterone, or the synthetic P, R5020. As well, this response of HSP90 was not seen in non-estradiol target tissues such as the spleen. Ramachandran et al. (1988) also measured P, receptors in myometrium of those mice which were ovariectomized and treated with estrogen. P, receptor levels increased 3.67 times above that of controls following 24 hours of estradiol treatment. Similarly, Olazabal et al. (1992) found that estradiol treatment of ovariectomized rats increased HSP90 levels 2-3 times, 18 hours following treatment. Both HSP70 and HSP90 were found to change during the menstrual cycle and to be differentially regulated in the endometrium and myometrium (Komatsu et al. 1997). In the human myometrium, mRNA and protein expression of HSP70 and HSP90 were found to be higher in the proliferative phase compared to levels during the secretory phase (Komatsu et al. 1997). This same study found HSP70 levels in the endometrium to increase during the secretory phase, while HSP90 levels in this tissue remained unchanged during the menstrual cycle (Komatsu et al. 1997). Expression of estrogen receptors and P, receptors is known to be highest during the
proliferative phase in the human myometrium, and during the secretory phase estrogen receptors expression is down-regulated while P₄ receptor levels are maintained (Lessey et al. 1988). Estradiol treatment has been shown to increase HSP90 and HSP70 in the sheep endometrium and myometrium (Wu et al. 1996b). In these sheep P₄ treatment increased HSP levels but not to the extent of estradiol treatment alone, and the combination of both estrogen and P₄ treatment resulted in an even lesser response than P₄ alone (Wu et al. 1996b).

Estrogen receptors appear to form only transient associations with HSP90. Sabbah et al. (1996) suggested that at physiological temperature, HSP90 may stabilize an active form of the receptor in accordance with its general molecular chaperone role. When temperatures are elevated or the tissue is under some other environmental stress, the increased cellular concentration of HSP90 interferes negatively with estrogen receptor-dependent transcription, in accordance with inhibition of gene transcription attributed to HSP90 after heat-shock (Sabbah et al. 1996). Therefore, under stress conditions the binding of estrogen receptors to the estrogen response element on a target gene is inversely dependent on the relative concentration of HSP90 in vitro. If a labouring uterus is considered a 'stressed' tissue than HSP would apparently act to inhibit estrogen effects, but if not, than HSP would confer their normal chaperone function on these receptors.

The time from heat-shock to activation of HSP response can be detected within minutes (Morimoto et al. 1992). Estrogen up-regulation of HSP in vivo can be detected within 18-24 hours (Komatsu et al. 1997; Ramachandran et al. 1988). Therefore, with respect to time of response, changing levels of HSP at the time of labour could be rapidly altered and thus play an important role in altering the contractility of the uterus.

2.3.5 Interaction of heat-shock proteins with progesterone receptors
A number of studies have examined the specific interaction of HSP with P₄ receptors. A single unoccupied P₄ receptor is found in complex with several other proteins, a number of which are heat-shock proteins (Goodman and Hodgen 1996). Time course studies on in vitro assembly of P₄ receptor complexes have suggested that HSP70 and p48 are among the earliest components associated with P₄ receptors, followed by HSP90 and p60 (Prapapanich et al. 1996). The binding
of proteins such as p23, FKBP52, FKBP54, and CyP40 to HSP90, as well as 3 components of the MAP kinase signaling system Src, Raf, and Mek [see (Pratt 1998) for a review] are also necessary to create a mature P₄ receptor complex (Smith et al. 1990). P₄ receptors within this complex do not form dimers or bind to DNA (Goodman and Hodgen 1996). As well, these complexes are in a steady state of assembly/disassembly with a half-life of 5 minutes, which is independent of whether hormone is bound or not (Smith 1993). After the conformational change produced by ligand binding (and perhaps enhanced by concurrent phosphorylation of the P₄ receptors), some of these proteins (including HSP90 but not HSP70) rapidly dissociate from the P₄ receptors (Goodman and Hodgen 1996). The dissociation of these proteins from the receptor can be caused by P₄ treatment (Smith et al. 1992). Following hormone binding, P₄ receptors form dimers. DeMarzo et al. (1991) suggested that HSP90 may repress DNA-binding activity of steroid receptors indirectly by blocking receptor dimerization, and hence HSP90 release from the receptor may be a key step in regulating dimerization. Figure 2.5 depicts the interaction of HSP with P₄ receptors.
Figure 2.5: Schematic diagram outlining the proposed mechanism of progesterone ($P_4$) receptor action in humans. The following abbreviations are used in the figure: p (phosphorylation); HSP90 (heat-shock protein 90); HSP70 (heat-shock protein 70); CBP [cAMP response element binding protein (CREB)-binding protein/p300]; PCAF (p300/CBP associated factor); p160 [which includes SRC1 (steroid receptor coactivator); TIF2 (transcription intermediary factor 2); DNA (deoxyribonucleic acid); PRE (progesterone response element); TFs (transcription factors); GTFs (general transcription factors); RNA (ribonucleic acid); mRNA (messenger RNA), AIB1 [amplified in breast cancer 1, the human homologue of PCAF (p300/CBP activation factor) in the mouse].

Once formed, the structure of the $P_4$ receptor is modified through phosphorylation. The binding of HSP, immunophilins, and other such proteins, further modifies receptor structure. Ligand binding causes a further change in receptor structure, the majority of HSP are removed from the receptors, and another wave of phosphorylation takes place. The next step involves either the binding of coregulatory proteins then dimerization of the receptors, or dimerization of the receptors followed by the binding of coregulatory proteins. All coactivator proteins have histone acetyltransferase activity which helps break down or loosen nucleosomal structure. The fully functional receptor then binds to the PRE of its target DNA. The receptor/coregulatory protein complex the interacts with GTFs and RNA polymerase to effect transcription. A more detailed description of these events is provided in the text.
2.3.6 Heat-shock protein levels *in vivo*

Our knowledge of HSP levels *in vivo* is somewhat limited. Blake et al. (1990) examined mRNA expression of HSP70 in brain, liver, lung and skin of rats exposed to elevated ambient temperatures. In brain, lung, and skin tissues induction was transient with maximum HSP70 expression occurring at 1 hour, and returning to baseline 3 hours after removal of the animals from heat stress. In liver, maximum HSP70 expression occurred 6 hours after heat stress.

Of the *in vivo* studies which have been performed on human tissues, a limited number have described HSP in the uterus (Koshiyama et al. 1995; Nanbu et al. 1996; Tabibzadeh et al. 1996; Tang et al. 1995; Wu et al. 1996b). Komatsu et al. (1997) described HSP mRNA and protein in human endometrium during the normal menstrual cycle. HSP90 and the inducible form of HSP70 did not change notably during the cycle. HSP70 (constitutive form) was found to be greater in the secretory phase than in the proliferative phase of the menstrual cycle (Komatsu et al. 1997; Tabibzadeh et al. 1996). As mentioned in section 2.3.4, in the myometrium, Komatsu et al. (1997) found both HSP90 and HSP70 levels to be highest in the proliferative phase, and lower in the secretory phase (Figure 2.6). Estrogen and P₄ receptor levels in the myometrium are high during the proliferative phase when estradiol and P₄ levels are low. This is in comparison to the secretory phase, when these steroid levels are higher and consequently, estrogen receptor are down-regulated. P₄ receptors however, remain relatively constant in the myometrium through the menstrual cycle despite changing P₄ levels. Therefore, HSP levels in the myometrium during the menstrual cycle appear to follow levels of estrogen receptors.
Figure 2.6:  

a) Maternal circulating levels of 17β-estradiol and P₄ during the menstrual cycle [modified from (Aron and Tyrrell 1994)].  
b) Estrogen and P₄ receptor (A and B isoforms combined; protein, immunostaining) in the human myometrium during the menstrual cycle [modified from (Lessey et al. 1988)].  
c) Relative amounts of HSP90α and HSP70 in the human myometrium during the menstrual cycle. Data for both HSP are the same for the proliferative and the secretory phase [modified from (Komatsu et al. 1997)].
2.3.7 Alterations in heat-shock response

HSP levels in response to stress appear to change with age as well as disease state of the organism. An increase of HSP70 levels in response to stress appears to be attenuated with senescence in rats (Kregel et al. 1995; Pahlavani et al. 1995). This suggests that an aging animal has a reduced ability to properly maintain cellular function and integrity after a thermal challenge. Wikland and Wiqvist (1984) examined HSP27 levels in human uterine leiomyomas and adjacent healthy myometrium, and found that leiomyomas had higher concentrations of HSP27 than did healthy myometrium. Takahashi et al. (1995) found estrogen receptor-positive human breast carcinomas to preferentially express HSP27, and HSP70, while estrogen receptors-negative carcinomas preferentially expressed p53. Somewhat in contrast to these results, Nanbu et al. (1996) found HSP70 and p53 expression to be correlated with a loss of estrogen and 

2.4 Steroid receptor coregulators

Binding of a ligand to the ligand-binding domain of steroid receptors results in a conformational change which creates the surface required for interaction of various proteins with the AF and DNA binding regions of the receptors (Voegel et al. 1996; Xu et al. 1996). Such proteins are referred to as coregulators. Coregulators may be defined as limiting factors which enhance the transcriptional activity of nuclear receptors without altering their basal transcription (Horwitz et al. 1996). For example, Onate et al. (1994) found that purified human P4 receptors expressed as a full-length protein in a baculovirus system were able to bind hormonal ligand and to dimerize, but the receptor's ability to bind DNA and affect transcription was greatly decreased. When nuclear extracts from several cellular sources were added DNA binding activity was restored, thus confirming that P4 receptors require accessory protein(s) for efficient interaction with specific DNA sequences (Onate et al. 1994). It is possible that certain clinical syndromes involving partial hormone resistance in which receptors are intact may in part be due to an impairiment of nuclear receptor coactivators (Xu et al. 1998).
Coregulators may exert their effects on transcriptional activity by stimulating steroid receptor DNA-binding, by stimulating other activators (Onate et al. 1994), by affecting chromatin structure (Kwon et al. 1994), by mediating activator-initiation complex interactions (Kee et al. 1996), and/or by some yet undescribed mechanism.

To date a number of coregulator (coactivators and corepressors) proteins have been described. The major coactivators known to act through the AF2 domain include: RIP140 (receptor interacting protein 140) (Cavailles et al. 1995); TIF1 (transcription intermediary factor 1) (LeDouarin et al. 1995); TRIP1 (thyroid receptor interacting protein 1)/SUG1 (Lee et al. 1995); ARA70 (androgen receptor coactivator 70) (Yeh and Chang 1996); Trip230 (thyroid receptor interacting protein 230) (Chang et al. 1997); ERAP140 (estrogen receptor associated protein 140); and related proteins of 160 kilodaltons (p160) named SRC1 (steroid receptor coactivator)/NCoA-1 (nuclear receptor coactivator-1) (Onate et al. 1995); TIF2 (transcription intermediary factor 2)/GRIP1 (glucocorticoid receptor interaction protein)/NCoA-2 (nuclear receptor coactivator-2) (Lyerly and DiMaio 1993; Voegel et al. 1996); AIB1 (amplified in breast cancer) (Li et al. 1997)/ACTR (Chen et al. 1997)/RAC3 (receptor associated coactivator) (Li et al. 1997)/pCIP/pCAF (p300/CBP/co-integrator-associated protein) (Anzick et al. 1997; Torchia et al. 1997); and CBP (cAMP response element binding protein (CREB)-binding protein)/p300 (Kamei et al. 1996). Elimination of the AF2 domain has been shown to abolish the interaction of the steroid receptor with its various coregulators and consequently transcriptional activation by that receptor (Jeyakamar et al. 1997; Masuyama et al. 1997; Voegel et al. 1998).

Coactivators can act through areas other than the AF2 region of nuclear receptors. For example, p300/CBP associated factor, PCAF (human)/GCN5 (yeast), associates with the DNA-binding domain (Blanco et al. 1998; Smith et al. 1998), and p300/CBP binds to the AF1 region as well as the AF2 region (Ogryzko et al. 1996).

Coactivators which act through the AF2 domain are believed to exhibit a number of specific characteristics: 1) they interact in vivo with nuclear receptors in an agonist- and AF2-integrity-dependent manner; 2) they bind directly to the ligand-binding domains of nuclear receptors in an agonist and AF2 integrity-dependent manner in vitro; 3) they harbor an autonomous transcriptional activation function; 4) they relieve nuclear receptor autosquelching (the transcriptional
autointerference generated by expressing increasing amounts of steroid receptor); and 5) they enhance the activity of some nuclear receptor AF2s when over-expressed in mammalian cells (Voegel et al. 1996).

The proteins that act through the AF2 domain of the steroid receptor all have at least one, if not more copies of a LXXLL motif in which L denotes leucine and X denotes any amino acid (Ding et al. 1998; Voegel et al. 1998). This motif, also known as a nuclear receptor (NR) box, or nuclear box, appears to be both necessary and sufficient for interaction of the coregulator with steroid receptors (Heery et al. 1997; LeDouarin et al. 1996; Torchia et al. 1997). The multiple motifs on each coregulator appear to differentially contribute to the binding of these proteins to various nuclear receptors. At present, the reasons behind the multiple motifs are not entirely clear. The multiple motifs may allow coregulators to interact with a broad range of nuclear receptors. They may also allow each coregulator molecule to interact with more than one nuclear receptor monomer (Ding et al. 1998).

Knowing that coactivators enhance the ability of steroid receptors to transactivate their target genes, they are believed in some way to enhance the assembly of basal transcription factors into a stable unit termed a preinitiation complex. This is a group of proteins including the steroid receptor, steroid receptor coactivators, TFIIB, and other complexing proteins (Figure 2.5). Transcription in eukaryotic nuclei is regulated by RNA polymerase. The majority of studies have focused on RNA polymerase II (RNAPII) because it is a multisubunit protein that transcribes all protein-encoding genes (Chilton and Hewetson 1998). The preinitiation complex thus in some way increases transcription initiation rates via RNA polymerase II (Ptashne 1988).

Along with coregulators and RNA polymerase II, mRNA synthesis is also regulated in part through general transcription factors (GTFs). GTFs (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFI IH) mediate binding of RNA polymerase II to the promoters, but also have important functions in later steps of the initiation process. In cell-free transcription systems GTFs and RNAPII are sufficient to initiate basal levels of transcription from TATA-only promoters (Bastian and Nordeen 1991).

Activators bind to promoters or enhancers in a sequence-specific manner and thereby stimulate the rate of transcription initiation at the target promoter above basal levels. The underlying
mechanisms of transcriptional stimulation are not well understood, but many activators have been shown to interact with various components of the initiation complexes. These interactions are thought to help to recruit RNAPII GTFs or the RNAPII holoenzyme complex, to stabilize initiation complex intermediates, or to induce conformational changes within components of the initiation complex that may activate subsequent steps [for review see (Zawel and Reinberg 1995)].

2.4.1 Histone acetyltransferase activity

Many of the steroid receptor coactivators described to date possess intrinsic histone acetyltransferase/acetylase (HAT) activity. Nucleosomes consist of double-helix DNA surrounding an octamer of histones. Histone deacetylation appears to favor interactions between adjacent nucleosomes leading to the compaction/condensation of the chromatin which are transcriptionally inactive (Bestor 1998). Histone acetylation is therefore needed to break-up nucleosomes rendering the DNA accessible for transcriptional activation (Bestor 1998). Thus, breaking down or loosening nucleosomal structure via histone acetylation by transcription factors and cofactors is an important step leading to steroid receptor transactivation of gene expression (Spencer et al. 1997; Wade and Wolffe 1997).

2.4.2 SRC1

SRC1 (NCoA-1) is expressed primarily in the nucleus of a variety of cell lines and tissues (Kamei et al. 1996; Yao et al. 1996). It binds glucocorticoid receptors, estrogen receptors, androgen receptors, mineralocorticoid receptors, P₄ receptors, vitamin D receptor, retinoic acid receptors (RAR and RXR), and thyroid receptors (Ding et al. 1998; Olson and Koenig 1997; Onate et al. 1995). SRC1, however, appears to have different effects on these different receptors. For example, SRC1 has been shown to augment the transcriptional activity of P₄ receptors, estrogen receptors, glucocorticoid receptors, thyroid receptors and retinoic acid receptors (Onate et al. 1995). Olson and Koenig (Olson and Koenig 1997) found that SRC1 only minimally augmented transcriptional activity by glucocorticoid receptors, while it had a much greater effect on transcriptional activation by P₄ receptors. SRC1 has been found to be essential for RAR signaling (Klakhoven et al. 1998).
SRC1’s effects on transcriptional activation appear to be limited to the receptor being bound to an agonist; SRC1 does not interact efficiently with P₄ receptors in the presence of an antagonist (Onate et al. 1995).

Xu et al. (1998) have developed SRC1 knockout mice. They found both male and female homozygous mutants to be viable and fertile. In response to steroid hormones, SRC1 target organs such as the uterus, prostate, testis, and mammary glands had decreased growth and development. They suggested from their results that SRC1 does mediate steroid hormone response in vivo and loss of this coactivator results in partial resistance to hormone. In SRC1 target tissues of homozygous mutants Xu et al. (1998) found that TF2 RNA expression was increased compared to wildtype mice. They suggested that this was in part to compensate for the loss of SRC1 function in these tissues.

Two SRC1 isoforms have been recently identified. SRC1a and SRC1e diverge at their C-termini and are functionally distinct (Klakhoven et al. 1998). SRC1a contains a unique LXXLL motif in its C-terminus. The reason for the functional difference between the two isoforms appears to be due to a CBP independent activational domain which is present in both isoforms but appears to be suppressed in SRC1a (Klakhoven et al. 1998). Klakhoven et al. (1998) found that in intact cells, SRC1e enhanced the transcriptional ability of estrogen receptors to a much greater extent than did SRC1a. SRC1 is expressed as two RNAs of approximately 5.5 and 7.5 kilobases, with a protein of 125 kilodaltons, in a variety of tissues (Onate et al. 1995). It is possible that these two RNA bands correspond to the two recently described SRC1 isoforms.

The exact mechanism of SRC1 action has yet to be determined. We do know that SRC1 possesses intrinsic HAT activity and it also interacts with another HAT, pCAF (Spencer et al. 1997). The HAT activity of SRC1 maps to its carboxyl-terminal region and is primarily specific for histones H3 and H4. Acetylation by SRC1 and pCAF of histones bound at specific promoters may result from ligand binding to steroid receptors and could be a mechanism by which the activation functions of steroid receptors and associated coactivators enhance formation of a stable preinitiation complex, thereby increasing transcription of specific genes from transcriptionally repressed chromatin templates (Spencer et al. 1997). After ligand binding, SRC1 may act, in part, as an adapter protein that promotes the integration of amino- and carboxyl-terminal receptor
functions, allowing for full receptor activation (McInerney et al. 1996). SRC1 may be capable of enhancing the transcriptional activity of related nuclear receptor superfamily members by facilitating the productive association of AF2 and AF1 in these receptors (McInerney et al. 1996).

2.4.3 GRIP1/TIF2

GRIP1 (NCoA-2) is the mouse homologue of the coactivator TIF2 in humans. GRIP1 binds to the same steroid receptors as does SRC1 (Ding et al. 1998). It is known to increase the transcriptional activity of retinoic acid receptors (RXR and RAR), thyroid receptors, glucocorticoid receptors, estrogen receptors, mineralocorticoid receptors, and androgen receptors, (Berrevoets et al. 1998; Hong et al. 1997; Walfish et al. 1997). TIF2 is known to form stable complexes with P₄ receptors (McKenna et al. 1998), yet a direct effect of TIF2 on P₄ receptor transactivation has yet to be demonstrated. Hong et al. (1996) originally described a partial cDNA of GRIP1, and later reported the isolation of a full-length GRIP1 cDNA (4.9 kilobases) (Hong et al. 1997). The mRNA of TIF2 is ~9 kilobases, and the protein has a molecular weight of 160 kilodaltons (Voegel et al. 1996). TIF2 and GRIP1 exhibit partial sequence homology with SRC1 (Voegel et al. 1996), suggesting that these proteins are members of a family of coactivators. This family is referred to as the p160 coactivators.

TIF2 has a single nuclear receptor interaction domain (NID) and two autonomous activation domains, AF1 and AF2 (Voegel et al. 1998). The NID is made up of three NR-interacting modules which each contain the LXXLL motif/box (Voegel et al. 1998). Mutation of these three NR modules (I, II, and III) abolishes TIF2 interactions with nuclear receptors. NR module II is sufficient for both efficient interaction with ligand binding domains and stimulation of AF2 activity. NR modules I and III are poorly efficient on their own but synergistically can promote interaction with ligand binding domains and AF2 stimulation (Voegel et al. 1998). Interactions of GRIP1 with estrogen receptors were more strongly affected by mutations in NR module II whereas interactions with the androgen and glucocorticoid receptors were more strongly affected by NR module III mutations (Ding et al. 1998). Voegel et al. (1998) also identified a CBP interaction domain, CID, which is identical with AF1. Therefore, TIF2 AF1 activity is mediated through CBP. TIF2 AF2
activity does not appear to involve interaction with CBP (Voegel et al. 1998). Klakhoven et al. (1998) identified a similar functional domain structure in SRC1.

GRIPl and SRC1 exhibit similar, but not identical nuclear receptor binding preferences (Ding et al. 1998). SRC1 has been shown to be essential for RAR signaling (Klakhoven et al. 1998), but TIF2/GRIPl is not (Torshia et al. 1997). Ding et al. (1998) found that the androgen receptor bound well to GRIPl but poorly to SRC1. As mentioned, TIF2/GRIPl and SRC1 contain three copies of the NR binding motif LXXLL in their central regions. The isoform of SRC1, SRC1a, has an additional NR box (box IV) at its extreme C terminus with an NR binding preference somewhat different from that of the central NR binding domain of SRC1e. GRIPl has no NR box in its C-terminal region and therefore no c-terminal NR binding function (Ding et al. 1998). These differences may contribute to the functional differences seen between these two coactivators.

2.4.4 ARA70

ARA70 is a protein described in humans which interacts with androgen receptors and enhances androgen-receptor dependent transcription 10-fold (Yeh and Chang 1996). To date only ARA70 (Yeh and Chang 1996), TIF2 (Voegel et al. 1996), CBP and SRC1 (Ikonen et al. 1997) have been shown to enhance androgen-mediated transactivation. ARA70 has been shown to increase glucocorticoid receptor, P4 receptor, and estrogen receptor-dependent transcription 2-fold (Yeh and Chang 1996). ARA70 mRNA is ~3.6 kilobases, and its protein is 70 kilodaltons (Yeh and Chang 1996). ARA70 has been identified in mouse prostate, testis, adrenal gland, thymus, muscle, heart, kidney, lung, and fat tissues but not in seminal vesicle, spleen, liver or brain cortex (Yeh and Chang 1996). ARA70 has also been described in human breast cancer MCF-7 cells (Yeh and Chang 1996), murine gonadotropin-releasing hormone-secreting hypothalamic neuron GT1-7 cells (Belsham et al. 1998), as well as human prostate cancer DU145 cells (Yeh et al. 1998). Dihydrotestosterone (DHT) appears to be a more potent modulator of androgen receptor-ARA70 interaction than testosterone (Yeh and Chang 1996). While various coactivators can affect the transcriptional activity of androgen receptors in the presence of DHT, only ARA70 can induce
androgen receptor transcriptional activity in the presence of estradiol (Yeh et al. 1998b), suggesting that estradiol may be an important ligand for androgen receptors.

2.4.5 CREB and CBP/p300

Yet another coactivator is CBP/p300 (Klakhoven et al. 1998; Smith et al. 1996). They are functionally conserved proteins which have HAT activity (Ogryzko et al. 1996) along with several putative activation domains. CBP contains the leucine rich interaction motif, and binds/interacts directly with the nuclear receptor ligand-binding domain as well as with p160 proteins (Kamei et al. 1996). CBP appears to associate specifically with RNA polymerase II as well as the phosphorylated form of CREB and TFIIB, suggesting that this coactivator stimulates transcription, at least in part, thought its recruitment of RNA polymerase II to target gene promoters (Smith et al. 1996). Kamei et al. (1996) determined that CBP is essential for transcriptional activity by CREB, AF1, and nuclear receptors. These factors appear to compete with each other for limiting amounts of CBP within a cell (Kamei et al. 1996). The physiological importance of CBP is shown by people with Rubinstein-Taybi syndrome. These individuals are heterozygous for a truncated CBP transcriptional unit and consequently exhibit severe developmental defects (Petrij et al. 1995).

2.4.6 pCIP/pCAF/AIB1

A coactivator often found in cells in complex with CBP is pCAF (Torchia et al. 1997). It was first described in mouse tissues and believed to be in the same family of coactivators as SRC1 and GRIP1/TIF2, yet they are now believed to be functionally distinct proteins and thus pCAF belongs in a family separate from SRC1 and GRIP1/TIF2 (Torchia et al. 1997). As with SRC1, GRIP1/TIF2 and CBP it contains the LXXLL motif and has HAT activity (Torchia et al. 1997). The human homologue of the mouse pCAF is AIB1 (amplified in breast and ovarian cancer) (Anzick et al. 1997)/RAC3 (Chen et al. 1997)/ACTR (Li et al. 1997) which are alternate splice variants found by three groups independently. The yeast homologue of pCAF is GCN5 (Smith et al. 1998).
2.4.7 COPRA

The only cofactor so far identified which is believed to specifically effect P₄ receptor transactivation is cofactor of P₄ receptor activation (COPRA). The C-terminal sequences of P₄ receptors including AF2 region somehow mask the N-terminal region thus blocking the activation functions of DNA-bound receptor (Klotzbucher et al. 1997). COPRA is believed to somehow relieve this repression (Klotzbucher et al. 1997). In experiments involving baculovirus-expressed c-terminally truncated P₄ receptors lacking the AF2 region COPRA has no effect on transactivation since the truncated receptor is acting through AF1 and/or AF3 domains (Klotzbucher et al. 1997).

2.4.8 Other coactivators

There are a number of other coactivators which mediate steroid receptor transcriptional activation, yet a detailed discussion on them is beyond the scope of this review. Briefly then, notable coactivators which may be involved in P₄ action include the high-mobility group chromatin protein (HMG1) which is known to enhance the binding of P₄ receptors to PREs (Onate et al. 1994). HMG1 activity is increased by estradiol (Chau et al. 1998; Verrier et al. 1997). It directly interacts with p53 and stimulates p53 DNA binding (Jayaraman et al. 1998). It also appears to act in sequence with TAFII to promote estrogen receptor-estrogen response element binding (Verrier et al. 1997). Another coactivator is RSP5(yeast)/hRPF1(human) (Imhof and McDonnell 1996). Imhof and McDonnell (1996) found the expression of RSP5 in yeast and human cells potentiated human P₄ receptor and human glucocorticoid receptor transcriptional activity. The activity of RSP5/hRPF1 was enhanced by a protein known as SPT3 which interacts with the TATA box binding protein (TBP), suggesting a functional link between activated P₄ receptors, glucocorticoid receptors, and the general transcriptional apparatus (Imhof and McDonnell 1996).

Coactivators which have been identified but have not yet been associated with P₄ receptor transactivation include: thyroid hormone receptor coactivator (F-SRC1) a protein similar to SRC1 (Lipshitz 1986); peroxisome proliferator-activated receptor binding protein (PBP) serves as a coactivator, containing two LXXLL motifs (Zhu et al. 1997); receptor-associated coactivator 3 (RAC3) which is related to SRC1 and TIF2 (Li et al. 1997).
A number of other proteins, which may not necessarily be characterized as coactivators, also appear to affect $P_4$ receptor transcriptional activity. One such protein is receptor binding factor (RBF-1) which is a small (6-10 kilodalton) chromatin-associated protein (Zhuang et al. 1993). It is believed to enhance nuclear binding of $P_4$ receptors to DNA. RBF-1 has been identified in all cells that respond to $P_4$ including the rat uterus (Landers et al. 1994; Zhuang et al. 1993), yet it has also been identified in non-$P_4$ responsive tissues suggesting that it may play a diverse role in steroid receptor transcriptional activation (Landers et al. 1994).

### 2.4.9 Combined effects of coregulators

In addition to coactivators and other transcription factors which enhance steroid receptor transactivation of target DNA, there are corepressor proteins which have inhibitory effects on this transactivation (Jackson et al. 1997). McDonnell et al. (1992) identified and characterized a protein they named SSN6. They found this repressor specifically affects the transcriptional activity of the AF1 activation domain of the estrogen receptor. Mutation of SSN6 results in an increase of $P_4$ receptor and estrogen receptor transcriptional activity (McDonnell et al. 1992). SSN6 has not yet been shown to interact directly with DNA (McDonnell et al. 1992). Another corepressor, silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), is capable of inhibiting the activation of estrogen receptor transcriptional activation mediated by SRC1 (Smith et al. 1997). Yet another corepressor is the thyroid/retinoid acid receptor corepressor [hN-CoR (human)/mN-CoR (mouse)] which is known to inhibit estrogen receptor as well as $P_4$ receptor transactivation (Jackson et al. 1997). Both SMRT and N-CoR are known to interact with receptors in the absence of hormone (Jackson et al. 1997; Smith et al. 1997).

Steroid receptor corepressors favor histone deacetylation (Wolffe 1997). As discussed earlier, steroid receptor coactivators have histone acetyltransferase activity. The various corepressors and coactivators may have somewhat different histone deacetylation and acetyltransferase activities respectively (Korzus et al. 1998). These differences may be required in order to affect the transcriptional activity of various steroid receptors with apparently different interaction strengths (Chen et al. 1997; Korzus et al. 1998). Thus, a complex interaction of
coactivators and corepressors, along with the actions of various other transcription factors, receptor concentrations, and ligand levels, ultimately results in the level of transactivation in a given situation.

Coactivator and corepressor action on steroid receptors when they are bound to antagonists may help explain the presence of partial agonistic activities of some antagonists. For example, Jackson et al. (1997) characterized a novel hinge-hormone binding domain protein L7/SPA which, when coexpressed with RU486-occupied human P$_4$ receptor increased the partial agonist activity of RU486 3-10 fold, but had no effect on antagonist mediated transcription. Pure antagonists such as the antiprogestin ZK98299 cannot be up-regulated by L7/SPA (Jackson et al. 1997). Jackson et al. (1997) also found that SMRT, along with SRC1, suppress RU486 or tamoxifen-mediated partial agonist activity by more than 90% (Jackson et al. 1997). Thus, as mentioned earlier, the presence and/or abundance of various coactivators and corepressors can have a significant effect on the transcriptional activity of steroid receptors not only when they are agonist bound, but also when they are bound to antagonists with partial agonist activities.

2.5 Other mechanisms of steroid action

In humans maternal circulating P$_4$ levels do not drop prior labour initiation, neither does there appear to be a significant change in local levels, or in the production or metabolism of P$_4$. The amount of free circulating versus serum-protein bound P$_4$ does not change at this time. In addition, there does not appear to be a significant change in maternal circulating E/P$_4$ ratio with labour.

P$_4$ effects are mediated in part by its interaction with its steroid receptors. These effects can be affected by altering the levels of the A and B isoforms of the P$_4$ receptor, as well as altering the levels of the various proteins which are necessary and/or which affect the transcriptional activation of these receptors. In addition to effects mediated via steroid receptors, steroids, including P$_4$, have also been shown, under certain circumstances, to exert physiological effects through interactions with other receptor systems.

Classical/genomic mechanism of steroid action through nuclear receptors produces effects within hours or days. This mechanism can not therefore, account for the variety of rapid (seconds or minutes) steroid effects [see (Duval et al. 1983; McEwen 1991; O'Malley et al. 1995; Wehling
We are aware of only a few mechanisms by which $P_4$ may induce such rapid effects: by acting through sex hormone-binding globulin (SHBG) or corticosteroid binding globulin (CBG) membrane receptors; by opening plasma membrane Ca$^{2+}$ channels and thereby increasing intracellular calcium levels (Heery et al. 1997); or through the binding of $P_4$ metabolites to GABA$_A$ membrane receptors.

SHBG and CBG bind circulating steroids, and by doing so can regulate plasma concentration of free steroids (Hammond et al. 1991; Rosner 1990; Rosner 1996; Rosner et al. 1991; Scrocchi et al. 1993). A suggested model for SHBG-steroid system involves the binding of a steroid free SHBG molecule to its membrane receptor. The subsequent binding of a steroid to the SHBG molecule may potentiate cellular second messenger systems (Rosner 1990). While such a model has not yet been described in detail for CBG, it is believed to affect cellular second messenger systems as well. For example, Nakhala et al. (1988) found an increase in adenylate cyclase activity and an accumulation of cyclic adenosine monophosphate (cAMP) following the addition of CBG to MCF-7 (breast carcinoma) cells. $P_4$ does not significantly bind to SHBG, but does so to CBG [see (Rosner 1996; Westphal et al. 1977) for reviews]. It is possible that a portion of the non-genomic effects of $P_4$ may be mediated through CBG membrane receptors.

$P_4$ effects may in some cases be ligand-independent. For example, Power et al. (1991) found that dopamine could mimic the effect of $P_4$, causing translocation of the chicken $P_4$ receptor from the cytoplasm to the nucleus. Zang et al. (1994) suggested that there are numerous signaling pathways which can activate $P_4$ receptors independent of ligand binding. One of these mechanisms may involve phosphorylation of $P_4$ receptors, a process which is known to be essential for receptor action (discussed in section 22.4) (Beck et al. 1992; Sheridan et al. 1989a; Sheridan et al. 1989b). Activators of kinases stimulate the hormone-dependent response, and inhibitors of protein kinase A can inhibit hormone-dependent activation of $P_4$ receptors (Beck et al. 1992; Denner et al. 1990). Therefore, kinase and/or phosphatase activity is important for modulation of steroid receptor activity in the presence and absence of ligand.

$P_4$ and corticosteroids are metabolized to 5$\alpha$-products which directly bind $\gamma$-aminobutyric acid (GABA) receptors. Allopregnanolone/tetrahydroprogesterone (3$\alpha$-hydroxy-5$\alpha$-pregnane-20-one; AP) is one of the principle $P_4$ metabolites. Naturally occurring 3$\alpha$-hydroxy, 5$\alpha$-pregnane
metabolites such as AP, interact with GABA receptors, and are capable of producing an inhibitory effect similar to that mediated by GABA at these receptors (Harrison et al. 1987; Majewska and Vaupel 1991; Paul and Purdy 1992). For example, both GABA and AP have been shown to inhibit the synthesis and release of oxytocin (Kendrick et al. 1992). Thus, another mechanism through which the effects of P₄ may be attenuated at the time of labour may be altering the actions of one of its metabolites.

As mentioned earlier, the uterus contains GABAergic receptors. Many P₄ metabolites, particularly those of the 5β-reduced series, and AP of the 5α-series, are potent inhibitors of uterine contractility (Majewska and Vaupel 1991; Putnam et al. 1991). Such inhibition during pregnancy may be mediated by the binding of these metabolites to GABAergic receptors in the uterus (Putnam et al. 1991). GABA levels in the uterus drop during third trimester pregnancy (Erdo et al. 1989), but P₄ levels at this time are approximately 100 times the levels obtained during the luteal cycle in non-pregnant women (Ganong 1994). Erdo et al. (1989) found GABA concentrations in uterine tissue to be lower in pregnant (cesarean section at term) than in non-pregnant women (hysterectomy, comparable patient age). They also found the specific binding of ³H-GABA significantly increased in specimens from pregnant subjects (Erdo et al. 1989). Erdo et al. (1989) suggested some gestation-related functional role for the GABA system in the human uterus. Therefore, the GABA system may be important for the initiation of human labour by the interaction of GABAₐ receptors with AP, producing an inhibitory effect similar to that mediated by GABA at these receptors.

2.5.1 Progesterone synthesis and metabolism

P₄ can be formed in a number of ways: from pregnenolone by the enzyme 3β-hydroxysteroid dehydrogenase (Figure 2.3); from pregnenolone sulfate by the enzyme steroid sulfatase; or via re-synthesis from one of its metabolites, 20α-dihydroprogesterone, by the enzyme 20α-hydroxysteroid dehydrogenase. Riley et al. (1993) studied the abundance of 3β-hydroxysteroid dehydrogenase mRNA via northern blots in placenta and chorio-decidua obtained following elective cesarean section at term, after preterm labour, or after term or post-term vaginal
delivery. They concluded that the abundance of 3β-hydroxysteroid dehydrogenase mRNA does not change in the placenta or fetal membranes with labour. This is consistent with the view that changes in 3β-hydroxysteroid dehydrogenase gene expression and decreased P₄ production are unlikely to affect intrauterine paracrine/autocrine regulatory mechanisms leading to term or preterm labour in women. As discussed in section 2.1.14, Mitchell and Challis (1988) found an increase in the activity of the enzyme 17β-20α-hydroxysteroid dehydrogenase in fetal membrane with labour. P₄ may be metabolized through irreversible conversion to 5α-dihydroprogesterone (5α-pregnane-3,20-dione) by the enzyme 5α-reductase. It is this pathway which leads to the reversible production of AP from 5α-dihydroprogesterone by the enzyme 3α-hydroxysteroid dehydrogenase / 3α-ketoreductase (Penning et al. 1996) (Figure 2.7).
Figure 2.7: Formation of allopregnanolone from progesterone via 5α-dihydroprogesterone. Allopregnanolone is then metabolized to pregnanediol, which is then excreted. While progesterone and 5α-dihydroprogesterone bind to intranuclear progesterone receptors, allopregnanolone binds, along with γ-aminobutyric acid, to γ-aminobutyric acid-A receptors.
Pregnanolone \rightleftharpoons \text{Progestosterone}

5\alpha\text{-Reductase}

5\alpha\text{-Dihydroprogesterone}

3\alpha\text{-Hydroxysteroid dehydrogenase}
\uparrow
3\alpha\text{-Ketoreductase}

\text{Allopregnanolone}

\rightarrow \text{Pregnanediol}

\rightarrow \text{URINE}

\rightarrow \text{GABA} \text{A receptor}

\rightarrow \text{\gamma-Aminobutyric acid (GABA)}
2.5.2 γ-aminobutyric acid and its receptor

GABA is an inhibitory neurotransmitter in the central and peripheral nervous system. Three major types of GABA receptors have been identified: GABA_A, GABA_B, and GABA_C. GABA_A receptors are ligand-gated Cl^- ion channels, and are the site of action of a variety of pharmacologically and clinically relevant drugs (Janis and Triggle 1986; Mathur et al. 1993; Milewich et al. 1977). By binding to GABA_A receptors GABA increases neuronal membrane conductance for Cl^-, resulting in membrane hyperpolarization and reduced neuronal excitability (Lambert et al. 1995; Sieghart 1992). GABA_B receptors are not directly linked to Cl^- channels. They appear to be associated with Ca^{2+} or K^+ channels via second messenger systems (Bowery 1989; Cooper et al. 1991). GABA binding to GABA_B receptors on a cell results in an increase in K^+ conductance and a decrease in Ca^{2+} conductance within the cell (Bowery 1989; Cooper et al. 1991). GABA_C receptors, like GABA_A receptors are ligand-gate Cl^- ion channels (Bormann and Feigenspan 1995). GABA_C receptors are selectively activated by cis-4-aminocrotonic acid, a partially folded GABA analogue (Bormann and Feigenspan 1995). While steroids are known to modulate GABA_A receptors, they are not known to modulate GABA_B (Cooper et al. 1991) or GABA_C receptors (Bormann and Feigenspan 1995).

The activity of the GABA_A receptor can be inhibited by competitive and noncompetitive antagonists, such as the convulsants bicuculline and picrotoxin, respectively. The activity of the GABA_A receptor can be enhanced by a variety of structurally diverse agents such as: ethanol; behavioral depressant barbiturates; anxiolytic, anticonvulsant, muscle relaxant, and sedative-hypnotic benzodiazepines; general anesthetic agents; as well as anxiolytic, anticonvulsant and hypnotic steroids (Johnston 1996; Sieghart 1992). All these compounds which act on the GABA_A receptor appear to bind to it via separate allosteric binding sites on the receptor (Sieghart 1992).

The GABA_A receptor is a hetero-oligomer of 220-400 kilodaltons. It is composed of two to four different polypeptides/subunits, α1-α6; β1-β3; γ1-γ3; δ; ε; and/or π, which are about 55 kilodaltons each (Macdonald and Olsen 1994). Numerous structurally different combinations are possible, for example, αβγδ or αβ (Sieghart 1992). It is likely that each of these subunits have
different affinities for GABA and various other ligands (Olsen and Tobin 1990). Some compounds such as benzodiazepines exhibit an absolute GABA<sub>A</sub> receptor subunit specificity. Some studies suggest that steroid action at the GABA<sub>A</sub> receptor may be slightly influenced by subunit composition, while others do not (Lambert et al. 1995; Prince and Simmonds 1993). The only subunit which appears to affect steroid potentiation of GABA-evoked currents is the α subunit. While there is no clear consensus among studies investigating the role of the α subunit [see (Lambert et al. 1995) for a review], GABA<sub>A</sub> receptors with the α6β1γ2 subunit combination exhibit a reduced steroid effect when compared to receptors containing other α units (Puia et al. 1993). The α6 subunit is confined to granule cells of the cerebellum, and consequently will not affect steroid and GABA<sub>A</sub> interactions in the uterus.

Cellular receptors for GABA are present in the central nervous system as well as in the periphery [see Erdo and Wolff (1990) for a review]. It was through electrophysiological studies with the synthetic steroidal general anesthetic agent, alphaxalone, that the rapid non-genomic action of certain steroids upon GABA<sub>A</sub> receptors was first appreciated (Harrion and Simmonds 1984). Anxiolytic effects of pregnane steroids acting at GABA<sub>A</sub> receptors in the central nervous system have received considerable attention (Bitran et al. 1991a; Bitran et al. 1991b; Bitran et al. 1993; Crawley et al. 1986; Im et al. 1990; Johnston and Gluckman 1983; Wieland et al. 1991), and P₄ metabolites are known to modulate GABA-ergic neurotransmission (Harrion et al. 1987; Hiernke et al. 1991; Majewska et al. 1986; Majewska et al. 1988; Puia et al. 1990; Purdy et al. 1990b). With respect to the female reproductive system, GABA<sub>A</sub> receptors have been identified in the ovary, oviduct, uterus and placenta (Brann et al. 1990; Erdo et al. 1985; Erdo et al. 1989; Erdo and Wolff 1990; Gimeno et al. 1986; Majewska and Vaupel 1991). GABA<sub>A</sub> receptors in tissues such as the gut, vas deferens, urinary bladder, fallopian tubes and uterus are known to mediate contractile responses (Gimeno et al. 1986). GABA<sub>A</sub> receptors in peripheral blood vessels may be involved in the regulation of vasoconstrictor tone and consequently the modulation of tissue blood flow (Anwar and Mason 1982).
2.5.3 Interaction of 5α-reduced metabolites of progesterone with the γ-aminobutyric acid receptor

Studies examining the interaction of 5α-reduced metabolites of P₄ at GABA_A receptors have found that they enhance GABA-stimulated Cl⁻ conductance by prolonging the open time of the Cl⁻ channel (Twyman and Macdonald 1992). This action is similar to the functional activity of barbiturates and other GABA agonists (Friedman et al. 1993; Majewska et al. 1986; Peters et al. 1988). In addition, steroids increase the frequency of single channel openings. In this respect, their molecular mechanism resembles that of benzodiazepines (Macdonald and Olsen 1994).

A number of studies have shown anxiolytic and sedative/hypnotic effects following administration of AP (Beck et al. 1996; Bitran et al. 1991a; Bitran et al. 1993; Brot et al. 1997; Wieland et al. 1991). Majewska et al. (1986) found AP to be 600 times more potent than the most potent barbiturate in its modulation of brain GABA_A receptors. There is a correlation between the pharmacological potencies of GABA agonists such as picrotoxin and barbiturates, and their ability to displace the ³⁵S-labeled convulsant t-butylbicyclophosphorothionate [³⁵S]TBPS, which labels close to or on the GABA Cl⁻ channel (Squires et al. 1982). Majewska et al. (1986) determined that AP inhibits [³⁵S]TBPS binding to the GABA_A-receptor complex, and potentiates the actions of GABA in activating Cl⁻ conductance in cultured rat hippocampal and spinal cord neurons at 10⁻⁷ to 10⁻⁵ M. This information, along with known levels of GABA both circulating and in fetal membranes and uterine tissues in third trimester pregnancy (Erdo et al. 1985; Erdo et al. 1989; Erdo and Wolff 1990), raises the question as to whether AP may act as an inhibitor of myometrial contractions during late pregnancy and whether its withdrawal at term may contribute to the contractions of labour.

Methods have been developed for measuring AP, but until recently they have not been sufficiently sensitive to measure AP in peripheral venous plasma. Thin-layer or paper chromatography to separate steroids, followed by gas-liquid chromatography has been used to measure AP in extracts of rat blood (Parkes et al. 1988; Skatrud 1989). Ichikawa et al. (1974) used this method to measure AP in ovarian venous blood during the estrous cycle and found that AP levels paralleled those of plasma P₄, with a maximum level of 450 ng/ml in ovarian venous plasma. Holzbauer et al. (1975) later reported that AP was secreted in adrenal venous plasma at levels
comparable to that produced by the ovary. Purdy et al. (1990a; unpublished) have developed antibodies for AP, but they are not sufficiently specific to measure AP without chromatography as a means of separating steroids prior to radioimmunoassay (RIA). Using high-pressure liquid chromatography (HPLC) followed by RIA, Purdy et al. (1990a) determined that circulating levels of AP in cycling women range up to 2.5 ng/ml, and closely parallel circulating levels of P₄. Bicikova et al. (1995) found similar results to those of Purdy et al. (1990a). Rapkin et al. (1997) found AP levels in cycling women range between 3.6 and 7.5 ng/ml. Women in third trimester pregnancy average 30 ng/ml, or approximately 100 nM circulating AP (Paul and Purdy 1992).

Ovarian secretion of estrogen may mediate an increase in the metabolism of P₄ to AP in the uterus prior to labour. Ovarian secretion of estradiol-17β and estrone increases sharply at the end of pregnancy in rats (Bertics et al. 1987; Heap et al. 1973). Estrogen treatment has been shown to increase in vitro metabolism of ³H-P₄ to AP by uteri of pregnant rats (Howard and Wiest 1972), and to increase in vitro formation of 5α-dihydroprogesterone and AP from ³H-P₄ by uteri of non-pregnant rats (Armstrong and King 1971). Therefore, high levels of ovarian estradiol-17β prior to labour in rats may result in increased metabolism of P₄ to AP in uterine tissues.

As mentioned earlier, Erdo et al. (1989) examined gestational changes in GABA levels and GABA binding in the human uterus. GABA levels decreased during pregnancy in humans while GABA_A receptor binding affinity increased (Erdo et al. 1989). GABA_A receptors in the rabbit uterus mediate inhibitory contractile responses. Thus, the higher density of GABA_A receptors in the human uterus may represent an increased target for GABAergic inhibition of uterine contractility during pregnancy even though the amount of GABA is reduced in the pregnant uterus. Since GABA is known to be synthesized by the human term placenta (Erdo et al. 1985), it is possible that GABA of placental origin could modulate uterine contractility (Erdo et al. 1989).

One of P₄’s important roles in the maintenance of pregnancy is the inhibition of uterine contractility (Biswa and Craigo 1994). Many of the metabolites of P₄, such as AP, are actually more potent than P₄ in inhibiting uterine contractility (Putnam et al. 1991). A number of researchers have examined the effects of AP on myometrial contractility in non-pregnant rats and have found that it inhibits uterine motility in vitro (Csapo and Resch 1979; Kubli-Garfias et al. 1979). Lofgren et al. (1992) examined the in vitro effects of P₄ and two 5α-reduced progestins on
contracting human myometrium at term. They concluded that 5α-reduced P₄ metabolites such as AP are not potent inhibitors of contracting human term myometrium in vitro. Their testing was limited to myometrial samples from women undergoing cesarean sections who were not in active labour at the time of sampling. Lofgren and Backstrom (1994) later examined whether P₄ and the two P₄ metabolites, AP and 5α-dihydroprogesterone, had different effects on myometrial strips in vitro when the strips were continuously exposed to P₄ from the time of sampling to testing. They found that the continuous presence of P₄ facilitated human myometrial contraction frequency once contractions had started in tissue from women not in active labour at the time of sampling, but the addition of AP or 5α-dihydroprogesterone did not inhibit these contractions. Thus, Lofgren and Backstrom (1994) and Lofgren et al. (1992) did not find that AP inhibited uterine contractions, regardless of whether the myometrial strips were continuously exposed to P₄ from the time of biopsy or not. However, this does not preclude AP's role in the inhibition of myometrial contractions in humans. Lofgren and Backstrom (1994) and Lofgren et al. (1992) added only P₄ and the steroid to be tested. AP may require the presence of GABA before contractions are inhibited through the GABAA receptor complex (Twyman and Macdonald 1992).

2.5.4 5α-reduction

Two different genes encode the isozymes 5α-reductase type 1 and 5α-reductase type 2 (Anderson and Russell 1990; Jenkins et al. 1992; Labrie et al. 1992; Levy et al. 1995; Russell and Wilson 1994; Thigpen et al. 1993). Both of these isozymes are capable of converting testosterone and P₄ into their 5α-reduced metabolites (Russell and Wilson 1994) (Figure 2.3). The type 1 isozyme is found primarily in the liver, skin, and adrenal gland (Thigpen et al. 1993). No mutations in this gene have yet been described in humans. The 5α-reductase type 2 gene is normally expressed in the urogenital tract and liver (Labrie et al. 1992). Mutations in the type 2 gene result in male pseudohermaphroditism (Andersson et al. 1991). Mahendroo et al. (1996) developed a line of mice with a null mutation in the 5α-reductase type-1 gene. In the absence of 5α-reductase type 1, androgens are converted to estrogens rather than 5α-reduced androgens (Figure 2.3). While male mice lacking the 5α-reductase type-1 gene (Srd5a1) appeared normal, females with the same
mutation had a number of defects in gestation (Mahendroo et al. 1996). Females homozygous for the 5α-reductase mutation had a significant rate of fetal death midgestation (Mahendroo et al. 1997). Mahendroo et al. (1997) suggested that fetal loss was primarily due to an excess of estrogen of maternal origin. Another parturition defect associated with the absence of the 5α-reductase type 1 gene in the mice was that 67% of pregnant females failed to deliver their young at term (Mahendroo et al. 1996). This parturition defect could be overcome by systemic administration of 5α-reduced androgens, in particular, androstanediol (5α-androstan-3α,17β-diol) (Mahendroo et al. 1996).
3. The importance of 5α-reduced progesterone metabolites at labour in humans

3.1.1 Introduction

The effects of \( P_4 \) at labour in humans may be overcome by decreasing the actions of some endogenous myometrial relaxant. The major route of \( P_4 \) metabolism in the uterus is via 5α-reduction, initially to 5α-dihydroprogesterone (5α-pregn-3,20-dione; DHP) and subsequently to allopregnanolone (3α-hydroxy-5α-pregnane-20-one; AP; Figure 2.7). Studies in the central nervous system have shown that AP binds γ-aminobutyric acid-A receptors (GABA\( _\alpha \)), enhancing GABA stimulated chloride conductance (Paul and Purdy 1992). Activation of GABA\( _\alpha \) receptors inhibits smooth muscle contractions (Majewska and Vaupel 1991). Thus, by binding GABA\( _\alpha \) receptors in the myometrium, AP could play a role in inhibiting myometrial contraction. GABA levels in the uterus drop during the third trimester of pregnancy (Erdo et al. 1989), consistent with the view that GABA is probably not a primary inhibitor of uterine contractility during this period. However, \( P_4 \) concentrations at this period are approximately 100 times those observed during the luteal phase in non-pregnant women (Anderson et al. 1985; Boroditsky et al. 1978), and, consequently, intrauterine levels of \( P_4 \) metabolites such as AP could also be elevated during late gestation. Preliminary studies have found that AP levels in maternal serum during pregnancy are higher than those observed during the normal menstrual cycle (Paul and Purdy 1992). In late pregnancy, therefore, a decrease in 5α-reductase activity, and hence AP levels, could lead to disinhibition of uterine contractions, resulting in labour.

We have thus determined the concentrations of AP, DHP, as well as \( P_4 \), 17β-estradiol, and estriol in human maternal serum, umbilical cord serum, and amniotic fluid, in samples obtained from normal women between 38-40 weeks of pregnancy. Since testosterone and \( P_4 \) are competitive substrates for the 5α-reductase enzyme (Kerian et al. 1994), we have also measured the 5α-reduced metabolite of testosterone, androstanediol (androstanediol-3-glucuronide), in maternal serum obtained from normal women between 38-40 weeks of pregnancy. Samples from women who were
in active, spontaneous labour were compared to samples from those who were not in labour at the time of delivery.

3.1.2 Methods

3.1.2.1 Sample collection

Samples were collected from selected patients admitted to Mt. Sinai Hospital, Toronto, Canada. Informed consent was obtained from each patient and the collections were approved by the University of Toronto’s Review Committee on the Use of Human Subjects. Samples of human amniotic fluid, maternal serum, and umbilical cord serum were collected at the time of delivery from women in established, spontaneous labour (vaginal births, n=19) as well as from women at the same stages of pregnancy (38-40 weeks gestation) who were not in labour at the time of sampling (cesarean section deliveries, n=19). All pregnancies and deliveries were unremarkable. Maternal infection was not suspected in any case. Cesarean sections were classified as either elective or repeat cesarean sections. The exact time of labour initiation (for vaginal deliveries) and birth were noted. In some cases, the membranes were ruptured by the attending physician and/or labour was augmented by oxytocic administration. Membrane rupture and oxytocic administration were noted and included as separate variables in the statistical analysis of the data: neither significantly affected mean steroid levels in the vaginal delivery group (see below). Women in labour and women not in labour at the time of birth did not significantly differ with respect to gravidity, parity, or maternal age (Student’s t-test, p>0.05). Gestational age and sex of the infant were noted.

The amniotic fluid, maternal blood, and umbilical cord blood samples were collected within 15 minutes of birth. Maternal blood was obtained via either a cubital vein, or a superficial dorsal hand vein. Umbilical cord blood was obtained from the cord vein after it was cut from the infant. During vaginal births, amniotic fluid which flowed out vaginally immediately following the birth of the fetus was collected into sterile sample jars. Samples of amniotic fluid which contained visible blood or meconium were omitted from the analyses. During cesarean section deliveries, amniotic fluid was collected following uterine incision by inserting a syringe through the fetal membranes.
into the amniotic sac prior to rupture of the membranes and removal of the fetus. Care was taken to obtain amniotic fluid samples that appeared free from blood, or meconium.

Blood was collected into 7 ml plain vacuum tubes and allowed to clot for approximately 3 hours at 4°C. Blood was centrifuged at 7000 rpm at 4°C for 30 minutes and serum then withdrawn and stored at -20°C until assayed. Amniotic fluid samples were also collected (cesarean sections) or transferred (vaginal deliveries) to 7 ml plain vacuum tubes and stored at 4°C for approximately 3 hours before being centrifuged at 7000 rpm at 4°C for 30 minutes. The supernatant was separated from the pellet of cells which formed at the bottom of the tube. Amniotic fluid free from cells was then stored at -20°C until assayed.

3.1.2.2 Allopregnanolone antibody

The antiserum was prepared by Dr. R.H. Purdy against a 3β-hydroxy-20-oxo-5α-pregnan-11-α-yl carboxymethyl ether bovine serum albumin conjugate, whose synthesis has been previously described (Purdy et al. 1990a). The immunization procedure in Purdy et al. (1990a) involved rabbits, while the antiserum used here was prepared in a male sheep. Initial multiple intradermal injections were made with 200 μg of the steroid-albumin conjugate in Freund's complete adjuvant emulsion. These were followed by five successive subcutaneous booster injections of 200 μg conjugate in Freund's incomplete adjuvant every six weeks, followed by a final intravenous injection of 200 μg conjugate in saline, prior to the collection of serum one month later.

The AP antibody obtained from Dr. R.H. Purdy was characterized in our lab. To determine cross-reactivity of this antibody with P₄ and DHP, concentrations of these steroids (P₄ and DHP from Steraloids, Wilton, NH) ranging from 30 pg/tube to 15 ng/tube were assayed in triplicate with the AP antibody and ³H-AP as tracer (the RIA method is described in detail in the following section). Results were analyzed by computer assisted least-squares curve fitting to determine the relative displacements with each steroid. The anti-ovine AP antibody cross reacts 14.3% with P₄ and 15.3% with DHP.
3.1.2.3 Allopregnanolone and 5α-dihydroprogesterone measurements

Preliminary purification of samples prior to high performance liquid chromatographic separation of AP, P₄ and DHP was carried out as follows. Serum (0.2 ml) or amniotic fluid (0.4 ml) were mixed with a recovery standard of ³H-labeled P₄ [1,2-³H(N), (0.1 ml, 16,000 cpm)], and distilled water (to a final volume of 1.0 ml) in glass culture tubes and vortexed. Diethyl ether (3 ml) was added to each sample to extract endogenous steroids. The extraction was repeated twice and the combined extracts evaporated to dryness under a stream of nitrogen. The dry residue was reconstituted in approximately 0.5 ml of 5% ethanol in dichloromethane, and added to 1.0 ml Silica Gel 60 columns prepared in eppendorf pipette tips plugged with glass wool. Columns were eluted with a further 3.0 ml of 5% ethanol in dichloromethane and the material flowing through the column was collected and evaporated to dryness. This step ensured that any lipids which might have affected the transport of the samples through the high performance liquid chromatography column were removed prior to the injection of the sample.

The high performance liquid chromatography apparatus was fitted with a silica column (Nova-Pack silica steel column, 3.9 mm x 150 mm, Waters, Canada) with a mobile phase of dichloromethane containing 0.2% ethanol at a flow rate of 1 ml/min. Purified sample extracts were solubilized in dichloromethane (0.075 ml) and injected. Samples were collected into culture tubes at 1 minute intervals for 30 minutes. Under these conditions 5α-dihydroprogesterone and AP eluted at approximately 8 and 12 minutes respectively. The internal P₄ standard eluted at approximately 17 minutes. Figure 3.1 shows the separation of cross-reacting steroids 5α-dihydroprogesterone and P₄ from AP by high performance liquid chromatography. Sample tubes were dried under a stream of nitrogen then reconstituted in phosphate buffered saline, pH 7.0 (0.75 ml). These tubes were then incubated at 37°C for 1 hour. Of the reconstituted samples, 0.125 ml was counted on a B-counter to determine the amount of ³H-P₄, and consequently the recovery of the steroids, through extraction, purification and high performance liquid chromatography. Mean recovery through the entire sample preparation and high performance liquid chromatography procedure was 68.5±1.4 % (mean±standard error of the mean).

RIA procedure for both AP and DHP measurements involved the addition of AP antibody [0.25 ml, 1:12,000 dilution (antibody:buffer with bovine serum albumin) working dilution], and ³H-
AP [New England Nuclear, 50 Ci/mmol, 5α-[9,11,12-3H(N)], (0.25 ml, 3,000 dpm)] to each tube containing 0.25 ml of the sample in buffer (duplicate tubes were prepared). Tubes were vortexed for 30 seconds, then incubated at room temperature for 1 hour. Tubes were cooled on ice for 20 minutes, and unbound steroid was separated from that bound to the antibody with 200 µl ice-cold dextran-coated charcoal [0.6% (wt/vol) charcoal (Sigma, Oakville, ON), 0.006% (wt/vol) dextran (Sigma, Oakville, ON), in phosphate buffered saline, pH 7.4]. Tubes were vortexed for 30 seconds and incubated with charcoal for 20 minutes. Tubes were then centrifuged at 2000 rpm for 40 minutes, at 4°C, and the supernatant counted in a β-counter.

AP purchased from Steraloids (Wilton, New Hampshire), was used for the standard curve for both AP and DHP assays. The amount of AP in each sample was determined by summing the immunoreactivity in the area of the AP peak (Figure 3.1, tubes 12-14). The amount of DHP in each sample was determined by summing the immunoreactivity in the area of the DHP peak (Figure 3.1, tubes 7-9), then correcting the DHP values for the cross reactivity of the AP antibody used with DHP.

3.1.2.4 Progesterone, Estradiol-17β, and Estriol measurements

Total P₄, estradiol-17β (E₂) and estriol (E₃) were measured in unextracted serum with commercially available solid-phase, competitive binding, ¹²⁵I radioimmunoassay kits (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA), following the instructions of these kits. These P₄, E₂ and E₃ kits are highly specific; there is very low cross-reactivity between the antisera and other compounds which may be present in the patient samples such as steroids or therapeutic drugs.

3.1.2.5 Assay specifics

The lower limit of assay sensitivity was set at 90% of total binding. This resulted in a limit of 20.1 pg/ml for AP assays, 87.8 pg/ml for P₄ assays, 0.67 ng/ml for E₂ assays, and 3.69 ng/ml for E₃ assays. The upper limit of sensitivity was set at 10% total binding which resulted in a limit of 7.41 ng/ml for AP assays, 36.3 ng/ml for P₄ assays, 544 ng/ml for E₂ assays, and 4526 ng/ml for
E₃ assays. Inter-assay variances (determined from a sample pool which was analyzed in all RIAs performed), for the AP, P₄ and E₃ assays were 10.6%, 7.9% and 7.7% respectively. All E₂ determinations were made in a single assay. Intra-assay variances (for each steroid n=4 multiple samples were analyzed in one RIA), were 5.5% for AP assays, 3.6% for P₄ assays, 4.9% for E₂ assays, and 4.1% for E₃ assays.
Figure 3.1: A representative graph of the samples which eluted through the high performance liquid chromatography (HPLC) apparatus. The left axis refers to the immunoreactivity of a sample of umbilical cord blood which was extracted, purified, injected into a high performance liquid chromatography apparatus then radioimmunoassayed. That which eluted through the high performance liquid chromatography apparatus was collected into culture tubes at a rate of 1 tube/minute, with a solvent flow rate of 1 ml/minute (1 ml/tube). The collection continued for 30 minute (tubes). A radioimmunoassay was then performed on the first 15 tubes using an antibody for allopregnanolone which cross reacts with 5α-dihydroprogesterone and progesterone. $^3$H-progesterone was added to all samples prior to extraction to determine recovery through to radioimmunoassay. The left axis refers to the radioactivity measured in these tubes following high performance liquid chromatography, but prior to radioimmunoassay.
3.1.2.6 *Androstanediol measurements*

Levels of androstanediol-3-glucuronide were measured in maternal serum using an radioimmunoassay kit from Diagnostic Systems Laboratories Inc. (Webster, Texas). The lower limit of assay sensitivity was 0.13 ng/ml, while the upper limit was 49.7 ng/ml. All measurements of androstanediol were made in a single assay (coefficient of variance 5.2%).

3.1.2.7 *Data analyses*

Data were analyzed using three statistical program packages (SPSS for Windows; Sigmastat and SAS for Macintosh). Gestational age was compared between women in labour and those not in labour at the time of birth using Student's t-test. Contingency table analysis was used to determine whether the ratio of males and females born differed for labour versus not in labour samples. Comparisons between steroid concentrations in samples obtained from women in labour and women not in labour were made initially using multiple ANOVA, with labour, chemical identity of the steroid measured, and tissue compartment, as independent variables. Where ANOVA revealed significant labour by steroid interactions, further comparisons between individual group steroid concentrations in samples from labouring and non-labouring patients were made using Student's t-tests. For samples obtained from women who delivered vaginally, labour augmentation (augmented versus non augmented), and membrane rupture (spontaneous versus artificial) were examined to determine whether these variables affected the outcome of comparisons between hormonal concentrations in labouring and non-labouring women using stepwise linear regression. For those variables that significantly predicted the variance associated with the hormonal concentrations measured in the in labour samples, Student's t-tests were repeated with these variables entered into the analysis independently.

Skew or inhomogeneity of variance, where detected in data to be analyzed by ANOVA or t-tests, were eliminated by log transformation. When means are presented they are accompanied by the standard error of the mean. Power analysis was performed to determine the probability of type II errors.
3.1.3 Results

Women who were not in labour at the time of delivery had a mean gestation length of 275.6±1.8 days. This differed by only a week from the mean gestation length of the women who were in labour at the time of delivery [283.0±1.3 days (t=3.44, p=0.001, d.f.=36)]. The ratio of males and female infants born did not significantly differ for labour versus not in labour samples (χ²=2.33, p=0.234, d.f.=1).

Levels of AP, DHP, E₂, E₃, and androstanediol tended to be higher in samples collected from women in labour than in those collected from women not in labour (Figure 3.2, and Figure 3.3). There was a similar trend towards higher levels of P₄ in amniotic fluid and cord serum, but not in maternal serum (Figure 3.2). These small differences were not large enough to result in significant effects being observed between the in labour and not-in-labour groups by ANOVA, for any one of the three fluids sampled (p>0.05 for all). In both maternal serum and amniotic fluid, significant interactions between labour state and steroid identity were observed, reflecting the fact that several, but not all steroids, tended to be present at higher concentrations during labour. In no case, however, were the concentrations of any individual steroid measured found to be statistically different between the two sample groups (women not in labour versus women in labour; p>0.05 by Student’s t-tests). Progesterone concentration was significantly correlated only with DHP concentrations in maternal serum (Figure 3.4). Power analysis revealed a high probability of committing a type two error.

For samples obtained from women who delivered vaginally, labour augmentation (augmented versus not augmented), and oxytocics (administered verses not administered) were found not to affect the outcome (Stepwise Regression, p>0.1 for all hormones in all compartments measured). Membrane rupture (spontaneous verses artificial) significantly entered the stepwise regression model only with respect to one measurement (P₄ in cord serum: R²=0.626, F=13.398, d.f.=1,7). When hormones measured in the samples obtained from women in labour in which the membranes had been ruptured artificially, were compared to concentrations measured in women who were not in labour, the same trends as those discussed in the previous paragraph were found:
levels of AP, DHP, E₂, E₃, and androstediol tended to be higher in samples collected from women in labour than in those collected from women not in labour; and a similar trend towards higher levels of P₄ in amniotic fluid and cord serum, but not in maternal serum. The situation was similar for samples obtained from women in labour in which the fetal membranes spontaneously ruptured. Thus, while membrane rupture (spontaneous or artificial) may affect the concentrations of P₄ in cord serum, this variable does not appear to affect comparisons between in labour and not in labour groups of women. Therefore, for all comparisons in this study both categories of membrane rupture (spontaneous verses artificial), labour augmentation (augmented versus not augmented), and oxytocics (administered verses not administered) for in labour samples are grouped.
Figure 3.2: Allopregnanolone, 5α-dihydroprogesterone, progesterone, estradiol-17β, and estriol measured in amniotic fluid, umbilical cord serum and maternal serum collected from women in and not in labor at the time of delivery. In each boxplot, the bottom, middle and top lines of the box represent the 25th, 50th, and 75th percentiles respectively. The bottom of the vertical line which intersects the box represents the 5th percentile, and the top of this line the 95th percentile. Points above and below this line indicate values outside this range. Sample size varies among plots and are listed above the x-axis.
Figure 3.3  Boxplots of concentrations of androstanediol glucuronide measured in maternal serum collected from women in \(n=13\) and not in labor \(n=15\) at the time of delivery. In each boxplot, the bottom, middle and top lines of the box represent the 25th, 50th, and 75th percentiles respectively. The bottom of the vertical line which intersects the box represents the 5th percentile, and the top of this line the 95th percentile. Points above and below this line indicate values outside this range.
Figure 3.4: Allopregnanolone and 5α-dihydroprogesterone concentrations in amniotic fluid, umbilical cord serum, and maternal serum, plotted against concentration of progesterone in the same samples. Progesterone concentration was significantly correlated only with dihydroprogesterone concentrations in maternal serum, these regression lines
[solid line, in labor: (dihydroprogesterone) = 4.153 + 0.0199(progesterone);
dashed line not in labor: (dihydroprogesterone) = 4.112 + 0.00436(progesterone)] are shown on the graph. Note that the axis scales differ between plots.
○ Not in labor

+ In labor

Allopregnanolone (ng/ml)

progesterone (ng/ml)

Amniotic fluid

Cord serum

Maternal serum

94a
3.1.4 Discussion

The sensitivity of the uterus to \( P_4 \) could decline late in gestation as a result of either changes in the \( P_4 \)-responsive receptor systems in the myometrium, or alterations in intrauterine \( P_4 \) metabolism. The potential for \( P_4 \) metabolites, such as AP, to act as mediators of \( P_4 \) responses in the uterus was initially suggested by observations on the effects of these steroids on uterine contractility (Putnam et al. 1991). In uteri from non-pregnant rats, AP inhibits myometrial contractility \textit{in vitro}, (Csapo and Resch 1979; Kubli-Garfias et al. 1979). In contracting human myometrium at term, however, Lofgren et al. (1992) concluded that AP is not a potent inhibitor of myometrial contractions. These experiments were limited to myometrial samples from women undergoing cesarean sections who were not in active labour at the time of sampling. Subsequently, Lofgren and Backstrom (1994) examined the effect of AP on myometrial strips \textit{in vitro} under continuous \( P_4 \) exposure from the time of sampling to testing. In tissue from women not in active labour at the time of sampling they found that \( P_4 \) facilitated myometrial contraction frequency, once contractions had started. The addition of AP did not inhibit these contractions. These data do not however, preclude a role for AP in the inhibition of myometrial contractions in humans \textit{in vivo}. Since the effects of AP on GABA\( _A \) receptors in the brain are synergistic with those of GABA, it is possible that GABA and AP might have to be present simultaneously, as they are in normal pregnancy, (Erdo et al. 1989; Paul and Purdy 1992) in order for there to be significant inhibitory effects on myometrial contractility.

If AP does indeed mediate inhibitory effects of \( P_4 \) on uterine contractility, one might expect circulating concentrations of this steroid to decline in active labour. Until very recently, this hypothesis could not be tested. Methods for measuring AP are only now becoming sufficiently sensitive for assays to be performed in peripheral venous plasma. In the present study, using a new, more specific antibody against AP coupled with a high performance liquid chromatography-based sample purification scheme modified from that previously employed by Purdy et al. (1990a), we have obtained the first direct estimates of cord and maternal AP concentrations in labouring and non-labouring women at term. In confirmation of previous work, (Purdy et al. 1990a) levels of AP
in maternal serum were found to be higher than those reported earlier for non-pregnant women. AP concentrations in both maternal and cord serum, as determined by this study, are high enough to raise the possibility of synergistic effects between AP and GABA. Studies using rat brain membranes have shown that concentrations of AP as low as 1-10 nM may potentiate the effects of GABA on the GABA-benzodiazepine-chloride channel complex (Majewska et al. 1986). These effects of AP could contribute to the actions of P₄ on the fetal and maternal central nervous systems, as well as on the myometrium during pregnancy. A number of studies have shown anxiolytic and sedative/hypnotic effects following administration of AP (Bitran et al. 1991a; Bitran et al. 1993; Brot et al. 1997; Komeyev and Costa 1996; Wieland et al. 1991).

Purdy et al. (1990a), Schmidt et al. (1994), and Bicikova et al. (1995) found levels of AP in the serum of normal cycling females to be correlated with levels of P₄. Our data do not show such a tight correlation between AP and P₄ concentrations. There is considerable variation between women with respects to levels of circulating steroids near the end of gestation (Anderson et al. 1985; Boroditsky et al. 1978; Cousins et al. 1977; Mathur et al. 1980). This variation could explain the lack of correlation between P₄ and AP levels measured in this study since we obtained individual samples from a number of different women at the time of birth. Further studies on samples collected sequentially throughout the third trimester will be required to resolve this issue.

The hypothesis that circulating AP concentrations might fall with the onset of labour is also not substantiated by our results. If anything, a trend in the opposite direction, towards increased AP levels, was observed in the samples taken from patients in active labour. A similar trend was also observed in the measured concentrations of DHP, the immediate precursor to AP, as well as in maternal serum androstanediol glucuronide levels, suggesting that labour may in fact be associated with an increase rather than a decrease in steroid metabolism via the 5α-reductase pathway. However, whatever the direction of any change in 5α-reductase activity associated with labour, its overall magnitude is clearly small and there is considerable overlap between the steroid profiles observed in labouring and non-labouring patients: statistical significance was observed in the preliminary ANOVA only with respect to the interaction terms; and none of the individual group comparisons between the cesarean section and vaginal delivery patients for each steroid reached statistical significance using Student’s t-test.
Whether or not androstanediol may play a specific role in mammalian pregnancy is presently unclear. Mahendroo, et al. (1996) found it to be effective in triggering labour in female mice lacking the gene for the 5α-reductase type-1 enzyme and suggested that it might be acting as either a P₄ receptor antagonist, or potentially via some as yet unidentified receptor system (Mahendroo et al. 1996). The results presented here suggest that androstanediol glucuronide is present in maternal serum during parturition at quite high concentrations, within the range observed in normal men, higher than those in premenopausal women (Miles et al. 1992; Paulson et al. 1986). The concentrations of this steroid do not, however, differ markedly between labouring and non-labouring women.

Interpretation of these results is complicated by the one week difference in mean gestation lengths between the two sample groups. Since the primary goal of sample collection was to obtain samples from normal pregnancies, some difference in gestational age was inevitable; elective cesarean sections, which provided the samples from the non-labouring patients, were performed approximately one week prior to the expected delivery date. Cesarean-delivered patients were not in active labour at the time of sampling, and since cervical dilation and effacement were not determined, it is difficult to know when these patients might have gone into spontaneous labour if their pregnancies had been allowed to continue. Since AP has been found in the adrenals (Meikle and Odell 1986) it is also possible that changes in the hypothalamic-pituitary-adrenal axis associated with labour or surgery could have contributed significantly to measured circulating AP concentrations.

Nevertheless, the overall pattern of the results is consistent with previous work in this field. Increased estrogenic stimulation of the myometrium at the end of pregnancy is believed to play an important role in initiating labour. Estrogens promote gap junction formation, stimulate uterine prostaglandin production, decrease the resting potential of uterine smooth muscle cells, and promote synthesis of receptors for oxytocin and relaxin (Crawley et al. 1986). Estrogen treatment has been shown to increase in vitro metabolism of ^3^H-P₄ to AP by uteri of pregnant rats, (Danforth and Ueland 1986) and to increase in vitro formation of DHP and AP from ^3^H-P₄ by uteri of non-pregnant rats (Howard and Wiest 1972). An increase in maternal estrogenic activity in the
myometrium at term might therefore be expected to increase, rather than decrease, 5α-reduction of 
P₄ and/or testosterone.

Although these data do not support the hypothesis that 5α-reductase activity changes 
dramatically with the onset of labour in women, they do not necessarily disprove the view that 
alterations in P₄ and/or androgen metabolism may contribute to the mechanisms involved in 
initiating uterine contractions. Fetal membranes could modulate the concentrations of the steroids 
reaching the myometrium in ways that might not be obvious from measurements made in maternal 
serum samples. Increased local P₄ metabolism might not be immediately apparent in maternal 
circulating levels of P₄ or AP, since the intrauterine (membrane) compartment may not be fully 
equilibrated with the bloodstream. The observation that amniotic fluid P₄ concentrations decrease in 
late gestation in women, at the same time as P₄ levels are rising in maternal peripheral blood 
(Armstrong and King 1971), is consistent with this hypothesis.

In summary, these data confirm that AP is present in maternal and cord blood at the end of 
gestation at concentrations sufficient to potentially affect GABA-sensitive systems in both the 
myometrium and central nervous system. They also demonstrate that androstenediol-3-glucuronide, 
a major product of testosterone metabolism via the 5α-reductase pathway, is present at relatively 
high concentrations in maternal serum at term. A dramatic change in the concentrations of 5α-
reduced steroids in the serum or amniotic fluid does not, however, appear to occur at the time of 
labour, which makes it difficult to envisage a dramatic shift in steroid 5α-reduction as the 
mechanism underlying increased myometrial contractility occurring at parturition.
4. A and B progesterone receptor isoforms in the myometrium at labour

4.1 Introduction

The importance of P4 in pregnancy maintenance is suggested by the following findings: rats ovariectomized during pregnancy go into labour and deliver their young preterm; administration of P4 to ovariectomized rats or non-ovariectomized rats prevents labour from occurring; and administration of the P4 antagonist RU486 precipitates labour [see (Challis and Lye 1994; Mahajan and London 1997) for reviews]. P4's primary role in the pregnant uterus is believed to be the maintenance of uterine quiescence. Since P4 is essential for pregnancy maintenance one would expect its levels to decrease at the time of labour initiation. In species such as the rat, there is a decrease in maternal circulating P4 levels prior to the onset of labour, yet in humans, maternal circulating levels of P4 remain relatively constant through the last stages of gestation, dropping only once the placenta is delivered. Therefore, unless the role of P4 in the human is quite different from that in other species, some mechanism must ultimately result in the attenuation of P4 effects at the time of labour in humans.

There are a variety of mechanisms by which the effects of P4 could be attenuated at the time of labour in humans. Maternal circulating levels of P4 are maintained, as is the ratio of P4 bound to serum binding proteins verses that of free/unbound in maternal serum at the time of labour initiation. Only free P4 in the serum is available for transport into target cells. Thus, these mechanisms do not appear to contribute to a functional P4 withdrawal in humans. P4 action could also be altered by affecting P4 nuclear receptors which function as ligand-modulated transcription factors. As discussed earlier in Section 2.2.2, there are at least two functional isoforms of the P4 receptor, the A and B isoforms. These two isoforms are transcribed from different start sites on the same gene, and they differ with respect to their binding affinities, as well as transcriptional effects. Therefore, the relative levels of P4 receptor isoforms A and B may modulate tissue response to P4.
Since $P_4$ receptor isoform A can repress transcriptional activity of the B isoform, we hypothesized than an increase in the ratio of myometrial A:B $P_4$ receptor isoforms at term might affect a functional $P_4$ withdrawal. We therefore examined the expression of the $P_4$ receptor A and B isoforms at both the mRNA and protein levels in pregnant human myometrium before or during labour, both preterm and at term. To determine whether any changes in the $P_4$ receptor A and B isoforms are restricted to species such as humans which do not exhibit a $P_4$ withdrawal, we also examined the expression of $P_4$ receptor A and B isoforms in the rat, a species in which there is a fall in maternal plasma $P_4$ prior to labour.

4.2 Methods

4.2.1 Sample collection

4.2.1.1 Human

Samples were collected from patients admitted to Mt. Sinai Hospital, Toronto, Canada. Informed consent was obtained from each patient and the collections were approved by the University of Toronto's Review Committee on the Use of Human Subjects. Uterine samples were collected from the uterus at the incision site during cesarean sections on women who were not in labour as well as from women who were in labour at the time of section. The majority of samples were collected from the lower region of the uterus. These samples were approximately 2 x 3 cm, and taken from the upper (cranial) border of the incision. None of the labouring women received tocolytics, oxytocin or prostaglandins during the course of their labour. A small set of samples were collected from cesarean sections following a classical (longitudinal; fundal to lower uterus region) incision in the uterus. Fundal to lower uterine samples were obtained from these women ($n=4$). Pregnant samples were collected from women who delivered preterm (24-36 weeks) as well as at term (37-42 weeks). In addition, non-pregnant uterine samples were collected from pre-menopausal women following hysterectomy.
Following excision, uterine tissue was rinsed in a phosphate buffered saline, decidual/endometrial and stromal layers were separated, and the remaining myometrium was placed in liquid nitrogen and stored \(-80^\circ\text{C}\) until use.

4.2.1.2 Rat

Individually housed Wistar rats (Charles River Co., St. Constance, Canada) were maintained under 14 hour light, 10 hour dark lighting conditions. These rats were fed Purina rat chow (Ralston Purina, St. Louis, MO), and water \textit{ad libitum}. Virgin female rats (approximately 250 g) were mated, and the day on which a vaginal plug was observed was designated day 1 of gestation. Under such conditions these rats delivered on the morning of day 23 of gestation. All experiments were approved by the Institutional Animal Care Committee.

Pregnant rats were killed on days 15 (d15), 21 (d21), 22 (d22), 23 (d23; during labour), or one day post partum (pp1). All animals were sampled following decapitation. Rats on day 23 during labour were sampled following the delivery of at least 2 pups with at least one pup remaining in utero. Uteri were removed and endometrium was separated from myometrium. Myometrial tissue was rinsed in phosphate buffered saline, frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\) until use.

Myometrium was also obtained from non-pregnant ovariectomized rats. Five days following ovariectomy these rats were either treated with estrogen [silastic capsules (inside diameter 1.47 mm, outside diameter 1.96 mm, 1 cm in length, Dow Corning) with 10\% 17\% estradiol diluted with cholesterol] or with cholesterol alone. Eight days following ovariectomy, myometrial tissue was collected from these rats following decapitation. Myometrial tissue was also collected from cycling rats. Myometrium was collected from all these rats as described in the previous paragraph.

4.2.1.3 Cell culture

\(P_4\) receptors have been most extensively characterized in T47D cells (human breast carcinoma) (Sartorius et al. 1994a; Sheridan et al. 1989b), and thus, for all experiments in this chapter T47D cells served as a positive control. T47D cells (a gift from Dr. I. Andrulis, Samuel...
Lunenfeld Research Institute, Toronto, ON) were maintained in RPMI with phenol red (Gibco BRL, Burlington, ON) plus 100 U/ml penicillin (Gibco BRL, Burlington, ON), 100 μg/ml streptomycin (Gibco BRL, Burlington, ON), and 10% (vol/vol) fetal bovine serum (Gibco BRL, Burlington, ON). Cell medium was changed to 10 ml of phenol red-free RPMI with antibodies and 10% (vol/vol) fetal bovine serum, steroid free, 48 hours prior to steroid treatment. [Serum was made steroid free by adding 0.065% (wt/vol) charcoal (Sigma, Oakville, ON) to fetal bovine serum and stirring for 24 hours at 4°C. Charcoal was separated from serum by centrifugation at 13,000 RPM for 60 minutes. Supernatant (stripped serum) was then sterilized by passage through 0.22 μm filter (Millipore, Bedford, MA).] Cells were treated with 10 μl of ethanol (EtOH) containing 1x10^{-6} M 17β-estradiol for 24 hours, then sampled.

PC3 cells (human prostate adenocarcinoma; ATCC, CRL1435) express very low levels of P₄ receptors and thus served as a negative control for all experiments in this chapter examining P₄ receptors. PC3 cells were maintained in RPMI with phenol red (Gibco BRL, Burlington, ON) plus 100 U/ml penicillin (Gibco BRL, Burlington, ON), 100 μg/ml streptomycin (Gibco BRL, Burlington, ON), and 10% (vol/vol) fetal bovine serum (Gibco BRL, Burlington, ON). Cell medium was changed to phenol red-free RPMI with antibodies and 10% (vol/vol) fetal bovine serum steroid free, for 48 hours prior to sampling.

### 4.2.2 Ribonucleic acid blots

Total RNA was extracted from frozen human myometrium, T47D cell plates, and PC3 cell plates using Trizol (Gibco BRL, Burlington, ON), following the manufacturer’s suggested protocols for tissue and cell culture. From human myometrium and PC3 cells 30 μg of RNA, and 10 μg of RNA from T47D cells were separated on 1% (wt/vol) agarose, 3.7% (wt/vol) formaldehyde denaturing gels (according to the method of Dr. D. Belsham, University of Toronto, Canada). RNA on these gels was then transferred onto a nylon membrane (GeneScreen, DuPont, NEN Research Products, Boston, MA). The membranes were cross-linked by UV irradiation, then baked at 75°C for 2 hours in a vacuum oven.

The RNA on the membranes was hybridized to a complementary DNA (cDNA) probe for human P₄ receptor (full length; 2578 base pairs, a gift from Dr. B.F. Mitchell, University of Alberta,
Probes were labeled with [α-32P]dCTP (NEN Research Products, Boston, MA) using the multiprime DNA labeling system (Amersham, UK). 32P-labeled cDNA was separated from free [α-32P]dCTP by precipitation in a final concentration of 0.7M ammonium acetate, 1.07 M Tris, 1.07 M EDTA, 0.003% (wt/vol) sodium dodecyl sulfate, 10 μg tRNA, and 68% (vol/vol) EtOH. Precipitate was reconstituted in water and boiled. The reconstituted probe was added to the hybridization buffer [0.325 M NaP, 0.07% (wt/vol) sodium dodecyl sulfate, 32.5% (vol/vol) formamide, and 1% (wt/vol) bovine serum albumin fraction V] and filtered through a 0.45 μm syringe filter before addition to membrane. The membranes were hybridized with the radiolabeled probe for 20 hours at 58°C. Following hybridization unbound probe was washed from membranes with 150 mM NaP, 0.1% (wt/vol) sodium dodecyl sulfate buffer, followed by 30 mM NaP, 0.1% (wt/vol) sodium dodecyl sulfate buffer until wash buffer was free of unbound radioactivity. Membranes were then exposed to x-ray film (Reflection, DuPont, NEN Research Products Boston, MA) for an appropriate length of time (approximately two weeks for P4 receptor probe) at room temperature. Radiolabeled probe was then removed from blots by placing them in a boiling solution of 0.1% (wt/vol) sodium dodecyl sulfate, 0.1% (vol/vol) SSC (0.15 M sodium chloride, 0.015 M sodium citrate) for two times 20 minutes. Blots were then hybridized with a cDNA probe for 18S (a gift from Dr. D. Denhardt, Rutgers University, Piscataway, NJ), following a method similar to that for the P4 receptor probe. The hybridization buffer for the 18S probe consisted of 0.35 M sodium phosphate, 0.07% (wt/vol) sodium dodecyl sulfate, 30% (vol/vol) formamide, and 1% (wt/vol) bovine serum albumin fraction V. Hybridization occurred at 55°C for 16 hours. Exposure to x-ray film was for approximately one hour at room temperature.

X-ray films were scanned using an Epson flat bed scanner at a resolution of 300 dpi. The intensities of the sample bands on these digital images were analyzed densitometrically using NIH Image 1.61 software. The optical density for P4 receptor for a given sample on a given membrane was divided by the optical density of 18S for the same sample on the same membrane. Thus, 18S served as an internal control. Multiple exposures were obtained for each probe to ensure that densitometry was calculated within the linear range.

Table 4.1 describes the samples used for northern blot analysis presented.
Table 4.1: Samples used in northern blot analyses. Results from these samples presented in Figure 4.3.

<table>
<thead>
<tr>
<th>Sample ID #</th>
<th>Gestational age (weeks)</th>
<th>Preterm or term</th>
<th>Labour status (not vs. in)</th>
<th>Dilation (cm)</th>
<th>Reason for cesarean section or hysterectomy</th>
<th>Sample description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1322</td>
<td>29</td>
<td>preterm</td>
<td>not</td>
<td>-</td>
<td>-fetal distress</td>
<td></td>
</tr>
<tr>
<td>2324</td>
<td>32</td>
<td>preterm</td>
<td>not</td>
<td>-</td>
<td>-fetal distress</td>
<td></td>
</tr>
<tr>
<td>2400</td>
<td>34</td>
<td>preterm</td>
<td>not</td>
<td>-</td>
<td>repeat/oblique</td>
<td></td>
</tr>
<tr>
<td>2674</td>
<td>29</td>
<td>preterm</td>
<td>not</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1315</td>
<td>32</td>
<td>preterm</td>
<td>in</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2343</td>
<td>29</td>
<td>preterm</td>
<td>in</td>
<td>5</td>
<td>breech</td>
<td></td>
</tr>
<tr>
<td>2344</td>
<td>36</td>
<td>preterm</td>
<td>in</td>
<td>2</td>
<td>breech</td>
<td></td>
</tr>
<tr>
<td>3539</td>
<td>29</td>
<td>preterm</td>
<td>in</td>
<td>-</td>
<td>breech/premature rupture of membranes</td>
<td></td>
</tr>
<tr>
<td>2271</td>
<td>39</td>
<td>term</td>
<td>not</td>
<td>0</td>
<td>breech</td>
<td></td>
</tr>
<tr>
<td>1433</td>
<td>38</td>
<td>term</td>
<td>not</td>
<td>-</td>
<td>breech</td>
<td></td>
</tr>
<tr>
<td>1088</td>
<td>41</td>
<td>term</td>
<td>not</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2805</td>
<td>39</td>
<td>term</td>
<td>not</td>
<td>0</td>
<td>repeat</td>
<td></td>
</tr>
<tr>
<td>3309</td>
<td>41</td>
<td>term</td>
<td>in</td>
<td>-</td>
<td>face presentation</td>
<td></td>
</tr>
<tr>
<td>3332</td>
<td>41</td>
<td>term</td>
<td>in</td>
<td>4</td>
<td>fetal distress</td>
<td></td>
</tr>
<tr>
<td>3420</td>
<td>39</td>
<td>term</td>
<td>in</td>
<td>7</td>
<td>breech</td>
<td></td>
</tr>
<tr>
<td>3430</td>
<td>39</td>
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<td>in</td>
<td>6</td>
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<td></td>
</tr>
<tr>
<td>3153</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>leiomyoma</td>
<td>hysterectomy, lower region</td>
</tr>
</tbody>
</table>
4.2.3 Reverse transcription -- Polymerase Chain Reaction

Total RNA was extracted from human myometrium, T47D, and PC3 cells with 4 M guanidium isothiocyanate according to a method described by Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Reverse transcription was performed in a 40 µl volume containing 8 mM dNTP (2 mM each of dATP, dCTP, dGTP, dTTP, Pharmacia Biotech), 39.1 U RNase Inhibitor (RNAguard, Pharmacia Biotech), 200 ng Random Hexamers (Pharmacia Biotech), 200 U Moloney Murine Lukemia Virus Reverse Transcriptase (M-MLV RT, Gibco BRL), 7.5 mM MgCl, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 2 µg of total RNA. Reverse transcription (RT) involved the incubation of this mixture at 25°C for 10 minutes, 42°C for 30 minutes, 99°C for 5 minutes, then 4°C for 5 minutes. Reverse transcription products were stored at -20°C until use in polymerase chain reaction (PCR). A tube containing all RT reagents with the exception of water for RNA was run with each RT reaction as a control.

PCR methods varied for each amplified product (see Table 4.2). Generally, the reactions contained 10 µl of reverse transcription product, Taq DNA polymerase (Gibco BRL), sense and antisense primers (synthesized by Gibco BRL), and H₂O up to 40 µl. PCR reaction was as follows: pre-incubation at 95°C for 5 minutes; denaturation at 94°C for 1 minute; primer annealing at 55°C for 1 minute; extension at 72°C for 1 minute, [denaturation, annealing and extension steps repeated for appropriate number of cycles (see Table 4.2)]; long extension at 72°C for 10 minutes, then PCR products were maintained at 4°C. A tube containing all PCR reagents with the exception of water for RT product was run with each PCR reaction as a control.
Table 4.2: Primers used to identify the four mRNA species of interest. Concentrations of the various components of the reverse transcription reaction are listed. As well, cycles used to identify the polymerase chain reaction (PCR) product of interest, and the size of the product amplified, are listed. The amplified regions for both P₄ receptor A+B and P₄ receptor B lie within exon 1. The location of these amplified regions is shown in a schematic illustration of the progesterone receptor, Figure 2.4. For human calponin the region amplified was 719 bases of the total 1496 base cDNA. Human calponin mRNA is translated as a single open reading frame encoding for 297 amino acids (33 kilodaltons) (Maguchi et al. 1995). The amplified region of β-actin is located within an exon (Nakajima-Iijima et al. 1985).

<table>
<thead>
<tr>
<th>Product name</th>
<th>Primer sequence</th>
<th>Primer length (base pairs)</th>
<th>Nucleotide position</th>
<th>Final primer concentration (µM)</th>
<th>Final Taq concentration (U)</th>
<th>Final Mg concentration</th>
<th>Cycle numbers</th>
<th>Size of PCR product (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₄ receptor A+B</td>
<td>5'-ACGGCAGCTGCCCATATAAGTGCTG-3'</td>
<td>24</td>
<td>1275-1298</td>
<td>0.9</td>
<td>2.5</td>
<td>1.9</td>
<td>25,27,29</td>
<td>300</td>
</tr>
<tr>
<td>5'-CGCTGAGAGCGGGAATCTCTTT-3'</td>
<td>24</td>
<td>1551-1574</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>P₄ receptor B</td>
<td>5'-ATGACTGAGCTGAGGAAGGCGCAGCTG-3'</td>
<td>24</td>
<td>744-767</td>
<td>0.5</td>
<td>5</td>
<td>1.9</td>
<td>24,26,28</td>
<td>300</td>
</tr>
<tr>
<td>5'-ACTGAGACCTGCTGCTCCAGCACC-3'</td>
<td>24</td>
<td>1026-1049</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calponin</td>
<td>5'-GATGGCATTATTGTTGCAC-3'</td>
<td>20</td>
<td>240-259</td>
<td>0.4</td>
<td>2.5</td>
<td>5.6</td>
<td>17,19,21</td>
<td>719</td>
</tr>
<tr>
<td>5'-TTGTAGTAGTTGCTGCGTG-3'</td>
<td>20</td>
<td>939-958</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-AAGAGAGGCATCCTCCACCT-3'</td>
<td>20</td>
<td>222-241</td>
<td>0.4</td>
<td>2.5</td>
<td>1.9</td>
<td>19,21,23</td>
<td>218</td>
</tr>
<tr>
<td>5'-TACATGGCTGGGTGTTGAA-3'</td>
<td>20</td>
<td>420-439</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
4.2.3.1 Semi-quantitative analyses of RT-PCR:

Of the PCR products 10 µl was run on an agarose gel stained with ethidium bromide. Agarose gels were photographed with Polaroid positive/negative black and white film (#665). The resulting negatives were then scanned using an Epson flat bed scanner at a resolution of 600 dpi. The intensities of the sample bands on these digital images were analyzed densitometrically using NIH Image 1.61. β-actin is a common ‘housekeeping gene’ for PCR analyses of muscle tissues (Nakajima-Iijima et al. 1985), and thus it was included in these analyses. However, for myometrial tissues we also examined calponin, a smooth muscle specific gene (Winder and Walsh 1996). The inclusion of calponin analyses helped determine whether smooth muscle cell density differed between samples.

An examination of numerous PCR cycles for each product of interest resulted in an exponential curve typical of PCR (Chevillard et al. 1996; Crotty et al. 1994; Reischl and Kochanowski 1995; Wang and Mark 1990) (Figure 4.1). From these curves we chose three cycle numbers from within the curve’s linear range (Figure 4.1, Table 4.2). We tested the ability of these cycles to detect the product of interest by diluting the RT product entered into the PCR. The cycles chosen for the semi-quantitative analyses varied in their sensitivity but generally resulted in a positive amplification of DNA diluted 1/4.

RNA was confirmed free of genomic DNA by two methods: RNA was first run on a denaturing formamide gel and lanes examined for traces of DNA near loading wells; the second method involved running the RNA in a β-actin PCR reaction without prior reverse transcription. No positive amplification of DNA was observed.

The identity of all PCR products was confirmed by sequence analyses.

P2 receptor A and B, P4 receptor B, calponin, and β-actin DNA amplification through PCR are shown for T47D cells (P2 receptor abundant), PC3 cells (P4 receptor deficient), and human myometrium in Figure 4.2. This experiment was performed to confirm the specificity of the primers used and the relative abundance of the amplicons in these tissues/cells.
Figure 4.1: Densitometric analysis of polymerase chain reaction (PCR) performed on a pool of reverse transcriptase products obtained from human myometrial RNA. For each sequence of interest [progesterone (P₄) receptor A and B isoforms, P₄ receptor B isoform, β-actin and calponin], the PCR reaction was stopped following a range of cycles. These PCR products were then loaded onto a agarose gels and stained with ethidium bromide. Bands on these gels were analyzed densitometrically, and the resultant values were graphed with their corresponding cycle number. Regression lines were then fit to these points. Three cycle numbers from the linear ranges of these curves were chosen for subsequent PCR analyses.
Figure 4.2: Polymerase chain reaction (PCR) was used to amplify progesterone (P₄) receptor A and B isoforms, P₄ receptor B isoform, β-actin and calponin DNA. This DNA was reverse transcribed from RNA extracted from: a P₄ receptor abundant cell line, T47D cells; a P₄ receptor deficient cell line, PC3 cells; myometrial tissue obtained from a woman in labour at term; myometrial tissue obtained from a woman not in labour at term; and finally, from a woman who was not pregnant at the time of her hysterectomy. As expected P₄ receptor isoforms were found to be high in T47D cells, and low in PC3 cells. T47D cells are a breast cancer cell, and PC3 cells are a prostate cancer cell, thus, they did not express smooth muscle specific calponin. The size of each PCR amplified product [base pairs (bp)] is noted on the right of each gel image.
Table 4.3a (term deliveries) and Table 4.3b (term and preterm deliveries) describe all samples used for RT-PCR analysis presented.

Multiple regression analyses confirmed (p<0.05) that the amount of amplicon produced was not dependent upon the position (row or column) of the samples in the PCR machine, or their position (row or lane) in the agarose gel.

4.2.4 Immunoblots

Frozen tissue was pulverized in liquid nitrogen with a mortar and pestle on dry ice. Tissue was homogenized with a polytron homogenizer in RIPA lysis buffer [50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% (vol/vol) Triton X-100, 1% (vol/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate] with protease inhibitors [100 μM Na2VO3, and protease inhibitor cocktail tablets (Boehringer Mannheim, "Complete™, Mini, EDTA-free")]. For T47D and PC3 cells, media was removed from plates. Plates were washed twice in ice-cold phosphate buffered saline, and RIPA lysis buffer was added to each plate. Cells were scraped from plates and transferred to 1.5 ml eppendorf tubes. Cells were homogenized with a polytron homogenizer. Homogenates from both cultured cells and frozen tissue were spun for 15 minutes at 4°C. Protein containing supernatants were removed. Bio-rad protein assay (Bio-rad, CA, USA) was used to determine protein concentration. The concentrations of all samples to be run together on a gel were determined in the same protein assay.

Appropriate amounts of protein (50 μg for T47D cells, 150 μg for human myometrium and PC3 cells, 100 μg for rat myometrium) were loaded on 4-12% tris-glycine gels (Novex, Helix Technologies, Scarborough, Canada). Loading dye [100 mM Tris/HCL pH 6.8, 200 mM dithiothreitol, 4% (vol/vol) sodium dodecyl sulfate, 0.2% (wt/vol) bromophenol blue, 20% (vol/vol) glycerol] was added to samples, 1:1 (vol:vol), and samples were boiled for 3 minutes prior to their loading onto the gels. SeeBlue pre-stained standards (15 μl per lane, Novex, Helix Technologies, Scarborough, Canada) were loaded onto the first lane of each gel. Gel running buffer consisted of 250 mM Tris, 1.92 M glycine, 0.1% (wt/vol) sodium dodecyl sulfate. Gels were run at 120 V until the 36 kilodalton marker ran off the bottom of the gel (approximate time of
Table 4.3a: Samples used in the examination of progesterone receptor RNA via reverse transcription-polymerase chain reaction. All samples were from term deliveries. BS refers to Bishop’s score. Results are presented in Figure 4.4.

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Table 4.3b: Samples used in the examination of progesterone receptors RNA via reverse transcription-polymerase chain reaction. Samples were obtained from preterm as well as term deliveries. BS refers to Bishop's score.

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- hysterectomy, middle of uterus
- hysterectomy, lower region
- hysterectomy, fundal region
- hysterectomy, fundal region
- hysterectomy, lower region
- hysterectomy, fundal region
- hysterectomy, fundal region
- hysterectomy, lower region
- hysterectomy, lower region
2.5 hours. Gels were then transferred onto Immobilon-P (polyvinylidene difluoride, Millipore, Bedford, MA). Transfer buffer consisted of 50 mM Tris, 380 mM glycine, 0.1% (wt/vol) sodium dodecyl sulfate, 20% methanol. Gels were transferred overnight at 100 mA.

Membranes were blocked with 5% (wt/vol) skim milk powder in TBST buffer [50 mM Tris, 150 mM NaCl, 0.2% (vol/vol) Tween 20, pH 7.4], overnight at 4°C. Membranes were then incubated with the rabbit polyclonal primary antibody PR(C-20) (SantaCruz Biotechnology, Inc., CA, USA), at 1:100 dilution in blocking solution for 1 hour at room temperature with gentle agitation. This antibody was raised against a 20 amino acid peptide (LRPDSEASQSPQYSFESLPO) which correspond to amino acids 545-564 of the human P4 receptor, and consequently recognizes both the A and B isoforms (see Figure 2.4). After primary antibody incubation, membranes were washed with TBST three times for 10 minutes. Membranes were next incubated with a secondary antibody, goat-anti-rabbit horse radish peroxidase (Amersham, UK), at a 1:3000 dilution in blocking solution for 1 hour at room temperature with gentle agitation. Membranes were then washed with TBST three times for 10 minutes. The membranes were exposed to ECL reagent (Amersham, UK), then exposed to x-ray film (X-omat blue XB-1, Kodak, NEN Life Sciences, Boston, MA) for approximately 20 minutes.

X-ray films were scanned using an Epson flat bed scanner at a resolution of 600 dpi. The intensities of the sample bands on these digital images were analyzed densitometrically using NIH Image 1.61 software.

T47D cells are known to be abundant in P4 receptor isoforms A and B (Savouret et al. 1991a), and previous studies have confirmed the size and location of these isoforms on western gels (Table 2.2). T47D cells were thus loaded on each western gel probed for P4 receptors. To identify the bands which correspond to the P4 receptor A and B isoforms in the samples examined, the following three methods were employed: the location of the P4 receptor isoforms for T47D cells; the banding pattern obtained following exposure of blots to PR(C-20) following preabsorption of the antibody with its immunizing peptide PR(C-20) (SantaCruz Biotechnology, Inc., CA, USA); as well as the banding pattern obtained following exposure of the blot to goat-anti-rabbit horse radish peroxidase secondary antibody, without prior incubation of the blot with primary antibody PR(C-20).
Table 4.4 describe all samples used for western blot analysis presented.

4.2.5 Data analyses

Data were analyzed using two microcomputer-based statistical program packages (Sigmasstat for Windows, and Statview for Macintosh). Optical densities for each set of PCR primers were compared as follows: for each human the optical densities determined for the three cycle numbers were fitted to a linear curve using regression analysis; the formula for this line (y=mx+b) was then used to determine the 'predicted' optical density for the second (middle) cycle number examined. For example, PCR of P4 receptor A+B was amplified for 25, 27, and 29 cycles (see Table 4.2). For a given RT sample this resulted in bands (on film of agarose gel containing the PCR products) with optical densities (y) of 11.39, 22.65, and 33.91, at cycles (x) 1, 2 and 3 respectively. A linear regression of these three points and the substitution of x=2 into the formula for the line results in the ‘predicted’ optical density (y) of 18.08. This procedure was repeated for each human sample, and for each set of PCR primers. For each human the predicted optical density for P4 receptor A+B, P4 receptor B, and calponin were then divided by the predicted optical density for β-actin. Statistical analyses were then performed on these relative optical density values. Student’s t-test was used to compare relative optical density (± standard error) of PCR bands between myometrial samples obtained from women not in labour, and women in labour at the time of delivery. A one-way analysis of variance (ANOVA) was used to compare not in labour and in labour samples which were collected from women during preterm as well as term deliveries. Due to the limited number of samples obtained from different regions of the uterus, as well as the variance in the regions from which the samples were obtained, no attempt was made to analyze the results from these samples statistically.

With respect to P4 receptors at the protein level, the optical density of 5 bands between 120 and 79 kilodaltons was determined and compared between groups using a one-way ANOVA (with x=sample groups, and y=optical density of a specific P4 receptor band). For rats, comparison was made between pregnant and non-pregnant samples using a Student’s t-test. No suitable control protein could be identified, therefore, all protein results are presented as mean (± standard error) of
the optical density. When a significant ANOVA was identified (p<0.05), significance between individual x pairs was determined using Fisher’s post-hoc test.

Skew or inhomogeneity of variance, where detected in data to be analyzed by ANOVA or t-tests, were eliminated by log transformation. If it was not possible to eliminate skew or inhomogeneity of variance in the data non-parametric comparisons were performed. In no instance however, were the results obtained via non-parametric analysis different from that obtained via parametric analysis. Results of parametric tests are thus presented. When means are presented they are accompanied by the standard error of the mean.
Table 4.4: Samples used in the examination of progesterone receptor protein via western blots. Samples were obtained from preterm as well as term deliveries. BS refers to Bishop’s score.

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4.3 Results

4.3.1 $P_4$ receptor mRNA

Figure 4.3 is a northern blot probed for $P_4$ receptor mRNA. T47D cell banding pattern for $P_4$ receptor mRNA (bands at 11.4, 6.1, 5.2, 4.5, 3.2, and 2.5 kilobases) is similar to that published by other groups (Table 2.1) (Wei et al. 1990; Wei et al. 1988; Wei and Miner 1994). $P_4$ receptor isoform expression for pregnant human myometrial samples however, was very low and banding was unclear and not easily linked to T47D cell bands. Thus, analysis of $P_4$ receptor isoform expression in human myometrium by northern blotting proved inconclusive. Semi-quantitative RT-PCR was then performed to analyze the mRNA levels of the A and B isoforms of the $P_4$ receptor in human myometrial tissues at the time of labour.

Semi-quantitative RT-PCR analysis of $P_4$ receptors showed a slight but non-significant decrease in $P_4$ receptors A+B, and calponin values in samples obtained from women in labour when compared to women not in labour (Student’s t-test: p>0.05, Figure 4.4). There was a significant decrease in $P_4$ receptor B isoform with labour (Student’s t-test: t=-2.25, p=0.03, d.f.=27, Figure 4.4). When samples from preterm deliveries were considered along with samples from term deliveries no significant differences were found between samples (One-way ANOVA: p>0.05, Figure 4.5).

Samples from upper regions of the uterus were obtained from 4 humans. Two of these were not pregnant at the time of their hysterectomies. The other 2 women had hysterectomies postpartum, one of these was in labour, and the other not in labour at the time of delivery. Figure 4.6 shows the relative levels of $P_4$ receptors A and B, and $P_4$ receptor B isoforms, and calponin in samples obtained from the fundal, middle and/or lower/cervical regions of these uteri. No overall trends were observed between the variables measured in different regions of the uteri.
Figure 4.3: Northern blot containing RNA from human myometrium sampled from women who were not in labour as well as from those who were in labour at the time of cesarean section. Samples were collected from preterm as well as term deliveries. The human breast cancer cell line T47D cells, known to be abundant in progesterone (P$_4$) receptors were used as a positive control. This blot was probed for P$_4$ receptor protein using a $^{32}$P labeled full-length P$_4$ receptor cDNA probe.
Progesterone receptor bands (kilobases)

11.4
6.1 5.2
4.5
3.2
2.5

Progesterone receptors

Preterm not labour (n=4)  Preterm in labour (n=4)  Term not labour (n=6)  Term in labour (n=4)  T47D Cells (n=2)
Figure 4.4:  
a) Representative photographs (positive images) of agarose gels loaded with polymerase chain reaction (PCR) products for the RNA species of interest: progesterone (P₄) receptor A and B isoforms, P₄ receptor B isoform, calponin, and β-actin. The size of the amplified products [base pairs (bp)] are listed. Samples were obtained from human myometrium collected from women who were not in labour as well as from those who were in labour at the time of cesarean section. All deliveries were at term (37-42 weeks). The PCR cycle numbers used to amplify the reverse transcriptase products are listed above their corresponding bands.

b) Relative optical density of P₄ receptor A and B isoforms, P₄ receptor B isoform, and calponin PCR products. There was a non-significant decrease in P₄ receptor A+B and calponin values in samples obtained from women in labour when compared to women not in labour (Student’s t-test: p>0.05). There was a significant decrease in P₄ receptor B isoform with labour (t=-2.25, p=0.03, d.f.=27).
a) The figure shows a gel electrophoresis analysis of various DNA fragments including:
- P4 receptor A+B at 300 bp
- P4 receptor B at 300 bp
- Calponin at 719 bp
- Beta-actin at 218 bp

The gel is marked with PCR cycle numbers on the right. Below the gel, there is a lane labeled as 'Pool lane control'.

b) The bar chart compares the relative optical density of:
- P4 receptor A+B
- P4 receptor B
- Calponin

The chart is divided into two groups: Not labour (n=16) and In labour (n=13). The y-axis represents the relative optical density, ranging from 0 to 2.5.
Figure 4.5: Relative optical density of progesterone (P₄) receptor A and B isoforms, P₄ receptor B isoform, and calponin polymerase chain reaction products. Samples were obtained from human myometrium sampled from women who were not in labour as well as from those who were in labour at the time of cesarean section. Samples were collected from preterm as well as term deliveries. No significant differences were noted between the groups (One-way ANOVA, p>0.05).
Figure 4.6: Optical density of progesterone (P₄) receptor A and B isoforms, P₄ receptor B isoform, calponin, and β-actin polymerase chain reaction products. Samples were obtained from more than one region of each uterus. Two women were not pregnant, while two women were pregnant at the time of sampling. No statistical analyses were performed on these data.
Not pregnant - hysterectomy (3274/3267/3271)
Not pregnant-hysterectomy (3277/3280)
Pregnant term-in labour (1230/1225/1224)
Pregnant Term-not labour (1350/1359)

Progestosterone receptor A+B / β-actin

Progestosterone receptor B / β-actin

Calponin / β-actin

Relative optical density

Upper region ← Lower region
(fundal)

Region of uterus from which sample was obtained
4.3.2 \( P_4 \) receptor protein

4.3.2.1 Human

Figure 4.7 shows the location of the \( P_4 \) receptor isoforms for T47D cells, the banding pattern obtained with exposure of blots to PR(C-20) following preabsorption of the antibody with its immunizing peptide PR(C-20)P, as well as the banding pattern obtained following exposure of the blot to goat-anti-rabbit horse radish peroxidase without prior incubation of the blot with primary antibody. The antibody PR(C-20) recognized a number of bands (Figure 4.7). Investigators examining \( P_4 \) receptor protein using western blots have identified A and B isoform bands at varying sizes dependent in part upon the antibody used and the tissue examined (Table 2.2). In the present study, for T47D cells, human myometrium, and rat myometrium, 5 bands could be identified in the region between 120 and 79 kilodaltons. These bands were identified at 119, 106, 96, 89, and 79 kilodaltons. T47D cells were highly abundant in two receptor isoforms at 119 and 89 kilodaltons. Human and rat myometrium were much less abundant in these isoforms. An additional band was abundant in T47D cells at 68 kilodaltons.

In the blots examining \( P_4 \) receptors in human myometrium, bands were identified at 119 (faint), 106, 96, and 89 kilodaltons (Figure 4.8). The 89 kilodalton form was significantly higher in non-pregnant myometrium than pregnant not in labour or in labour myometrium (One-way ANOVA: \( F=3.96, p=0.03, d.f.=2,25 \)). No significant difference in the amount of any \( P_4 \) receptor isoform was noted between myometrial samples collected from women not in labour and from women in labour. In samples obtained from pregnant women, the level of isoform abundance from greatest to least was: 96, 106, 89, 119 kilodalton. Human samples had less \( P_4 \) receptor protein than rat myometrial samples, and much less than T47D cells (Figure 4.8).
Figure 4.7: a) Western blot probed first with the polyclonal antibody PR(C-20), then secondary antibody, followed by a chemiluminescent detection reagent and exposed to film for 30 minutes. b) An identical blot was prepared and run in parallel with the previously described blot (a). This blot was probed with PR(C-20) which had been pre-incubated with its immunizing peptide PR(C-20)P, then secondary antibody, followed by a chemiluminescent detection reagent and exposure to film for the same amount of time as blot (a). c) The (a) blot was then stripped and probed with secondary antibody, followed by chemiluminescent detection reagent and exposure to film for 30 minutes. The blots contained protein from T47D progesterone (P₄) receptor abundant cells, PC3 P₄ receptor deficient cells, and samples obtained from human and rat myometrium. Bands which appear on the second (b) and third (c) blots are non-specific for P₄ receptors.
a) Western blot probed with PR(C-20) polyclonal primary antibody

b) Western blot probed with PR(C-20) preabsorbed with PR(C-20) immunizing peptide [PR(C-20)P]

c) Western blot probed with secondary antibody (anti-rabbit horse radish peroxidase), without prior probing with PR(C-20)
Figure 4.8:  

a) A representative western blot showing the bands which appear in the region expected for progesterone (P₄) receptor isoforms A and B. Specific bands are present at 119, 106, 96, 89, and 79 kilodaltons (kDa).

b) Mean optical density of the 119, 106, 96, and 89 kDa bands in protein extracted from the following samples: T47D, P₄ receptor abundant cells; human myometrium samples obtained from women who were not in labour at the time of cesarean section, as well as from those who were in labour at the time of section; human myometrium from women who were not pregnant at the time of their hysterectomies; myometrial tissue from rats who were at day 22 of pregnancy (delivery on day 23). The 89 kilodalton form was significantly higher in non-pregnant myometrium than pregnant not in labour or in labour myometrium (One-way ANOVA: F=3.96, p=0.03, d.f.=2,25). No significant difference in the amount of any P₄ receptor isoform was noted between myometrial samples collected from women not in labour and from women in labour. Human samples had less P₄ receptor protein than rat myometrial samples, and much less than T47D cells. n=4 blots (all 4 blots were prepared and treated at the same time).
Western blot of progesterone receptor A and B isoforms in human myometrium

Protein marker (kDa)

Potential progesterone receptor bands (kDa)

- 119 kDa
- 106 kDa
- 96 kDa
- 89 kDa

T47D Cells
Not in labour
In labour
Not preg.
Rat day 22 preg.

Human myometrium

Optical density

- T47D cells
- Not in labour
- In labour
- Not pregnant (hysterectomy)
- Rat day 22 pregnancy

Human myometrium
4.3.2.2 Rat

In the blots examining P₄ receptors in rat myometrium, bands were identified at 119 (very faint), 106, 96, 89, and 79 kilodalton (Figure 4.9). Very close examination of the 89 kilodalton isoform band reveals two bands of similar size. It was not possible with the methods employed to distinguish between the bands constituting this doublet densitometrically. In samples obtained from pregnant rats, the 119 kilodalton band was not measurable densitometrically, and the 96 kilodalton form was the most abundant (Figure 4.9). The only band which was found to be significantly different between days of pregnancy was the 96 kilodalton form (One-way ANOVA: F=23.57, p=0.0001, d.f.=8,26). When analyzed as a group, rats that were not-pregnant had significantly higher levels of each P₄ receptor band than did pregnant rats (Student’s t-test: p<0.05 for each) (Figure 4.9).
Figure 4.9: a) A representative western blot showing the proteins bands which appear in the region expected for progesterone (P₄) receptor isoforms A and B. Specific bands are present at 119, 106, 96, 89, 79, and 68 kilodaltons (kDa). Samples examined are protein extracted from rat myometrium. Tissues were collected at various days (d) of gestation [d15, d21, d22, d23 (labour) and one day post partum (pp1)], as well as from ovariectomized non-pregnant rats, and ovariectomized non-pregnant rats that were estradiol treated. Protein extracted from P₄ receptor abundant T47D cells were included on the gel as well.

b) Mean optical density of the 106, 96, 89, and 79 kDa bands identified in a). The only band which was found to be significantly different between days of pregnancy was the 96 kilodalton form (One-way ANOVA: F=23.57, p=0.0001, d.f.=8.26). When analyzed as a group, rats that were not-pregnant had significantly higher levels of each P₄ receptor band than did pregnant rats (Student’s t-test: p<0.05 for each). Bars with the same letters are significantly different (p<0.05). n=4 blots (all 4 blots were prepared and treated at the same time).
Western blot of progesterone receptor A and B isoforms in rat myometrium

a) Protein marker (kDa)

Potential progesterone receptor bands (kDa)

- 106 kDa
- 96 kDa
- 89 kDa
- 79 kDa

b) Optical density

- d15
- d21
- d22
- d23
- pp1
- Ovx
- Ovx Cycle T47D
- Ovx + E2
- Cycle
- T47D cells

Pregnant
Not-pregnant

n=4 blots
4.4 Discussion

With no decrease in maternal circulating levels of P₄ at the time of labour initiation in humans, some other mechanism must result in the attenuation of P₄'s inhibitory affects on the myometrium. We found P₄ receptor A and B isoform mRNA decreased with the onset of labour in humans, yet overall, this decrease was not found to be significant. Neither was a decrease in these isoforms found with labour at the protein level. P₄ receptor isoforms in rats at the protein level did not show any significant physiologically relevant changes with the onset of labour.

This study highlighted some of the difficulties associated with examining P₄ receptors during pregnancy. The first of these being the low levels of P₄ receptors present at this time. This is due to the fact that P₄ down-regulates its own receptors. Therefore, during third trimester pregnancy when maternal circulating levels of this steroid are approximately 100 times those observed during the luteal phase in non-pregnant women (Anderson et al. 1985; Boroditsky et al. 1978), P₄ receptor levels in the myometrium are lower than any other time during pregnancy or during the cycle (Giannopoulos and Tulchinsky 1979; Padayachi et al. 1990; Perrot-Applanat et al. 1994). While a subtle change in the levels of P₄ receptor isoforms present in the myometrium could result in the attenuation of P₄ effects resulting in the initiation of labour, such a change is not easily detectable due to the low abundance of these receptors.

Another difficulty in measuring P₄ receptor isoforms is that there appear to be multiple RNA species. Northern blot results obtained for human P₄ receptors in this study are similar to those published by Wei et al. (Wei et al. 1990; Wei et al. 1988; Wei and Miner 1994) for T47D cells, and human endometrial tissue. The multiple faint banding pattern obtained however, did not allow for reliable densitometric analysis. Wei et al. (Wei et al. 1990; Wei et al. 1988; Wei and Miner 1994) suggested that the 2.5 and 5.2 kilobase northern blot bands encode for the P₄ receptor A isoform. The 3.2, 4.5, 6.1, and the 11.4 I (Wei et al. identified 4 bands at 11.4 kilobases which they referred to as 11.4 I, II, III, and IV) and II kilobase species could code for either A or B P₄ receptor isoforms, and the 11.4 III, and IV bands do not code for either A or B (Wei et al. 1990;
Wei et al. 1988; Wei and Miner 1994). Therefore, it would be difficult to correlate and potential results from a northern blot of P4 receptors with levels of functional P4 receptors.

Examining P4 receptors at the protein level is also difficult in part because the published sizes of P4 receptor A and B isoforms are not consistent with either the antibody used, or the tissue in which the bands are identified (see Table 2.2). The A isoform band has been identified between 72 and 95 kilodaltons, and B isoform band has been identified between 86 and 120 kilodaltons (references in Table 2.2). The majority of human P4 receptor antibodies available have been characterized for T47D cells, not for native human tissues. This, along with the inconsistencies in size identification, makes it difficult to determine the exact size of protein bands which correspond to the A and B isoforms, and whether other bands present are varying forms of these receptors.

In the present study multiple bands between 79 and 119 kilodaltons were identified on western blots that appear specific to the P4 receptor; these bands disappeared when the primary antibody PR(C-20) was incubated with the immunizing peptide PR(C-20)P, and did not appear when the blot was incubated with secondary antibody only. The antibody used recognizes a 20 amino acid region at the end of the A/B region and the beginning of the DNA binding domain. This amino acid sequence is unique to P4 receptors; it does not recognize any other amino acid sequence published to date. The 5 bands identified appear in the human and rat samples where 150 µg of protein was loaded onto the gels, as well as in T47D cells for which only 50 µg of protein was loaded. Thus, it is unlikely that the concentration of protein on the gel is the result of any non-specific binding of the primary antibody in the region of interest. In support of this, we also tried probing for P4 receptors on western blots using a number of other antibodies: mouse monoclonal anti-human P4 receptor antibody from Affinity Bioreagents (Golden, CO); mouse monoclonal anti-human P4 receptor antibody [PR(AB-52)] from Santa Cruz (Santa Cruz, CA, recognizes A and B isoforms); mouse monoclonal anti-human P4 receptor antibody [PR(B-30)] from Santa Cruz (Santa Cruz, CA, recognizes B isoform only); and mouse monoclonal anti-human P4 receptor antibody from Novacastra (Newcastle, UK, recognizes A and B isoforms). Banding on western blots using these antibodies was faint compared to that for PR(C-20), yet the banding pattern that was discernible included the bands found using PR(C-20). It is possible that the five bands clearly
identified using PR(C-20) are different processing and/or phosphorylation forms of the A and/or B isoforms.

The multiple bands found at the mRNA and protein level in this study may be variants of P₄ receptors which have yet to be characterized. Multiple forms are first suggested at the mRNA level where there are 7 forms capable of coding for the A isoform, and 5 capable of coding for the B isoform (Wei et al. 1990; Wei et al. 1988; Wei and Miner 1994). At the protein level there are 3 methionine start sites which are known to result in the A, B and C isoforms (Figure 2.5) (Sartorius et al. 1994b). Differential post-translational processing of these three forms could potentially result in a number of receptors of differing sizes. As well, certain antibodies, such as the one used in this study, will recognize more than two bands between 72 and 120 kilodaltons (see Table 2.2). Therefore, it is not presently clear whether multiple bands in the size region of the A and B isoforms are different transcription products, or whether they are the result of post-translational modifications.

Sherman et al. (1976) identified 5 forms of the P₄ receptor in chick oviduct cytosol, which they numbered I through V, in order of their elution from agarose gel columns. All 5 forms contained the steroid-binding site. They found forms II and III corresponded to the B and A isoforms. They termed forms IV and V, the smallest forms, mero-receptors (Sherman et al. 1976). Further characterization of mero-receptors (Miller et al. 1975; Niu et al. 1981; Sherman and Diaz 1977; Sherman et al. 1978) found that they range from 20 to 40 kilodaltons, and while they contained the steroid-binding site, they lacked the nuclear binding domain. Thus, it is unclear what role these receptors may have, if any, in P₄ responses. These forms are likely proteolytic breakdown products. No recent studies have further examined these forms. The existence of mero-receptors does however, give further evidence of the complexity which exists in P₄ receptor signaling.

A variance in P₄ receptor distribution through the various layers, and/or regions of the human uterus may affect P₄ response, yet we did not find differences in regional expression of P₄ receptor A and B isoforms in human uteri. Wikland et al. (1984) found a regional difference in the response of myometrial strips to prostaglandins. Before labour both prostaglandin E₂ and F₂α stimulated contraction in myometrial strips obtained from the lower segment of human uteri, while
uterine segments obtained from the fundal region were relatively insensitive to such treatment. Fundal segments collected during labour however, were stimulated by both prostaglandin E₂ and prostaglandin F₂α, while the lower uterine segments relaxed in response to prostaglandin E₂ and were slightly stimulated by prostaglandin F₂α. Wikland et al. (1984) thus suggested that there is a complex interaction between prostaglandins and the various regions of the uterus, which is dependent in part, upon whether the uterus is labouring or not. How et al. (1995) did not find difference in estrogen or P₄ receptors between samples which were collected from the fundal and lower uterine segments of the same uterus. These samples were obtained from two women who delivered preterm and who were not in labour at the time of classical cesarean delivery (How et al. 1995). Our limited number of regional samples provided no clear evidence for a change in P₄ receptor distribution with the initiation of labour.

It is possible that a variance, if one exists, may be between the various layers of the uterus, not simply between different regions. The inner circular muscle layer of the human uterus is known to contract differently in response to stimuli than the external longitudinal layer (Breuiller et al. 1988; Breuiller et al. 1987; Daels 1974; Pinto et al. 1967c). It is unclear whether the study of Wikland et al. (1984) examined full thickness samples from both the fundus and the lower region of the uterus. The samples obtained in the present study from the fundal as well as the lower cervical region were full thickness. It would be interesting to determine P₄ receptor mRNA and protein levels within the specific layers of the myometrium to see whether they differed in non-labouring and labouring samples.

P₄ action at the time of labour may be altered by affecting a mechanism of P₄ action which does not involve P₄ receptors. This may, in part, explain why we do not see a significant decrease in the levels of P₄ receptors with labour. We do not know to what extent the actions of P₄ are mediated through such non-genomic or ligand-independent mechanisms in the uterus during gestation. Yet, it is possible that the effects of P₄ may be attenuated at the time of labour by altering such effects, leaving the relative abundance of the P₄ receptor isoforms unaltered.

While P₄ is important for the maintenance of pregnancy, the mechanisms by which its effects are overcome at the time of labour remain to be elucidated. P₄ responses are undoubtedly the result of a complex interaction of genomic and non-genomic steroid effects, which may be
ligand dependent or independent. It is not clear how such effects may be altered at labour. It does not appear as if an alteration in the level of the various $P_4$ receptor isoforms is responsible. This conclusion is independent of whether the species has a drop in maternal circulating levels of $P_4$ at the time of labour as in the rat, or whether maternal circulating levels of $P_4$ are maintained as in the human. It remains possible that $P_4$ genomic effects may be altered by affecting the levels of various cellular proteins which are necessary to render the receptor active.
5. **Heat-shock and steroid receptor coactivator proteins in the myometrium at labour**

5.1 **Introduction**

There are a number of ways in which the effects of P₄ might be attenuated at the time of labour in humans independent of a change in maternal circulating levels. The study presented in Chapter 4 suggests that there is no significant decrease in the levels of P₄ receptor isoforms in human myometrium with labour. As discussed in Section 2.3 the P₄ receptor interacts with a series of other proteins which can enhance and/or suppress its ability to interact with DNA. Thus, changes in the expression of any of these proteins at the time of labour might modulate P₄ receptor signaling and hence effect a withdrawal of P₄ action on the myometrium. Amongst these interacting proteins are Heat-shock proteins (HSP) and steroid receptor coactivators.

As discussed in detail in section 2.3, HSP are a group of highly conserved proteins found in nearly all cells in all organisms. HSP are constitutively expressed and their levels are increased in response to stress. The precise functions of these proteins are largely unknown but, overall, they are believed to be involved in maintaining cellular homeostasis. The association of HSP with P₄ receptors is essential for the correct three-dimensional conformation, location, as well as activation/inactivation, of these receptors (Figure 2.5). Several different HSP exist. The two major groups are the 68-73 kilodalton size class (HSP70), and the 80-90 kilodalton size class (HSP90). It is these two groups which have been the most extensively characterized with respect to steroid receptor function.

Nuclear receptors such as those for P₄ can inhibit or enhance transcription as well as interfere or squelch the activity of other steroid receptors by recruiting a variety of proteins to the transcription complex. Such proteins are referred to as coactivators or corepressors. Thus, the amount of, or ratio of coactivators to corepressors that are bound to nuclear receptors under specific conditions can produce an array of receptor activity ranging from complete transcriptional inhibition to maximal transcriptional activation.
A number of coactivators have been described to date, and they are described in detail in section 2.4. Of these, there are at least three which might specifically affect P₄ and/or estrogen receptor signaling, and for which there is sufficient information in vitro to allow us to examine them in vivo. The first of these is steroid receptor coactivator 1 (SRC1), which can increase receptor-dependent transcription greater than 14-fold in the presence of ligand, but has little effect in the absence of hormone (Onate et al. 1995). A second important coactivator with respect to P₄ action is transcriptional intermediary factor 2 (TIF2). The third important steroid receptor coactivator is ARA70. ARA70 is an androgen-receptor-associated protein which functions as an activator to enhance androgen receptor transcriptional activity (Yeh and Chang 1996). ARA70 can enhance the transcriptional activity of the androgen receptor 10 fold, and the activity of the glucocorticoid, estrogen and P₄ receptors 2-fold.

The role of coactivators in the myometrium or in pregnancy has yet to be examined in any species. It is possible that alterations in some or all of these coactivators could affect steroid transactivation in the human myometrium such that labour may be precipitated without a significant change in other steroidal variables such as steroid circulating levels or steroid receptor levels.

We wished to determine whether the effects of P₄ on the myometrium at the time of labour may be overcome by altering the relative abundance of HSP (HSP90α, HSP90β, or HSP70), or coactivators (TIF2, ARA70, or SRC1) present in the myometrium. mRNA expression, through northern blot techniques, of the three HSP and the three coactivators, was examined in human myometrial tissues collected from labouring and non-labouring women who delivered both preterm and at term. In situ hybridization was used to examine the cellular distribution of ARA70 within human myometrial tissues. Time limited the analysis of other coactivators via this method. To determine whether these proteins changed with the onset of labour in a species which has a maternal decrease in circulating P₄ levels, the expression of the HSP and coactivators was examined in rat myometrial tissue samples. As a positive control we measured the expression of HSP90α, HSP90β and HSP70 in sheep myometrium under conditions similar to those described by Wu et al. (1996a).
5.2 Methods

5.2.1 Sample collection

5.2.1.1 Human

Samples were collected as described in section 4.2.1.1. Table 5.1 lists the samples collected from not in labour and in labour cesarean section deliveries, preterm and at term, which were used in northern blot analyses of HSP and coactivators. Table 5.2 lists the samples collected from different regions of the uteri of various women who underwent either a longitudinal incision cesarean section, post-partum hysterectomy, or a hysterectomy while not pregnant.

Following excision, uterine tissue was rinsed in a phosphate buffered saline, endometrium and stromal layers were separated, and the remaining myometrium was placed in liquid nitrogen and stored -80°C until use.

5.2.1.2 Rat

Individually housed Wistar rats (Charles River Co., St. Constance, Canada) were maintained under 14 hour light, 10 hour dark lighting conditions. These rats were fed Purina rat chow (Ralston Purina, St. Louis, MO), and water ad libitum. Virgin female rats (approximately 250 g) were mated, and the day on which a vaginal plug was observed was designated day 1 of gestation. Under such conditions these rats delivered on the morning of day 23 of gestation. All experiments were approved by the Institutional Animal Care Committee.

Samples were collected from pregnant rats or from rats in which we have altered, through various endocrine or physiological manipulations, the onset of labour and/or myometrial expression of proteins associated with the contraction of the uterus.
Table 5.1: Samples used in the examination of heat-shock protein 90α, 90β, and 70, as well as coactivators SRC1, TIF2, and ARA70, by northern blots. Samples were obtained from preterm as well as term deliveries. BS refers to Bishop’s score.

<table>
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<th>Preterm or term</th>
<th>Labour status (not vs. in)</th>
<th>Dilatation (cm)</th>
<th>Reason for cesarean section</th>
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</tr>
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<td>2123</td>
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<td>not</td>
<td>-</td>
<td>-breech</td>
</tr>
<tr>
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<td>-</td>
</tr>
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<td>-</td>
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<td>-</td>
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</tr>
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<td>-</td>
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<td>32</td>
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<td>not</td>
<td>-</td>
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<td>-</td>
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<td>term</td>
<td>not</td>
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<td>-</td>
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<td>in</td>
<td>4</td>
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<td>7</td>
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<td>in</td>
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<td>37</td>
<td>term</td>
<td>in</td>
<td>4</td>
<td>-breech</td>
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Samples used in the examination of heat-shock protein 90α, 90β, and 70, as well as coactivators SRC1, TIF2, and ARA70, by northern blots. Samples were obtained from preterm and term deliveries, as well as from non-pregnant uteri following hysterectomy. BS refers to Bishop’s score.

<table>
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<th>Gestational age (Weeks)</th>
<th>Preterm or term</th>
<th>Labour status (not vs. in)</th>
<th>Dilatation (cm)</th>
<th>Reason for cesarean section or hysterectomy</th>
<th>Sample description</th>
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<td>-</td>
<td>hysterectomy, fundal and lower region samples</td>
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</tr>
<tr>
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<td>-</td>
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<td>fibroids</td>
<td>hysterecomy, middle of uterus</td>
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<td>classical section -</td>
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<td>classical section -</td>
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<td>classical section-</td>
</tr>
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<td>not</td>
<td>-</td>
<td>conjoined twins</td>
<td>classical section-</td>
</tr>
<tr>
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<td>in</td>
<td>-</td>
<td>hysterectomy, middle region</td>
<td></td>
</tr>
<tr>
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<td>in</td>
<td>-</td>
<td>hysterectomy, fundal region</td>
<td></td>
</tr>
</tbody>
</table>
5.2.1.2.1 *Pregnancy study*

Pregnant rats were killed on days 21 (d21), 22 (d22), 23 (d23; during labour), or one day post partum (pp1). All animals were sampled following decapitation. Rats on day 23 during labour were sampled following the delivery of at least 2 pups, with at least one pup remaining in utero. Uteri were removed and endometrium was separated from myometrium. Myometrial tissue was rinsed in phosphate buffered saline, frozen in liquid nitrogen, and stored at -80°C until use.

5.2.1.2.2 *Progesterone treatment*

For the \( P_4 \) treatment study on pregnant rats, hormone treatment began on day 20. The rats were injected daily with either 5 mg of \( P_4 \) in 0.2 ml mineral oil, or 0.2 ml mineral oil without \( P_4 \). The \( P_4 \) treated rats were sampled in the mornings of either day 21, 22, 23, or 24. The administration of \( P_4 \) prevented the onset of labour in these rats. The control rats were sampled on either day 21, 22 or 23 (during labour) of gestation.

5.2.1.2.3 *Progesterone treatment of ovariectomized rats*

The previous experiment was repeated with the exception that rats were ovariectomized on day 18 of gestation. Surgery was performed under general anesthesia [Ketamine:Xylazine, 2:1, 1mg/kg body weight (Smith et al. 1990)]. \( P_4 \) treatment or control injections began on day 18. Rats were sampled on days 19, 20 or 21 of gestation.

5.2.1.3 *Sheep samples*

The gestation length of the mixed breed ewes was 145-147 days. Term control sheep myometrial samples were collected from ewes just prior to the onset of labour at term. Term in labour sheep myometrial samples were collected from ewes during labour immediately following delivery. For the collection of pre-term myometrial samples surgery was performed on ewes under
general anesthesia on days 119-122 of gestation. Following the method of Jeffray et al. (Jeffray et al. 1998), a catheter was inserted into the fetal jugular vein. Following a 5 day recovery period fetuses were infused in utero with either cortisol (4-pregnen-11β,17,21-triol-3,20-dione, Steraloids, Inc., Wilton, NH; 5 μg/minute), or an equal volume of saline [3 ml/hour, with 2% (vol/vol) ethanol (EtOH)] for 96 hours. Animals were killed with an overdose of Euthanyl (24% sodium pentobarbital; MTC Pharmaceuticals, Cambridge, Ontario). Ewes with glucocorticoid infused fetuses as well as those with saline infused fetuses were not in labour. Samples of the myometrium were rapidly collected and frozen in liquid nitrogen, then stored at -80°C until use.

5.2.2 RNA Blots

Total RNA was extracted from human myometrium, untreated pregnant rat samples, and sheep samples using Trizol (Gibco/BRL, Burlington, ON), following the manufacturer’s suggested protocol. RNA blots were prepared as described in Section 4.2.2.

Samples collected from the P₄ treated pregnant rat studies (both ovariectomized and non-ovariectomized), and the rat mechanical stretch study, were extracted of total RNA with 4 M guanidium isothiocyanate according to a method described by Chomczynski and Sacchi (1987). 20 μg of RNA was separated on 1% (wt/vol) agarose, 3.7% (vol/vol) formaldehyde denaturing gels. RNA on these gels was then transferred onto a nylon membrane (GeneScreen, DuPont, NEN Research Products, Boston, MA). The membranes were cross-linked by ultraviolet irradiation. The human, rat, and sheep northern membranes were then baked at 75°C for 2 hours in a vacuum oven.

Table 5.3 lists the northern blot made, and the probes used on these blots.

The RNA on the membranes was hybridized to a complementary DNA (cDNA) probe (see Table 5.4). Probe preparation, pre-hybridization, hybridization, washing, membrane stripping, exposure to film, and film analysis (band densitometry) procedures are as described in section 4.2.2.

The identity of SRC1, ARA70, and TIF2 probes was confirmed by sequence analyses.
Table 5.3: A list of species from which RNA was obtained for northern blots, the experimental and/or sample conditions, as well as specific mRNAs probed for on these blots. A check-mark (✓) means that the particular probe was used on the blot, and an ‘x’ means that it was not. The corresponding Figure identification number is listed as well.

| Species | Sample description | Probes used: | | | | | | Figure # |
|---------|--------------------|--------------|--------------|--------------|--------------|-------------|----------|
| Human   | Term and preterm deliveries | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 5.1          |
|         | -samples from not and in labour cesarean sections both preterm and at term | | | | | | | and 5.2      |
| Human   | Regional samples | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 5.3          |
|         | -samples taken from various regions of the uterus | | | | | | | and 5.4      |
|         | -samples from not and in labour cesarean sections both preterm and at term | | | | | | |          |
| Rat     | Pregnancy | ✓ | ✓ | ✓ | x | x | x | ✓ | 5.5          |
|         | -day 20,21,22,23,1 day post partum | | | | | | |          |
| Rat     | Progesterone treated & pregnant | ✓ | ✓ | x | x | x | x | ✓ | 5.6          |
|         | -blot prepared by A. Orsino | | | | | | |          |
| Rat     | Pregnant ovariectomized model | ✓ | ✓ | ✓ | x | x | x | ✓ | 5.7          |
|         | -rats either ovariectomized or not on day 18 of pregnancy -rats ovariectomized either given or not given P₄ treatment -blot prepared by A. Orsino | | | | | | |          |
| Sheep   | Term and preterm deliveries | ✓ | ✓ | ✓ | x | x | x | ✓ | 5.8          |
|         | -early pregnancy (control and glucocorticoid-induced labour) -term (not in labour and in labour) -samples from Dr. J.R. Challis’ lab | | | | | | |          |
Table 5.4: List of the cDNA used to identify the 8 RNA species of interest on northern blots. Size of probe, method by which probes obtained/prepared, as well as the size of the RNA band the probe identifies on a northern blot, are listed. All probes are based on human cDNA sequences.

<table>
<thead>
<tr>
<th>cDNA probe</th>
<th>Probe length</th>
<th>How probe obtained/prepared</th>
<th>Length of mRNA (kilobases) that probe recognizes</th>
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<td>4.5 kilobases</td>
<td>Dr. P.G. Walfish, Samuel Lunenfeld Res. Inst., Toronto, ON</td>
<td>5.5 and 7.5</td>
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<tr>
<td>ARA70</td>
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<td>Dr. T. Brown, University of Toronto, ON</td>
<td>3.6</td>
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<tr>
<td>TIF2</td>
<td>772 bases</td>
<td>PCR product ligated and transformed in pBluescript SK+/- (Promega, Madison, WI)</td>
<td>9</td>
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<tr>
<td>Cxn-43</td>
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<td>Dr. David Paul, Department of Anatomy and Cell Biology, Harvard Medical School, Boston, MA</td>
<td>3.1</td>
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<td>18 S</td>
<td>260 bases</td>
<td>Dr. D. Denhardt, Rutgers University, Piscataway, NJ</td>
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5.2.3 \textit{In situ} hybridization

Frozen blocks of human myometrium were cut at -20 °C into 12 μm thick sections. Sections were mounted onto positively charged glass microscope slides (Probe-on, Fisher, Canada). Each slide contained a myometrial sample from a woman non in labour and from a woman in labour. Duplicate slides were made from each set of samples; one was used with the antisense probe, and the second with the sense probe. RNAase-free conditions were maintained throughout the experiment.

Slides were quickly transferred from the cryostat to 4% (wt/vol) paraformaldehyde in phosphate buffered saline (PBS; pH 7.4). Slides remained in the fixative for 15 minutes at 4°C. Slides were moved into PBS at room temperature for 5 minutes. Tissue was dehydrated by dipping the slides in the following EtOH concentration for 3 minutes each: 70%, 95%, 100%, 100%.

Sections were left to air dry then they were placed in a vacuum desiccator overnight at room temperature. The next morning tissues were pretreated by dipping slides into the following solutions at room temperature: 0.1% (vol/vol) triton + 2xSSC (0.15 M NaCl, 0.015 M NaCitrate, pH 7.0) for 30 minutes; 2xSSC for 5 minutes room temperature; 2xSSC for 5 minutes; 4% (wt/vol) paraformaldehyde in PBS, pH 7.4 for 5 minutes; PBS for 5 minutes; triethanolamine solution [0.1 M triethanolamine hydrochloride (Sigma, Oakville, ON, C₆H₁₃NO₃·HCl), 0.05 M NaOH] for 2 minutes; triethanolamine solution with 0.002% (vol/vol) acetic anhydride for 10 minutes; 2xSSC for 5 minutes; 2xSSC for 5 minutes; 50% EtOH for 3 minutes; 70% EtOH for 3 minutes; 95% EtOH for 3 minutes; 100% EtOH for 3 minutes; 100% EtOH for 3 minutes. Slides were then air dried.

\textit{In situ} hybridization was used to examine the cellular localization of ARA70 mRNA human myometrium. ARA70 cDNA was obtained from Dr. T.J. Brown (University of Toronto, ON). The cDNA probe was 698 base pairs long. The identity of this probe was confirmed through sequence analyses.

Probe was prepared for \textit{in situ} first by linearization (SacI or EcoR1). Linearized probe was treated for 1 hour at 27°C with proteinase-K (0.5 µg/µl linearized product, Sigma, Oakville, ON).
DNA was then phenol/chloroform purified and reconstituted through EtOH precipitation. This cDNA was then used to transcribe the cRNA probe. The following reagents (RNA transcription kit, Stratagene, LaJolla, CA) were added to 2 µl (1 µg) of DNA: 5 µl 5x transcription buffer, 1 µl rATP, 1 µl rGTP, 1 µl rCTP, 1 µl of 0.75 M DTT, 1 µl (10 U) RNA polymerase (T7 for Sacl cut, and T3 for EcoR1 cut), and 8 µl diethylpolycarbonate (DEPC) treated H2O. Next 5 µl (100 µCi) 35S-UTP was added. This mix was incubated for 1 hour at 37°C. Unbound 35S-UTP was separated from bound by passage through a G-50 sephadex quick spin column for radiolabeled RNA purification (Boehringer Mannheim, Germany). Radioactivity was quantified. A 20 ml volume of hybridization buffer contained the following: 12.5 ml formamide; 5.0 ml 50% dextran sulfate (35 ml DEPC H2O and 25 g dextran sulfate); 1.5 ml 5 M NaCl; 0.5 ml 50x Denhardt’s solution (1 g Ficol, 1 g polyvinylpyrrolidone, 1 g bovine serum albumin fraction V); 0.25 ml 1 M Tris-HCL pH 8.0; and 50 µl 0.5 M EDTA pH 8.0. Prior to the addition of radioactive probe the following were combined to yield 1 ml of hybridization solution: 800 µl hybridization buffer; 20 µl of 25 mg/ml tRNA (Gibco BRL, Burlington, ON); 10 µl of 1M DTT; radioactive cRNA probe for a concentration of 10 million counts per minute per ml of hybridization buffer; and DEPC H2O.

The hybridization buffer containing the radioactive cRNA probe was heated at 65°C for 10 minutes, then placed on ice. Pretreated slides were placed in a hybridization chamber lined with 2.5xSSC in formamide (Ultra Pure, Gibco, Gaithersburg, MD). 75 µl of the hybridization/cRNA mix was placed on each slide. Slides were coverslipped. The hybridization chamber was then sealed and placed at 57°C for 20 hours. Following this incubation time the hybridization chamber was allowed to sit at room temperature for 30 minutes. Slides were removed from the chambers and immersed in 4xSSC for 30 minutes, with gentle agitation. Sense and antisense slides were processed in separate containers. Coverslips separated from slides during this period. To remove excess probe slides were then immersed in 4xSSC for 5 minutes (repeated 3 times). To remove non-specifically bound probe tissues were treated in an RNase buffer [0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, 20 µg/ml RNase A (from bovine pancreas, Boehringer Mannheim, Germany)], 30 minutes at 37°C with agitation. Slides were processed through the following buffers at room temperature with agitation: 2xSSC with 0.001 M dithiothreitol (DTT, Gibco, Gaithersburg, MD); 1xSSC with 0.001 M DTT; 0.5xSSC with 0.001 M DTT. Slides were then placed into preheated
0.1×SSC with 0.001 M DTT at 85°C for 30 minutes. Slides were rinsed in 0.1×SSC with 0.001 M DTT. Tissue was then dehydrated through the following steps at room temperature for 3 minutes each: 50% EtOH with 0.001 M DTT in SSC; 70% EtOH with 0.001 M DTT in SSC; 95% EtOH with 0.001 M DTT in SSC; 100% EtOH; then 100% EtOH. Slides were allowed to dry. Slides were exposed against film (BioMax MR, Kodak, NEN Life Sciences, Boson, MA) for 2 weeks. Film was removed from the slides and developed.

Under safe-light conditions slides were coated with (exposed to) NTB-2 Autoradiography emulsion (Kodak, NEN Life Sciences, Boson, MA), and kept light-safe at 4°C for 10 weeks. Following emulsion exposure slides were developed in D-19 developer for 3.5 minutes (Kodak, NEN Life Sciences, Boson, MA). Slides were next rinsed in water before fixed in 5% sodium thiosulfate for 10 minutes. Slides were washed in running water for 30 minutes. Tissue was counterstained by the following method: 50% EtOH for 2 minutes; 70% EtOH for 2 minutes; 80% EtOH for 2 minutes; 95% EtOH for 2 minutes; 100% EtOH for 2 minutes; 100% EtOH for 2 minutes; cresyl violet (Sigma, Oakville, ON) for 15 minutes; 70% EtOH for 1 minutes; 70% EtOH with 0.0004% (vol/vol) glacial acetic acid for 30 seconds; 80% EtOH for 1 minute; 95% EtOH for 2 minutes; 100% EtOH for 2 minutes; then xylene for 5 minutes. Slides were coverslipped.

Images were recorded digitally using a Leica microscope (LEICA DMR) with Northern Eclipse Image Analysis Software 2.1 for the IBM, (Empix Imaging Inc.).

5.2.4 Data analyses

Data were analyzed using two microcomputer-based statistical program packages (Sigmastat for Windows, and Statview for Macintosh). Relative optical density values for HSP and steroid receptor coactivator mRNA were compared between not in labour and in labour samples obtained either preterm or at term using a One-way ANOVA (x=preterm not in labour, preterm in labour, term not in labour, or term in labour; y=relative optical density) as well as a 2-way ANOVA (x1=preterm or term, x2=in labour or not in labour; y=relative optical density). Due to the limited number of samples obtained from different regions of human uteri, as well as the variance in the regions from which these samples were obtained, no attempt was made to analyze the results from these samples statistically. Relative optical density values for HSP, and cxn-43 mRNA were
compared between myometrial samples from rats sampled on days 21 to one day post partum using a One-way ANOVA. Two-way ANOVA was used to compare between P₄ treated and control rats on days 21 to 23 of gestation. To compare between rats that were either ovariectomized or sham operated on day 18 of gestation and/or were treated with P₄ on days 18-21 of gestation a 2-way ANOVA was used (x₁=+/- ovariectomy, x₂= +/- P₄ treatment; y=relative optical density). Relative optical density values for HSP mRNA in sheep myometrium were compared between not in labour and in labour samples obtained either preterm or at term using a One-way ANOVA (x=preterm not in labour, preterm in labour (glucocorticoid infused), term not in labour, or term in labour; y=relative optical density) as well as a 2-way ANOVA (x₁=preterm or term, x₂=in labour or not in labour; y=relative optical density). When a significant One-way ANOVA was identified (p<0.05), significance between individual x pairs was determined using Fisher’s post-hoc test.

Skew or inhomogeneity of variance, where detected in data to be analyzed by ANOVA or t-tests, were eliminated by log transformation. When means are presented they are accompanied by the standard error of the mean.
5.3 Results

5.3.1 Northern blots

5.3.1.1 Human

Human myometrium showed a significant increase in HSP90α and HSP90β mRNA with term labour (One-way ANOVA, p<0.02 for each, Figure 5.1), and a significant increase from preterm not in labour to term in labour for SRC1, and TIF2 (One-way ANOVA, p<0.02 for each, Figure 5.2). No significant interaction however, were found between time of gestation (preterm vs term) and the labour state (not in labour vs in labour) (2-way ANOVA, p>0.05). Figure 5.3 shows HSP and coactivator mRNA levels in different regions of the uterus. In some humans the level of message differs depending on the region from which the samples were obtained, however, there does not appear to be an overall pattern to this variation. A similar distribution was noted for SRC1 (7.5 and 5.5 kilodalton bands), and ARA70 in various regions of the uterus (Figure 5.4).
Figure 5.1: a) Representative northern blot containing human myometrial RNA. Myometrium was collected from women who were not in labour as well as from those who were in labour at the time of cesarean section. Samples were collected from women who delivered preterm as well as term. Blot was probed for heat-shock protein (HSP) 70, HSP90α, HSP90β as well as for 18 S.

b) Mean relative optical densities (HSP optical density / 18 S optical density) for the samples described in a). Human myometrium showed a significant increase in HSP90α, and HSP90β with term labour (One-way ANOVA, p<0.02 for each). For each HSP examined, variables with the same letter are not significantly different (that is, they are statistically similar) as determined by a One-way ANOVA and Fisher PLSD test.
Heat-shock protein mRNA in human myometrium

a)

HSP 90α

HSP 90β

HSP 70

18S

Preterm not in labour
Preterm in labour
Term not in labour
Term in labour
Lane control

b)

ROD (HSP/18s)

HSP 90α

HSP 90β

HSP 70

Preterm not in labour (n=8)
Preterm in labour (n=8)
Term not in labour (n=14)
Term in labour (n=11)
Figure 5.2: a) Representative northern blot containing human myometrial RNA. Myometrium was collected from women who were not in labour as well as from those who were in labour at the time of cesarean section. Samples were collected from women who delivered preterm as well as term. Blot was probed for the following coactivators: ARA70, SRC1, and TIF2, as well as for 18 S.

b) Mean relative optical densities (coactivator optical density / 18 S optical density) for the samples described in a). Human myometrium showed a significant increase in SRC1 and TIF2 mRNA with increasing gestation (One-way ANOVA, p<0.02 for each). For each coactivator, variables with the same letter are not significantly different (that is, they are statistically similar) as determined by a one-way ANOVA and Fisher PLSD test.
Steroid receptor coactivator mRNA in human myometrium

a)

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</tr>
</tbody>
</table>

b)

![Graph showing ROD (ARA70, SRC1) and ROD (TIF2) measurements for different groups.](image-url)

- ARA70
- SRC1 (7.5 kilobase band)
- SRC1 (5.5 kilobase band)
- TIF2

Legend:
- Preterm not in labour (n=8)
- Preterm in labour (n=8)
- Term not in labour (n=14)
- Term in labour (n=11)
Figure 5.3: Relative optical density (ROD) from a northern blot of heat-shock protein (HSP)90α, HSP90β, and HSP70 RNA. Samples were obtained from more than one region of each uterus. Three women were not pregnant, while three women were pregnant at the time of sampling. Each point on the graph represents one sample from one woman. Therefore, each line on the graph represents one woman, and each point on this line represents a sample taken from a different region of her uterus. These data were not analyzed statistically due to the limited sample size, and the potential variation in the uterine regions from which these samples were obtained.
Human myometrium regional variation heat shock proteins

ROD (HSP 96/186a)

Upper region (fundal)  Lower region

Region of uterus from which sample was obtained
Figure 5.4: Relative optical density from a northern blot of the coactivators SRC1 (7.5 and 5.5 kilobase bands), and ARA70 RNA. Samples were obtained from more than one region of each uterus. Three women were not pregnant, while three women were pregnant at the time of sampling. Each point on the graph represents one sample from one woman. Therefore, each line on the graph represents one woman, and each point on this line represents a sample taken from a different region of her uterus. These data were not analyzed statistically due to the limited sample size, and the potential variation in the uterine regions from which these samples were obtained.
Human myometrium regional variation
steroid receptor coactivators

Region of uterine from which sample was obtained

Upper region (funial)  Lower region

- Not pregnant-hysterectomy (3274)/3277/3271)
- Not pregnant-hysterectomy (3277/3280)
- Pregnant preterm-no labour (2721)/2722/2723/2724/2725/2726)
- Pregnant term-no labour (2796/2797/2798/2799/2800)
- Pregnant term-in labour (1230)/1225/1224)
5.3.1.2 Rat

HSP mRNA did not differ significantly in the rat from day 21 of gestation to 1 day post partum (One way ANOVA, p>0.05, Figure 5.5). Connexin-43 (cxn-43) mRNA was found to significantly differ between pregnancy days (One-way ANOVA, F=4.7, p=0.006, d.f.=5,23, Figure 5.5), with a significant increase on day 23 from all other days sampled (p<0.05 Fisher’s post hoc test), which is consistent with previously published results (Lye et al. 1993; Orsino et al. 1996). P₄ treatment of pregnant rats did not change the pattern in HSP message observed across days 21, 22 or 23 of pregnancy (Figure 5.6); P₄ treatment did not significantly increase the levels of HSP message found (Student’s t-test, p>0.05). Ovariectomy, or ovariectomy followed by P₄ treatment, did not significantly alter the levels of HSP message detectable in the myometrium of these rats (ANOVA, p>0.05, Figure 5.7).
Figure 5.5:  

a) Representative northern blot containing rat myometrial RNA. Myometrium was collected from rats on days 21 (d21), 22 (d22), 23 (d23, labour), and one day post partum (pp1). Samples were also collected from ovariectomized non-pregnant rats (ovx). Blot was probed for heat-shock protein (HSP) 70, HSP90α, HSP90β as well as for connexin-43 (cxn-43), and 18 S.

b) Mean relative optical densities (ROD; HSP or cxn-43 optical density / 18 S optical density) for the samples described in a). HSP mRNA did not differ significantly in the rat from day 21 of gestation to 1 day post partum (One-way ANOVA, p>0.05). Cxn-43 mRNA was found to significantly differ between pregnancy days (One-way ANOVA, F=4.7, p=0.006, d.f.=5,23). Variables with the same letter are not significantly different (that is, they are statistically similar) as determined by a One-way ANOVA and Fisher PLSD test. n=4 for each day of pregnancy.
Pregnant rat myometrium

a)

HSP90α

HSP90β

HSP70

Cxn-43

18S

d21  d22  d23  pp1  ovx

b)

HSP90α  HSP90β  HSP70  Cxn-43

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<td>0.25</td>
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<td>0.00</td>
</tr>
</tbody>
</table>

Legend:
- d21 (n=4)
- d22 (n=4)
- d23 (n=4)
- pp1 (n=4)
- ovx (n=4)
Figure 5.6:  

a) Representative northern blot containing rat myometrial RNA. Myometrium was collected from rats on days 21 (d21), 22 (d22), and 23 (d23, control rats were sampled during labour on this day, treated rats were not in labour). From day 20 of gestation onwards, rats were injected with either 0.5 mg progesterone/day in mineral oil, or with mineral oil without steroid as a control. Blot was probed for heat-shock protein (HSP) 90α, and HSP90β as well as for 18 S.

b) Mean relative optical densities (ROD; HSP optical density / 18 S optical density) for the samples described in a). A 2-way ANOVA failed to detect any difference in the level of HSP90α or HSP90β between treatment groups or the days in which the animals were sampled. n=3 rats on each day for controls, and n=3 rats on each day for progesterone treatment.
Progesterone treated pregnant rat myometrium

a) Control vs. Progesterone treated

Day of Pregnancy

21 22 23 21 22 23 21 22 23

HSP90α

HSP90β

18 S

b) Bar graph

Control

(n=3/day of pregnancy)

Progesterone treated

(n=3/day of pregnancy)

Day of pregnancy

21 22 23 21 22 23
Figure 5.7:  

a) Representative northern blot containing rat myometrial RNA. Rats were ovariectomized on day 18 of gestation. Myometrium was collected from these rats on days 19 (d19), 20 (d20), and 21 (d21). From day 18 of gestation onwards, rats were injected with either 0.5 mg progesterone/day or with mineral oil as a control. Blot was probed for heat-shock protein (HSP) 90α, HSP90β, and HSP70, as well as for 18 S.

b) Mean relative optical densities (ROD; HSP optical density / 18 S optical density) for the samples described in a). A 2-factor ANOVA failed to identify any significant interaction between treatment groups and/or HSP examined. n=3 animals on each day of pregnancy, for each treatment.
Pregnant ovariectomized rat model

a)

HSP 90α
HSP 90β
HSP 70
18 S

Day of pregnancy: 19 20 21 19 20 21 19 20 21
Treatment: Sham Ovx Ovx+P₄

Myo cont.

n=3 animals on each day of pregnancy, for each treatment

b)

Graph showing the changes in ROD for HSP 90α/18 S and HSP 70/18 S with different treatments (Sham, Ovx, Ovx+P₄) across different days of pregnancy (19, 20, 21).
5.3.1.3 Sheep

HSP message in sheep myometrium was greatest in samples collected from sheep during normal term labour. For HSP90α and HSP70 this difference was significant (One-way ANOVA: HSP90α, \( F=14.00, p=0.003, \text{d.f.}=3,15 \); HSP70, \( F=11.96, p=0.0006, \text{d.f.}=3,15 \), Fisher PLSD significance at 95%, Figure 5.9). A 2-way ANOVA found a significant interaction between labour state (no in labour vs in labour) and gestation time (preterm vs term) for HSP 90α (\( F=13.55, p=0.03, \text{d.f.}=1,12 \)) and HSP90β (\( F=7.28, p=0.02, \text{d.f.}=1,12 \)). These results confirmed those of Wu et al. (Wu et al. 1996b). There however, was not found to be an increase in HSP with glucocorticoid induced labour, which is in contrast to the results of Wu et al. (1996b).

5.3.2 In situ hybridization

Figure 5.10 shows the distribution of ARA70 message in the tissue examined. ARA70 mRNA was most abundant in smooth muscle cells surrounding vessels. In situ hybridization did not show a difference in message expression of ARA70 between myometrium collected from women not in labour and that collected from women in labour at the time of cesarean section (Figure 5.11).
Figure 5.8:  

a) Northern blot containing sheep myometrial RNA. Sheep were sampled either preterm [controls (not in labour, n=4), glucocorticoid infused (not in labour, n=4)], or at term [not in labour (n=4), or in labour (n=4)]. The blot was probed for heat-shock protein (HSP) 90α, HSP90β, and HSP70, as well as for 18 S.

b) Mean relative optical densities (ROD; HSP optical density / 18 S optical density) for the samples described in a). HSP90α, HSP90β and HSP70 mRNA were significantly different between sample groups (One-way ANOVA: HSP90α, F=31.23, p=0.0001, d.f.=3,15; HSP90β, F=22.20, p=0.0001, d.f.=3,15; HSP70, F=16.18, p=0.0002, d.f.=3,15). Results of pairwise comparisons are noted on the graph; for each HSP examined, delivery states with the same letter are not significantly different (that is, they are statistically similar) (Fisher PLSD, p>0.05). A 2-way ANOVA found a significant interaction between labour state (not in labour vs in labour) and gestation time (preterm vs term) for HSP90α (F=13.55, p=0.03, d.f.=1,12) and for HSP90β (F=7.28, p=0.02, d.f.=1,12).
Heat-shock protein message in sheep myometrium

a)

HSP 90α

HSP 90β

HSP 70

18s

Early controls
Not in labour

Glucocorticoid
Infused fetuses
Not in labour

Term controls
Not in labour

Term
In labour

b)

<table>
<thead>
<tr>
<th></th>
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<td><img src="image14" alt="Graph" /></td>
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Relative optical density
Figure 5.9: Representative images of *in situ* hybridization of ARA70 RNA in human myometrium. This tissue was collected from a women who underwent a repeat/routine cesarean section at term while not in labour. The three images on the left are of tissues probed with sense cRNA control, while the images on the right were probed with antisense cRNA probe. 

a) images of the uterine tissue, 1000X magnification, brightfield.  
b) images of the uterine tissue, 1000X magnification, darkfield.  
c) images of the uterine tissue, magnified 2.5X, on autoradiographic film.  

The 'L' marks the lumen of a blood vessel, the red line marks the wall of this blood vessel, and the yellow line marks an area of high ARA70 mRNA expression surrounding the vessel wall.
ARA70 ISH in human myometrium

a) Sense

b) Antisense

c)
Figure 5.10: Radiographic film exposed to microscope slides with human myometrial tissues which were probed for ARA70 with either a sense or anti-sense probe. Tissues were collected from women who were not in labour (n=3, tissues on the left side of the slide) or from women who were in labour (n=3, tissue on the right side of the slide) at the time of cesarean section. Film was exposed for two weeks at room temperature. Each marking on the ruler represents 1 mm.
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<td></td>
<td>not in labour</td>
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5.4 Discussion

To our knowledge this is the first study to characterize the expression of HSPs and steroid receptor coactivators in the human pregnant myometrium. Of significance we found increased expression of HSP90α and HSP90β with the onset of term labour, and increased expression of SRC1 and TIF2 with increasing gestation and during preterm labour. Since these proteins can modify the transcriptional activities of steroid receptors it is possible that such changes could influence the myometrial expression of contraction-associated proteins such as cxn-43, and receptors for oxytocin and prostaglandins, and hence contractile activity of the myometrium at term and during labour.

The increase in HSP90 in human myometrium during term labour is consistent with the report of Wu et al (1996) who found increased HSP expression in the sheep myometrium during term labour, data which we confirmed in this study. The consequences of such an increase remain to be determined. It has been suggested that HSP90 might maintain steroid receptors in an unbound state and thus may inhibit ligand binding to steroid receptors. HSP90 interacts with P4R and therefore, increased expression of HSP90 at labour may contribute to a functional P4 withdrawal. A more likely scenario however, is that HSP alters the conformation of the steroid receptors in favor of ligand binding, and HSP90 bound to steroid receptors keeps the receptors from interacting with DNA in the absence of ligand, that in the presence of ligand HSP90 favors steroid receptor activation. Whether HSP90 favors P4R and/or estrogen receptor action would depend on the relative affect which HSP90 has on these receptors. To our knowledge no study has examined this aspect of HSP90 action [see Gehring (1998) for a review].

There are a number of mechanisms which may be responsible for an increase in HSP with labour. Wu et al. (1996) demonstrated that HSP90α and HSP70 expression in non-pregnant ovariectomized ovine myometrium was regulated positively by estrogen and that the effects of estrogen could be blocked by P4. Such a finding is consistent with the report of Komatsu et al. (1997) who found higher HSP mRNA expression in the myometrium during the proliferative than the secretory phase of the menstrual cycle (see Figure 2.6). Cytokines such as transforming
growth factor-β and interleukin-6 have also been shown to increase HSP90 and/or HSP70 expression in various cell types (Stephanou et al. 1997; Takenaka and Hightower 1992). It is therefore possible that cytokines expressed in intrauterine tissues at term may regulate expression of myometrial HSPs, though this remains to be demonstrated. HSPs were first identified because of their increased levels following heat stress (Catelli et al. 1985; Hightower and White 1981; O'Malley et al. 1985). We now know that various metabolic 'stressors' can result in an increase in HSP levels (Peetermans 1995; Papizet 1995). It is possible therefore, that inflammation, ischemia, hypertrophy, and/or stretch of myometrial tissues at the end of gestation and labour may also contribute to an increase in HSP levels.

In contrast to the study of Wu et al. (1996) we did not find an increase in HSP in glucocorticoid infused sheep. This may be due to the use of synthetic glucocorticoids by Wu et al. (Wu et al. 1996) rather than cortisol in our study, or more likely, reflects the fact that sheep from Wu et al. (1996) were labouring whereas ours were not.

While our northern blots identified major bands at the predicted molecular size for each HSP [i.e. HSP90α mRNA, 2.95 kilobases; HSP90β, 2.7 kilobases; and HSP70 (constitutive), 2.8 kilobases], additional hybridization bands were also observed that were slightly larger than the band of interest. Udelsman et al. (1994) also noted additional bands in blots for HSP70 in the rat aorta and adrenal, as did Ehrenfried et al. (1996) in the rat ileum. Figures contained within the report of Wu et al. (1996b) in the sheep also contained similar bands although these were not commented on. The nature of the bands remains to be determined, although it is possible that they could represent other isoforms of the HSPs.

In contrast to our data in humans and sheep, we found no change in HSP expression in the rat myometrium. It is possible that since P₄ levels fall and estrogen levels increase at term in this species a HSP-mediated functional withdrawal of P₄ is not required. However, such an explanation may be simplistic since changes were shown in the myometrium of sheep, which also experience decreased P₄ levels during labour.

HSPs binds to a number of different signal transducers in addition to steroid receptors (Rutherford and Zuker 1994; Stepanova et al. 1996; Xu and Lindquist 1993). It is therefore
possible that the HSPs which we are measuring in the myometrium at the time of labour are interacting with proteins other than steroid receptors.

Changes in the expression of steroid receptor coactivators, as reported here, may enable changes in estrogen and/or P₄ receptor actions even in the absence of changes in the circulating/tissue levels of these steroids. Under certain circumstances steroid receptors fail to induce transcription even in the presence of elevated steroid levels, a phenomenon known as autosquelching. Coactivators such as SRC1 and TIF2 can relieve this squelching and permit transcriptional activity from the previously ‘inactive’ receptors. This may be particularly important in human labour where no changes in estrogen or P₄ levels occur.

The consequences of increased coactivator mRNA expression in the human myometrium must be interpreted with caution for a number of reasons. Firstly, it remains to be demonstrated whether the changes in mRNA reported here are paralleled by changes in protein. Secondly, we do not know what the ultimate effect of the interaction of multiple coactivators with multiple receptors might be. Previous in vitro studies have only examined interactions between individual coactivators with single receptors yet a much more complicated situation occurs in vivo. More information is needed to determine to what extent the target of coactivators in vivo is the estrogen receptor and/or P₄ receptor. However, given that contraction associated genes (e.g. cxn-43, oxytocin receptors, or prostaglandin receptors) in other species are regulated positively by estrogen and negatively by P₄, we might speculate that an increase in SRC1 and TIF2 at term might increase estrogen induced transcriptional activity on contractile associated gene expression. To date no studies have investigated the regulation of steroid receptor coactivators in cells or tissues.

While androgen receptors are present in the non-pregnant human myometrium (Mertens et al. 1996; Wilson and McPhaul 1996), to our knowledge, they have not been identified in the pregnant myometrium of any species. We found no change in ARA70 with labour in humans, suggesting that ARA70 does not play a significant role in P₄, estrogen, or androgen receptor transactivation at this time in the myometrium.

Data on single samples taken from different regions of the uterus do not suggest a regional variation in HSP mRNA expression. For steroid receptor coactivators there does appear to be a regional difference in some of the uteri examined, however, the pattern of expression for the
samples taken from the lower uterus is not consistent with the mean obtained for multiple samples from this same region (see Figure 5.3). More samples in clearly defined conditions of labour and not-in labour need to be examined, yet such samples are extremely difficult to obtain.

In conclusion, we have observed increases in the myometrial expression of proteins that interact with steroid receptors during pregnancy and with the onset of labour. While the precise consequences of these changes remains to be determined, it is possible that they might modify the transcriptional activity of the estrogen and P₄ receptor systems and hence modulate myometrial expression of contraction associated proteins and the regulation of myometrial contractility during labour. Moreover, it is possible that such activities could occur independent of changes in steroid hormone levels.
6. General discussion

Eutherian mammals require P₄ for the maintenance of pregnancy and its inhibitory effects on uterine contractility must be overcome at the end of gestation for labour to ensue. The most consistent endocrine change across species associated with the onset of labour is a fall in maternal circulating P₄ levels. Unlike most animal species however, there is no fall in plasma or tissue levels of P₄ prior to the onset of human labour, nor is there a change in the ratio of P₄ circulating free, verses that which is bound to steroid binding globulins in humans. Nevertheless, P₄ is essential for the maintenance of human pregnancy as evidenced by the induction of labour following administration of RU486 (Cadepond et al. 1997; Mahajan and London 1997), or following ovariectomy at 7 weeks gestation (which is prior to the shift in primary P₄ production from the corpora lutea to the placenta) [see Csapo (1969) for a review]. Therefore, there is no evidence to suggest that P₄ is acting on the myometrium in any way different to that of other species studied. Thus, some mechanism must exist by which the effects of P₄ might be attenuated at the time of labour. This thesis examined a number of ways by which this might be achieved.

It is possible that the effects of P₄ on the human myometrium may be altered by changing the relative abundance of one of its primary metabolites. We therefore hypothesized that fluid levels of the 5α-reduced P₄ metabolite AP would decrease with labour, releasing the uterus from its inhibitory effects on contraction through agonistic binding to GABA₆ receptors. However, we did not find a decrease in maternal circulating, amniotic fluid, or umbilical cord blood levels of AP with the onset of labour in humans, suggesting that this is not the mechanism responsible for the attenuation of P₄ effects at labour.

The second study hypothesized that a change in the relative levels of the two P₄ receptor isoforms A and B may result in the attenuation of P₄ effects by affecting the action of P₄ via its receptors. No significant changes however, were found in the levels of P₄ receptor A and/or B isoforms at either the RNA or protein levels. P₄ receptor A and B isoforms were also examined for the first time in rat myometrium but were not found to change with the onset of labour. Therefore, it does not appear as if a change in the levels of P₄ receptor isoforms is the mechanism by which the effects of P₄ on the human myometrium are attenuated at labour. This result is independent of whether there is a decrease in P₄ levels with the onset of labour such as the rat, or whether the
species maintains maternal circulating levels of $P_4$ through to the delivery of the placenta as do humans.

The third study presented in this thesis examined the hypothesis that a change in the amounts of HSPs, HSP90 and HSP70, and/or a change in the amounts of the steroid receptor coactivators SRC1, TIF2 or ARA70 might occur with the onset of labour, and that this would modify steroid receptor transactivation. Such changes could result in an increase in estrogen effects and a decrease in $P_4$ effects on the myometrium, ultimately resulting in myometrial activation and the onset of labour. Not only did we identify for the first time that HSPs were present in the pregnant human myometrium, but that there was a significant increase in human myometrial levels of HSP90α and HSP90β levels with the onset of term labour. No such increase was seen in HSP90α, HSP90β or HSP70 with labour in the rat. However, we did observe an increase in HSP90α, HSP90β and HSP70 levels with term labour in sheep. This suggests that the increase in HSPs is not necessarily related to maternal circulating levels of $P_4$ at labour, and/or is species specific.

In this thesis we demonstrated that the steroid receptor coactivators SRC1, TIF2, and ARA70 are expressed in human myometrium. We also demonstrated for the first time in any tissue that expression of the coactivators was subject to regulation in that SRC1 and TIF2 were increased with gestational age and with the onset of preterm labour. This result, together with the increase in HSP90α and HSP90β with labour in humans, may be an important mechanism by which $P_4$ and estrogen responses are altered in the myometrium such that labour occurs at the end of gestation.

Therefore, of the mechanisms examined in this thesis which may lead to the attenuation of $P_4$ effects in the human uterus at the time of labour, those involving HSPs and steroid receptor coactivators seem most promising. The mechanisms, however, by which a change in these factors may result in an increase in estrogen and a decrease in $P_4$ effects at the time of labour are not yet clear.

HSP90 interacts with non-ligand bound steroid receptors and is thought to participate in receptor folding, keeping them in a non-active, non-DNA bound state in the absence of ligand. Once ligand is bound, HSP90 quickly dissociates and the receptor-ligand complex can bind DNA. HSP70 participates in receptor folding but is also important for the transport of steroid receptors
within the cell [see Gehring (1998) for a review]. Wu et al. (1996) suggested that increased expression of HSP90 and HSP70 might inhibit \( P_4 \) receptors in the myometrium and stimulate estrogen receptor function. In developing a model by which an increase in HSPs might attenuate \( P_4 \) actions, let us assume that HSP90 in the human myometrium preferentially binds estrogen receptors rather than \( P_4R \). Increased levels of HSP90 may then maintain stability of the unbound form of estrogen receptors, thereby increasing the probability of estrogen binding to its receptor. Since \( P_4R \) must be bound to ligand for coactivator association, an increase in coactivators would have no affect on unliganded non-HSP-bound \( P_4R \). Therefore, the increase in steroid receptor coactivators would result in an increase in estrogen rather than \( P_4 \) transactivation. This would lead to an increase in those genes associated with myometrial contractions, and thus the onset of labour.

Another interpretation of the results presented in this thesis is one which makes no assumption about relative preference of HSP for \( P_4R \) or estrogen receptors. Segnitz (1997), and Smith (1995) suggested that HSP90 is required for estrogen, \( P_4 \), glucocorticoid, and androgen binding to their cognate receptors. In these studies the hormone binding ability of estrogen, \( P_4 \), glucocorticoid, and androgen receptors was inhibited by the treatment of cells with geldanamycin, a specific HSP90 blocking agent. Therefore, in this model HSP90 is needed for the functional conformation of both the \( P_4R \) and estrogen receptor, and an increase in HSP90 would enhance ligand binding and thus steroid receptor action. One would assume that these receptors could interact with coactivators such as SRC1 and TIF2. Therefore, the balance of \( P_4R \) action and estrogen receptor action would be determined by relative affinity of coactivators for \( P_4R \) or estrogen receptors. Enhancement of \( P_4R \) would lead to predominance of an inhibitory/quiescence state in the myometrium, whereas enhancement of estrogen receptors would lead to activation of myometrial contractions and hence labour.

\( P_4 \) induced inhibition of the myometrium during labour might initially seem inappropriate, but there are now increasing data to suggest regionalization within the myometrium during labour. These data suggest that in the lower segment of the uterus there is an increase in expression of genes that likely lead to myometrial relaxation, [e.g. CRH receptor subtype 1 (Stevens et al. 1998), PGE receptor subtype 4 (EP4), and cxn26 (Teoh 1996)]. In contrast, in the fundus there is an increase in expression of genes that lead to activation of myometrium [e.g. cxn43, cyclooxygenase
isoform 2, (N. Europe-Finner, personal communication)). Since the samples in which we observed an increase in HSP90 and coactivators were from the lower segment, it is possible that these changes might promote \( P_4 \) action rather than attenuate it.

Finally, there is the possibility that changes in HSPs are merely the consequence of changes in the myometrium during labour rather than a causative factor. Since estrogen but not \( P_4 \) is known to increase the transcription of HSP (Yang et al. 1995), an increase in HSPs during labour may reflect an already estrogen dominated state within the myometrium. Also, as mentioned earlier, the increase in HSP during labour may be the result of various stressors associated with the labour process on the myometrium.

A limitation of the studies presented in sections 4 and 5 resides in the collection of human myometrial samples. Variation is associated with the non-labouring samples since we do not know the exact time in which these women would have gone into labour had their pregnancies been allowed to continue. With respect to the samples collected from labouring uteri, they were not all at the same stage of labour at the time of cesarean section. Given these limitations however, we ensured that all term non-labouring samples were obtained from booked sections which were routinely scheduled 1 week prior to expected delivery date, and term and preterm labouring samples were all obtained from women who had spontaneous onset of non-augmented clinical labour.

Messenger RNA and protein levels of a particular substance are often but not always correlated. Only mRNA levels of HSP and coactivators were measured in section 5. Komatsu et al. (1997) examined HSP90 and HSP70 mRNA and protein in human myometrium and endometrium during the menstrual cycle. They found mRNA and protein levels to be correlated. Therefore, a correlation likely exists between mRNA and protein levels of HSP in the human myometrium during pregnancy as well. To be confident of this however, protein levels of HSP in these tissues would have to be measured. With respect to steroid receptor coactivators, very little information is available on their expression \textit{in vivo}, and as of yet there is no evidence to support a correlation between mRNA and protein levels in tissues. Antibodies for SRC1, TIF2, and ARA70 are not yet commercially available, thus measuring protein levels of these coactivators would involve the development of such antibodies.
In summary, labour is a complex physiological event. Its initiation must however, in some way involve an attenuation or overriding of P₄ effects. The series of experiments presented in this thesis contribute to our knowledge of this process in humans. It does not appear as if a change in the levels of P₄ receptor isoforms, or a decrease in maternal circulating, amniotic fluid, or umbilical cord blood levels of the 5α-reduced P₄ metabolite AP are the mechanisms through which the effects of P₄ on the human myometrium are attenuated at labour. An increase in HSPs, along with an increase of SRC1 and TIF2 with labour, may be important factors regulating P₄ and estrogen receptor action and might thus contribute to the regulation of myometrial gene expression and the onset of labour.
7. Future directions

In order to determine which if any of the models presented in section 6 are valid, further research is required. Such studies will likely be very difficult since they must take into account the multiple receptors, HSPs, coactivators, and other interacting proteins that are present within the myometrium. The following experiments however, might begin to address these issues:

1) A critical issue not directly addressed by this thesis is whether or not there is indeed a functional withdrawal of P₄ in the human myometrium during labour. This is a very difficult question to address. One approach however, might be to determine whether there is a change in the transactivational activity of P₄ receptors in the human myometrium with labour, irrespective of what factors are involved. To examine this, myometrial cells from women either prior to labour or during active spontaneous labour could be transfected with a construct in which the luciferase coding region (Luc) driven by a viral promoter [e.g. cytomegalovirus (CMV) promoter] is placed downstream of one or more PRE. This construct would provide a ‘read out’ of P₄ receptor function. We would hypothesize that if there is a functional withdrawal of P₄ action during labour then stimulation of P₄ increase in Luc activity would be less in myocytes from women in labour than from women not in labour. There are a number of possible difficulties with such an experiment, most notably possible loss of the endogenous receptor function when cells are placed in culture.

2) A second major issue is to define the role of HSP90 in regulating P₄ within the myometrium. Using a similar approach to that described in (1), myometrial cells collected from women during cesarean sections, would be transfected with the PRE-CMV-Luc construct. These cells would also be transfected with or without a vector containing a full length HSP90 cDNA. We would then compare P₄-induced luciferase activity in the presence or absence of HSP90. If myometrial cells do not maintain P₄ responsiveness it may be necessary to conduct this study in myometrial cell lines which have been stably transfected with P₄ receptors or other P₄ responsive cell lines such as T47D cells.
It may also be informative with respect to the role of HSP90 in regulating P₄ within the myometrium, to measure HSP90-P₄ receptor binding in myometrium (tissue and/or primary cell culture) collected from women during cesarean sections, and determine whether the levels of P₄ receptor-bound HSP90 versus unbound HSP90 changes with the onset of labour.

3) We suggested that the increase in expression of the coactivators SRC1 and TIF2 might modulate estrogen and/or P₄ responses in the late pregnant/labouring myometrium. However, this hypothesis needs to be tested experimentally. A possible approach would be to overexpress SRC1 or TIF2 in steroid responsive myometrial cells which have been co-transfected either a PRE-reporter construct and/or an estrogen response element reporter construct. We would then assess the effects of SRC1 and TIF2 on appropriate reporter constructs.

4) It has recently been demonstrated that steroid receptor coactivators require other proteins for their action. Two such proteins have been identified as AIB1 and CBP (Blanco et al. 1997; Kamei et al. 1996; Ogryzko et al. 1996) (see Figure 2.5). Mutations in the yeast homologue of AIB1 (GCN5) have been shown to abolish the transcription of the steroid receptor coactivator complex by blocking histone acetyltransferase activity (Dr. P. Walfish, personal communication). It would be interesting to determine whether AIB1 and CBP proteins are present in the myometrium and whether their expression changes with gestation and/or with the onset of labour.

5) As discussed in section 6 there is accumulating evidence that the fundal region of the human uterus is responsive to contractile stimuli, while the lower region of the uterus is responsive to relaxational stimuli. While results from this thesis do not suggest that there is regionalization in P₄R, HSP90α, HSP90β, HSP70, SRC1, or ARA70 expression in the human myometrium, it would be informative to analyze the expression of these genes as well as the activity of PRE in samples which were taken from pregnancies devoid of complications. Such samples would be obtained from women following transverse incisions in the lower uterus where myometrium was collected via curettage of both the fundal and lower uterine segments. As well, regionalization of these mRNAs
could also be analyzed in multiple samples taken from a longitudinal section of non-human primate uteri.
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