INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI®

UNIVERSITY OF ALBERTA

THE REGULATION OF OXYTOCIN AND ITS RECEPTOR IN LATE GESTATION IN RATS

BY

<u>XIN FANG</u>

©

A thesis submitted to the Faculty of Graduate Studies and Research as a requirement for the degree of Doctor of Philosophy

Medical Sciences - Medicine

Edmonton, Alberta
Spring 2000

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-59956-6

Canada

University of Alberta

Library Release Form

Name of Author: Xin Fang

Title of Thesis: The Regulation of Oxytocin and its Receptor in Late Gestation in Rats

Degree: Doctor of Philosophy

Year this Degree Granted: 2000

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly, or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

#206 11121-82 AVE
Edmonton, Alberta
Canada T6G 0T4

Date: *Dec. 23, 1999*

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The Regulation of Oxytocin and its Receptor in Late Gestation in Rats" submitted by Xin Fang in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences – Medicine.
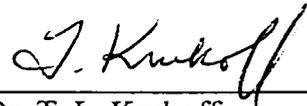
Dr. T. L. Krukoff
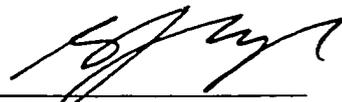
Dr. S. J. Lye
(External Examiner)
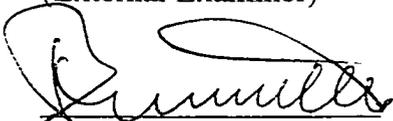
Dr. B. F. Mitchell
(Supervisor)
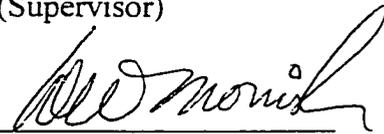
Dr. D. W. Morrish
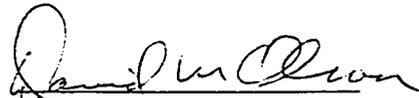
Dr. D. M. Olson

Date: Dec 21, 1999

For my family

and

my best friend, Dr. Margaret C Finlayson

ABSTRACT

Oxytocin (OT) and its receptor (OTR) are synthesized in the endometrium and myometrium of the pregnant rat during late gestation. In both tissues, OT and OTR may be regulated by estradiol (E_2) and progesterone (P_4), and the immune system. The P_4 receptor (PR) has at least three major isoforms, PR-A, PR-B and PR-C. In general, PR-B is the stronger transactivator, and PR-A and PR-C act as inhibitors of PR-B and other nuclear receptors. PR gene expression is also responsive to E_2 or P_4.

Maternal serum E_2 increased and P_4 declined throughout late gestation. Uterine OT mRNA increased on approximately day 16. Uterine OTR peptide increased on the morning of delivery. Uterine OT peptide increased only during parturition. Parturition was significantly delayed by 24 h by the estrogen antagonist tamoxifen (TAM). TAM inhibited the increase in OT mRNA and peptide. With TAM, the significant increases in uterine OTR and PGE_2 were significantly delayed, but still occurred before the delayed parturition. The P_4 antagonist RU486 caused preterm parturition approximately 27 h after treatment and significantly blocked the increase in OT mRNA. OTR mRNA and peptide, as well as PGE_2 and $PGF_{2\alpha}$ were significantly increased within 12 hours after RU-486.

PR-total (PR-A, PR-B and PR-C) mRNA increased significantly with maximal levels on the day of parturition. PR-B mRNA did not change significantly, suggesting an increase in PR-A and/or PR-C in late gestation. TAM inhibited the increase in PR-total mRNA and RU486 increased PR-total mRNA.

Administration of lipopolysaccharide during late gestation was extremely toxic to pregnant rats and gave extremely variable results. Interlukin-6 caused a significant increase in OTR mRNA level in uterine explants from pregnant rats but not in tissues from non-pregnant animals.

We conclude that: 1) E_2 stimulates the synthesis of OT and OTR within the rat uterus and is essential for normal parturition; 2) withdrawal or antagonism of P_4 increases uterine OTR and PGs; 3) PR-A and/or PR-C expression increases in late gestation; and 4) The pro-inflammatory cytokine IL-6 increases expression of OTR in uterine explants. We speculate that these mechanisms may be involved in the regulation of the timing of parturition.

ACKNOWLEDGMENTS

I would like to acknowledge the following people for their assistance with this thesis. Dr. Bryan F Mitchell, my Ph.D. supervisor for his encouragement and patience; My supervisory committee; Susan Wong for her technical help and useful discussions; and finally my husband for his understanding. I am grateful for the financial support for this study that was provided by the Medical Research Council of Canada, and the 75-anniversary graduate student award from University of Alberta.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	adenylyl cyclase
ACTH	adrenocorticotropin
AF	activation function
ANOVA	analysis of variance
AP-1	activator protein-1
APRE	acute-phase response element
APRF	acute-phase response factor
AVP	arginine vasopressin
BUS	B-upstream segment
CAP	contraction-associated protein
CD	cytoplasmic domain
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
CRH	corticotropin-releasing hormone
Cx-43	connexin-43
DAG	diacylglyceride
DEPC	diethylopyrocarbonate
DBD	DNA-binding domain
DNA	deoxyribonucleic acid
E_2	estradiol
ED	extracellular domain
EDTA	ethylenediaminetetraaetic acid
EIA	enzyme immunoassay
ER	estrogen receptor
ERE	estrogen response element

ET-1	endothelin-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-protein	GTP-binding protein
HBD	hormone-binding domain
hsp	heat shock protein
IF	inhibitory function
IL-1	interleukin-1
IL-6	interleukin-6
IP_3	inositol 1,4,5-triphosphate
kb	kilobase
kDa	kilodalton
KLCK	myosin light chain kinase
Km	Michaelis-Menten constant
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
NF-IL6	nuclear factor interleukin-6
NP-I	neurophysin-I
OT	Oxytocin
OTR	OT receptor
OTX	carboxyl-extended forms of OT
P_4	progesterone
PBS	phosphate buffered saline
PG	prostaglandin
PGDH	15-hydroxyprostaglandin dehydrogenase
PGHS	prostaglandin-endoperoxide H synthase
pH	negative log of hydrogen ion concentration
PIP_2	phosphatidylinotol 4,5-bisphosphate
PKA	protein kinase A

PKC	protein kinase C
PLC	phospholipase C
poly-A tail	polyadenylate tail
PR	progesterone receptor
PVN	paraventricular nucleus
RARE	retinoic acid response element
RIA	radioimmunoassay
RU486	17β-hydroxy-11β-(4-methylanminophenol)-17α-(prop-1-ynyl)estra-4,9-diene-3-one
SD	standard deviation
SE; SEM	standard error of the mean
SON	supraoptic nuclei
TAM	tamoxifen, 1-*p*-*β*-dimethylaminoethoxyphenyl-*trans*-1,2-diphenylbut-1-ene
TGFα	transforming growth factor α
TGFβ	transforming growth factor β
Th	T helper cell
TMD	transmembrane domain
TNFα	tumour necrosis factor-alpha
tRNA	transfer ribonucleic acid
VP	vasopressin
VPR	vasopressin receptor
V*max*	maximal velocity

1.　INTRODUCTION

1.1. RATIONALE OF STUDY

Oxytocin (OT) is a nonapeptide (9-amino acid molecule) that was discovered nearly a century ago. Its physiological functions are still not fully determined. Until the past decade, most researchers believed that OT was produced exclusively in the supraoptic and paraventricular nuclei of the hypothalamus. Its physiological functions were to augment milk-ejection by stimulation of the myoepithelial cells of the mammary gland or to stimulate uterine contractions. In 1991, synthesis of OT mRNA was demonstrated in human the decidua and amnion of late human pregnancy (1). Subsequently, investigations revealed similar findings in various species including the rat (2, 3). Although an endocrine role for OT in the initiation of labour remains very controversial, these new findings led investigators to study the biological mechanisms and physiological functions of intrauterine OT during pregnancy and at parturition. This thesis represents some of these studies.

OT acts through a specific cell membrane receptor. OT receptors (OTR) can be found in both human and rat intrauterine tissues (4, 5). In essentially all species, the concentration of OTR in intrauterine tissues is increased at the time of parturition (4, 6). However, OTR may play another role rather than direct stimulation of myometrial contractions. OTR can stimulate other powerful uterotonic agents, prostaglandins (PGs), in uterine tissues. The relationships among intrauterine OT, OTR and PGs in late gestation will be examined in this thesis.

Despite extensive study, the regulation of OT and OTR remains unclear. Most research suggests that the sex steroids, estrogen and progesterone (P_4), are important in controlling the production of local OT and OTR in late gestation. P_4 is produced during pregnancy by the corpus luteum in the rat and

1

luteolysis in late gestation causes a significant decrease in P_4 levels (7). In contrast, P_4 is produced predominantly by the placenta in human pregnancy. Diurnal and/or local variations may cause an increase in the estrogen/P_4 ratio in the human uterus (8, 9). These changes in the estrogen/P_4 ratio may influence gene expression of uterine OT and its receptor. Inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-alpha (TNFα) accumulate in intrauterine tissues and amniotic fluid near the time of labour onset and this is particularly significant in pregnancies complicated by intrauterine infection. The promoter of the OTR gene in several species shows many response elements that may interact with inflammatory cytokines. This suggests that immune modulators may have an important role in controlling the synthesis of OT and/or OTR in late gestational intrauterine tissues. The nature and roles of these mediators will be further investigated in this thesis.

The normal length of human gestation is 38 to 42 weeks. Labour initiated prior to 37 completed weeks of gestation is considered preterm. Preterm delivery occurs in approximately seven per cent of all births in Canada. However, 75 per cent of perinatal deaths and long term disabilities are related to preterm births, mainly because the organ systems in preterm babies have not matured sufficiently to adapt to extrauterine existence. The financial and emotional costs that these bring to the families and to the health care system are tremendous. One of the unresolved questions of reproductive biology is, "What initiates labour?" Until the mechanisms that initiate human labour are fully understood, it is unlikely that a rational approach to the development of effective preventative or therapeutic strategies for prevention of preterm labour will be devised. Some of the studies in this thesis will address the role of intrauterine OT and OTR in a model of preterm labour.

Recently, OT antagonists that block signal transduction at the OTR have been developed. These may be useful in preventing preterm birth in pregnant

2

women. This thesis provides new data that may permit better understanding of the role of OT, OTR and their related factors in the process of parturition. This information may help to design better strategies for potential clinical use of OT antagonists and other pharmacological agents.

1.2. HYPOTHESIS AND PURPOSE

Our **hypothesis** is that intrauterine OT and its receptor play important roles in the initiation of labour. Further, the synthesis of these factors in intrauterine tissues is regulated by various factors including sex steroids and mediators of the immune system. The **purpose** of the studies contained in this thesis is, through development of a rat model, to investigate the paracrine or autocrine roles and the mechanisms of regulation of OT and OTR at the time of parturition. This information may lead to better understanding of the physiological mechanisms that regulate the timing of parturition either at term or preterm.

1.3. SPECIFIC AIMS

The specific aims of the studies in this thesis are:

1) To determine, during late rat gestation and parturition, the uterine concentrations of mRNA and protein for OT, OTR and their potential regulators, including receptors for estrogen and P_4.

2) To determine the effects of the estrogen antagonist, tamoxifen (TAM) on rat gestation and to investigate the changes in uterine OT and OTR gene expression and other related factors after TAM treatment in late gestation.

3

3) To determine the effects of the P_4 antagonist, RU-486, on rat gestation and to investigate the changes in uterine OT and OTR gene expression and other related factors after RU-486 treatment in late gestation.

4) To determine the mRNA levels for P_4 receptor isoforms in rat uterine tissues in late gestation and to determine the effects of sex steroid antagonists on isoform gene expression.

5) To explore the effects of IL-6 or endotoxin on OT and OTR gene expression in late gestation rat uterus.

6) To evaluate the rat as a potential model for human parturition

1.4. THE ANIMAL MODEL USED IN THIS THESIS

The choice of the rat as experimental animals in this thesis study was dictated by several considerations:

1) availability and the relatively low price for animals;

2) relatively short gestational length and ease of experimental manipulation;

3) ability to produce data with relatively large n values;

4) availability of information concerning reproductive endocrine, paracrine and autocrine systems including sex steroids and OT gene expression;

5) availability of necessary reagents for experimental assays.

4

1.5. REVIEW OF SELECTED LITERATURE

1.5.1. Historical Perspectives

Physiological research at the beginning of this century indicated the existence in the neurohypophysis of substances that caused vasoconstriction and "rapid birth" in animal models. In 1906, the British scientist Sir Henry Dale demonstrated for the first time that intravenous injection of posterior pituitary extract stimulated frequent uterine contractions in gravid cats (10). Blair-Bell used this extract clinically to treat the serious haemorrhage during a cesarean section, proposing it as a cure for postpartum uterine atony (11). About the same time, several investigators recognized that neurohypophysial extract also had a powerful "galactogogic effect"(milk secretion activity) on the mammary gland (12, 13). It was not until 1953 that Du Vigneaud (14) purified and chemically characterized hormones from the posterior pituitary extract and termed them oxytocin (from the Greek *oxus*, meaning sharp and *tokos*, referring to childbirth) and vasopressin (VP).

In the 1940's, the posterior pituitary extract was introduced into clinical practice to stimulate labour (15). In 1953, Du Vigneaud (14, 16) synthesized a product that was chemically and physiologically identical to the biological hormone. For this contribution, that marked the first synthesis of a polypeptide hormone, he was awarded the Nobel Prize in 1955. The structure of the OT gene was elucidated 30 years later (17). Until the last decade, investigators believed that OT was synthesized solely in the hypothalamus. It was carried in neurosecretory granules, stored in the axon terminals in the neurohypophysis and released into the circulation on appropriate stimulation (18). The principal biologic properties of OT were its uterotonic and milk-ejection effects. Fuchs *et al.* (19) first proposed that OT was indeed involved in regulation of human parturition based on their own work and that of many others.

5

1.5.2. Oxytocin

1.5.2.1. Synthesis and Release of OT

1.5.2.1.1. OT in the Brain

Traditionally, OT is considered an endocrine hormone that is synthesized in the cell bodies of the magnocellular neurons in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. The neurosecretory material is transported in vesicles along the axon, stored in secretory granules in the neurohypophysis and released into the circulation to stimulate uterine contraction during late pregnancy and labour or milk-ejection during lactation. Exocytosic secretion is regulated by physiological signals, such as distension of the birth canal at the time of fetal expulsion during labour (20) or suckling during lactation (18).

OT is secreted from the neurohypophysis in a pulsatile fashion with a definite circadian rhythm pattern. Its half-life in the blood is approximately 90 seconds (21). In human and non-human primates, the highest plasma concentration of OT is observed at night. In the rat, a nocturnal animal, OT secretion has been found to increase during the day and to fall during the night (22). In late pregnancy, there is a variation in the circadian rhythm of OT secretion in the human (8) and the rhesus monkey (23). Some investigators have suggested that variations in the pulsatile secretion pattern are responsible for the onset and maintenance of human labour (24) (see below).

In addition to the hypothalamus, OT is also found in many areas of the central nervous systems. Some investigators found that in the human brain the highest concentration of OT outside the hypothalamus was in the locus coeruleus (25), the area from which many of the noradrenergic pathways of the brain originate. The functions of OT from these varied brain regions may involve the process of memory (26) and several behavioral characteristics including sexual and maternal instincts (27, 28).

6

1.5.2.1.2. OT in Reproductive Tissues

Recently, OT mRNA and peptide synthesis have been found in human decidual (29) and rat endometrial (2) cells in late pregnancy. OT mRNA in rat uterus is essentially the same as that expressed in the hypothalamus but has a shorter polyadenylate (poly-A) tail (30). In eukaryotic genes, the poly-A tail has a role in increasing mRNA stability and translational efficiency. Modification of the length of the poly-A tail of OT mRNA in rat hypothalamus has been observed during pregnancy and lactation, relating to elevation of OT secretion and biosynthesis in these periods (30). The processes of OT secretion and post-translational processing appear to be similar in intrauterine tissues and in the hypothalamo-neurohypophysial system.

OT is expressed in the corpus luteum of the ovary in ovine (31), bovine (32) and human (33) species. In the bovine, OT is believed to mediate luteolysis by promoting the release of luteolytic $PGF_{2\alpha}$ from uterine endometrium (34). The role of OT in human ovarian function is not known with certainty and the changing OT levels in the peripheral blood of women during the menstrual cycle originate from the pituitary and not the ovary (35). Some studies suggest that ovarian OT can act in a paracrine manner to regulate steroidogenesis (36).

Several research groups reported that OT is present in the Leydig cells of the testis of several species including man but in much smaller quantities than in the ovary (37, 38). The presence of OT mRNA indicated local synthesis (39). The studies suggested that local hormone synthesis in the testis is responsible for promoting the propulsion of spermatozoa (40).

7

1.5.2.1.3. OT in Other Tissues

Adrenal gland

Both mRNA and peptide for OT have been identified in human and rat adrenal glands (37). The functional significance of adrenal OT is not yet certain. There is some evidence that OT may be involved in adrenocortical function since perfusion of the isolated rat adrenal gland with OT specifically inhibited acetyl choline-stimulated aldosterone production (41).

Thymus:

OT is found in the human thymus in surprisingly large amounts (42). It is present in the nurse cells and mRNA studies have revealed that it is synthesized in the thymic epithelium (43). It has been reported that OT has lymphokine activity *in vitro* (44).

Pancreas

High concentrations of OT have been identified in the human and rat pancreas (45) but there is no evidence to show actual synthesis of the peptides within the organ. The concentration of OT in pancreatic tissues is enough to stimulate the release of glucagon but not insulin, into the medium (46). Several studies in rats, dogs and humans indicated that pancreatic OT was involved in regulation of blood glucose and glucagon levels (47, 48). However, physiological functions of OT in the pancreas remain to be further investigated.

1.5.2.2. Gene Structure of OT

The OT precursor contains the nonapeptide OT as well as a 10 kDa protein, OT-associated neurophysin I (NP-I). The sequences of OT genes for the rat (49, 50), the human (51), the bovine (52), and the mouse (53) have been

8

determined. Restriction analysis of genomic DNAs has revealed only one copy of the OT gene. The gene contains three exons encoding the principal functional domains of the polypeptide precursors (Fig. 1-1a). The first exon (A) comprises the 5'noncoding promoter region, a putative signal peptide, OT nonapeptide, and the NH_2-terminal, variable region of NP-I. The second exon (B) encodes the central, conserved region of NP and the third exon (C) encodes the remaining COOH terminus of NP-I and the untranslated sequence.

The gene encoding the related peptide arginine VP (AVP) has a very similar organization. In fact, these two genes are closely linked with an 11 or 9 kb intergenic DNA sequence in the rat (50) or human genomes (51, 54). They are oriented in such a way that transcription of the two genes occurs on opposite DNA strands. Both OT and AVP are nonapeptides that differ from one another in only two positions, at amino acids 2 and 8 though they control quite different functions. Similar to OT, the VP precursor also contains an associated polypeptide termed NP-II. Comparisons of the mRNA sequences (55) and protein sequences (39) for the OT and AVP precursor hormones from all species presently sequenced indicate a remarkably high degree of homology in the part encoding the central region of the NPs.

The OT gene promoter region contains several elements that resemble known transcription factor binding sites (Fig. 1-1b). Most of these are conserved among species such as human, mice, rats, and cows. In both rat and human, promoter regions of OT gene contain several response elements for the estrogen receptor (ER) (56, 57), retinoic acid receptor (58), and thyroid hormone receptor (59). Human OT promoter contains an imperfect palindromic sequence (at -164) and several functional half palindromes for ER. The composite at −164 is also capable of interacting with a third member of the steroid receptor superfamily, mainly the orphan receptor COUP-TF. Very recent studies showed that the orphan receptors interact with a much higher

9

affinity to the estrogen response element (ERE) located in human OT gene promoter than do the steroid receptors (60). In rat OT gene promoter, three EREs are identified but only two of them are functional (located between −188 and -54) (57). A DNA sequence identical to one of three EREs is also present in the human OT encoding gene. Moreover, one enhancer conferring glucocorticoid responsiveness to a reporter gene is located at −2449 to −2464 (57).

1.5.2.3. Posttranslational Modification and OT Precursors

OT is separated from NP-I by a tripeptide spacer, Gly-Lys-Arg (49). In the rat, during post-translational processing, OT-gly-lys-arg is cleaved from NP-I. The basic amino acids arginine and lysine are then serially cleaved by carboxypeptidase B to yield OT-gly. The OT-gly is transformed by α-amidating enzyme to mature, biologically active amidated OT (as reviewed in (61)) (Fig.1-2). The intermediate forms containing 10, 11 or 12 amino acids are collectively referred to as carboxy-extended forms of OT (OTX).

In the rat hypothalamus, the processing from the initial translational product through to mature OT in the adult is very similar to that in the fetus (62). However, this processing efficiency in the fetus is very low and incomplete, resulting in the accumulation of OTX (63). The ability to form the fully processed amidated form of OT increases during late fetal and early neonatal development (62). In sheep plasma, the concentration of OTX forms is 35 fold higher than OT during the second and third trimesters of pregnancy (64). However, by term, though the concentration of OTX forms remains several-fold higher than that of OT, the concentration of OTX forms has declined to one half and OT has increased several-fold. In human umbilical plasma, a similar pattern of maturation occurs (65).

Recently, we have measured rat uterine OT and its OTX forms using two antisera, one recognizing OT and OTX forms, the other recognizing only mature amidated OT. We found that uterine tissue concentration of OTX forms were 5- to 10-fold greater that those of OT and both increased progressively and significantly through late gestation. However, the ratio of OT to its extended forms did not change significantly. Administration of antagonists of estrogen (TAM) or P_4 receptors (RU486) reduced concentrations of OTX forms by > 90% and of OT by 50 %. Furthermore, using a muscle bath preparation, OTX forms were weak uterine stimulants and did not alter the OT concentration-response curves. OTX forms were two or three orders of magnitude less able than OT to displace radiolabeled OT from late-gestational uterine binding sites. These data suggest that rat uterine carboxyl-extended OT prohormones are regulated in part by estrogen and P_4. However, in contrast to our original hypothesis, OTX forms do not compete with OT for binding to OTR, and have little direct biological activity. Their role in the process of parturition may be confined to acting as substrates for OT synthesis (66).

1.5.2.4. Catabolism of OT

The metabolism of OT was first described by Page *et al.* a half century ago (67). They documented that serum from pregnant women had the ability to catabolize and inactivate oxytocin. They termed this activity 'oxytocinase'. This enzymatic activity was predominantly found in the cytosolic fraction from human placenta.

The OT molecule is a nonapeptide with a disulfide bridge between the cystine at position 1 and 6 resulting in a ring structure at the N-terminus that is essential for biological activity. It can be degraded either at the N- or C-terminal ends (Fig. 1-3). The most common form of degradation is by aminopeptidase activity that opens the ring by cleaving the bond between cys 1

11

and tyr 2, and then cleaves amino acids from the N-terminus of the molecule (68). A second method of catabolism occurs by the action of post-proline endopeptidase that cleaves between the proline and leucine thus splitting the terminal leucine-glycine amide dipeptide from the molecule. Other possible routes of OT breakdown could involve the action of a carboxypeptidase enzyme cleaving amino acids from C-terminus or by enzymatic disruption of the disulfide bond. However, neither of these latter pathways appears to be a major method of OT metabolism.

Our laboratory has detected cystine aminopeptidase activity in human chorion, placenta and decidua, mainly in the microsomal subcellular fractions of these tissues (69). The greatest activity is located in the placenta and is significantly less in the decidua than in the chorion. The dominant enzyme in decidua appears to be a post-proline endopeptidase. There are no significant differences in the K_m or apparent V_{max} values for either enzyme between tissues collected before or after labour (69), suggesting that the regulation of OT metabolism is unlikely to be a major factor in the process of labour initiation.

Recently, we have characterized the metabolism of OT in late pregnant rat uterine tissues (70). We found that OT was actively metabolized in both uterine and placental tissues. Total oxytocinase activity was similar in the two tissues. In uterine tissues, activity was greater in the cytosolic fractions, while in placenta, activity was evenly distributed between the cytosolic and microsomal fractions. The cytosolic fractions of each tissue contained predominantly post-proline endopeptidase activity whereas the microsomes contained predominantly aminopeptidase activity. There was a slight trend to decreasing oxytocinase activity with advancing gestation in both subcellular fractions but this was statistically significant only in the microsomal fraction. The maximal decline in activity was only 25 – 50%. Treatment of TAM had

12

no effect on oxytocinase activity. Based on these data, we concluded that rat uterine and placental tissues contains post-proline endopeptidase and aminopeptidase activities that metabolize OT. However, it is doubtful that changes in these activities are major factors in regulating the increase in OT concentrations measured in rat intrauterine tissues at the time of parturition (70).

1.5.3. OT Receptor

1.5.3.1. Family of OT/VP Receptors

All the diverse central and peripheral neuropeptide actions are mediated by a family of VP and OT receptors. Though two distinct subtypes of OTR on myometrium and endometrium were postulated (5), most studies have indicated the presence of a single class of OT-binding sites. So far, there is no consensus whether or not more than one OTR exists. However, based on the second messenger systems to which they are coupled, VPRs have been subdivided into two main subclasses: V1 and V2 receptors (V1R and V2R) (71). Based on the use of specific agonists and antagonists, V1 receptor subtypes have been identified and termed V1a and V1b receptors (V1aR and V1bR) (72).

OT is a relatively specific ligand for the OTR, but VP is more promiscuous and binds to VPRs as well as to OTRs with high affinity (as reviewed in (73)). In the rat, OT is able to release corticotrophin from pituitary cells, which is mediated by V1bRs not by OTR (74). This suggests that among the VPRs, the V1bR seems to interact with OT.

One question has been asked by a few investigators. Is it possible that in the absence of OT, VP would be an effective substitute for OTR? Notably, magnocellular VP neurons are also activated (75) and VP release from posterior pituitary is increased during parturition in several species including

13

the rat (76). Since VP can activate OTRs, its actions would be expected to supplement those of OT during parturition. Some effects of VP in intrauterine tissues are similar to OTs. For example, VP is quite effective at stimulating $PGF_{2\alpha}$ production by the decidua (5). It is possible that, if in the absence of endogenous OT, the OTRs are upregulated, the actions of VP alone will indeed provide an effective substitute for the absent OT.

1.5.3.2. Distribution of OTR and VPR

Within the uterus, OTRs are present on myometrium and endometrium during pregnancy in various species (see below). OTRs and VPRs have also been identified in other reproductive and non-reproductive tissues.

Mammary gland

OTRs have been identified in the mammary gland membrane and OT induces milk ejection by contraction of mammary myoepithelial cells (77). The expression of the OTR gene reaches maximal shortly after parturition and remains at high levels throughout the period of lactation (6). OTRs have also been found in breast cancer tissues and in breast cancer cell lines (78, 79). The OT in several tumor cell lines has an inhibitory function on cell proliferation (79).

Ovary

Through binding studies, OTRs have been identified in human and bovine ovaries (80). In both species, the concentration of OTRs reaches highest level during the luteal phase (80). The function of OT may involve ovulation and luteolysis. Using RT-PCR technique, OTR mRNA has been found in both species of ovaries, suggesting the local production of OTR.

14

Pituitary

By using a specific rat OTR cDNA, Breton *et al.* have found that pituitary OTR gene expression is restricted to lactotrophs (81). This gene expression is markedly increased at the end of gestation and in response to estrogen treatment (81). The function of OT in the pituitary is related to stimulation of the release of prolactin, gonadotrophin and corticotrophin (74). Interestingly, studies with specific antagonists demonstrated that the OT effect on corticotrophin is mediated via the V1bR (74). Recently, the cloning human V1bR cDNA confirmed that the V1bR is encoded by a separate gene that is expressed in pituitary (82).

Brain

OTR gene has been expressed in the rat olfactory system, basal ganglia, parts of the amygdaloid nucleus, the ventromedial nucleus of the hypothalamus as well as areas in the brain stem and spinal cord (83, 84). V1aR mRNA and AVP binding sites correspond to V1aRs have been found in rat olfactory nucleus, the limbic system, the hypothalamus, thalamus, the chorioid plexus, the circumventricular organs, the brain stem and spinal cord (83). No V2R mRNA could be detected in rat brain (85). The OTR- and VPR-mediated function may involve some specific learning, maternal and sexual behavious (86, 87). Studies demonstrated that concentrations of brain OTR mRNA and binding sties are induced by estrogen (84, 86, 88). In contrast to genomic effects of estrogen, the effects of P_4 on hypothalamic OTR gene are non-genomic. P_4 can potentiate estrogen-induced behavioral modifications (89).

15

Kidney

A line of studies has shown that OTRs expressed in kidney (90, 91) and OT plays an important role as a natriuretic hormone (92). Physiological elevations of plasma OT levels lead to a natriuresis that is blocked by specific OT antagonists (93). In pregnant rats, renal OTR mRNA levels undergo a more than 3-fold decrease at term, while binding studies reveal a concomitant decrease in renal OT-binding sites (91). Estrogen strongly upregulates OTR mRNA levels and OT-binding sites in kidney, suggesting OT may be an important physiological regulator of sodium and fluid homeostasis specially during high estrogen states (91, 94).

There is a high gene expression of V2R in renal tubular cells (95). Activation of the renal V2R increases water reabsorption from urine to blood. In situ hybridization studies demonstrated V1aR mRNA in vascular bundles of the renal medulla and in segments of cortical distal tubules (95). The function of renal V1aR is still little known.

Thymus

The VP/OT system may also be involved in immunomodulation. OTRs and VPRs have been identified on thymocytes (96) and VP- and OT-like immunoreactivity has been detected in thymic nurse cells (97). Interestingly, using immature pre-T-cell and mature cytotoxic T-cell lines, Martens *et al.* reported that, during T-cell maturation, there was a switch from the expression of V1aRs in immature T-cells to expression of OTRs in mature T-cell (97). This evidence suggests that both VP and OT may play a role in modulation of T-cell maturation and function.

16

Smooth muscle cells

VlaRs have been found on vascular smooth cells (98). These vascular VlaRs are upregulated by estrogen (99) and downregulated by glucose (100). Interestingly, the expression of OTR gene but not VPRs has been found in human vascular endothelial cells. These OTRs are structurally identical to the uterine and mammary OTRs. The OTRs in vascular endothelial cells produce a calcium-dependent vasodilatory response (101).

1.5.3.3. Gene Structure of OTR

The human OTR gene was first cloned by Kimura *et al.* (102) in 1992 from a library derived from a *Xenopus* oocyte. There are two sizes of mRNA, 3.6 kb in breast and 4.4 kb in ovary, uterine endometrium and myometrium. Subsequently, rat, pig and sheep OTR have been cloned and sequenced (103-105). The human OTR gene is located on chromosome 3 (106). The rat OTR gene contains three and the human OTR gene contains four exons (Fig. 1-4a). In the rat, a 97-bp intron is present in the 5' untranslated region and a >12-kb intron interrupts the coding region between transmembrane domains (TMD) 6 and 7. The predicted amino acid sequences derived from the coding region of the rat and the human OTR shows that 92.8% are identical. Conversely, the rat OTR gene has only 48% and 38% sequence identity to the rat VlR and V2R (103).

In rat OTR genes, the promoter region contains sequence elements that correspond to the consensus sequences for binding sites of nuclear factor-interleukin 6 (NF-IL6) and to the acute phase response factor (103). There are no palindromic estrogen response element (ERE) in the first 3500 nucleotides upstream from the transcription start site but there are several copies of the 5'- or 3'-half response elements in the proximal promoter region (103). However, after isolating and sequencing additional 2 kb of upstream sequence missed

17

from the studies of Rozen *et al.*, Bale *et al.* (107) have identified several response elements including a palindromic ERE, half-serum response element, and several AP-1, AP-2, AP-3 and AP-4 sites. There is no P_4 response element (PRE) or glucocorticoid response element (GRE) in either human or rat OTR promoters (Fig. 1-4b).

The encoded human and rat OTRs are typical G protein-coupled receptors, 389- (human) and 388- (rat) amino acid polypeptide with seven TMDs (102, 103). OTR and its related receptor molecules, VPRs contains four extracellular domains (EDs) and four cytoplasmic domains (CDs). These domains include an extracellular N-terminal domain and intracellular C-terminal domain as well as three extracellular and intracellular loops connecting the TMDs (Fig. 1-5). The areas of greatest similarity between OTR and VPRs include TMD 2, 3 and 6 and ED 2 and 3. The strongest divergence occurs in the N- and C-terminal domains as well as in CD3.

1.5.3.4. OTR as a G-protein Receptor

OT is one of many compounds that influence myometrial contractility by binding to myometrial cell membrane receptors that activate effector systems within the cell. The link between the hormone-receptor complex and the effector system is usually a regulatory GTP-binding protein (G-protein) (Fig. 1-5). G-proteins, consisting of α, β, and γ subunits, are classified and grouped into several families according to their α subunit. Some are substrates for ADP-ribosylation by bacterial exotoxins. Human myometrium contains several types of G-proteins, G_s, G_i, G_{i3}, G_q, G_{11} and G_z. These proteins may regulate the activity of adenylyl cyclase (AC) or phospholipase C (PLC), two enzymes that are important effector enzymes that generate second messengers. They also may be linked to voltage-dependent ion channels or to phospholipase

18

A_2 that can generate arachidonic acid, a precursor for PG synthesis within the cell.

The G-proteins responsible for PLC activation in human myometrium are of at least two types: a pertussis resistant G-protein G_q or G_{11} and a pertussis toxin sensitive protein G_i. OT stimulates myometrial contractility by binding to cell membrane receptors coupled to G-proteins, both G_q or G_{11} and G_i pathways (108). OT provokes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), generating two second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglyceride (DAG). IP_3 mobilizes Ca^{++} from the sarcoplasmic reticulum that in turn activates myosin light chain phosphorylation. DAG activates protein kinase C (PKC) resulting in the phosphorylation of several target proteins. DAG can be recycled into PIP_2 or be hydrolyzed by diacyl- and monoacylglycerol lipases allowing the release of arachidonic acid (as reviewed in (109)) (Fig. 1-5).

The responsiveness of the tissue to OT depends on the number of activated receptors for OT, on the degree of coupling between the hormone-receptor complex and the G-protein, and on the degree of coupling between the G-protein and the effector system. Furthermore, OT increases intracellular $[Ca^{++}]$ also by directly activating Ca^{++} channels in the plasma membrane (110).

The ability of OT to increase endogenous IP_3 levels has been reported in fresh human smooth muscle slices (111). Also, the time course studies agree with the rise in intracellular $[Ca^{++}]$ in human myometrial cells challenged with OT (108). However, there is no difference in IP_3 response to OT between myometrial tissue obtained at term but not in labour compared to tissues obtained during spontaneous labour (109).

1.5.4. Parturition

1.5.4.1. Three Phases of Parturition

The pregnant myometrium is relatively quiescent until parturition begins. Casey and MacDonald (112) referred this first 95% of pregnancy period as uterine phase 0 of parturition. The precise point at which parturition begins cannot be discerned but there is agreement among investigators that the process of parturition can be further divided into three separate but interrelated physiological phases: phase 1 (*activation*), phase 2 (*stimulation*) and phase 3 (*involution*) (112, 113). At the end of phase 0 of parturition, the uterus is awakened and prepared for labour. This period of uterine awakening, which clearly precedes the onset of active labour, is referred to as phase 1 of parturition. During this phase, the uterus is prepared to give maximal response to a stimulant. It is not clear whether this phase involves active preparation of the uterus or whether it is due to an escape from the inhibitory mechanisms maintaining uterine quiescence prior to activation. This phase is characterized by increases in populations of receptors for stimulants such as OTR and PG, increases in the area of gap junctions between myometrial cells and increases in the synthesis and functional activity of ion channels. In addition, the cervix undergoes a process of softening and ripening. It may take several days to complete and reach the next phase. During the phase 2 (*stimulation*), the myometrium is sensitive to all stimulants, such as OT and PGs, and can generate a maximal response to facilitate delivery of the fetus and placenta. The duration of this phase is much shorter than the previous phase. Finally, in phase 3 (*involution*) the fetus, placenta and fetal membrane are delivered and eventually, the uterine tissues return to their non-pregnant state. These three phases must be developed in an orderly sequence and during a defined period of gestation. When the sequence is delayed or prematurely activated, or dysregulated, pregnancy complications occur (as reviewed in (114)).

1.5.4.2. Mechanism of Myometrial Action

Uterine muscle cells contain two types of protein, myosin and actin. Myosin is made of two heavy and four light polypeptide chains. The two heavy chains form a globular head that interacts with actin and also has enzymatic activity to hydrolyze ATP and to provide energy for contraction. The light chains are attached the globular head and provide regulatory sites for Ca^{++} binding and phosphorylation. Ca^{++} interact with calmodulin to activate myosin light chain kinase (MLCK) which phosphorylates a 20-kDa light chain of myosin. Phosphorylated myosin interacts with actin resulting in activation of myosin ATPase and causing a contraction. Contraction of the myometrial cells can be increased either by activating MLCK or by inhibiting activity of myosin phosphatase (as reviewed in (109)) (Fig. 1-5).

Contraction in these muscle cells is controlled by intracellular free $[Ca^{++}]$ and the extent of myosin light chain phosphorylation. Increases in intracellular $[Ca^{++}]$ can be achieved by increasing uptake from outside the cell through Ca^{++} channels in plasma membrane or by mobilizing Ca^{++} from intracellular stores in the sarcoplasmic reticulum. Relaxation in uterine muscle can be achieved by lowering intracellular $[Ca^{++}]$ or reducing the sensitivity of MLCK to Ca^{++}. There is also a mechanism for rapid lowering of intracellular $[Ca^{++}]$ by extrusion of Ca^{++} from the cell or by reuptake into the sarcoplasmic reticulum (as reviewed in (109)).

1.5.4.3. Switch from Myometrial Contractures to Contractions in Late Pregnancy

There are two types of myometrial activity that occur throughout pregnancy in most species (115). Although uterine contractile activity is inhibited during pregnancy, the myometrium is not totally quiescent. Long lasting, low frequency and low amplitude myometrial contractile activities

21

termed *"contractures"* are observed in a number of species including man (116), monkey (117), sheep (118), guinea pig (119) and rabbit (120). At labour, the short duration, high frequency and high amplitude myometrial *"contractions"* take over.

The switch from contractures to contractions tends to occur at night and is related to alteration in maternal plasma estrogen concentrations. In the rhesus monkey, 24-h infusion of androstenedione elevates estrogen concentration in maternal plasma and stimulates the switch from contractures to contractions. Interestingly, this only occurs at night (115). Wilson *et al.* (121) adapted the androgen-infused model used in the rhesus monkey to the pregnant baboon and demonstrated that infusion of DHEAS led to a switch from contractures to contractions. Using this paradigm, they reported that inhibition of the conversion of androgen to estrogen by aromatase inhibitors also prevented the switch in myometrial contraction patterns. In the pregnant sheep, the rise in maternal plasma estrogen level is very abrupt, occurring only during the last 24 h of pregnancy (122). This rapid increase in estrogen concentration may explain the observation that sheep switch from contractures to contractions only once, about 6 h before delivery in contrast to non-human primates in which the repetitive switch is gradual over several days to weeks (115). These studies support the hypothesis that maternal estrogen prepares the myometrium for a periodic signal that causes the switch from contractures to contractions.

1.5.4.4. Contraction-associated Proteins

During the activation phase of parturition, the expression of contraction-associated proteins (CAP) increases. Three of these proteins are commonly used as the markers of uterine activation: OTR, PG synthesis protein (both discussed below) and gap junction proteins. The major gap junction protein is

22

connexin 43 (Cx-43). Synthesis of Cx-43 and assembly of gap junctions are essential to facilitate the myometrial cell-cell coupling that enables the strong coordinated contractions of labor. Studies by Lye and coworkers demonstrated a number of key points regarding Cx-43 in both rat and human uterus. Cx-43 gene expression increases in myometrium with rat gestational age (123) and human onset of labour (124). In humans and rats, it is regulated by estrogen (stimulatory) and P_4 (inhibitory) (123, 125). Estrogen appears to stimulate an increase in Cx-43 coincident with increased OTR (123), suggesting that the increased uterine contractile response to OT at term is associated with increased gap junctions. Additionally, in the rat, uterine stretch also is a stimulus to Cx-43 mRNA synthesis (126).

1.5.4.5. OT as an Endocrine Hormone at Parturition

With knowledge of the potent and specific uterine contractile property of OT, early researchers attempted to define the role of OT in the initiation of labour. Experiments from several research groups have showed controversial results.

Several lines of research findings cast doubt on the role of OT in the initiation of human parturition. Some investigators failed to find a rapid increase in serum concentration of OT before labour onset but after only the fetal expulsive stage of labour (127). The levels of serum OT are very low throughout pregnancy and remain undetectable during early labour stage in 70 % of pregnant women (128). Additionally, the required association constant of OTR (129) to maximize uterine activity is 10 - 100 time higher than the actual concentration of OT in maternal serum at the time of parturition. Finally, women with known posterior pituitary dysfunction, and therefore thought to be deficient in OT, have apparently normal labour (130).

23

There are few studies of serum OT concentrations in rat late pregnancy. Later, using an intrauterine sensor to detect the uterine contractility before and after labour onset, while collecting the serum samples, Higuchi *et al.* (131, 132) found no significant increase in serum OT concentration until fetal expulsion of labour. The peak levels of serum OT were noted during milk ejection. Using pelvic neurectomy (which abolished the Ferguson's reflex) in the rat, the same research group noted that the duration of labour was prolonged and serum OT concentration decreased, without affecting the onset of labour (133). Furthermore, immunoneutralization of circulating OT by specific antisera slightly prolonged the delivery time (133). Electrolytic lesions of rat median eminence at day 13 of pregnancy had no effect on parturition but completely suppressed the milk ejection reflex (134). All these studies suggested that, in the rat as in the human, circulating OT is unlikely to play an important role in the initiation of parturition, though it may play a role once labour has been instituted.

Several investigators offered explanations for these controversial results. They speculated that the failure to find increases in circulating OT concentrations may have been due to technical difficulties in measuring OT in the serum, particularly at the time of parturition. The high intravascular catabolism and the low concentration of serum OT in late human pregnancy have concerned many investigators. In late human pregnancy, maternal serum contains a high concentration of oxytocinase that rapidly degrades OT after sample collection (67). Moreover, the pulsatile pattern of secretion of OT into the circulation (24) and the short plasma half-life (approximately 90 sec to 3 min) of OT (21) made it even more difficult to obtain valid data. Furthermore, recent reports have shown that some antibodies used for radioimmunoassay (RIA) of OT have cross-reactivity with OTX (OT-gly), resulting in an artificially high measurement of serum OT (135).

24

Thornton et al. (128) addressed these concerns. Using a protocol to minimize OT degradation following sample collection (rapid acidification, chilling, EDTA and phenanthrolone), a one-minute sampling protocol, and a sensitive and specific RIA, they still failed to find a significant change in maternal serum OT concentration until the stage of fetal or placental. On the contrary, Dawood et al. (127) and Fuchs et al. (24) reported an increase in OT concentrations and in OT pulse frequency and duration at the beginning of labour in women. Luckman et al. (136) reported that the inhibition of the activation of the neurohypophysial OT system in parturient rats resulted in a significant prolongation of parturition.

Several investigators also explored the possible role that OT derived from the fetus might play in the initiation of labour. The human fetus produces and stores OT in the hypothalamus throughout pregnancy. The concentration of OT in the fetal pituitary increases progressively from week 14 of gestation up to term (130, 137). At term pregnancy, a considerable amount of OT can be demonstrated in amniotic fluid (138) and in cord blood (139). Concentrations of OT are higher in umbilical serum than in maternal serum and higher in the umbilical artery than umbilical vein (139). The highest levels are found at the time of vaginal delivery, with significantly lower levels at cesarean section when the patient is not in labour. However, normal spontaneous deliveries occur with an anencephalic fetus that lack fetal OT synthesis (140). Therefore, most investigators have questioned whether fetal OT plays any role in regulating the timing of human parturition.

Recent studies indicate that the pattern of OT secretion is more important than the absolute levels of OT. In the last few weeks of pregnancy in the rhesus monkeys (141, 142) and human (143), there is a unique nocturnal increase in uterine contractions. These nocturnal contractions correlate with the circadian rhythm of pulsatile OT secretion, with an acrophase at night, in

25

both the rhesus monkey (23, 142) and human (8). The nocturnal surges of uterine activity near term can be abolished in rhesus monkeys by use of specific OT antagonists (142, 144). Thus, the controversy continues as to whether OT from the posterior pituitary is just a facilitative factor for the procession of labour or a pivotal hormone in the mechanism of initiation of parturition.

1.5.4.5 OT as a Paracrine or Autocrine Hormone at Parturition

During the last ten years, a non-neuronal production of OT peptide has been suggested in several endocrine glands and peripheral tissues. It has been postulated that OT may have a role in regulating the timing of parturition in a paracrine mechanism rather than or, in addition to, an endocrine process. Paracrine synthesis and biological activity of OT may occur without any change in the maternal circulation, thus rationalizing a significant role for OT in the initiation of parturition despite the previously noted negative findings.

1.5.4.6.1. OT in Intrauterine Tissues in Late Gestation

The first suggestion that OT may be synthesized in reproductive tissue was made after measurement of high OT concentrations in bovine corpus luteal tissue (33). Two years later a high level of mRNA encoding the OT gene was found in the same bovine tissues (32). Further investigations suggested an interaction involving ovarian OT and uterine $PGF_{2\alpha}$ in regulation of ovine luteolysis (145).

Chibbar *et al.* (1) first reported that mRNA encoding OT was produced in human late gestational intrauterine tissues, namely in the decidua, chorion and amnion. They also found a greater concentration of OT mRNA in tissues obtained after the onset of spontaneous labour at term in comparison with

26

tissues obtained at term but before labour onset, suggesting an increase in OT mRNA synthesis around the time of the onset of labour.

Several studies in rat intrauterine tissues have shown a similar pattern of synthesis of mRNA encoding OT in late gestation. Lefebvre *et al.* (2) demonstrated that OT mRNA was synthesized mainly in the epithelial layer of the endometrium during pregnancy. Between days 14 and 18 of pregnancy, there was a rapid increase in OT mRNA accumulation in the uterus. These investigators estimated that the total mRNA encoding OT in the day 21 pregnant uterus was approximately 70-fold higher than in hypothalamus. The amnion (3) and placental (146) also produced a small amount of OT mRNA. In contrast to rat uterine level of OT mRNA, the placental concentration of OT mRNA showed a decrease before parturition (146). They also noted that the tissue levels of mRNA in the late pregnant rat were much higher than in humans (2).

To investigate whether OT peptide exists in rat uterine tissues, Lefebvre *et al.* (2) assayed the amounts of immunoreactive OT (ir-OT) in the late pregnant uterus. They found that the day 21 pregnant uterus contains a 35-fold higher level of ir-OT, compared with that in the nonpermanent uterus. Subsequently, they also detected an increase in ir-OT level in amnion prior to parturition. However, using a selective OTR ligand, they revealed a high number of OT binding sites in rat uterus, but not in amniotic or placental membranes (2).

The findings that intrauterine OT mRNA and peptide increase tremendously in late gestation in both humans and rats, and OT gene expression occurs in, or anatomically close to the target tissues, the endometrium and myometrium, support the hypothesis that OT may act in a paracrine or autocrine mechanism to regulate the timing of parturition.

1.5.4.6.2. OTR in Intrauterine Tissues in Late Gestation

Within the rat or human uterus, OTRs are present on both myometrial and endometrial epithelial cells. Most studies have indicated the presence of a single class of OT-binding sites. Chen *et al.* (5) postulated two distinct subtypes of OTR in rat myometrium and endometrium. They showed that two different OT analogues block OT-induced myometrial activity but have agonistic activity on endometrial activity. Moreover, some OT agonists elicit myometrial contractions but have no effect on the endometrium. However, to date, there has been no satisfactory evidence supporting the existence of more than one OTR gene or transcript in either rat or human.

One of the most consistent findings in the study of parturition in several species is the dramatic increase in OTR before the onset of labour. Soloff *et al.* (6) were the first to report a gestational increase in OT binding by uterine cells in rats. Subsequently a similar pattern increase was demonstrated in human uterus (4). The same investigators have concluded that the increase in myometrial sensitivity to OT was due to an increase in OT binding to uterine OTR in both humans (4) and rats (6). The K_d of the OTR did not change with advancing gestation.

Larcher *et al.* (147) have shown that uterine mRNA levels for OTR increased more than 25-fold during gestation, reached a peak at parturition and fell rapidly by 85% within 24 h following parturition. Using *in situ* hybridization, they noted that OTR mRNA was localized in the myometrial cells but not in the endometrial cells (147). Others demonstrated that OTR was present in the plasma membrane of both myometrium and endometrial epithelium (4, 148). Chen *et al.* (5) reported that stimulation of endometrial OTRs led to production and secretion of PGs, while stimulation of myometrial OTRs elicited myometrial contraction.

28

1.5.4.7. Pharmacological and Genetic Studies of OT in Parturition

1.5.4.7.1. OT Antagonists

Studies using specific antagonists of OT may help clarify the role of OT in the initiation of labour. More importantly, the development of suitable OT antagonists as new and effective tocolytic agents might be of therapeutic value in the prevention of preterm labour. Manning *et al.* and others have synthesized a series of analogues of OT primarily by changing or substituting amino acids. In general, these modifications have involved alteration of the tyrosine moiety at position 2 along with substitution for the amino acids at position 4 and 8. This results in greater selectivity and antagonism for OTR. Results from studies using these agents to prevent or delay parturition in rat have been conflicting. Chan *et al.* (5) and Hahn *et al.* (149) have shown that certain OT antagonists do not delay labour onset, but significantly lengthen the course of labour. One explanation is that those OT antagonists block the OT-induced uterine activity in myometrium but, meanwhile, stimulate the synthesis of prostaglandins in endometrium (150). Conversely, others reported a delay in the initiation of labour and a prolongation of the process of labour by using different agents known to block both endometrial and myometrial OTRs (151).

Atosiban (1-deamino [2-0-ethyl-D-tyrosine, 4-threonine] ornithine vasotocin) was synthesized ten years ago by Melin *et al.* and is the only OT antagonist that has been tested in clinical trials as tocolytic agent for the prevention of preterm delivery (152). It is a nonselective blocker, effective in inhibition of uterine contractility via antagonism of both OTR and V1aR (153). In the human non-pregnant myometrium, atosiban is the most potent AVP antagonist, and in pregnant tissue atosiban shows the most pronounced inhibition of OT (152). Atosiban can inhibit the switch from contractures to contractions and may be of value in reducing the occurrence of premature birth in pregnant primates including human (154).

29

Recently, novel OT antagonists have been developed with the aim of improving bioavailability and effective duration (155). In addition, non-peptide OT antagonists that can be administered orally have been developed (156). Clearly, more studies are required to ascertain the role of OT antagonists in clinical practice.

1.5.4.7.2. Knockout Mouse Studies

Recently, investigators obtained surprising results from their knockout mouse studies (157, 158). Nishimori *et al.* (157) developed a colony of mice deficient in OT (deletion of exon A of OT gene) using embryonic stem cell technology. Young *et al.* (158)eliminated, by homologous recombination, most of the first intron (A) and the last two exons (B and C) of the OT gene in mice. Female mice lacking OT were of normal fertility. Surprisingly, homozygote pregnant mice had a normal duration of gestation and an apparently normal parturition of normal timing and duration. However, though the dams produced milk, all offspring died shortly after birth due to failure of the dam to lactate and nurse properly. They concluded that OT plays an essential role only in milk ejection in the mouse. However, in mice there is no evidence concerning the presence of an intrauterine paracrine system and the extent to which these findings apply to other species where such a paracrine system exists is not known.

Gross *et al.* (159) recently have generated mice deficient in one of prostaglandin synthases, prostaglandin-endoperoxide H synthase-1 (PGHS-1) or both OT and PGHS-1. Mice with targeted disruption of the PGHS-1 gene have impaired luteolysis and delayed parturition resulting in neonatal death. However, a normal parturition occurred in the combined OT and PGHS-1-deficient mice. Investigators concluded from this experiment that PGHS-1 is

30

essential for normal labour in the mouse, with critical function being to overcome the luteotrophin action of OT in late gestation (159).

1.5.4.8. Prostaglandins

1.5.4.8.1. Synthesis and Metabolism of PGs

PGs are a group of regulatory factors derived from arachidonic acid (AA), a polyunsaturated acid containing 20 carbon atoms. AA is oxygenated through PGG_2 to PGH_2 by the enzyme PGHS, also known as Cox. PGHS exists in two isoforms: a constitutive form PGHS-1 and an inducible form PGHS -2. PGH_2 is metabolized to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 by specific enzymes, respectively. AA is an essential component of the structural phospholipids of cell membranes. Therefore, it is present in every cell of the body. Due to normal metabolic turnover of membrane phospholipids, free AA is continuously released in small amount in the cell (as reviewed in (160)). There are two major enzymes involved in liberation of free AA from cellular sources: phospholipase A_2 (PLA$_2$) and phospholipase C (PLC). In most cells PLA$_2$ catalyses the hydrolysis of AA from the 2-position of phosphatidylcholine, phosphatidylethanolamine or plasmalogens. AA also arises by an indirect mechanism. Through the sequential action of PLC, a diglyceride lipase and a monoglyceride lipase, AA is released from the second glyceryl carbon of phosphatidylinositol (as reviewed in (160)).

The major metabolizing enzyme for PGs is 15-hydroxyprostaglandin dehydrogenase (PGDH) which catalyzes oxidation of the 15-OH group of PGE and PGF series to form 15-keto,13,14-dehydro metabolites. Both PGHS and PGDH may be equally important in regulating PG levels in intrauterine tissues during pregnancy.

31

1.5.4.8.2. PGs in the Initiation of Labour

Administration of PGE_2 or $PGF_{2\alpha}$ at any stage of pregnancy causes abortion or labor (161), and blockage of PG synthesis prolongs gestation, reduces myometrial activity and increases the duration of labour (162, 163). Furthermore, the maternal plasma concentration and urinary excretion of PG metabolites increase at labour, indicating the increase in production of PGs. These findings suggest that PGs are critical factors in controlling the timing of parturition.

A wide range of tissues may contribute PGs during pregnancy including the decidua (164), fetal membranes (164, 165), placenta (166), and myometrium (167). In the human and higher primates, PG levels in amniotic fluid are higher near term than in early and mid pregnancy (168). With the onset of labour, amniotic fluid concentrations of PG rise sharply and continue to increase in parallel with cervical dilatation (169, 170). The most likely source of amniotic fluids PGs is the fetal membranes. In late pregnancy, amnion produces a large amount of PGE_2, while the decidua is identified as the main uterine source of $PGF_{2\alpha}$ (164).

Olson and colleagues have shown that the activity of PGHS and the output of PGs increases in human fetal membranes, amnion and chorion with labour, and that this correlates significantly and exclusively with a rise in the expression of the PGHS-2 isoforms in these tissues (171-173). The chorion, the layer positioned between the amnion and the decidua, contains both PGDH (174) and PGHS (173), but the metabolizing PGDH predominates (175). This capacity of PG metabolism in chorion is little changed in late gestation and with the onset of labour (164).

Since the chorion contains high activity of PGDH (175), the amnion might not be involved in initiating labour. The decidua lying adjacent to the myometrium is likely to be involved, as it is a rich source of $PGF_{2\alpha}$. There is

32

controversy in the literature regarding the role of decidual PGs at term parturition. Mijovic *et al.* (173) have shown no change in PGSH specific activity in decidua with labour. Others have shown that the content of PGs in decidua significantly increased with onset of labour (164, 176, 177). Moreover, the PG production within decidua is possibly due to the population of macrophage-like cells (178). Furthermore, over 40 % of decidual cells are bone marrow-derived cells, carrying CD45 surface antigen and these cells are shown to enhance the synthesis of PGs at labour (177). Activation of decidua could therefore result in multiple stimulatory signals comprising both PGs and cytokines, perhaps, further stimulating interaction between PGs and the action of OT, which could stimulate the myometrium (discussed later).

In addition, PGs driving the myometrium can be generated within myometrium itself. Some data suggest that human parturition is associated with increase in expression of PGHS -2, but not PGHS -1 in the myometrium (179). However, using western immunoblotting with specific antibodies for rat PGHS -1 and PGHS -2, Myatt *et al.* (180) have shown that in rat myometrium the concentration of PGHS -1 not PGHS -2 increased significantly from day 16 to a maximum at the time of delivery on day 22 and decreased immediately afterward.

1.5.4.8.3. Regulation of PGs

The regulation of PG synthesis in intrauterine tissues has been extensively studied. Most studies have been done in *in vitro* systems such as cells or tissues. Among steroids, glucocorticoids demonstrated a stimulating effect on PG output of amnion tissues (181), while P_4 or estrogen showed no effect on PG production in gestational tissues of women at term (as reviewed in (114)). Furthermore, adrenocorticotropin (ACTH) and corticotropin-releasing hormone (CRH) increase the PG output of all human intrauterine

33

tissues at term (182, 183). Moreover, other factors also affect PG output and PGHS activity *in vitro* intrauterine tissues. OT has been shown to stimulate PG production of human decidual (4) or rat endometrial (184) tissues. Several cytokines, such as TNFα and IL-1β also affect PG output in both human and rat gestational intrauterine tissues (185, 186).

Although the lines of *in vitro* information relating the regulation of PG production are known, little is known about the factors and mechanism that control human intrauterine PG production *in vivo*. None of the hormones or paracrine factors which affect the PG output of human gestational tissues *in vitro* have been shown to be the physiological factors of the increasing intrauterine PG levels observed at labour in women. However, there is some information from the results of animal experiments. Wilson *et al.* have carried out extensive studies to determine whether ovariectomized pregnant rats under a mimicking P_4 withdrawal phenomenon in the presence or absence of estradiol could change uterine PG levels. They found that P_4 withdrawal significantly elevated only rat uterine PGE_2 but not $PGF_{2\alpha}$, while P_4 withdrawal with exogenous administration of estradiol increased $PGF_{2\alpha}$ but not PGE_2 levels (187). These findings suggest there is a difference of regulation of PG synthesis between humans and other animals.

Furthermore, artificial rupture of membranes or distension of the cervix lead to a rise of PG levels in maternal circulation and in the amniotic fluid, suggesting the certain mechanical stimulation *in vivo* appears to play a role in regulation of PG output of human gestational tissues (as reviewed in (114)).

34

1.5.4.9. Other Stimulants

Endothelin-1 (ET-1)

Another potential candidates for a physiologically important uterine stimulant is ET-1. Like OT, the receptor for ET-1 is coupled via PLC/IP$_3$ to Ca^{++} mobilization. The mechanism of Ca^{++} channel opening by ET-1 is probably similar to that for OT and PGF$_{2a}$. Human endometrial and amnion cells synthesize ET-1. A higher level of ET-1 is found in amniotic fluid at term than that at early or mid gestation. However, the chorion contains an active catabolizing enzyme, enkephalinase, which prevents ET-1 from crossing to the myometrium. It is possible that ET-1 acts indirectly by stimulation of other bioactive substances (188).

Growth Factors

Growth factors such as epidermal growth factor (EGF) and transforming growth factor α (TGFα) are polypeptides that are implicated in the autocrine or paracrine regulation of growth and differentiation in tissues and are synthesized in the uterus (189). EGF and TGFα are shown to accumulate in the amniotic fluid in late pregnancy and at labour. Fetal kidney is the most likely source of these growth factors. *In vitro* studies showed that EGF enhanced the PGE$_2$ output from amnion cells (190). Transforming growth factor β (TGFβ), another growth factor present in fetal membranes has anti-progesterone activity on several P$_4$ responsive genes. These data suggest that some growth factors are also involved in the regulation of gestational tissue function and are important in the timing of labour onset (as reviewed in (191)).

35

Catecholamines

Catecholamines, most likely from fetal urine, are present in the amniotic fluid. The level of catecholamines increases as gestation advances. Alpha-adrenoceptors and beta-adrenoceptors are present on myometrial cells. The excitatory effects of catecholamines on the uterus are mediated by alpha-adrenoceptors. The human amnion responds to beta-adrenergic stimulation with increased AA release and PGE_2 production. However, the physiological role of catecholamines in pregnancy and parturition is not understood (as reviewed in (192, 193)).

1.5.5. Regulation of the Timing of Parturition

1.5.5.1. Estrogen and Progesterone

1.5.5.1.1. Estrogen,Progesterone, and the Estrogen/Progesterone Ratio

In 1956 Csapo proposed the "the progesterone block theory". He suggested that delivery could not take place before a fall in maternal P_4 levels because P_4 blocked the activity of the myometrium (194). In some species (including rat, mouse, rabbit, etc.) the corpus luteum is maintained throughout gestation to produce P_4 that maintains uterine quiescence. The removal of corpora lutea or ovaries leads to abortion. Luteolysis just before labour in these species causes P_4 withdrawal to trigger labour onset (7).

In sheep, the P_4 block theory also appears to be operative. In the pregnant sheep, the placenta produces P_4 and estrogen. One or two days before labour onset there is a rapid decrease in maternal serum P_4 and a switch from P_4 to estrogen domination. These changes are regulated by the fetal hypothalamo-pituitary-adrenal axis. Specifically, after the fetal hypothalamo-pituitary-adrenal axis is matured, fetal cortisol secretion increases. This stimulates induction of the 17-hydroxylase/17, 20-desmolase gene in the placenta. Activation of this enzyme results in metabolism of P_4 into estrogen

36

precursors that are subsequently converted in the placenta to estrogen (as reviewed in (195)). These changes result in a decrease in maternal serum P_4 and an increase in serum estrogen.

In the human, maternal serum P_4 levels increase progressively toward term (196). P_4 is predominantly produced in the corpora lutea in early pregnancy (until approximately 6-8 weeks menstrual age) and in the placenta for the remainder of pregnancy. Removal of corpora lutea or ovaries before six weeks menstrual age causes an abortion but has no effect later in gestation. PR antagonists, such as RU486 increase myometrial contractility and induce the abortion. However, some studies have shown that administration of exogenous P_4 does not delay the parturition at term (197, 198). There is no good evidence to show that systemic maternal P_4 withdrawal is a prerequisite for the initiation of primate parturition. However, a modest rise in estrogen in late pregnancy is associated with parturition.

In most species, parturition is accompanied by an increase in production of maternal serum concentration of estrogen. In humans, estrogen production involves the feto-placental unit, an interaction between the fetus and placenta. In the fetal adrenal gland pregnenolone is derived largely from LDL-cholesterol and thus converted to dehydroepiandrosterone sulfate (DHAS), estrogen precursor. DHAS is converted in the placenta to estrone or estradiol or 16-hydroxylated in the fetal liver for subsequent conversion in the placental to estriol. Estriol has less biological activity and estradiol has highest activity. The androgenic precursors for estrone and estradiol also are synthesized in large amount in maternal side. The precursors for these estrogens are derived in approximately equal amounts from both the fetal and maternal adrenal glands.

37

1.5.5.1.2. Estrogen Receptor and Progesterone Receptor

The classical actions of estrogen and P_4 are manifested through their initial interaction with specific high-affinity intracellular receptors. The estrogen receptor (ER) and P_4 receptor (PR) are members of a superfamily of nuclear receptors that includes receptors not only for steroid and thyroid hormones but certain vitamins and many orphan receptors whose ligands have yet to be identified. Within this family, the architecture of members has a similar feature containing multiple functional domains, including a highly conserved central DNA-binding domain (DBD), a moderately conserved C-terminal hormone-binding domain (HBD), and a poorly conserved N-terminal region whose function is largely unknown. These receptors regulate the transcription of target genes through domains called activation functions (AFs), mediated by the basal transcription machinery either directly or indirectly (199). For many steroid receptors, two AFs have been identified: one (AF1) located in the N-terminal region and the other (AF2) in the C-terminal HBD. AF1 is constitutively active when AF2 is removed, whereas AF2 is a ligand-dependent activator (200).

Prior to ligand binding the receptor exists as an inactive complex that contains a group of proteins including the heat-shock protein 90 (hsp90), hsp70 and other associated proteins. The hsp90 is involved in the maintenance of the receptor in an inactive state in the absence of ligand and also is important for folding of the receptor and/or transport across membranes, but the function of the other proteins is unknown. Upon hormone binding the complex is dissociated from hsp90 and the receptor is activated by a conformational change. The activated receptors dimerize before binding to DNA at specific hormone response elements. Activated receptors may interact directly with response elements of DNA or may interact with other transcription factors to influence gene transcription.

38

ER and PR are found in many tissues of essentially all animal species. This discussion will focus on those that are found in human or rat decidua, fetal membranes, placenta and myometrium. In human decidua, the concentrations of ER mRNA incresae 3 to 4-fold around the time of labour onset, whereas PR mRNA does not change (201). In human myometrial tissue, levels of ER and PR are absent or low throughout pregnancy, perhaps due to the relatively low sensitivity of the methods used (202). However, in the rat myometrium, there is no change in nuclear ER from day 16 to 20 of pregnancy and PR from day 16 to 18, nuclear PR concentrations begins to fall from day 18 onwards and nuclear ER increases sharply from day 20 to 22 (delivery), indicating that an alteration of the nuclear receptor balance away from P_4 dominance towards estrogen on the day parturition (180). These data suggest that a change in the biological action of estrogen or P_4 might be accomplished by changing concentrations of receptors rather than the ligands themselves. This is a concept that we have addressed further in chapter 5 of this thesis.

1.5.5.1.3. Isoforms of PR and ER

PR:

PR exists in target tissues as several distinct isoforms. In the human, the 933-amino acid PR-B contains a N-terminal 164-amino acid upstream segment (BUS) which is missing in the truncated 769-amino acid PR-A (203-205). The two isoforms are synthesized from a single gene but initiated from two promoters giving rise to two different RNA transcripts (205, 206). Both PR-A and PR-B have AF1 and AF2 regions. Only PR-B processes AF3 in the BUS and this requires an intact DBD to be transcriptionally active. In general, PR-B transactivation is stronger than PR-A, probably due to transcriptional synergism between AF3 and one of the other AFs (207).

PR-A can inhibit the activities of PR-B as well as other members of the steroid receptor family (208) including the androgen receptor and glucocorticoid receptor (GR). Recently, an inhibitory function domain (IF) has been found in both isoforms (209). The IF is located in a 292-amino acid segment lying upstream of AF1. It represses the activity of PR-A but not PR-B due to constraints imparted by the BUS which contains AF3 (209). These observations may explain why only PR-B can activate transcription in the presence of some anti-progestins. The IF is functionally independent and strongly represses transcription. When the IF is fused into the N-terminal region of ER, it silences ER-dependent transcription.

Though the two PR isoforms are transcribed from the same gene, two functionally distinct promoters have been found in both humans (205) and rats (206). By using the human PR gene 5'-flanking sequences as promoter regions in a chimeric gene, Kastner *et al.* (205) showed that the first functional promoter (located between -711 and +31) directed initiation of transcription of hPR-B mRNAs from starting sites located at +1 and +15 (ATG for hPR-B translation located at +744). More importantly, there is second promoter located between +464 and +1105 to initiate hPR-A mRNAs and transcription start site is located between +737 and +842 (ATG for hPR-A translation located at +1236). The rat PR gene 5'-region is structurally similar to its human homologies, although the rat PR 5'-untranslation region is approximately 15 % smaller. Two functionally promoters (-131/+65 and +461/+675) also have been found in rat PR gene (206). (Fig. 1-6)

A third PR isoform, PR-C has recently been reported in human breast cancer cell line (210). PR-C is also a N-terminally truncated product but smaller than PR-A. It has only the second zinc finger of the DBD but has an intact HBD. It is possible that PR-C has a specific P_4 binding function (210,

40

211) and acts as a selective suppressor of P_4 action by binding to P_4 to curtail P_4-binding to active receptor forms.

Although expression of PR expression is known to be regulated by estrogen, the molecular mechanisms underlying this regulation are not well understood. In the human PR promoter, no classical estrogen responsive element is detected in the corresponding sequences but both promoters are estrogen inducible (205). In rats, there are four weak, but functional, imperfect EREs as well as several half-sites, widely spaced and spread in the 5'-flanking region and the 5'-untranslated region (212).

In spite of the importance of P_4 throughout pregnancy, there is little information concerning the concentrations of PR-A, PR-B and PR-C in pregnant uterine tissue. Ogle *et al.* (213) reported that PR-C was the dominant form in rat decidual basalis in early gestation. Studies in this thesis report the gestational changes and differential expression of PR in rat uterine tissues in late pregnancy (see chapter 5). These types of studies may be important because in primates, including the human, there is no decrease in maternal plasma P_4 levels before parturition occurs. A change in the relative expression of the PR isoforms, could achieve a "P_4 withdrawal" within intrauterine tissues without changes in concentrations of P_4 or in total binding of P_4 to uterine tissues. This thesis provides some data addressing this hypothesis which may help us to better understand the role of P_4 in controlling the labour onset.

ER:

The original human ER (now known as ERα) gene is localized on chromosome 6. In 1995, a second isoform of ER, ERβ was discovered and localized on chromosome 14 (214). The ERβ protein is smaller than the ERα but possesses the modular structure of distinct functional domains characteristic of the members of the superfamily of nuclear receptors. The

41

amino acid sequence in both DBD and HBD demonstrates considerable homology between ERα and ERβ (215). The specific physiological actions of ERβ and its functional interaction with ERα have not yet been resolved, although studies indicate that ERα and ERβ can interact *in vitro*, cross-signaling with each other (216).

Relative binding studies *in vitro* have shown that many ligands have a similar affinity for both isoforms but some ligands, for example, 17β-estradiol has an ERα-selective agonist potency and 16β,17α-epiestriol has an ERβ selective agonist potency (217). Furthermore, some anti-estrogens, such as tamoxifen (TAM) and 4-OH-TAM, have partial ERα-selective agonist/antagonist function but a pure antagonist effect through ERβ (217).

Estrogens regulate the transcription of target genes by two types of mechanisms. The classical type of response consists of receptor homodimers, occupied by ligand, binding to ERE to stimulate transcription. The ER may also mediate gene transcription from an activator protein 1 (AP1) enhancer element that requires ligand and the AP1 transcription factors, Fos and Jun for transcriptional activation. However, ERα and ERβ function differently at AP1 sites. When activated by 17β-estradiol, ERα stimulated transcription whereas ERβ inhibited transcription (218).

Some biologic responses may only be elicited by a specific receptor isoform. Young *et al.* (219) found that OTR was increased several-fold in response to estrogen treatment in the wide-type mouse brain, but this increase in OTR binding was completely abolished in knockout mice lacking ERα. These data suggest that ERα is absolutely essential for the induction of OTR binding, at least in the brain tissues (219).

42

1.5.5.1.4. Effects of Sex Steroids on OT and OTR Gene Expression

Estrogen, Progesterone and the OT gene

Several studies have demonstrated the importance of estrogen and P_4 in regulating intrauterine OT gene expression. Both rat and human OT genes contain ERE and additional half ERE. Richard *et al.* demonstrated that these EREs are estrogen sensitive in transfected cells (56). However, very recent studies by Stedronsky *et al.* (60) showed that ER has little or no interaction with ERE located in the human OT gene in estradiol-stimulated breast cancer cells. Furthermore, the promoter of the OT gene has no P_4 responsive element (PRE), suggesting that the effects of P_4 might be through indirect or non-genomic mechanisms. Estrogen significantly increases OT mRNA and OT peptide in late gestational human amnion-chorion-decidua tissues using an *in vitro* explant system (201). P_4 appears to have a variable effect but in one study it appeared to block the estrogen-induced increase in mRNA encoding OT (201). Lefebvre *et al.*, (220) using non-pregnant rats treated with estrogen, P_4 or both steroids over several days, demonstrated that estrogen stimulated OT gene expression in uterine tissues. P_4 alone had little or no effect on OT gene expression. However, if P_4 was combined with estrogen, it had a strong synergistic stimulatory effect on uterine OT gene expression.

In both humans (221) and rats (222), increased secretion of OT from the pituitary has been correlated with high concentrations of maternal serum estradiol. Exogenous estrogen can increase plasma OT concentration in women (221). However, the effects of estrogen on hypothalamic OT gene expression *in vivo* have not been consistent. Estrogen administration to ovariectomized rats has been reported to reduce (223), increase (224), or leave unchanged (225) OT mRNA in OT neurons of the hypothalamus. P_4 has been shown to increase OT mRNA in the PVN (226). In contrast, another study demonstrates that exogenous P_4 implants decrease OT mRNA in the PVN and

43

SON in late rat gestation (227). Amico *et al.* (227) treated ovariectomized rats with implants initially with estrogen and P_4 and later removed only the P_4 to mimic steroid changes in late gestation. They found OT mRNA was increased in the hypothalamus of animals receiving this regimen compared to sham treated cohorts, but no increase was found in OT mRNA, if estrogen was not administrated or if P_4 was not withdrawn prior to sacrifice. These data suggest that both estrogen and P_4 are important but P_4 withdrawal plays a pivotal role in induction of the hypothalamic OT gene in the estrogen-primed rat.

Estrogen, Progesterone and the OTR gene

There is considerably more evidence for sex steroid regulation of OTR, compared to the evidence for that of OT. In 1975, Soloff *et al.* reported that estrogen increased the number of OTR in rat uterine tissues and this could be blocked by concurrent administration of P_4 (129). Similar results have been obtained using rat uterine explants (228) and in human myometrial cells in primary culture (229). Withdrawal of P_4 from estrogen-pretreated pregnant rats results in prompt resumption of OTR synthesis (230).

Most OT binding sites are upregulated by estrogen. This includes hypothalamic (88), pituitary (231), uterine (232) and renal (94) OT binding sites. Furthermore, treatment of immature ovariectomized rats with estradiol significantly increases uterine OTR mRNA levels (4- to 5-fold) and OT binding (6-fold), while cotreatment with P_4 strongly reduces OT binding by 75 % but does not significantly affect the estrogen-induced rise in OTR mRNA (147, 232). However, P_4 alone has no effect on either OTR mRNA or OT binding in immature rats (147). In non-pregnant sheep, P_4 alone acts as an inhibitor for expression of myometrial OTR gene (233)

Evidence suggests that the effects of estrogen and P_4 are mediated in different ways. The rat OTR gene contains several sites corresponding to ERE

and half ERE palindromes (103, 107) and there is no PRE in the OTR gene. Soloff's laboratory (234) demonstrated that the estrogen-induced up-regulation of OTR protein required ongoing protein synthesis but the specific P_4-induced down-regulation was also observed in the presence of protein synthesis inhibitors. This information suggests that estrogen regulation occurs, at least in part, via increased receptor biosynthesis or involves synthesis of protein mediators. The estrogen effect might also be mediated by an increase in OTR mRNA stability. Conversely, the effects of P_4 on OTR may involve protein-protein interactions between preformed proteins or nongenomic mechanisms.

Studies in the human nonpregnant uterus have given more confusing results. Myometrial OTR concentrations were lowest in both menopausal women who have a very low estrogen concentrations and in cyclic women at mid-cycle who have high estrogen concentrations (235). There was no correlation with urinary estrogen concentrations. The highest myometrial OTR concentrations were found in the late luteal phase and were positively correlated with urinary P_4 metabolite concentrations, suggesting that P_4 does not suppress myometrial OTR levels in the late luteal phase.

The data from rats and primates suggest that the regulation of OTR may be different between these two species. Alternatively, it is possible that there is a paracrine system within the human uterus wherein estrogen and P_4 concentrations are not reflected in serum or urinary measurements.

1.5.5.1.5. Sex Steroid Antagonists

Antagonists

Hormone antagonists are compounds that compete with hormones for receptor binding but fail to trigger the normal response and thereby inhibit the action of the hormones.

45

Antiestrogens:

The first synthetic antiestrogen to be described was a triphenolic compound called MER25. Although this antiestrogen is too toxic to be used clinically, it led to the synthesis of clomiphene, another antiestrogen used in induction of ovulation in subfertile women. Later, numerous antiestrogens have been developed, particularly for treatment of advanced breast cancer. The main therapeutic antiestrogen that has been used to date is TAM.

TAM has a triphenyl-ethylene structure with two rings corresponding to the A and D rings of estradiol. TAM is converted, mainly in the liver, to 4-hydroxytamoxifen, and this converted form increases its affinity for the ER, and consequently its potency by approximately 100-fold (236). The alkylamide side-chain of TAM is essential for its antiestrogenic activity. Although TAM has turned out to be an effective drug for the treatment of hormone dependent breast cancer, it was recognized that it was actually a partial agonist. The relative antagonistic and agonistic activities depend on the physiological response. For example, in the high estrogen milieu, such as late pregnancy, TAM acts as a strong antagonist.(237). The binding site for TAM overlaps with that of estrogen binding site, so it acts as a competitive inhibitor of estrogen action. TAM binding allows the receptor to dimerize and bind to DNA with high affinity, but blocks transcriptional activity. The agonistic activity is thought to be derived primarily from AF1, which appears to function even when TAM is bound to the receptor. (as reviewed in (238))

More recently developed antiestrogens such as ICI 182780 are completely devoid of agonistic activity and are referred to as pure antiestrogens. The effectiveness of ICI 182780 as antiestrogen depends on an alkylamine side chain with an optimum length of 16 -18 carbon atoms at the 7α position in the B ring in the steroid. The mechanisms of action of this pure antiestrogen may be two-fold. First, it prevents nuclear uptake of the ER so

46

that during the process of nucleocytoplasmic shuttling the receptor accumulates in the cytoplasm and is degraded, thus causing a decrease in the cellular content of receptor protein by markedly reducing its half-life (239). Second, ICI 182780 disrupts dimerization, thus inhibiting DNA binding. *In vitro* studies showed that this interfering with dimerization was due to the 7α long carbon side chain (238).

Estrogens are known to produce an increase in uterine blood flow and volume as well as an increase in uterine weight (240). Estrogens are also essential for fetal growth during pregnancy (240). The mechanisms of action of estrogen in inducing increased blood flow could be through their receptors present in the uterus or by a direct effect on vascular smooth muscle. Increases in uterine blood flow and the stimulation of various macromolecular syntheses in the uterus-fetal compartment result in both uterine and fetal growth. Majid *et al.* (241) have shown that in the rat, a single injection of estrogen resulted in a peak in uterine blood flow 3 hours later and increased uterine wet and dry weights 24 hours after the injection. Pretreatment with TAM inhibited or reduced significantly the uterine weight and uterine blood flow responses to estrogen. Furthermore, TAM inhibited endometrial cell division in the immature rat (242) and the process of decidualization in pseudopregnant rat (243). However, there is little information about the effects of TAM during late gestation. Whether TAM can affect the gestational length and the fetal growth need further investigation. In chapter 3 of this thesis, further data is presented regarding the effects of TAM on rat fetal growth and in the process of parturition.

Antiprogesterone:

After the discovery of the PR, ideas began to form about a molecule that could compete with P_4 for its binding site on the receptor, and at the same time

47

block the receptor in a non-functional formation. In 1975, a mid-cycle form of contraception by administration of an antiprogesterone was proposed, but it was difficult to use. The search for a better P_4 antagonist was based on studies that had previously produced efficient anti-estrogens such as nafoxidin and TAM. New P_4 antagonists were tested, among which Roussel Uclaf's RU486 (mifepristone) turned out to be the most interesting one (244). RU486 binds with high affinity to the receptors for progestins and glucocorticoids, and antagonizes both their activities. Its structure resembles that of P_4 and cortisol but contains an 11β-aryl substitution. RU486 interacts directly with the HBD of PR or GR and competitively inhibits P_4 or glucocorticoid binding. Upon hormone binding, RU486 promotes displacement of heat shock proteins, permits dimerization and facilitates association of the receptor with DNA. However, DNA binding does not trigger transcriptional activity.Recently, some studies have shown that the binding of RU486-PR complexes to DNA may exhibit agonistic activity in some tissues, such as the primate endometrium (as reviewed in (245)).

A subsequent antiprogesterone compound (ZK98299; onapristone) has been extensively studied. Onapristone and RU486 show differences in various *in vitro* and *in vivo* models with regard to pharmacodynamic and pharmacokinetic properties. The binding affinity of onapristone to PR and GR is a little lower than that of RU486. The molecular mechanisms of action of onapristone seem to differ from RU486 in that onapristone impairs the binding of the PR complexes to the PRE in the promoter of a PR-regulated gene. Thus, onapristone may be considered as a "pure" P_4 antagonist (as reviewed in (246)).

Antiprogesterone effects during pregnancy vary according to species and stage of pregnancy. Species with physiological P_4 withdrawal at term, such as rats and sheep respond to RU486 during late pregnancy by preterm

delivery shortly following administration (247). Primate species appear to respond better to onapristone (as reviewed in (246)). RU486 alone did not successfully induce preterm parturition in monkeys but it was very effective in inducing deliveries in combination with OT. In contrast, onapristone alone could effectively induce parturition in non-human primates. In humans, RU486 is not effective alone in inducing second trimester abortions, but is highly efficacious in combination with a prostaglandin (245). The scant clinical data available show that term labour can be induced with RU486 alone in approximately 50% of women.

To further investigate the mechanisms underlying the mechanisms of antiprogesterone, we have studied the effects of RU486 on uterine sex steroid receptors, OT, OTR and prostaglandins (PGs) in late rat gestation (Chapter 4).

1.5.5.2. Interactions between OT and PGs

Several previous studies have suggested an interesting relationship between PGs and OT. Flint *et al.* found a role for luteal OT as well as $PGF_{2\alpha}$ in the control of luteolysis in ruminants, proposing that a positive feedback mechanism operates between luteal OT and uterine $PGF_{2\alpha}$ (31). In pregnant intrauterine tissues, OT stimulation of the myometrial OTR leads to an immediate influx of Ca^{++} into the myocyte cytoplasm from both extracellular and intracellular sites, resulting in a contractile response (108). However, Wilson *et al.* reported that OT at physiological concentrations also stimulated the immediate release of free arachidonic acid (AA) in dispersed human decidual cells in a perfusion system. This AA may be used to enhance prostaglandin synthesis (248). Furthermore, Fuchs *et al.* (249)showed that OT causes a significant increase in the production of both PGE and PGF in decidua and in the production of PGE in amnion but had little effect on myometrial PGF or PGE production. Similar findings have been reported in rat

49

endometrial cells (184). These data suggest that OT is a physiological stimulatory factor for PG production in rat endometrium and human fetal membranes.

Several studies suggest that PGs are important stimulants of OTR synthesis. Using the rat model, Alexandrova *et al.* (250) found that the administration of $PGF_{2\alpha}$ to day 18 pregnant dams resulted in premature delivery on day 20 and this $PGF_{2\alpha}$-induced labour was associated with a rise in the concentration of myometrial OTR. The PG synthesis inhibitor naproxen markedly attenuated *in vitro* PGE_2 and $PGF_{2\alpha}$ release and delayed the increase in OTR concentration by 24 h in term rats. However, co-administration of $PGF_{2\alpha}$ reversed the suppressive effects of naproxen on OTR, suggesting the late gestational increase on OTR can be blocked with PG synthesis inhibitors (251). Therefore, it is possible that there is also a positive feedback system between OTR and PGs within intrauterine tissues. This paracrine interaction within the pregnant uterus may increase myometrial contractility at the time of parturition.

1.5.5.3. Immune Mediators

Recent evidence supports an association between the occurrence of an intrauterine inflammatory response and labour onset, either at term or preterm. However, the precise mediators connecting these two events are unknown. In the past few years, three lines of evidence suggest that the immune system plays some role in the process of parturition. First, around the time of labour onset, there is influx of bone marrow-derived cells of the immune system into the decidual lining of the pregnant uterus (252). These cells account for 47% of total decidual cells at term and include macrophages (18 %), large granular lymphocytes (3 %) and T cells (8 %). The remaining cells, the proportion of which varied between individuals, are CD16-positive granulocytes. Second,

the concentrations of cytokines in amniotic fluid are elevated at the time of term or preterm and markedly increased in labour accompanied by intrauterine infection. Third, human decidua is capable of synthesizing cytokines. Decidual cells produce PGs in response to many of these cytokines (253).

1.5.5.3.1. Inflammatory Cytokines

Cytokines are a group of polypeptides that act non-enzymatically to regulate the growth, differentiation, and function of cells. Generally, cytokines are produced in relatively small quantities to act locally in a paracrine or autocrine role, with a few being released into circulation in measurable amounts. Therefore, they are crucially important in the local as well as the systemic reactions of the acute phase response, and in both the cellular and humoral arms of the immune system.

Cytokines in intrauterine tissues:

Abundant recent data implicate the inflammatory cytokines IL-1β, IL-6 and TNFα in the mechanism of human parturition. In the amniotic fluid of preterm women associated with clinically evident infection, the concentration of TNFα (254), IL-1β (255) and IL-6 (256) are markedly increased. These levels are also increased at normal term labour and preterm labour in the absence of infection (257). Maternal serum IL-6 concentrations increase significantly at the time of normal term parturition (257). Human decidua has been shown to be a major source of all three inflammatory cytokines (258-260). However, only a very small fraction of cytokines synthesized in the decidua (e.g. 4 - 7 % for IL-6) may be transmitted across the fetal membranes intact, suggesting that the cytokines in amniotic fluid are unlikely to be a reflection of decidual cytokine production (261). It is likely that the majority of cytokine production in intrauterine infection is from inflammatory cells that

51

have infiltrated to the amnion and not from local decidual cells. Furthermore, the syncytiotrophoblast of placental villi also are a potential source of cytokines. Chorion secretes more IL-6 than decidua under basal conditions and this increases in response to IL-1β stimulation (as reviewed in (262)). The increases in decidua or chorion IL-6 production in response to IL-1β and TNFα can be demonstrated at the mRNA level (260, 262).

There may be many interactions among cytokines, involving positive feedback mechanisms within intrauterine tissues, particularly in decidual tissue, that amplify local production of inflammatory mediators. As noted previously, IL-1β can induce expression of TNFα and IL-6, while TNFα also induces the expression of IL-6 and IL-1β (260). These two cytokines can also enhance their own production. However, there is no evidence showing that IL-6 has any positive effect on TNFα or IL-1β production but inhibitory effects on the secretion of these two cytokines in response to LPS.

Cytokines and parturition:

Each of the three inflammatory cytokines was shown to stimulate the rate of production of PGE_2 by amnion cells and decidual cells *in vitro* (as reviewed in (262)). The concentrations of these cytokines required for such stimulatory actions are within the ranges measured in amniotic fluid of women with intra-amniotic infections. Infusion of IL-1β into the amniotic fluid of rhesus monkey in the preterm period resulted in significant increases in intra-amniotic TNFα as well as PGE_2 and $PGF_{2\alpha}$ accompanied by preterm labour (263). The mechanisms whereby cytokines stimulate PG biosynthesis have been extensively studied. There is evidence that TNFα and IL-1β can induce fibroblast PGHS protein (185, 186). Cytokine-induced stimulation can be mediated by transcription factors, such as nuclear factor-IL6 (NF-IL6) (see later). It has been suggested that TNFα may also directly stimulate AA release

in human neutrophils (264). Moreover, recent studies have shown that both IL-1 and TNFα increased endothelin, another uterotonic agent produced by amnion and this stimulatory effect was noted at the mRNA level. Endothelins, however, do not cross the fetal membranes to any significant extent (262).

Several research groups have investigated whether certain cytokines or LPS can induce preterm birth in animal models. Romero *et al.* reported that administration of IL-1α to day 15 pregnant C3H/HeJ inbred mice (genetically endotoxin resistant) induced preterm labour within 24 hours (265). Others also found that preterm parturition occurs after intra-amniotic administration of IL-1α and TNFα in pregnant rabbit (266). Moreover, intraperitoneal injection of LPS to C3H/HeN pregnant mice at 15 days of gestation (70 % gestation) induced preterm delivery associated with large increases in maternal serum concentrations of TNFα , IL-6, and IL-1α and in amniotic fluid concentrations of IL-6 and IL-1 (267).

Based on these data, we have proposed that certain cytokines in intrauterine tissues are important mediators of parturition and have initiated experiments to determine their effects on expression of OTR in the rat uterus (Chapter 6).

1.5.5.3.2. Transcription Factors

Recently, the mechanisms of cytokines in regulation of the target gene in the local reproductive tissues have been extensively studied. The effects of inflammatory cytokines on myometrial activation are likely to be mediated by transcription factors. The transcription factors may further regulate the expression of the CAP such as OTR, connexin 43 and PGHS.

Nuclear factor IL6 (NF-IL6):

NF-IL6 is a transcription factor that regulates transcription of many genes in the immune system. Originally, it was identified as a mediator binding to the IL-1-response element of the IL-6 gene. Recently, it was found to induce a number of acute-phase proteins in response to LPS, IL-1, IL-6, TNFα and nerve growth factor in many cells. It is activated by phosphorylation with PKC and/or other kinases. Following phosphorylation, NF-IL6 translocates to the nucleus and dimerizes at the leucine zipper domain. Thus, the carboxy-terminal basic domain interacts with target DNA. Target gene transcription can be stimulated or inhibited by NF-IL6. The amino terminal region of NF-IL6 protein is responsible for transactivation and this can be inhibited by MAP kinase mediated by phosphorylation of the internal portion of the molecule (as reviewed in (268)).

The consensus response element for NF-IL6 is T(G/T)NNGNAA(G/T). Rat OTR promoters contain six (twice in reverse orientation) and three of these motifs respectively. The role of NF-IL6 in regulation of OTR gene expression has not been studied. The human PGHS -2 gene promoter also has two NF-IL6 response elements (269) that may mediate the TNFα-inducted stimulation of PGHS -2 expression (185). NF-IL6 is also a major regulatory factor of PGHS -2 transcription in rat smooth muscle cells (270) and human pancreatic islets (271).

Acute-phase response factor (APRF) and NF-1-related factor:

APRF is a member of the STAT family of transcription factors. Rat OTR has four acute-phase response elements (CTGGGA), recognized by APRF. Three motifs are centered upstream of the cap site and one motif is within intron one. The motif is present and functional in a series of other IL-6-regulated acute-phase response genes. Rat OTR also contains a TGGCA

54

motif, an element recognized by an NF-1-related factor that is found associated with IL-1-responsive promoters (103).

Activator protein 1 (AP-1):

Estrogen is a primary regulator of OTR through direct controlling expression of OTR gene in human and rat tissues. In fact, there are one palindromic ERE in the 5' flanking region as well as several widely spaced half-palindromic EREs (TGACC or GGTCA) in the rat OTR gene 5' flanking region, with one located at 25 bp 5' to the cap site. It is possible that these half EREs may confer estrogen responsiveness, as is the case with the chicken ovalbumin gene (272). An alternate or additional possibility is that the sex steroid effects are indirect and mediated by other factors. One of possibilities is through AP-1, another transcription factor. This mechanism involves protein-protein interactions with the ER modulating the activity of AP-1. AP-1 consists of homo- or heterodimeric of Fos and Jun oncoproteins. Both rat and human OTR promoters contain AP-1 binding sites, suggesting potential regulation by c-Fos/c-Jun heterodimers. Fos and Jun are expressed at low levels in myometrial tissue but their mRNAs can be increased by estrogen treatment (273, 274). P_4 can inhibit the estrogen-induced increase in c-*fos* in rat uterus (275). The promoter region of Cx-43 gene, a major gap junction protein also has two AP-1 sites. The estrogen-induced increase in Cx-43 in late pregnancy is preceded by an increase in c-*fos* and c-*jun* expression, associating with activation of the myometrium in humans (276) and rats (277). Inflammatory cytokines IL-1β and TNFα can stimulate AP-1 gene expression in human hepatoma cells (278). There is little information about the interactions among the inflammatory cytokines, AP-1 and the gene expression for contraction associated proteins.

55

Interactions among transcription factors:

Adding to the complexity of control mechanisms for gene expression are the permutations of possible protein-protein interactions among transcription factors. The basic leucine zipper domain of NF-IL6 can interact with other members of the bZIP family, including Fos and Jun. Estrogen receptors also can directly interact with NF-IL6 and modify their interaction with DNA. NF-IL6 homodimers can bind to both NF-IL6 and AP-1 sites, whereas Fos and Jun cannot bind to most NF-IL6 sites. Furthermore, some studies indicate that NF-IL6 also binds to acute phase response elements (CTGGGA). Many acute phase protein genes harbor both NF-IL6 and STAT binding motifs in their promoter region, suggesting that these two families synergistically activate acute phase protein genes (as reviewed in (268)).

Based on this information, there is a speculation that the interactions between the cytokine related transcription factors and response elements in promoter regions of the genes for contraction associated proteins have great potential for positive feedback mechanisms that may be important in the evolution of labour.

1.5.5.3.3. Ratio of Th-2 to Th-1 Specific Cytokines in Parturition

Within the immune system, the activation of B and cytotoxic cells depends on a variety of cytokines that are synthesized and secreted by several kinds of T helper (Th) cells. During normal human and mouse pregnancies there exists a dominance in Th-2 specific cytokines within the uterine environment, wherein Th-2 activity is increased and Th-1 activity is decreased (279). Th-2 cells produce cytokines, such as IL-4, IL-5, IL-6, IL-10 and IL-13, which stimulate antibody generation. Th-1 cells, however, produce cytokines, such as IL-2, TNFβ and INFγ, that are needed for cellular responses. Throughout most of pregnancy, there is an enhanced maternal humoral

56

immune response and a diminished cellular response. Normally, Th-2 cytokines are beneficial to both mouse and human trophoblast, whereas Th-1 cytokines lead to fetal demise in the mouse (280). According to the current theory, the Th-2 cell activity dominates during pregnancy because Th-2 specific cytokines are required for the maintenance of pregnancy and perhaps for the normal development of the fetus, whereas Th-1 cytokines may be detrimental to pregnancy, and their actions may be responsible for labour onset.

Mosmann *et al.* (281) reported that IL-10 (a Th-2 product) can down-regulate the function of Th-1 cells, and INFγ (a Th-1 product) can inhibit the function of Th-2 cells. PGE_2 is thought to be involved in the Th2/Th-1 responses, but the role is still uncertain. Although PGE_2 stimulates cAMP in both Th-1 and Th-2 cells, this results in inhibition of the function only of Th-1 cell (282). Additionally, some studies indicate that the Th-2 cytokines (e.g. IL-4) may suppress PG release by inhibiting the secretion of the PG-stimulating Th-1 cytokines (283). This adds increasing strength to the theory that Th-2 cytokines maintain pregnancy, while Th-1 cytokines serve to terminate the pregnancy.

1.5.6. Summary

Parturition is a complex process. It involves a multifactorial system of interconnected positive-feedback loops. OT is a powerful stimulant of the myometrium and it has been used clinically to induce labour in women for half a century. However, there is still controversy over its role in the endogenous mechanisms responsible for the initiation of term and preterm labour. It is possible that OT secreted by maternal and fetal hypothalamus plays little role in the onset of labour, but intrauterine produced OT, as part of an intrauterine paracrine system, could regulate myometrial contractility. Interaction between

57

PGs and OT at the time of parturition could be an important component of this paracrine system. The inflammatory cytokines accumulated in the amniotic fluid in pregnancies complicated with intrauterine infection and inflammation may have a primary role in activation of this paracrine system. They also may play an important role in the physiological process of normal parturition. Other factors are also present in the uterus and influence certain aspects of uterine function in late pregnancy, but their physiological roles remain to be investigated.

Estrogen and P_4 are mainly concerned as regulators of this paracrine system. The ratio of estrogen to P_4 in the uterus possibly influences the contractility of the myometrium at the time of labour onset. The regulation of estrogen and P_4 could be through several levels in the uterine paracrine network: controlling the synthesis of uterotonic factors (OT, PGs and etc.) and their receptors, influencing the quantities or differential expression of the isoforms of their own receptors, and modulating the interactions among intrauterine paracrine systems. There could be important non-genomic as well as classical genomic mechanisms of these steroids.

The involvement of OT in initiation of normal and preterm labour and the potential regulators of OT and OTR in this paracrine network requires further investigation. The information provided in this thesis hopefully will provide a better understanding of biological and physiological mechanisms of rat parturition at term or during the preterm period. This may form a basis to design better strategies for prevention or treatment of disorders of the timing of parturition in the human.

Figure 1-1   Rat OT gene and promoter region.

a. The schemes to show the structural organization of rat OT gene. Protein-coding regions are shown as boxes: SP, signal peptide; NP-I, neurophysin-1.

b.   Promoter region of the rat OT gene. Two imperfect palindromic ERE, four retinoic acid response elements (RARE) and two thyroid response elements (TRE) are indicated. Translation start site is indicated.

60
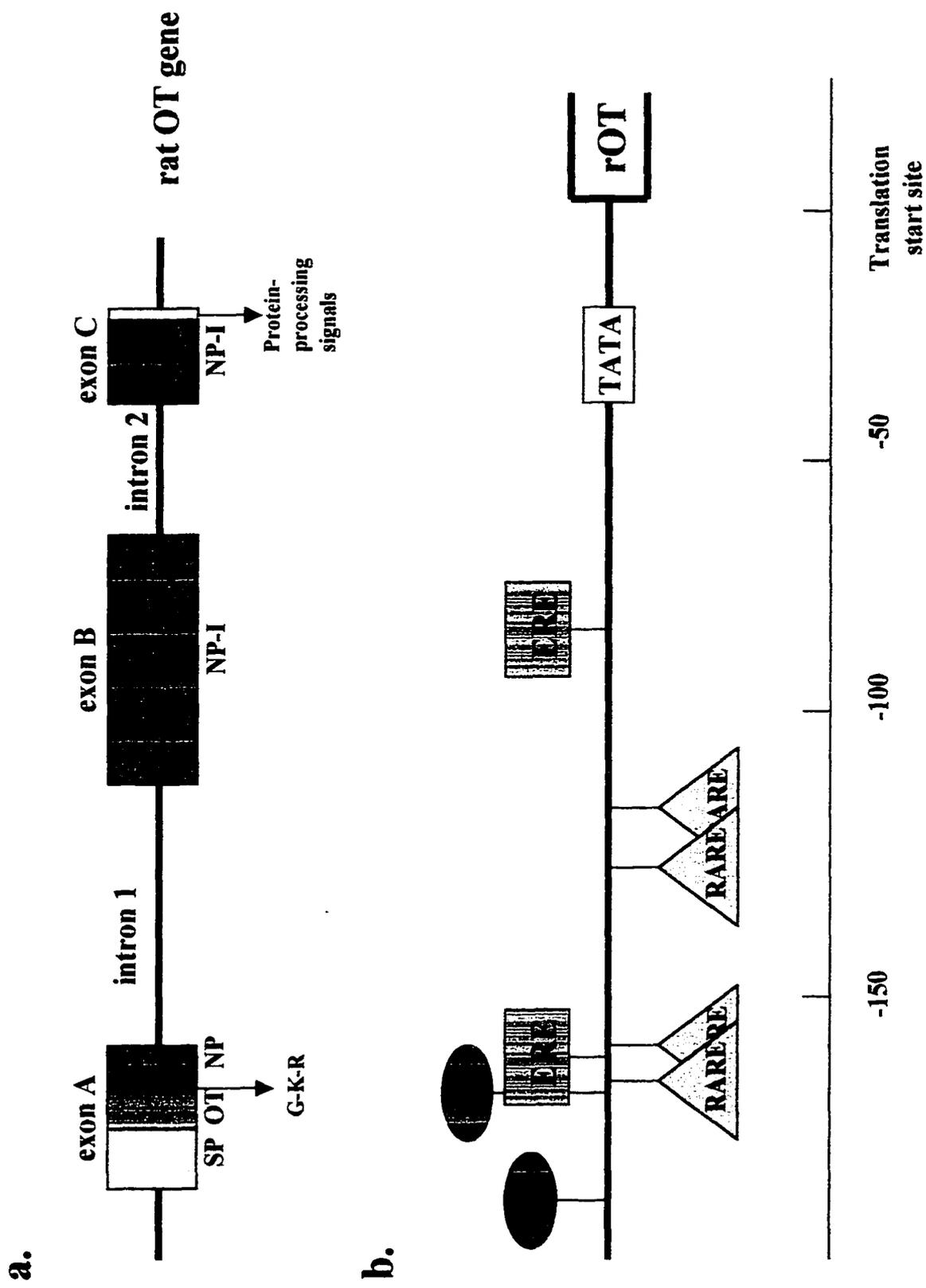
Figure 1-2   Posttranslational processing of the OT-NP-1 preprohormone.
The initial translation product is cleaved serially by endopeptidase and
carboxypeptidase B enzymes and the resultant OT-gly is acted on by α-
amidating enzyme to form mature amidated oxytocin.

Oxytocin-Gly-Lys-Arg-Neurophysin I

$\downarrow$ *Endopeptidase*

Oxytocin-Gly-Lys-Arg + **Neurophysin I**

$\downarrow$ *Carboxypeptidase B*

Oxytocin-Gly + **lysine** + **arginine**

$\downarrow$ *α-Amidating enzyme*

Oxytocin (NH$_2$)
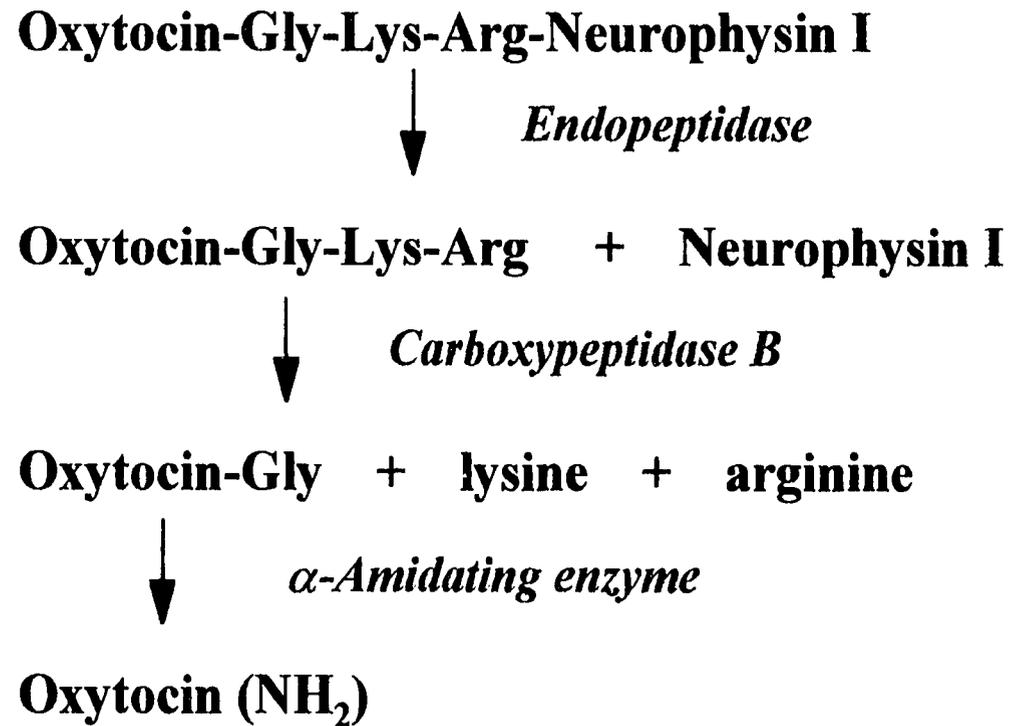
Figure 1-3   Metabolism of the OT molecule by enzymes measured in human decidua or rat uterus. OT is metabolized through two major enzymes: cystine aminopeptidase which initially cleaves the cys^1-tyr^2 bond to open the ring structure and then cleaves amino acids from the amino terminal of the molecule, and post-proline endopeptidase which cleaves between the Pro^7 and Leu^8 amino acids.

Cystine aminopeptidase

Cys¹ Cys^1

S–S

Tyr² Tyr^2

Ile³ Ile^3

Gln⁴ Gln^4

Asn⁵ Asn^5

Cys⁶ — Pro⁷ $Cys^6 - Pro^7$

Leu⁸ — Gly⁹-NH₂ $Leu^8 - Gly^9\text{-}NH_2$

Post-proline endopeptidase

64
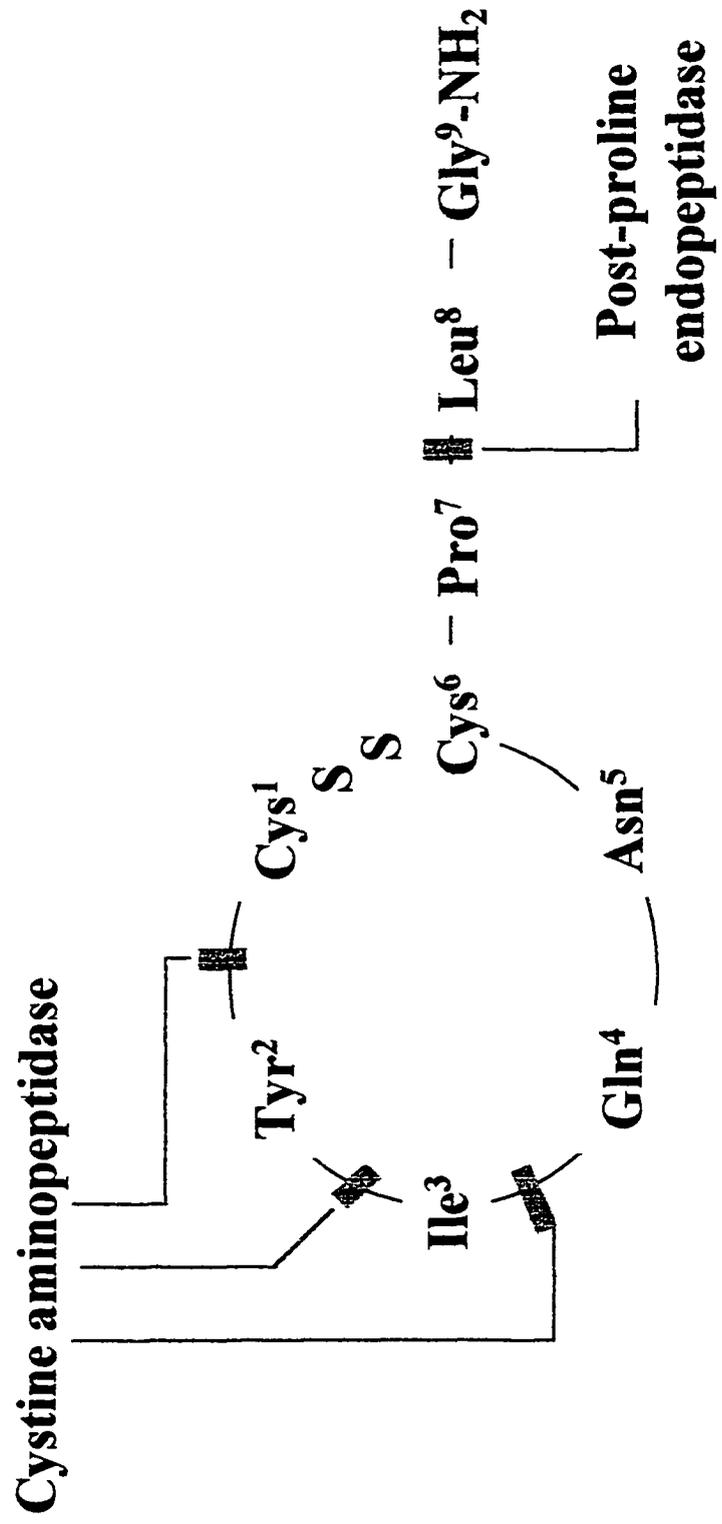
Figure 1-4   OTR gene and promoter region.

a.   Comparison of the human and the rat OTR genes. Exons are
         represented as boxed and numbered. Percentages indicate
         identity of the regions indicated.

a.

intron >12 kb

rOTR

hOTR

66
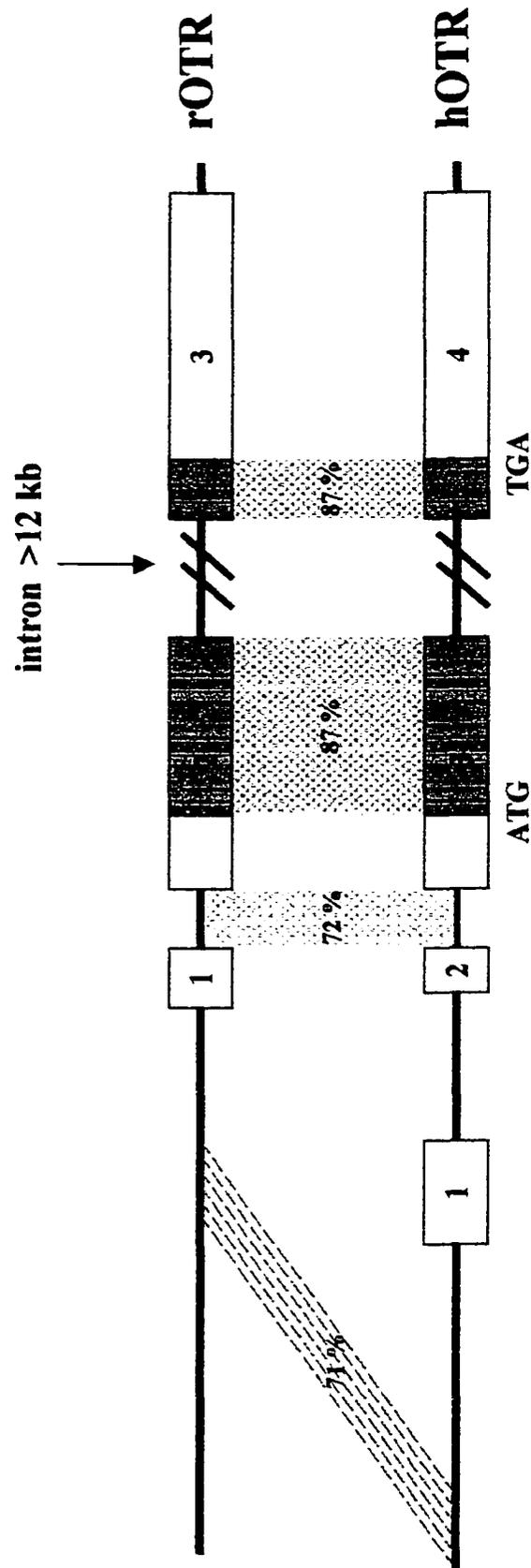
Figure 1-4   OTR gene and promoter region.

        b.   The rat OTR gene promoter region. The main translation start

site is indicated. Sequence elements corresponding to six NF-IL6 sites, acute-

phase response elements (APRE), a potential Sp1, and several AP-1 sites are

indicated. Several half ERE and a palindromic ERE are indicated. Translation

stop site is indicated.

b.

OTR

Translation
start site

NF- IL6

NF- IL6

NF- IL6

NF- IL6

NF- IL6

NF- IL6

NF- IL6

APRE

APRE

APRE

APRE

SP1

2000

3000

4000

2000

1000

68
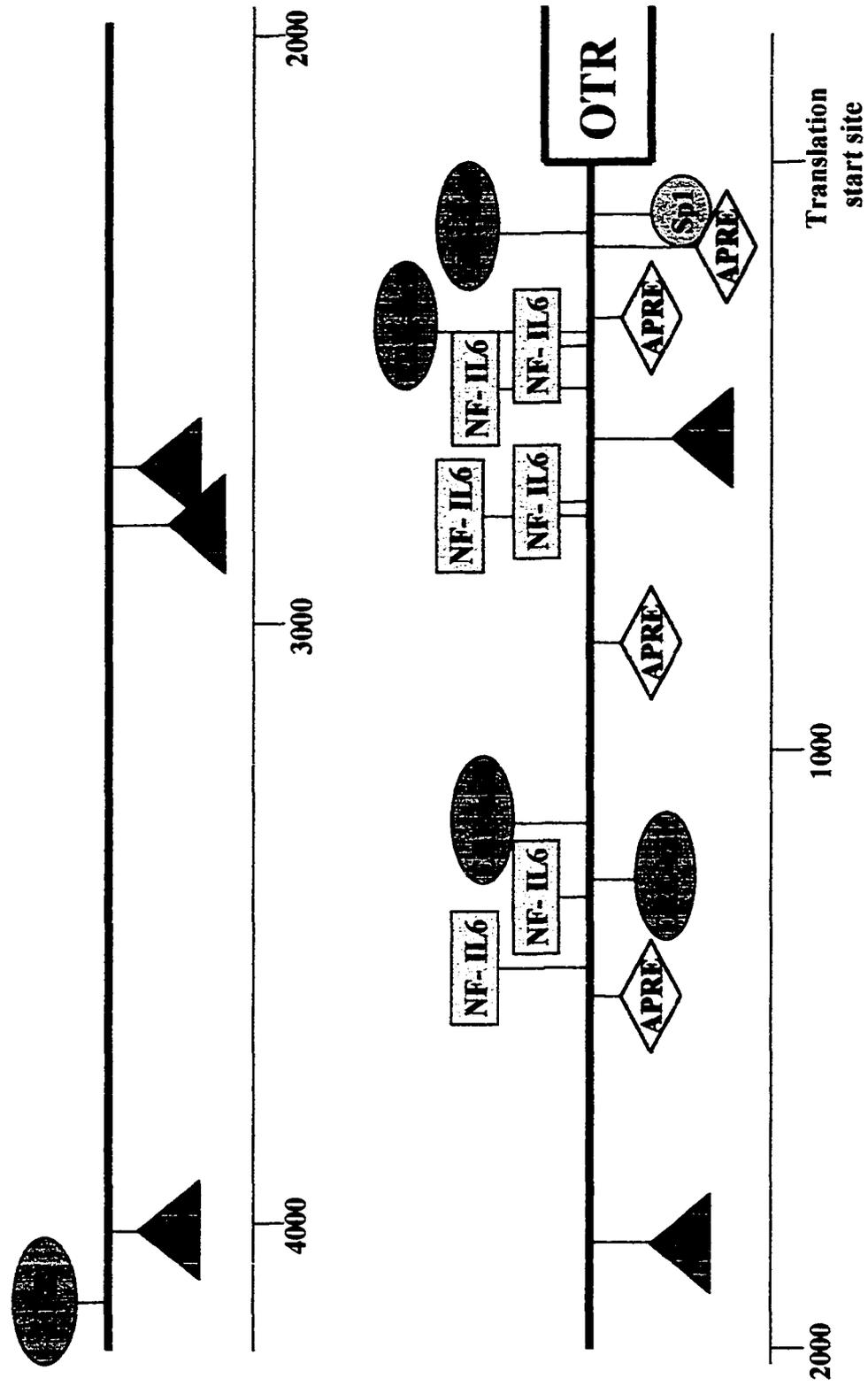
Figure 1-5   Signal transduction mechanism for the OTR in the myometrium. This receptor is a classic 7-transmembrane domain receptor linked through a G-protein to PLC. After receptor stimulation with OT, the α-subunit of the heterotrimeric G-protein hydrolyses GTP to GDP and releases the $\beta\gamma$-subunit to stimulate PLC. This enzyme converts phosphatidyl ionositides to DAG and IP_3. DAG stimulates PKC activity causing phosphorylation of substrates which will characterized the response of the specific cell type. The IP_3 stimulates flux of Ca^{++} into the cytoplasm through calcium channels, principally from the sarcoplasmic reticulum but also from the extracellular space. The increased Ca^{++} will combine with calmodulin to stimulate MLCK to produce myometrial contractions. In the decidua, a characteristic response would be synthesis and release of PGs.

cell membrane

phosphatidyl inositol

G-protein

IP$_3$

diacylglycerol

sarcoplasmic reticulum

Ca^{++}

Ca^{++} calmodulin
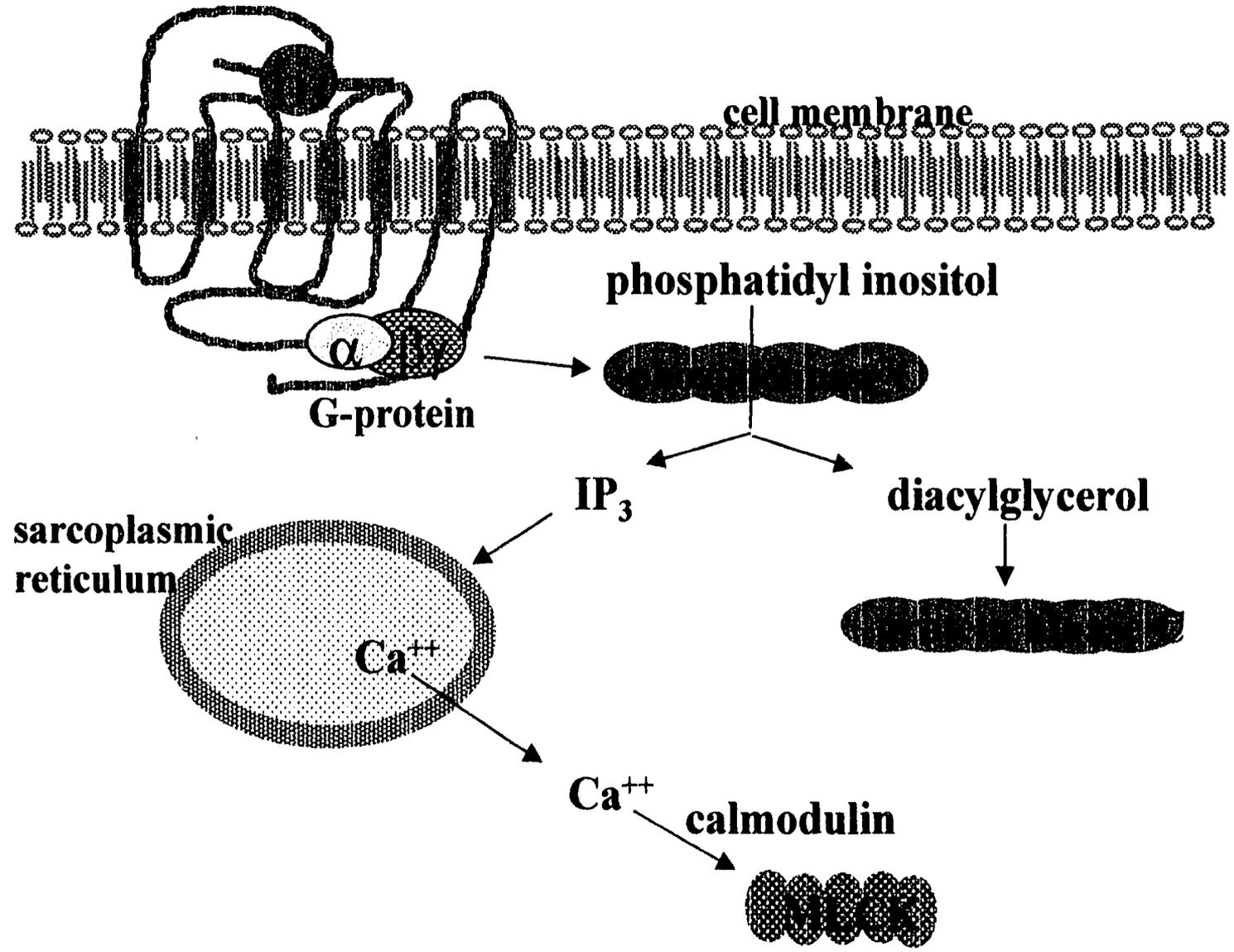
Figure 1-6   The structure of rat PR gene. Two promoters, promoter B
(purple) and promoter A (yellow) are indicated. The Exons are presented as
boxes and numbered. The translation start sites (ATG) for each isoform are
indicated. Rat PR exists three isoforms and transcribes from the same gene.
A/B, C, D, and E/F regions are indicated. DBD, DNA-binding domain; HBD,
hormone-binding domain.

rat PR gene

rPR-B mRNA

rPR-A mRNA

rPR-C mRNA
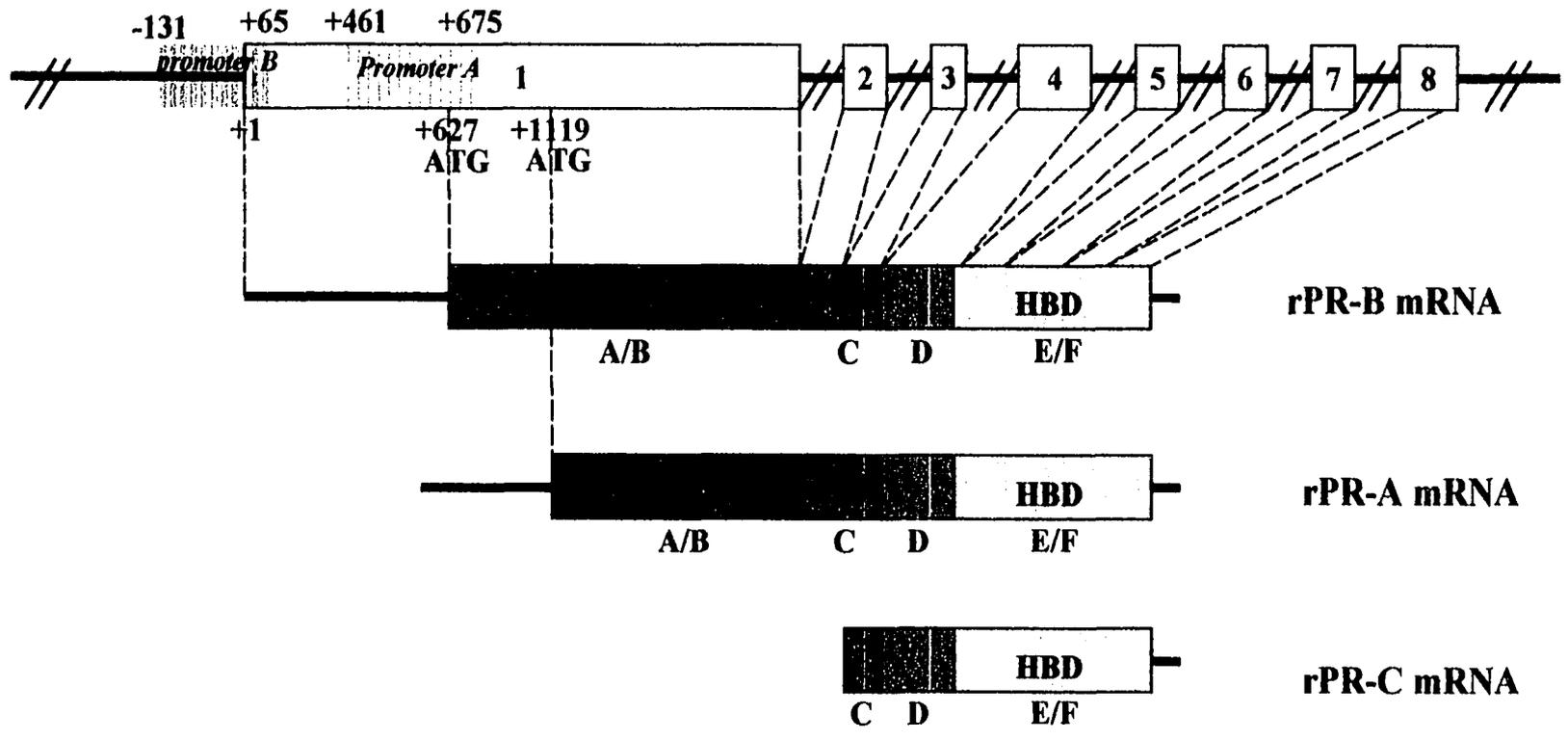
1.6. REFERENCES

1. **Chibbar R, Miller FD, Mitchell BF** 1991 Oxytocin is synthesized in human fetal membranes. Proceedings of the 38th Annual Meeting of the Society for Gynecologic Investigation, San Antonio TX, March 18-21, abstract #7, p102

2. **Lefebvre DL, Giaid A, Bennett H, Lariviere R, Zingg HH** 1992 Oxytocin gene expression in rat uterus. Science 256:1553-5

3. **Lefebvre DL, Lariviere R, Zingg HH** 1993 Rat amnion: a novel site of oxytocin production. Biol Reprod 48:632-9

4. **Fuchs AR, Fuchs F, Husslein P, Soloff MS, Fernstrom MJ** 1982 Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor. Science 215:1396-8

5. **Chen DL, Chan WY, Manning M** 1994 Agonist and antagonist specificities of decidual prostaglandin-releasing oxytocin receptors and myometrial uterotonic oxytocin receptors in pregnant rats. J Reprod Fertil 102:337-43

6. **Soloff MS, Alexandrova M, Fernstrom MJ** 1979 Oxytocin receptors: triggers for parturition and lactation? Science 204:1313-5

7. **Csapo AI, Wiest WG** 1969 An examination of the quantitative relationship between progesterone and the maintenance of pregnancy. Endocrinology 85:745-6

8. **Fuchs AR, Behrens O, Liu HC** 1992 Correlation of nocturnal increase in plasma oxytocin with a decrease in plasma oestradiol/progesterone ratio in late pregnancy. Am J Obstet Gynecol 167:1559-63

9.   **Mitchell BF, Wong S** 1993 Changes in 17 beta,20 alpha-hydroxysteroid dehydrogenase activity supporting an increase in the estrogen/progesterone ratio of human fetal membranes at parturition. Am J Obstet Gynecol 168:1377-85

10.   **Dale HH** 1906 On some physiological actions of ergot. J Physiol 34:163-206

11.   **Blair-Bell W** 1909 The pituitary body and the action of pituitary extract in shock, uterine atony, and intestinal paresis. Br Med J Part 2:1609-13

12.   **Ott I, Scott JC** 1911 The action of infundibulin upon the mammary secretion. Proc Soc Exp Biol Med 8:48-9

13.   **Schafer EA, Mackenzie K** 1911 The action of animal extracts on milk secretion. Proc R Soc Lond [Biol] 84:16-22

14.   **Du Vigneaud V, Ressler C, Swan JW, Roberts CW, Katsoyannis PG, Gordon S** 1953 The synthesis of an octapeptide amide with the hormonal activity of oxytocin. J Am Chem Soc 75:4879-80

15.   **Theobold GW, Brahm A, Cappbell J, Grange PD, Driscoll WJ** 1948 The use of posterior pituitary extracts in physiological amounts in obstetrics. Br Med J 2:123-7

16.   **Du Vigneaud V, Lawler HC, Popenoe EA** 1953 Enzymatic cleavage of glycinamide from vasopressin and a proposed structure for this pressor antidiurenctic hormone of the posterior pituitary. J Am Chem Soc 75:4880-1

17. **Land H, Grez M, Ruppert S, Schmale H, Rehbein M, Richter D, Schutz G** 1983 Deduced amino acid sequence from the bovine oxytocin-neurophysin I precursor cDNA. Nature 302:342-4

18. **Brownstein MJ, Russell JT, Gainer H** 1980 Synthesis, transport, and release of posterior pituitary hormones. Science 207:373-8

19. **Fuchs AR, Goeschen K, Husslein P, Rasmussen AB, Fuchs F** 1983 Oxytocin and initiation of human parturition. III. Plasma concentrations of oxytocin and 13,14-dihydro-15-keto-prostaglandin F2 alpha in spontaneous and oxytocin-induced labor at term. Am J Obstet Gynecol 147:497-502

20. **Ferguson JKW** 1939 A study of the motility of the intact uterus at term. Surg, Gynec and Obst 359-66

21. **Thornton S, Davison J, Baylis PH** 1990 Effect of human pregnancy on metabolic clearance rate of oxytocin. Am J Physiol 259:21-4

22. **Windle RJ, Forsling ML** 1993 Variations in oxytocin secretion during the 4-day oestrous cycle of the rat. J Endocrinol 136:305-11

23. **Seron-Ferre M, Taylor NF, Martin MC, Leake RD** 1991 Development of a circadian variation of plasma oxytocin concentration in the late gestation rhesus monkey. J Clin Endocrinol Metab 72:1323-7

24. **Fuchs AR, Husslein P, Fuchs F** 1991 Oxytocin secretion and human paturition: pulse frequency and duration increase during spontaneous labor in women. Am J Obstet Gynecol 164:1515-23

25. **Jenkins JS, Ang VTY, Hawthorn J, Rossor MN, Iversen LL** 1984 Vasopressin, oxytocin and neurohypophysins in the human brain and spinal cord. Brain Res 291:111-7

26. **Bohus B, Kovacs G, Wied D** 1978 Oxytocin, vasopressin, and memory: opposite effects on consolidation and retrieval process. Brain Res 157:414-7

27. **Hughes AM, Everitt BJ, Lightman SL, Todd K** 1987 Oxytocin in the central nervous system and sexual behaviour in male rats. Brain Res 414:133-7

28. **Pederson CA, Ascher JA, Monroe YL, Prange AJ** 1982 Oxytocin induces maternal behaviour in virgin female rats. Science 216:648-9

29. **Chibbar R, Miller FD, Mitchell BF** 1993 Synthesis of oxytocin in amnion, chorion, and decidua may influence the timing of human parturition. J Clin Invest 91:185-92

30. **Zingg HH, Lefebvre DL** 1989 Oxytocin mRNA: increase of polyadenylate tail size during pregnancy and lactation. Mol Cell Endocrinol 65:59-62

31. **Flint APF, Sheldrick EC** 1982 Ovarian secretion of oxytocin is stimulated by prostaglandin. Nature 297:587-8

32. **Ivell R, Richter D** 1984 The gene for the hypothalamic peptide hormone oxytocin is highly expressed in the bovine corpus luteum: biosynthesis, structure and sequence analysis. EMBO J 3:2351-4

33. **Wathes DC, Swann RW** 1982 Is oxytocin an ovarian hormone? Nature 297:225-7

34. **Auletta F, Flint APF** 1988 Mechanisms controlling corpus luteum function in sheep, cows, non-human primates and women, especially in relation to the time of luteolysis. Endocr Rev 9:88-105

35.   **Shukovski L, Healy DL, Findlay JK** 1989 Circulating immunoreactive oxytocin during the human menstrual cycle comes from the pituitary and estradiol-dependent. J Clin Endocrinol Metab 68:455-60

36.   **Bennegard B, Hahlin M, Dennefors B** 1987 Antigonadotrophic effect of oxytocin in the isolated human corpus luteum. Fertility & Sterility 47:431-5

37.   **Nicholson HD, Swann RW, Burford CD, Wathes DC, Porter DG, Pickering BT** 1984 Identification of oxytocin and vasopressin in the tesis and in adrenal tissue. Regul Pept 8:141-6

38.   **Guldenaar SEF, Pickering BT** 1985 Immunocytochemical evidence for the presence of oxytocin in rat tesis. Cell Tissue Res 240:485-7

39.   **Rehbein M, Hillers M, Mohr E, Ivell R, Morley S, Schmale H, Richter D** 1986 The neurohypophyseal hormones vasopressin and oxytocin. Precursor structure, synthesis and regulation. Biol Chem Hoppe Seyler 367:695-704

40.   **Worley RTS, Nicholoson HD, Pickering BT** 1985 Testicular oxytocin: an initiator of seminiferous tubule movement? *In* Resent Progress in Cellular Endocrinology of the Testis. pp205-12 Sez J M, Forest MG, Dazord A & Bertrand J. (editors). Inserm, Paris.

41.   **Porter ID, Whitehouse BJ, Taylor AH, Nussey SS** 1988 Effect of arginine vasopressin and oxytocin on acetylcholine-stimulation of corticosteroid and catecholamine secretion from the rat adrenal gland perfused *in situ* Neuropeptides 12:265-71

42.   **Geenen V, Legros JJ, Franchimont P, Baudrihaye M, Defresne M-P, Boniver J** 1986 The neuroendocrine thymus: coexistence of oxytocin and neurophysin in the human thymus. Science 232:508-11

43. **Geenen V, Legros JJ, Franchimont P, Defresne MP, Boniver J, Ivell R, Richter D** 1987 The thymus as a neuroendocrine organ. Synthesis of vasopressin and oxytocin in human thymic epithelium. Ann NY Acad Sci 496:56-66

44. **Johnson HM, Torres BA** 1985 Regulation of lymphokine production by arginine vasopressin and oxytocin. Modulation of lymphocyte function by neurohypophysial hormones. J Immunol 135:7730-50

45. **Amico JA, Finn FM, Haldar J** 1988 Oxytocin and vasopressin are present in human and rat pancreas. Am J Med Sci 296:303-7

46. **Dunning BE, Moltz J, Fawcet CP** 1984 Actions of neurohypophyseal peptides on pancreatic hormone release. Am J Physiol 246:E108-14

47. **Dunning BE, Verbalis JG, Fawcett CP** 1985 Evidence for participation of the neuro-hypophysial hormones in the hyperglucagonemic response to haemorrhage in the rat. Neuroendocrinology 41:385-9

48. **Vihardt H, Krarup T, Holst JJ, Bie PG** 1986 The mechanism of the effect of oxytocin on plasma concentration of glucose, insulin, and glucagon in conscious dogs. J Endocrinol 108:293-8

49. **Ivell R, Richter D** 1984 Structure and comparison of the oxytocin and vasopressin genes from rat. Proc Natl Acad Sci U S A 81:2006-10

50. **Mohr E, Schmitz E, Richter D** 1988 A single rat genomic DNA fragment encodes both the oxytocin and vasopressin genes separated by 11 kilobases and oriented in opposite transcriptional directions. Biochimie 70:649-54

51. **Sausville E, Carney D, Battey J** 1985 The human vasopressin gene is linked to the oxytocin gene and is selectively expressed in a cultured lung cancer cell line. J Biol Chem 260:10236-41

52. **Rupper SD, Scherer G, Schutz G** 1984 Recent gene conversion involving bovine vasopressin and oxytocin precursor gene suggested by nucleotide sequence. Nature 308:554-7

53. **Hara Y, Battey J, Gainer H** 1990 Structure of mouse vasopressin and oxytocin genes. Brain Res Mol Brain Res 8:319-24

54. **Summar ML, Phillips JAI, Battey J** 1990 Linkage relationships of human argnine vasopressin-neurophysin-II and oxytocin-neurophysin-I to prodynorphin and other loci on chromosome 20. Mol Endocrinol 4:947-50

55. **Young WS, 3rd** 1992 Expression of the oxytocin and vasopressin genes. J Neuroendocrinol 4:529-40

56. **Richard S, Zingg HH** 1990 The human oxytocin gene promoter is regulated by estrogens. J Biol Chem 265:6098-103

57. **Mohr W, Schmitz E** 1991 Functional characterization of estrogen and glucocorticoid responsive elements in the rat oxytocin gene. Mol Brain Res 9:293-8

58. **Richard S, Zingg HH** 1991 Identification of a retinoic acid response element in the human oxytocin promoter. J Biol Chem 266:21428-33

59. **Adan RA, Cox JJ, van Kats JP, Burbach JP** 1992 Thyroid hormone regulates the oxytocin gene. J Biol Chem 267:3771-7

60.   **Stedronsky K, Walther N, Ivell R** 1999 The -160 hormone response element of the human oxytocin gene is not a natural target for estrogen receptors. 1999 World Congress on Neurohypophysial Hormones, Edinburgh, Scotland, August 28 - September 2 , abstract O4, p40

61.   **Acher R** 1993 Neurohypophysial peptide systems: processing machinery, hydroosmotic regulation, adaptation and evolution. Regul Pept 45:1-13

62.   **Altstein M, Whitnall MH, House S, Key S, Gainer H** 1988 An immunochemical analysis of oxytocin and vasopressin prohormone processing in vivo. Peptides 9:87-105

63.   **Altstein M, Gainer H** 1988 Differential biosynthesis and posttranslational processing of vasopressin and oxytocin in rat brain during embryonic and postnatal development. J Neurosci 8:3967-77

64.   **Morris M, Castro M, Rose JC** 1992 Alterations in oxytocin prohormone processing during early development in the fetal sheep. Am J Physiol 263:R738-40

65.   **Mueller-Heubach E, Morris M, Rose JC** 1995 Fetal oxytocin and its extended forms at term with and without labor. Am J Obstet Gynecol 173:375-81

66.   **Mitchell BF, Fang X, Wong S** 1997 Metabolism of oxytocin in rat uterus and placenta in late gestation. Biol Reprod 57:807-12

67.   **Page EW** 1946 The value of plasma pitocinase determinations in obstetrics. J Obstet Gynecol 52:1014-22

68.   **Burbach JPH, Lebouille JLM** 1982 Proteolytic conversion of arginine-vasopressin and oxytocin by brain synaptic membranes. J Biol Chem 258:1487-94

69.   Mitchell BF, Chibbar R 1995 Synthesis and metabolism of oxytocin in late gestation in human decidua. Adv Exp Med Biol 395:365-80

70.   Mitchell BF, Fang X, Wong S 1998 Role of carboxy-extended forms of oxytocin in the rat uterus in the process of parturition. Biol Reprod 59:1321-7

71.   Jard S 1983 Vasopressin isoreceptors in mammls: relation to cAMP dependent and cAMP-independent transducer mechanisms. Current Topics in Membrane Transsportport 18:225-85

72.   Jard S, Gaillard RD, Guillon G, Marie J, Schoenenberg P, Muller AF, Manning M, Sawyer WH 1986 Vasopressin antagonists allow demonstration of a novel vasopressin receptor in the rat adenohypophysis. Mol Pharmacol 30:171-7

73.   Zingg HH 1996 Vasopressin and oxytocin receptors. Baillieres Clin Endocrinol Metab 10:75-96

74.   Schlosser SF, Almeida OFX, Patchev VK, Yassouridis A, Elands J 1994 Oxytocin-stimulated release of adrenocorticotropin from the rat pituitary is mediated by arginine vasopressin receptors of the V1b type. Endocrinology 135:2058-63

75.   Lin SH, Miyata S, Itoh T, Kawarabayashi T, Nakashima T, Kiyohara T 1995 Fos expression in the hypothalamic magnocellular neurons of rats during pregnancy, parturition and lactation. Neuroscience Res 23:29-34

76.   Hartman RD, Rosella-Dampman LM, Emmert SE, Summy-Long JY 1986 Inhibition of release of neurohypophyseal hormones by endogenous opioid peptides in pregnant and parturient rats. Brain Res 382:352-9

77. **Soloff MS, Fernstrom MA, Fernstrom MJ** 1989 Vasopressin and oxytocin receptors on plasma membranes from rat mammary gland. Demonstration of vasopressin receptors by stimulation of inositol phosphate formation, and oxytocin receptors by binding of a specific 125I-labeled oxytocin antagonist, d(CH2)5(1)[Tyr(Me)2, Thr4,Tyr-NH2(9)]OVT. Biochem Cell Biol 67:152-62

78. **Ito Y, Kimura T, Kobayashi T** 1995 Expression of the oxytocin receptor in human breast cancer tissue. Abstracts of the Hanseatic Endocrine Conference on Oxytocin, A91

79. **Cassoni P, Sapino A, Negro F, Bussolati G** 1994 Oxytocin inhibits proliferation of human breast cancer cell lines. Int J Pathol 425:467-72

80. **Fuchs AR, Behrens O, Helmer H, Vargsted A, Ivanisevic M, Grifo J, Barros C, Fields M** 1990 Oxytocin and vasopressin binding sites in human and bovine ovaries. Am J Obstet Gynecol 163:1961-7

81. **Breton C, Pechoux C, Morel G, Zingg HH** 1995 Oxytocin receptor messenger ribonucleic acid: characterization, regulation, and cellular localization in the rat pituitary gland. Endocrinology 136:2928-36

82. **De Keyzer Y, Auzan C, Lenne F, Beldjord C, Thibonnier M, Bertagna X, Clauser E** 1994 Cloning and characterization of the human V3 pituitary vasopressin receptor. FEBS Lett 356:215-20

83. **Tribollet E** 1992 Vasopressin and oxytocin receptors in the rat brain. Handbook of Chemical Neuroanatomy 11:289-320

84. **Breton C, Zingg HH** 1997 Expression and region-specific regulation of the oxytocin receptor gene in rat brain. Endocrinology 138:1857-62

85. **Ostrowski NL, Lolait SJ, Bradley DJ, O'Carroll AM, Brownstein MJ, Young WS** 1992 Distribution of V1a and V2 vasopressin receptor messenger ribonucleic acids in liver, kidney, pituritary and brain. Endocrinology 131:533-5

86. **McCarthy MM, Kleopoulos SP, Mobbs CV, Pfaff DW** 1994 Infusion of antisense oligodeoxynucleotides to the oxytocin receptor in the ventromedial hypothalamus reduces estrogen-induced sexual receptivity and oxytocin receptor binding in the female rat. Neuroendocrinology 59:432-40

87. **Ostrowski NL** 1998 Oxytocin receptor mRNA expression in rat brain: implication for behavioral integration and reproductive success. Psychoneuroendocrinology 23:989-1004

88. **Tribollet E, Audigier S, Dubois-Dauphin M, Dreifuss JJ** 1990 Gonadal steroids regulate oxytocin receptors but not vasopressin receptors in the brain of male and female rats. An autoradiographical stidy. Brain Res 496:66-81

89. **Schumacher M, Coirini H, Pfaff DW, McEwen** 1990 Behavioural effects of progesterone associated with rapid modulation of oxytocin receptors. Science 250:691-4

90. **Stoeckel ME, Freund-mercier MJ** 1989 Autoradiographic demonstration of oxytocin binding sites in the macula densa. Am J Physiol 257:F310-4

91. **Breton C, Neculcea J, Zingg HH** 1996 Renal oxytocin receptor messenger ribonucleic acid: characterization and regulation during pregnancy and in response to ovarian steroid treatment. Endocrinology 137:2711-7

92. **Brimble MJ, Balment RJ, Smith CP, Windle RJ, Forsling ML** 1991 Influence of oxytocin on sodium excretion in the anaesthetized Brattleboro rat. J Endocrinol 129:49-54

93. **Verbalis JG, Mangione MP, Stricker EM** 1991 Oxytocin produces natriuresis in rats at physiological plasma concentration. Endocrinology 128:1317-22

94. **Ostrowski NL, Young WS, Lolait SJ** 1995 Estrogen increases renal oxytocin receptor gene expression. Endocrinology 136:1801-4

95. **Ostrowski NL, Young WS** 1993 Expression of vasopressin V1a and V2 receptor messenger ribonucleic acid in the liver and kidney of embryonic, developing, and adult rats. Endocrinology 133:1849-59

96. **Elands J, Resink A, De Kloet ER** 1988 Neurohypophysial receptors in the rat thymus, spleen and lymphocytes. Endocrinology 126:2703-10

97. **Martens H, Robert F, Legros JJ** 1992 Expression of functional neurohypophyseal peptides receptors by murine immature and cytotoxic T-cell lines. Prog NeuroEndocrinImmunol 5:31-9

98. **Geisterfer AA, Owens GK** 1989 Arginine vasopressin induced hypertrophy of cultured rat aortic smooth muscle cells. Hypertension 14:413-20

99. **St. Louis J, Parent A, Lariviere R, Schiffrin EL** 1986 Vasopressin responses and receptors in the mesenteric vasculature of estrogen-treated rats. Am J Physiol 251:H885-9

100. **Williams B, Tsai P, Schrier RW** 1992 Glucose-induced downregulation of angiotensin II and arginine vasopressin receptors in cultured rat aortic vascular smooth muscle cells. Role of protein kinase C. J Clin Invest 90:1992-9

101. **Thibonnier M, Conarty DM, Preston JA, Plesnicher CL, Dweik RA, Erzurum SC** 1999 Human vascular endothelial cells express oxytocin receptors. Endocrinology 140:1301-9

102. **Kimura T, Tanizawa O, Mori K, Brownstein MK, Okayama H** 1992 Structure and expression of a human oxytocin receptor. Nature 356:526-9

103. **Rozen F, Russo C, Banville D, Zingg HH** 1995 Structure, characterization, and expression of the rat oxytocin receptor gene. Proc Natl Acad Sci U S A 92:200-4

104. **Gorbulev V, Buchner H, Akhundova A, Fahrenholz F** 1993 Molecular cloning and functional expression of V2[8-lysine] vasopressin and oxytocin receptors from a pig kidney cell line. Eur J Biochem 215:1-7

105. **Riley PR, Flint APF, Abayasekara DRE, Stemard HJ** 1995 Structure and expression of an ovine endometrial oxytocin receptor cDNA. J Mol Endocrinol 15:196-202

106. **Inoue T, Kimura T, Azuma C** 1994 Structure and organization of the human oxytocin receptor gene. J Biol Chem 269:32451-6

107. **Bale T, Dorsa D** 1997 Cloning, novel promoter sequence, and estrogen regulation of a rat oxytocin receptor gene. Endocrinology 138:1151-8

108. **Phaneuf S, Europe-Finner GN, Varney M, MacKenzie IZ, Watson SP, Lopez Bernal A** 1993 Oxytocin-stimulated phosphoinsitide hydrolysis in human myometrial cells: involvement of pertussis toxin-sensitive and -insensitive G-proteins. J Endocrinol 136:497-509

109. **Lopez Bernal A, Rivera J, Europe-Finner GN, Phaneuf S, Asboth G** 1995 Parturition: activation of stimulatory pathways or loss of uterine quiesence? Adv Exp Med Biol 395:435-51

110. **Mironneau J** 1990 Ion channels and excitation-contraction coupling in myometrium. *In* Uterine Contracility pp9-19 Garfied RE. (editor) Serono Synposia USA. Norwell, MA.

111. **Chilvers ER, Batty IH, Challiss RA, Barnes PJ, Nahorski SR** 1991 Determination of mass changes in phosphatidylinositol 4,5-bisphosphate and evidence for agonist-stimulated metabolism of inositol 1,4,5-trisphosphate in airway smooth muscle. Biochem J 275:373-9

112. **Casey ML, MacDonald PC** 1993 Human parturition: Distinction between the initiation of parturition and the onset of labor. Sem Reprod Endocrinol 11:272-84

113. **Lye SJ, Challis JRG** 1989 Paracrine and endocrine control of myometrial activity. *In* Advances in Fetal Physiology: Reviews in Honour of G. C. Liggins. pp361-75 Gluckman PD, Johnston BM & Nathanielsz PW. (editors) Advances in Perinatal Medicine (VIII). Perinatology Press. Ithaca, NY.

114. **Zakar T, Mitchell BF** 1996 The endocrinology of late pregnancy and parturition. *In* Advances in Organ Biology: Pregnancy and Parturition 1:pp153-73 Bittar EE. (editor) JAI Press INC. Greenwich, Connecticut, London, England.

115. **Nathanielsz PW, Giussani DA, Mecenas CA, Wu W, Winter JA, Garcia-Villar R, Baguma-Nibasheka M, Honnebier MBOM, McDonald TJ** 1995 Regulation of the switch from myometrial contractures to contractions in late pregnancy: studies in the pregnant sheep and monkey. Reprod Fertil Dev 7:595-602

116. **Csapo AL, Sauvage J** 1968 The evolution of uterine activity during human pregnancy. Acta Obstet Gynecol Scandinavia 47:181-212

117. **Germain G, Cabrol C, Visser A, Sultan C** 1982 Electrical activity of the pregnant uterus of the cynomolgus monkey. Am J Obstet Gynecol 142:448-57

118. **Nathanielsz PW, Balley A, Poore ER, Thorburn GD, Harding R** 1980 The relationship between myometrial activity and sleep state and breathing in fetal sheep throughout the last third of gestation. Am J Obstet Gynecol 138:653-9

119. **Porter DG** 1971 Quantiative changes in myometrial activity in the guinea-pig during pregnancy. J Reprod Fertil 27:217-26

120. **Demianczuk N, Towell ME, Garfield RE** 1984 Myometrial electrophysiological activity and gap junctions in the pregnant rabbit. Am J Obstet Gynecol 149:485-91

121. **Wilson L Jr, Pak SC, Santolava J, Parsons M** 1994 Induction of estradiol synthesis and nocturnal uterine contractions by dehydroepiandrosterone and their inhibition by an aromatase inhibitor. Proceedings of the 41st Annual Meeting of the Society for Gynecologic Investigation, Chicago. IL, Abstract #14.

122. **Challis JRG** 1971 Sharp increase in free circulating oestrogens immediately before parturition in sheep. Nature 229:208

123. **Lye SJ, Nicholson BJ, Mascarenhas M, MacKenzie L, Petrocelli T** 1993 Increased expression of connexin-43 in rat myometrium during labour is associated with an increase in the plasma estrogen: progesterone ratio. Endocrinology 132:2380-6

124. **Chow L, Lye SJ** 1994 Expression of the gap junction protein connexin-43 is increased in the human myometrium toward term and with the onset of labor. Am J Obstet Gynecol 170:788-95

125. **Anderson J, Grine E, Eng CLY, Zhao K, Barbieri RL, Chumas JC, Brink PR** 1993 Expression of connexin-43 in human myometrium and leiomyoma. Am J Obstet Gynecol 169:1266-76

126. **Ou C, Orsino A, Lye SJ** 1997 Expression of connexin-43 and connexin-26 in the rat myometrium during pregnancy and labor is differentially regulated by mechanical and hormonal signals. Endocrinologgy 138:1398-407

127. **Dawood MY, Raghavan KS, Pociask C, Fuchs F** 1978 Oxytocin in human pregnancy and parturition. Obstet Gynecol 51:138-43

128. **Thornton S, Davison J, Baylis PH** 1992 Plasma oxytocin during the frist and second stages of spontaneous human labour. Acta Endocrinol 126:425-9

129. **Soloff MS** 1975 Uterine receptor for oxytocin: effects of estrogen. Biochem Biophys Res Commun 65:205-12

130. **Chard T** 1989 Fetal and maternal oxytocin in human parturition. Am J Perinatol 2:145-52

131. **Higuchi T, Honda K, Fukuoka H, Negoro H, Wakabayashi K** 1985 Release of oxytocin during suckling and parturition in the rat. J Endocrinol 105:339-46

132. **Higuchi T, Tadokoro Y, Honda K, Negoro H** 1986 Detailed analysis of blood oxytocin concentrations during suckling and parturition in the rat. J Endocrinol 110:251-6

133. **Higuchi T, Uchide K, Honda K, Negoro H** 1986 Oxytocin release during parturition in the pelvic-neurectomized rat. J Endocrinol 109:149-54

134. **Gale CC, MaCann SM** 1961 Hypothalamic control of pituitary gonadotrophins. Impairment in gestation parturition and milk ejection following hypothalamic lesions. J Endocrinol 22:107-17

135. **Amico JA, Hempel J** 1990 An oxytocin precursor intermediate circulates in the plasma of humans and rhesus monkeys administered estrogen. Neuroendocrinology 51:437-43

136. **Luckman SM, Antonijevic I, Leng G, Dye S, Douglas AJ, Russell JA, Bicknell RJ** 1993 The maintenance of normal parturition in the rat requires neurohypophysial oxytocin. J Neuroendocrinol 5:7-12

137. **Khan-Dawood FS, Dawood MY** 1984 Oxytocin content of human fetal pituitary glands. J Obstet Gynecol 150:501-5

138. **Seppala M, Aho I, Tissari A, Ruoslahti E** 1972 Radioimmunoassay of oxytocin in amniotic fluid, fetal urine and meconium during late pregnancy and delivery. Am J Obstet Gynecol 114:788-95

139. **Chard T, Hudson CN, Edwards CRW, Boyd NRH** 1971 Release of oxytocin and vasopressin by human foetus during labour. Nature 234:352-4

140. **Otsuki Y, Yamaji K, Fujita M** 1983 Serial plasma oxytocin concentrations during pregnancy and labor. Acta Obstet Gynecol Scandinavia 62:15-8

141. **Ducsay CA, Cook MJ, Walsh SW, Novy MJ** 1983 Circadian patterns and dexamethasone-induced changes in uterine activity in pregnant rhesus monkeys. Am J Obstet Gynecol 145:389-96

142. **Honnebier MBOM, Figueroa JP, Rivier J, Vale W, Nathanielsz P** 1989 Studies on the role of oxytocin in late pregnancy in the pregnant rhesus monkey: plasma concentrations of oxytocin in the maternal circulation throughout the 24-h day and the effect of synthetic oxytocin antagonist [1-beta-Mpa(beta-$(CH_2)_5)_D$,(Me(Tyr^2, Orn^8))] oxytocin on spontaneous nocturnal myometrial contractions. J Dev Physiol 12:225-32

143. **Zahn V** 1984 Uterine contractions during pregnancy. J Perinat Med 12:107-13

144. **Hirst JJ, Haluska GJ, Cook MJ, Hess DL, Novy MJ** 1991 Comparison of plasma oxytocin and catecholamine concentrations with uterine activity in pregnant rhesus monkeys. J Clin Endocrinol Metab 73:804-10

145. **Flint APF, Sheldrick EL** 1983 Evidence for a systemic role for ovarian oxytocin in luteal regression in sheep. J Reprod Fertil 67:215-25

146. **Lefebvre DL, Giaid A, Zingg HH** 1992 Expression of the oxytocin gene in rat placenta. Endocrinology 130:1185-92

147. **Larcher A, Neculcea J, Breton C, Arslan A, Rozen F, Russo C, Zingg HH** 1995 Oxytocin receptor gene expression in the rat uterus during pregnancy and the estrous cycle and in response to gonadal steroid treatment. Endocrinology 136:5350-6

148. **Chan WY, Chen DL, Manning M** 1993 Oxytocin receptor subtypes in the pregnant rat myometrium and decidua: pharmacological differentiations. Endocrinology 132:1381-6

149. **Hahn DW, Demarest KT, Ericson DE, Homm SRE, Capetola RJ, McGuire JI** 1987 Evaluation of 1-deamino-[D-Tyr(Oethyl)^2, Thr^4,Orn^8] vasotocin, an oxytocin antagonist, in animal models of uterine contractility and preterm labor: A new tocolytic agent. Am J Obstet Gynecol 157:977-82

150. **Chan WY, Berezin I, Daniel EE, Russell KC, Hruby VJ** 1991 Effects of inactivation of oxytocin receptor and inhibition of prostaglandin synthesis on uterine oxytocin receptor and gap junction formation and labor in the rat. Can J Physiol Pharmacol 69:1262-7

151. **Antonijevic IA, Douglas AJ, Dye S, Bicknell RJ, Leng G, Russell JA** 1995 Oxytocin antagonists delay the initiation of parturition and prolong its active phase in rats. J Endocrinol 145:97-103

152. **Melin P, Trojnar J, Johnsson B, Vilhardt H, Akerlund M** 1986 Synthetic antagonists of the myometrial response to vasopressin and oxytocin. J Endocrinol 111:125-31

153. **Manning M, Miteva K, Pancheva S, Stoev S, Wo NC, Chan WY** 1995 Design and synthesis of highly selective in vitro and in vivo uterine receptor antagonists of oxytocin: comparisons with Atosiban. Int J Pept Protein Res 46:244-52

154. **Akerlund M, Stromberg P, Hauksson A, Andersen LF, Lyndrup J, Trojnar J, Melin P** 1987 Inhibition of uterine contractions of premature labour with an oxytocin analogue. Br J Obstet Gynaecol 94:1040-4

155. **Manning M, Cheng LL, Klis WA, Stoev S, Przybylski J, Bankowski K, Sawyer WH, Barberis C, Chan WY** 1995 Advances in the design of selective antagonists, potential tocolytics, and radioiodinated ligands for oxytocin receptors. Adv Exp Med Biol 395:559-83

156. **Pettibone DJ, Guidotti M, Harrell CM, Jasper JR, Lis EV, O'Brien JA, Reiss DR, Woyden CJ, Bock MG, Evans BE, Freidinger RM, Williams PD, Murphy MG** 1995 Progress in the development of oxytocin antagonists for use in preterm labor. Adv Exp Med Biol 395:601-12

157. **Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR** 1996 Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. Proc Natl Acad Sci U S A 93:11699-704

158. **Young WS, 3rd, Shepard E, Amico J, Hennighausen L, Wagner KU, LaMarca ME, McKinney C, Ginns EI** 1996 Deficiency in mouse oxytocin prevents milk ejection, but not fertility or parturition. J Neuroendocrinol 8:847-53

159. **Gross G, Imamura T, Luedke C, Vogt SK, Olson LM, Nelson DM, Sadovsky Y, Muglia LJ** 1998 Opposing actions of prostaglandins and oxytocin determine the onset of murine labor. Proc Natl Acad Sci U S A 95:11875-9

160. **Funk CD** 1993 Molecular biology in the eicosanoid field. Prog Nucleic Acid Res Mol Biol 45:67-98

161. **Keirse MJNC** 1990 Clinical use of eicosanoids for cervcial ripening before induction of labour. In Eicosanoids in Reproduction. Mitchell, M D. (editor) Boca Raton, FL, CRC Press:pp223-47

162. **Novy MJ, Cook MJ, Manaugh L** 1974 Indomethacin block of normal onset of parturition in primates. Am J Obstet Gynecol 118:412-6

163. **Hsu HW, Figueroa JP, Honnebier MBOM, Wentworth R, Nathanielsz PW** 1989 Power spectrum analysis of myometrial electromyogram and intrauterine pressure changes in the pregnant rhesus monkey in late gestation. Am J Obstet Gynecol 161:467-73

164. **Skinner KA, Challis JRG** 1985 Changes in the synthesis and metabolism of prostaglandins by human fetal membranes and decidua at labour. Am J Obstet Gynecol 151:519-23

165. **Olson DM, Skinner K, Challis JRG** 1983 Prostaglandin output in relation to parturition by cells dispersed from human intrauterine tissues. J Clin Endocrinol Metab 57:694-9

166. **Thorburn GD** 1991 The placenta, prostaglandins and parturition. Reprod Fertil Dev 3:277-94

167. **Bamford D, Jogee M** 1980 Prostaglandin formation by the pregnant human myometrium. Br J Obstet Gynaecol 87:215-8

168. **Hillier K, Calder AA, Embrey MP** 1974 Concentrations of prostaglandin F2a in amniotic fluid and plasma in spontaneous and induced labour. J Obstet Gynaecol British Commonw 81:257-63

169. **Romero R, Baumann P, Gonzales R, Gomez R, Rittenhouse L, Behnke E, Mitchell MD** 1994 Amniotic fluid prostanoid concentrations increase early during the course of spontaneous labor at term. Am J Obstet Gynecol 171:1610-13

170. **Romero R, Munoz H, Gomez R, Parra M, Polanco M, Valverde V, Hasbun J, Garrido J, Ghezzi F, Mazor M, Tolosa JE, Mitchell MD** 1996 Increases in prostaglandin bioactivity precedes the onset of human parturition. Prost Leuk Essen Fatty Ac 54:187-91

171. **Hirst JJ, Teixeira T, Zakar T, Olson DM** 1995 Prostaglandin endoperoxide-H-synthase-1 and -2 messenger reibonucleic acid levels in human amnion with spontaneous labor onset. J Clin Endocrinol Metab 80:517-23

172. **Teixeira FJ, Zakar T, Hirst JJ, Guo F, Sadowsky D, Machin G, Demianczuk N, Resch B, Olson DM** 1994 Prostaglandin endoperoxide H synthase (PGHS) activity and immunoreactive PGHS-1 and -2 levels in human amnion throughout gestation and at labor. J Clin Endocrinol Metab 79:1396-402

173. **Mijovic J, Zakar T, Nairn T, Olson DM** 1997 Prostaglandin-endoperoxide H synthase-2 expression and activity increases with term labor in human chorion. Am J Physiol 272:E832-40

174. **Cheung PYC, Challis JR** 1989 Prostaglandin E_2 metabolism in the human fetal membrance. Am J Obstet Gynecol 161:1580-5

175. **Cheung PYC, Walton JC, Tai HH, Riley SC, Challis JRG** 1990 Immunocytochemical distribution and localization of 15-hydroxyprostaglandin dehydrogenase in human fetal membranes, decidua and placenta. Am J Obstet Gynecol 163:1445-9

176. **Willman EA, Collins WP** 1976 The concentrations of prostaglandin F_{2a} in tissues with the fetoplacental unit after spontaneous or induced labor. Br J Obstet Gynaecol 183:786-9

177. **Norwitz ER, Starkey PM, Lopez Bernal A** 1992 Prostagladin D2 production by term human decidua: Cellular origins defined using flow cytometry. Obstet Gynecol 80:440-5

178. **Norwitz ER, Starkey PM, Lopez Bernl A, Turnbull AC** 1991 Identification by flow cytometry of the prostaglandin-producing cell populations of term human decidua. J Endocrinol 131:327-34

179. **Challis JRG** 1997 Prostaglandins and Parturition. Society for Gynecological Investigation, Postgraduate Course.

180. **Myatt L, Langdon G, Brockman DE** 1994 Identification and changes in concentrations of prostaglandin H synthase (PGHS) isoform in rat myometrium at parturition. Prostaglandins 48:285-96

181. **Potestio F, Zakar T, Olson DM** 1988 Glucocorticoids stimulate prostaglandin synthesis in human amnion cells by a receptor-mediated mechanism. J Clin Endocrinol Metab 67:1205-10

182. **Jones SA, Challis JRG** 1990 Effects of corticotrophin-releasing hormone (CRH) and adrenocorticotrophin (ACTH) on prostaglandin output by human placenta and fetal membranes. Gynecol Obstet Invest 29:165-8

183. **Benedetto C, Petraglia F, Marozio L, Chiarolino L, Florio P, Genazzani AR, Massobrio M** 1994 Corticotropin-releasing hormone increases prostaglandin F2 activity on human myometrium. Am J Obstet Gynecol 171:126-31

184. **Chan WY** 1980 The separate uterotonic and prostaglandin-releasing actions of oxytocin. Evidence and comparison with angiotensin and methacholine in the isolated rat uterus. J Pharmacol Exp Ther 213:575-9

185. **Yamamoto K, Arakawa T, Taketani Y, Takahashi Y, Hayashi Y, Ueda N, Yamamoto S, Kumegawa M** 1997 TNFα-dependent induction of cycloocygenase-2 mediated by NF-κB and NF-IL6. Adv Exp Med Biol 407:185-9

186. **Raz A, Wyche A, Siegel N, Needleman P** 1988 Regulation of fibroblast cyclooxyenase synthesis by interleukin-1. J Biol Chem 263:3022-8

187. **Wilson L Jr, Lindsey R** 1987 Effects of progesterone withdrawal on uterine prostaglandin levels in the ovariectomized pregnant rat. Prostaglandins Leukot Med 29:95-105

188. **Mitchell MD** 1991 Endothelin in perinatal biology. Sem Perinatol 15:79-85

189. **Brigstock DR, Heap RB, Brown KD** 1989 Polypeptide growth factors in uterine tissues and secretions. J Reprod Fertil 85:747-58

190. **Zakar T, Olson DM** 1988 The action of epidemal growth factor on human amnion prostaglandin E2 output. Can J Physiol Pharmacol 66:769-75

191. **Olson DM, Zakar T, Mitchell BF** 1993 Prostagland synthesis regulation by intrauterine tissues. *In* Molecular Aspects of Placental and Fetal Membrane Autocoids. pp56-95. Rice GE and Brennecke SP (editors) CRC Press Inc. Boca Raton, FL.

192. **Huszar GB, Walsh MP** 1991 Relationship between myometrial and cervical functions in pregnancy and labor. Sem Perinatol 15:97-117

193. **Bleasdale JE, Direnzo GC** 1989 Fetal influence on human parturition. *In* Fetal Medicine Reviews. 1:pp. 242-88. Rodeck C (editor) Blackwell Scient., London.

194. **Csapo AI** 1956 Progesterone "block". Am J Anatomy 98:273-91

195. **Challis JRG, Lye SJ** 1994 Parturition. Oxf Rev Reprod Biol 8:61-129

196. **Tulchinsky D, Hobel CY, Yeager E, Marshall YR** 1972 Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnany. 1. Normal pregnancy. Am J Obstet Gynecol 112:1095-100

197. **Hendricks CH** 1970 The control of labor. Gynecol Invest 1(Suppl):37-54

198. **Haluska GL, Novy MJ** 1993 Hormonal modulation of uterine activity during primate parturition. Sem Reprod Endocrinol 11:261-71

199. **Tsai MJ, O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451-86

200. **Webster NJG, Green S, Jin J-R, Chambon P** 1988 The hormone-binding domains of the estrogen and glucocorticord receptors contain an inducible transcription activation function. Cell 54:194-207

201. **Chibbar R, Wong S, Miller FD, Mitchell BF** 1995 Estrogen stimulates oxytocin gene expression in human chorio-decidua. J Clin Endocrinol Metab 80:567-72

202. **Khan-Dawood FS, Dawood MY** 1984 Estrogen and progesterone receptor and hormone level in human myometrium and placenta in term pregnancy. Am J Obstet Gynecol 150:501-5

203. **Horwitz KB, Alexander PS** 1983 *In situ* photolinked nuclear progesterone receptors of human breast cancer cells: subunit molecular

97

weights after transformation and translocation. Endocrinology
113:2195-201

204. **Krett NL, Wei LL, Francis MD, Nordeen SK, Gordon DF, Wood WM, Horwitz KB** 1988 Human progesterone A-receptors can be synthesized intracellularly and are biologically functional. Biochem Biophys Res Commun 157:278-85

205. **Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P** 1990 Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 9:1603-14

206. **Kraus WL, Montano MM, Katzenellenbogen BS** 1993 Cloning of the rat progesterone receptor gene 5'-region and identification of two functionally distict promoters. Mol Endocrinol 7:1603-16

207. **Sartorius CA, Melville MY, Hovland AR, Tung L, Takimoto GS, Horwitz KB** 1994 A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. Mol Endocrinol 8:1347-60

208. **Vegeto E, Shahbaz MM, Wen DX, Goldman ME, O'Malley BW, McDonnell DP** 1993 Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. Mol Endocrinol 7:1244-55

209. **Hovland AR, Powell RL, Takimoto GS, Tung L, Horwitz KB** 1998 An N-terminal inhibitory function, IF, suppresses transcription by the A-isoform but not the B-isoform of human progesterone. J Biol Chem 10:5455-60

210. **Wei LL, Miner RS** 1994 Evidence for a third progesterone receptor isoform in human breast cancer cells T47D. Cancer Res 54:340-3

211. **Wei LL, Gonzalez-Aller C, Wood WM, Miller LA, Horwitz KB**
1990 5'-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated "C"-receptor and unique A-receptor messages. Mol Endocrinol 4:1833-40

212. **Kraus WL, Montano MM, Katzenellenbogen BS** 1994
Identification of multiple, widely, spaced estrogen-responsive regions in the rat progesterone receptor gene. Mol Endocrinol 8:952-69

213. **Ogle TE, Dai D, George P, Mahesh VB** 1997 Stromal cell progesterone and estrogen receptors during proliferation and regression of the decidua basalis in the pregnant rat. Biol Reprod 57:495-506

214. **Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson J-A** 1997 Human estrogen receptor-beta gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 82:4258-65

215. **Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A** 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93:5925-30

216. **Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, Ouchi Y, Muramatsu M** 1998 The complete primary structure of human estrogen receptor-beta and its heterodimerization with ER-alpha *in vivo* and *in vitro*. Biochem Biophys Res Commun 243:122-6

217. **Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J-A, Nilsson S** 1998 Differential response of estrogen receptor-alpha and estrogen-beta to partial estrogen agonists/antagonists. Mol Pharmacol 54:105-12

218. **Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson J-A, Kushner PJ, Scablan T** 1997 Differential ligand activation of estrogen receptors ER-alpha and ER-beta at AP1 sites. Science 277:1508-10

219. **Young LJ, Wang Z, Donaldson R, Rissman EF** 1998 Estrogen receptor α is essential for induction of oxytocin receptor by estrogen. Neuroendocrinology 9:933-6

220. **Lefebvre DL, Farookhi R, Giaid A, Neculcea J, Zingg HH** 1994 Uterine oxytocin gene expression. II. Induction by exogenous steroid administration. Endocrinology 134:2562-6

221. **Amico JA, Seif SM, Robinson AG** 1981 Oxytocin in human plasma: correlation with neurophysin and stimulation with estrogen. J Clin Endocrinol Metab 52:988-93

222. **Yamaguchi K, Asaishi T, Negoro H** 1979 Effect of oestrogen treatment on plasma oxytocin and vasopressin in ovariectomized rats. Endocrinologica Japonicum 26:197-205

223. **Caldwell JD, Brooks PJ, Jirikowski GF, Barakat AS, Lund PK, Pedersen CA** 1989 Estrogen alters oxytocin mRNA levels in the preoptic area. J Neuroendocrinol 1:1-7

224. **Chung S, McCabe J, Pfaff D** 1991 Estrogen influences on oxytocin mRNA expression in preoptic and anterior hypothalamic regions studied by *in situ* hybridization. J Comp Neurol 307:281-95

225. **Burbach JPH, Adan RAH, van Tol HHM, Verbeeck MAE, Axelson JF, van Leewuen FW, Beekman JM, Ab G** 1990 Regulation of the rat oxytocin gene by estradiol. J Neuroendocrinol 2:633-9

226. **Kawata W, McCabe JT, Chung SK, Dutt A, Kazunari Y, Hirakawa M, Kumamoto K, Hirayama Y, Pfaff DW** 1991 The effect of progesterone on oxytocin mRNA in hypothalamic neurons of estrogen-treated female rats studied with *in situ* hybridization. Biomed Res 12:405-15

227. **Amico JA, Crowley RS, Insel TR, Thomas A, JA OK** 1995 Effect of gonadal steroids upon hypothalamic oxytocin expression. Adv Exp Med Biol 395:23-35

228. **Soloff MS, Fernstrom MA, Periyasamy S, Soloff S, Baldwin S, Wieder M** 1983 Regulation of oxytocin receptor concentration in rat uterine explants by estrogen and progesterone. Can J Biochem Cell Biol 61:625-30

229. **Adachi S, Masataka O** 1995 The regulation of oxytocin receptor expression in human myometrial monolayer culture. J Smooth Muscle Res 31:175-97

230. **Fuchs AR, Periyasamy S, Soloff MS** 1983 Systemic and local regulation of oxytocin receptors in the rat uterus, and their functional significance. Can J Biochem Cell Biol 61:615-24

231. **Antoni AF** 1986 Oxytocin receptors in rat adenohypophysis: evidence from radioligand binding studies. Endocrinology 119:2393-5

232. **Fuchs AR, Periyasamy S, Alexandrova M, Soloff MS** 1983 Correlation between oxytocin receptor concentration and responsiveness to oxytocin in pregnant rat myometrium: effects of ovarian steroids. Endocrinology 113:742-9

233. **Ma XH, Wu WX, Nathanielsz PW** 1999 Down-regulation of oxytocin receptor (OTR) mRNA by progesterone (P4) in the myometrium (Myo) of non-pregnant ovariectomized (OVX) sheep.

Supplement to: Journal of the Society for Gynecologic Investigation, 46th annual Meeting, Atlanta, Georgia. 6:abstract 709, p226A

234. **Soloff MS, Wieder MH** 1983 Oxytocin receptors in rat involuting mammary gland. Can J Biochem Cell Biol 61:631-5

235. **Maggi M, Magini A, Fiscella A, Giannini S, Fantoni G, Taffoletti F, Giambattista M, Serio M** 1992 Sex steroid modulation of neurohypophysial hormone receptors in human nonpregnant myometrium. J Clin Endocrinol Metab 74:385-92

236. **Jordan VC, Collins MM, Rowsby L, Prestwich G** 1977 A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J Endocrinol 75:305-

237. **Jordan VC, Allen KE, Dix CJ** 1980 Pharmacology of tamoxifen in laboratory animals. Cancer Treatment Reports 64:745-59

238. **Parker MG** 1995 Structure and function of estrogen receptors. Vitamins & Hormones 51:267-87

239. **Gibson MK, Nemmers LA, Beckman WC Jr, Davis VL, Curtis SW, Korach K** 1991 The mechanism of ICI 164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. Endocrinology 129:2000-10

240. **Pepe GJ, Albrecht ED** 1995 Actions of placental and fetal adrenal steroid hormones in primate pregnancy. Endocr Rev 16:608-48

241. **Majid E, Senior J** 1982 Anti-oestrogen modification of uterine responses to oestrogen in the rat. J Reprod Fertil 66:79-85

242. **Jordan VC, Dix CJ** 1979 Effect of oestradiol benzoate, tamoxifen and monohydroxytamoxifen on immature rat uterine progesterone receptor synthesis and endometrial cell division. J Steroid Biochem 11:285-91

243. **Barkai U, Kidron T, Kraicer PF** 1992 Inhibition of decidual induction in rats by clomiphene and tamoxifen. Biol Reprod 46:733-9

244. **Philbert D, Moguilewsky M, Mary M, Lecaque D, Tournemine C, Secchi J, Deraed R** 1985 Pharmacological profile of RU 486 in animals. *In* The Antiprogesterone Steroid RU 486 and Human Fertility Control. pp49-68. Baulieu EE & Segal SJ (editors). Plenum Press. New York.

245. **Lebeau M-C, Baulieu EE** 1994 Steroid antagonists and receptor-associated proteins. Hum Reprod 9:437-44

246. **Chwalisz K, Stockemann K, Fuhrmann U, Fritzemeier KH, Einspanier A, Garfield RE** 1995 Mechanism of action of antiprogestins in the pregnant uterus. Ann NY Acad Sci 202-23

247. **Chwalisz K** 1994 The use of progesterone antagonists for cervical ripening and as an adjunct to labour and delivery. Hum Reprod 9(Suppl. 1):131-61

248. **Wilson T, Liggins GC, Whittaker DJ** 1988 Oxytocin stimulates the release of arachidonic acid and prostaglandin F_{2a} from human decidual cells. Prostaglandins 35:771-80

249. **Fuchs AR, Husslein P, Fuchs F** 1981 Oxytocin and the initiation of human parturition. II. Stimulation of prostaglandin production in human decidua by oxytocin. Am J Obstet Gynecol 141:694-7

250. **Alexandrova M, Soloff MS** 1980 Oxytocin receptors and parturition. III. Increases in estrogen receptor and oxytocin receptor concentrations in the rat myometrium during prostaglandin F2 alpha-induced abortion. Endocrinology 106:739-43

251. **Chan WY, Berezin I, Daniel EE** 1988 Effects of inhibition of prostaglandin synthesis on uterine oxytocin receptor concentration and myometrial gap junction density in parturient rats. Biol Reprod 39:1117-28

252. **Vince GS, Starkey PM, Jackson MC, Sargent IL, Redman CWG** 1990 Flow cytometric characterization of cell populations in human pregnancy decidua and isolation of decidual macrophages. J Immunol Methods 132:181-9

253. **Mitchell MD, Edwin S, Romero RJ** 1990 Prostaglandin biosynthesis by human decidual cells: effects of inflammatory mediators. Prostagladins Leukotrienes and Essential Fatty Acids 41:35-8

254. **Romero R, Mazor M, Sepulvada W, Avila C, Copeland D, Williams J** 1992 Tumor necrosis factor in preterm and term labour. Am J Obstet Gynecol 166:1576-87

255. **Romero R, Brody DT, Oyarzun E** 1989 Infection and labour. III. Interleukin-1: a signal for the onset of parturition. Am J Obstet Gynecol 160:1117-23

256. **Romero R, Avila C, Santhanam U, Sehgal PB** 1990 Amniotic interleukin-6 in preterm labor. J Clin Invest 85:1392-400

257. **Opsjon S-L, Wathen NC, Tingulstad S, Wiedswang G, Sundan A, Waage A, Austgulen R** 1993 Tumor necrosis factor, interleukin-1, and interleukin-6 in normal human pregnancy. Am J Obstet Gynecol 169:397-404

258. **Casey ML, Cox SM, Beutler B, Milewich L, MacDonald PC** 1989 Cachectin/tumor necrosis factor-α formation in human decidua. J Clin Invest 83:430-6

259. **MacDonald PC, Koga S, Casey ML** 1991 Decidual activation in parturition: examination of amniotic fluid for mediators of the inflammatory response. Ann NY Acad Sci 622:315-30

260. **Dudley DJ, Trautman MS, Araneo BA, Edwin SS, Mitchell MD** 1992 Decidual cell biosynthesis of interleukin-6: regulation by inflammatory cytokines. J Clin Endocrinol Metab 74:884-9

261. **Kent ASH, Sullivan MHF, Elder MG** 1994 Transfer of cytokines through human fetal membranes. J Reprod Fertil 100:81-4

262. **Mitchell MD, Trautman MS, Dudley DJ** 1993 Immunoendocrinology of preterm labour and delivery. Ballieres Clin Obstet Gynaecol 7:553-75

263. **Baggia S, Gravett MG, Witkin SS, Haluska GJ, Novy MJ** 1996 Interleukin-1β intra-amniotic infusion induces tumor necrosis factor-α, prostaglandin production, and preterm contractions in the pregnant rhesus monkey. Journal of the Society for Gynecologic Investigation 3:121-6

264. **Atkinson YH, Murry AW, Krilis S, Vadas MA, Lopez AF** 1990 Human tumour necrosis factor-alpha (TNFα) directly stimulates arachidonic acid release in human neutrophils. Immunology 70:82-7

265. **Romero R, Mazor M, Tartakovsky B** 1991 Systemic administration of interleukin-1 induces preterm parturition in mice. Am J Obstet Gynecol 165:969-71

266. **Bry K, Hallman M** 1993 Transforming growth factor-β2 prevents preterm delivery induced by interleukin-1α and tumor necrosis factor-α in the rabbit. Am J Obstet Gynecol 168:1318-22

267. **Fidel PL Jr., Romero R, Wolf N, Cutright J, Ramirez M, Araneda H, Cotton DB** 1994 Systemic and local cytokine profiles in endotoxin-induced preterm parturition in mice. Am J Obstet Gynecol 170:1467-75

268. **Akira S, Kishimoto T** 1997 NF-IL6 and NF-κB in cytokine gene regulation. Adv Immunol 65:1-46

269. **Tazawa R, Xu X-M, Wu KK, Wang L-H** 1994 Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin synthase-2 gene. Biochem Biophys Res Commun 203:190-9

270. **Chen G, Kamal M, Hannon R, Warner TD** 1998 Regulation of cyclo-oxygenase gene expression in rat smooth muscle cells by catalase. Biochem Parmacol 55:1621-31

271. **Sorli CH, Zhang HJ, Armstrong MB, Rajotte RV, Maclouf J, Robertson RP** 1998 Basal expression of cyclooxygenase-2 and nuclear factor-interleukin 6 are dominant and coordinately regulated by interleukin 1 in the pancreatic islet. Proc Natl Acad Sci U S A 95:1788-93

272. **Tora L, Gaub MP, Mader S, Dierich A, Bellard M, Chambon P** 1988 Cell-specific activity of a GGTCA half-palindromic ooestrogen-responsive element in the chicken ovalbumin gene promoter. EMBO J 7:3771-8

273. **Loose-Mitchell DS, Chippetta Stancel GM** 1988 Estrogen regulation of c-fos messenger ribonucleic acid. Mol Endocrinol 2:949-51

274. **Weisz A, Cicatiello L, Persico E, Scalona M, Bresciana F** 1990 Estrogen regulates expression of c-*jun* protooncogene. Mol Endocrinol 4:1041-50

275. **Kirkland JL, Murthy L, Stancel GM** 1992 Progesterone inhibits the estrogen-induced expression of c-*fos* messenger ribonucleic acid in the uterus. Endocrinology 130:3223-30

276. **Geimonen E, Boylston E, Royek A, Andersen A** 1998 Elevated connexin-43 expression in term human myometrium correlates with elevated c-*jun* expression and is independent of myometrial estrogen receptors. J Clin Endocrinol Metab 83:1177-85

277. **Piesanti M, Lye SJ** 1995 Increase in messenger ribonucleic acid encoding the myometrial gap junction protein, connexin-43, requires protein synthesis and is associated with increased expression of the activiator protein-1, c-*fos*. Endocrinology 136:3571-8

278. **Wang S, Evers BM** 1998 Cytokine-mediated differential induction of hepatic activatorprotein-1 gene. Surgery 123:191-8

279. **Wegmann TG, Lin H, Guilbert L, Mosmann TR** 1993 Bidirectional cytokines interactions in the maternal-fetal relationship: is successful pregnancy a T$_H$2 phenomenon? Immunol Today 14:353-6

280. **Chaouat G** 1993 The roots of the problem: "the fetal allograft". *In* Immunology of Pregnancy. pp1-17. Chaouat G. (editor) CRC Press, Boca Raton, FL,

281. **Mosmann TR, Coffman RL** 1989 Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv Immunol 46:111-47

282. **Betz M, Fox BS** 1991 Prostaglandin E_2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J Immunol 146:108-13

283. **Hart PH** 1989 Potential antiinflammatory effects of interleukin-4: suppression of human monocyte tumor necrosis factor alpha, interleukin-1 and prostaglandin E2. Proc Natl Acad Sci U S A 86:3803-7

2. MATERIALS AND METHODS

2.1. ANIMALS

All experiments were approved by the Institutional Animal Care committee. Time-mated, primigravid Sprague-Dawley rats (approximately 250 grams each) were transferred from Charles River Canada to our animal facility at day 12 of pregnancy. Food and water were available ad libitum. The light:dark cycle was 12h:12h. Euthanasia of the rats was performed via intraperitoneal injection of Euthanyl™ at 100 mg/kg body weight. The blood was collected by heart puncture and allowed to clot at 4° C. The clotted blood was centrifuged at 1,000 x g for 15 min. Sera were collected, frozen and stored at -70° C. The uteri were removed immediately after euthanasia. Uterine tissues were frozen in liquid nitrogen and stored at -70° C until RNA extraction or homogenization for radioimmunoassay (RIA) and enzyme immunoassay (EIA) or binding assays.

2.2. METHODS

2.2.1. Tissue Preparation for Cytosolic and Microsomal Fractions

Each frozen tissue sample was crushed in a mortars precooled with liquid nitrogen and placed on dry ice. Approximately one gram of crushed frozen tissue was homogenized in 3 ml tris-EDTA buffer containing monothioglycerol at 3 x 5 s setting 5 (Brinkman Polytron) on ice. After homogenization, the sample was centrifuged at 1,000 x g for 15 min and the supernatant was transferred to a fresh centrifugation tube. The cytosolic fraction and microsomes were separated by centrifugation at 105,000 x g for 60 min at 4° C. The resulting microsomal pellet was resuspended in 25 mM Tris buffer and hand homogenized to form a suspension for OT binding assay. The supernatant as cytosolic fraction was collected for PGE_2, ER and PR

109

assays.

2.2.2. Radioimmunoassay

Serum estradiol (E_2) and Progesterone (P_4), OT, and PGE_2 were measured by RIAs. The RIA procedures and antisera for the E_2 and P_4 assays have been described previously (1). The samples were extracted from sera and incubated with radiolabeled estradiol or progesterone and antibody overnight at 4° C. Free and bound steroids were separated with dextran-coated charcoal. After centrifugation at 3,000 x g for 15 min, the supernatant was counted by scintillation spectrometry. TAM did not interfere in the E_2 RIA.

OT peptide was determined by RIA according to the procedure described previously (2). The antibody for OT was generously provided by Dr. A. P. F. Flint. It does not cross-react significantly with vasopressin or other known peptides. Briefly, one gram of frozen uterine tissue was homogenized in 4 ml acid buffer (5% formic acid, 10% trifluoroacetic acid and 1% NaCl in 1.0 N HCl) and centrifuged at 1,000 x g for 30 min. The supernatant or serum treated with acid buffer was passed through C-18 Sep-pak cartridges (Waters, Milford, MA). OT was eluted from the column with 75% acetonitrile in 0.01 M trifluoroacetic buffer. The extracts were dried and then dissolved in 0.5 ml RIA buffer containing 50 mM sodium phosphate and 10 mM ethylenediaminetetraacetic acid (EDTA). A 25 - 50 µl aliquot was incubated with 5,000 cpm [^{125}I]OT (New England Nuclear-DuPont, Boston, MA) and OT antibody overnight at 4° C. After incubation with a second antibody (rabbit anti-sheep serum) and normal sheep serum, the samples were centrifuged at 1,000 x g for 15 min. The supernatant was aspirated and the pellet was counted in a Geiger spectrometer.

The PGE_2 assay has been described previously (3). The antiserum specific for PGE_2 was provided by Dr. Leslie Myatt from the University of

110

Cincinnati. The cytosolic fraction after high speed centrifugations was used for the assays. Briefly, 0.5 ml samples with 3 ml citrate buffer containing 15 % ethanol were passed through C-18 Sep-pak cartridges pre-equilibrated with 4 ml 95% ethanol and 4 ml double distilled H_2O. The column was washed with 2 ml H_2O and 3 ml petroleum ether. PGE_2 was eluted from the column with 3 ml ethylacetate. The eluted PGE_2 was dried and reconstituted with sodium phosphate buffer containing gelatin. A 50 - 100 µl aliquot was incubated with [^{3}H]PGE_2 (New England Nuclear Corp., Boston, MA) and antibody overnight at 4° C. Free and bound PGE_2 were separated with dextran-coated charcoal. After centrifugation at 3,000 x g for 15 min, the supernatant was counted by scintillation spectrometry.

2.2.3. Enzyme-immunoassay

The ER concentrations in uterine tissue cytosols were measured by EIA using a monoclonal antibody kit (Abbott Laboratories, Mississauga, ON). This antibody measures both occupied and unoccupied receptors and the ER measurements are not affected by the presence of physiologic concentrations of other steroids, including TAM at 10^{-9} M. Higher concentrations of TAM (= 10^{-7} M) may result in overestimation of ER by 40 - 100% (manufacturer's information sheet). ER-EIA assay was performed according to the manufacturer's instruction. Briefly, 200 µl of 0, 5, 25, 100, and 250 fmol/ml standards and 100 µl cytosol samples and control with 100 µl of Specimen Diluent were added into assigned wells. An ER bead was added to each well, gently tapped and incubated at 4 C for 18 h. After washing the ER beads, 200 µl of ER Conjugate were dispensed into each well and incubated in a 37° C water bath for 60 min. Finally, the beads were transferred into the reaction tubes, mixed with 300 ml of OPD Substrate Solution and incubated at room temperature for 30 min . After adding 1 ml of 1 N sulfuric acid to each tube,

111

all the standards, control and samples were read at 492 nm in spectrophotometer (Abbott Laboratory, North Chicago, IL).

$PGF_{2\alpha}$ concentration in uterine tissue cytosols were measured by EIA. The assay used an ACE^{TM} EIA kit (Cayman Chemical Co., Ann Arbor, MI) and was performed according to the manufacture's instruction. Briefly, samples were prepared with adding 5000 cpm of $[^3H]$-$PGF_{2\alpha}$ and 2 ml of ethanol, and passed through C-18 Sep-Pak. After the cartridge was rinsed with water and HPLC grade hexane, $PGF_{2\alpha}$ was eluted with ethyl acetate containing 1 % methanol. After samples were evaporated, 1 ml of EIA buffer was added to each. 500 µl of samples were aliquoted for scintillation counting and the remaining 500 µl samples for the EIA analysis. Each sample was assayed at two dilutions, and each dilution was assayed in duplicate. After samples or standards with tracer and antiserum were pipeted into the designed wells of a plate, the plate was incubated for 18 h at room temperature. When ready to develop, the wells were rinsed five times and added 200 µl of Ellman's reagent to each well. The plate was read at 412 nm.

2.2.4. Progesterone Binding Assay

The cytosol PR in uterine tissues was measured using a radioligand binding assay with the same sample as for cytosol ER. Samples were incubated with a mixture containing increasing concentrations of $[^3H]$promegestone ($[^3H]$R-5020, 0.2 nmol/L to 5 nmol/L; New England Nuclear-DuPont, Boston, MA), in the presence of 10-fold concentrations of dexamethasone and dihydrotestosterone to prevent binding to glucocorticoid or androgen receptors. Nonspecific binding was measured following the addition of 50 nmol/L nonradioactive R-5020. After incubation overnight, free and bound ligand were separated with dextran-coated charcoal. Following centrifugation, the supernatants were counted in a scintillation spectrometer.

112

2.2.5. Oxytocin Binding Assay

OTR in uterine tissues was measured using a modification of a published OT binding assay method (4). Briefly, the pellet obtained following the 105,000 x g centrifugation step was washed, resuspended in Tris buffer and incubated for 1 h with 0.6 nmol/L [^{3}H]OT (New England Nucear-DuPont, Boston, MA) and increasing concentrations of non-radioactive OT (0.0 to 9.4 nmol/L). Nonspecific binding was measured following the addition of 100 nmol/L nonradioactive OT. Incubation was terminated by filtering the suspension through a glass microfibre filter (GF/C, Whatman, Springfield Mill, Maidstone, England) and rinsing with cold Tris buffer. The filters (carrying receptor-bound [^{3}H]OT) were counted by scintillation spectrometry. The total number of OTR and K_d in each sample was determined by Scatchard analysis performed using 6 concentrations of OT.

2.2.6. Ribonuclease Protection Assay
2.2.6.1. Total RNA Preparation

Two methods were used for total RNA preparation: a single-step method by guanidinium thiocyanate phenol-chloroform (5) and a Trizol^{TM} method.

In the single-step method, approximately one gram of frozen uterine tissue was homogenized in 10 ml of denaturing solution containing 4 M. guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyal amd 0.1 M 2-mercaptoethanol. Sequentially, each homogenized sample was mixed with 10 ml of phenol (water saturated) and 0.2 ml of chloroform-isoamyl alcohol (49:1 chloroform and) at acidic conditions (pH 4.0). After the suspensions were shaken vigorously for 10 sec and cooled on ice for 15 min, samples were centrifuged at 10,000 x g for 20 min at 4° C. Finally, the aqueous phase containing total RNA was transferred to a fresh centrifuging tube, mixed with

113

an equal volume of isopropanol and placed at -20° C for 1 h to precipitate the RNA. After precipitation, the sample was sedimentated at 10,000 x g for 20 min to obtain a RNA pellet and redissolved with 0.5 ml of denaturing solution. The solubilized RNA was transferred into an Eppendorf tube and reprecipitated with 0.5 ml of isopropanol at -20° C for overnight. After 10 min centrifugation at 4° C, the RNA pellet was washed with 75 % ethanol once and redissolved in 0.1 - 0.3 ml of TE buffer containing 10 mM tris-Cl and 1 mM EDTA. The concentration of RNA was determined by spectrophotometry at A260.

In the Trizol method, 0.1 -0.2 gram of frozen uterine tissue was homogenized in 1 ml of Trizol reagent. The homogenized samples were mixed with 0.2 ml of chloroform, shaken vigorously by hand for 15 sec and incubated on ice for 5 min. After incubation, the samples were centrifuged at 12,000 x g for 15 min at 4° C. Following centrifugation, the mixture was separated in a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube, the RNA precipitated by mixing with an equal volume of isopropyl alcohol and incubated at -70° C for 15 min or -20° C for 2 h. After centrifugation at 12,000 x g for 10 min at 4° C, the supernate was removed and discarded. The RNA pellets were washed in 75 % ethanol and centrifuged at 7,500 x g for 5 min at 4° C. After briefly drying the pellets, RNA was redissolved in 0.5 ml TE buffer containing 10 mM tris-Cl and 1 mM EDTA.

2.2.6.2. Complementary DNA (cDNA) templates

OT

A specific cDNA to generate complementary RNA (cRNA) for rat OT

mRNA was prepared from a rat genomic clone (kindly provided by Dr. Hartwig Schmale from the University of Hamburg (6)). The antisense cRNA probe corresponded to exon C and part of the second intron of the rat OT gene.

OTR

The complementary DNA for OTR was prepared from a rat genomic clone and subcloned into pCR™II plasmid in E.coli bacteria (kindly provided by Dr. Stephen J. Lye from the University of Toronto) and corresponded to the exon 2 and 3 of OTR genes which encode transmembrane domains 3 to 7. After regeneration of bacteria, the vectors were extracted and linearized with AccI restriction enzyme for OTR cRNA probe.

ER, PR-total and PR-B

The rat ER cDNA was prepared using reverse transcription-polymerase chain reaction (RT-PCR) with primers to portions of exons 4 and 6 (cDNA Cycle Kit, Invitrogen Corp., San Diego, CA). The resultant 439 nucleotide probe was subcloned using pCR-Script AmpSK(+) cloning kit (Stratagene, La Jolla, CA).

The rat PR-B cDNA was generated by RT-PCR with primer sets flanking the part of the 5'-untranslated region and N-terminal region of B form (390 bp). The rat PR-total cDNA was prepared by primers flanking the steroid-binding domain for all A, B and C forms (320 bp) (for detail see chapter 5).

RT-PCR and Subcloning

The RT reaction of the first-strand cDNA was generated by random primers using day 21 pregnant uterine total RNA (1μg) and PCR was performed for 30 cycles of 1 min at 95° C, 2 min at 56° C and 3 min at 72° C.

115

The RT-PCR products were screened on 1 % agrose gel.

The amplified DNA was cloned using pPCR-Script™ Amp Cloning kit (Stratagene, La Jolla, CA). Restriction and sequence analyses were preformed to confirm the sequence of the inserted PCR product. The PCR-generated rat ER, PR-B and PR-total cDNA clone in pPCR-Script plasmid is linearized by Not I to generate an anti-sense cDNA template and EcoR I by to generate a sense template. In the ribonuclease protection assay (RPA), after synthesis of cRNA, hybridization of day 21 pregnant uterine total RNA (20 µg) with sense probe, along with hybridization of tRNA (10 µg) with antisense probe were performed as the negative controls. The optimal concentrations of total RNA (µg) were determined by increasing concentrations of total RNA (from 5 µg to 80 µg). The minimal radioactivity of ^{32}P-CTP labeled probes was determined by increasing the amount of radioactivity (from 0.125 to 2.0 cpm x 10^6) titrated with 20 µg total RNA (the optimal concentration) in RPA

2.2.6.3. Ribonucease Protection Assay

OT, OTR, ER and PR-total and PR-B mRNA levels in the uterine tissues were measured by an RPA previously described (7). Briefly, 10 µg - 40 µg total RNA (10 µg for OT, 40 µg for OTR or 20 µg for ER, PR-total or PR-B) was hybridized to gel-purified antisense ^{32}P-labelled RNA probe in 80% formamide and 5 x salts containing 200 mM 1,4-piperazinediethanesulfonic acid, 2 M NaCl and 5mM EDTA for 18 h at 55° C. Following incubation with 0.75 µg ribonuclease A and 300 units ribonuclease T_1 (both from Boehringer Mannheim Canada, Laval, Québec) for 30 min at 30° C, protected fragments were analyzed on 6 % or 8% denaturing polyacrylamide gels. A RNA sample from one animal at each time point from each group, chosen at random, was run on each gel. The gel was exposed to XAR x-ray film (Eastman Kodak, Rochester, NY) for 1 h. A rat glyceraldehyde-3-phosphate-dehydrogenase

116

(GAPDH) probe or β-actin probe (both from Ambion Inc. Austin, TX) was used in all samples as an internal control. Autoradiograms were quantitated using laser densitometry and data were presented as the ratio of arbitrary densitometric units of OT mRNA to GAPDH mRNA or β-actin mRNA.

2.3. References

1. **Mitchell BF, Wong S** 1993 Changes in 17 beta,20 alpha-hydroxysteroid dehydrogenase activity supporting an increase in the estrogen/progesterone ratio of human fetal membranes at parturition. Am J Obstet Gynecol 168:1377-85

2. **Chibbar R, Wong S, Miller FD, Mitchell BF** 1995 Estrogen stimulates oxytocin gene expression in human chorio-decidua. J Clin Endocrinol Metab 80:567-72

3. **Mitchell BF, Rogers K, Wong S** 1993 The dynamics of prostaglandin metabolism in human fetal membranes and decidua around the time of parturition. J Clin Endocrinol Metab 77:759-64

4. **Soloff MS, Alexandrova M, Fernstrom MJ** 1979 Oxytocin receptors: triggers for parturition and lactation? Science 204:1313-5

5. **Chomcynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. Anal Biochem 162:156-9

6. **Ivell R, Richter D** 1984 Structure and comparison of the oxytocin and vasopressin genes from rat. Proc Natl Acad Sci U S A 81:2006-10

7. **Chibbar R, Miller FD, Mitchell BF** 1993 Synthesis of oxytocin in amnion, chorion, and decidua may influence the timing of human parturition. J Clin Invest 91:185-92

3. RELATIONSHIPS AMONG SEX STEROIDS, OXYTOCIN, AND THEIR RECEPTORS IN THE RAT UTERUS DURING LATE GESTATION AND PARTURITION

(This paper has been published in Endocrinology, 1996)

3.1. INTRODUCTION

OT is a potent stimulus to myometrial contractility in essentially all species. In both humans and rats, OT mRNA is synthesized within human intrauterine tissues, principally the decidua, with increasing concentrations around the time of parturition (1, 2). In explants of human chorio-decidua, estrogen increases OT mRNA concentration (3). We have shown that estrogen is synthesized in fetal membranes and decidua, and increases around parturition (4, 5). Furthermore, we have demonstrated mRNA for ER and PR in human amnion, chorion, and decidua with a similar tissue distribution as OT mRNA. There is a significant increase in ER mRNA around the time of parturition (3). These findings support the existence of a paracrine system within late gestational human intrauterine tissues involving sex steroids and OT that may regulate the timing of labour onset. This paper presents data from our further studies of this paracrine system using an animal model that can be manipulated experimentally.

In the rat, OT mRNA is synthesized in the epithelial layer of the endometrium (2). Between day 14 - 18 of pregnancy, there is a rapid increase in OT mRNA accumulation, and the highest level of OT expression is restricted to the last 3-4 days of gestation. There is also a corresponding increase in immunoreactive OT in uterine tissues during pregnancy, with the highest level occurring at the time of parturition. Estrogen stimulates OT release into the rat circulation (6) and also enhances the synthesis of uterine OT

in non-pregnant animals (7). These findings support the presence of a similar paracrine system within the rat uterus and suggest that the rat may be a relevant model in which to study the role of locally produced OT in the initiation of labour.

Uterine contractility is also effected by the sensitivity of the myometrium to OT. In both humans and rats, myometrial sensitivity to OT increases through late gestation in parallel with an increase in myometrial OTR concentrations (8-11). Estrogen increases OT binding in ovariectomized pregnant rats and P4 completely inhibits this estrogen-induced rise (11). This increase in OTR was correlated with the concentration of ER. These findings suggest that OTR is regulated by ovarian steroids in a manner related to ER activation. Furthermore, it has been shown that OT stimulates the secretion of uterotonic PGs from the pregnant rat endometrium (12) and human decidua (8, 13).

In this report, we have further characterized the relationships among sex steroids, OT and their receptors to test the hypothesis that local concentrations of OT and OTR in the uterus of the late gestation pregnant rat are regulated by sex steroids in a receptor mediated fashion. In this regard, we have determined the effects of the estrogen receptor blocker TAM on serum levels of estrogen and P4 and on the synthesis of ER, PR, OT and OTR in late gestation rat uterine tissues. Our secondary aim was to evaluate the rat as a potential model for human parturition.

3.2. MATERIALS AND METHODS

3.2.1. Animals and Treatment

The animals used in this study are described in detail in Chapter 2. The rats were divided into 2 groups. In this strain of rats, parturition usually occurs early in the afternoon of day 22 when the day of mating is considered day 0.

120

The experimental group was treated daily with TAM 200 μg/rat (Sigma, St. Louis, MO) with 0.4 ml oil, from day 15 of pregnancy until being euthanized. This dose was chosen since it has been shown to have maximal antagonistic effects with high estrogen levels as seen in late gestation (14). Subcutaneous injections were performed into the back of the rat using a 26.5 gauge needle. Euthanasia was at pregnancy day 19, 21, 21.5 (the evening of day 21) and 22 of pregnancy, and during labour (following delivery of first pup). The control group received only daily injections with vehicle (0.4 ml oil). The rats were injected between 0900 and 1000 hours and euthanasia was also performed at this time or, in the case of the 21.5 day groups, between 2100 and 2200 hours. Five rats were used at each time point in each group. Samples were taken for measurement of serum concentrations of E_2, P_4 and OT, uterine OT mRNA and peptide levels, uterine cytosol concentrations of ER and PR, and uterine levels of PGE_2 and OTR.

3.2.2. RIAs for E_2, P_4, OT and PGE_2

The RIA procedures and antisera for the E_2 and P_4 assays have been characterized previously (5). OT peptide was determined by RIA according to the procedure described previously (3). The cytosol PGE_2 assay has been described previously (15). The protocol is described in detail in Chapter 2.

3.2.3. OT mRNA Assay

A special probe for rat OT mRNA prepared from a rat genomic clone kindly provided by Dr. Hartwig Schmale from the University of Hamburg (16). RPA for the measurement of OT mRNA levels in human decidual tissues has previously been reported (1). The protocol (Chapter 2) was found to be optimal for use with rat uterine tissue.

121

3.2.4. ER Assay

The ER concentrations in uterine cytosols were measured by EIA. The methodology is described in detail in Chapter 2.

3.2.5. PR Assay

The cytosol PR in uterine tissues was measured using a radioligand binding assay with the same sample as that used to determine cytosol ER. This methodology was designed and validated as part of these studies and is described in detail in Chapter 2.

3.2.6. OTR Assay

OTR in uterine tissues was measured using a modification of a published OT binding assay (9). The methodology was modified and validated for use in these studies and is described in detail in Chapter 2.

3.2.7. Statistical Analysis

Data are presented in text and graphs as the mean ± standard error of the mean. The results were first analyzed by one-way analysis of variance (InStat, GraphPad Software, San Diego, CA) to detect changes with advancing gestational age. Post-hoc comparisons of the means were performed using the Tukey-Kramer Multiple Comparisons Test. Differences between the experimental and control groups were sought using the two-tailed unpaired Student's t test. Differences were considered to be significant when a P value < 0.05 was obtained. If Bartlett's test revealed non-homogeneity of variance, the corresponding non-parametric test was utilized.

122

3.3. RESULTS

3.3.1. Parturition

In our rat strain, spontaneous delivery occurs early in the afternoon of day 22 of gestation. In the control group, the duration of pregnancy was 539.9 ± 1.1 h (calculated from midnight on the day of mating). TAM treatment significantly prolonged the duration of pregnancy by 24 h to 563.3 ± 2.6 h. The average fetal weight in the TAM treated dams was significantly less than in controls on either day 21 or 22 of pregnancy. However, when the treated dams finally did go into labour, the birth weight of the litter was not significantly different from that in the control group (Table 3-1).

3.3.2. Serum E_2 and P_4

Serum concentrations of E_2 increased significantly, whereas the level of P_4 declined significantly in late pregnancy before labour (Table 3-1). Between day 19 and the time of labour, serum P_4 decreased to one-third, and the estrogen to P_4 ratio (E/P ratio) increased 5-fold. In the TAM-treated group, E_2 levels after day 21 were significantly less than those in controls and the increase in E/P ratio was not statistically significant.

3.3.3. Uterine Cytosol ER and PR

The concentration of ER in uterine cytosol increased significantly after day 19 of pregnancy (Fig. 3-1a) from a level of 18.4 ± 0.9 to 88.2 ± 11.4 fmol/mg tissue protein. TAM treatment blocked the increase. The concentrations of cytosolic PR in uterine tissues did not significantly change during late gestation, and there was no apparent effect of TAM (Fig. 3-1b).

123

3.3.4. Uterine OT mRNA and Peptide

There was a 3- to 4-fold increase in uterine OT mRNA between day 16 and 19 of pregnancy (Fig. 3-2) with no further significant change throughout gestation. TAM significantly inhibited this increase in the OT mRNA concentration, resulting in levels 5- to 7-fold lower than control values. There was no effect of gestational age or TAM on GAPDH gene expression. In controls, the concentration of OT peptide in uterine tissues was significantly higher during labour than at earlier points (Fig. 3-2b). This increase was not observed in the TAM-treated animals. In this group, uterine tissue OT peptide concentrations were consistently lower than in controls, but this achieved statistical significance only during labour. In the maternal circulation, serum concentrations of OT were quite variable (range, 50 - 300 pmol/liter; data not shown) and did not change significantly through late gestation. TAM had no significant effect on serum OT.

3.3.5. Uterine OTR

In the control group, there was an abrupt 5-fold increase in uterine OTR that occurred only a few hours before parturition (Table 3-2). TAM almost completely inhibited this increase on day 22 of pregnancy. However, despite continued TAM treatment, a significant increase in OTR to control levels occurred some time before the delayed parturition in the experimental group.

3.3.6. Uterine PGE_2

Uterine tissue concentrations of PGE_2 increased significantly in late pregnancy, with peak levels occurring by day 21.5 of pregnancy (Table 3-2). Treatment with TAM resulted in significantly lower PGE_2 concentrations on day 21 and 21.5. However, peak PGE_2 tissue concentrations at the time of the

124

TAM-delayed parturition were similar to those in the control animals at normal parturition.

3.4. DISCUSSION

These data are the first to correlate the sequence of events relating to sex steroids and uterine OT around the time of parturition in the rat. Normal parturition in this species occurs in the early afternoon of day 22. The first significant change we detected was an increase in OT mRNA in uterine tissues between days 16 and 19. This is in agreement with previous findings (2), which also demonstrated that OT mRNA was located in the endometrial epithelial cells and increased greatly before day 18, then again at parturition. Contrary to our expectations, we detected no further increase in uterine OT mRNA after day 18 when expressed as a ratio to GAPDH. The difference between these results and the previous study (2) may be explained by several methodological differences: Our studies used 10 µg total RNA in a ribonuclease protection assay with a cRNA probe, whereas the previous study used 100 µg poly-A RNA in a Northern assay with an oligonucleotide probe. Additionally, uterine wet weight as well as total RNA increase dramatically around this time. Therefore, it is likely that the total mass of OT mRNA actually did increase, but the concentration relative to GAPDH or total RNA did not change. In the present studies, we did not attempt to separate endometrium from myometrium because we were interested in potential paracrine interactions within the entire tissue. Examination of isolated tissue layers may give a different profile of OT mRNA concentration with advancing gestation.

TAM treatment completely prevented the increase in OT mRNA noted in controls before day 19. Even at the time of labour and delivery, which was delayed 24 hours, uterine OT mRNA levels remained very low. This suggests

125

either that tissue concentrations of OT mRNA are not determinants of uterine contractility or that there are other mechanisms that can be evoked to bring about parturition after a significant delay.

The blockade of the late gestational increase in OT mRNA by TAM supports previous findings that OT gene expression is regulated by estrogen. Endometrial OT mRNA is increased in both humans and rats by estrogen treatment (3, 7). The OT gene promoter region contains estrogen response elements that have been shown to confer estrogen responsivity *in vitro* (18). However, it is noteworthy that the levels of OT mRNA did not parallel serum E_2 concentrations. Unfortunately, we did not measure serum E_2 levels before day 19, but others have shown that serum E_2 levels are relatively constant at that time (19). There was no further increase in OT mRNA after day 21, when serum E_2 increased markedly. These finding suggest that serum estrogen may not be the most important source of estrogenic activity in the pregnant rat endometrium and allows speculation that locally produced estrogen may be more important.

The peak concentrations of OT peptide in uterine tissues occurred at the time of parturition, several days after the peak in OT mRNA. One possible reason for the increase in OT peptide is increased translational efficiency. This may be regulated by an increase in serum E_2, a decline in P_4, or an increase in the E/P ratio. As the increase in OT peptide was absent in the TAM group despite normal serum P_4 withdrawal, we suggest that the increase in E_2 was the most likely mechanism. Alternatively, increased OT peptide may have been due to decreased OT catabolism in the uterine tissues. The rates of OT metabolism in uterine tissues around parturition in the rat are unknown, but there is no change in OT metabolism in human decidua around the time of labour onset (20). It is also possible that initial translation of OT mRNA results in the formation of extended forms of OT that are not measured in our

RIA (21). The final increase in OT peptide would occur only when the tissues had the capability to convert the extended forms to mature OT. A final explanation could be that increase in tissue OT peptide may simply reflect the increasing serum OT levels that occur at this time (22). This explanation is unlikely because the increase was not seen in the TAM-treated group in which our serum OT levels were not different from the control values.

TAM acts by binding to ER and has the ability to induce a spectrum of hormonal effects. In rats, it displays both agonist and antagonist properties (23). In this study, we saw no evidence of agonist activity. Indeed, not only did TAM appear to act as an estrogen antagonist, it also significantly suppressed serum E_2 concentrations. Estrogen is known to increase uterine blood flow, and this can be prevented by TAM (24). E_2 in rat serum during pregnancy is produced from luteal tissues, but its precursor androgen is of placental origin (19). We suggest a reduction of uterine blood flow caused by TAM results in decreased production of E_2 precursors, thus decreasing the synthesis of E_2. A reduction in uterine blood flow also would explain the significant reduction of fetal growth rate in the TAM treated animals. This reduction in serum E_2 concentration was not observed when ICI182780 was used as an estrogen antagonist in the late gestation rat (25). This difference may be explained by different profiles of activity for the two antagonists or may be related to dose. It is possible that the changes in hormone receptors caused by TAM were secondary to the reduced uterine blood flow, rather than by direct estrogen antagonism at the receptor gene level. The known direct effects of estrogen antagonism on these receptors in other tissues makes this an unlikely possibility.

In agreement with previous studies (19), there was a significant decline in maternal serum P_4 concentrations in the days immediately preceding delivery. The serum E/P ratio increases throughout this time. Treatment of

127

rats with TAM did not influence the late gestation decline in serum P_4, but did attenuate the increase in the E/P ratio. Despite the persistent low estrogen levels and continued TAM treatment, parturition did occur. This suggests that the decline in P_4 is more important than the rise in serum estrogen. This would support the P_4 block concept of parturition (26). Although we observed no changes in PR levels during late gestation, we measured only cytosol levels of PR. Others have detected significant decreases in nuclear PR levels or in the ratio of ER to PR in the nucleus at the time of parturition in the rat (27,28).

The increase in uterine ER concentration was parallel to the increase in serum E_2 and the E/P ratio. These findings are in agreement with previous data showing that ER concentrations in both cytosol and nuclear fractions of rat myometrium increased with E_2 (9, 27). In agreement with others, our studies found that TAM decreased the uterine cytosol level of ER (28).

There was a 5-fold rise in uterine OTR concentrations just a few hours before delivery. The mean K_d of the OTR is in the range of 1-3 nmol/liter and did not change with advancing gestation or TAM treatment. TAM inhibited the increase in receptor number on day 22. Interestingly, the promoter region of the OTR gene of several species has been sequenced and is lacking a complete palindromic concensus estrogen response element sequence, although there are several half-sequences present (29, 30). It is not yet clear whether the effects of estrogen and TAM on OTR gene expression are direct or indirect. The much greater increase in OTR compared to that of OT peptide or mRNA, and its proximity to the onset of labour suggest that the concentration of OTR, rather than that of OT itself, may be the limiting determinant of the timing of parturition. It is also noteworthy that in the TAM treatment group, parturition did not occur in the absence of OTR, in contrast to the low levels of OT mRNA and peptide in uterine tissues. In two additional animals, we doubled the dose of TAM and obtained similar findings. Other estrogen antagonists

128

also have been shown to cause prolonged gestation in the rat associated with a decrease in the synthesis of connexin 43 and gap junction formation (25).

Uterine tissue concentrations of PGE_2 rose during late pregnancy, with maximal levels achieved in the evening before parturition. TAM treatment resulted in significantly lower concentrations than control values on the day preceding delivery. As OT stimulates endometrial PGE_2 production (8, 12, 13), this decrease in PGE_2 may have resulted from interruption of the estrogenic stimulus to OT or OTR synthesis in the endometrium.

The data from the control and TAM-treated animals indicate three events that occurred before parturition in both models: the serum P_4 withdrawal and the increase in tissue PGE_2 and OTR concentrations. Several previous studies have suggested an interesting relationship among PGs, OT and OTR. OT stimulates production of PGE_2 from human decidua and rat endometrium (8, 12, 13). In the ovine corpus luteum, PGs stimulate the synthesis of OT (31). In rat endometrium, OTR synthesis is increased by PG (32) and suppressed by PG synthesis inhibitors (33). Therefore, there may be a positive feedback amplification circuit involving OT and PGs that could influence myometrial contractility. Such a mechanism could help explain why parturition eventually occurs 24 hours later than normal in the TAM-treated animals. In this group, an estrogen-independent increase in uterine PG synthesis may cause a sufficient increase in OT peptide and OTR to initiate a positive feedback loop that culminates in parturition even in the presence of estrogen antagonism. Our data support the hypothesis that P_4 withdrawal is the predominant regulator of this positive feedback loop.

A secondary objective of these experiments was to establish if the rat was a suitable model to study intrauterine paracrinology at parturition. As in most animal models, rat parturition is preceded by an increase in estrogen and decline in P_4 in maternal serum. This is in contrast to the human, in whom no

129

significant changes are noted before parturition. However, we demonstrated that both estrogen and P_4 are synthesized in human chorio-decidual tissue and that the local synthetic mechanisms may bring about an increase in the E/P ratio within these tissues around the time of labour onset (4, 5). This may be reflected in the amniotic fluid, where there is an increase in E/P ratio before labour onset (34). Trends in tissue levels of OT mRNA and peptide, PG, and OTR are similar in the rat and human, and at least some of the regulatory mechanisms appear to be the same. Thus, for the study of paracrine mechanisms, the rat appears to be a good model, although much additional experimental work in both species will be required to completely validate this. It should be noted that in our studies, we have defined the beginning of labour as the birth of the first pup. Clearly, labour began some time before this. Thus, it is possible that some of the changes we have measured occur after labour onset and are a consequence, rather that a cause, of labour. Electromyographic leads or intrauterine pressure-sensing devices will be required to more precisely define the timing of the initiation of labour. Whereas larger animal models allow frequent sampling from chronic preparations, permitting more detailed endocrine studies, they have no advantage as a source of the intrauterine tissues necessary to study paracrine relationships.

Our data suggest an important role for OTR in parturition. Results from studies using OT antagonists in rat pregnancy have been conflicting. Chan *et al* (32) and Hahn *et al* (35) have shown that OT antagonists do not delay labour onset, but significantly lengthen the course of labour. Conversely, Wilson *et al* (36) and Antonijevic *et al* (37) reported a delay in the initiation of labour and also a prolongation of the process of labour. The explanation for these conflicting results may be that there are different OTR subtypes in decidua and myometrium (38). Both may result in uterine contractions. However OTR

130

antagonists may inhibit one subtype while actually stimulating the other (38). Initial clinical studies in women in preterm labour suggest that OT receptor antagonists may be useful in suppressing uterine contractions (39). Clearly, more studies are required to understand the role of OT in both the initiation and progress of labour in the two species.

In summary, we have demonstrated time-course relationships among estrogen P_4, OT and their receptors in uterine tissues preceding parturition in the rat and investigated the effects of an estrogen antagonist on these parameters. The data confirm the importance of estrogen in ensuring normal fetal growth and in regulating several important factors in parturition, including estrogen itself, ER, OT and OTR. The TAM studies also suggest the presence of an estrogen-independent positive feedback loop involving OT, OTR and PGs that may be predominantly under the negative control of P_4. The reported data provide further support for the hypothesis that paracrine events within the pregnant uterus are important in regulating the timing of parturition. Finally, the data suggest that the rat model may have considerable similarity and relevance to the human.

131

Table 3-1. Fetal weight and serum estrogen and progesterone levels through late gestation and labor (mean ± s.e.m.) in the rat.

	day of gestation				
	19	21	21.5	22	labor
fetal weight (gm per fetus)					
control*	1.6 ± 0.06	3.9 ± 0.3	4.5 ± 0.2	6.0 ± 0.2	6.1 ± 0.1
tamoxifen*	1.4 ± 0.01	$3.5^{\dagger}$ ± 0.2	4.2 ± 0.3	$5.1^{\dagger}$ ± 0.2	5.9 ± 0.1
serum estradiol (pmol/L) '					
control*	324.0 ± 27.7	296.5 ± 25.5	447.0 ± 95.9	372.8 ± 30.4	548.0 ± 66.0
tamoxifen	183.1 ± 6.9	238.6 ± 60.4	$351.0^{\dagger}$ ± 90.6	$263.2^{\dagger}$ ± 78.2	$122.2^{\dagger}$ ± 35.8
serum progesterone (nmol/L)					
control*	186.0 ± 13.1	149.1 ± 37.6	60.0 ± 5.5	61.2 ± 7.8	59.0 ± 4.8
tamoxifen	226.0 ± 52.3	136.0 ± 49.2	125.4 ± 27.2	117.2 ± 47.2	31.6 ± 8.5
serum estradiol/prog. ratio (x 1000)					
control*	1.54 ± 0.21	2.51 ± 0.80	6.53 ± 1.21	5.39 ± 0.42	8.02 ± 0.46
tamoxifen	0.84 ± 0.20	2.13 ± 0.68	$3.43^{\dagger}$ ± 1.76	$2.43^{\dagger}$ ± 0.55	$4.20^{\dagger}$ ± 1.66

* statistically significant change with increasing gestational age by ANOVA ($P < 0.05$)

† statistically significant difference between tamoxifen and control groups ($P < 0.05$)

132

Table 3-2. Rat uterine OTR and PGE_2 levels through late gestation and labor (mean ± s.e.m.).

	day of gestation				
	19	21	21.5	22	labor
tissue OTR (fmol/mg protein)					
control*	23.2 ± 5.8	26.8 ± 5.5	29.1 ± 7.5	127.5 ± 7.8	147.4 ± 11.6
tamoxifen*	24.1 ± 5.1	31.1 ± 5.1	14.2 ± 6.2	18.0† ± 2.1	110.5 ± 22.4
tissue PGE₂ (fmol/mg protein)					
control*	478 ± 54	766 ± 71	1343 ± 205	1111 ± 73	992 ± 135
tamoxifen*	388 ± 87	532† ± 65	691† ± 17	897 ± 114	1248 ± 292

* statistically significant change with increasing gestational age by ANOVA ($P < 0.05$)

† $P < 0.05$ comparing the control to the tamoxifen group

133

Figure 3-1   The effects of TAM on cytosol concentrations of (a) ER and (b)
PR in pregnant rat uterine tissues. ER was measured Iby enzyme immunoassay
and PR by radioligand binding assay. (* $p < 0.05$ comparing the two groups at
the same time point.)

a.

ER (fmol/mg tissue protein)

Control
TAM

19   21   21.5   22   labour

Days of gestation

b.

PR (fmol/mg tissue protein)

Control
TAM

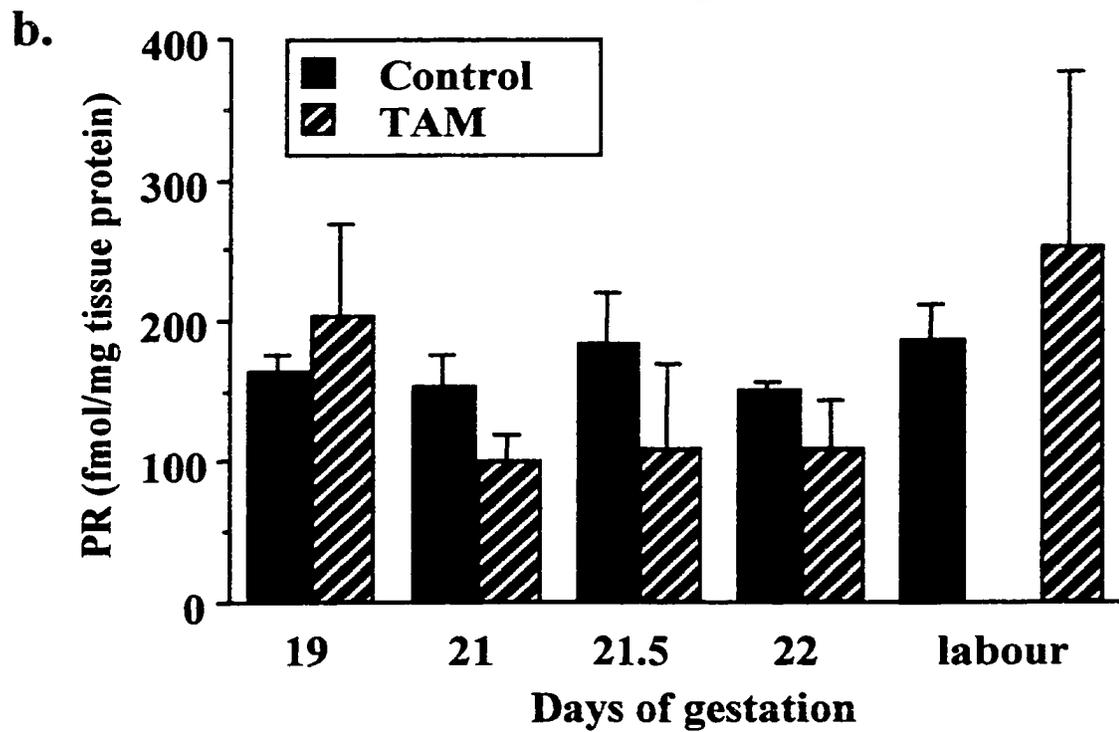19   21   21.5   22   labour
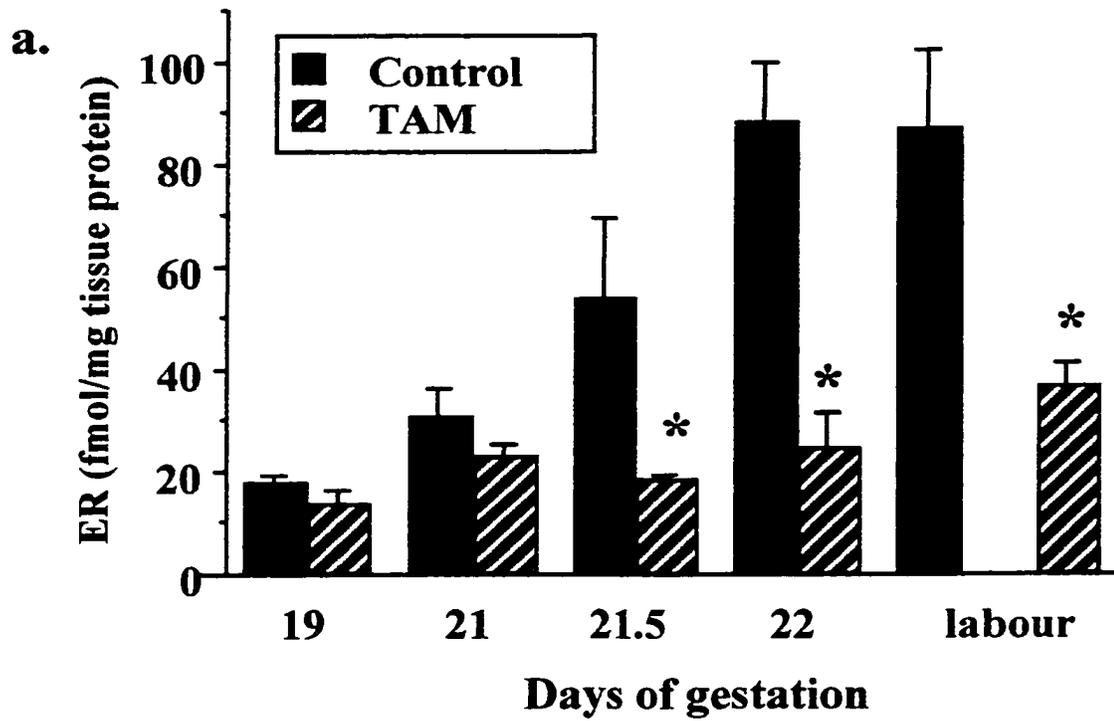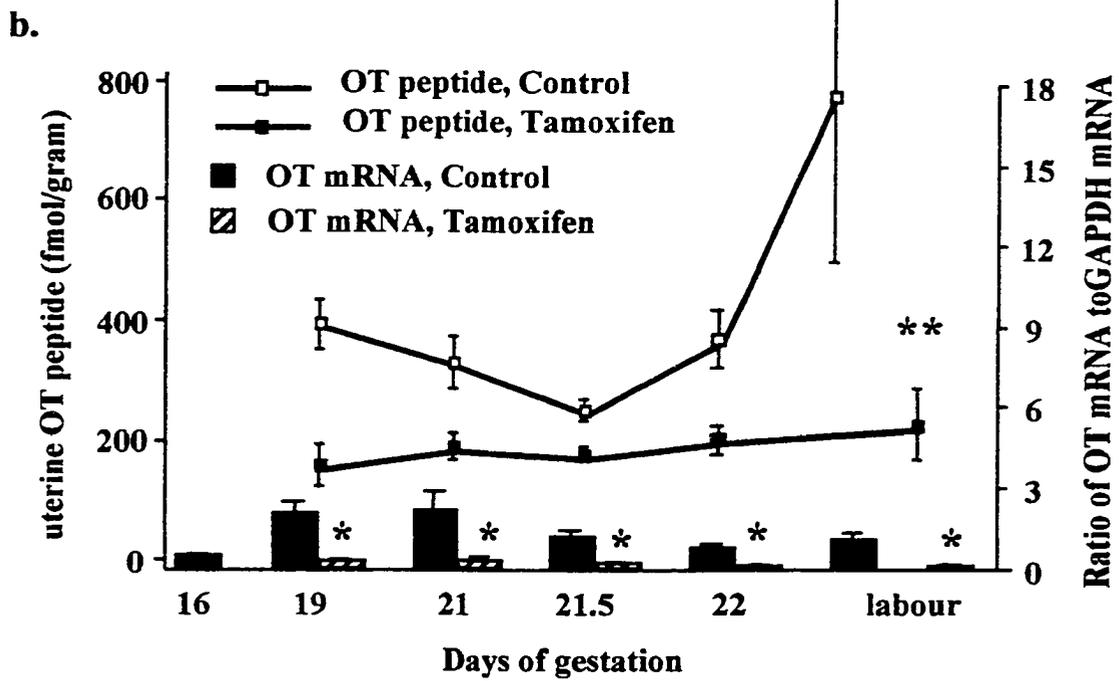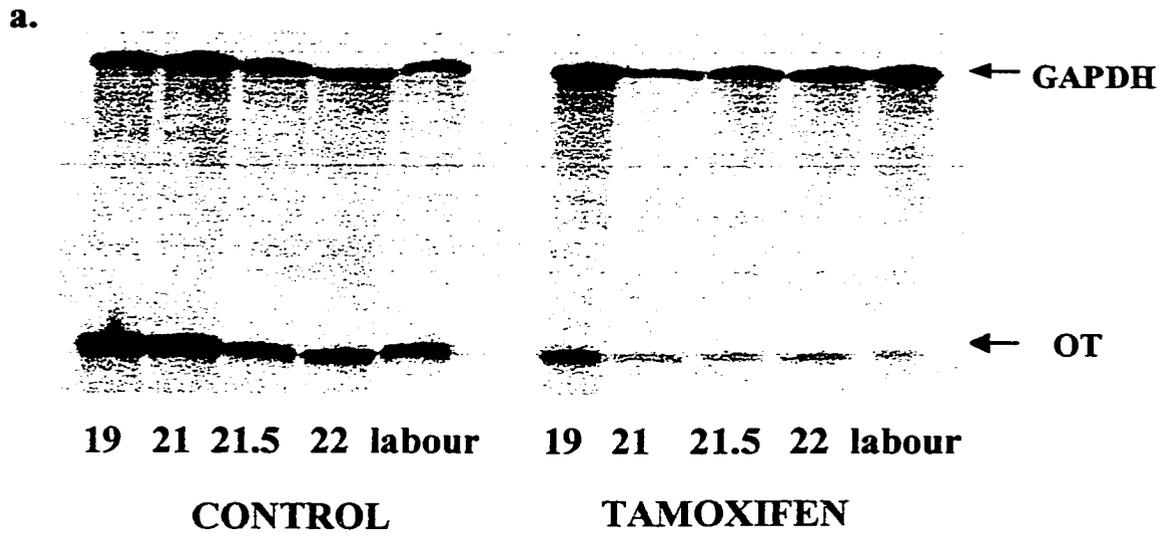
Days of gestation

135

Figure 3-2.　Uterine OT mRNA and OT peptide concentrations in control and TAM-treated rats in late gestation.

　　　　a.)　Representative ribonuclease protection assays showing OT mRNA levels in control animals compared to TAM treated animals in late gestation. Each lane contains RNA from the uterus of one animal.

　　　　b.)　Line graphs illustrate OT peptide levels in uterine tissues measured by radioimmunoassay. The histograms demonstrate the ratio of OT to GAPDH mRNA measured by laser densitometry. (* $p < 0.05$ comparing the two groups at the same time point.)

a.

19 21 21.5 22 labour   19 21 21.5 22 labour

CONTROL   TAMOXIFEN

b.

□— OT peptide, Control
■— OT peptide, Tamoxifen
■ OT mRNA, Control
▨ OT mRNA, Tamoxifen

Days of gestation

137

3.5. REFERENCES

1. **Chibbar R, Miller FD, Mitchell BF** 1992 Synthesis of oxytocin in amnion, chorion and decidua may influence the timing of human parturition. J Clin Invest 91:185-92

2. **Lefèbvre DL, Giaid A, Bennett H, Larivière R, Zingg HH** 1992 Oxytocin gene expression in rat uterus. Science 256:1553-5

3. **Chibbar R, Wong S, Miller FD, Mitchell BF** 1995 Estrogen stimulates oxytocin gene expression in human fetal membranes and decidua. J Clin Endocrinol Metab 80:567-72

4. **Mitchell BF, Cross J, Hobkirk R, Challis JRG** 1984 Formation of unconjugated estrogens from estrone sulfate by dispersed cells from human fetal membranes and decidua. J Clin Endocrinol Metab 58:845-50

5. **Mitchell BF, Wong S** 1993 Changes in $17\beta,20\alpha$-hydroxysteroid dehydrogenase activity supporting an increase in the estrogen/progesterone ratio of human fetal membranes at parturition. Am J Obstet Gynecol 168:1377-85

6. **Yamaguchi K, Asaishi T, Negoro H** 1979 Effect of oestrogen treatment on plasma oxytocin and vasopressin in ovariectomized rats. Endocrinol Japon 26:197-205

7. **Lefèbvre DL, Farookhi R, Giaid A, Neculcea J, Zingg HH** 1994 Uterine oxytocin gene expression. II. Induction by exogenous steroid administration. Endocrinology 134:2562-6

8. **Fuchs AR, Fuchs F, Husslein P, Soloff MS, Fernström MJ** 1982 Oxytocin receptors and human parturition: A dual role for oxytocin in initiation of human labour. Science 215:1396-8

9. **Soloff MS, Alexandrova M, Fernstrom MJ** 1979 Oxytocin receptors: triggers for parturition in the rat? Science 204:1313

10. **Zingg HH, Rozen F, Chu K, Larcher A, Arslan A, Richard S, Lefèbvre D** 1994 Oxytocin and oxytocin receptor gene expression in the uterus. Recent Progress in Hormone Research 50:255-73

11. Fuchs **AR, Periyasamy S, Alexandrova M, Soloff MS** 1983 Correlation between oxytocin receptor concentration and responsiveness to oxytocin in pregnant rat myometrium: effect of ovarian steroids. Endocrinology 113:742-9

12. **Chan WY** 1980 The separate uterotonic and prostaglandin-releasing actions of oxytocin. Evidence and comparison with angiotensin and methacholine in the isolated rat uterus. J Pharmacol Exp Ther 213:575-9

13. **Wilson T, Liggins GC, Whittaker DJ** 1988 Oxytocin stimulates the release of arachidonic acid and prostaglandin $F_{2\alpha}$ from human decidual cells. Prostaglandins 35:771-80

14. **Black LJ, Goode RL** 1980 Uterine bioassay of TAM, trioxifene and a new estrogen antagonist (LY117018) in rats and mice. Life Sciences 26:1453-8

15. **Mitchell BF, Rogers K, Wong S** 1993 The dynamics of prostaglandin metabolism in human fetal membranes and decidua around the time of parturition. J Clin Endocrinol Metab 77:759-64

16. **Ivell R, Richter D** 1984 Structure and comparison of the oxytocin and vasopressin genes from rat. Proc Natl Acad Sci USA 81:2006-10

17. **Tso JY, Sun XH, Kao T, Reece KS, Wu R** 1985 Isolation and characterization of rat and human glyceraldehyde-3-phosphate

dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. Nucleic Acids Res 13:2485-502

18. **Richard S, Zingg HH** 1990 The human oxytocin gene promoter is regulated by estrogen. J Biol Chem 265:6098-103

19. **Gibori G, Sridaran R** 1981 Sites of androgen and estradiol production in the second half of pregnancy in the rat. Biol Reprod 24:249-56

20. **Mitchell BF, Wong S** 1995 Metabolism of oxytocin in human decidua, chorion and placenta. J Clin Endocrinol Metab 80:2729-33

21. **Amico J, Hempel J** 1990 An oxytocin precursor intermediate circulates in the plasma of humans and rhesus monkeys administered estrogen. Neuroendocrinology 51:437-43

22. **Higuchi T, Tadokoro Y, Honda K, Negoro H** 1986 Detailed analysis of blood oxytocin levels during suckling and parturition in the rat. J Endocrinol 110:251-6

23. **Jordan VC, Dix CJ** 1979 Effect of oestradiol benzoate, TAM and monohydroxy-TAM on immature rat uterine progesterone receptor synthesis and endometrial cell division. J Steroid Biochem 11:285-91

24. **Majid E, Senior J** 1982 Anti-oestrogen modification of uterine responses to oestrogen in rat. J Reprod Fert 66:79-85

25. **Hendrix EM, Myatt L, Sellers S, Russell PT, Larsen WJ** 1995 Steroid hormone regulation of rat myometrial gap junction formation: effects on cx-34 levels and trafficking. Biol. Reprod 52:547-60

26. **Csapo AI, Knobil E, Van der Molen HJ, Weist WG** 1971 peripheral plasma progesterone levels during human pregnancy and labour. Am J Obstet Gynecol 110:630-2

27. **Myatt L, Langdon G, Brockman DE** 1994 Identification and changes in concentrations of prostaglandin H synthase (PGHS) isoforms in rat myometrium at parturition. Prostaglandins 48:285-96

28. **Clark JH, Anderson JN, Peck EJ Jr** 1973 Estrogen receptor-antiestrogen complex: Atypical binding by uterine nuclei and effects on uterine growth. Steroids 22:707-18

29. **Rozen F, Russo C, Banville D, Zingg HH** 1995 Structure, characterization, and expression of the rat oxytocin receptor gene. Proc Natl Acad Sci 92:200-4

30. **Inoue T, Kimura T, Azuma C, Inazawa J, Takemura M, Kikuchi T, Kubota Y, Ogita K, Saji F** 1994 Structural organization of the human oxytocin gene. J Biol Chem 269:32451-6

31. **Flint APF, Sheldrick EL** 1982 Ovarian oxytocin secretion is stimulated by prostaglandin. Nature 297:287-8

32. **Chan WY, Berezin I, Daniel EE, Russell KC, Hruby VJ** 1991 Effect of inactivation of oxytocin receptor and inhibition of prostaglandin synthesis on uterine oxytocin receptor and gap junction formation and labour in the rat. Can J Physiol Pharmacol 69:1262-7

33. **Chan WY, Berezin I, Daniel EE** 1988 Effects of inhibition of prostaglandin synthesis on uterine oxytocin receptor concentration and myometrial gap junction density in parturient rats. Biol Reprod 39:1117-28

141

34. **Romero R, Scoccia B, Mazor M, Wu YK, Benveniste R** 1988 Evidence for a change in the progesterone/estrogen ratio in human parturition at term. Am J Obstet Gynecol 159:657-60

35. **Hahn DW, Demarest KT, Ericson DE, Homm SRE, Capetola RJ, McGuire Jl** 1987 Evaluation of 1-deamino-[D-Tyr(Oethyl)^2, Thr^4, Orn^8] vasotocin, an oxytocin antagonist, in animal models of uterine contractility and preterm labour: A new tocolytic agent. Am J Obstet Gynecol 157:977-82

36. **Wilson L Jr, Parsons MT, Ouano L, Flouret G** 1990 A new tocolytic agent: Development of an oxytocin antagonist for inhibiting uterine contractions. Am J Obstet Gynecol 163:195-202

37. **Antonijevic IA, Douglas AJ, Dye S, Bicknell RJ, Leng G, Russell JA** 1995 J Endocrinol 145:97-103

38. **Chen DL, Chan WY, Manning M** 1994 Agonist and antagonist specificities of decidual prostaglandin-releasing oxytocin receptors and myometrial uterotonic oxytocin receptors in pregnant rats. J Reprod Fertil 102:337-43

39. **Akerlund M, Stromberg P, Hauksson A, Andersen LF, Lyndrup J, Trojnar J, Melin P** 1987 Inhibition of uterine contractions of premature labour with an oxytocin analogue. Results from a pilot study. Br J Obstet Gynaecol 94:1040-4

4. EFFECTS OF RU486 ON STROGEN, PROGESTERONE, OXYTOCIN AND THEIR RECEPTORS IN THE RAT UTERUS DURING LATE GESTATION

(This paper has been published in Endocrinology, 1997)

4.1. INTRODUCTION

OT is a potent and specific stimulus to myometrial contractility and is thought to be important in the mechanism of labour initiation. Although classically described as being synthesized in the hypothalamus, then stored and secreted from the neurohypophysis, it is now known that this nonapeptide hormone is synthesized in the epithelial layer of rat endometrium and in the chorio-decidual lining of late human pregnancy (1, 2). In the rat, this synthesis increases rapidly between day 14 and day 18 of pregnancy and remains at a high level during the last 3 - 4 days of gestation. However, uterine OT peptide concentrations increase only during parturition. In the human, chorio-decidual tissues obtained after the spontaneous onset of labour contain higher OT mRNA than those obtained at term but before labour. Concentrations of OTR rise in parallel with OT sensitivity at the time of labour in essentially all species studied. These findings support the hypothesis that locally produced OT and OTR participate in a paracrine network regulating the timing of parturition in both rat and human.

In the rat, synthesis of OT and OTR is primarily regulated by estrogen and progesterone (3-5). In most animal species including the rat, parturition is preceded by a significant increase in estrogen and decrease in progesterone concentrations resulting in a marked increase in the estrogen/progesterone ratio

143

(5-7). This change favors synthesis of OT and OTR within intrauterine tissues. Evidence also suggests that PGs play a role in the regulation of the uterine activity, and it has been proposed that part of the role of OT is mediated by stimulation of decidual PG synthesis (8).

We recently demonstrated that administration of an anti-estrogen will significantly delay the increase in uterine OT mRNA and peptide and OTR and, consequently, delay parturition (7). The present studies were undertaken to determine the effects of an anti-progestin compound (RU486) on intrauterine levels of OT, OTR and PGs and to observe the effects on the process of parturition in the rat. A clearer understanding of the intrauterine paracrine network involving sex steroids, OT, OTR and PGs may yield new strategies for controlling the time of birth.

4.2. MATERIALS AND METHODS

4.2.1. Animals and Treatment

The animals used in this study are described in detail in Chapter 2. The rats were divided into 2 groups. The experimental group was treated once with RU486 (2.5 mg /rat in 0.4 ml oil) at day 15 of pregnancy. Subcutaneous injections were performed into the back of the rat using a 26.5 gauge needle. Animals (n = 5) were euthanized at 0, 6, 12, 24 or 48 h after treatment and during labour (after delivery of first pup). The control group received only injections with vehicle (0.4 ml oil). The rats were injected between 0900 and 1000 hours. Samples were taken for measurement of serum concentrations of E_2 and P_4, uterine OT mRNA and peptide levels, uterine cytosol concentrations of ER, ER mRNA and PR, uterine OTR mRNA and protein levels, and uterine PGE_2 and $PGF_{2\alpha}$ levels. For the final results, there were 5 animals in each group except the 6 h controls (n = 3) and the 48 h RU486 group (n = 4) and the delivery group (n = 6).

144

4.2.2. RIAs

The RIA procedures and antisera for the serum E_2 and P_4 have been characterized previously (7). Uterine OT peptide and uterine cytosol PGE_2 were determined by RIA according to the procedure described previously (7). A detail methodology is described in Chapter 2.

4.2.3. EIA

$PGF_{2\alpha}$ and ER concentrations in uterine cytosols were measured by EIA. A detail procedure is described in Chapter 2.

4.2.4. Receptor Binding Assay

OTRs in uterine tissues were measured using a modification of a published OT-binding assay. P_4 receptors were measured in uterine cytosol fraction. Both binding assays have been characterized previously (7) and are described in Chapter 2.

4.2.5. OT, OTR and ER mRNA Assay

A specific probe for rat OT mRNA was prepared from a rat genomic clone kindly provided by Dr. Hartwig Schmale from the University of Hamburg (9). The cRNA probe for OTR was prepared from a rat genomic clone (kindly provided by Dr. Stephen J. Lye from the University of Tornoto). The rat ER cRNA probe was prepared using RT-PCR. Uterine OT, OTR and ER mRNA concentrations were measured by RPA. The details of the probes and procedure are described in Chapter 2.

4.2.6. Statistical Analysis

Data are presented in text and graphs as the mean ± standard error of the mean. The results were first analyzed by one-way analysis of variance

145

(InStat, GraphPad Software, San Diego, CA) to detect changes with advancing gestational age. Post-hoc comparisons of the means were performed using the Fisher's Protected LSD Test (SuperANOVA, Abacus Concepts Inc., Berkeley, CA). Differences between the experimental and control groups were sought using the two-tailed unpaired Student's t test. Differences were considered to be significant when a P value < 0.05 was obtained. If Bartlett's test revealed non-homogeneity of variance, the corresponding non-parametric test was utilized.

4.3. RESULTS

4.3.1. Parturition

Most RU486-treated dams started vaginal bleeding by 12 h after treatment. The mean time to delivery of the first fetus was 27.0 ± 1.2 h after treatment. All treated animals except four, delivered within 48 h from treatment. The remaining four animals were killed at 48 h. None of the control animals underwent spontaneous labour and delivery.

4.3.2. Serum E_2 and P_4

Serum concentrations of E_2 and P_4 did not change significantly in the control group throughout the 48 hours of observation (Fig. 4-1). However, in the RU486-treated animals, there was a significant increase in serum E_2 and P_4 by the time of delivery of the first fetus, compared to time zero. In the treated group that had not delivered by 48 h, the serum E_2 increase was similar to that of the group that had delivered. However, serum P_4 concentrations in the treated, but undelivered animals, did not increase as did the group that delivered prematurely.

146

4.3.3. ER and PR

The concentrations of ER in uterine cytosol at time zero was 35.6 ± 6.1 fmol/mg tissue protein. This did not change throughout the 48 h in the control group (32.1 ± 5.9 at 48 h), and there was no significant difference after treatment with RU486 (36.2 ± 4.8 in the group at delivery). Tissue concentrations of ER mRNA also were unchanged through 48 h in the controls (Fig. 4-2) and did not change significantly after RU486 treatment. The levels of PR were low, but detectable, and did not change in the control group, remaining in the range of 200 fmol/mg tissue protein (data not shown). Levels of PR were undetectable in the RU486-treated group.

4.3.4. OT mRNA and Peptide

There was a two-fold increase in OT mRNA between 0 h and 24 h in the control group (day 15 and day 16 of pregnancy) (Fig. 4-3a), but concentrations of OT peptide remained unchanged (Fig. 4-3b). RU486 abolished this increase in OT mRNA, and levels remained low after treatment, even during premature labour and delivery. There was no effect of RU486 treatment on uterine OT peptide concentrations. Expression of GAPDH did not change with gestational age or RU486 treatment.

4.3.5. OTR mRNA and Peptide

In the control group, the concentrations of OTR mRNA in uterine tissues were low (Fig. 4-4a). By 6 hours after RU486 treatment, OTR mRNA concentrations increased significantly and remained high until delivery. The measurements of OTR binding increased in parallel to the levels of OTR mRNA (Fig. 4-4b). In the group that did not deliver by 48 h, OTR mRNA and peptide levels were increased to a level similar to those animals that delivered.

147

4.3.6. PGE_2 and $PGF_{2\alpha}$

Uterine PGE_2 and $PGF_{2\alpha}$ remained a constant low levels in the control group (Fig. 4-5). In contrast, the concentrations of PGE_2 in the RU treated group increased gradually, achieving maximal levels after 24 h of treatment and remaining high until delivery. This increased concentration after RU486 was even more pronounced for $PGF_{2\alpha}$, where a 16-fold increase was observed between 12 h and 24 h after treatment (Fig. 4-5).

4.4. DISCUSSION

These results demonstrate that a single injection of RU486, to pregnant rats on day 15, causes preterm delivery. This confirms the findings of Garfield *et al.* (11), who demonstrated that RU486 induced premature birth in rats within 72 h. They also are in agreement with the previous observation of an increase in myometrial activity after RU486 (12). Because RU486 acts by blocking P_4 receptors (13), our data support the classical concept that P_4 is an important hormone in the maintenance of pregnancy and the initiation of parturition (14).

Parturition in most animal models proceeds normally only when P_4 concentrations decrease. However, in our experiments, both maternal serum E_2 and P_4 concentrations were increased at the time of premature parturition in the RU486-treated animals. This is in keeping with the data of Garfield *et al.*, though the changes in that study did not achieve statistical significance (11). We interpret the increase in sex steroids to indicate that the anti-progestin has interfered with a negative feedback loop and upregulated ovarian production of E_2 and P_4. This would be compatible with previous studies where treatment with an anti-P_4 monoclonal antibody increased serum E_2 levels in early pregnancy in the hamster (15). RU486 also may have a direct effect on ovarian

P_4 synthesis, at this stage of pregnancy, by stimulating activity of 3β-hydroxysteroid dehydrogenase activity (16).

On day 15 of pregnancy, uterine cytosol concentrations of PR were low but detectable. After RU486, PR was undetectable. We are uncertain whether this effect is an artifact caused by the ability of RU486 to compete for $[^3H]P$ binding to rat uterine cytosolic PR (17) or whether the decrease is real. However, in T47D human breast cancer cells, RU486 caused a transient reduction in basal levels of PR mRNA and protein (18). Additionally, our assay measured only cytosolic PR, and the apparent decrease could have been caused by an RU486-induced shift of PR into the nuclear subcellular fraction.

We detected no change in cytosolic ER or ER mRNA after RU486. Indeed, there was a consistent trend to decreased ER mRNA with RU486, though this did not reach statistical significance in any group. This was a surprising result, because it is known that P_4 interferes with replenishment of cytosolic ER (19). P_4 also increases the turnover of nuclear ER in hamster decidual cells (20). When rhesus macaques were treated with RU486 in late gestation, cytosolic and nuclear ER levels increased significantly in both decidua and myometrium, changes that are not usually seen in normal spontaneous term labour (21). Our failure to demonstrate an RU486-induced increase in ER in rats during late pregnancy may be caused by limiting our measurements to the cytosolic compartment. However, the lack of change in ER mRNA suggests that this is not the case. It also is possible that our relatively short observation period did not permit evaluation of the full affects of RU486, though it is noteworthy that the biological affect of parturition did occur. Finally, it is possible that there are interspecies differences in the influence of RU486 on ER during late pregnancy.

Concentrations of OT mRNA within the uterus of the control group increased significantly between day 15 and day 17 of pregnancy without any

change in uterine OT peptide concentrations. This confirms our previous findings (7) of increased concentrations of OT mRNA between day 14 and day 18 of pregnancy without changes in peptide levels. RU486 blocks this normal increase in OT mRNA, suggesting that P_4 may have a positive influence on uterine OT synthesis. This would support previous findings in the non-pregnant rat, where E_2 and P_4 had a synergistic effect on uterine OT gene expression (3). Our previous studies (22), using term human chorio-decidua, demonstrated that E_2 stimulates synthesis of OT mRNA, but P_4 alone was without effect. In concert, these data support the contention that uterine OT gene expression depends on a synergistic action between E_2 and P_4.

Tissue concentrations of OT remained constant in the control rats despite an increase in OT mRNA on day 16. We have noted this disparity between OT mRNA and OT peptide concentrations previously (7). The initial translation product from OT mRNA requires further modification before synthesis of mature amidated OT recognized by our RIA (23). Thus, the discrepancy between OT mRNA and peptide levels may be secondary to delays in translation or post-translational processing of OT mRNA.

There is a marked increase in OTR approximately at the time of parturition in the rat, and this parallels an increase in the sensitivity of the myometrium to OT (24). In the human, OTR also increases markedly as parturition approaches (25), and a similar increase occurs in uterine OTR mRNA concentrations (26). In the present studies, a significant increase in OTR occurred within 6 h of RU486 treatment, and this was immediately followed by an increase in OTR peptide. This strongly suggests that P_4 plays an important role in inhibition of OTR synthesis and confirms earlier findings (4, 5). Recently, Larcher *et al* (27) have demonstrated that P_4 treatment results in a much greater suppression of OTR binding than of OTR mRNA. Conversely, our results with RU486 reveal a greater relative effect on OTR

150

mRNA (approximately 10-fold increase), compared to OTR binding (approximately 5-fold increase). The reason for the differences is not clear, but their studies were performed in ovariectomized, non-pregnant rats using a synthetic OT analogue as ligand, whereas our studies used radiolabeled OT with tissues from intact pregnant animals. In any case, these data suggest that control of OTR translation may be an important regulatory step and that the mechanisms may be different in the pregnant and non-pregnant states. It is noteworthy that in both of our experimental models [prolonged gestation by TAM (7) and preterm labour by RU486], parturition occurred when OTR peptide reached a threshold value without the changes in uterine OT gene expression.

The mechanisms mediating the effects of sex steroids on OTR gene remain unclear. Recently, the promoter region of the OTR gene in several species has been sequenced, and several "half-palindromes" of estrogen response elements were found (28). It is possible that these half-EREs confer estrogen sensitivity. However, despite strong evidence that P_4 down-regulates OTR gene expression, the OTR promoter region is devoid of response elements that are known to interact with PR. This suggests that the antagonism of P_4 on uterine OTR gene expression is mediated by an indirect genomic or a nongenomic mechanism. As noted by Larcher *et al.* (27), the disparate effects of P_4 on OTR mRNA and binding measurements suggests that at least part of the effect of P_4 may be at the translational or post-translational level.

Basal uterine tissue levels of $PGF_{2\alpha}$ and PGE_2 were similar. However, after RU486, PGE_2 increased approximately 4-fold and $PGF_{2\alpha}$ increased more than 10-fold. The time course of the increases seemed to follow those for OTR. This is in agreement with previous studies showing that PGs increased in rats after RU486 only in those that were aborting and only after labour had started (12). Rat endometrium in late gestation produces PGE_2 and $PGF_{2\alpha}$ in

151

response to OT stimulation (8) and thus, in our experiments, may be a result of the increased OTR. These PGs may play a role in parturition by being directly utertonic or by stimulating further synthesis of OTR (29). Earlier studies by Chan *et al.* (8, 29) have demonstrated a potential positive feedback loop between PGs and OTR. Our results do not allow conclusions as to whether the rise in PGs is a cause or consequence of labour. Finally, our data are compatible with previous findings that RU486 stimulates production of PGE_2 and $PGF_{2\alpha}$ by human endometrial stromal cells (30).

All four RU486-treated animals that remained undelivered at 48 h were bleeding vaginaly at the time they were killed. Their uterine concentrations of OT, OTR and PGs were indistinguishable from those who delivered. Indeed, the only way in which they differed from the delivered group was that their serum P_4 levels were significantly reduced, compared to the treated animals at the time of delivery or with the controls at 48 h. We speculate that these animals had completed the biochemical changes that initiated labour, were in active labour, and would have delivered shortly after the end of the experiment at 48 h. This distribution of delivery times would be similar to that observed previously in rats administered the same dose of RU486 at a similar time in gestation (11).

In summary, these data have demonstrated that the anti-progestin RU486 stimulates premature delivery in the rat by evoking changes in OTR and PGs similar to those seen in normal term parturition. Our findings strongly support a major role for P_4 in suppressing OTR gene expression in late pregnancy, before myometrial activation, around the time of labour onset. The data also suggest that the increase in PGs occurs secondarily to the increase in OTR. The process of RU486-induced premature delivery occurred without an increase in uterine OT gene expression, suggesting that regulation of the receptor is a more critical step than regulation of the ligand. Further

152

investigation into regulation of uterine OTR may provide information on which
to develop improved methods for controlling the timing of parturition.

153

Figure 4-1   a.) Concentrations of E_2 in maternal serum at time intervals following treatment with RU486 beginning at day 15 of pregnancy.

b.) Concentrations of P_4 as in 1a.

Each point represents the mean ± SEM from 3 - 6 animals. The histogram represents the concentrations of E_2 or P_4 in animals following delivery of the first fetus (27.0 ± 1.2 h). Points within the same treatment group but having different letters are significantly different from each other by analysis of variance and Fisher's Protected LSD test.   * denotes significant difference between the RU486 and control groups using Student's unpaired t test.
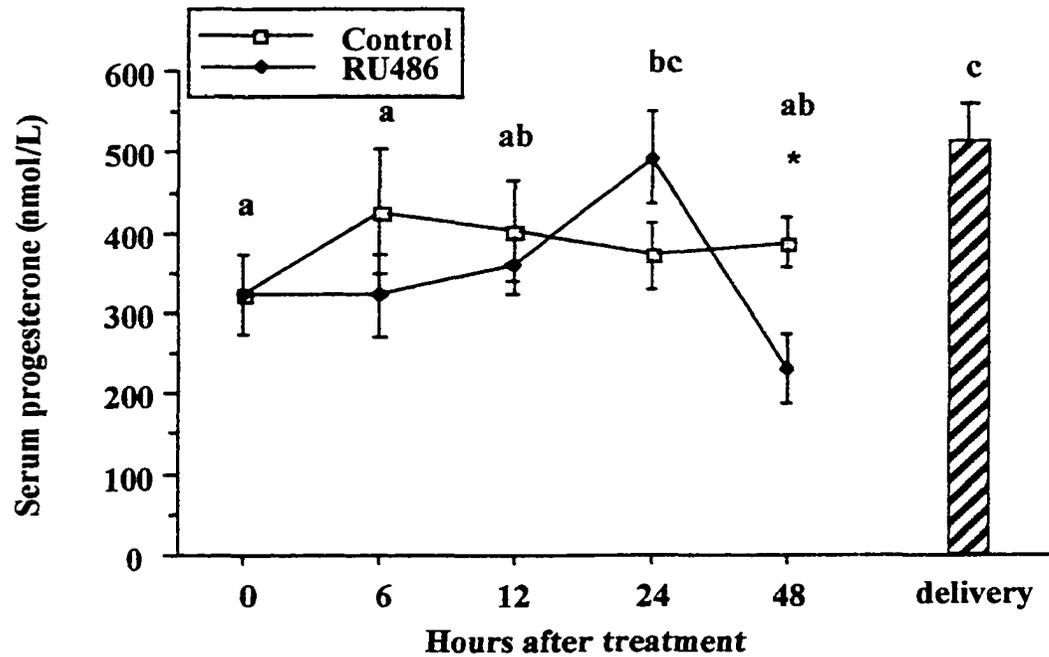
a.

b.

155
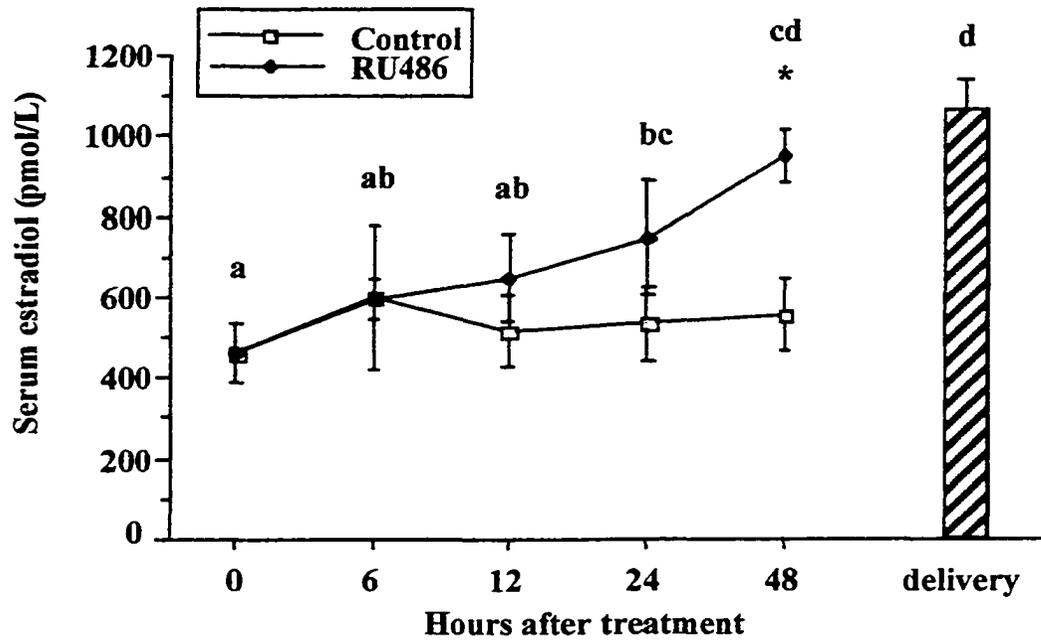
Figure 4-2   Uterine ER mRNA concentrations as a ratio of GAPDH mRNA at time intervals following treatment with RU486 beginning at day 15 of pregnancy. Each point represents the mean $\pm$ SEM from 3 - 6 animals. RNA was measured using ribonuclease protection assays. The histogram represents the ratio of OT/GAPDH mRNA of animals following delivery of the first fetus (27.0 $\pm$ 1.2 h).
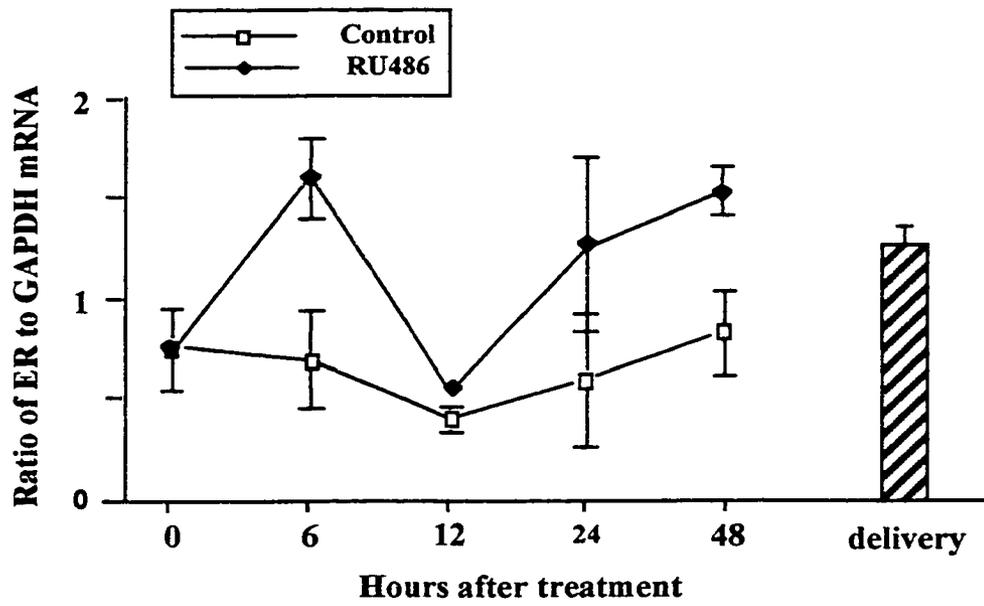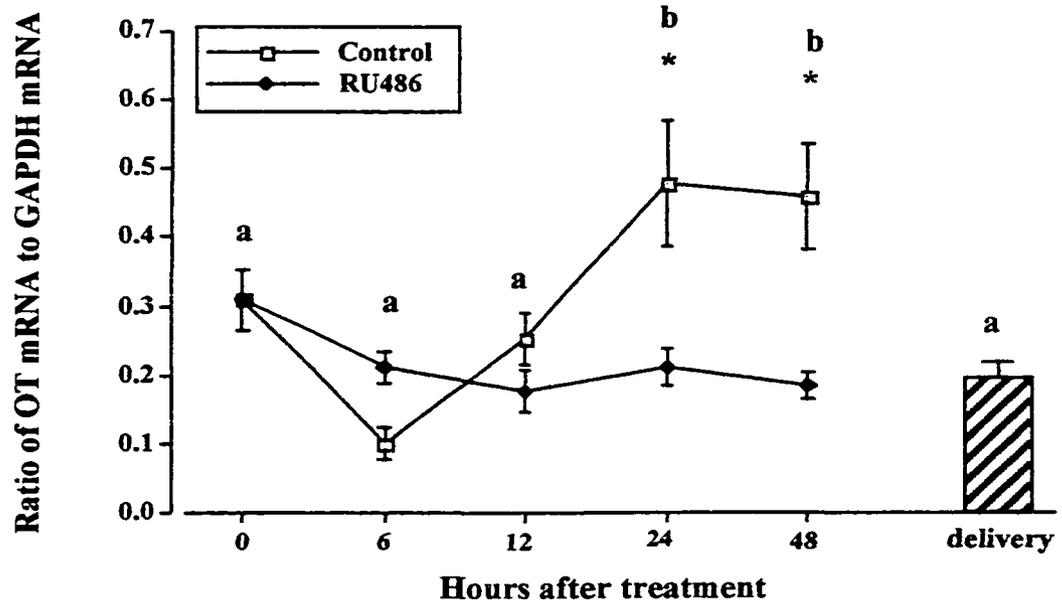
157

Figure 4-3   a.) Uterine OT mRNA as a ratio of GAPDH mRNA at time intervals following treatment with RU486 beginning at day 15 of pregnancy. RNA was measured using ribonuclease protection assays.

b.) Uterine tissue OT peptide concentrations measured by RIA for the same animals as in 2a.

Each point represents the mean ± SEM from 3 - 6 animals. The histogram represents the ratio of OT/GAPDH mRNA or OT peptide concentrations of animals following delivery of the first fetus (27.0 ± 1.2 h). Points within the same treatment group but having different letters are significantly different from each other by analysis of variance and Fisher's Protected LSD test. * denotes significant difference between the RU486 and control groups using Student's unpaired *t* test.
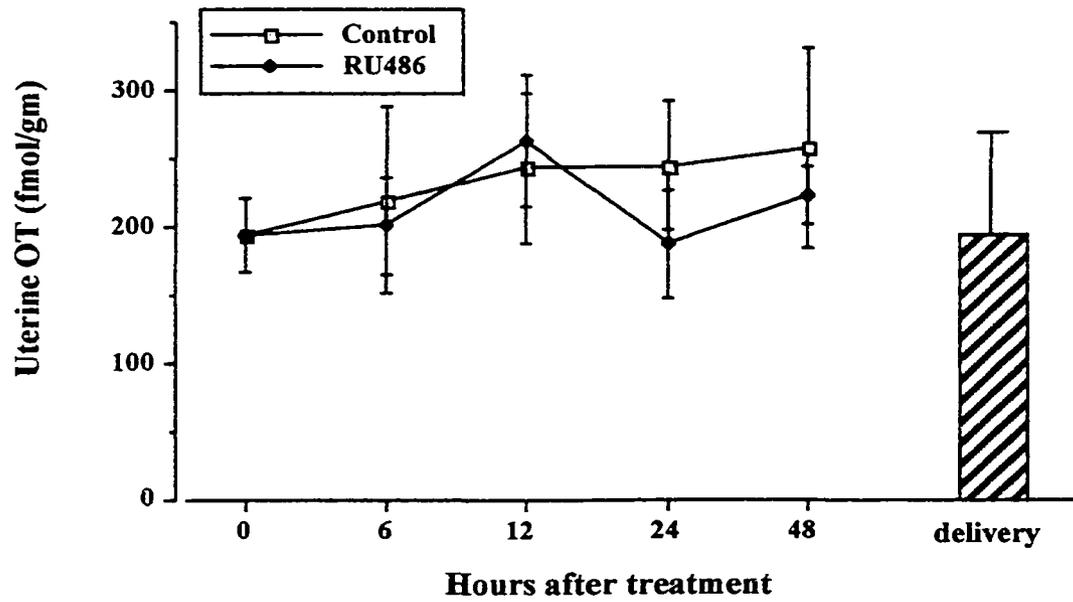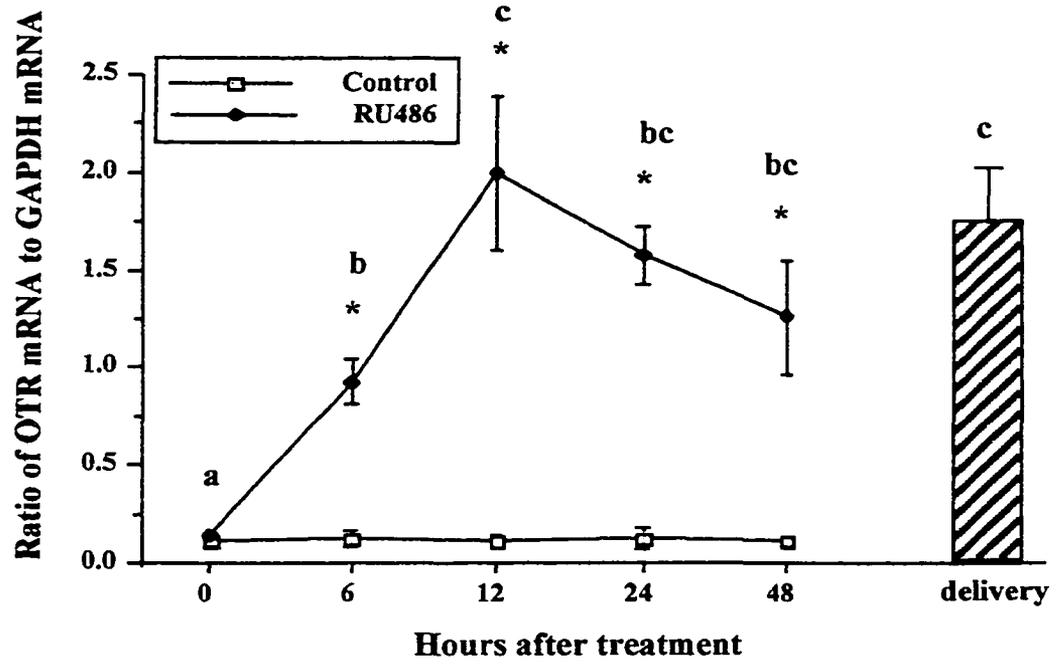
a.

b.

Figure 4-4   a.) Uterine OTR mRNA as a ratio of GAPDH mRNA at time

intervals following treatment with RU486 beginning at day 15 of pregnancy.

b.) Uterine tissue OTR peptide concentrations measured using a

ligand binding assay for the same animals as in 3a.

Each point represents the mean ± SEM from 3 - 6 animals measured using

ribonuclease protection assays. The histogram represents the ratio of

OTR/GAPDH mRNA or OTR peptide concentrations of animals following

delivery of the first fetus (27.0 ± 1.2 h). Points within the same treatment

group but having different letters are significantly different from each other by

analysis of variance and Fisher's Protected LSD test. * denotes significant

difference between the RU486 and control groups using Student's unpaired t

test.

a.

b.

161
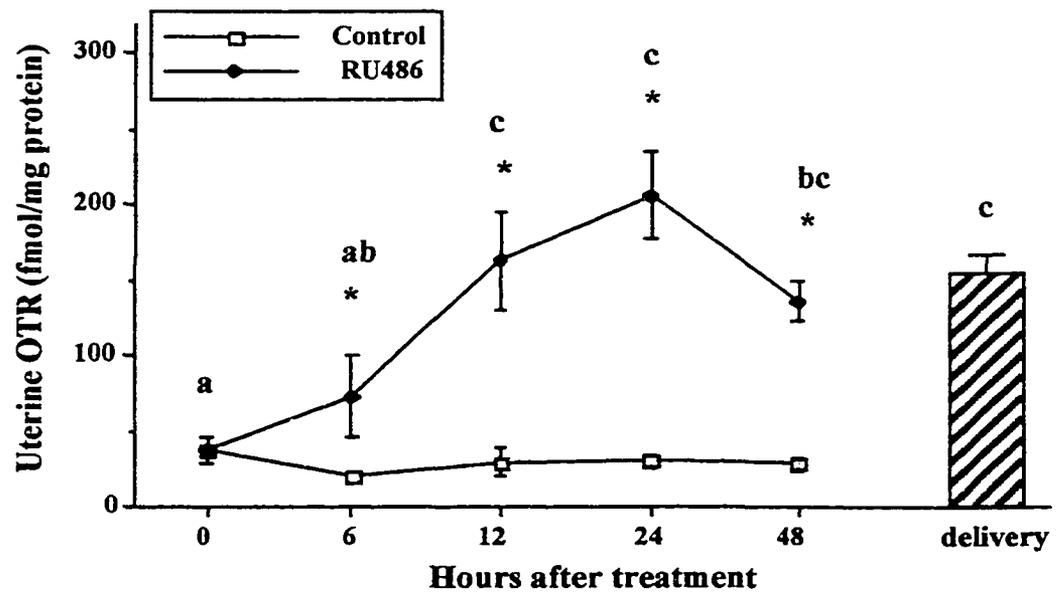
Figure 4-5   a.) Concentrations of PGE_2 in rat uterine tissue at time intervals following treatment with RU486 beginning at day 15 of pregnancy.

b.) Concentrations of PGF_{2a} as in 5-4a.

Each point represents the mean $\pm$ SEM from 3 - 6 animals. The histogram represents PGE_2 or PGF_{2a} levels in animals following delivery of the first fetus (27.0 $\pm$ 1.2 h). Points within the same treatment group but having different letters are significantly different from each other by analysis of variance and Fisher's Protected LSD test. * denotes significant difference between the RU486 and control groups using Student's unpaired t test.
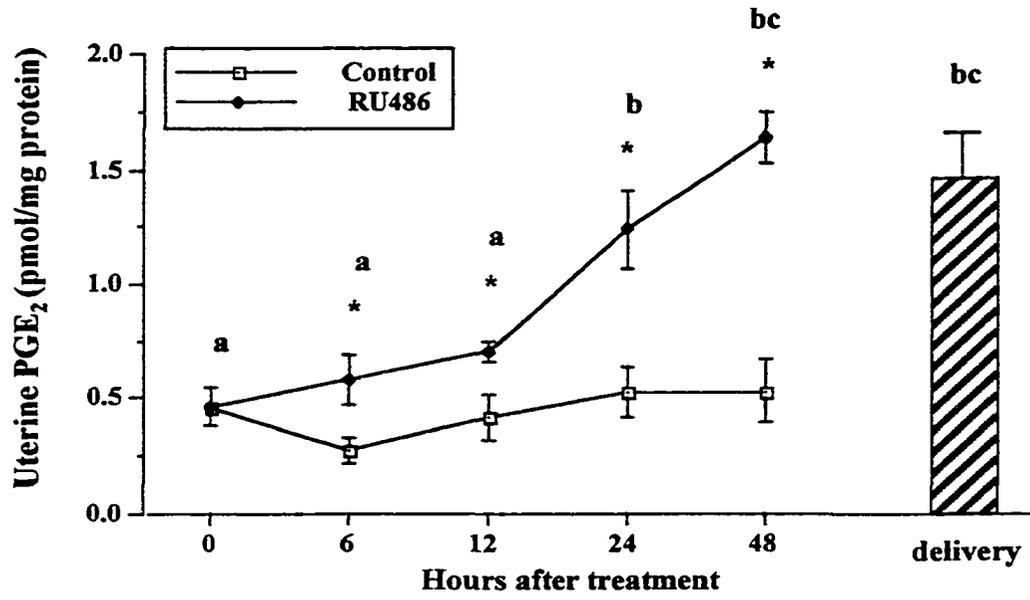
a.

b.

163
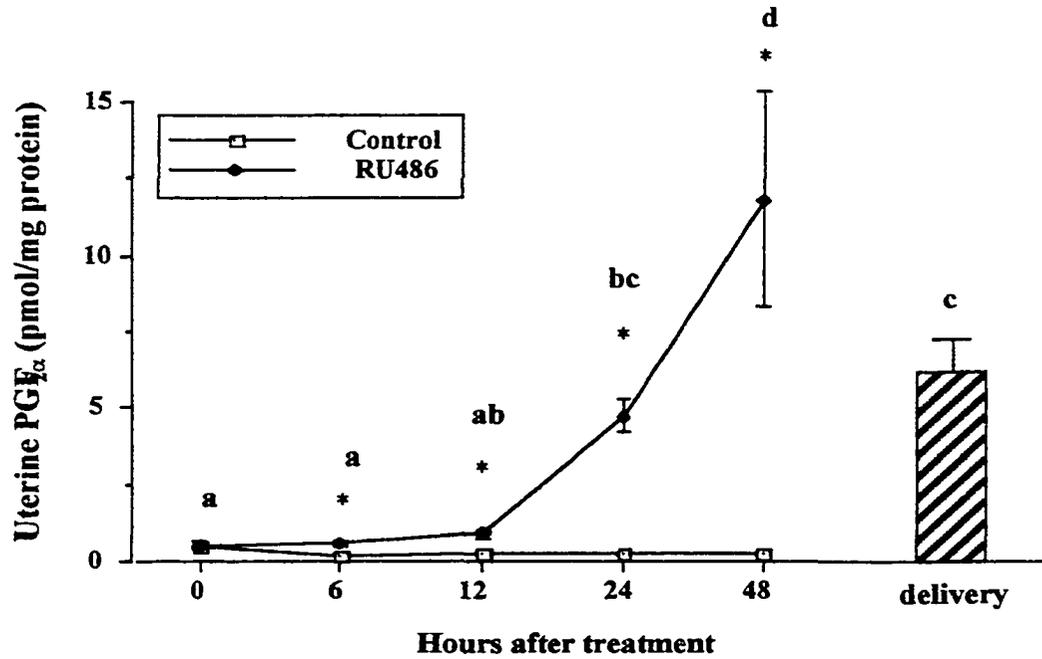
4.5. REFERENCES

1. **Lefèbvre DL, Giaid A, Bennett H, Larivière R, Zingg HH** 1992
    Oxytocin gene expression in rat uterus. Science 256:1553-5

2. **Chibbar R, Miller FD, Mitchell BF** 1992 Synthesis of oxytocin in
    amnion, chorion and decidua may influence the timing of human
    parturition. J Clin Invest 91:185-92

3. **Lefèbvre DL, Farookhi R, Giaid A, Neculcea J, Zingg HH** 1993
    Uterine oxytocin gene expression. II. Induction by exogenous steroid
    administration. Endocrinology 134(6):2562-6

4. **Fuchs AR, Periyasamy S, Alexandrova M, Soloff MS** 1983
    Correlation between oxytocin receptor concentration and responsiveness to
    oxytocin in pregnant rat myometrium: effect of ovarian steroids.
    Endocrinology 113:742-9

5. **Alexandrova M, Soloff MS** 1980 Oxytocin receptors and parturition. I.
    Control of oxytocin receptor concentration in the rat myometrium at term.
    Endocrinology 106:730-48

6. **Challis JRG, Olson DM.** 1988 Parturition. In: The Physiology of
    Reproduction. Eds. Knobil E, Neill J. Raven Press, New York. pp 2177-
    234

7. **Fang X, Wong S, Mitchell BF** 1996 Relationships between sex steroids,
    oxytocin and their receptors in the rat uterus during late gestation and at
    parturition. Endocrinology 137:3213-9

8. **Chan WY** 1980 The separate uterotonic and prostaglandin-releasing
    actions of oxytocin. Evidence and comparison with angiotensin and
    methacholine in the isolated rat uterus. J Pharmacol Exp Ther 213:575-9

9. **Ivell R, Richter D** 1984 Structure and comparison of the oxytocin and vasopressin genes from rat. Proc Natl Acad Sci USA 81:2006-10

10. **Tso JY, Sun XH, Kao T, Reece KS, Wu R** 1985 Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. Nucleic Acids Res 13:2485-502

11. **Garfield RE, Casc JM, Baulieu EE** 1987 Effects of the antiprogesterone RU 486 on preterm birth in the rat. Am J Obstet Gynecol 157:1281-5

12. **Arkaravichien W, Kendle KE** 1992 Fetal viability and fetal growth after prolonged uterine contractions induced by progesterone withdrawal in late pregnancy in rats. J Reprod Fert 96:299-308

13. **Baulieu EE** 1991 On the mechanism of action of RU 486. Ann N Y Acad Sci 626:545-60

14. **Csapo AI** 1956 Progesterone block. Am J Anat 98:273-81

15. **Greenwald GS, Wang MW** 1991 A monoclonal antibody to progesterone interrupts pregnancy in the hamster by curtailing secretion of the luteotropic complex of prolactin and follicle-stimulating hormone. Endocrinology 129:1735-43

16. **Telleria CM, Deis RP** 1994 Effect of RU486 on ovarian progesterone production at pro-oestrus and during pregnancy: a possible dual regulation of the biosynthesis of progesterone. J Reprod Fert 102:379-84

17. **Okulicz WC** 1987 Effect of the antiprogestin RU486 on progesterone inhibition of occupied nuclear estrogen receptor in the uterus. J Steroid Biochem 28:117-22

18. **Read LD, Snider CE, Miller JS, Greene GL, Katzenellenbogen BS**
1988 Ligand-modulated regulation of progesterone receptor messenger ribonucleic acid and protein in human breast cancer cell lines. Mol Endocrinol 2:263-71

19. **Hsueh AJ, Peck Jr. EJ, Clark JH** 1975 Control of uterine estrogen receptor levels by progesterone. Endocrinology 98:438-44

20. **Takeda A, Leavitt WW** 1986 Progestin induced down regulation of nuclear estrogen receptor in uterine decidual cells: analysis of receptor synthesis and turnover by the density shift method. Biochem Biophys Res Commun 135:98-105

21. **Haluska GJ, Weat NB, Novy MJ, Brenner RM** 1989 Uterine estrogen receptors are increased by RU486 in late pregnant rhesus macaques but not after spontaneous labour. J Clin Endocrinol Metab 70:181-6

22. **Chibbar R, Wong S, Miller FD, Mitchell BF** 1995 Estrogen stimulates oxytocin gene expression in human fetal membranes and decidua. J Clin Endocrinol Metab 80:567-72

23. **Gainer H** 1983 Precutsors of oxytocin and vasopressin. Prog Brain Res 60:205-15

24. **Fuchs AR, Periyasamy S, Alexandrova M, Soloff MS** 1983 Correlayion between oxytocin receptor concentrations and responsiveness to oxytocin in pregnant rat myometrium: effects of ovarian steroids. Endocrinology 113:742-9

25. **Fuchs AR, Fuchs F, Husslein P, Soloff MS, Fernström MJ** 1982 Oxytocin receptors and human parturition: A dual role for oxytocin in initiation of human labour. Science 215:1396-8

26. **Kimura T, Takemura M, Nomura S, Nobuunaga T, Kubota Y, Inoue T, Hashimoto K, Kumazawa I, Ito Y, Ohashi K, Koyama M, Azuma C, Kitamura Y, Saji F** 1995 Expression of oxytocin receptor in human pregnant myometrium. Endocrinology 137:780-5

27. **Larcher A, Neculcea J, Breton C, Arslan A, Rozen F, Russo C, Zingg HH** 1995 Oxytocin gene expression in the rat uterus during pregnancy and the estrous cycle and in response to gonadal steroid treatment. Endocrinology 136:5350-6

28. **Rozen F, Russo C, Banville D, Zingg HH** 1995 Structure, characterization, and expression of the rat oxytocin receptor gene. Proc Natl Acad Sci 92:200-4

29. **Chan WY, Berezin I, Daniel EE** 1988 Effects of inhibition of prostaglandin synthesis on uterine oxytocin receptor concentration and myometrial gap junction density in parturient rats. Biol Reprod 39:1117-28

30. **Kelly RW, Healy DL, Cameron MJ, Cameron IT, Baird DT** 1986 The stimulation of prostaglandin production by two antiprogesterone steroids in human endometrial cells. J Clin Endocrinol Metab 62:1116-23

5. PROGESTERONE REEPTOR ISOFORMS IN LATE GESTATION RAT UTERUS: THE INFLUENCE OF ESTROGEN AND PROGESTERONE ANTAGGONISTS

5.1. INTRODUCTION

Progesterone (P_4) is responsible for uterine quiescence throughout pregnancy. P_4 withdrawal is an important step to initiate parturition in most species including rat (1).

The effects of P_4 are manifested classically in cells containing specific intranuclear receptors that are members of the steroid/thyroid nuclear receptor superfamily. Our previous studies detected no significant change in the binding of P_4 to rat uterine tissues around the time of parturition (2). The progesterone receptor (PR) has several isoforms, all originating from the same gene (3). The rat uterus contains PR-A, PR-B and PR-C isoforms (4). PR-A is a truncated form of PR-B arising from a different transcription start site and lacking a 164 amino acid of N-terminal transactivating region of PR-B (Figure 5-1) (5). In general, PR-B is the stronger transactivator and PR-A is a dominant inhibitor of PR-B and other nuclear receptors (5, 6). PR-C is also an N-terminal truncated transcriptional product but much smaller than PR-A (7). It has only a second zinc finger of the DNA-binding domain but an intact hormone-binding domain. It appears to act as a selective suppressor of P_4 action by binding P_4 in the cytosolic fraction to curtail progesterone-binding to active receptor forms (3, 7). In human breast cancer cells PR-C was a minor isoform (7). However, Ogle *et al* reported that it was the predominant form in decidual basalis in early gestation (4).

The PR-A and PR-B isoforms each have a distinct promoter region (5, 8). Uterine PR may be regulated by estrogen or P_4. Estrogen increases PR mRNA levels (5, 9, 10) and P_4 binding (11, 12) whereas P_4 decreases the number of its own receptors (13, 14). There is no information regarding the

168

effect of estrogen or P_4 on the relative quantities of the PR isoforms in the late pregnant uterus. It is possible that the changing concentrations of estrogen and P_4 in maternal rat serum around the time of parturition could affect the expression of PR-A, PR-B or PR-C in uterine tissues.

In this study, our objectives are to measure the concentrations of mRNAs encoding the PR isoforms in rat uterus through late gestation and to determine the effects of the estrogen antagonist tamoxifen (TAM) and the PR antagonist RU 486 on differential expression of PR isoforms.

5.2. MATERIALS AND METHODS

5.2.1. Animals and Treatments

The animals used in this study are described in detail in Chapter 2.

5.2.1.1. Normal Pregnant Animals

In this strain of rats, parturition usually occurs early in the afternoon of day 22 when the day of mating is considered day 0. The rats (n = 3 – 9 at each time point) were killed between 0900 and 1000 h on day 13, 15, 16, 18, 19, 20, 21, 22 of pregnancy, after delivery of the first pup (delivery) and postpartum day 1.

5.2.1.2. Steroid Antagonist Groups

The animals (n = 3 – 5 at each time point) in the TAM group were treated daily with TAM (200 µg per rat, subcutaneously; Sigma Chemical Co., St. Louis, MO) in 0.4 ml oil from day 15 of pregnancy until death. Control animals received only oil. Animals were killed on days 19, 21, 21.5 (the evening of day 21 of pregnancy), day 22 of pregnancy and during labour (after delivery of the first pup).

169

The animals in the RU486 group (n = 4 – 6 at each time point) were injected once with RU486 (2.5 mg per rat) on day 15 of pregnancy and killed at time 0 h, 6 h, 12 h and 24h after treatment, and during labour.

5.2.2. Rat PR-isoform mRNA Measurement

Rat PR isoform probes were generated using RT-PCR. The primer sets are shown in Fig 5-1. The P_{B1}/P_{B2} primer set flanks part of the 5'-untranslated region and N-terminal region of the B form (390 bp). The P_{T3}/P_{T4} set flanks the hormone-binding domain for all A, B and C isoforms (320bp). The reverse transcription products were generated by using random primers, and PCR was performed for 30 cycles of 1 min at 95° C, 2 min at 56° C and 3 min at 72° C. The RT-PCR products with primer sets of P_{B1}/P_{B2} and P_{T3}/P_{T4} were generated using total RNA (1μg) from a day 21 pregnant rat uterus. The mRNA measured using the probe from the 5'-untranslated region is referred to as PR-B and the mRNAs measured using the probe from the 3' region is referred to as PR-total.

The amplified DNA (PR-B or PR-total) was cloned using pPCR-Script™ Amp Cloning kit (Stratagene, La Jolla, CA). Restriction and sequence analyses were performed to confirm the sequence of the inserted PCR product. The PCR-generated rat PR-B and PR-total cDNA clone in pPCR-Script plasmid was linearized using the restriction enzyme, Not I to generate an antisense DNA template transcribed by T7 polymerase.

Uterine tissues were homogenized and total RNA extracted using Trizol™. PR-total and PR-B mRNA levels in the uterine tissues were measured using RPA The procedure of preparation of RNA and RPA are described in Chapter 2. In this assay, negative controls were performed by hybridization of day 21 pregnant uterine total RNA (20 μg) with a sense probe, along with hybridization of tRNA (10 μg) with the antisense probe.

170

5.2.3. Statistical Analysis

Data are presented in graphs as the mean ± standard error of the mean (SEM). The results were first analyzed by one-way ANOVA (Instat; GraphPad Software, San Diego, CA) to examine changes with advancing gestational age. *Post hoc* comparisons of the means were performed using the Tukey-Kramer test. Differences between the experimental and control groups were sought using two-way ANOVA (Prism; GraphPad Software) to detect changes. If any significance occurred, the two-tailed unpaired Student's *t* test was performed between two groups at the same time point. Differences were considered to be significant when a *P* value less than 0.05 was obtained. If Bartlett's test revealed non-homogeneity of variance, the corresponding nonparametric test was used.

5.3. RESULTS

5.3.1. PR Isoform mRNA Levels in Late Normal Gestation

The PR-total mRNA in the normal pregnant uterus changed significantly through late gestation with maximal levels on day 22 of pregnancy ($p < 0.01$), the day of parturition (Fig. 5-2a & 5-2b). There was no significant change in PR-B mRNA. The ratio of PR-total to PR-B increased similarly with a peak occurring on the day of parturition (Fig. 5-2c).

5.3.2. Effects of TAM on PR Isoforms

Treatment with TAM significantly prolonged the duration of pregnancy by 24 ± 1.2 h ($P < 0.01$) (2). As in the normal pregnancy group above, there was a significant increase in the ratio of PR-total to β-actin mRNA on day 22 of pregnancy in the control animals (Fig. 5-3a). Treatment with TAM completely prevented this increase. PR-B mRNA levels did not change in the control animals and were not influenced by treatment with TAM (Fig. 5-3b).

5.3.3. Effect of RU486 on PR Isoforms

In the animals treated with RU486, the mean time to delivery of the first fetus was 27.0 ± 1.2 h after treatment (15). None of the control animals underwent spontaneous labour and delivery. There was a significant increase in PR-total mRNA in the animals treated with RU486 ($P < 0.05$) with peak levels occurring at the time of delivery (Fig. 5-4a). The level of PR-B mRNA did not change significantly after RU-486 administration (Fig. 5-4b).

There was no effect of gestational age or of steroid antagonists on expression of the β-actin gene that was used as an internal control (Fig. 5-2a).

5.4. DISCUSSION

These data report the mRNA concentrations of uterine PR isoforms in rat late pregnancy. As with our previous studies, we have elected not to attempt to separate endometrium from myometrium. We believe that there may be important paracrine interactions between these tissues that may influence parturition. By not separating the tissues, we have avoided disruption of this potential paracrine network and have also avoided potential artifacts due to tissue trauma.

The mRNA levels detected by the probe for PR-total approximate the total PR mRNA (PR-A, PR-B and PR-C). Unfortunately, we have no method to distinguish the mRNA for PR-A or PR-C separately. Since the radiolabeled probe may hybridize with different efficiency to the different isoforms, we have avoided attempting to calculate PR-A plus PR-C by simply subtracting PR-B from the PR-total. However, it appears that PR-B accounts for approximately half of the total PR mRNA before day 22. This suggests that it is the predominant isoform in the rat uterus through late gestation. By day 22 of pregnancy, just a few hours before delivery, the predominance of PR-B

172

declines. PR-A and/or PR-C increase significantly and become the predominant isoforms.

Although there is similar ligand-binding affinity for each of these isoforms (7, 16), they may have different functions. PR-A has been demonstrated to have an inhibitory influence on PR-B and other members of the steroid receptors including the glucocorticoid receptor and estrogen receptor (17). Recently, an inhibitory function domain was found in PR-A and PR-B. However, only PR-A can independently and strongly repress the transcription of the target genes, due to lack of N-terminal upstream segment which contains the additional activation function (AF_3) (18). PR-C lacks one of the zinc fingers necessary for association with DNA response elements and may acts as a depressor of P_4 actions by binding P_4 or dimerizing with the two other PR isoforms (19).

In human breast cell lines PR-C was a minor isoform (7). However, in early pregnant rat decidua, PR-C is the dominant form (20). Our data suggest that PR-B becomes the dominant isoform in late gestation but PR-A and/or PR-C become dominant prior to delivery. This change may be an important aspect of preparation of the gravid uterus for parturition in that it may effectively diminish the ability of P_4 to maintain uterine quiescence.

In most animal models, including the rat, parturition proceeds normally only when there is a withdrawal from the influence of P_4 (1). In most cases, this is accomplished by a decrease in the production rate of P_4 with a corresponding decline in maternal serum P_4 concentrations. Maternal serum P_4 concentrations in this species decline in late gestation and reach their lowest levels by day 22 (2). A switch in PR isoform expression would augment the P_4 withdrawal. This mechanism could be particularly important in primate species where there appears to be no significant decline in maternal P_4 concentrations around the time of parturition (21).

173

Administration of the estrogen antagonist TAM resulted in a significant delay in parturition. Although TAM may have mixed agonistic and antagonistic properties, it acts as a strong antagonist in the high estrogen milieu of late pregnancy (22). We have demonstrated that TAM administration does not significantly change maternal serum P_4 concentrations (2). It is possible that the effect of TAM on the timing of parturition is mediated by a change in the relative expression of PR isoforms. The data from the TAM-treated animals suggest that the increase in PR-A/PR-C prior to parturition is dependent on estrogen. The 5'-untranslated and 5'-flanking regions of the rat PR gene contain five imperfect palindromic estrogen response element (ERE) and several widely-spaced half-sites (10). Estrogen up-regulates PR in breast cancer cells (23, 24) rat uterus (9) and sheep endometrial and myometrial cells (25). The estrogen-induced isoform ratios also appear to be a tissue-specific. Estrogen stimulated mRNA for both PR- A (10 fold) and PR-B (5-fold) in human endometrial stromal cells (26) but only increased PR-B in T47D cells (23). Estrogen increased PR-A protein levels 5-fold greater than PR-B in rhesus monkey endometrium (27). These findings support our data and suggest that the PR-A isoform may account for most of the estrogen-dependent increase in PR mRNA that we demonstrated prior to parturition.

The administration of the PR antagonist RU486 on day 15 of gestation caused markedly premature parturition approximately a day following administration. The present data demonstrate that in this model, changes in PR isoform expression appear to be similar to those observed at normal parturition. Treatment with RU-486 significantly increased the concentration of PR-total mRNA but not PR-B, suggesting that P_4 has a tonic inhibitory effect on PR-A and/or PR-C expression and this is released by the P_4 antagonist.

The effects of P_4 appear also to be tissue specific and isoform-expression specific. For example, within human endometrium P_4 increased the

174

PR-A and PR-B mRNA by 2- to 10-fold in the stromal cells but reduced them in glandular epithelial cells (26). Different effects were also found in different tissues from the rhesus macaque. Ogle *et al.* reported that RU-486 treatment reduced decidual PR-A and PR-B but not PR-C protein levels in day 9 pregnant ovariectomized rat (20). They also reported that P_4 had different effects on PR mRNA between day 10 and day 14 gestational tissues. The differences between their findings and ours may relate to the difference in tissue types as well as the difference in gestational age.

These experiments were designed to study the influence of sex steroid antagonists on relative expression of PR isoforms. We acknowledge that it is highly likely that estrogen and P_4 influence transcription of many other genes that play an important role in the mechanisms of parturition. Similarly, the steroids, and potentially their antagonists, may have important non-genomic effects including interaction with membrane receptors, such as oxytocin receptors (28), that may influence uterine contractility. It is not clear how important the changes in PR isoform expression are in relation to the complex mechanisms regulating the timing of parturition. However, this physiologic phenomenon does offer an explanation that could underlie a mechanism for significant "P_4 withdrawal" in the absence of changes in P_4 concentrations or total P_4 binding to uterine tissues. It will be important to demonstrate corresponding changes in protein expression using Western analysis or immunohistochemical techniques.

Little is known regarding the regulation of expression of PR isoforms. Though the three isoforms are transcribed from the same gene, at least two functionally distinct promoters have been found on this gene. Consensus progesterone response elements have not been identified. Whereas estrogen may influence gene transcription directly through the weak ERE sites in the promoters, it is likely that there are more important indirect or non-genomic

175

mechanisms of steroid hormone action. In addition to tramscriptional regulation, these mechanisms may influence mRNA stabillity, translational activity or post-translational modifications of the PR genee products.

In summary, these data have provided information regarding the uterine concentrations of mRNAs for PR isoforms through late raat gestation and around the time of parturition. The results strongly suppoort a role for estrogen and P4 in the regulation of PR isoform expression. Our ffindings provide another mechanism that may contribute to the withdrawall of P_4 that is essential in the mechanism of parturition in many species. Further investigation of the changes in PR isoforms at translational and posttranslatiornal levels may provide more information regarding the roles of PR isofoarms at parturition.

Figure 5-1   a.) Diagrammatic scheme of cDNA for the rat PR-A, PR-B and

PR-C isoforms. The PR-A isoform is an N-terminally truncated naturally

occurring variant of the B isoform. The PR-C isoform is also a N-terminally

truncated transcriptional product but much smaller than PR-A. The locations

of the PCR primers used to generate cDNA templates (orange areas) for cRNA

probes used for ribonuclease protection assays are indicated.

        b.) The sequences of primers used to generate the probes for

PR-B mRNA (P_{B1}/P_{B2})and for PR-total mRNA (P_{T3}/P_{T4}). The latter includes

mRNAs for PR-A plus PR-B plus PR-C.

a.

nt

rPR-B

rPR-A

rPR-C
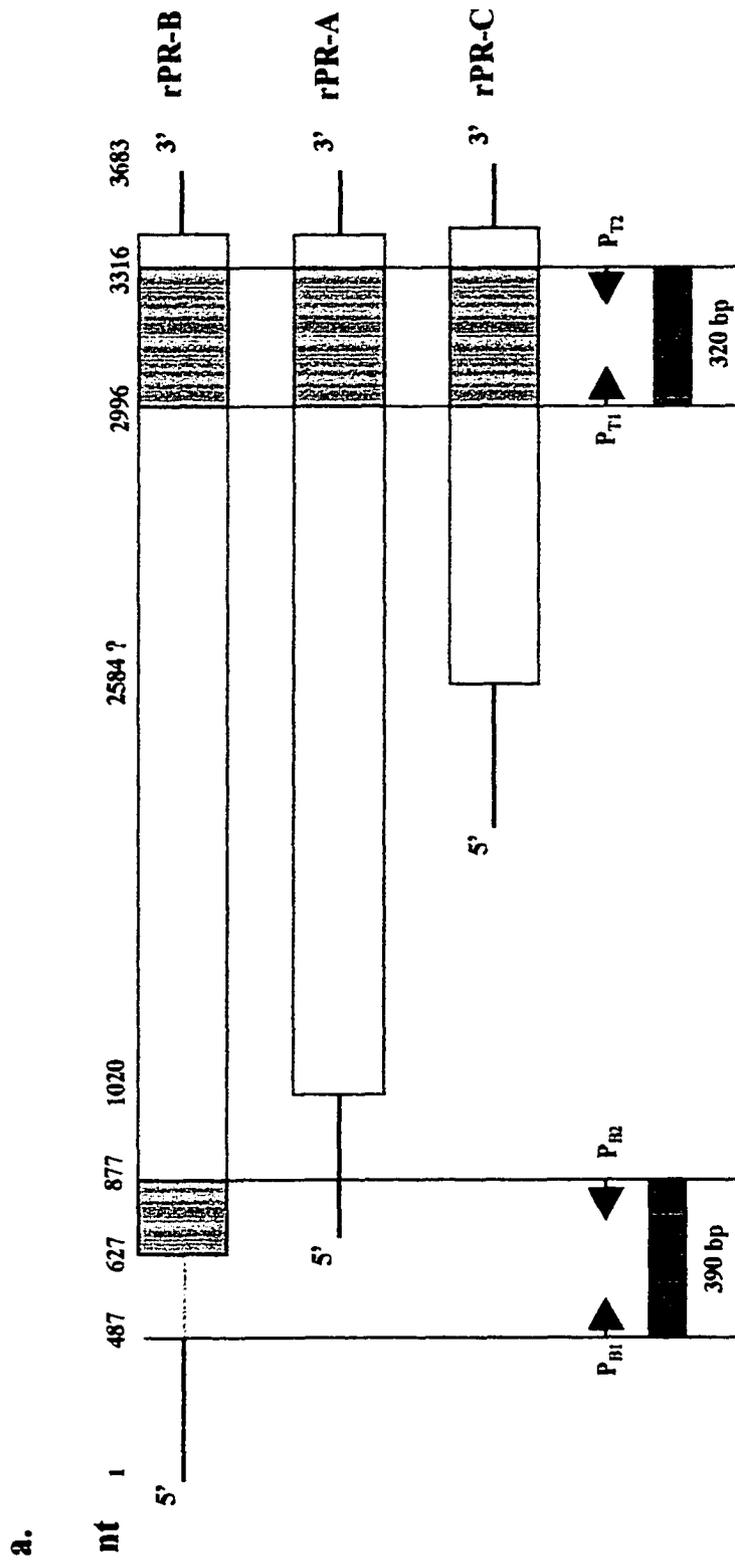
b.

P_{B1} : 5'-GAGGTGGAGATCCCACGGGTC-3'
P_{B2} : 5'-ACGTCGGACAGCGACTGCTG-3'
P_{T1} : 5'-CCATGTGGCAGATCCCACAGGCGTT-3'
P_{T2} : 5'-TGGAAATTCAACACTCAGTGCCCGG-3'

178

Figure 5-2   Uterine PR-total and PR-B mRNA concentrations in rat uterine tissues obtained through late gestation from day 13 of pregnancy to during delivery (del'y) and postpartum day 1 (pp-1). Each point represents the mean ± SEM from 3 - 9 animals. Histograms representing PR-total in panel b. and histograms in panel c. with different letters are significantly different each other by ANOVA and Tukey-Kramer tests. ($P < 0.05$)

a.) Representative ribonuclease protection assay showing PR-total, PR-B and β-actin mRNA levels in uterine tissue. Each lane contains RNA (20 µg) from the uterus of one animal.

b.)   The ratio of PR-total or PR-B to β-actin mRNA.

c.)   The ratio of PR-total to PR-B mRNA.

a.

PR-total

PR-B

β–actin

Day   13   15   16   18   19   20   21   22   del'y   pp-1

b.

Ratio of PR to β-actin mRNA

c.

Ratio of PR-total to PR-B

Gestational age

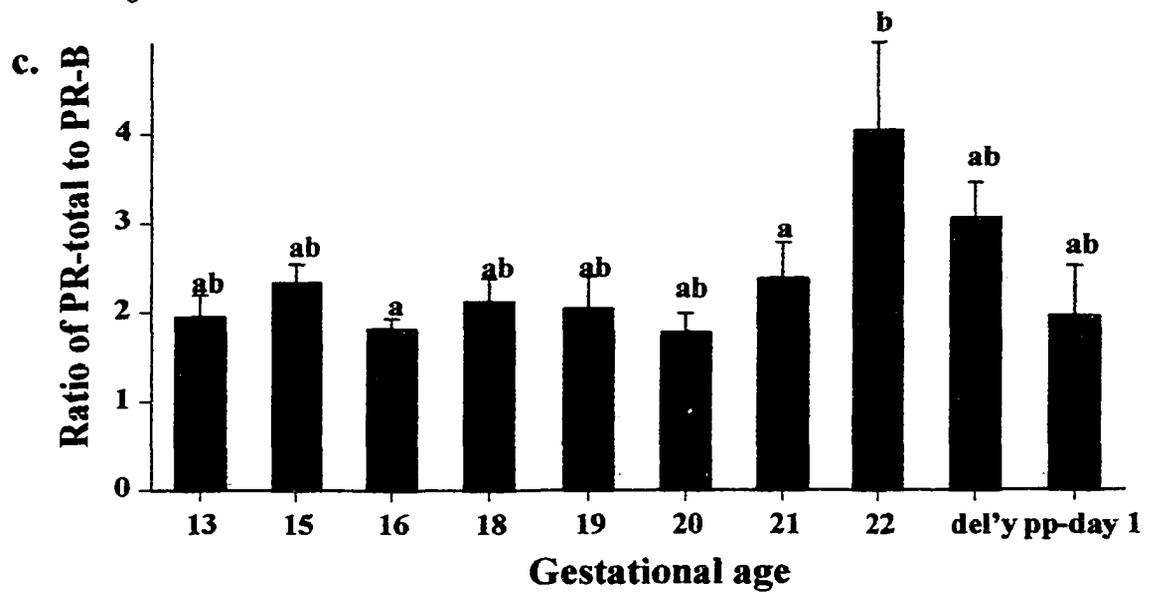13   15   16   18   19   20   21   22   del'y   pp-day 1

180

Figure 5-3   Effects of TAM on uterine PR-total and PR-B mRNA concentrations in rat uterine tissues through late gestation and delivery (del'y). Each point represents the mean ± SEM from 3 - 5 animals. The histograms with different letters are significantly different from each other by ANOVA and Tukey-Kramer tests. The asterisk (*) indicates a statistically significant difference between the control and TAM-treated groups ($P < 0.05$).

a.) The ratio of PR-total to β-actin mRNA in controls and TAM treated animals.

b.) The ratio of PR-B to β-actin mRNA in controls and TAM treated animals.

a.

Ratio of PR-total to β-actin mRNA

Gestational age: 19, 21, 21.5, 22, del'y Control, del'y TAM

b.

Ratio of PR-B to β-actin mRNA

Gestational age: 19, 21, 21.5, 22, del'y Control, del'y TAM
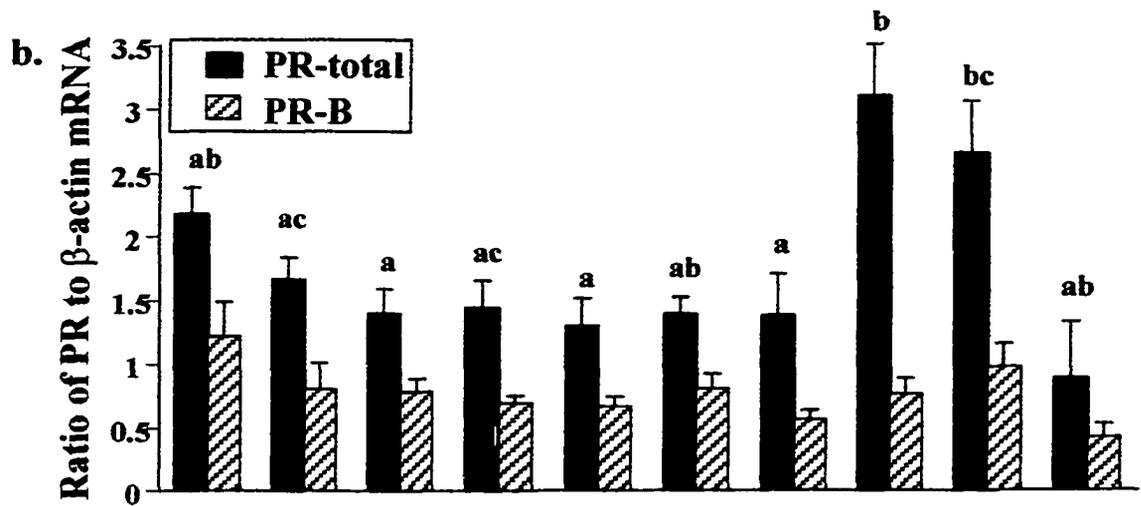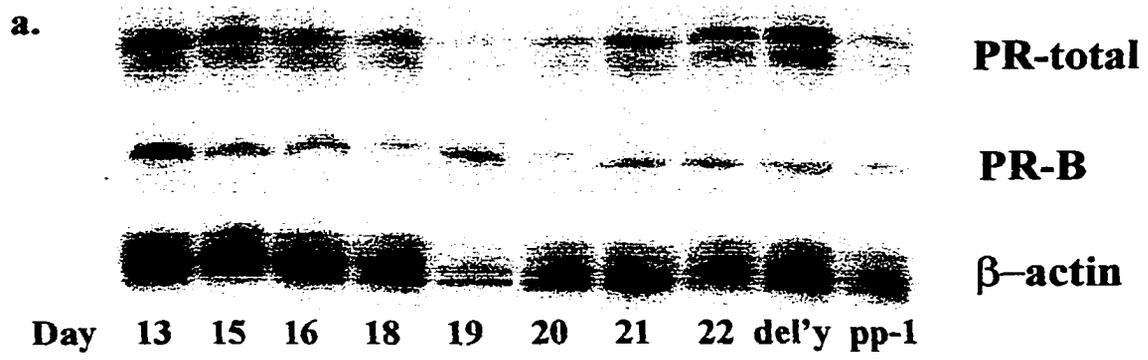
Gestational age

182

Figure 5-4   Effects of RU486 on uterine PR-total and PR-B mRNA
concentrations in rat uterine tissues through late gestation and delivery (del'y).
Each point represents the mean ± SEM of 4 - 6 animals. The histograms with
different letters are significantly different from each other by ANOVA and
Tukey-Kramer tests. The asterisk (*) indicates a statistically significant
difference between the control and RU486-treated groups ($P < 0.05$).

a.)   The ratio of PR-total to β-actin mRNA in controls and RU486
treated animals.

b.)   The ratio of PR-B to β-actin mRNA in controls and RU486
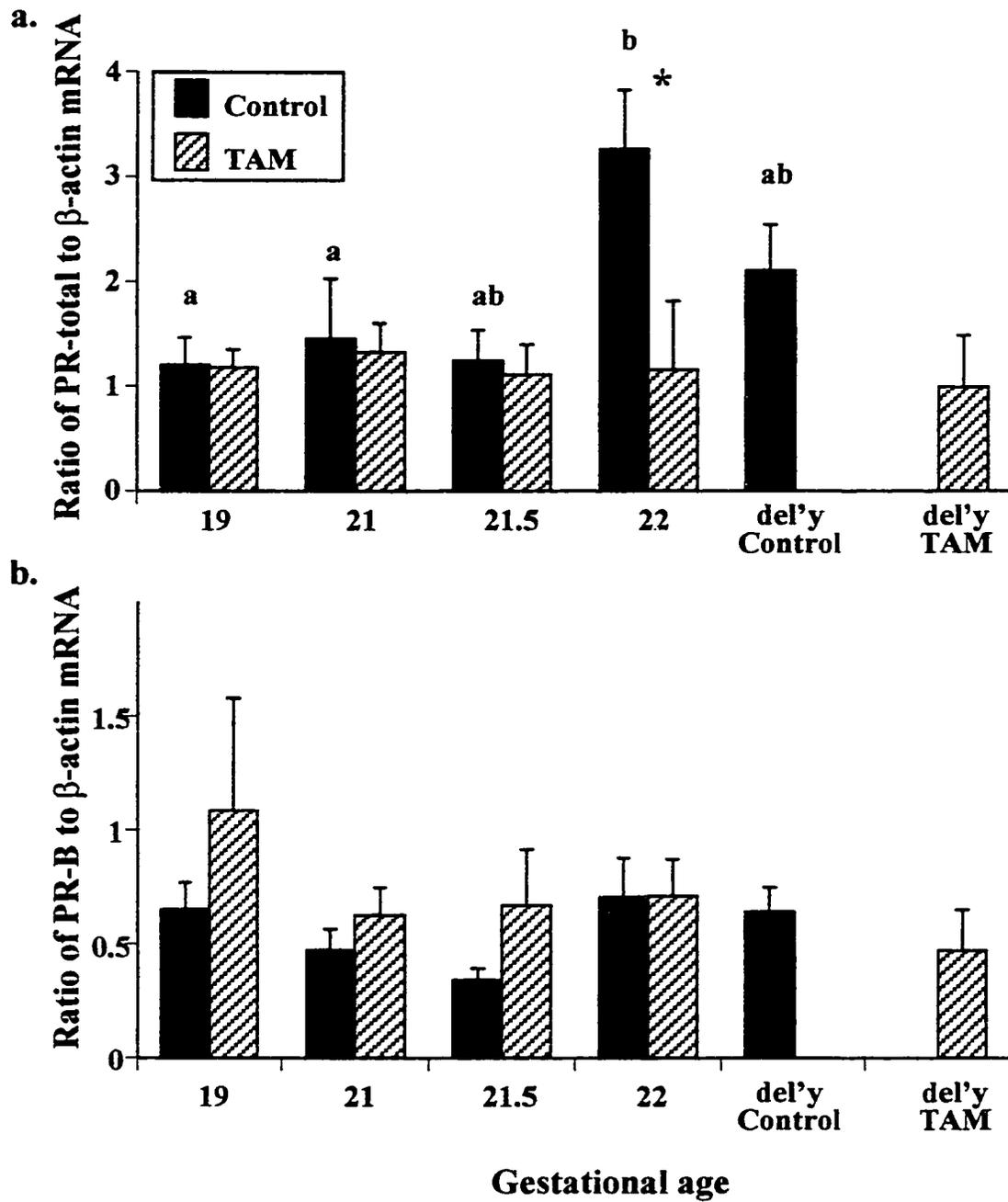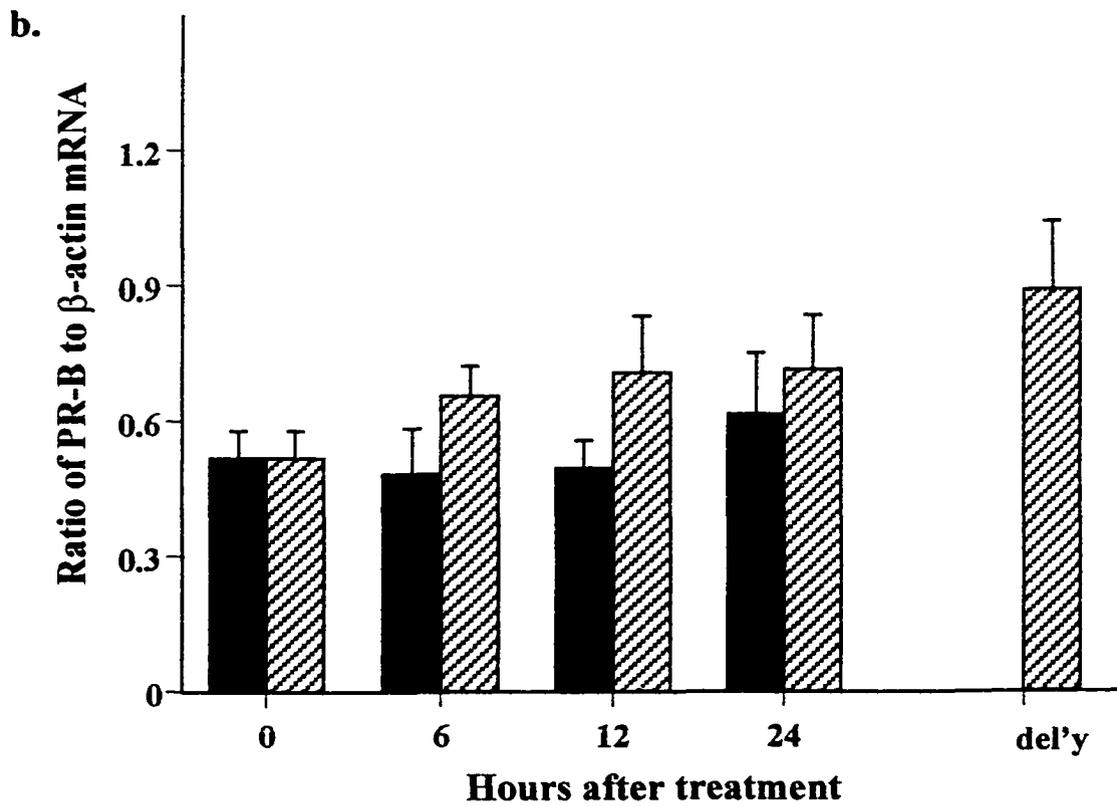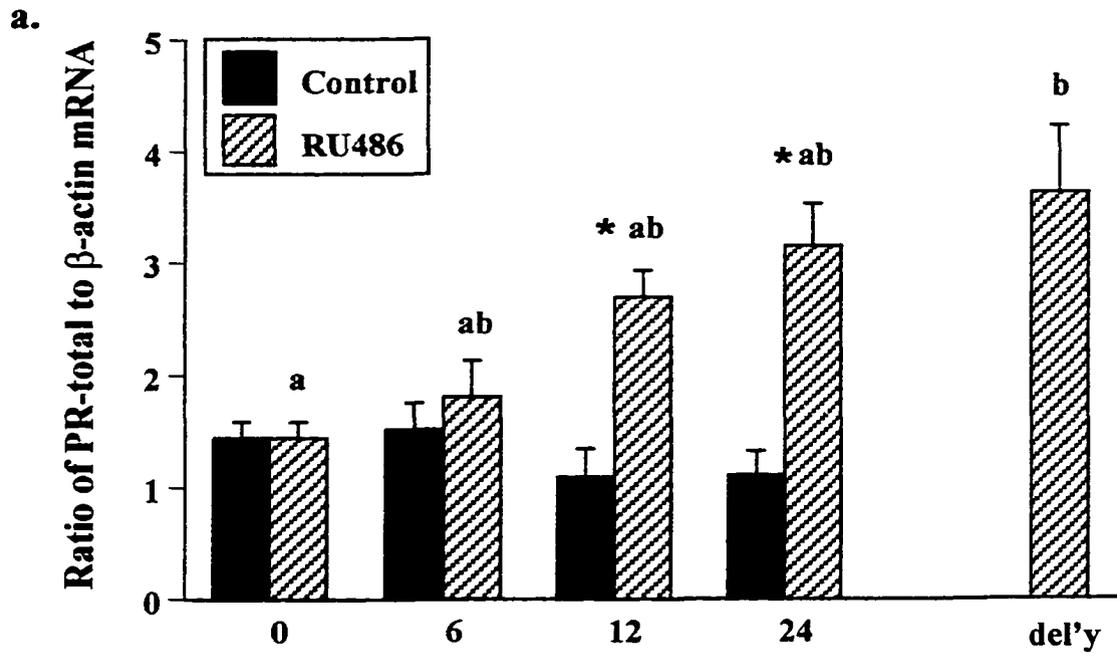treated animals.

a.

b.

Hours after treatment

5.5. REFERENCES

1. **Csapo AI, Wiest WG** 1969 An examination of the quantitative relationship between progesterone and the maintenance of pregnancy. Endocrinology 85:745-6

2. **Fang X, Wong S, Mitchell BF** 1996 Relationships among sex steroids, oxytocin, and their receptors in the rat uterus during late gestation and at parturition. Endocrinology 137:3213-9

3. **Wei LL, Gonzalez-Aller C, Wood WM, Miller LA, Horwitz KB** 1990 5'-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated "C"-receptor and unique A-receptor messages. Mol Endocrinol 4:1833-40

4. **Ogle TE, Dai D, George P, Mahesh VB** 1997 Stromal cell progesterone and estrogen receptors during proliferation and regression of the decidua basalis in the pregnant rat. Biol Reprod 57:495-506

5. **Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P** 1990 Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 9:1603-14

6. **Tung L, Mohamed MK, Hoeffler JP, Takimoto GS, Horwitz KB** 1993 Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors. Mol Endocrinol 7:1256-65

7. **Wei LL, Miner RS** 1994 Evidence for a third progesterone receptor isoform in human breast cancer cells T47D. Cancer Res 54:340-3

8. **Kraus WL, Montano MM, Katzenellenbogen BS** 1993 Cloning of the rat progesterone receptor gene 5'-region and identification of two functionally distict promoters. Mol Endocrinol 7:1603-16

9. **Kraus WL, Katzenellenbogen BS** 1993 Regulation of progesterone receptor gene expression in rat uterus: modulation of estrogen action by progesterone and sex steroid hormone antagonists. Endocrinology 132:2371-9

10. **Kraus WL, Montano MM, Katzenellenbogen BS** 1994 Identification of multiple, widely, spaced estrogen-responsive regions in the rat progesterone receptor gene. Mol Endocrinol 8:952-69

11. **Milgrom E, Luu Thi MT, Atger M, Bauleu EE** 1973 Mechanism regulating the concentration and conformation of progesterone receptor(s) in the uterus. J Biol Chem 248:6366-74

12. **Leavitt WW, Chen TJ, Allen TC, Johnston JO** 1977 Regulation of progesterone receptor formation by estrogen action. Ann NY Acad Sci 286:210-25

13. **Vu Hai MT, Logear F, Warenbourg M, Migrom E** 1977 Hormonal control of progesterone receptors. Ann NY Acad Sci 286:199-209

14. **Read LD, Snider CE, Miller JS, Greene GL, Katzenellenbogen BS** 1988 Ligand-modulated regulation of progesterone receptor messenger ribonucleic acid and protein in human breast cancer cell lines. Mol Endocrinol 2:263-71

15. **Fang X, Wong S, Mitchell BF** 1997 Effects of RU486 on estrogen, progesterone, oxytocin, and their receptors in the rat uterus during late gestation. Endocrinology 138:2763-8

186

16. **Carbajo P, Christensen K, Edwards DP, Skafar DF** 1996 Binding of [3H]progesterone to the human progesterone receptor: differences between individual and mixed isoforms. Endocrinology 137:2339-46

17. **Vegeto E, Shahbaz MM, Wen DX, Goldman ME, O'Malley BW, McDonnell DP** 1993 Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. Mol Endocrinol 7:1244-55

18. **Hovland AR, Powell RL, Takimoto GS, Tung L, Horwitz KB** 1998 An N-terminal inhibitory function, IF, suppresses transcription by the A-isoform but not the B-isoform of human progesterone. J Biol Chem 10:5455-60

19. **Wei LL, Norris BM, Baker CJ** 1997 An N-terminally truncated third progesterone receptor protein, PRc, forms heterodimer with PR_B but interferes in PR_B-DNA binding. J Steroid Biochem Mol Biol 62:287-97

20. **Ogle TF, Dai D, George P, Mahesh VB** 1998 Regulation of progesterone receptor and estrogen receptor in decidua basalis by progesterone and estradiol during pregnancy. Biol Reprod 58:1188-98

21. **Tulchinsky D, Hobel CY, Yeager E, Marshall YR** 1972 Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnany. 1. Normal pregnancy. Am J Obstet Gynecol 112:1095-100

22. **Jordan VC, Allen KE, Dix CJ** 1980 Pharmacology of tamoxifen in laboratory animals. Cancer Treatment Reports 64:745-59

23. **Graham JD, Roman SD, McGowan E, Sutherland RL, Clarke CL** 1995 Preferential stimulation of human progesterone receptor B

expression by estrogen in T-47D human breast cancer cells. J Biol Chem 270:30693-700

24. **Wei LL, Krett NL, Francis MD, Gordon DF, Wood WM, O'Malley BM, Horwitz KB** 1988 Multiple human progesterone receptor messenger ribonucleic acids and their autoregulation by progestin agonists and antagonists in breast cancer cells. Mol Endocrinol 2:62-72

25. **Wathes DC, Mann GE, Payne JH, Riley PR, Stevenson KR, Lamming GE** 1996 Regulation of oxytocin, oestradiol and progesterone receptor concentrations in different uterine regions by oestradiol, progesterone and oxytocin in ovariectomized ewes. J Endocrinol 151:375-93

26. **Tseng L, Zhu HH** 1997 Regulation of progesterone receptor messenger ribonucleic acid by progestin in human endometrial stromal cells. Biol Reprod 57:1360-6

27. **Bethea CL, Widmann AA** 1998 Differential expression of progestin receptor isoforms in the hypothalamus, pituitary, and endometrium of rhesus macaques. Endocrinology 139:677-87

28. **Grazzini E, Guillon G, Mouillac B, Zingg HH** 1998 Inhibition of oxytocin receptor function by direct binding of progesterone. Nature 392:509-12

6. EFFECTS OF LIPOPOLYSACCHARIDE AND INTERLEUKIN-6 ON OXYTOCIN RECEPTOR IN NON-PREGNANT AND PREGNANT RAT UTERUS

6.1. INTRODUCTION

OT is a potent stimulus to myometrial contractility in essentially all species. We and others have demonstrated that OT and OTR are synthesized within intrauterine tissues of the rat and the human during late gestation (1-3). OT mRNA is increased significantly between day 14 and day 18 of pregnancy and there is a corresponding increase in immunoreactive OT that occurs just before labour. Myometrial sensitivity to OT parallels the rapid increase in OTR mRNA and protein that occurs in the few hours immediately preceding normal parturition (1, 3, 4). Little is known regarding the regulation of OT and OTR around the time of parturition. Expression of OT and OTR genes in the rat uterus may be regulated by estrogen and progesterone (5, 6).

Recent evidence suggests a role for the immune system in the regulation of parturition. Amniotic fluid concentrations of IL-1, IL-6 and TNFα are elevated at the time of human preterm or term labour (7, 8). Human decidual and mouse uterine tissues synthesize those cytokines (9-12). The rat OTR gene promoter contains 3 copies of the acute phase response element that can be activated by IL-6 and 3 copies of the response element for NF-IL6 that is involved in mediation of many immune reactions (13). These data support the hypothesis that the inflammatory cytokines may play a role in parturition through regulation of OTR gene transcription.

The objective of the present studies was to investigate the interactions

between the immune system and parturition in the rat with particular reference to changes in serum estradiol and progesterone concentrations and intrauterine expression of OT and OTR. Specifically, we report our attempts to establish a rat model of preterm labour using LPS *in vivo* to stimulate the immune system in late gestation. We also report our studies to establish an *in vitro* uterine explant system to determine the effects of the pro-inflammatory cytokines on uterine tissue concentrations of OT and OTR mRNA. We include here our data investigating the effects of IL-6 on uterine OTR mRNA levels.

6.2. MATERIALS AND METHODS

6.2.1. Animals and Treatments

The animals used in this study are described in detail in Chapter 2. The uteri were removed immediately after death. Vaginal smears were used to determine the estrus phase in non-pregnant animals.

In vivo studies

In total, 40 pregnant animals were used in these studies. In preliminary studies, varying doses of LPS (10 - 400 µg/rat, with appropriate saline controls) were injected intraperitoneally at day 18 of pregnancy to determine the effects on the length of gestation. Some animals received a repeated doses of LPS after 8 hours the first dose given. To determine the time course of changes in estradiol and progesterone, animals were injected with 200 µg LPS. Serum and tissue samples were collected at time 0 h, 4 h, and 10 h after treatment and during labour (after delivery of the first pup). The control group received only vehicle and the animals were killed at the same time points as the experimental group. Samples were collected, frozen in liquid nitrogen and stored at $-80°$ C.

190

In vitro **explant system**

Uterine tissues were collected from pregnant rats at day 16 of gestation or from non-pregnant rats (ten-week old virgin) on proestrus. Three or four whole uterine tissues were pooled from the pregnant or non-pregnant animals and chopped into 0.5 cm squares under sterile conditions. Three or four such squares (total of 0.15 to 0.2 gram tissue) were randomly distributed into each of 60 x 15 mm petri dishes containing 4 ml of DMEM-Ham's F:12 medium with supplements and 10ml/L 100x lyophilized antibiotics-antimycotics. The supplements included 0.24 % $NaHCO_3$, 25 mM HEPES, and 10 mM MEM non-essential amino acids solution. Incubations were performed in duplicate, with appropriate controls, in the presence of rat IL-6 at concentrations of 0, 1, 10, or 100 ng/ml for 0, 10, 24, or 48 hours at 37 C in 95% air:5% CO_2. The medium was changed after 24 h. At the end of the incubation period, tissues were collected, frozen in liquid nitrogen and stored at $-80°$ C.

6.2.2. RIA for Serum E_2, P_4 and Uterine OT

The RIA procedures and antisera for E_2 and P_4 assays have been discribed previously (14). OT peptide was determined by RIA according to the procedure described previously (2). The protocol is described in detail in Chapter 2.

6.2.3. RNA Preparation and mRNA Measurement for OT and OTR

Frozen uterine samples or explant tissues were homogenized in 1ml Trizol™ and total RNA was extracted and purified by following the manufacturer's instructions. OT mRNA and OTR mRNA levels in the uterine tissues were measured by RPA. The assay was performed as previously described (2). The details of the probes and procedure of RPA are decribed in Chapter 2. A sample of RNA from one animal at each time point from each group, chosen at random, was included on each gel. The gel was exposed to

191

XAR x-ray and autoradiograms were quantitated by analyzing the density of each blot using NIH-imagine analysis software, after scanning in a high performance scanner.

6.2.4. OT Binding Assay for OTR

A binding assay to measure OTR in uterine tissues was developed using a modification of published OT binding assay (1) and are described in Chapter 2.

6.2.5. Statistical Analysis

Data are presented in graphs as the mean ± SEM. Sequential data were first analyzed using one-way ANOVA (Instat; GraphPad Software, San Diego, CA). *Post hoc* comparisons of the means were performed using the Fisher's Protected LSD test. Differences between the experimental and control groups were sought using two-way ANOVA (Prism; GraphPad Software). The two-tailed unpaired Student's t test was performed to compare two groups at the same time point. Differences were considered significant when a P value less than 0.05 was obtained. If Bartlett's test revealed nonhomogeneity of variance, the corresponding nonparametric test was used.

6.3. RESULTS

6.3.1. *In vivo* Studies

In preliminary studies to establish the most effective dose of LPS to stimulate preterm parturition, there was marked variability in the responses observed. There appeared to be no relationship between the dose of LPS and the occurrence of preterm delivery (Table 6-I). When preterm labour did occur, the first pup was delivered between 24 h and 48 h after LPS treatment. There was a high maternal mortality rate after LPS treatment and, again, this

appeared to have no relationship to the dose of LPS. Interestingly, in two of the animals receiving lower doses of LPS, parturition was delayed by a day.

Because of the variability of the response and the high mortality rate with maternal intraperitoneal LPS injections, it was elected to abandon further attempts to establish this model. However, tissues were obtained from several of the animals in the planned time course study and these data are presented in Figure 6-1. Only 2 or 3 experiments were completed at each time point so statistical analyses were not attempted. Although we are unable to make valid conclusions, the data demonstrate some interesting trends. As expected, serum concentrations of estradiol and progesterone did not change in the control group. In contrast, in the LPS- treated rats, serum estradiol remained unchanged until labour when it was quite low and serum progesterone appeared to decline immediately after treatment with LPS. Whereas OT mRNA and peptide remained unchanged in the control animals, there appeared to be a decline in both in the LPS-treated group. Concentrations of OTR mRNA and binding activity remained constant in the controls but increased markedly after LPS injection, beginning at approximately 10 hours and achieving levels approximately 20-fold increased over basal levels at the time of delivery.

6.3.2. *In vitro* Studies

Using uterine explants from the rats at day 16 of gestation, incubation with IL-6 resulted in a significant increase in the tissue concentration of mRNA for OTR. The maximal response was observed at IL-6 concentrations of 10 ng/ml. At concentrations of 100 ng/ml, there was a decline in OTR mRNA to baseline levels and the explants appeared in poor condition by 24 hours (Fig. 6-2).

In the uterine explant system, OTR mRNA concentrations in the tissues from day 16 pregnant animals tended to increase with time in culture but this

193

did not achieve statistical significance. However, in the presence of IL-6 (10 ng/ml), OTR mRNA levels were stimulated significantly over time with maximal tissue concentrations (3 - 5-fold increase over controls) detected at 48 h after treatment (Fig.6-3). The concentration of OTR mRNA in uterine tissues obtained from rats on proestrous was approximately 2-fold higher than in pregnant uterus at day 16. IL-6 had no effect on OTR mRNA in proestrous uterine tissues.

6.4. DISCUSSION

A growing body of evidence suggests that host-derived cytokines are important in the mechanism of parturition. Based on the model of LPS-induced preterm labour in mice (15), we expected that administration of LPS also would precipitate delivery in the rat. Cytokines such as IL-1, TNFα and IL-6 are elevated in amniotic fluid of women with preterm labour or term labour (7, 8). Human decidua and murine endometrium have been shown to produce these three cytokines (9-12). Further, only 4-7 % of cytokines, including IL-6, can traverse the amnion and chorion intact (16), suggesting that the synthesis of cytokines by the decidua may be much higher than expected from amniotic fluid concentrations. Furthermore, preterm labour can be induced by intra-amniotic injection of IL-1β in the rhesus monkey (17), by systemic injection of IL-1α or LPS in mice (15, 18) or by intra-amniotic administration of IL-1α and TNFα in the rabbit (19). However, in the present studies, the systemic administration of LPS to pregnant rats at day 18 gave extremely variable and unpredictable results. Many rats injected with LPS appeared severely ill and the maternal mortality rate was extremely high. Neither the delivery rate nor the degree of maternal response appeared to be dose-dependent. Because of this, we considered that this model would be unstable and likely of little physiologic relevance. Therefore, we discontinued

194

attempts to establish an *in vivo* model of LPS-induced preterm labour in the rat. However, it should be noted that we used only one lot of LPS and it may be inappropriate to generalize to all preparations of endotoxin.

Little is known concerning the relationships between cytokines and the intrauterine expression of OT or OTR in late rat gestation. Our preliminary data showed an increase in the concentrations of OTR mRNA and OT binding which began within hours after injection of the endotoxin. This increase is similar to our previous findings following the injection of the progesterone antagonist RU486 to the mother on day 15 of gestation (20). Interestingly, in the present studies, it appeared that maternal serum progesterone levels began to decline before the OTR mRNA levels increased. Since the corpus luteum is the principal source of progesterone in rat gestation (21), these findings would be compatible with the hypothesis that the primary mechanism of LPS is to induce luteolysis. The low levels of estradiol at the time of delivery would support this hypothesis. Though we did not measure the concentration of IL-6 in uterine tissues after LPS treatment, others have found that concentrations of IL-1, IL-6, and TNFα increase markedly within 10 h after LPS treatment to pregnant mice (15). Again, this would be compatible with the hypothesis that the primary mechanism of LPS is to induce luteolysis.

The increased OT binding demonstrated in these preliminary data following LPS treatment is likely due to translation of the increased OTR mRNA. Alternatively, it could result from the decrease in P_4, a hormone that recently has been demonstrated to compete with OT for binding to OTR (22).

As in our previous studies, parturition occurred despite a decline in local OT mRNA and peptide (1, 20). This suggests that OT is less important than OTR in the regulation of myometrial contractility. It also raises the intriguing possibilities that there may be another ligand for OTR or that the receptor may be activated in the absence of ligand. In the *in vivo* experiments, the levels of OT mRNA and peptide were reduced following LPS. This

195

suggests that LPS or its mediators may suppress OT gene expression. Interestingly, in two of the animals that received lower doses of LPS, pregnancy was extended for 24 hours. Although these observations are quite preliminary and cannot form the basis of valid conclusions, they suggest that the interaction between the pro-inflammatory cytokines and uterine contractility may be complex and variable according to dose.

Though luteolysis may explain some of the effects of LPS injection, our results from the uterine explant experiments demonstrate that IL-6 also has a direct effect on local synthesis of OTR mRNA in the tissues obtained in late pregnancy. This is similar to previous report demonstrating that IL-6 increased OTR mRNA in cultures of immortalized human myometrial cells (23). This culture system allows the study of intact tissues within their endogenous matrix and avoids the potentially artifact-producing procedures of cell dispersion and culture. Using this system with human tissues, we have demonstrated that the cells remain metabolically, endocrinologically and histologically intact for at least five days (24). During the incubation period, there was a gradual decline in the abundance of the "housekeeping" β-actin mRNA accompanied by an increase in OTR mRNA. We added IL-6 to the cultures immediately following tissue preparation without a recovery period. The incubation medium was changed only once every 24 hours and it is possible that toxin products from the damaged tissues may be responsible for the decline in mRNA for β-actin. However, these data suggest that the cytokine-induced increase in OTR is mainly transcriptionally regulated. We do not know whether the induced levels of OTR mRNA are through increase in synthesis or stability of mRNA. OT binding was not measured in these experiments. The optimal concentration of IL-6 for stimulating OTR mRNA was approximately 10 ng/ml in day 16 uterine explants. At 100 ng/ml, there was no increase in OTR mRNA and the mRNA levels for the reference gene

(β-actin) were decreased. The explants appeared in unhealthy condition by 24 hours suggesting a non-specific toxic effect on the tissues.

There was no change in OT mRNA after administration of IL-6, compared to the control (data not shown) indicating that the mechanisms for regulating OT and OTR gene expression in these tissues are different

Since our objective has been to study paracrine interactions between tissue layers within the pregnant uterus, we made no attempt to separate the various tissues within the uterus in either the *in vivo* nor *in vitro* experiments. Both myometrium and endometrium contain OTR that may be important in the regulation of parturition (25). Our present data do not allow conclusions as to whether one or more tissues or cell types respond to IL-6 with increasing concentrations of OTR mRNA.

In contrast to the tissues from pregnant rats, uterine OTR mRNA levels were not stimulated in tissues from the non-pregnant animals. This suggests that the effects of cytokines in uterine tissues may be mediated by other factors that are present during pregnancy. Such factors could include IL-6 receptors or a variety of transcription factors that are commonly associated with mediation of the effects of the inflammatory cytokines (26, 27). In this regard, both the rat and human OTR promoters contain NF-IL6 response elements (13, 28). One of them is quite close to the transcription start site and one is closely associated with two acute phase response elements. Other transcription factors such as NF-κB have been shown to regulate the enzyme prostaglandin H synthase-2 which is important in the mechanism of parturition (29). There is no information about the concentrations of these transcription factors nor the role they play in regulating myometrial contractility in the non-pregnant or pregnant uterus.

Based on consideration of the response elements present in the promoter region of the OTR gene, we chose to use IL-6 in our initial studies of the regulation of OTR by cytokines. It will be important to explore the role of

other cytokines, particularly those associated with the pro-inflammatory response, on the regulation of uterine OTR concentrations.

In summary, our results show that LPS and IL-6 stimulate OTR gene expression in the late gestation rat uterus. They suggest that LPS may induce luteolysis *in vivo* but also demonstrate a direct effect of IL-6 on uterine tissues *in vitro*. The data also demonstrate that LPS is extremely toxic to the pregnant dam and is an unreliable and unpredictable method to induce preterm parturition. These studies suggest a potentially important role for the immune system in regulation of uterine contractility in late gestation and emphasize the possibility that pro-inflammatory cytokines could be therapeutic targets in the strategy to prevent or reverse preterm labour. Further studies exploring these interactions between the immune system and the late gestation uterus may yield valuable information regarding the physiology and pathophysiology of parturition.

Table 6-I: The effect of maternal intraperitoneal LPS on parturition

LPS dose /rat (μg)	No. of animal	maternal death	preterm delivery
400	2	2	-
200	5	2	2 (20) (48)*
200 + 200**	1	1	-
100	2	1	-
100 + 100**	2	2	-
50	5	4	1 (30)
50 + 50**	2	1	1 (29)
25	4	2	1 (47)
10 + 10**	2	-	1 (46)
10 + 25**	2	-	-

* numbers in parenthesis indicate the hours after treatment when preterm delivery occurred
** these animals were administered repeated doses of LPS - the second dose given 8 hours after the first

Figure 6-1   The effects of administration of LPS on serum estradiol and progesterone, uterine OT and ratio of OT mRNA to GAPDH mRNA, and uterine OTR and ratio of OTR mRNA to GAPDH mRNA. Histograms represent levels in the control (closed) and LPS-treated (cross-hatched) groups. In these preliminary experiments, there were 2 or 3 animals in each group at each time point.

Serum estradiol (pg/ml)

Serum progesterone (ng/ml)

OT (pg/gram tissue)

Ratio of OT/GAPDH mRNA

OTR (fmol/mg protein)

Ratio of OTR/β-actin mRNA

■ Control   ▨ LPS treated

Hours after treatment
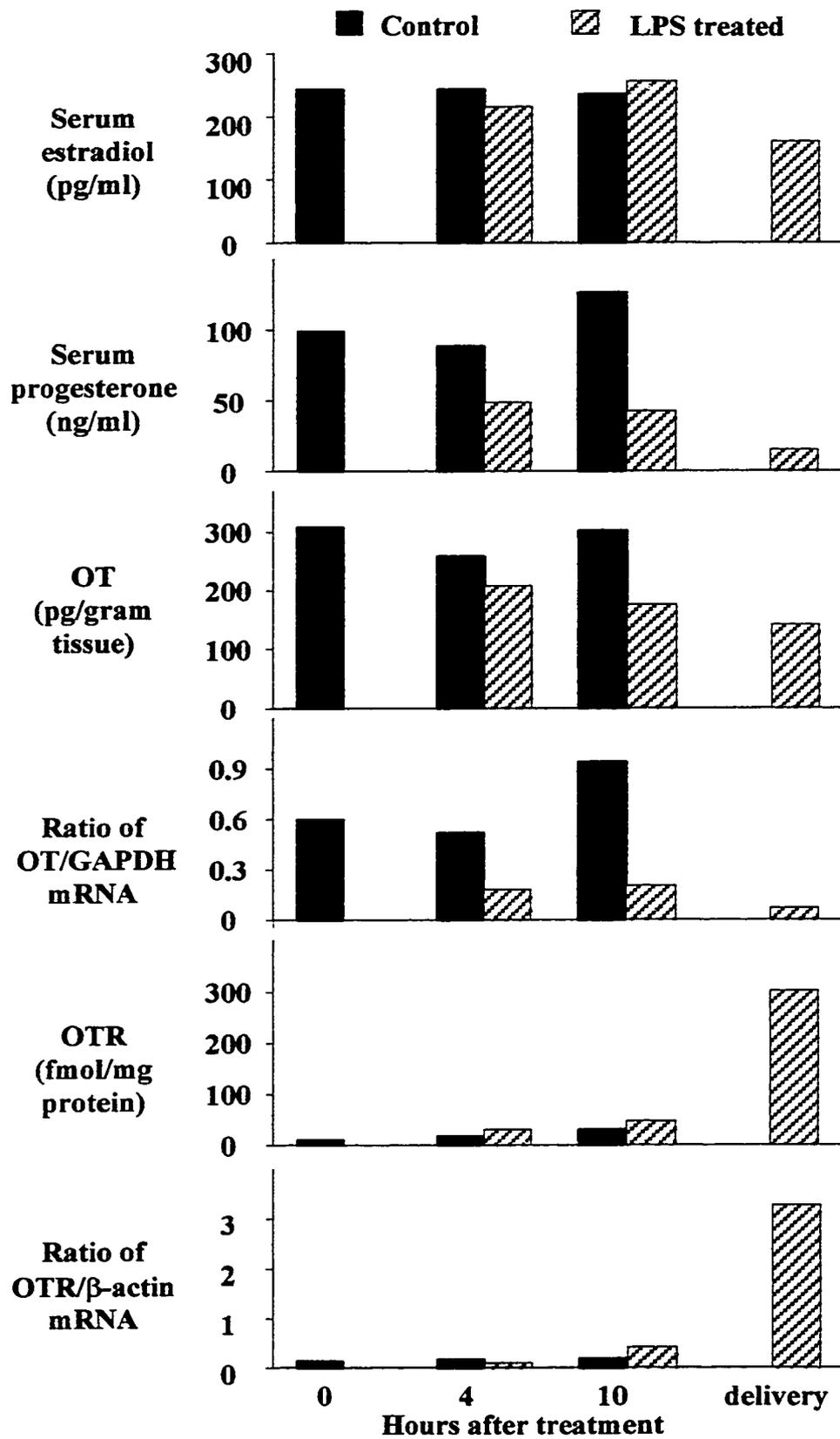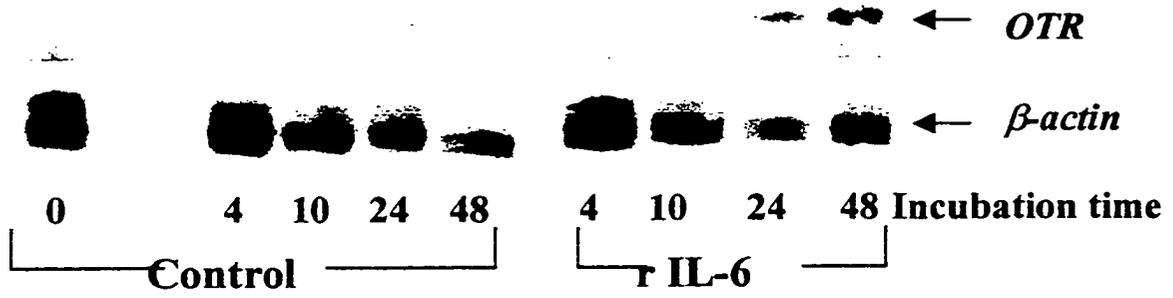
0   4   10   delivery

201

Figure 6-2   Dose response curves of IL-6 in uterine explants obtained from pregnant rats on day 16. Tissues were incubated for 48 h. Each point represents the mean ± SEM from 3-6 animals. The different letters are significantly different from each other by Fisher's Protected LSD test. ($P <$ 0.05)

.

a.
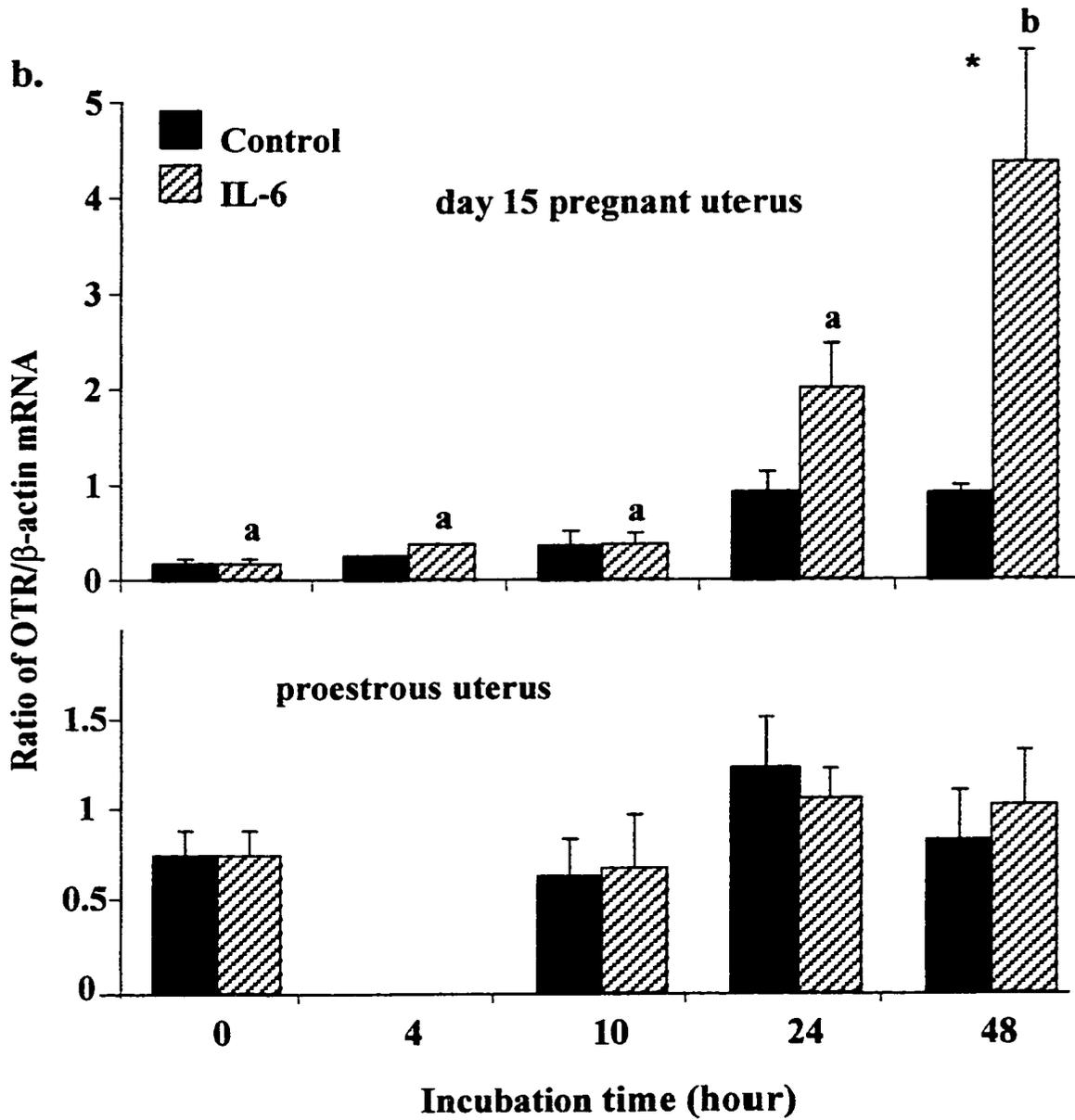

b.

Incubation time (hour)

Figure 6-3   The effects of IL-6 on pregnant and proestrous uterine OTR mRNA.

a.)   Representative ribonuclease protection assay showing uterine OTR and β-actin mRNA levels in control and IL-6 treated rat uterine explants. Each lane contains RNA (40 µg) from day 16 pregnant uterus of one animal.

b.)   the effects of IL-6 on day 16 and proestrous uterine OTR mRNA.

Each point represents the mean ± SEM from 6 animals. The different letters are significantly different from each other by Fisher's Protected LSD test. The asterisk denotes $P < 0.05$, comparing the two groups at the same time point.
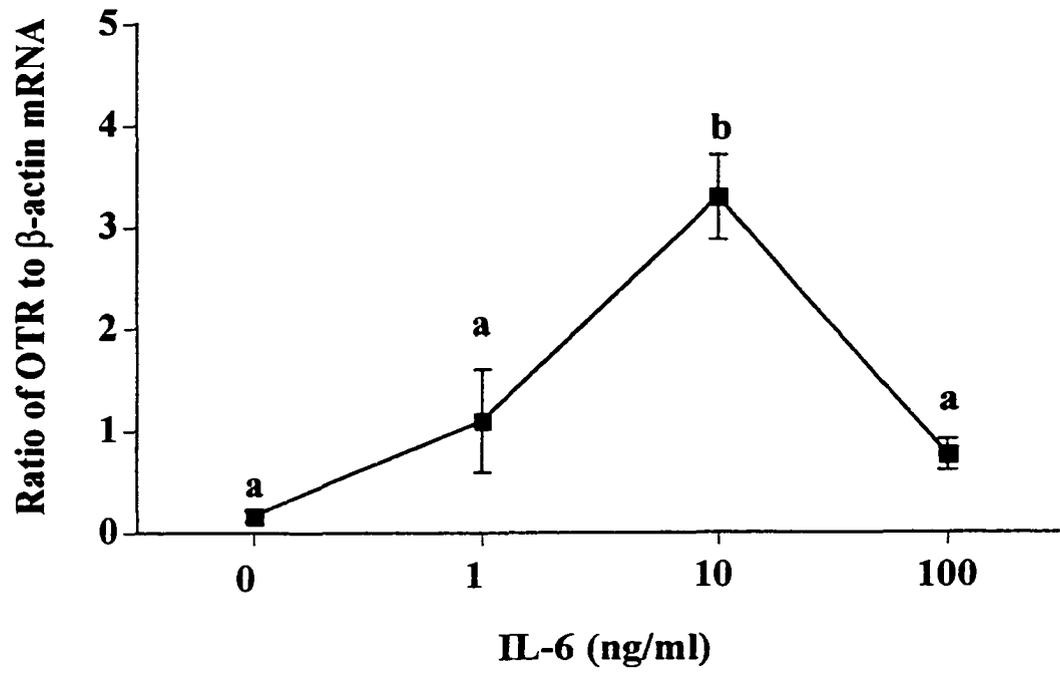
6.5. REFERENCES

1. **Fang X, Wong S, Mitchell BF** 1996 Relationships among sex steroids, oxytocin, and their receptors in the rat uterus during late gestation and at parturition. Endocrinology 137:3213-9

2. **Chibbar R, Wong S, Miller FD, Mitchell BF** 1995 Estrogen stimulates oxytocin gene expression in human chorio-decidua. J Clin Endocrinol Metab 80:567-72

3. **Soloff MS, Alexandrova M, Fernstrom MJ** 1979 Oxytocin receptors: triggers for parturition and lactation? Science 204:1313-5

4. **Hirst JJ, Haluska GJ, Cook MJ, Hess DL, Novy MJ** 1991 Comparison of plasma oxytocin and catecholamine concentrations with uterine activity in pregnant rhesus monkeys. J Clin Endocrinol Metab 73:804-10

5. **Fuchs AR, Periyasamy S, Alexandrova M, Soloff MS** 1983 Correlation between oxytocin receptor concentration and responsiveness to oxytocin in pregnant rat myometrium: effects of ovarian steroids. Endocrinology 113:742-9

6. **Lefebvre DL, Farookhi R, Giaid A, Neculcea J, Zingg HH** 1994 Uterine oxytocin gene expression. II. Induction by exogenous steroid administration. Endocrinology 134:2562-6

7. **Hillier SL, Witkin SS, Krohn MA, Watts DH, Kiviat NB, Eschenbach DA** 1990 The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamnionitis, and chorioamnion infection. Obstet Gynecol 81:941-8

8. **Opsjon S-L, Wathen NC, Tingulstad S, Wiedswang G, Sundan A, Waage A, Austgulen R** 1993 Tumor necrosis factor, interleukin-1,

206

and interleukin-6 in normal human pregnancy. Am J Obstet Gynecol 169:397-404

9. **Casey ML, Cox SM, Beutler B, Milewich L, MacDonald PC** 1989 Cachectin/tumor necrosis factor-α formation in human decidua. J Clin Invest 83:430-6

10. **MacDonald PC, Koga S, Casey ML** 1991 Decidual activation in parturition: examination of amniotic fluid for mediators of the inflammatory response. Ann NY Acad Sci 622:315-30

11. **Dudley DJ, Trautman MS, Araneo BA, Edwin SS, Mitchell MD** 1992 Decidual cell biosynthesis of interleukin-6: regulation by inflammatory cytokines. J Clin Endocrinol Metab 74:884-9

12. **De M, Sanford TH, Wood GW** 1992 Detection of interleukin-1, interlukin-6, and tumor necrosis factor-α in the uterus during the second half of pregnancy in the mouse. Endocriology 131:14-

13. **Rozen F, Russo C, Banville D, Zingg HH** 1995 Structure, characterization, and expression of the rat oxytocin receptor gene. Proc Natl Acad Sci U S A 92:200-4

14. **Mitchell BF, Wong S** 1993 Changes in 17 beta,20 alpha-hydroxysteroid dehydrogenase activity supporting an increase in the estrogen/progesterone ratio of human fetal membranes at parturition. Am J Obstet Gynecol 168:1377-85

15. **Fidel PL Jr., Romero R, Wolf N, Cutright J, Ramirez M, Araneda H, Cotton DB** 1994 Systemic and local cytokine profiles in endotoxin-induced preterm parturition in mice. Am J Obstet Gynecol 170:1467-75

16. **Kent ASH, Sullivan MHF, Elder MG** 1994 Transfer of cytokines through human fetal membranes. J Reprod Fertil 100:81-4

17. **Baggia S, Gravett MG, Witkin SS, Haluska GJ, Novy MJ** 1996 Interleukin-1β intra-amniotic infusion induces tumor necrosis factor-α, prostaglandin production, and preterm contractions in the pregnant rhesus monkey. Journal of the Society for Gynecologic Investigation 3:121-6

18. **Romero R, Mazor M, Tartakovsky B** 1991 Systemic administration of interleukin-1 induces preterm parturition in mice. Am J Obstet Gynecol 165:969-71

19. **Bry K, Hallman M** 1993 Transforming growth factor-β2 prevents preterm delivery induced by interleukin-1α and tumor necrosis factor-α in the rabbit. Am J Obstet Gynecol 168:1318-22

20. **Fang X, Wong S, Mitchell BF** 1997 Effects of RU486 on estrogen, progesterone, oxytocin, and their receptors in the rat uterus during late gestation. Endocrinology 138:2763-8

21. **Csapo AI, Wiest WG** 1969 An examination of the quantitative relationship between progesterone and the maintenance of pregnancy. Endocrinology 85:745-6

22. **Grazzini E, Guillon G, Mouillac B, Zingg HH** 1998 Inhibition of oxytocin receptor function by direct binding of progesterone. Nature 392:509-12

23. **Helmer H, Knofler M, Hakl T, Husslein, P** 1998 Cytokine induced responses of primary and transformed human myometrial cells. Gynecol Invest 5:179A (abstract #T574)

24. **Mitchell BF, Powell WA** 1984 Progesterone production by human fetal membranes: an in vitro incubation system for studying hormone production and metabolism. Am J Obstet Gynecol 148:303-9

25. **Chan WY, Chen DL, Manning M** 1993 Oxytocin receptor subtypes in the pregnant rat myometrium and decidua: pharmacological differentiation. Endocrinology 132:1381-6

26. **Akira S, Kishimoto T** 1997 NF-IL6 and NF-κB in cytokine gene regulation. Adv Immunol 65:1-46

27. **Kishimoto T, Taga T, Akira S** 1994 Cytokine signal transduction. Cell 76:253-62

28. **Inoue T, Kimura T, Azuma C** 1994 Structure and organization of the human oxytocin receptor gene. J Biol Chem 269:32451-6

29. **D'Aquisto F, Iuvone T, Rombola L, Sautebin L, Di Rosa M, Carnuccio R** 1997 Involvement of NF-κB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. FEBS Lett 418:175-8

7.   CONCLUSIONS

The information we have collected during the course of our experiments allow us to provide at least partial answers to three major questions regarding the role of OT and its receptor in the mechanisms of parturition in the rat:

1.   Are OT and its receptor important in the process of rat parturition?

The answer is yes. Based upon all current information, it is becoming clear that the process of parturition involves multiple phases controlled by multiple factors, and these factors have to be stimulated and coordinated in an appropriate fashion. In the rat, OT and its receptor are a part of these complicated components and may play a key role in regulating the process of labour. Our data clearly demonstrate that a rapid increase in uterine OTR production occurs prior to parturition at term or at the time of preterm labour induced by anti-progestin (RU486) treatment (Chapter 3 and 4). This increase in uterine OTR is also present in the preliminary data following the injection of endotoxin (Chapter 6). In addition, when OTR remained at a low level following treatment with the anti-estrogen TAM, parturition was delayed until the uterine concentration of OTR increased to levels seen at normal term labour (Chapter 3). Based on these data, OTR is definitely associated with the process of parturition, although we can not definitively conclude that there is a cause-effect relationship.

From the literature, other studies indicate a positive relationship between OT levels and uterine contractile activity in late gestation. There is increased pulsatile release of OT into nocturnal maternal plasma in late pregnancy in rhesus monkeys and humans (1-3). This increase is paralleled with an increase in the nocturnal myometrial contractility. Furthermore, in rhesus monkeys (4) and baboons (5), an OTR blocker can diminish this

210

myometrial contractility and responsiveness and delay the occurrence of parturition in rats (6).

In our studies, uterine concentrations of OT remained low even at the time of delayed parturition in the TAM-treated animals or at the time of RU486-induced preterm parturition. These data indicate that high concentrations of OT are not necessary for parturition. However, the concentration of uterine OT on day 21 of pregnancy is approximately 30-fold increased over that in the nonpregnant uterus (7). Although TAM and RU486 decrease OT concentrations in the uterus, they are still much higher than that in the non-pregnant uterus. Therefore, the uterine concentrations of OT peptide in TAM- and RU486-treated animals may provide sufficient ligand to stimulate myometrium. Conversely, since in all animal models of normal, preterm or delayed parturition, OTR concentrations increase markedly before delivery, it is possible that myometrial activity is controlled at the receptor level, rather than the ligand level. However, it also raises the intriguing possibilities that there may be another ligand for OTR or that the receptor may be activated in the absence of the ligand.

Collectively, the literature, in addition to our own results, leads us to conclude that OTR does have an important role in rat parturition although the importance of OT needs further investigation.

2.   Are OT and its receptor responsible for the initiation of parturition?

The answer to this is no. Recently, Nishimori *et al* and Young *et al* have developed a null mutation "knockout" strain of mice deficient in OT. Parturition occurred normally in these mice but the offspring did not survive due to lack of maternal nursing. From this it can be concluded that OT is required for nursing but is not essential for parturition (8, 9). Although it is possible that there are interspecies differences, other data from human and

animal experiments do not support an obligatory role for OT in the initiation of parturition.

Most human and rat data indicate that OT in the maternal circulation increases significantly only after active labour is well established. Therefore circulating OT may only be responsible for fetal expulsion but unlikely stimulate the initiation of labour. However, the recent discovery of an OT paracrine or autocrine system within the pregnant rat or human uterus may help rationalize a role for OT even in the initiation process. A much higher concentration of OT mRNA and OT in uterine tissues than that in hypothalamus supports the existence of an intrauterine paracrine or autocrine system for OT during pregnancy. Within this system, OT can directly stimulates myometrial activity, but more likely, several factors such as OT, PGs and their receptors cooperate and interact with each other to stimulate myometrial contractility, and eventually initiate labour. This concept is in keeping with our data in which mRNA encoding OTR is increased at 6 h, OTR is elevated at 12 h, $PGF_{2\alpha}$ is elevated at 24 h and RU-induced preterm delivery occurs at a mean of 27 h after injection (Chapter 4). This is also compatible with recent experiments in a PGF receptor gene "knockout" mouse that does not undergo parturition. In this model, OTR concentrations remain undetectable and the uterus is unresponsive to OT (10).

The process of parturition begins some time before the clinical signs of labour. There is no way to determine the exact moment when parturition begins. Initiation of parturition may involve multiple changes in biochemical and physiological events, including maternal and/or fetal hormone changes. In general, P_4 is responsible for uterine relaxation during pregnancy. In contrast, estrogen is believed to promote uterine contractility. P_4, acting as a gene suppressor, down-regulates a number of genes that appear to be essential for uterine contractions including OTR, Cx-43, and calcium channels, while

212

estrogen up-regulates these genes in late gestation. In late rat pregnancy, luteolysis causes the withdrawal of P_4 and precedes the initiation of parturition. Our anti-progestin RU486 experiments support the hypothesis that P_4 has an inhibitory effect on OTR and PGs, while withdrawal of P_4 can induce preterm labour in this species (Chapter 4). This is also compatible with our findings following the injection of endotoxin LPS (Chapter 6).

3.   What are the major regulators controlling synthesis of OT and its receptor in late pregnancy?

Based on the literature and the data from this thesis, it appears that sex steroids are major regulators of the intrauterine paracrine system. In particular, this thesis has provided interesting results regarding the role of sex steroids in regulation of OT and OTR.

Administrating the anti-estrogen TAM, the increased mRNA and peptide levels for OT normally seen prior to parturition is completely abolished. This is compatible with experiments from human decidual explants in which estrogen increases the OT mRNA level approximately 4-fold (11). These data strongly suggest that estrogen stimulates OT synthesis. In the TAM-treated animals, OTR mRNA and OTR remain low at the time when normal parturition usually occurs but, at the time of delayed parturition, do increase to normal parturition concentrations, suggesting that estrogen is not required for the pre-parturition increase in OTR. We have hypothesized that the increased OTR may be stimulated by PGs, even in the absence of estrogen.

When the P_4 antagonist RU486 is administered on day 15 of rat pregnancy, uterine OT mRNA and OT decrease slightly. These results are in keeping with studies in non-pregnant rats, in which estrogen and P_4 have a synergistic stimulatory effect on uterine OT gene expression (12). Our data showing that OTR mRNA and peptide are increased markedly after treatment

213

with RU486 support that P_4 plays an important role in regulation of synthesis of uterine OTR, and also confirms earlier findings (13, 14).

The effects of the sex steroids also can be regulated by affecting biosynthesis of their own receptors. Our results from anti-estrogen experiments showed that TAM decreased uterine cytosolic level of ER, suggesting that estrogen has a positive influence on uterine ER synthesis. Furthermore, recent studies indicate that differential expression of isoforms of ER and PR may be important for achievement of the function of these sex steroids. Our data demonstrate that mRNA levels for PR-A and/or PR-C increase significantly in the rat uterus just before term labour (Chapter 5). Since PR-A and PR-C have an "inhibitory" influence over PR-B, this change in isoform expression could effectively result in a P_4 withdrawal that may then influence myometrial contractility. Though the animals treated with TAM or RU486 do not show a change in uterine cytosol total PR, TAM decreases and RU486 increases the mRNA encoding PR-A and/or PR-C (Chapter 5), strongly suggesting that estrogen and P_4 can regulate uterine PR functional level by controlling the synthesis of their own isoforms. However, further studies are necessary to explore the protein levels of these isoforms for an understanding of the mode of action of these isoforms in the uterine paracrine system.

Accumulating evidence indicates that the inflammatory mediators are involved in synthesis of intrauterine contraction associated proteins including OTR. We have started to explore this unfamiliar field by injecting the bacterial endotoxin, LPS into pregnant rats, and by administering one of the pro-inflammatory cytokines, IL-6 to rat uterine explants. Both experiments show a marked increase in uterine OTR gene expression, supporting our hypothesis that inflammatory mediators, such as endotoxin or IL-6 play a positive role in regulation of uterine OTR (Chapter 6). However, further studies are needed to clarify the mechanisms of these cytokines in this intrauterine paracrine system.

214

As noted previously, several studies demonstrate a positive interaction between OTR and PGs in intrauterine tissues (15, 16). The inflammatory cytokines also stimulate PG production (17, 18) and it is possible that there is an important synergistic network involving cytokines and PGs that regulate the synthesis of intrauterine OTR. We speculate that when OTR concentrations reach a threshold value in this paracrine system, parturition occurs (Fig 7-1).

In summary, estrogen and P_4 are the major regulators of intrauterine OT and its receptor. Estrogen appears to upregulate both, whereas P_4 appears to have a positive effect on OT but a strongly negative effect on OTR. Estrogen and P_4 also regulate their own receptor synthesis within this paracrine system. Estrogen increases its own receptor and also increases PR-A and/or PR-C mRNA level, whereas P_4 inhibits PR-A and/or PR-C mRNA in rat uterine tissues.

Finally, the work in this thesis ha helped address another question of importance to researchers in this field.

Is the rat model suitable for human parturition studies?

Though rat and human are two completely different species, there are still many physiological similarities. In the human, estrogen and P_4 are produced predominantly by the corpus luteum during the first 6 - 8 weeks of gestation after which the placenta is the major producer of steroids. In contrast, in rats these two hormones are produced by the corpus luteum throughout pregnancy. However, we are interested particularly in intrauterine paracrine or autocrine systems, which affect OT and its receptor gene expression and influence parturition onset. Intrauterine hormone changes appear to be similar in both species prior to labour onset: a local increase in E_2/P_4 ratio caused by luteolysis in rats and by local synthetic changes in humans. Tissue contributions and concentrations of mRNA and peptide for

215

OT and OTR also are similar in rats and in humans in late pregnancy. OT is mainly synthesized in the decidua in the human (19) and endometrial layer in the rat (7). OTR is produced by decidua and endometrium, and myometrium in both rats and humans. These data suggest that there is a paracrine or autocrine network in both human and rat intrauterine tissues. Additionally, there are several other advantages to using the rat model rather than large animals to study the intrauterine paracrine system. These include a short gestational age, relative low cost and ease of manipulation. Therefore, the rat appears to be a good model for studying the role of the intrauterine paracrine system in the mechanism of parturition, although more experiments are required in both species to completely validate this.

Figure 7-1   Schematic diagram of the hypothesized paracrine network of

interaction between sex steroids, OT, PGs and their receptors with rat or

human intrauterine tissues. When a withdrawal of P_4 in rats or a local increase

in E_2/P_4 ratio in humans occurs in the late pregnant uterus, ER and PR-A

and/or PR-C synthesis are increased to enhance the estrogen stimulating action

and to diminish the P_4 inhibitory action. The changes in estrogen and P_4 action

lead to increases in both OTR and PG levels. The positive interactions between

OTR and PGs amplify this effect. Additionally, inflammatory cytokines,

including IL-6, may stimulate synthesis of OTR and PG in this paracrine

network. When OTR and PG concentrations reach a threshold value, the

parturition occurs. Effects of antiestrogen TAM (green), antiprogestin RU486

(red) and endotoxin LPS (blue) on the paracrine pathway are indicated.
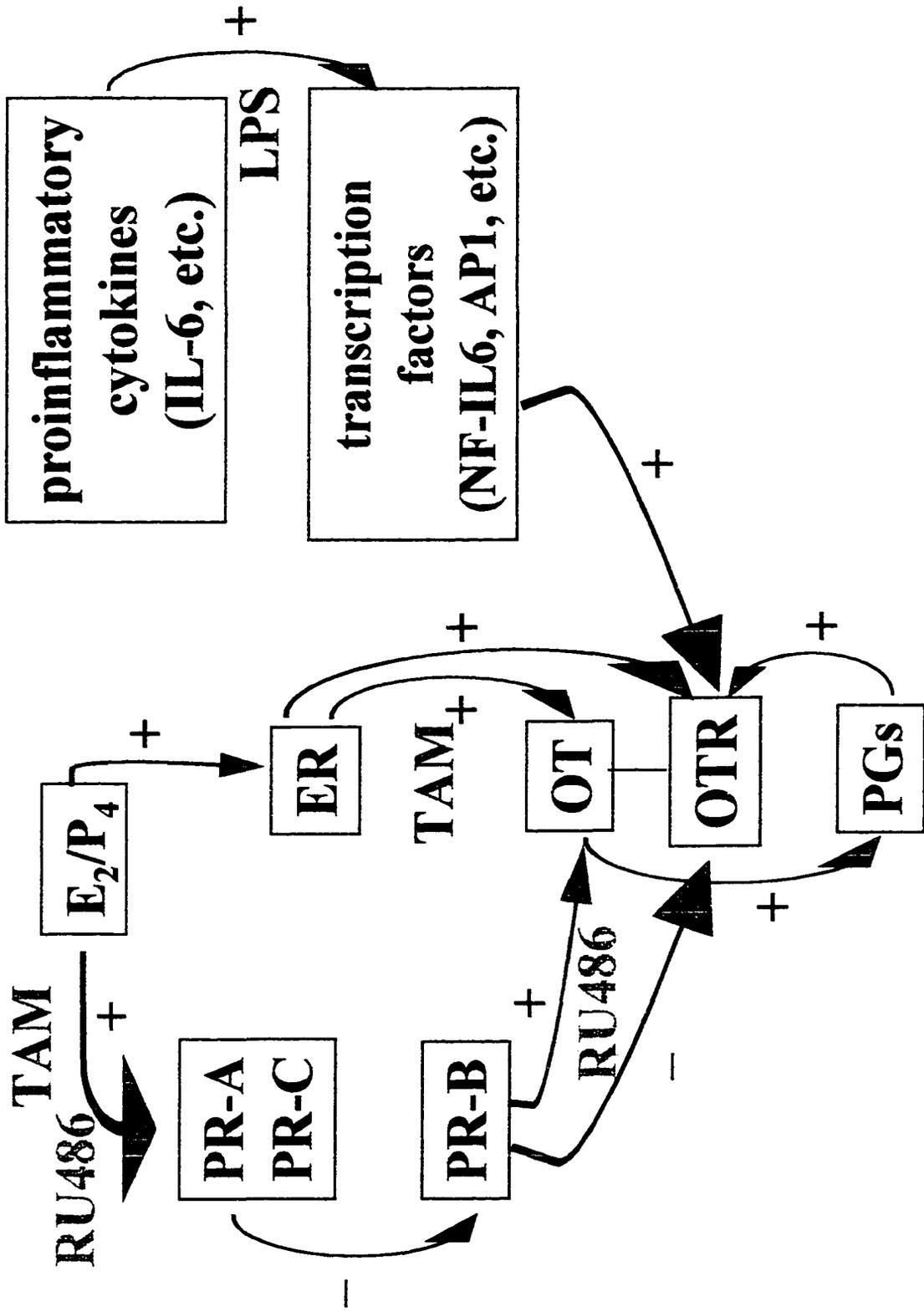
7.1. REFERENCES

1. **Honnebier MBOM, Figueroa JP, Rivier J, Vale W, Nathanielsz P** 1989 Studies on the role of oxytocin in late pregnancy in the pregnant rhesus monkey: plasma concentrations of oxytocin in the maternal circulation throughout the 24-h day and the effect of synthetic oxytocin antagonist [1-beta-Mpa(beta-$(CH_2)_5)_D$,(Me(Tyr^2, Orn^8))] oxytocin on spontaneous nocturnal myometrial contractions. J Dev Physiol 12:225-32

2. **Seron-Ferre M, Taylor NF, Martin MC, Leake RD** 1991 Development of a circadian variation of plasma oxytocin concentration in the late gestation rhesus monkey. J Clin Endocrinol Metab 72:1323-7

3. **Fuchs AR, Behrens O, Liu HC** 1992 Correlation of nocturnal increase in plasma oxytocin with a decrease in plasma oestradiol/progesterone ratio in late pregnancy. Am J Obstet Gynecol 167:1559-63

4. **Giussani DA, Mecenas CA, Jenkins SL, Winter JA, Derks JB, Nathanielsz PW** 1994 The oxytocin antogonist Atosiban abolishes androsteredione (Δ^4-A)-induced, premature myometrial contractions in chronically-instrumented rhesus monkey at 0-8 of gestation. J Physiol 483:113p

5. **Wilson L Jr, Pak SC, Santolava J, Parsons M** 1994 Induction of estradiol synthesis and nocturnal uterine contractions by dehydroepiandrosterone and their inhibition by an aromatase inhibitor. Proceedings of the 41st Annual Meeting of the Society for Gynecologic Investigation, Chicago. IL, Abstract #14.

6. **Antonijevic IA, Douglas AJ, Dye S, Bicknell RJ, Leng G, Russell JA** 1995 Oxytocin antagonists delay the initiation of parturition and prolong its active phase in rats. J Endocrinol 145:97-103

7.   **Lefebvre DL, Giaid A, Bennett H, Lariviere R, Zingg HH** 1992
        Oxytocin gene expression in rat uterus. Science 256:1553-5

8.   **Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR** 1996 Oxytocin
        is required for nursing but is not essential for parturition or reproductive
        behavior. Proc Natl Acad Sci U S A 93:11699-704

9.   **Young WS, 3rd, Shepard E, Amico J, Hennighausen L, Wagner
        KU, LaMarca ME, McKinney C, Ginns EI** 1996 Deficiency in
        mouse oxytocin prevents milk ejection, but not fertility or parturition. J
        Neuroendocrinol 8:847-53

10.   **Sugimoto Y, Yamasaki A, Segi E, Tsuboi K, Aze Y, Hishimura T,
        Oida H, Yoshida N, Tanaka T, Katsuyama M, Hasumoto K,
        Murata T, Hirata M, Ushikubi F, Negishi M, Ichkawa A, Narumiya
        S** 1997 Failure in parturition in mice lacking the postaglandin F
        receptor. Science 227:681-3

11.   **Chibbar R, Wong S, Miller FD, Mitchell BF** 1995 Estrogen
        stimulates oxytocin gene expression in human chorio-decidua. J Clin
        Endocrinol Metab 80:567-72

12.   **Lefebvre DL, Farookhi R, Giaid A, Neculcea J, Zingg HH** 1994
        Uterine oxytocin gene expression. II. Induction by exogenous steroid
        administration. Endocrinology 134:2562-6

13.   **Fuchs AR, Periyasamy S, Alexandrova M, Soloff MS** 1983
        Correlation between oxytocin receptor concentration and
        responsiveness to oxytocin in pregnant rat myometrium: effects of
        ovarian steroids. Endocrinology 113:742-9

14.   **Alexandrova M, Soloff MS** 1980 Oxytocin receptors and parturition.
        I. Control of oxytocin receptor concentration in the rat myometrium at
        term. Endocrinology 106:730-5

15. **Flint APF, Sheldrick EC** 1982 Ovarian secretion of oxytocin is stimulated by prostaglandin. Nature 297:587-8

16. **Wilson T, Liggins GC, Whittaker DJ** 1988 Oxytocin stimulates the release of arachidonic acid and prostaglandin F_{2a} from human decidual cells. Prostaglandins 35:771-80

17. **Yamamoto K, Arakawa T, Taketani Y, Takahashi Y, Hayashi Y, Ueda N, Yamamoto S, Kumegawa M** 1997 TNFα-dependent induction of cycloocygenase-2 mediated by NF-κB and NF-IL6. Adv Exp Med Biol 407:185-9

18. **Raz A, Wyche A, Siegel N, Needleman P** 1988 Regulation of fibroblast cyclooxyenase synthesis by interleukin-1. J Biol Chem 263:3022-8

19. **Mitchell BF, Chibbar R** 1995 Synthesis and metabolism of oxytocin in late gestation in human decidua. Adv Exp Med Biol 395:365-80