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Effect of Progesterone on the release of Prostaglandin F2 alpha from uterine endometrial epithelial cells in ruminants

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Résumé

La progestérone (P4) et l'oxytocine (OT) stimulent la sécrétion de la prostaglandine $F_2\alpha$ ($PGF_2\alpha$) des cellules épithéliales de l'endomètre et il y a une réponse différentielle de ces cellules à OT pendant le cycle oestral des ruminants. Pour déterminer comment P4 influence cette réponse chez la vache, nous avons utilisé des utérus de bovin prélèvés au début du cycle (jour 1-3) afin d'effectuer une culture de cellules épithéliales de l'endomètre.

La chromatographie liquide sous haute pression et la spectrophotométrie de masse (HPLC) ont montré que les cellules en culture ont métabolisé P4 et que les métabolites étaient soit 5 α- soit 5 β- prégnanédione. Pour déterminer si ces métabolites peuvent modifier la synthèse de $PGF_2\alpha$, les cellules ont été incubé avec P4, 5 α- ou 5 β- prégnanédione. Les résultats ont prouvé que seule P4 et non l'OT causent une augmentation significative de la synthèse de $PGF_2\alpha$. Les analyses de liaison de l'OT à son récepteur n'ont montré aucun effet significatif sur la concentration en récepteur d'oxytocine (OTR). L'augmentation de la synthèse de $PGF_2\alpha$ au niveau des cellules traitées avec la P4 est probablement due à une augmentation des enzymes impliquées dans la synthèse de $PGF_2\alpha$. Comme RU 486, un anti-progestérone, diminue la synthèse de $PGF_2\alpha$ et la concentration en OTR, nous avons traité nos cellules avec RU 486. L'anti-progestérone a augmenté significativement la synthèse de $PGF_2\alpha$ ($P<0.05$) et l'effet maximal était à une concentration de 1μM tandis que la concentration des OTR était diminuée. Afin de comparer l'effet de RU 486 sur la synthèse des PG et sur la concentration des OTR avec celui d'un antagoniste pur, des cellules ont été traité avec ZK 137316 et des résultats semblables ont été obtenus. D'autres expériences ont montré que l'AMPc et le 5α-dihydrotestosterone (DHT) stimulent la sécrétion de $PGF_2\alpha$ par les cellules épithéliales de l'endomètre de bovin via différentes voies. Ainsi, OT n'est pas le seul stimulant de la synthèse de $PGF_2\alpha$ dans les cellules épithéliales de l'endomètre de bovin. Nous concluons qu'il existe d'autres facteurs capables de modifier la capacité de l'OT à augmenter la synthèse de $PGF_2\alpha$. Le traitement des cellules avec de

l'indométhacine, un inhibiteur non sélectif de COX, induit une diminution de la synthése de $PGF_2\alpha$ ainsi que de la concentration des OTR. Nous suggérons que l'effet paracrine de $PGF_2\alpha$ sur les cellules épithéliales de l'endométre serait de moduler le nombre d'OTR.

Mots clés :

Bovin, utérus, cellules épithéliales de l'endomètre, progestérone, Mifepristone (RU 486), prostaglandine, androgène.

Summary:

Progesterone (P4) and oxytocin (OT) stimulate secretion of prostaglandin $F_2\alpha$ ($PGF_2\alpha$) from the endometrial epithelial cells but there is differential responsiveness of these cells to OT during the estrus cycle in ruminants. To determine how P4 influences this responsiveness in the cow, we used bovine uteri collected early in the cycle (days1-3) for the culture of endometrial epithelial cells. HPLC and mass spectrophotometry showed that these cells metabolized P4 and that the metabolites were either 5α- or 5β-pregnanedione. To determine if these metabolites of P4 were able to modify $PGF_2\alpha$ synthesis, cells were incubated with P4, 5α- or 5β-pregnanedione. Results showed that only P4 caused significant increase in $PGF_2\alpha$ synthesis but not OT stimulation of $PGF_2\alpha$ synthesis. OT binding assays showed no significant effect on oxytocin receptor (OTR) concentration. The increase in $PGF_2\alpha$ synthesis in P4 treated cells is probably due to an increase in the enzymes involved in PG synthesis. Since RU 486, an antiprogesterone, has been shown to decrease $PGF_2\alpha$ synthesis as well as OTR concentration, we treated our cells with RU 486. This antiprogesterone increased $PGF_2\alpha$ synthesis significantly ($P<0.05$) and the maximal effect was at a concentration of 1μM while OTR was down regulated. In order to compare the effect of RU 486 on PG synthesis and OTR concentration with that of a pure antagonist, cells were treated with ZK 137316 and the similar results were obtained. Further experiments revealed that cAMP and 5α-dihydrotestosterone (DHT) stimulated $PGF_2\alpha$ secretion from the bovine endometrial epithelial cells via different pathways. Thus, OT is not the only stimulant of $PGF_2\alpha$ synthesis in the bovine endometrial epithelial cells. We conclude that there are other factors that are able to modify the ability of OT to increase synthesis of $PGF_2\alpha$. The treatment of the cells with indomethacin, a non selective COX-inhibitor, showed that the inhibitory effect of indomethacin on COX enzymes leads to decreased synthesis of $PGF_2\alpha$ and a decrease in OTR. We postulate that a paracrine effect of $PGF_2\alpha$ on the endometrial epithelial cells may be able to modulate OTR number.

Keywords:

Bovine, uterus, endometrial epithelial cells, progesterone, Mifepristone (RU 486), prostaglandin, androgen.

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List of abbreviations

5β-DHP	5β-dihydroxyprogesterone
AA	arachidonic acid
ACAT	acyl-CoA cholesterol acetyltransferase
AMP	adenosine monophosphate
AP	antiprogesterone
AR	androgen receptor
ARC	cytosol androgen receptor
AVP	arginine vasopressin
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CREP	cAMP-response element binding protein
CE	cholesteryl ester
CL	corpus luteum
CoA	co-activators
COX	cyclooxygenase
COX 1	cyclooxygenase 1
COX 2	cyclooxygenase 2
$cPLA_2$	cytosolic PLA_2
DAG	diacylglycerol
dbcAMP	dibutyryl cyclic adenosine monophosphate
DHP	dihydroprogesterone
DHT	5α-dihydrotestosterone
E_2	estradiol-17β
EL	endometrial lumenal
enJSRVs	endogenous Jaagsiekte sheep retroviruses

ER	estradiol recetor
ERC	cytosol estrogen receptor
ERα	estrogen receptor alpha
ERE	estrogen response element
FBS-DC	fetal bovine serum dextran-charcoal extraction
FCS	fetal calf serum
FGF-7	fibroblast growth factor- 7
FGF-10	fibroblast growth factor- 10
FP receptor	$PGF_2\alpha$ receptor
GABA	γ-Amino butyric acid
GC/MS	gas chromatography/mass spectrometry
GE	glandular epithelia
GPCR	G-protein-coupled receptor superfamily
GR	glucocorticoid receptors
HBSS	Hank's balanced salt solution
HGF	hepatocyte growth factor
HMG-CoA reductase	3-hydroxy-3-methylglutaryl (HMG)-CoA reductase
HPLC	high pressure liquid chromatography
HSD	hydroxysteroid dehydrogenase
Hsp90	heat shock protein 90
IFN-τ	interferon-tau
IMP	intra myometrial pressure
iNOS	nitric oxide synthase
IP3	inositol (1, 4, 5)-triphosphate
LBD	ligand-binding domain
LDL	low-density lipoprotein

LH	lutinizing Hormone
LVA10	vasopressin -receptor-specific ligand
MAPK	mitogen-activated protein kinase
MUC-1	mucin glycoprotein one
NA	noradrenaline
NBCS	new born calf serum
NLS	Nuclear localization sequences
OT	oxytocin
OTR	oxytocin receptor
P_4	progesterone
PG	prostaglandin
PGE_2	prostaglandin E_2
PGES	prostaglandin E synthase
$PGF_2\alpha$	prostaglandin F_2 alpha
PGFM	15-keto-13, 14-dihydroprostaglandin $F_2\alpha$ metabolite
PGFS	prostaglandin F synthase
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGHS	prostaglandin G/H synthase
PIP_2	phosphatidylinositol-4, 5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PR	progesterone receptor
PRC	cytosol progesterone receptor
PREs	progesterone-responsive elements
PXR	pregnane X receptor

RBAs	relative binding affinities
RU 486	Mifepristone
SH	steroid hormones
SPRMs	selective progesterone receptor modulators
SR	steroid hormone receptor
TM	transcription machinery
TNF-α	tumor Necrosis Factor-α
V1a	vasopressin 1a
V1aR	vasopressin 1a receptor
VEGF	vascular endothelial growth factor

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Introduction

Initially, because of the emphasis placed on the importance of pituitary support for the maintenance of the corpus luteum, it was considered that withdrawal of pituitary luteotrophins such as lutinizing hormone (LH) and/or prolactin might cause the cyclic regression of the corpus luteum. However, with the advent of more sophisticated methods for measuring circulating pituitary gonadotropins, it became apparent that, at the time of corpus luteum regression, measurable levels of these hormones were present in ruminants (1-2).

Loeb, who first demonstrated that hysterectomy in the cyclic guinea pig abolished cycles and caused abnormal persistence of the corpora lutea, reported the importance of the uterus in the control of corpus luteum regression (3). In the 1970s researchers discovered that degradation of the corpus luteum results from a luteolysin secreted by the uterus. Soon afterwards it was confirmed that the luteolysin is prostaglandin (PG) $F_2\alpha$ secreted from endometrial epithelium (4). Similar effects were subsequently observed in the cyclic sheep, cow, pig, and mare and in the pseudopregnant hamster, rabbit, and rat (5).

Prostaglandins are important regulators of reproductive events including ovulation, implantation, parturition, luteolysis and recognition of pregnancy (6-7). In ruminants the role of uterine prostaglandin $F_2\alpha$ ($PGF_2\alpha$) in luteolysis or the regression of the corpus luteum (CL) during late diestrus is well established (8). This role of $PGF_2\alpha$ as a uterine luteolytic hormone was supported by the finding that systemic administration of indomethacin, an inhibitor of PG synthesis (9), or the intrauterine administration of indomethacin (10) delayed or prevented luteal regression in several

species. Also, immunization against $PGF_2\alpha$, either passively (11-12) or actively (13-14), delayed regression of the corpus luteum in the sheep.

Luteolysis is characterized by an initial decline of progesterone secretion that is commonly designated as functional luteolysis as distinct from structural or morphological luteolysis which, as the name suggests, signifies the subsequent change in the cellular structure of the gland and its gradual involution in the ovary to form a small scar composed of connective tissue known as corpus albicans which often persists in the ovary for several weeks (15).

Release of $PGF_2\alpha$ from the uterus in a pulsatile fashion on days 17–18 of the estrous cycle is essential to induce regression of the CL in ruminants (16-17). It has been clearly shown that $PGF_2\alpha$, the luteolytic hormone that is released cyclically from the uterus at the time of corpus luteum regression, is elevated as a series of pulses in uterine venous blood during luteolysis in cow (18). Also, it has been demonstrated that levels of PGFM in the peripheral blood of cows show pulsatile increases with a pattern of several series of pulses of short duration for 2-3 days during and after luteolysis (19), thus supporting the role of $PGF_2\alpha$ as a luteolytic hormone (figure 1).

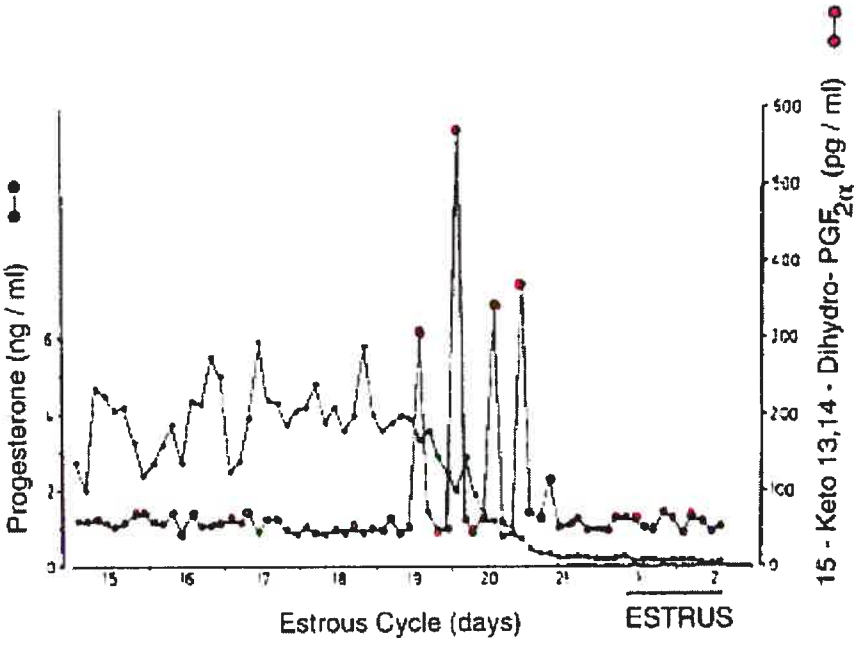

Figure 1: Concentration of 15-keto-13, 14-dihydroprostaglandin $F_2\alpha$ metabolite (PGFM) in the peripheral plasma of a cyclic cow during luteolysis. Pulses of PGFM representing uterine secretion of $PGF_2\alpha$ coincide with the decline of progesterone during luteolysis.

Taken from: Kindahl et al. (1984) Levels of prostaglandin $F_2\alpha$ metabolites in blood and urine during early pregnancy. *Anim. Reprod. Sci;* 7: 133-48.

When $PGF_2\alpha$ is administered in a pulsatile fashion (20), the CL appears to be particularly sensitive to the luteolytic effects of $PGF_2\alpha$. Although continuous exposure of target cells to a ligand would down-regulate responses (desensitization), the pulsatile exposure of cells to the ligand may prevent desensitization and, in consequence, enhance or maintain cellular responses (21).

It is generally accepted that $PGF_2\alpha$ is secreted primarily from the luminal epithelium of the endometrium (22). In addition to uterus-derived $PGF_2\alpha$, the functional CL of the cow produces and secretes at least three kinds of PGs, such as $PGF_2\alpha$, PGE_2, and 6-keto-$PGF_1\alpha$, the stable inactive metabolite of prostacyclin (PGI_2)(23, 24, 25, 26).

In the bovine endometrium, epithelial and stromal cells have specific morphological and functional properties. Epithelial cells preferentially produce $PGF_2\alpha$, whereas stromal cells produce mainly PGE_2 (27, 28, 29, 30). In bovine endometrial cells in primary culture, $PGF_2\alpha$ content is increased after stimulation with oxytocin in epithelial cells but not in stromal cells (31, 32).

In contrast, exposure to interferon-tau (IFN-τ), the pregnancy recognition signal produced by the embryo in ruminants, increases PGE_2 production by both epithelial and stromal cells when used at high physiological concentrations (28).

The first step involved in prostaglandin formation is the hydrolytic release of arachidonic acid mediated by members of the phospholipase A_2 family of enzymes (figure 2).

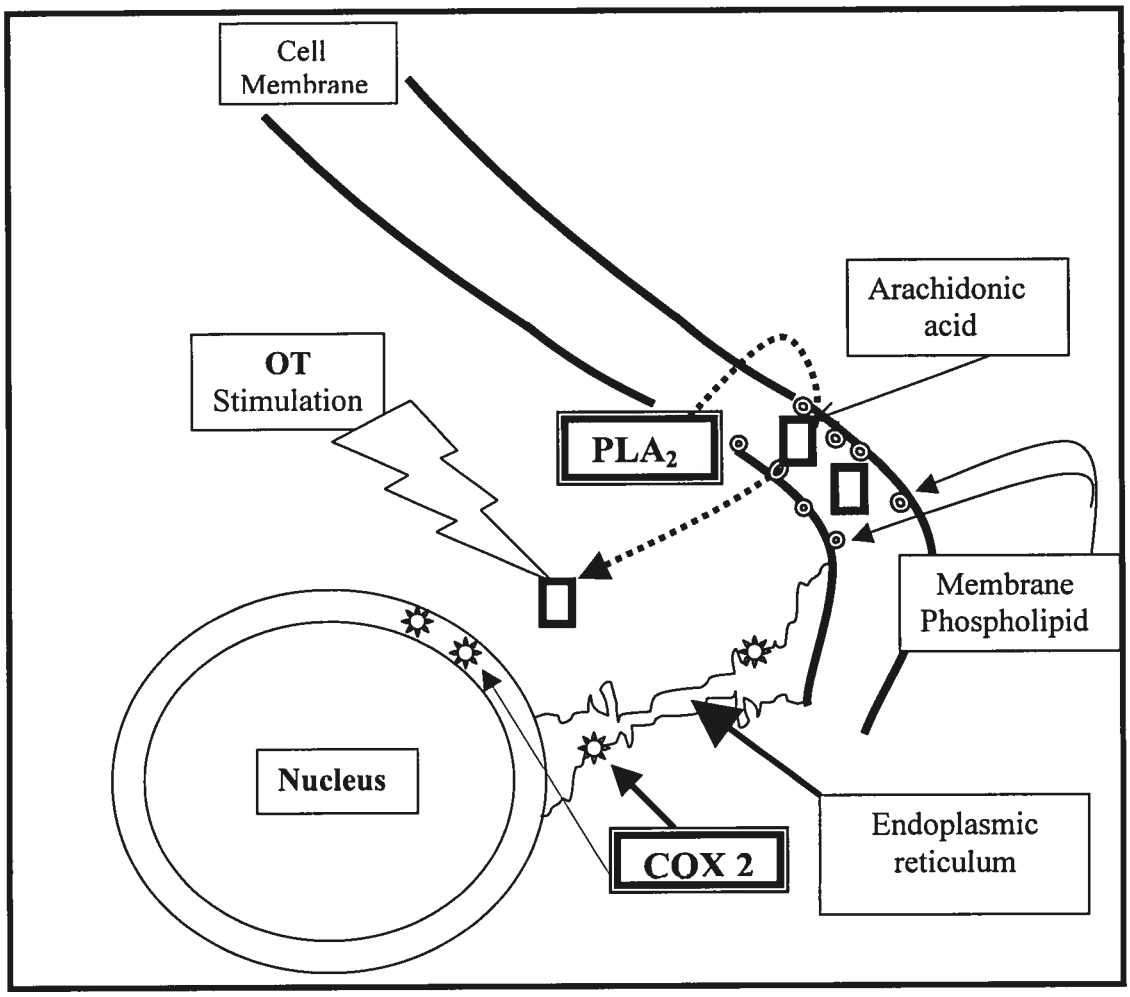

Figure 2: Location of the enzymes involved in the synthesis of prostaglandin $F_2\alpha$ in the cells.

Following its release, arachidonate is converted to PGH_2 by the action of prostaglandin G/H synthase (PGHS), which is situated on the luminal surface of the endoplasmic reticulum and the outer envelope of the nuclear membrane (33). Numerous studies have demonstrated the existence of two distinct genes encoding two isoforms of PGHS, named PGHS-1 and PGHS-2. Both enzymes possess a PGG_2-synthetic cyclooxygenase activity that has resulted in their being colloquially referred to as cyclooxygenase-1 and -2 (or COX-1 and -2), but they are also responsible for the rapid conversion of PGG_2 to PGH_2 via a peroxidase activity (34). Under basal

conditions, $PGF_2\alpha$, the principal prostaglandin produced by epithelial cells (29, 31), would be produced through constitutively expressed COX-1. An increase in COX-2 in response to stimulators of prostaglandin production (29) supports its identification as an inducible enzyme. This is further supported by the observation that expression of COX-2 protein in response to oxytocin or arachidonic acid treatment closely matched the production of prostaglandins (35).

Sensitivity to oxytocin (OT) in a variety of target tissues is widely regarded to be controlled through changes in OT receptor (OTR) population density resulting from corresponding regulation of OTR gene transcription (16, 36). The ontogeny of endometrial expression of OTR in sheep and cattle is associated with development of uterine responsiveness to OT, resulting in OT-induced secretion of uterine $PGF_2\alpha$. (37, 38), which is critical for promoting corpus luteum regression in ewes (20, 39) and which leads to further follicular development, estrous behavior, ovulation, and the opportunity for mating and conception to occur. If mating and conception occur, then the initiation of endometrial OTR expression is blocked during early pregnancy (16, 40, 41); sensitivity to OT is abrogated, luteolysis is prevented, and pregnancy is established. Endometrial sensitivity to OT is markedly reduced during early pregnancy in sheep (42, 43) and cattle (41, 44). In pregnant ruminants, this reduced sensitivity is controlled through inhibition of OTR expression (16, 40, 41). Although there are reports, especially in the cow, that OT might not be involved in luteolysis (45), there is no conclusive evidence for other factors being involved in the regulation of pulsatile secretion of $PGF_2\alpha$. Thus, the acquisition of responsiveness to OT by the endometrial epithelium determines when endogenous secretion of $PGF_2\alpha$ will occur during the estrous cycle, and this appears to require the coordinated action of P_4 and E_2. In ruminants, three hormones—P_4, oxytocin, and estradiol (E_2)—may play major roles in regulating uterine secretion of $PGF_2\alpha$ (46). Progesterone administered early in the estrous cycle, before the normal rise in plasma P_4, results in premature luteolysis (47), whereas delaying the action of P_4 on the endometrium by the use of an antagonist delays luteolysis. Thus, the length of time the endometrium is exposed to P_4 determines the length of the luteal phase (48).

Oxytocin is an acute stimulus for $PGF_2\alpha$ secretion. It is secreted from the pituitary and the CL acting via the OTR to stimulate the pulsatile release of $PGF_2\alpha$ from the luminal epithelium of the endometrium (15, 49).

P_4 and E_2 regulate uterine secretion of $PGF_2\alpha$, in part, by controlling both the timing and the magnitude of uterine secretory responsiveness to oxytocin (50). Lafrance and Goff showed that under the influence of P_4, estradiol enhances the OT-induced release of $PGF_2\alpha$ and suggest a possible synergistic action of these hormones for the induction of luteolysis in heifers (51).

It is widely accepted that P_4 downregulates OTR during the mid luteal phase (52-53) and that upregulation of the OTR is initiated by an increase in ER (54). However, studies in the cow have shown that there is an increase in OTR before an increase in ER is observed (55). This, together with the fact that prolonged treatment of ovariectomised ruminants with P_4 alone is able to induce the response to OT (51), suggests that the initial increase in OTR is not brought about by changes in E_2 or ER. It is therefore possible that changes in the action of P_4 results in the initial upregulation of OTR and that this is further increased when P_4 decreases and ER increases.

Prostaglandin Synthesis

In general, there are two large subgroups within the phospholipase family. The first is a group of small (approximately 14 kd), extensively disulfide cross-linked, secreted enzymes sharing a high degree of homology (56). The second subgroup of PLA_2 enzymes is best characterized by the type IV cytosolic PLA_2 ($cPLA_2$), an 85-kd enzyme without homology with other PLA_2 enzymes (57). The nuclear envelope and endoplasmic reticulum are the primary sites for arachidonic acid (AA) metabolism initiated by $cPLA_2$ in activated cells. These are also the primary subcellular locations for the COX enzymes, 5-lipoxygenase that catalyzes conversion of arachidonic acid

to leukotrienes (58) and some of the terminal synthases like PGE synthase (PGES) and PGF synthase (PGFS). The downstream enzymes, PGES and PGFS, catalyze the conversion of PGH_2 to PGE_2 and $PGF_2\alpha$, respectively (59). Many studies indicate that PGFS and PGES are highly expressed in endometrium during mid- and late luteal phases of the bovine estrous cycle (59, 60, 61, 62, 63).

Following its release, arachidonic acid is converted to PGH_2 by the action of prostaglandin G/H synthase (PGHS), also known as cyclooxygenase (COX), which is situated on the luminal surface of the endoplasmic reticulum and the inner and outer membranes of the nuclear envelope (33).

COX proteins are rate-limiting enzymes for the conversion of arachidonic acid into PGH_2, the common precursor of all prostaglandins. There are two isoforms of cyclooxygenase: COX-1 and COX-2 (34). Numerous studies have demonstrated that COX-1 and COX-2 are encoded by two distinct genes (64). Both enzymes possess a PGG2-synthetic cyclooxygenase activity and a peroxidase activity that converts PGG2 to PGH2. Despite catalytic and structural similarities, COX-1 and -2 differ in most other respects, including gene structure and regulation and mRNA stability (7, 34). After biosynthesis of PGH2, this endoperoxide is converted to one of several possible prostanoids by a terminal synthase. Prostaglandin $F_2\alpha$ is synthesized via three pathways from PGD2, PGE_2, or PGH2 by PGD 11-ketoreductase, PGE 9-ketoreductase, or PGH 9-, 11-endoperoxide reductase, respectively (65).

The constitutive enzyme, COX-1, is expressed in most nucleated cells. On the other hand, the inducible COX-2 is present only after induction by a variety of factors such as cytokines and tumor promoters (29) and plays a role in various pathological and some physiological conditions. The same authors proposed that the two different enzymes could be associated with distinct pools of arachidonic acid and different downstream enzymes (34, 66).

Arosh and his colleagues demonstrated that during the bovine estrous cycle, COX-1 is not expressed either at the mRNA or at the protein level, but that COX-2 is expressed throughout the cycle with maximal expression between day 16 and day 18

in the bovine endometrial cells (59). Asselin et al. reported an increase in expression of COX-2 mRNA and $PGF_2\alpha$ production after stimulation with oxytocin (29).

In a study conducted by Parent et al. on primary bovine epithelial cells, expression of COX-1 and COX-2 proteins was measured by western blot analysis after treatment of epithelial cells with optimal concentrations of oxytocin or arachidonic acid, both of which are stimulators of prostaglandin production. Under non-stimulated conditions, expression of COX-1 protein by epithelial cells was high, whereas expression of COX-2 was low. Stimulation of the same cells with oxytocin or arachidonic acid at concentrations known to stimulate prostaglandin production increased the amounts of COX-2 but did not affect COX-1 (35).

Oxytocin

All neurohypophysial hormones including OT are nanopeptides with a disulfide bridge between Cys residues 1 and 6. This results in a peptide constituted of a 6-amino acid cyclic part and a COOH-terminal α-amidated three-residue tail. Based on the amino acid at position 8, these peptides are classified into vasopressin and OT; OT contains a neutral amino acid at this position and the vasopressin contains a basic amino acid (lysine, arginine) at this position. Isoleucin in position 3 is essential for stimulating OTRs and Arg. or Lys. in position 8 for acting on vasopressin receptors. The difference in the polarity of these amino acids residues is believed to enable the vasopressin and OT peptides to contact with the respective receptors (67).

The actual source of neurohypophysial hormones including OT is the nerve cells in the supraoptic and paraventricular nuclei of the hypothalamus. From here the hormones are carried to the neurohypophysis by way of the axoplasm in the nerve fibers that pass from the hypothalamus to the neurohypophysis of the pituitary, where they are stored until released (68).

The finding that prostaglandin causes a release of oxytocin from the posterior

pituitary (69) has been supported by the report of Schams et al. in the cow showing that the administration of several $PGF_2\alpha$ analogs caused a large elevation of oxytocin in jugular venous blood that reached a peak within 15–20 min. (70).

Regulation of secretory function of hormone secreting neurons is via feedback mechanisms of hormones from the anterior hypophysis, adrenal cortex, thyroid and gonads or by neural pathways in the hypothalamus (71). However, OT is also synthesized in peripheral tissues, e.g., uterus, placenta (72), corpus luteum (73, 74), testis (75) and heart (76).

It is well known that the bovine CL is a site of P_4, prostaglandin and OT production (74). In several species, the ovary has been shown to contain OT and may be a site of local OT production (77). Functional OTRs have been detected in bovine granulosa cells, suggesting that OT may be an autocrine factor during follicular growth (78).

The sensitivity of bovine endometrium to oxytocin varies during the estrous cycle. The stimulatory effects of oxytocin on $PGF_2\alpha$ secretion by the bovine endometrium were observed at the follicular phase, estrus, and early luteal stages of the estrous cycle (days 1-4), whereas oxytocin had no effect during the mid-to-late luteal stages (days 5-17) (79). These results confirmed the previous reports that the high responsiveness of the endometrium to oxytocin was maintained during the period of luteal regression until the early luteal stage of the next estrous cycle (40, 80, 81). However, there is increasing evidence that oxytocin is not essential for the initiation of $PGF_2\alpha$ output during luteolysis in the cow (82, 83, 84). In fact, concentrations of oxytocin in blood (85), and in intact and microdialyzed CL (86) are extremely low at the time of spontaneous luteolysis. Moreover, the blockade of uterine oxytocin receptors with a specific oxytocin antagonist from Days 15 to 22 of the cycle affected neither luteolysis nor the duration of the estrous cycle in heifers (83). Therefore, $PGF_2\alpha$ secretion by the endometrium may be regulated not only by oxytocin but also by one or more other factors in cattle. Moreover, since the blockade of oxytocin receptors decreased the magnitude of $PGF_2\alpha$ release without preventing an increase of PGFM in blood (84), oxytocin may play a supportive and modulatory role as a regulator of the amplitude of pulsatile $PGF_2\alpha$ secretion after the initiation of luteolysis in cattle (87).

Wathes et al. showed that OT concentration in the CL of bovine ovary increased from stage 1 (early luteal phase days 1-4) to stage 2 (mid-luteal phase days 5-10) but declined during stage 3 (late luteal phase days 11-17) and were low in follicles, whole ovaries and pregnancy corpora lutea (88). Binding of OT to its specific receptors in the uterus activates phospholipase C and release triphosphoinositol (IP3) and diacyloglycerol (DAG). The DAG stimulates protein kinase C (PKC) which might lead to several actions to increase contractility. The IP_3 stimulates Ca^{++} release from intracellular stores. This intracellular mobilization of Ca^{++} is followed by increases in $PGF_2\alpha$ secretion (89, 90). The mechanism of action of oxytocin in myometrium is illustrated in figure 3:

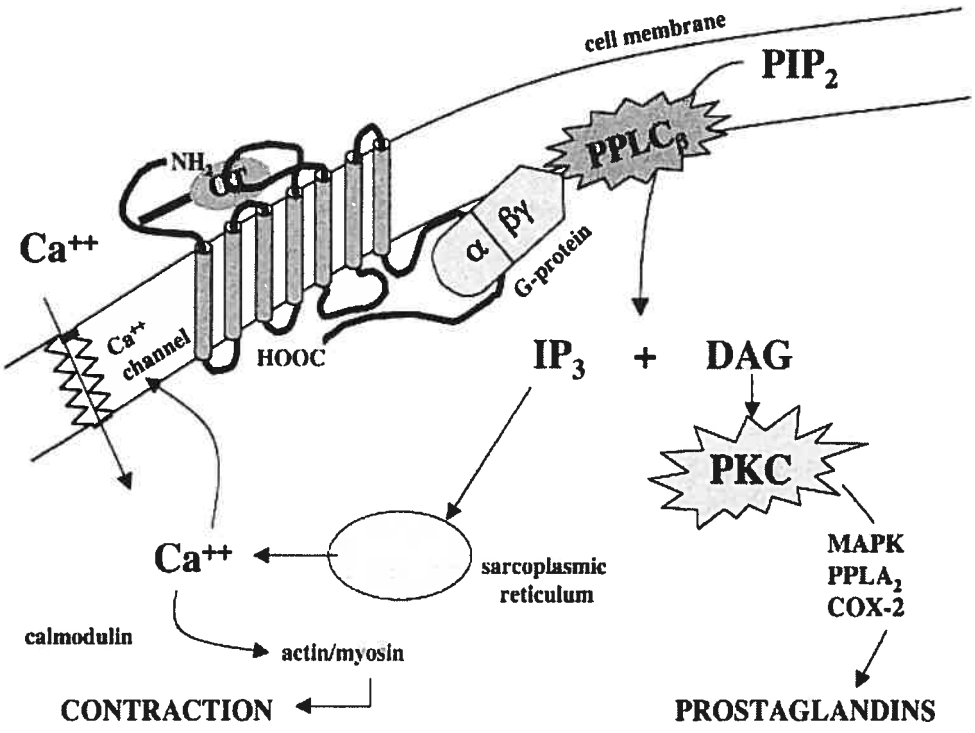

Figure 3: Mechanism of action of oxytocin (OT). Interaction of OT with its specific membrane receptor triggers G-protein ($\alpha/\beta\gamma$) mediated activation of phospholipase C (PPLC) resulting in production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). The DAG stimulates protein kinase C (PKC) which might lead to several actions to increase contractility. The IP_3 stimulates Ca^{++} release from intracellular stores. The oxytocin receptor (OTR) might also be connected directly to a receptor-activated Ca^{++} channel. The increase in intracellular Ca^{++} stimulates actin/myosin coupling resulting in muscle fiber contraction. COX-2 = cyclooxygenase 2; PIP_2 = phosphatidylinositol-4, 5-bisphosphate; MAPK = mitogen-activated protein kinase; PPLA = phospholipase A.

Taken from: Mitchell B.F, Schmid B (2001) Oxytocin and its receptor in the process of parturition, *J Soc Gynecol Investig;* 8: 122-33.

Lutinizing hormone (LH), $PGF_2\alpha$ and tumor necrosis factor-α (TNF-α) are among the other regulators of endometrial $PGF_2\alpha$ secretion (87). A brief discussion of their effects follows:

There is conflicting evidence that LH is involved in uterine functions, since LH is traditionally known to drive progesterone synthesis of CL. Nevertheless, since LH-induced endometrial COX-2 expression and increased $PGF_2\alpha$ release by bovine - endometrial cells, LH has been suggested to be involved in the regulation of $PGF_2\alpha$ release by bovine endometrium during the late luteal phase. In support of this, it was found that the concentration of the uterine LH/hCG receptor varied during the estrous cycle, with higher values at Days 15 to 17 and lower values at Days 2 to 4 (91, 92). Moreover, Canino *et al.* (93) demonstrated that LH stimulated $PGF_2\alpha$ secretion in the cow. However, the effect of LH on $PGF_2\alpha$ synthesis in bovine endometrium appears to be limited to a specific time frame, namely the mid-to-late luteal phase of the cycle (93). Therefore, LH may play a reinforcing role rather than an initial role in luteolysis in cattle (92).

Wade and Lewis (94) demonstrated that exogenous $PGF_2\alpha$ stimulates the utero-ovarian release of $PGF_2\alpha$ in the ewe, suggesting that the utero-ovarian $PGF_2\alpha$ auto-amplification unit is a component of the luteolytic mechanism of $PGF_2\alpha$. Furthermore, a $PGF_2\alpha$ analogue increased $PGF_{2\alpha}$ release from the bovine uterus on day 18 of the estrous cycle (84). It has been shown that $PGF_2\alpha$ activates PKC and increases intracellular calcium mobilization (21), which may in turn stimulate $PGF_2\alpha$ production in the endometrium (95, 96, 97). Therefore, the endogenous $PGF_2\alpha$ may be an essential component in the mechanism regulating its own production (87).

Some recent studies indicate the presence of functional TNF-α receptors in the bovine cyclic endometrium and suggest a possible role of TNF-α in the regulation of endometrial $PGF_2\alpha$ production in cattle (85, 97, 98, 99). TNF-α stimulated $PGF_2\alpha$ production only in the stromal cells *via* the activation of PLA_2 and nitric oxide synthase (98) (figure 4). Although both oxytocin and TNF-α affected $PGF_2\alpha$ output at the follicular stage, TNF-α, in contrast to oxytocin, also affected $PGF_2\alpha$ output at the mid and late luteal stages.

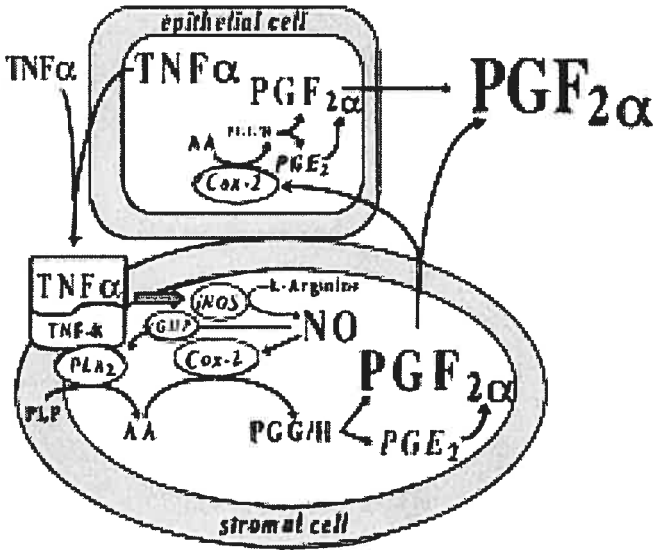

Figure 4: Hypothetical model for TNF-α control of $PGF_2α$ synthesis in bovine endometrial cells during luteolysis. TNF-α that is produced by endometrial epithelial cells and/or uterine macrophages stimulates $PGF_2α$ production only in the stromal cells *via* the activation of phospholipase A_2 (PLA_2) and nitric oxide (NO) synthase. TNF-α induced $PGF_2α$ output from stromal cells may be the first component of an auto-amplification cascade within the bovine endometrium and switches on the positive feedback loop between the uterine $PGF_2α$ and the luteal oxytocin to complete luteolysis. **Taken from:** Okuda K (2002) Regulation of endometrial prostaglandin $F_2α$ synthesis during luteolysis and early pregnancy in cattle. *Domestic Animal Endocrinology;* 23: 255-64.

Moreover, just before luteolysis, there are dramatic increases in TNF-α gene expression (100) as well as TNF-α release (101) from the CL. Therefore, the overall findings lead us to hypothesize that endometrial and/or luteal TNF-α may be a trigger

for the output of $PGF_2\alpha$ from the uterus in the initiation of luteolysis. Since $PGF_2\alpha$ is produced preferentially by epithelial cells (31, 32, 98, 102), TNF-α-induced $PGF_2\alpha$ output from stromal cells may be the first component of an auto-amplification cascade within the bovine endometrium and switch on the positive feedback loop between the epithelial $PGF_2\alpha$ and the luteal oxytocin to complete luteolysis (See Fig. 4). The endometrium apparently consists of many more stromal cells than epithelial cells, so the TNFα-stimulated $PGF_2\alpha$ from stromal cells could be sufficient to play a role in the initiation of luteolysis. Alternatively, TNFα-induced $PGF_2\alpha$ may stimulate $PGF_2\alpha$ production in both stromal and epithelial cells as a paracrine and autocrine regulator, as has been suggested to occur in the ovine corpus luteum at luteolysis (103). Consistent with this auto-amplification cascade, there are reports that $PGF_2\alpha$ treatment acutely increased $PGF_2\alpha$ output from the bovine (104) and ovine (94) uterus. Therefore, along with these findings, Skarzynski et al suggested that TNFα-induced $PGF_2\alpha$ from the stromal cells can initiate luteolysis in cattle. Furthermore, TNFα-induced $PGF_2\alpha$ from the stromal cells may switch on the positive feedback loop between epithelial $PGF_2\alpha$ and luteal OT that completes luteolysis in cattle (70). It should be noted that TNF-α induces the output of both $PGF_2\alpha$ (85, 98) and PGE_2 (97) in cultured bovine stromal cells. Moreover, it is well known that PGE_2 is converted into $PGF_2\alpha$ (105, 106). Thus, the potential capacity of the uterus to generate luteolytic $PGF_2\alpha$ in both epithelial and stromal cells from PGE_2 may be the next mechanism involved in TNF-α-induced luteolysis in cattle (87).

Oxytocin Receptor (OTR)

Peripheral vasopressin and OT receptors have been classified on the basis of both the second messenger system coupled to the receptors and the affinity of a series of vasopressin and OT analogues with enhanced selectivity for a certain receptor type. A

great number of molecular probes, including agonists and antagonists, and radio-labelled, fluorescent or photosensitive ligands make the OTR family a good model with which to study structure-function relationships. Today, OTR along with the vasopressin receptors have been cloned in mammals, lower vertebrates and invertebrates, and molecular cloning of this family has confirmed that vasopressin/OTR subtypes are members of the G-protein-coupled receptor (GPCR) superfamily, consisting of 7 hydrophobic transmembrane α-helices joined by alternating intracellular and extracellular loops, an extracellular N-terminal domain, and a cytoplasmic C-terminal domain. They display a high degree of sequence identity, showing about 102 invariant amino acids among the 370-420 amino acids in the human receptors. The appearance of OTRs in the endometrium of ruminants controls the onset of a feed back loop and episodic $PGF_2\alpha$ secretion. In ruminants, OT is produced in the CL and its release is stimulated by $PGF_2\alpha$. Thus a positive feedback loop is established that amplifies neural OT signals (107); neural OT stimulates $PGF_2\alpha$ secretion, which stimulates release of luteal OT, which in turn further stimulates release of $PGF_2\alpha$. Once concentrations of the OTRs have increased during late diestrus (38), the ability of OT to stimulate synthesis of uterine $PGF_2\alpha$ in ruminants may be mediated through a rapid increase in phospholipase C activity (108, 109). Activated PLC hydrolyses phosphoinositides (PI) presumably leading to the formation of DAG and inositol (1, 4, 5)-triphosphate (IP3). Synthesis of $PGF_2\alpha$ in the endometrium is stimulated by DAG which may act as a second messenger to activate PKC (110). Lafrance and Goff reported that stimulators of PKC activity (i.e. DAG analogues and phorbol ester 12-myristate 13 acetate) also stimulate $PGF_2\alpha$ secretion from the bovine endometrium to the same extent as OT. They concluded that stimulation of $PGF_2\alpha$ by OT is via the PKC effector pathway (111). Similar experiments in sheep have shown that oxytocin stimulated release of $PGF_2\alpha$ and activity of PLC in explants of ovine endometrial tissue in vitro. Second messengers associated with activation of PLC enhanced release of $PGF_2\alpha$ from ovine endometrial tissue (110).

By Northern blot analysis and in situ hybridization, Zingg et al. determined that, at term, the rat uterine epithelium represents a major site of oxytocin gene expression. Estrogens act as a strong inducer of uterine OT gene expression in vivo, and this effect is potentiated 7-fold by concomitant progesterone P_4 administration. Whereas OTR mRNA is strongly induced by estrogen, P_4 does not potentiate but slightly attenuates the estrogen-induced rise. However, estrogen-induced OT binding is completely reversed by concomitant P_4 administration, suggesting an additional post-transcriptional effect of progesterone (112).

Even if the receptors are downregulated in vivo, they show upregulation when explanted and cultured in vitro (113). This indicates that the OTR regulation is partly due to gene suppression in vivo. Despite the presence of steroid receptors in bovine endometrial cells, the level of OTR mRNA could neither be affected by progesterone or estradiol nor by a progesterone withdrawal protocol. The only factor that affected the OTR mRNA level was interferon-τ. As in vivo, this cytokine suppressed the OTR mRNA production (114).

As shown with knock-out mice, estradiol receptor α (ERα) is not necessary for basal OTR synthesis but is absolutely necessary for the induction of OTR binding in the brain by estrogen (115). However, it is unclear whether OTR gene transcription is predominantly regulated by estrogen. The continuous presence of receptors in certain brain regions after gonadectomy suggests the existence of alternate mechanisms of regulation (116).

While there is no estrogen response element (ERE) on the bovine or ovine OTR gene promoter region (117), ER can act through SP1 and possibly AP1 sites on gene promoters (118). This is a possible mechanism by which estradiol can up-regulate OTR and is supported by recent findings that ERα likely stimulates OTR promoter through both protein-DNA and protein-protein interactions with SP1 and AP-1 (119).

Gonadal steroids have an important influence on the uterine OT receptor mRNA accumulation in vivo. Estrogens administered to ovariectomized rats increased OT receptor binding sites and increased OT receptor mRNA accumulation several fold. Although progesterone leads to a marked decline of OT receptor binding sites, the mRNA levels of OT receptor were nearly unchanged (120). Estradiol treatment in

vivo induces an initial up-regulation of endometrial OTR expression in ewes (121, 122, 123) but if estradiol treatment is continued over several days, OTR concentration decreases (124, 125). Some researchers like Soloff et al., concluded these findings imply the involvement of nongenomic effects of progesterone (126).

Taken together, these results indicate that estradiol can induce a short-term up-regulation in OTR expression but that the effect cannot be maintained for more than 1–2 days. However, the exact mechanism of action of E_2 has been difficult to elucidate.

Steroids:

Five major classes of steroid hormones are derived from cholesterol: progestagens, glucocorticoids, mineralocorticoids, androgens, and estrogens. Hydroxylations by P450 monooxygenases that use NADPH and O_2 play an important role in the synthesis of steroid hormones and bile salts from cholesterol. P450 enzymes, a large superfamily, also participate in the detoxification of drugs and other foreign substances. Pregnenolone (C_{21}) is an essential intermediate in the synthesis of steroids. This steroid is formed by scission of the side chain of cholesterol. Progesterone (C_{21}), synthesized from pregnenolone, is the precursor of cortisol and aldosterone. Hydroxylation of progesterone and cleavage of its side chain yields androstenedione, an androgen (C_{19}). Estrogens (C_{18}) are synthesized from androgens by the loss of an angular methyl group and the formation of an aromatic A ring (127).

The following figure illustrates major metabolic pathways of the three principal steroids secreted by the gonads.

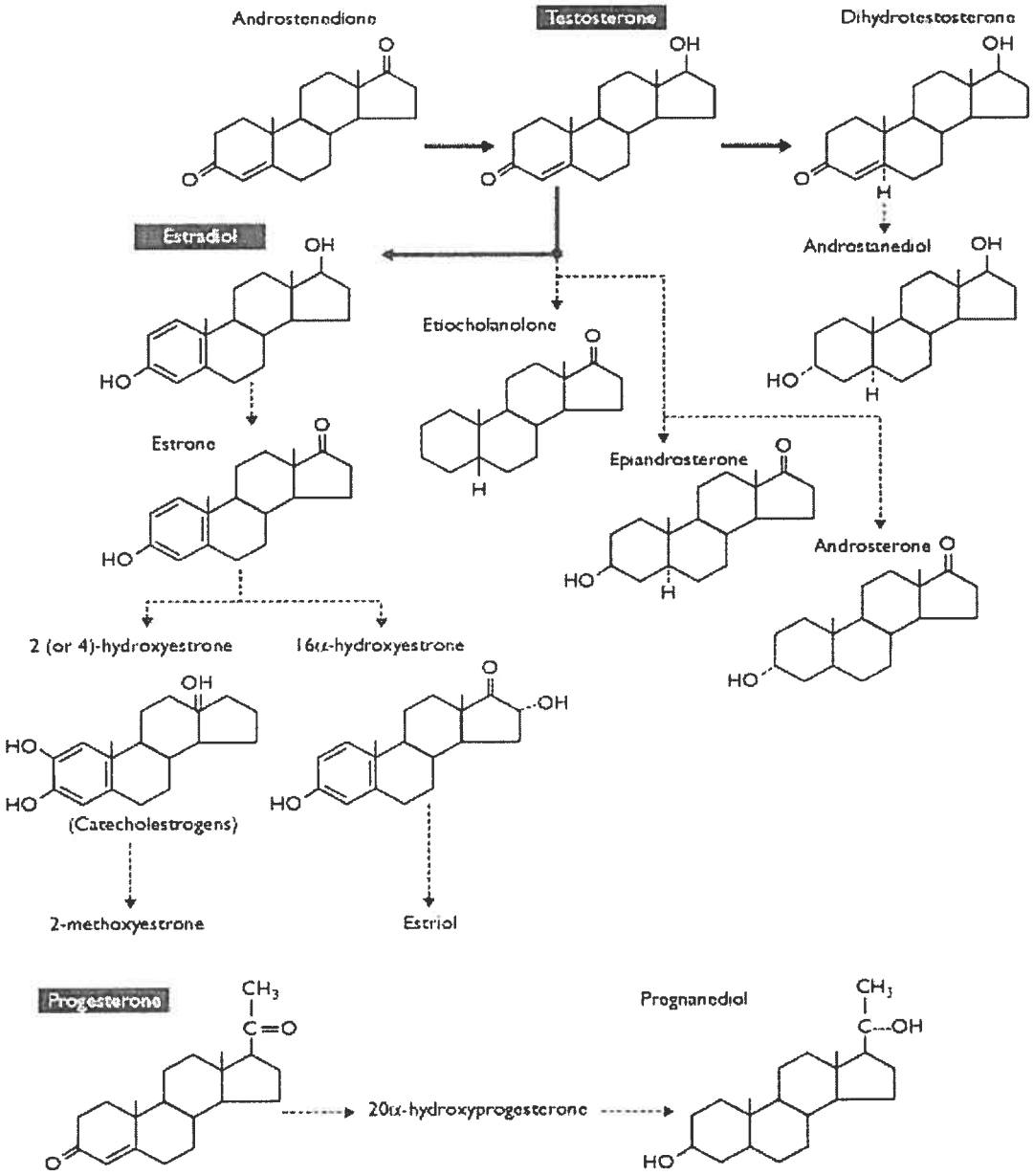

Figure 5: Major metabolic pathways of the three principal steroids (Progesterone, Estradiol and Testosterone) secreted by the gonads.

Taken from: Endocrinology: An Integrated Approach by Stephen Nussey, Saffron Whitehead © BIOS Scientific Publishers Ltd, (2001) *St. George's Hospital Medical School, London, UK.*

In addition to OT, E_2 and P_4 play a significant role in the initiation of luteolysis in ruminants. Uterine secretory responsiveness to OT increases at luteolysis, when endogenous, pulsatile secretion of $PGF_2\alpha$ normally begins. Therefore, the acquisition by the uterus of responsiveness to OT may determine when endogenous secretion of $PGF_2\alpha$ occurs during the estrus cycle. In the presence of P_4 uterine secretory responsiveness to OT develops slowly. Progesterone exerts two types of effects that contribute to the regulation of $PGF_2\alpha$ secretion. First, prolonged exposure to progesterone appears to promote uterine accumulation of arachidonic acid, prostaglandin endoperoxide synthase, and other substances needed for synthesis of $PGF_2\alpha$. Second, P_4 exerts a suppressive effect on secretion, which wanes after prolonged exposure. Together, these effects of P_4 ensure that $PGF_2\alpha$ is secreted only at the proper time to induce luteolysis (45).

In an in vivo study conducted by Mann et al, they exposed 8 long-term ovariectomized cows to prolonged treatment of P_4, but not estradiol. This led to the induction of responsiveness to OT, appearing within 2 days of progesterone treatment, reached a maximum by 6 days and was maintained until day 18. In ovariectomized ewes, while oestradiol treatment did induce temporary responsiveness to OT after 3 d treatment, progesterone treatment was required to induce sustained responsiveness that appeared by day 9 of treatment and was maintained up to day 12. Measurement of endometrial receptors for OT revealed a significant decline in OTRs by day 6 of treatment when responsiveness to OT was maximal, showing that receptor concentrations were not a limiting factor. The most likely mechanism by which progesterone treatment induces responsiveness to OT may be through the upregulation of post receptor signaling pathways and/or enzymes involved in prostaglandin synthesis (128). The results of some investigations show that there is a dose-dependent inhibition of OTR concentration by progesterone and a dose-dependent stimulation of basal $PGF_2\alpha$ release by estradiol (15, 46, 129, 130).

Mann (131) investigated the effect of progesterone and estradiol on basal and OT-stimulated $PGF_2\alpha$ production and on OTR concentrations in endometrium from long-term ovariectomized cows using an explant culture system. As a result he found that basal $PGF_2\alpha$ production was unaffected by progesterone treatment but was stimulated

by estradiol treatment in a dose-dependent manner. OTR concentration remained unchanged in control culture and were unaffected by treatment with estradiol while treatment with progesterone caused a dose-dependent inhibition. Responsiveness to OT in terms of increased PGF production developed spontaneously over the first 24 h of culture and was unaffected by treatment with progesterone and estradiol. The reason for spontaneous development of responsiveness to OT remains unknown but may result from the removal of tissue from the influence of an as yet unidentified inhibitory factor (131).

The effect of prolonged progesterone treatment of bovine endometrial cells resulted in an increase in OT-stimulated $PGF_2\alpha$ secretion but not in its basal secretion. It was demonstrated that estradiol had no effect on basal secretion of $PGF_2\alpha$ but did enhance the OT-stimulated secretion in cells exposed to progesterone for 17 and 21 days, but not in cells exposed to progesterone for only 10 days (132).

Overall, the action of progesterone was more cell type specific than receptor specific. The progesterone doses that are required to affect the signaling function of receptors are much higher than the progesterone levels found in plasma or in nonsteroidogenic tissues such as the myometrium. In steroidogenic tissues, however, huge amounts of progesterone have been measured. In steroidogenic cells as well as in their environment, progesterone might nongenomically influence the signaling of receptors. The molecular mechanisms underlying this progesterone action are not understood (133).

It is now clear that estradiol-17β and progesterone have multiple roles in controlling the luteolytic process, not only by regulating the enzymes necessary for the endometrial biosynthesis of $PGF_2\alpha$, but also by controling endometrial receptors for oxytocin in a number of species (15). Measurement of oxytocin in jugular plasma indicated that plasma levels of oxytocin increased markedly during hour-long bursts of intramyometrial pressure (IMP), whereas plasma levels of vasopressin remained unchanged. The concentration of oxytocin during the first large burst of IMP reached ~200 pg/ml of plasma, but peak concentration of oxytocin declined by ~50% during each subsequent burst of IMP (134, 135).

Other studies have also reported large pulses of oxytocin or its neurophysin during luteolysis in sheep (136), and cows (137). These results suggested that the large hour-long episodic releases of oxytocin in ruminants, interacting with rising levels of endometrial oxytocin receptors, evoked the large episodic pulses of uterine $PGF_2\alpha$ that cause luteolysis in these species. Moreover, evidence has accumulated that ovarian steroids also modulate the synthesis and secretion of oxytocin from the hypothalamic/posterior pituitary system, which explains, at least in part, the pulsatile nature of uterine $PGF_2\alpha$ secretion (15).

Progesterone is considered to be essential to maintain the uterine quiescence. Grazzini et al. (138) postulated that progesterone specifically binds to the rat OTR with high affinity (*K*d; 20 nM) and thereby inhibits receptor function. In case of the human OTR, a direct inhibitory interaction [inhibitory constant (*K*i) ~ 30 nM] with a progesterone metabolite, 5β pregnane-3, 20-dione, has been reported by the same authors. Grazzini et al. (138) claimed that progesterone could act as a negative modulator of the OTR and thus offered a plausible mechanism of how progesterone could contribute to uterine quiescence. However, these findings could not be reproduced in several other laboratories. Instead, the results of other investigations were totally different. Gimpl et al found that high concentrations of progesterone (>10μM) attenuated or blocked the signaling of several GPCRs, including the OTR. The progesterone effects occurred within minutes, were reversible, and could not be blocked by a protein synthesis inhibitor (139). Therefore, they concluded that these effects of progesterone probably were not mediated through genomic mechanisms, either activating or suppressing transcription of specific genes (e.g., the OTR or PGH2 synthase 2). In addition, this effect of progesterone is extremely transient and progesterone has no inhibitory effect on the response of cultured bovine uterine epithelial cells to OT when administered for 72 h and then withdrawn for 6 h immediately prior to OT exposure (140).

Progesterone receptor (PR)

Binding of steroid hormones to the specific steroid hormone receptor (SR) ligand-binding domain (LBD) induces a conformational modification of the receptor, followed by the separation of the receptor from cytoplasmic chaperone proteins such as heat shock protein 90 (Hsp90) and by the exposure of nuclear localization sequences. This allows nuclear translocation and homo/heterodimerization of the ligand-bound receptors, and their binding to steroid response elements (i.e. nucleotide sequences specifically recognized by SRs) on the promoter regions of the target genes, thus regulating gene expression by interacting with the transcription machinery (141, figure 6).

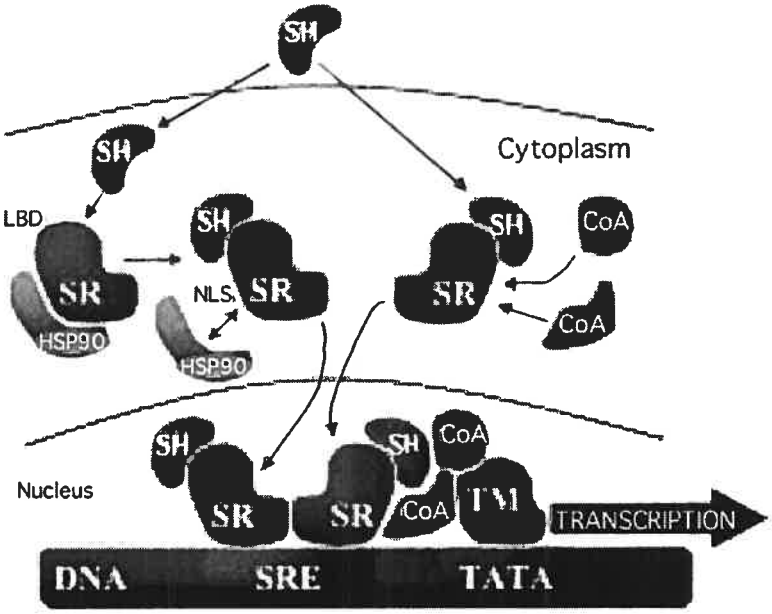

Figure 6: Steroid receptors' (SR) genomic actions. Binding of steroid hormones (SH) to the LBD of the SR induces a conformational modification of the receptor. This causes the separation of the receptor from cytoplasmic chaperone proteins such as Hsp90, the interaction with cell-specific co-activators (CoA), and the exposure of nuclear localization sequences (NLSs). This allows nuclear translocation and homo/heterodimerization of the ligand-bound receptors, and their binding to steroid

response elements (SREs, i.e. nucleotide sequences specifically recognized by SRs) on the promoter regions of the target genes, thus regulating gene expression by interfering with the transcription machinery (TM).

Taken from: Tommaso Simoncini and Andrea R Genazzani (2003) Non-genomic actions of sex steroid hormones. *European Journal of Endocrinology;* 148: 281–92.

The actions of progesterone are mediated by PR. Changes in the number of progesterone and oestradiol receptors in the endometrium are thought to play a role in the induction of luteolysis. The effect of oestradiol and progesterone on the regulation of their receptors in cultured bovine uterine epithelial and stromal cells was examined by Xiao et al. They showed that the number of PR was higher in the stromal cells than in epithelial cells, whereas the number of estradiol receptors was higher in the epithelial cells than in stromal cells. Estradiol upregulates its own receptor and increases the number of progesterone receptors in both cell types in vitro, whereas progesterone has little effect, but inhibits the effects of oestradiol on PRs. (142).

P_4 is the hormone of pregnancy and unequivocally required in all mammals for maternal support of conceptus (embryo/fetus and associated membranes) survival and development. However, the endometrial lumenal (LE) and glandular epithelia (GE) of a number of species exhibit a loss of PR expression prior to the stages of uterine receptivity and implantation. In sheep, PR expression becomes undetectable in the endometrial LE after Day 11 and then in the GE after Day 13. The actions of progesterone on endometrial epithelia during most of gestation appear to be mediated by the endometrial stroma that remains PR-positive throughout pregnancy. Stromal cells produce several growth factors, such as hepatocyte growth factor (HGF) and fibroblast growth factors-7 and -10 (FGF-7, FGF-10), that have receptors expressed specifically in the endometrial epithelia (143). These factors may be progesterone-responsive and mediate epithelial-mesenchymal interactions that are crucial for support of pregnancy. At estrus, estrogen increases PR expression in the endometrial epithelia (144). High levels of endogenous Jaagsiekte sheep retroviruses (enJSRVs) are expressed in the PR-positive endometrial LE and GE in response to increasing

progesterone and are hypothesized to stimulate trophoblast proliferation and production of interferon (IFN)-tau. IFN-tau, the pregnancy recognition hormone produced by the trophoblast from Days 10 to 21, acts in a paracrine manner on the PR-negative endometrial LE and superficial GE to inhibit transcription of estrogen receptor alpha (ERα) and OTR genes. These actions of IFN-tau maintain progesterone production from the corpus luteum by abrogating release of luteolytic pulses of $PGF_2\alpha$ from the endometrial epithelium. The antiluteolytic effects of IFN-tau are dependent on progesterone. Progesterone stimulation over 8-10 days suppresses expression of the PR gene in the LE and then GE. Loss of the PR in the LE is concomitant with decreases in mucin glycoprotein one (MUC-1), an inhibitor of blastocyst implantation. As the conceptus begins implantation on Day 15, the binucleate trophectodermal cells then differentiate and produce placental lactogen (PL), a member of the prolactin (PRL) and growth hormone (GH) family. PL stimulates GE proliferation and production of secretory proteins, such as UTMP and OPN. Interestingly, the effects of PL on the GE appear to require the absence of PR and prior exposure to IFN tau. During mid-pregnancy, the mononuclear trophectodermal cells produce GH that can also act on a progestinized uterus to stimulate GE hypertrophy and secretory function. The actions of this servomechanism are proposed to stimulate GE hyperplasia from Days 20 to 50 and then GE hypertrophy and maximal differentiated function after Day 50 when the majority of fetal growth and development occurs during gestation (143).

Mechanism of Action of Progesterone

Progesterone is an essential reproductive hormone that acts on the estrogen primed endometrium to induce conditions favorable for embryo implantation (145, 146), and the maintenance of pregnancy (147). The genomic effects of progesterone are mediated in target cells through interactions with specific intracellular progesterone

receptors (148). A well-known progesterone binding protein is the multidrug resistance P-glycoprotein (133). In addition to their role in detoxification, P-glycoproteins are involved in intracellular cholesterol transport. It is known that progesterone markedly interferes with the intracellular transport (and metabolism?) of cholesterol (see model in *Figure 7*). At concentrations in the micromolar range, it inhibits both the cholesterol esterification and the transport of cholesterol to and from the plasma membrane (149).

Paradoxically, at the same time, progesterone stimulates the activity of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, the key enzyme of de novo cholesterol biosynthesis. Hence, cholesterol precursors like lanosterol begin to enrich in the membranes of the cell (150). The OTR needs a cholesterol-rich microenvironment to become stabilized in its high-affinity state (151). Because the cholesterol precursors, particularly lanosterol, are completely inactive to support the OT receptor in its high-affinity state (152), the responsiveness of the OT system may not be fully operative during the continuous presence of high progesterone concentrations. According to this scenario, progesterone withdrawal would restore the cholesterol transport so that the highly enriched amounts of cholesterol precursors would now become rapidly converted to cholesterol (153). According to this postulated mechanism, progesterone could affect the signaling of all those receptors that are functionally dependent on cholesterol. It is important to note that the nongenomic actions of progesterone including its influence on the cholesterol transport require progesterone concentrations in the micromolar range. This suggests that the described effects may be limited to the steroidogenic tissues and to their environment. Most likely, progesterone acts in these tissues via both genomic and nongenomic pathways (summarized in *Figure 7 on the next page*) together with other steroids to control receptor activity (154).

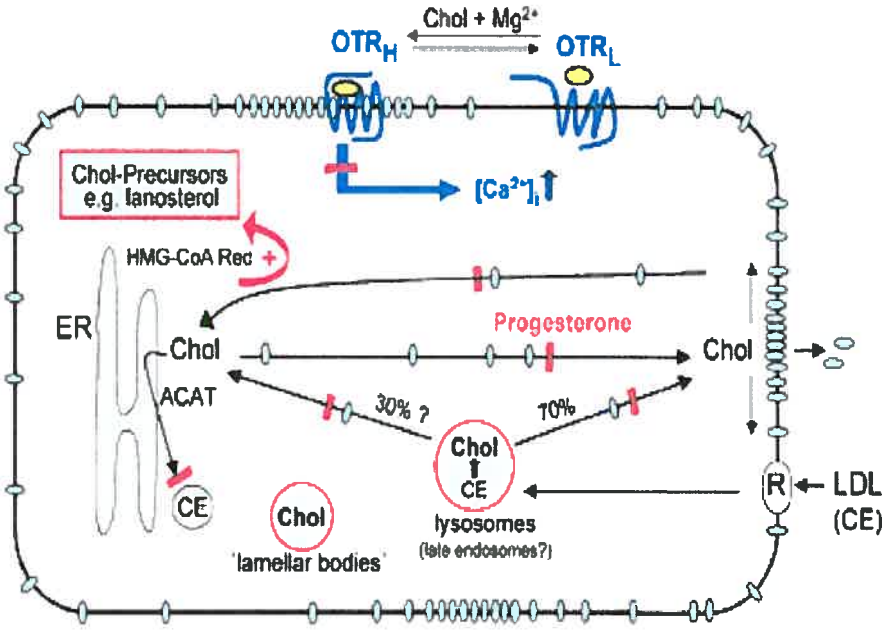

Figure 7: Schematic model of nongenomic inhibitory effects of progesterone. Progesterone inhibits both the signal transduction of G_q coupled receptors (as shown here for the OT receptor) and the intracellular trafficking of cholesterol. Principally, eukaryotic cells can obtain the required cholesterol (Chol, gray ellipses) by two sources: endogenously by de novo synthesis of cholesterol and exogenously by uptake of cholesteryl ester (CE)-rich low-density lipoprotein (LDL) particles via receptor-mediated (R) endocytosis. De novo synthesized cholesterol first arrives at cholesterol-rich domains in the plasma membrane (caveolae and/or "lipid rafts") that may function as cholesterol "sorting centers" within the plasma membrane, where most of the cellular cholesterol resides. Progesterone blocks several intracellular transport pathways of cholesterol (red bars) except for the LDL receptor-mediated uptake of cholesterol. Moreover, cholesterol esterification does not occur in the presence of progesterone, presumably due to the lack of cholesterol substrate for acyl-CoA cholesterol acetyltransferase (ACAT). As a consequence, unesterified cholesterol accumulates in lysosomes (or late endosomes) and lysosome-like compartments (designated as "lamellar bodies") (marked by red background). The key enzyme for the cholesterol de novo synthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA Red), is stimulated in the presence of progesterone (red arrow),

but the cholesterol biosynthesis stops at the level of precursors (e.g., lanosterol). Enzymes involved in the conversion of cholesterol precursors reside in the endoplasmic reticulum (ER), and progesterone most likely prevents sterol precursors localized in the plasma membrane from reaching the ER-resident enzymes, thereby preventing their conversion to cholesterol. Overall, progesterone induces a state of cholesterol auxotrophy. However, after progesterone withdrawal, the accumulated precursors will be rapidly converted to cholesterol. Thus cells will become overloaded with cholesterol for a certain period of time, after which the cholesterol homeostasis will be reestablished. We hypothesize that these reversible progesterone-induced changes of the cholesterol trafficking could have a strong influence on signal transduction processes, particularly in case of the OT receptor (OTR_H and OTR_L, high-affinity and low-affinity OT receptor, respectively; receptor in blue; OT in yellow).

Taken from: Gimpl G and Fahrenholz F (2001) The Oxytocin Receptor System: Structure, Function, and Regulation. *Physiol. Rev;* 81: 629-83.

Non-genomic actions of steroid hormones result from the recruitment of signaling pathways that are often associated with cell membrane receptors such as GPCRs, ion channels or enzyme-linked receptors (155). Through the recruitment of such pathways, steroids rapidly regulate multiple cellular functions, but can also modulate longer-term processes such as gene expression, protein or DNA synthesis and cell proliferation (156).

To name only a few of non-genomic effects of P_4 or its metabolites, we may mention induction of oocyte maturation (157) and interactions with the GABAA receptor (158). Other researchers have reported that P_4 might be acting via a non-genomic mechanism to regulate uterine production of OT in the rat (112). They have shown that regulation of uterine OT binding involves at least two different mechanisms: E_2-induced upregulation is accompanied by an increase in OTR mRNA accumulation, implying that the E_2 effect is mediated via increased OTR gene transcription and/or OTR mRNA stabilization. In contrast, P_4-induced OTR down-regulation occurs via a

novel non-genomic mechanism, involving a direct interaction of P_4 with the OTR at the level of the cell membrane. These effects are specific as signalling and binding functions of the closely related V1a vasopressin receptor remain unaffected by P_4, and as other, related steroids are devoid of any effect on OTR binding or signalling functions. The observation of a specific interaction of a steroid with a G-protein-linked receptor defines a new mechanism of non-genomic steroid action and uncovers a novel level of crosstalk between steroid and peptide hormone action (120). In a review article, Simoncini and Genazzani (2003) offered a tentative interpretation of genomic and non-genomic actions of steroid hormones (159). See figure 8:

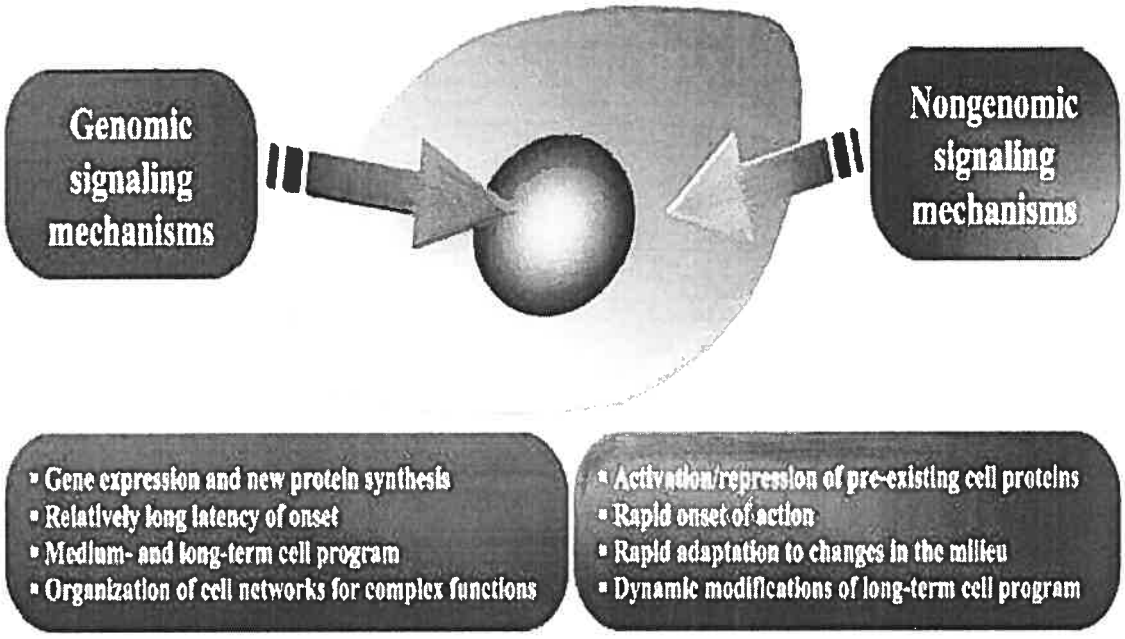

Figure 8: Genomic and non-genomic actions of steroid hormones: a tentative interpretation. Genomic actions of steroid hormones may serve to program cells, organs and systems for complex steroid hormone-regulated functions, providing the single cells and the cell networks with the tools to accomplish these tasks via gene expression and new protein synthesis. They are usually characterized by a relatively longer latency of action and could be meant to determine the medium- and long-term program of the cells. Non-genomic actions of steroids may instead serve to signal to the cells changes in the surrounding milieu, and to rapidly activate or repress the cellular functionalities needed to adapt to these changes, which are already present in the cells. The time of onset of these effects is usually extremely rapid, but the effects of these mechanisms may also serve to dynamically modulate the long-term cell program.

Taken from: Simoncini T and Genazzani A.R (2003) Non-genomic actions of sex steroid hormones. *European Journal of Endocrinology;* 148: 281–92.

While there is no evidence at present to show that P_4 is acting at the genomic level to regulate the bovine OTR, there has been a report to suggest that it might be acting at the cell membrane directly on the rat OTR protein (138). Grazzini et al showed that the effect of P_4 on uterine sensitivity to OT involves direct, non-genomic action of P_4 on the uterine OTR. P_4 inhibits OT binding to OTR-containing membranes in vitro, binds with high affinity to recombinant rat OTR expressed in CHO cells, and suppresses oxytocin-induced inositol phosphate production and calcium mobilization. These effects are highly steroid- and receptor-specific, because binding and signaling functions of the closely related human OTR are not affected by P_4 itself but by the P_4 metabolite 5β-dihydroprogesterone (138). They reported that addition of P_4 in vitro to membranes derived from a parturient rat uterus inhibited binding of the OTR-specific ligand OTA. P_4 also induced a dose-dependent reduction of specific OT binding to recombinant rat OTR expressed in CHO cells. By contrast, P_4 had no effect on binding of the V_1a vasopressin -receptor-specific ligand LVA10 to recombinant V_1a receptor (V1aR). Similar results were obtained with 3H-labelled natural agonists. P_4 coupled to bovine serum albumin also reduced oxytocin binding to intact OTR-expressing CHO cells, thereby excluding a cytoplasmic site of P_4 action. To determine whether the P_4 effect was ligand- or receptor-specific, Grazzini et al took advantage of the fact that arginine vasopressin (AVP) binds to both the OTR and the V_1aR with comparable affinity. [3H] AVP binding to the OTR was repressed by P_4, whereas [3H] AVP biding to V_1aR was unaffected, indicating that the specificity of the P_4 effect is determined by the receptor and not the ligand. Their results indicate that the P_4 interactions with the OTR are affected by guanine-nuclotide-induced changes of receptor conformation and that the observed effects of P_4 are highly receptor-and steroid-specific and are unlikely to be due to non-specific membrane interactions or to interactions with another unknown binding protein (138).

A similar effect of P_4 has been observed in the cow (140) and the sheep (160). However, this nongenomic action of P_4 on OTR is somewhat controversial because other groups have been unable to reproduce this effect of P_4 or a variety of other naturally occurring progesterone metabolites on the human OTR and the bovine OTR (139, 161) and importantly, there is no P_4 response element on the promoter region of

the OTR gene in bovine (117) or other species (162). Thus, the role of a nongenomic action of P_4 in the regulation of OT action in ruminants during the estrous cycle remains to be elucidated (163).

One of the other functions of progesterone is maintenance of uterine quiescence by decreasing uterine sensitivity to OT (164). And this, in turn, suppresses the ability of OT to induce endometrial secretion of $PGF_2\alpha$. This effect appears to be mediated through a direct influence in the interaction of OT with its own receptor (140).

Progesterone antagonists (PA)

Antihormones are potent antagonists of hormone action in vivo, but the mechanism underlying this antagonism is not fully understood. The antiprogesterone RU 486 (mifepristone) has partial agonistic and antagonistic actions. Interestingly, this compound displays partial progestational and glucocorticoid action and is therefore considered not to be pure antiprogestin (165). Hormone antagonists normally act by preventing the action of agonists like the effect of RU 486 that induces parturition in the rat.

Fang et at. has studied the changes in OT and OTR mRNA and peptide synthesis within the pregnant rat uterus during RU 486-induced parturition. Pregnant rats were given a single injection of RU 486 on day 15 of pregnancy (normal delivery occurs on day 22). The average time to delivery, after RU 486 injection, was 27 h. In controls, OT mRNA increased significantly, and this increase was blocked in the RU 486 treatment group. OTR mRNA levels increased within 6 h of RU- 486 and remained elevated until delivery. $PGF_2\alpha$ was increased 16-fold. They indicated that the mechanism of action of RU 486 is to inhibit the P_4 suppression of OTR synthesis, allowing increased expression of OTR, which may directly stimulate myometrial contractions or act indirectly through increased synthesis of prostaglandins (166).

Several steroid hormones transform (activate) their receptors from a cytosolic, non-

DNA binding 8S sedimentation form to a nuclear, DNA binding 4S form. A number of antiprogestins, including RU486, induce an equally dramatic, but distinct, structural alteration of the ligand binding domain. The distinction centers upon the final 30 to 40 amino acids at the carboxyl terminus. The conformational change can be induced by ligand prior to dissociation of the 8S complex and is not induced by heat shock protein removal in the absence of hormone (167). Using protease digestion and antibody mapping, Vegeto et al demonstrated that progesterone and RU 486 induce different conformational changes in the PR (168). Binding to progesterone receptors, RU 486 may change their conformation. In an investigation by Weigel et al, they have prepared a monoclonal antibody, C-262, to a synthetic peptide that contains the carboxy-terminal 14 amino acids from PR. This sequence is 100% conserved in all species of PRs that have been cloned to date, suggesting that this antibody will recognize all mammalian and avian PR. The C-262 antibody recognizes both native and denatured forms of the receptor. However, it does not recognize PR when they are bound to the hormone agonists, progesterone or R5020. Surprisingly the antibody does recognize PR when they are bound to the steroid antagonist RU 486. This suggests that progestin agonists induce a conformational change in the receptor that occludes the C-262 epitope in the carboxyl-terminus, whereas free receptors and receptors bound with RU 486 assume distinct conformation that leaves the C-terminal tail accessible to the C-262 antibody (169).

The actions of PAs as well as progesterone are mediated by the PR. In the target cell, progesterone produces a dramatic change in conformation of the PR that is associated with transforming (or activating) PR from a non-DNA binding form to one that will bind to DNA. This transformation is accompanied by a loss of associated heat shock proteins and dimerization. The activated PR dimer binds to the progesterone-responsive elements (PREs). The agonist-bound PR then activates transcription by associating with coactivators. The effect of the corepressors is blocked. With a progesterone antagonist the same process occurs initially. However, there is impaired interaction with coactivators. Corepressors are recruited in their place. This is the most likely explanation for the antagonist activity of steroidal antihormones (170). Androgen receptor (AR) antagonists are compounds that interfere in some way in the

biological effects of androgens. RU 486 has partial agonistic and antagonistic actions. Interestingly, this compound displays partial progestational and glucocorticoid action and is therefore considered not to be a pure antiandrogen. Binding of antagonists like RU 486, and not of an agonist, results in a conformational change of the LBD on androgen receptors appropriate for the interaction with a co-repressor and inappropriate for interaction with coactivators. Therefore, based on the conformational changes of the AR-LBD, induced by androgens or antiandrogens, it can be concluded that the different transcriptional activities displayed by either full agonists (testosterone, 5α-dihydrotestosterone, methyltrienolone), partial agonists (RU 486 and CPA) or full antagonists (hydroxyflutamide, bicalutamide) are the result of recruitment of a different repertoire of co-regulators (coactivators or co-repressors) as a consequence of these conformational changes (165).

In conclusion, RU 486 can act not only as a progesterone antagonist, a progesterone agonist but also induces morphological and molecular changes that are distinct from progesterone-mediated effects in PR-transfected cells. The non-progesterone-like effect of RU 486 may be mediated through a pathway that is different from the progesterone-mediated pathway, or it is the result of a blockade of certain critical step(s) in the progesterone-mediated pathway (171). Another feature of Mifepristone action is that it also binds with high affinity to glucocorticoid receptors (GR), resulting in antiglucocorticoid activity (172).

ZK137316 is another antigestagen, an estrogen receptor modulator introduced in March 27, 2001. It is a compound of Schering AG, Berlin, Germany, a 13b-methyl 19-nor-steroid like RU 486; however, in contrast to RU 486, ZK137316 exhibits only a weak antiglucocorticoid effect in vivo and 10-fold more potency than RU 486 in the implantation inhibitory test in the rat (173). Klein-Hitpass et al. (174) classified PAs into 2 different categories based on the results they obtained as: type I and type II. The former, represented by ZK 98299, does not induce specific binding of PR to PREs but competitively inhibits induction of DNA binding by progestines. While the latter, including RU 486, ZK 98734 and ZK 112993, induces stable, high-affinity binding of PR to PREs, analogous to progestines. The type II PA forms a stable

complex with DNA and can function as progesterone receptor agonist in the presence of activators (like cAMP) of the protein kinase A signaling pathway (175).

In non-human primates (rhesus monkey), ZK137316 inhibits endometrial proliferation accompanied by atrophy of spiral arteries, when administered chronically (176).

Androgen Receptors (AR)

The AR mediates the actions of the biologically active androgens, testosterone and 5α-dihydrotestosterone (DHT) in target cells. In an investigation on the distribution of AR in the pig, Cardenas and Pope concluded that immunoreactive AR is mainly present in luminal and glandular epithelia of the pig uterus and to a lesser extent in the myometrium, and does not change significantly during the estrous cycle or early pregnancy. Expression of the AR gene in the pig endometrium and myometrium appears to be regulated by E_2 and P_4 (177).

Bovine cytosol androgen receptor (ARC) concentrations were examined simultaneously in various regions of the uterus and in ovarian tissues of cows, and were related to cytosol estrogen (ERC) and progesterone receptor (PRC) concentrations and circulating steroid levels. ERC concentrations were 3-7-fold and PRC concentrations 13-29-fold those of ARC in bovine endometrial and myometrial tissues. When serum progesterone levels were low, both endometrial and myometrial ARC, endometrial ERC, and endometrial and myometrial PRC concentrations were higher ($p < 0.05$) than those observed during higher progesterone concentrations. Because serum DHT concentrations were higher during the luteal phase, it is possible that ARC was down-regulated by this natural ligand at this phase of the cycle. There were no differences between uterine horns in endometrial or myometrial ARC concentrations. Bovine cervical and ovarian stromal tissue also contained ARC, and the concentrations were about the same as in the endometrium and the myometrium.

The relative binding affinities (RBAs) of some steroid hormones towards ARC in vitro were: DHT (100%), testosterone (75%) while estradiol-17 beta, P_4 and dexamethasone had lower RBAs (2, < 1, < 1% respectively). Cytosol androgen receptor concentrations correlated significantly with cytosol progesterone (PRC) and estrogen receptor (ERC) concentrations, both in the endometrium and myometrium. These data show that androgens, such as DHT, may participate the endocrine regulation of bovine reproductive tissues (178).

There have been many reagents developed to modulate progesterone-dependent events, and they range from full PR antagonists to compounds with mixed agonist/antagonist actions, currently known as selective progesterone receptor modulators (SPRMs). In women and nonhuman primates, many PR antagonists suppress estrogen-dependent mitotic activity in the endometrial glands as well as block progestational development of the endometrium. These latter effects are tissue- and species-specific, are most dramatic in women and nonhuman primates, and are referred to as endometrial antiproliferative effects. Recent evidence suggests that the endometrial AR plays an important role in endometrial antiproliferative effect. For example, endometrial androgen receptors are increased by treatment with PR antagonists, and combination treatment with estrogen, a PR antagonist, and an antiandrogen (flutamide) prevents the endometrial antiproliferative effect (171).

Antiprogestins could bind endogenous androgens and mediate their own antiproliferative effects. There are other reports showing that antiprogestins including RU 486 and ZK 230211 also bind weakly to androgen receptors with mixed agonist/antagonist properties and may further modulate AR action (an evidence that AR plays a role in AP action in the primate endometrium) (179).The endometrium is a target tissue for androgen action. There is ample evidence of antiestrogenic effects of exogenous androgens *in vivo* (180, 181), and androstenedione can inhibit human endometrial cell growth and secretory activity *in vitro* (182). APs including RU 486 and ZK 137316 increased endometrial androgen receptor (AR) in macaques and women (183).

RU 486, as well as some other antiprogestins binds with high affinity to glucocorticoid receptors (GR), resulting in antiglucocorticoid activity (184). In a report by Attardi et al. they indicated that it is possible that mifepristone itself directly mediates these effects by interacting with AR, for which the relative binding affinity is 13% (185). The results of the investigations in the rat confirm that RU 486 possesses antiandrogenic as well as antiprogestational properties. It antagonized the inhibition produced by progesterone but had no effect on peroxidase induction by itself or in unprimed immature animals (186).

Hypothesis and Objectives:

Luteolysis is an important event in reproduction and steroids like P_4 play critical roles in that event. Although many aspects of action of P_4 in luteolysis have been discovered, the exact cause of the increase in OTR is not understood. It was thought that E_2 acting through increased ER resulted in increase in OTR and luteolysis. However, since there is no evidence of ER increasing before OTR and since prolonged treatment of cows with only P_4 can lead to increased OTR concentrations, further work is required into the mechanism of action of P_4 in the endometrium and specifically its role in this upregulation of the OTR.

The hypothesis for this study is that P_4 affects OTR number in ways other than the expected genomic action. Since OTR increases before the increase in ER, the initial increase in OTR concentration is caused by factor(s) other than estradiol. Our objectives in the present study were:

1- To determine if the endometrial epithelial cells are able to metabolize P_4. If so, examine the effect of specific metabolites on endometrial function.

2- To compare the effect of a pure P_4 antagonist (ZK 137 316) with that previously found with RU 486. Is RU 486 acting as an antagonist or partial agonist?

3- To determine if the reduced number of OTRs as one of the primary effects of P_4 is due to the decrease in prostaglandin synthesis or to the effect of RU 486.

Materials and Methods:

a. Chemicals and reagents

Tissue culture medium (RPMI 1640, phenol red free), HBSS (calcium and magnesium free), trypan blue (Grand Island, NY), Trypsin 2.5% (porcine), and Gentamicin reagent solution (10mg/ml) were purchased from Gibco. New born calf serum (NBCS) was purchased from ICN Boimedicals (Ohio, US), P_4, 5 alpha- and 5 beta-pregnane-dione, and Dihydrotestosterone from Sigma Chemical Co. (St. Louis, MO), OT was purchased from Vétoquinol (Lavatrice, Quebec), OT (Tyrosyl-2, 6-^{3}H), $PGF_2\alpha$- 3H was obtained from Perkin Elmer (Boston, MA), Matrigel was obtained from VWR Scientific (Montréal, Quebec, Canada). dbcAMP from Sigma and Biorad protein assay from Biorad®.

b. Preparation and culture of cells

Uteri from cows at Days 1–3 of the estrous cycle were collected at the slaughterhouse and after rinsing with water were transported on ice to the laboratory within one hour. Days 1–3 were selected because the stage of the estrous cycle can be accurately determined from slaughterhouse material due to the presence of the corpus hemorrhagicum. The stage of the estrus cycle was assessed by morphological observations of the reproductive tract (187). In this study, one uterus of the early estrous cycle (Days 1-3) was used for each of the experiments, and the endometrial epithelial cells were obtained as described previously (188). Briefly, the two horns of

each uterus were washed with sterile HBSS containing 50μg/ml gentamicin. The myometrial layers were dissected away and the horns were then everted to expose the epithelium. The everted horns were digested for 1.5–2 h in HBSS with 0.3% trypsin at 37°C in 5% CO_2:95% air. At the end of the incubation fetal calf serum (FCS) was added to a final concentration of 10% to block the action of trypsin. The cell suspension was centrifuged at 60×*g* for 5 min to separate epithelial cells from contaminating stromal cells. At the time of plating, the cell viability was greater than 95%. The homogeneity of the cell populations was examined by immunofluorescent staining for specific markers of epithelial cells (cytokeratin) and stromal cells (fibronectin) as previously described (189).

The pellet was washed three more times with HBSS and was then suspended in 25 ml RPMI medium containing 50 μg m^{-1}gentamicin supplemented with 10% fetal bovine serum depleted of steroids by dextran-charcoal extraction (FBS-DC). The cells were plated onto 100 mm×20 mm Nunclon Petri dishes (Gibco), and incubated at 37° C in 5% CO_2:95% air for 1-3 h to allow for attachment of contaminating stromal cells. At the end of incubation, the unattached epithelial cells were collected. After cell counting and viability determination by trypan blue exclusion, cells were plated into Matrigel coated 24-well plates (2×10^5 cells per well). 100μl of 1:8 Matrigel was added to each well of 24-well plates and the plates were dried overnight. At the time of plating, the cell viability was greater than 95%. Cells were cultured in RPMI medium (without phenol red) containing charcoal-treated NBCS at 37° C in 5% CO_2: 95% air. The medium was changed every 2 days with the medium supplemented with 5% FBS-DC until confluency was reached. Confluency of the epithelial cells isolated from endometrium early in the estrous cycle is generally reached after 6- 7 days in culture.

HPLC

To determine if the endometrial epithelial cells are able to metabolize P_4, the cells were treated with 3H pregnenolone or 3H progesterone for 6-24 hr. After incubation

the medium was collected and the steroids extracted and concentrated using C18 Sep-Pak Cartridge. Metabolites were separated using a Waters 2695 high-pressure liquid chromatography (HPLC) system fitted with either a Symmetry C18 reverse-phase column (3.5 μm, 4.6x 75mm) or a Radial-Pak reverse-phase cartridge. The solvent system was acetonitrile: water at a flow rate of 1 ml/min. Fractions (0.5ml) were collected, scintillation fluid added and radioactivity counted in a Waters 2695 counter. Substances in peak fractions were characterized by gas chromatography/mass spectrometry (GC-MS). Extraction of free and conjugated steroids was achieved using C18 Sep-Pak Cartridge, conjugated fractions were eluted with 0.2% methanol in water and the non-conjugated steroids eluted with 100% methanol.

Hormonal Treatment:

To investigate the effect of P_4 and its metabolites, 5α-pregane-dione and 5β-pregnane-dione on the OT stimulation of $PGF_2\alpha$, the medium was replaced with 1.0 ml of fresh RPMI-1640 without serum. Then the cells were cultured for 48 hr in the presence or absence of P_4 and its metabolites (100 ng ml^{-1}). Each group of cells was incubated for a further 4-6 hrs with or without OT (200ng ml^{-1}). The culture medium was collected for $PGF_2\alpha$ measurement at the end of the incubation and stored at -20°C until further processing.

In other experiments, we treated our epithelial cells with anti progesterones (RU 486 and ZK 137316) at different concentrations of 1, 5 and 10 μM, DHT at 100 ng ml^{-1} and dbcAMP at a concentration of 1mM for 48h.

Radio- Immunoassay (RIAs) of PGs:

Concentration of $PGF_2\alpha$ was measured in 100 μl aliquots of culture medium without dilution or after 5-fold dilution with assay buffer. Serial dilutions of medium samples (n=3) were parallel to the standard curve. The antibody was purchased from Assay

Statistical analysis

Each experiment was carried out using the cells from one uterus and was repeated with different uteri collected at different times from the slaughterhouse. For experiments involving the measurement of $PGF_2\alpha$ and OTR, all treatments were tested in triplicate. Effects of treatments were evaluated by least squares ANOVA. To examine the effect of P_4 on the OT stimulation of $PGF_2\alpha$, the data were analyzed by one way ANOVA, which included the main effects of P_4 treatment and OT, and all interactions. The comparison of different treatment groups with control was done by running Dunnett's test with a significant level of $P<0.05$. The other experiments were analyzed by two-way ANOVA, which included the main effects of OT, treatments and their interactions together with OT binding assay results. A probability of $P<0.05$ was considered to be statistically significant. The data were analyzed using the computer program JMP (SAS Institute Inc., Cary, NC).

Results:

1- Metabolism of progesterone and pregnenolone by the endometrial epithelial cells:

In order to see if endometrial epithelial cells metabolize P_4, we treated our cells with labelled pregnenolone and P_4 and then performed HPLC.

Designs, Inc. (Ann Arbor, MI); its cross-reactivity against 13, 14-dihydro-15-keto-$PGF_2\alpha$ (PGFM), 6-keto-$PGF_1\alpha$, PGD_2, PGE_2 and arachidonic acid were 0.07, 6.1, 0.6, 0.2, and 0.002%, respectively, at 50% displacement. The sensitivity of the assay was 62.5 pg ml^{-1}, and the intra- and interassay coefficients of variation were 9.2 and 12.3%, respectively.

Measurement of total protein

Total protein was measured in 10µl of cell suspension using the Bradford method (Bio-Rad Laboratories). Bovine serum albumin (BSA) was used as the standard (190). For this purpose the culture plates were used for the measurement of protein content of each well to standardize the results of hormone assays using Bio-Rad protein assay.

OT receptor and binding assay

At the end of the culture, the medium was aspirated, and 100µl buffer containing 3H-OT with or without a 100-fold excess of unlabeled OT were added and the cells incubated for 80 min at room temperature. At the end of the incubation, plates were put on ice and then washed 3 times with 1 ml of cold saline. Solubilization solution (250µl, 0.5% Triton X-100, 1M NaOH) was added into each well, and incubation was performed overnight at 37° C. The solubilized cells were neutralized with 62.5µl of 4M HCL and then counted in 4 ml of scintillation liquid.

1a. Metabolism of 3H-pregnenolone by endometrial epithelial cells:

In experiment 1, HPLC showed that pregnenolone was almost completely metabolized by the endometrial epithelial cells during 24h (Figure 9). Separation was performed using a Radial Pak column with a solvent system of acetonitrile: water (50:50) and a flow rate of 1ml/min.

Using P_4 and labeled pregnenolone as controls, HPLC showed that the extracted metabolite is different from progesterone (Figure 10)

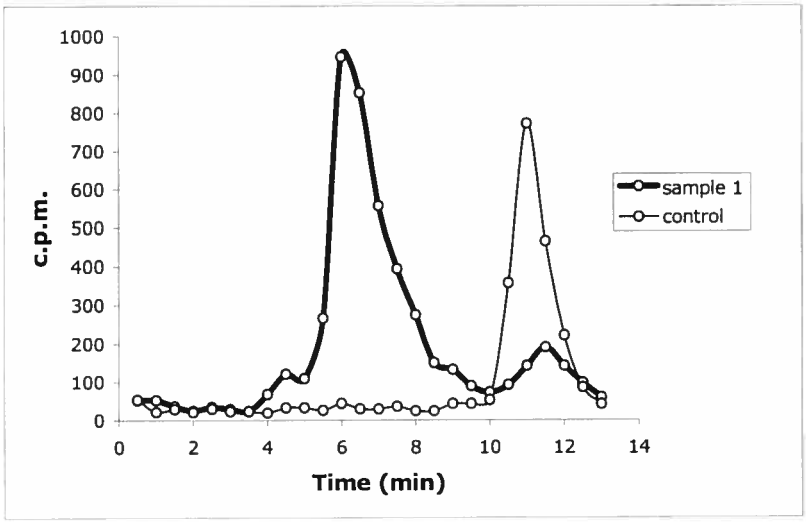

Figure 9: Metabolism of 3H-pregnenolone by the bovine endometrial epithelial cells (sample 1). 3H-pregnenolone without cells was used as control.

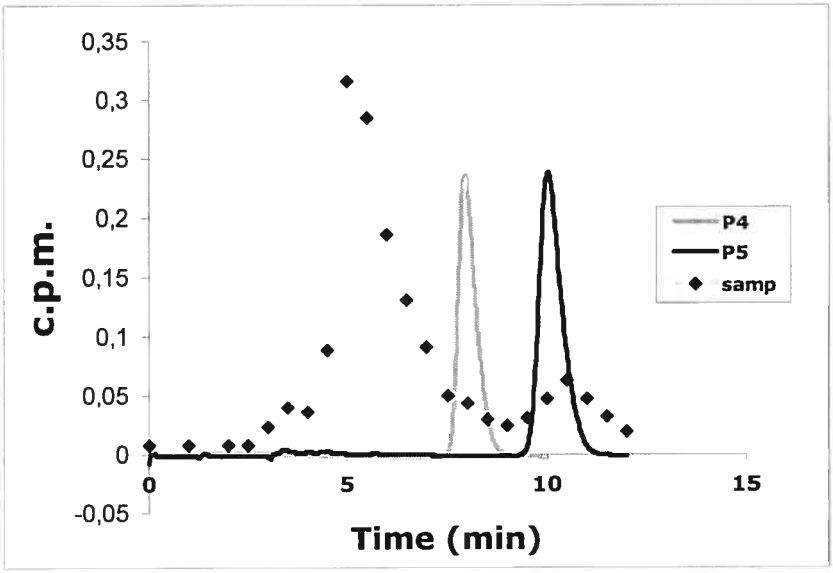

Figure 10: Profile of metabolized progesterone compared to 3H-progesterone (P_4) and 3H-pregnenolone (P_5) controls showing that the extracted metabolite is different from progesterone.

Changing the concentration of the solvent (AcN: water 40:60) to improve the resolution of the separation showed that the metabolite of pregnenolone was in fact at least 2 different metabolites. GC/Mass spectrometry showed that one of them was either 5α-pregnanolone or 5β-pregnanolone. Similar results were found for the metabolism of progesterone. Therefore, these two metabolites were used for the subsequent treatments of our cells. See figure 11:

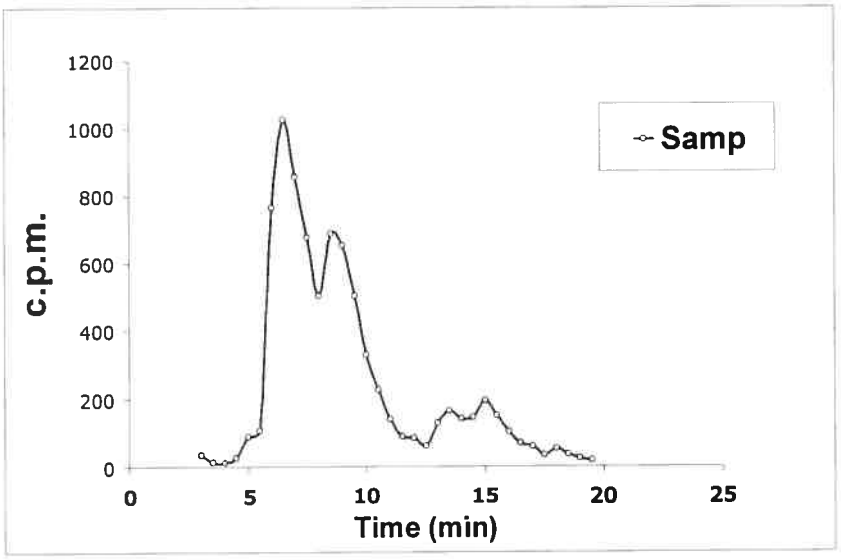

Figure 11: Appearance of two different metabolites of progesterone after changing the concentration of AcN: water from 50:50 to 40:60. Symmetry column AcN: water 40:60.

1b. Extraction of Free and Conjugated Steroids from C18 Sep-Pak Cartridge

The retention times for the metabolites of P_5 or P_4 indicated that these steroids were very polar. Thus, one possibility was that they could be conjugated steroids. To determine if this was the case, the metabolites of P_4 were separated into conjugated and free forms prior to separation by HPLC. The results of separation analysis showed that the 2 different metabolites of P_4 from our samples were mostly in the free form. See figure 12:

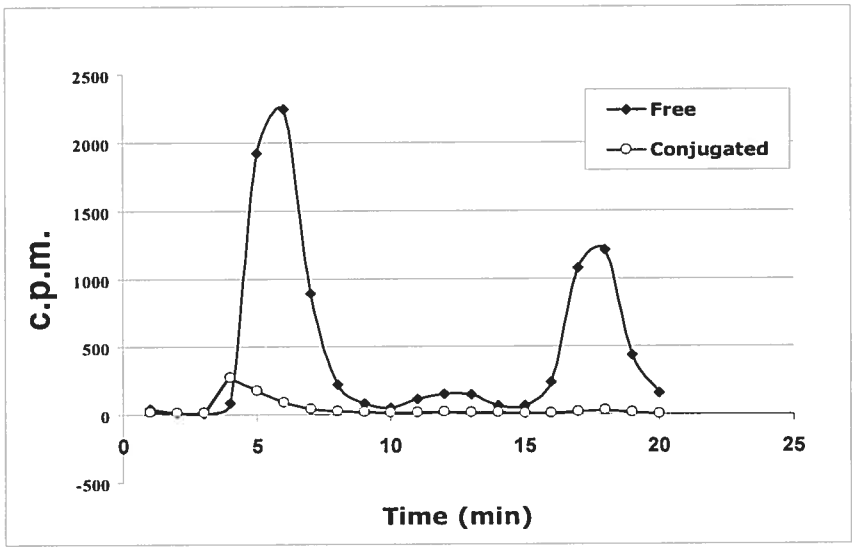

Figure 12: Separation of free (solid line) and conjugated progesterone (dotted line). Symmetry C18 reverse-phase column AcN: water 50:50.

2. The effect of progesterone and its metabolites on oxytocin stimulation of $PGF_2\alpha$:

5α- and 5β-pregnanedione were the metabolites we found from the results of our HPLC and GC/Mass spectrometry tests with labelled pregnenolone and P_4. We used these metabolites along with P_4 to treat our cells and see their effects on the release of $PGF_2\alpha$.

In experiment 2, P_4 at a concentration of 100ng ml^{-1} enhanced ($P<0.001$) the concentration of $PGF_2\alpha$ with or without stimulation of OT at a concentration of 200 ng ml^{-1} (Figure 13). The effect of P_4 metabolites (5α- and 5β-pregnane-dione at the concentration of 100 ng ml^{-1}) on the concentration of OT stimulated $PGF_2\alpha$ was not significant.

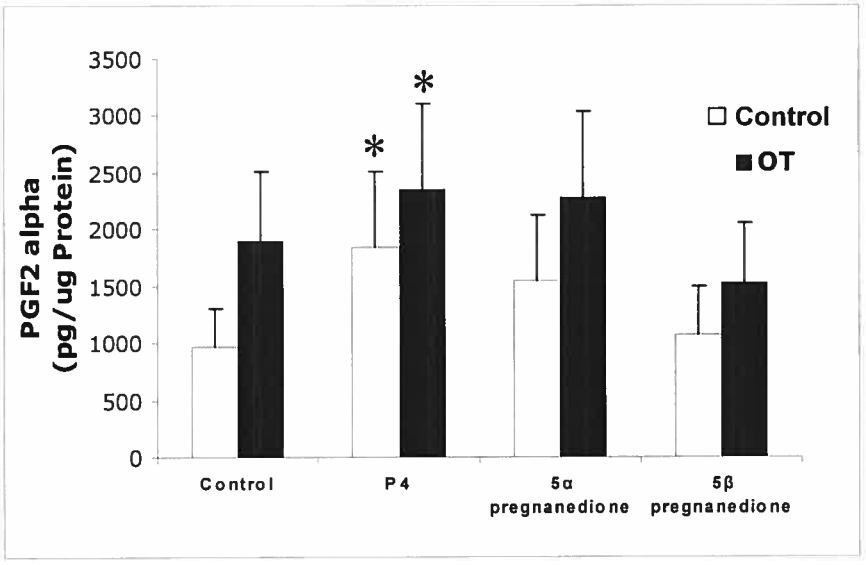

Figure 13: The effect of P_4 (100 ng ml^{-1}) and its metabolites on the release of OT-stimulated $PGF_2\alpha$ from the bovine endometrial epithelial cells. (*) ANOVA showed a significant effect of steroid and OT but no interaction. Subsequent analysis by running Dunett's test with a significant level of $P<0.05$ showed that only the P_4-treated cells were different from controls.

3. The effect of progesterone and its metabolites on OTR number:

In our attempts to study the effects of P_4 and its metabolites, 5α- and 5β-pregnanedione, on the concentration of OTRs of the endometrial epithelial cells, OT-binding assays were performed. The results of OT binding assays showed that 5α-pregnanedione tended to reduce the concentration of OTRs but statistical analysis revealed no significant effects of any of the steroids (Figure 14).

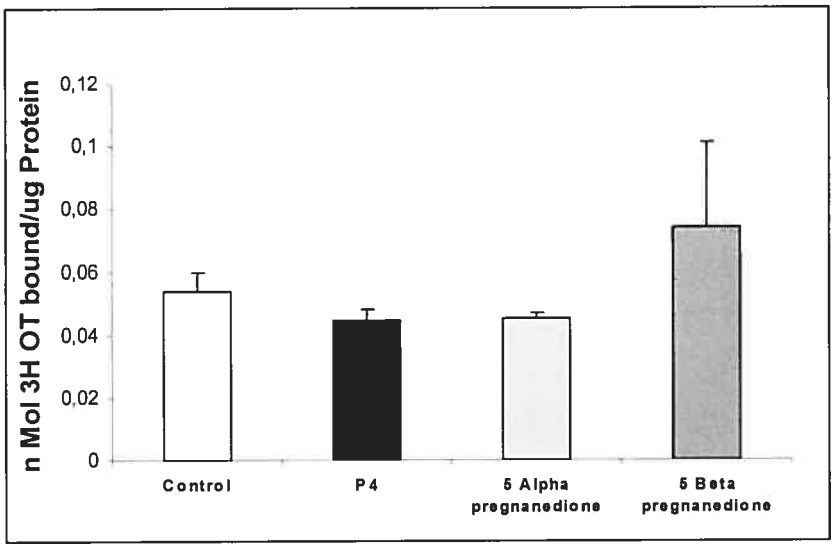

Figure 14: The effect of progesterone and its metabolites on the OTR concentrations. Endometrial epithelial cells were treated for 48 h in the presence of P_4, 5α- and 5β-pregnane-dione (100 ng ml^{-1}). At the end of the culture, the cells were incubated with 100 µl of 10 nM 3H-OT in the presence or absence of 100-fold molar excess of unlabeled OT in the binding buffer to measure the total and non-specific binding. Data are presented as specific binding, normalized to the respective protein content. Data are presented as the least square means and the S.E.M (n=3). None of the treatments significantly changed OTR number in cells.

4. The effect of antiprogestagens on the release of $PGF_2\alpha$:

After treatment of our cells with the antiprogesterones, RU 486 and ZK 137316, the concentration of $PGF_2\alpha$ produced by the cells and the concentration of OTRs were measured. There was a significant ($P<0.05$) increase in the concentration of $PGF_2\alpha$ by the addition of RU 486 and ZK 137316. However, RU 486 inhibited OT stimulated $PGF_2\alpha$ secretion (Figure 15).

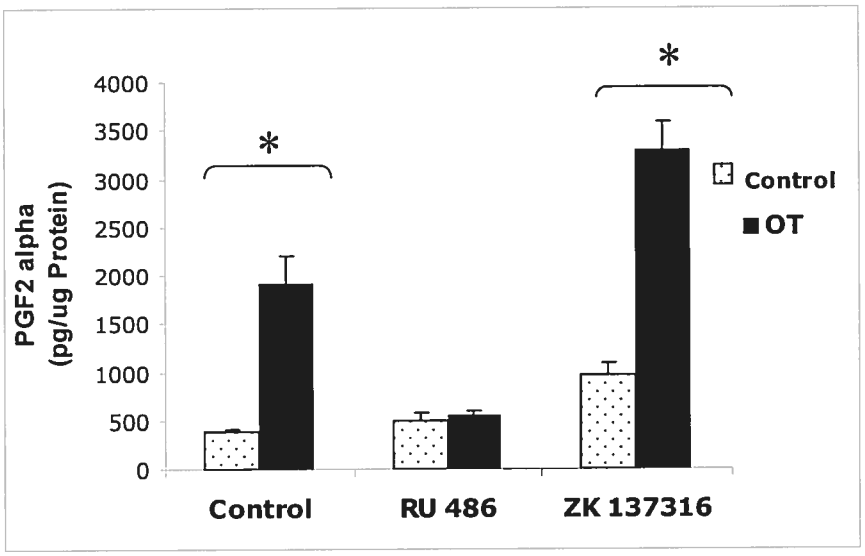

Figure 15: The effect of the antiprogesterones RU 486 (5μM) and ZK 137316 (1μM) on the release of $PGF_2\alpha$ from the bovine endometrial epithelial cells in the absence of P_4. (*) Asterisks show significant effects of OT.

To see the effect of RU 486 in the presence or absence of P_4 on the release of $PGF_2\alpha$ from the endometrial epithelial cells of the bovine uterus, the cells were treated with P_4 (100 ng/ml^{-1}) with or without co-treatment of RU 486 (5μM) for 48h. The results showed that RU 486 inhibited the effect of P_4 on the release of $PGF_2\alpha$ from the endometrial epithelial cells of the bovine uterus (Figure 16).

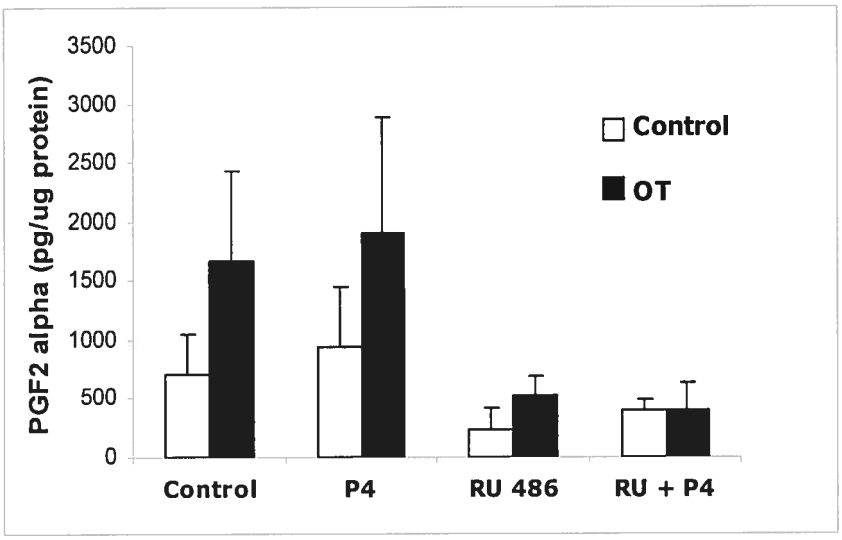

Figure 16: The effects of RU 486 (5μM) on the synthesis of $PGF_2\alpha$ with or without co-treatment of P_4 (100 ng ml^{-1}) on bovine endometrial epithelial cells.

In order to see the effect of ZK 137316 in the presence or absence of P_4 on the release of $PGF_2\alpha$ from the endometrial epithelial cells of the bovine uterus, the cells were treated with P_4 (100 ng ml^{-1}) with or without co-treatment of ZK 137316 (1μM) for 48h. The results showed that ZK 137316 alone significantly ($P<0.05$) increased the secretion of $PGF_2\alpha$ from OT-stimulated cells (Figure 17).

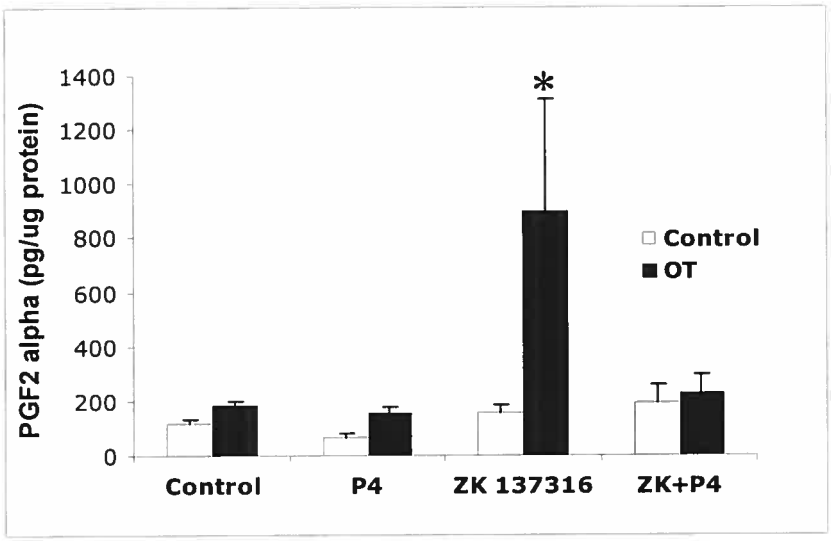

Figure 17: The effect of P_4 (100ng ml^{-1}) and ZK 137316 (1μM) on the synthesis of $PGF_2\alpha$ from bovine endometrial epithelial cells. ZK 137316 alone significantly increased the release of $PGF_2\alpha$ from OT-stimulated cells.

(*) Asterisks show significant effect of OT.

4a. The effect of different doses of antiprogestagens on the secretion of $PGF_2\alpha$:

After treating the bovine endometrial epithelial cells with RU 486 and ZK 137316 at concentrations of 1, 5 and 10 μM for 48 h, there was a significant increase in the level of $PGF_2\alpha$ by ZK137316 and RU 486 at a concentration of 1μM ($P<0.05$). At higher concentrations of 5 and 10μM there were no effects on the concentrations of $PGF_2\alpha$ either with or without OT stimulation (Figure 18, 19).

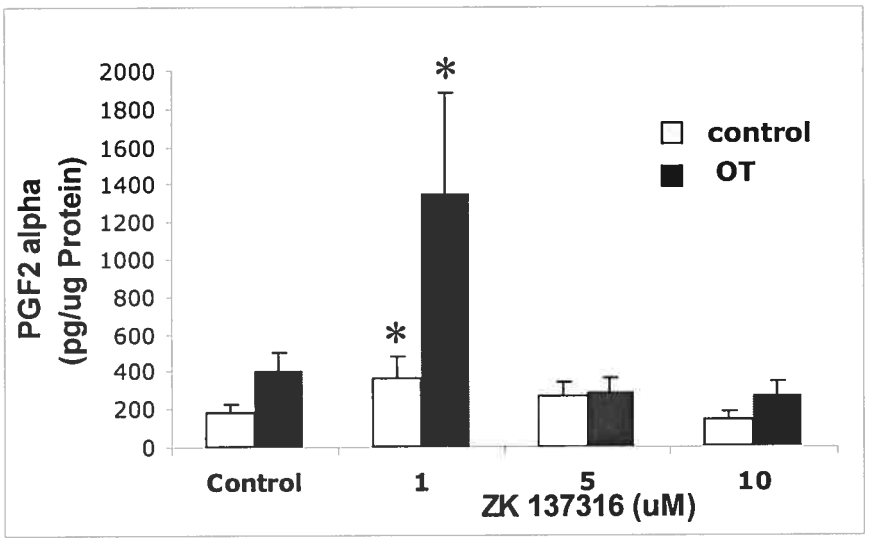

Figure 18: The effect of ZK 137316 dose response on $PGF_2\alpha$ concentration secreted by the bovine endometrial epithelial cells. There was a significant increase in the level of $PGF_2\alpha$ by ZK137316 at a concentration of 1μM ($P<0.05$). At higher concentrations of 5 and 10μM there were no effects on the concentrations of $PGF_2\alpha$ either with or without OT stimulation. (*) Asterisks show significant differences from the other groups.

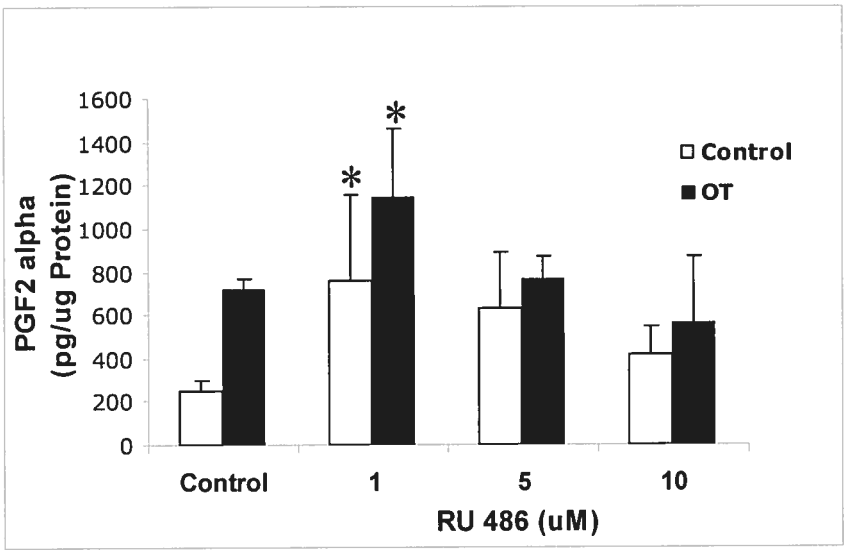

Figure 19: The effect of RU 486 dose response on the release of $PGF_2\alpha$ concentration. There was a significant increase in the level of $PGF_2\alpha$ by RU 486 at a concentration of 1μM ($P<0.05$). At a concentration of 5 μM, RU 486 enhanced the synthesis of $PGF_2\alpha$ in the presence or absence of OT stimulation. At 10 μM, the level of $PGF_2\alpha$ synthesis increased in the absence of OT stimulation but decreased with OT stimulation insignificantly. (*) Asterisks show significant differences from the other groups.

5. The effect of antiprogestagens on the OTR concentration:

OT binding assays demonstrated that both antiprogestins (RU 486 and ZK 137316) in the absence of P_4 decreased ($P<0.05$) the OTR concentration in the bovine endometrial epithelial cells (Figure 20).

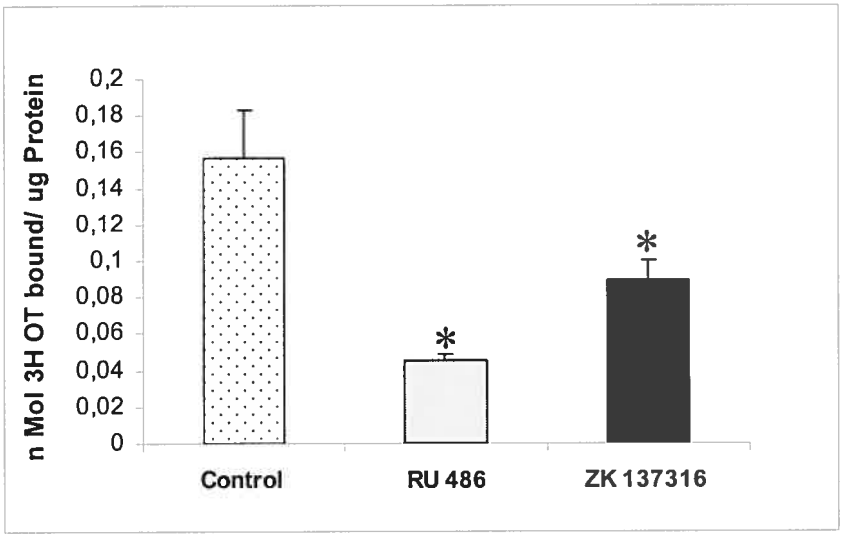

Figure 20: The effect of antiprogestagens RU 486 (5μM) and ZK 137316 (1μM) in the absence of P_4 on the OTR concentration from the bovine endometrial epithelial cells. (*) Asterisks show significant differences from controls ($P<0.05$).

To see the possible effects of ZK 137316 on OTR concentration in the epithelial endometrial cells at different doses, the cells were treated with ZK 137316 at 1, 5 and 10 μM. The results showed that ZK 137316 significantly reduced the OTR concentrations at all 3 doses (Figure 21).

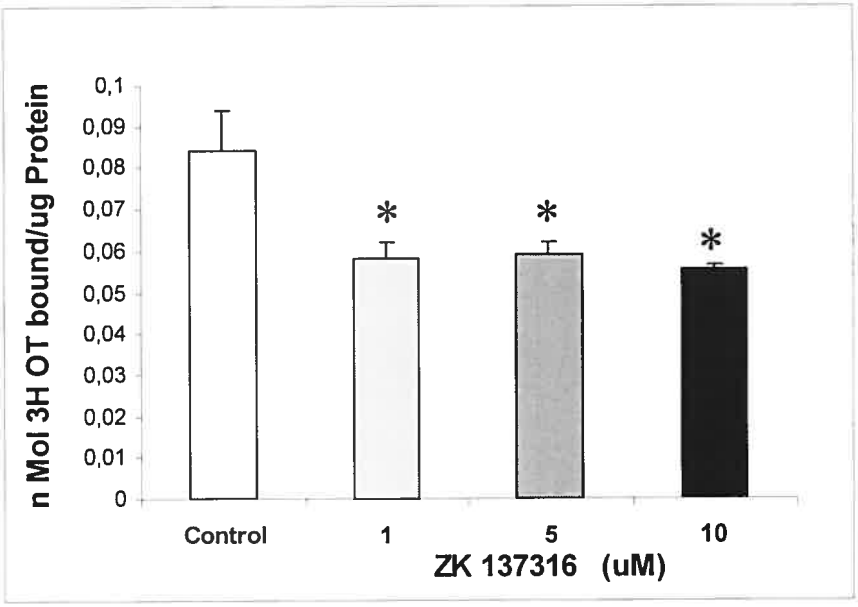

Figure 21: The effect of ZK 137316 dose response on OTR concentration in the bovine endometrial epithelial cells. And this reduction was significant ($P<0.05$).

(*) Asterisks show significant differences from control.

6. The effect of DHT on the release of $PGF_2\alpha$:

To investigate any other possible stimulant besides OT for the synthesis of $PGF_2\alpha$ and with respect to the fact that there are androgen receptors in the bovine endometrial cells, we treated our cells with a more potent androgen than testosterone, DHT, for 48 h to see its effect at different concentrations of 1, 100 and 500 ng/ml on the synthesis of $PGF_2\alpha$ by our cells. The results of radioimmunoassay assay show that DHT increases $PGF_2\alpha$ production in the bovine endometrial epithelial cells (Figure 22).

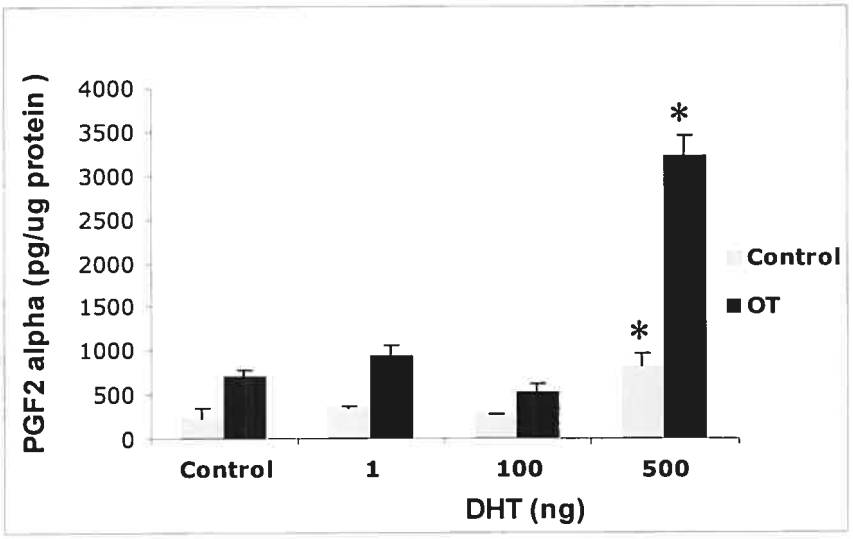

Figure 22: The effect of DHT dose response on the secretion of $PGF_2\alpha$ from bovine endometrial epithelial cells. There has been a significant increase in the release of $PGF_2\alpha$ at a concentration of 500ng/ml of DHT ($P<0.05$).

(*) Asterisks show significant differences from the other concentrations of DHT.

The results of OT binding assays showed no significant effect of DHT on the OTR concentrations in cells (data not shown).

7. The effect of Indomethacin on the release of $PGF_2\alpha$:

To determine if antiprogesterone-induced reduction in number of OTRs is due to the decrease in PG synthesis or to the effect of RU 486, we used indomethacin as an inhibitor of PG synthesis at different concentrations of 2, 4 and 8μM for 24 h. Indomethacin caused a dose dependent reduction in the release of $PGF_2\alpha$ from the cells (Figure 23).

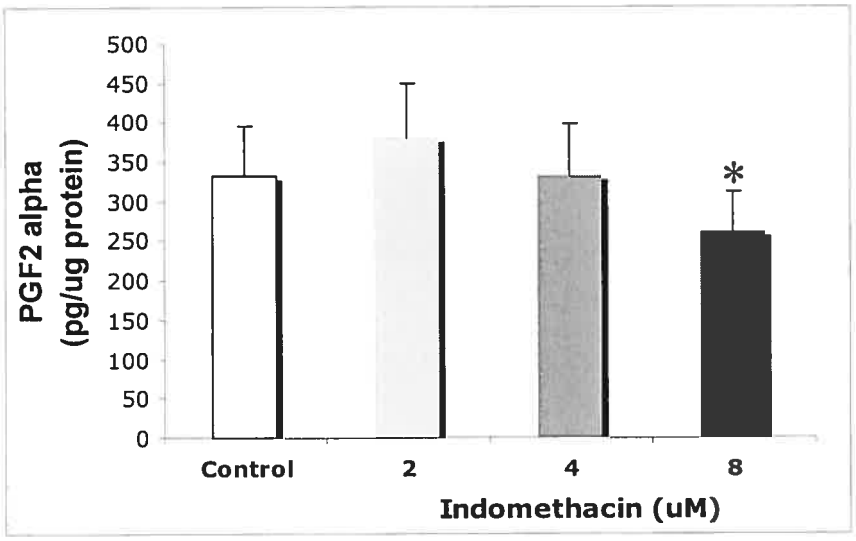

Figure 23: The effect of different doses of indomethacin on the release of $PGF_2\alpha$ from the bovine endometrial epithelial cells. (*) Asterisks show significant level from all other groups.

8. The effect of Indomethacin dose response on OT binding of the bovine endometrial epithelial cells:

After treating the bovine endometrial epithelial cells with Indomethacin for 24 h, there was a reduction in OTR concentration in the cells. The inhibition was significant at the concentration of 8μM (Figure 24).

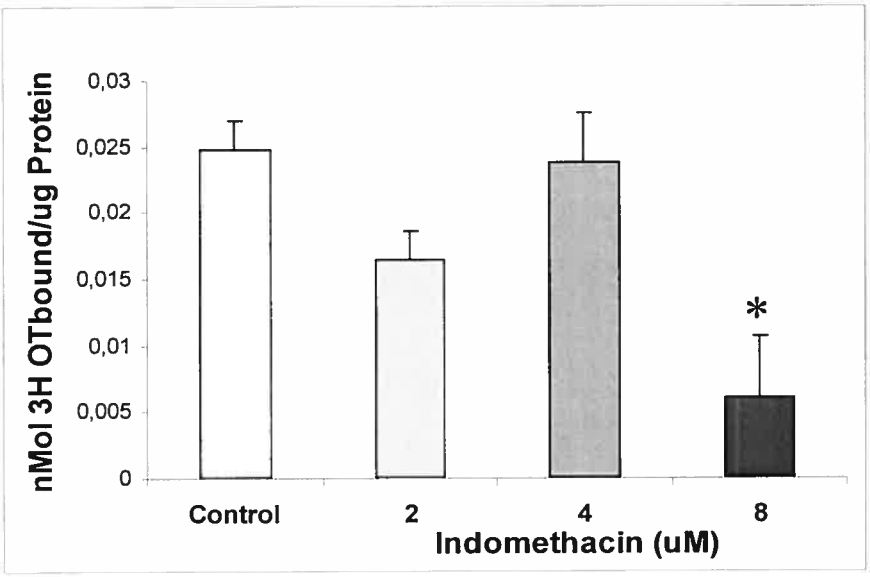

Fig 24: The effect of indomethacin dose response on the binding of OTR in the bovine endometrial epithelial cells. There is a reduction in the number of OTRs by indomethacin. (*) Asterisk shows significant difference from the other groups.

9. The effect of cAMP on the release of prostaglandin $F_2\alpha$:

Another possible pathway for the observed increase in the synthesis of $PGF_2\alpha$ in our endometrial cells that was caused by the action of antiprogesterones is via activation of cAMP. Addition of the cyclic nucleotide analog, dibutyryl-cAMP, at a concentration of 1mM enhanced the production of $PGF_2\alpha$ and the OT-stimulated secretion of $PGF_2\alpha$ in the presence of P_4. P_4 alone stimulated the synthesis of $PGF_2\alpha$ from the endometrial epithelial cells of the cow (Figure 25).

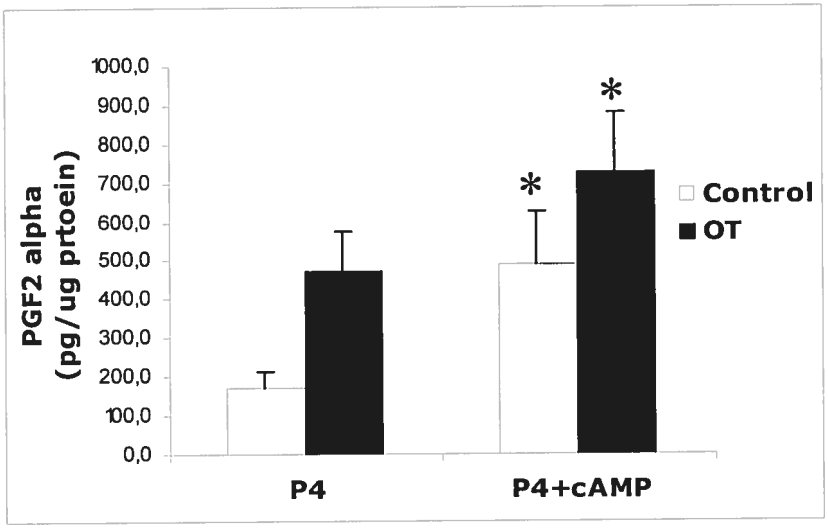

Figure 25: The effect of cAMP (1mM) on the release of $PGF_2\alpha$ from the bovine endometrial epithelium. (*) Asterisks show significant differences from the corresponding P_4 treated groups.

Discussion:

Our results with HPLC and GC/Mass spectrometry showed that pregnenolone was almost completely metabolized by the endometrial epithelial cells during 24h and one of the metabolites was either 5α- or 5β- pregnanedione that is consistent with results from previous studies on the metabolism of P_4 in the ovariectomized rats (191). There are other reports on P_4 metabolism in the human and rat indicating that uterine tissues contain mRNA and protein for 5β-reductase and for pregnane X receptor (PXR) (192). PXR regulates the expression of a network of hepatic and intestinal genes that encode the Phase I cytochrome P450 (CYP) enzymes, Phase II conjugating enzymes,

and drug transporters (193, 194). Acute in vitro treatment with 5β-dihydroxyprogesterone (5β-DHP) causes rapid uterine relaxation that is not mediated by PXR. Chronic in vivo administration of 5β-DHP to mice with intact PXR, but not in mice with disrupted PXR, causes an increased effect of 1400W, a specific inhibitor of inducible nitric oxide synthase (iNOS). Indeed, iNOS produces the potent smooth muscle relaxant nitric oxide. This suggests that 5β-DHP increased iNOS-modulated uterine tone, as occurs during pregnancy. It was concluded that metabolites of progesterone may act chronically through a PXR-mediated mechanism to regulate uterine contractility (192).

In all of our experiments, oxytocin stimulated $PGF_2\alpha$ secretion from bovine endometrial epithelial cells taken from cows at Days 1–3 of the estrous cycle, as previously reported (111, 132). Xiao et al., showed that OT increased the steady-state level of COX-2 mRNA, measured by Northern blot, which was maximal at 3 h (9-fold increase) and then decreased with time (80). OT also caused an increase in COX-2 protein, which peaked at 12 h (11-fold increase), as measured by Western blot. Moreover, other results showed that oxytocin stimulation of $PGF_2\alpha$ release by endometrium incubated in vitro increases as estrus approaches, reaching a maximum on the day of estrus. It was suggested that the high concentration of oxytocin receptors present in the Day 0 tissue permits oxytocin to recruit a much larger fraction of the total biosynthetic capacity of the tissue (37). Meyer et al. (195) reported an increase in oxytocin receptors during estrus in cattle, suggesting that the tissue may be more sensitive to oxytocin stimulation during this period. The intracellular mechanism involved in the stimulation of $PGF_2\alpha$ secretion appears to involve the protein kinase C effector pathway (111).

The results of the present study indicated that exposure to progesterone enhanced responsiveness of epithelial cells from bovine endometrium to OT. Such an effect of progesterone could be because of the fact that progesterone priming is necessary to induce the responsiveness of the bovine uterus to estradiol and oxytocin (51, 196, 197). Therefore, progesterone may directly affect $PGF_2\alpha$ synthesis in the bovine uterus, but estradiol may only modulate this process. Asselin et al reported that in epithelial cells, the basal production of $PGF_2\alpha$ was increased by P_4 (32). Although

progesterone stimulated basal $PGF_2\alpha$ production in bovine endometrial cells, it did not increase the expression of either COX-2 or PGFS mRNA (188). Therefore, the stimulatory action of progesterone on basal $PGF_2\alpha$ production may be associated with post-translational processes, i.e., progesterone may simply regulate the activity of COX-2 or PGFS proteins (196). Prolonged exposure to progesterone appears to promote uterine accumulation of arachidonic acid, prostaglandin endoperoxide synthase, and other substances needed for synthesis of $PGF_2\alpha$ that contributes to the regulation of $PGF_2\alpha$ secretion (45). Thus the increase in $PGF_2\alpha$ secretion in P_4-treated cells may be due to an increase in enzymes other than COX or PGFS. Our data is in agreement with the notion that isolated endometrial epithelial cells respond in a similar way to that observed in vivo and that interactions with other cell types within the endometrium are not essential for this response. In many systems prior exposure to E_2 is a determinant of the effects of P_4 and, although our cells were not exposed to E_2 in vitro before incubation with P_4, they would have been exposed to E_2 in vivo before removal of the cells.

In vivo, P_4 is responsible for the initial inhibition of the response to OT at the beginning of the estrous cycle. This effect was not observed in the present study, nor in other studies in vitro, and is possibly due to the spontaneous up-regulation of the response to OT when endometrial tissue is removed and incubated in vitro (113, 197). The factor(s) involved in this down-regulation of OTR in vivo remain to be further elucidated. Some preliminary studies have shown that RU 486 treatment of endometrial epithelial cells was able to decrease OTR and the OT-stimulated $PGF_2\alpha$ secretion (198). The main goal of this project was to elucidate the mechanism of action of RU 486, which would give important insight into the mechanism of action of progesterone in the endometrium. Our initial hypothesis was that the effect of P4 observed in vivo was not direct but an effect of a metabolite that was mimicked by RU 486. RU 486 can act as an antagonist or as a partial agonist depending on the tissue. To test this, the possible mechanism of P_4 and P_5 by endometrial tissue was examined.

The inability of progesterone and its metabolites to reduce OTR concentration and prostaglandin secretion in our experiments might be because our cells were not

treated with estradiol and so there was no combined action of P_4 and E_2 after removal of the cells from in vivo conditions. Thus, we may conclude that progesterone controls the $PGF_2\alpha$ secretory capabilities of the endometrium without necessarily upregulating OTR expression and probably, there might be other inhibitory factors that prevent progesterone from upregulating OTRs. In this regard, OTR regulation is partly due to gene suppression in vivo. Despite the presence of steroid receptors in isolated bovine endometrial cells, the level of OT receptor mRNA could neither be affected by progesterone or estradiol nor by a progesterone withdrawal protocol. The only factor that affected the OT receptor mRNA level was interferon-τ. As in vivo, this cytokine suppressed the OT receptor mRNA production (114). Therefore, the lack of any significant change in the concentration of OTRs after treatment of the cells with P_4 and its metabolites might be an indication of absence of any significant change in the OTR mRNA in these cells. Since regulation of uterine oxytocin receptor expression is multifactorial, and does not necessarily rely on gonadal steroids (199), the complex regulatory mechanisms of P_4 remain to be elucidated by further studies. It appears from our results that at least one of the metabolites of progesterone (5α- or 5β-pregnanedione) produced in the endometrium does not affect OTR and prostaglandin synthesis. Thus, it is unlikely that changes in P_4 metabolism during the estrus cycle are the cause of the observed changes in OTRs.

To try to determine if RU 486 plays the role of an antagonist or a partial agonist, the effect of RU486 treatment was compared with that of a pure antiprogesterone, ZK 137316. The present results showed that in bovine endometrial epithelial cell cultures both antiprogesterones increased $PGF_2\alpha$ concentration significantly with the highest effect at a concentration of 1μM of RU 486 and ZK 137316. This is in agreement with the results of the experiments conducted by Bouftila and Clabaut who assessed the effects of RU 486 at mid-pregnancy in the rat and reported that $PGF_2\alpha$ levels increased in both myometrium and uterus (200). Swahn and Bygdeman showed that treatment of rats with RU 486 suppressed progesterone action leading to increased $PGF_2\alpha$ levels in both myometrium and entire uterus (201). Moreover, our result is consistent with the study of Peplow. He treated ovariectomized rats with 2 antiprogesterone steroids (RU 486 and ZK 98299) and their uterine explants in

culture were found to produce significantly greater amounts of $PGF_2\alpha$ when compared to controls (202). 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 (162). 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 (166). As noted by Larcher *et al.* 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 posttranslational level (203). Therefore, it is concluded that RU 486 played the role of an antagonist. Our findings also show that the effect of the P_4-antagonists varies with dose and higher doses inhibit $PGF_2\alpha$ synthesis and OT-stimulated $PGF_2\alpha$ secretion. We suppose that the antagonistic effect of RU 486 will inhibit the suppressive effect of P_4 on the OTR expression and so OTR will be upregulated, but as we did not observe this result in our experiments with RU 486, the following possibilities might explain the observed downregulation of the OTR: 1- OTR has been upregulated but there is another inhibitory factor that reduced the number of OTRs in the endometrial cells. 2- °It is known that despite the 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 P4 on uterine OTR gene expression is mediated by an indirect genomic or a nongenomic mechanism (203). Further research in the uterus at the molecular level is needed.

Another possible pathway for the observed increase in the synthesis of $PGF_2\alpha$ in our endometrial cells is via activation by cAMP, so we treated our cells with cyclic nucleotide analog, dibutyryl cAMP at a concentration of 1 mM. As shown in the results, cAMP enhanced the production of $PGF_2\alpha$ and the oxytocin-stimulated secretion of $PGF_2\alpha$ in the presence of P_4. This means that in addition to our observation of the stimulating effect of P_4 on the release of $PGF_2\alpha$ and OT-stimulated $PGF_2\alpha$. Activation of PKA leads to further stimulation of $PGF_2\alpha$ secretion from the

endometrial epithelial cells of the bovine uterus. The cAMP activates PKA that in turn stimulates the expression of specific genes by phosphorylating a transcriptional activator called the cAMP-response element binding protein (CREP) (204). Tsai et al. evaluated the in vivo and in vitro regulation and temporal expression of messenger RNA for PGHS-2 and two specific prostaglandin receptors, $PGF_2\alpha$ receptor (FP receptor) and PGE receptor EP3 subtype (EP3 receptor), in bovine preovulatory follicular cells and luteal cells. In vitro culture of bovine granulosa cells using hCG or forskolin (an activator of adenylate cyclase) demonstrated that induction of FP receptor mRNA was mediated through PKA. PGHS-2 was acutely (< 12 h) increased by PKA and to a lesser extent by PKC. They also showed that PKA stimulates PGHS-2 and FP receptor mRNA by distinct mechanisms (205). Thus, we propose that the same pathway exists in uterine epithelial cells.

Studies on the mechanism of action of RU 486 have clearly shown that RU 486 is a partial agonist of androgen receptors and induces a conformational change of the AR-LBD different from that induced by other antiandrogens and androgens. Interestingly, also the ligand-dependent interaction between the AF-2 region in the LBD with the NH_2-terminal domain as measured in an in vitro pull-down assay is strongly agonist-dependent and does not occur with RU 486. It can be concluded that the different transcriptional activities displayed by either full agonists (testosterone, DHT, methyltrienolone), partial agonists (RU 486 and CPA) or full antagonists (hydroxyflutamide, bicalutamide) are the result of recruitment of a different repertoire of co-regulators (coactivators or co-repressors) as a consequence of these conformational changes. The differential recruitment of co-regulators can be considered as special form of ligand-selective modulation of the AR-LBD and can be applied in broader sense also to the tissue-selective modulation of androgen action, where levels of coactivators and co-repressors may ultimately determine the final activity (165). The observed stimulatory effect of DHT on $PGF_2\alpha$ synthesis in our cell cultures is possibly via binding of this androgen to the androgen receptors. Androgen receptors are present in the bovine endometrium and thus it is possible that androgens play a role in the regulation of $PGF_2\alpha$ synthesis. However, the stimulatory effect of DHT was only seen at relatively high doses, therefore may not be physiological. We

did not observe any significant increase in OTR concentration in our epithelial cells following their treatment with DHT.

It has been clearly demonstrated that an increase in PLA_2 activity is associated with $PGF_2\alpha$ production from the uterus in many species (6, 95, 206). Indeed, the stimulatory actions of both OT and noradrenaline (NA) were completely reduced by ACA (a PLA_2 inhibitor) in a study conducted by Skarzynski et al. (96). These findings suggest that PKC may affect PLA_2 activity. It is well known that the activation of PLA_2 is induced by increases of intracellular calcium concentration and of PKC activity (207). In contrast, the interaction between PKA and PLA_2 is not well understood. On the other hand, it is well established that PLA_2 stimulates intracellular arachidonic acid accumulation. Therefore, the failure of NA and OT to stimulate $PGF_2\alpha$ production in endometrial cells treated with ACA might be due to a lower accumulation of arachidonic acid, as a precursor of $PGF_2\alpha$, in the cells. Moreover, Kotwica et al (208) showed the possible actions of NA on endometrial $PGF_2\alpha$ output in heifers during luteolysis. Their experiment showed that when luteolysis was in progress, NA released OT and later increased PGF secretion. This may be a direct influence of NA on the uterus. NA was found to affect PGF release from the human uterus before and during ovulation (209, 210) and from the human deciduas (211).

In rats, catecholamines increase PGF secretion via β-adrenergic receptors (212). Moreover, blocking both α- and β-adrenergic receptors in the human uterus decreases catecholamine-induced PGF release (213).

NA affects $PGF_2\alpha$ production via the cAMP-PKA pathway (96). However, indirect actions of NA on PGF secretion by OT release are also possible (208). Previous experiments detected β-adrenergic receptors in bovine myometrium (214). Later there were reports indicating that NA and the stimulation of β-adrenergic receptors enhanced $PGF_2\alpha$ production from cultured bovine endometrial cells. These findings together with reports indicating that NA strongly stimulates P_4, OT, $PGF_2\alpha$ and PGE_2 secretion (96, 23, 215) support our conclusion that $PGF_2\alpha$ production is regulated by not only OT but also one or more other factors such as NA and/or cAMP.

The stimulation of $PGF_2\alpha$ release from bovine endometrial cells by forskolin implies that cAMP and PKA play important roles in regulating the endometrial release of

$PGF_2\alpha$. It is therefore assumed that PKA might mediate the stimulatory effect of NA on $PGF_2\alpha$ production. This supposition that the cAMP-PKA pathway is important in NA-stimulated $PGF_2\alpha$ production by bovine endometrial cells is supported by the results of some of the experiments conducted by Skarzynski et al. Rp-cAMP (a PKA inhibitor) completely inhibited NA-induced, but not OT-induced $PGF_2\alpha$ production. Furthermore, U-73122 (a PLC inhibitor) suppressed only the $PGF_2\alpha$ production stimulated by OT. These results suggest that the stimulatory effects of NA and OT on $PGF_2\alpha$ production may be mediated via separate signaling pathways: NA affects $PGF_2\alpha$ production via the cAMP-PKA pathway (96). Therefore, it is likely that the observed increase in the concentration of $PGF_2\alpha$ after the treatment of our cells with cAMP is due to the stimulation of PLA_2 activity via activation of PKA. However, cAMP treatment did not alter the OT-stimulation of $PGF_2\alpha$. Therefore, it is unlikely that the concentration of OTRs is influenced via the cAMP-PKA pathway.

The dose-dependent blockade of the synthesis of $PGF_2\alpha$ and OTRs under the effect of indomethacin, a non-selective COX-inhibitor (216) in our cells shows the inhibitory effect of indomethacin on COX enzymes that leads to decreased synthesis of $PGF_2\alpha$ by our cells. However, the inhibition by indomethacin was not as great as expected and higher concentration of indomethacin may be needed in endometrial cells for inhibition of prostaglandin synthesis. The treatment of the cells with indomethacin also resulted in a decrease in OTR. We postulate that a paracrine effect of $PGF_2\alpha$ on the endometrial epithelial cells is able to modulate OTR numbers.

Conclusion:

1- Bovine endometrial epithelial cells are able to metabolize progesterone.

2- Progesterone increases $PGF_2\alpha$ synthesis by the endometrial epithelial cells by increasing the responsiveness of endometrial cells to OT stimulation without a significant effect on the endometrial concentration of OTRs.

3- The antiprogesterones, RU 486 and ZK137316, both significantly ($P<0.05$) stimulated $PGF_2\alpha$ synthesis by endometrial cells and this stimulation was the strongest at the concentration of 1μM, but they both downregulated OTRs in the bovine endometrium, so we conclude that besides OT and OT stimulation there are other factors responsible for the increased synthesis of $PGF_2\alpha$.

4- Treatment of endometrial cells with the potent androgen DHT increased the secretion of $PGF_2\alpha$ without any significant increase in OTR concentrations. Because of the fact that androgen receptors are present in the bovine endometrium, it is possible that androgens play a role in the regulation of $PGF_2\alpha$ synthesis.

5- The treatment of endometrial cells with cAMP increased $PGF_2\alpha$ synthesis. These results reveal the existence of another pathway through activation of PKA by cAMP. So, OT is not the only stimulant of $PGF_2\alpha$ synthesis in the bovine endometrial epithelial cells.

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