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EFFECT OF INTERFERON-τ AND STEROID HORMONES ON THE EXPRESSION OF GENES INVOLVED IN THE PROSTAGLANDIN SYNTHESIS IN BOVINE ENDOMETRIAL CELLS

PAR

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SOMMAIRE

La lutéolyse et l'inhibition de la lutéolyse sont deux événements importants chez la vache qui cycle et la vache gravide. La lutéolyse est la destruction du corps jaune par l'intermédiaire de la sécrétion de prostaglandine (PG) par l'endomètre utérin. La synthèse des PG endométriales pendant le cycle oestral est sous la commande de l'estradiol (E2) de la progestérone (P4) et de l'ocytocine (OT). Chez l'animal gravide, l'interféron-τ (IFN-τ), qui est sécrété par l'embryon, inhibe la synthèse de PG. Cependant les mécanismes en jeu dans la régulation de la synthèse de PG sont mal connus au niveau cellulaire. Afin d'approfondir nos connaissances à cet égard, nous avons utilisé dans le cadre de ces études un système de culture primaire des cellules épithéliales et stromales. Nous avons postulé que 1) les hormones stéroïdiennes et l'interféron-τ (IFN-τ) ont des effets particuliers sur les différentes populations de cellules endométriales, 2) les hormones stéroïdiennes, l'OT et l'IFN-τ agissent sur la voie de synthèse des PG plus particulièrement sur la régulation de l'expression de la COX-2 et de la PGFS. L'objectif de cette étude était de mettre sur pied un système de culture primaire qui convienne aux cellules endométriales et qui soit sensible aux hormones stéroïdiennes, à l'ocytocine et à l'IFN-τ, d'examiner l'effet des hormones stéroïdiennes sur les récepteurs de l'E2 et de la P4 et enfin d'étudier les mécanismes par lesquels l'embryon modifie le rapport de la $PGF_{2\alpha}$ et de la PGE2.

Dans la première expérience, les effets des hormones stéroïdiennes sur la prolifération et la morphologie des cellules endométriales ont été examinés. L'ajout de P4 a altéré la morphologie des cellules stromales alors que l'ajout d'une combinaison de E2 et de P4 a augmenté la prolifération des cellules stromales, mais diminué le rapport protéine/ADN chez ces cellules pendant les 4 premiers jours. La morphologie des cellules épithéliales n'a pas été altérée par

l'ajout de stéroïdes. La prolifération de cellules épithéliales a été inhibée par l'ajout de E2 ($p<0,01$), mais pas par l'ajout de P4 ($p>0,05$). Les résultats ont démontré que l'E2 et la P4 avaient des effets différents sur la prolifération et la morphologie des cellules épithéliales et stromales *in vitro*.

Dans la deuxième expérience nous avons étudié l'effet des hormones stéroïdiennes sur le nombre de récepteurs. Les cellules ont été mises en culture pendant 4 ou 8 jours dans un milieu de culture additionné ou non de E2, P4 ou de E2+P4. L'analyse de saturation a démontré la liaison spécifique de la ^{3}H ORG 2058 et de ^{3}H E2 aux cellules épithéliales et stromales présentait de fortes affinités. Dans les cellules stromales, le E2 a augmenté la quantité de récepteurs de E2 (ER) et la quantité de récepteurs de P4 (PR) après 4 jours de culture ($p<0,01$), de manière proportionnelle à la dose et au temps. Utilisée seule, la P4 n'a eu aucun effet sur les ER ou les PR ($p>0,05$), cependant elle a inhibé l'effet stimulant de la E2. Chez les cellules épithéliales, la plus faible dose de E2 utilisé (0,1, 1 nM) a provoqué un accroissement du nombre de PR ($p<0,01$) après 4 jours de culture alors que la plus forte dose de E2 (10 nM), de P4 ou de P4+E2 (1 nM) utilisée n'a eu aucun effet. Par contre, le nombre d'ER a augmenté avec les concentrations de E2 ($p<0,01$). Ces résultats démontrent que le nombre de PR était plus élevé chez les cellules stromales que chez les cellules épithéliales alors que le nombre d'ER était plus élevé chez les épithéliales que chez les cellules stromales. E2 exerce une régulation en amont ('upregulation') de ces propres récepteurs et accroît le nombre de PR dans les deux types cellulaires *in vitro* alors que la P4 seule a plus d'effets mais inhibe l'effet de l'E2 sur les PR.

Dans la troisième expérience, l'effet des hormones stéroïdiennes et de l'interféron-τ bovin recombinant (rbIFN-τ) sur la production de PG et de cyclooxygénase-2 (COX-2) ainsi que sur l'expression du gène de la PGF synthétase (PGFS) ont été étudiés. L'E2 entraîne une diminution de la $PGF_{2\alpha}$ et

de la PGE2 alors que la P4 provoque un accroissement de la sécrétion de $PGF_{2\alpha}$ dans les cellules épithéliales. Les hormones stéroïdiennes n'ont eu aucun effet sur la production de PG dans les cellules stromales. Le rbIFN-τ a atténué la production de $PGF_{2\alpha}$ et PGE2 dans les cellules épithéliales et a accru leur production et leur rapport PGE2:$PGF_{2\alpha}$ dans les cellules stromales. Le transfert de Northern a permis de démontrer que l'E2 et le rbIFN-τ a amené une diminution de l'ARNm de COX-2 dans les cellules épithéliales. Inversement le rbIFN-τ a provoqué un accroissement de l'ARNm de COX-2 dans les cellules stromales. En outre, le rbIFN-τ a amené une diminution de l'ARNm de la PGFS dans les deux populations cellulaires, diminution qui était associée à une augmentation du rapport PGE2:$PGF_{2\alpha}$. Ces résultats démontrent que la régulation de la synthèse des PG par les hormones stéroïdiennes se déroulent différemment dans les cellules épithéliales et stromales du moins *in vitro*. L'inhibition de la sécrétion de $PGF_{2\alpha}$ par les cellules épithéliales et l'augmentation de la production de PGE2 par les cellules stromales par l'intermédiaire du rbIFN-τ est modulé par les hormones stéroïdiennes.

Dans la quatrième expérience, l'effet du rbIFN-τ sur le nombre de récepteurs de l'OT (OTR), la production de PG induite par l'OT et l'expression de la COX-2 et de la PGFS dans les cellules épithéliales endométriales en culture a été étudié. Les cellules épithéliales confluentes ont été incubées en présence ou en l'absence de 100 ng/ml d'OT ou d'OT plus 100 ng/ml de rbIFN-τ pendant 3, 6, 12 ou 24 heures. Le rbIFN-τ a inhibé la production de PG induite par l'OT et a réduit la fixation à l'OTR. L'OT a amené un accroissement de l'équilibre (steady-state level) de l'ARNm de COX-2 et a également provoqué l'augmentation de la production de protéines COX-2. Le rbIFN-τ à inhibé l'induction d'ARNm de COX-2 (89%, $p<0,01$) et la production de protéines COX-2 (50%, $p<0,01$) par l'OT et a amené une diminution de la production de PG induite par la phorbol 12-myristate 13-acetate (PMA). Nous avons conclu que l'inhibition par le rbIFN-

τ de la production de PG induite par l'OT serait due à une régulation en aval ('downregulation') de l'OTR, de la COX-2 et de la PGFS et que la diminution de COX-2 ne serait pas uniquement imputable à la diminution du nombre d'OTR.

En conclusion, ces résultats démontrent que l'E2 et la P4 ont des effets différents sur la régulation de la prolifération, sur la morphologie, sur le nombre de récepteurs de la PG et sur la sécrétion de cette hormone dans les différentes populations cellulaires. *In vitro,* l'IFN-τ a recours à deux mécanismes pour prévenir la lutéolyse. Il stimule la sécrétion de l'agent lutéoprotecteur, la PGE2, par les cellules stromales et inhibe la production de la lutéolysine, la $PGF_{2\alpha}$ par les cellules épithéliales. L'observation la plus importante qui ressort de ces études est que les différentes populations de cellules endométriales répondent de manière différente aux hormones stéroïdiennes OT et IFN-τ et que l'interaction entre ces types de cellules pourrait être nécessaire au fonctionnement normal des cellules épithéliales. Les résultats démontrent également que les hormones stéroïdiennes et l'IFN-τ peuvent altérer l'expression des enzymes en jeu dans la synthèse des PG. Cependant, on ne sait toujours pas si l'effet de l'IFN-τ sur la sécrétion de PG passe par d'autres facteurs comme l'oxyde nitreux ou les facteurs de croissance. Par conséquent, la mise au point d'un système adéquat de coincubation des cellules épithéliales et stromales est toujours nécessaire pour arriver à élucider les interactions et les voies du signal de transduction en jeu dans l'action de l'IFN-τ.

Summary

Luteolysis and the prevention of luteolysis are two important events in the cyclic and pregnant cow. Luteolysis, destruction of the corpus luteum, is caused by prostaglandin (PG) secretion from the uterine endometrium. Endometrial PG synthesis during the estrous cycle is regulated by estradiol (E2), progesterone (P4) and oxytocin (OT). In the pregnant animal, interferon-τ (IFN-τ), secreted from the embryo, inhibits PG synthesis. However, the mechanisms involved in the regulation of PG synthesis are not well understood at the cellular level. To further our understanding, a primary culture system of uterine epithelial and stromal cells was used in these studies. We hypothesized that 1) steroid hormones and IFN-τ have differential effects on the different endometrial cell types, 2) steroid hormones, OT and IFN-τ act on the PG synthetic pathway, in particular regulating cyclooxygenase-2 (COX-2) and PG F synthase (PGFS) expression. The objectives of this study were to establish an appropriate primary endometrial cell culture system which could respond to steroid hormones, OT and IFN-τ to examine the effect of steroid hormones on the receptors for E2 and P4, and to study the mechanisms by which the embryo modifies the ratio of $PGF_{2\alpha}$ to PGE2.

The effects of steroid hormones on proliferation and morphology of endometrial cells were first examined. Addition of P4 altered the morphology of stromal cells. E2 and P4 increased the proliferation ($P<0.001$) and decreased the protein to DNA ratios in stromal cells during the first 4 days. In contrast, the morphology of epithelial cells was not influenced by the addition of steroids. However, the proliferation of epithelial cells was inhibited by the addition of E2 ($P<0.01$), but not P4 ($P>0.05$). These results show that E2 and P4

have different effects on the proliferation and morphology of epithelial and stromal cells *in vitro*.

The effects of steroid hormones on their receptor numbers was determined. Cells were cultured for 4 or 8 days in medium alone or with E2, P4 or E2+P4. Saturation analysis showed that specific binding of both 16α-Ethyl-21-hydroxy-19-nor (6,7-^{3}H) pregn-4-ene-3,20-dione ([^{3}H] ORG 2058) and [^{3}H] E2 to epithelial and stromal cells exhibited high affinities. In the stromal cells, E2 increased E2 receptor (ER) and P4 receptor (PR) numbers after 4 days' culture ($p<0.01$) in a dose- and time-dependent manner. P4 alone had no effect on either ER or PR numbers ($p>0.05$), however, it did inhibit the stimulatory effect of E2. In epithelial cells, the lower doses of E2 used (0.1, 1 nM) stimulated the PR number ($p<0.01$) after 4 days' culture, whereas, the highest dose of E2 used (10 nM), P4 alone and P4 plus E2 (1 nM) had no effect. In contrast to the PR, ER numbers increased with the increase of E2 concentrations ($p<0.01$). The results show that PR numbers were higher in the stromal than in epithelial cells, whereas ER numbers were higher in epithelial cells than in stromal cells. E2 upregulates its own receptor and increases PR in both cell types *in vitro*, P4 alone had little effect but inhibited the effects of E2 on PR.

The effects of steroid hormones and recombinant bovine IFN-τ (rbIFN-τ) on PG production and COX-2 and PGFS gene expression were investigated. E2 decreased both $PGF_{2\alpha}$ and PGE2 whereas P4 increased $PGF_{2\alpha}$ secretion in epithelial cells. Steroid hormones had no effect on PG production in stromal cells. rbIFN-τ attenuated both $PGF_{2\alpha}$ and PGE2 production in epithelial cells and enhanced their production, and the ratio of PGE2 to $PGF_{2\alpha}$, in stromal cells. Northern blot analysis show that E2 and rbIFN-τ decreased COX-2 mRNA levels in epithelial cells. Conversely, rbIFN-τ increased COX-2 mRNA in stromal cells. Furthermore, rbIFN-τ decreased PGFS mRNA in both cell types and this

was associated with the increase in PGE2/$PGF_{2\alpha}$ ratio. These results show that the regulation of PG synthesis by steroid hormones is different in endometrial epithelial and stromal cells *in vitro*. The attenuation of $PGF_{2\alpha}$ secretion from epithelial cells and increased PGE2 production in stromal cells by rbIFN-τ are modulated by steroid hormones.

The effect of rbIFN-τ on OT receptor (OTR) number, OT-induced PG production, COX-2 and PGFS expression in cultured endometrial epithelial cells was investigated. Confluent epithelial cells were incubated in the presence or absence of either 100 ng/ml OT or OT plus 100 ng/ml rbIFN-τ for 3, 6, 12 and 24 h. rbIFN-τ inhibited the OT-induced PG production and reduced OTR binding. OT increased the steady-state level of COX-2 mRNA, and also increased COX-2 protein. rbIFN-τ suppressed the induction of COX-2 mRNA (89%, $p<0.01$) and COX-2 protein (50%, $p<0.01$) by OT and decreased PMA-stimulated PG production. OT increased PGFS mRNA, and this stimulation was attenuated by rbIFN-τ ($p<0.01$). It was concluded that rbIFN-τ inhibition of OT-stimulated PG production is due to downregulation of OTR, COX-2 and PGFS and that the decrease in COX-2 is not solely due to a decrease in OTR number.

In conclusion, our results show that E2 and P4 have differential effects on the regulation of proliferation, morphology, receptor number, and PG secretion in the different endometrial cell populations. *In vitro*, IFN-τ has a dual mechanism in the prevention of luteolysis. It stimulates the secretion of the luteoprotective agent, PGE2, by stromal cell, and inhibits the production of the luteolysin, $PGF_{2\alpha}$, by epithelial cells. The most significant findings in these studies are that the different endometrial cell types have differential responses to steroid hormones, OT and IFN-τ, and the interaction between cell types might be necessary for the normal function of epithelial cells. The results show that the steroids and IFN-τ can affect the expression of the enzymes involved in

PG synthesis. However, it is still not known if the effects of IFN-τ on PG secretion are mediated by other factors (such as nitric oxide or growth factors). Therefore, a suitable coincubation system of stromal and epithelial cells is needed for the further elucidation of interactions and the signal transduction pathways involved in IFN-τ action.

AVANT-PROPOS (PREFACE)

Cette thèse comprend une introduction générale, qui renferme l'hypothèse de départ, une revue de littérature générale; 4 articles comprenant chacun une introduction, une section Matériel et méthodes, des résultats, une discussion et des références; une discussion générale ainsi qu'une conclusion générale.

This thesis comprises a general introduction, which presents the overall hypothesis for the study; a general literature review; four publishable articles, each of which contains specific introduction, materials and methods, results, discussion and references; a general discussion and general conclusion.

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2-5 [A]	2',5'-oligoadenylate
bGCP-2	Bovine granulocyte chemotactic protein-2
bIFN-τ	Bovine interferon-τ
BSA	Bovine serum albumin
bTP-1	Bovine trophoblast protein-1
CL	Corpus Luteum
COX	Cyclooxygenase
DEPC	Diethyl pyrocarbonate
E2	Estradiol-17β
EGF	Epidermal growth factor
ER	Estradiol receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hank's Buffered Saline Solution
HCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
IgG	Immunoglobulin Gamma
ICBSP	Interferon consensus sequence binding protein
IFN	Interferon

IL-1	Interleukin-1
IRE	Interferon response element
IRF	Interferon regulatory factor
ISGF3	IFN-stimulated gene factor-3
KGF	Keratinocyte growth factor
LF	Lactoferrin
LH	Luteinizing hormone
LPS	Lipopolysaccharide
LSIRF	Lymphoid-specific IRF
MDBK	Madin-Darby bovine kidney
NBCS	New-born calf serum
NO	Nitric oxide
NOS	Nitric oxide synthase
OT	Oxytocin
oTP-1	Ovine trophoblast protein-1
OTR	Oxytocin receptor
P4	Progesterone
PAF	Platelet activating factor
PGF2α	Prostaglandin F2α
PGFS	Prostaglandin F synthase
PGE2	Prostaglandin E2
PGH2	Prostaglandin H2
PGHS	Prostaglandin G/H synthase
PLA2	Phospholipase A2
PMA	Phorbol 12-myristate 13-acetate
PR	Progesterone receptor
rbIFN-τ	recombinant bovine interferon-τ

SDS	Sodium dodecyl sulfate
TGFα	Transforming growth factor-α
TNF	Tumor necrosis factor
UCRP	Ubiquitin cross-reactive protein
VSV	Vesicular stomatis virus

1. Introduction

Luteolysis and the prevention of luteolysis are two key events in the reproductive process of ruminants. The luteal regression at the late stage of the luteal phase in cyclic ruminants is caused by an episodic release of prostaglandin (PG) $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterine endometrium, which is evoked by oxytocin (OT) after binding to specific endometrial receptors (Bazer *et al.*, 1991). OT receptor (OTR) number, which is regulated by ovarian steroid hormones [estrogen (E2) and progesterone (P4)], determines the sensitivity of uterine endometrium to OT stimulation. The minor increase in OTR numbers at the late stage of the luteal phase is believed to be responsible for initiating the pulsatile release of $PGF_{2\alpha}$ from the endometrium (Mann and Lamming, 1994; Lamming and Mann, 1995). E2 increases, and P4 inhibits, receptors for E2, P4 and OT. Prolonged (approx. 10 days) exposure of endometrium to high levels of P4 completely inhibits P4 receptors (PR) and thus releases the inhibitory effect of P4 on E2 receptor (ER) numbers ('progesterone block'). Without the P4 inhibition, receptors for E2 and OT increase in response to E2 (McCracken *et al.*, 1984).

In pregnant ruminants, the establishment of pregnancy requires that the conceptus modify the secretion of $PGF_{2\alpha}$ from the endometrium, so that regression of the corpus luteum (CL) does not occur and P4 secretion is maintained. P4, the hormone of pregnancy, is responsible for maintaining endometrial functions that permit early embryonic development, implantation and successful fetal/placental development. The luteolytic release of $PGF_{2\alpha}$ during late diestrus is reduced in the presence of a conceptus (Hooper *et al.*, 1986; Zarco *et al.*, 1988). Reduced responsiveness of endometrium to OT during

early pregnancy (McCracken, 1980; Fairclough *et al.*, 1984; Mirando *et al.*, 1990a; Mirando *et al.*, 1990b) is attributed to an inhibition of the increase in endometrial ER and OTR (McCracken *et al.*, 1984; Sheldrick and Flint, 1985) and also probably to the post-receptor mechanisms.

Type-I trophoblast interferon-τ (IFN-τ) is the antiluteolytic protein secreted by conceptuses of sheep and cattle during maternal recognition of pregnancy (Bazer *et al.*, 1991). Ovine IFN-τ (oIFN-τ) inhibits OT-induced endometrial $PGF_{2\alpha}$ secretion (Vallet *et al.*, 1988; Mirando *et al.*, 1990a) when infused into the uterine lumen of cyclic ewes. Treatment with natural or recombinant bovine IFN-τ (bIFN-τ) reduced the OT-induced secretion of $PGF_{2\alpha}$ and PGE2 by cultured bovine endometrial epithelial cells (Danet-Desnoyers *et al.*, 1994; Asselin *et al.*, 1997). However, the complete mechanism whereby IFN-τ inhibits endometrial responsiveness to OT and prevents CL regression during early pregnancy is not well known.

Overall, what is presently believed to occur during luteolysis and early pregnancy is as follows. E2 increases the numbers of PR, ER and OTR, while P4 decreases them. During the first 10 days of the luteal phase, P4 keeps the ER number low. At the late stage of luteal phase, with the completion of P4 block, ER number increases and subsequently OTR number increases due to the stimulation by E2. The endometrium becomes sensitive to OT, $PGF_{2\alpha}$ secretion by uterus increases in response to OT and luteolysis is initiated. In the pregnant cow, IFN-τ attenuates $PGF_{2\alpha}$ secretion by inhibiting ER and OTR expression. However, the effect of steroid hormones and IFN-τ on the post-receptor mechanisms of PG synthesis, such as regulation of PG G/H synthase (or cyclooxygenase, COX) and PG F synthase (PGFS) are not well understood. Furthermore, due to the complex interactions *in vivo*, the respective roles of the

different uterine endometrial cells in the initiation and prevention of luteolysis have not been clarified. This study will establish an appropriate primary endometrial cell culture system and examine for the first time the regulation of gene expression involved in prostaglandin synthesis by steroid hormones and embryonic signal, IFN-τ. The information gained will be important to the understanding of the pathophysiology of pregnancy establishment and maintenance failure and provide new strategies for improvement of domestic animal reproduction.

2. Literature Review

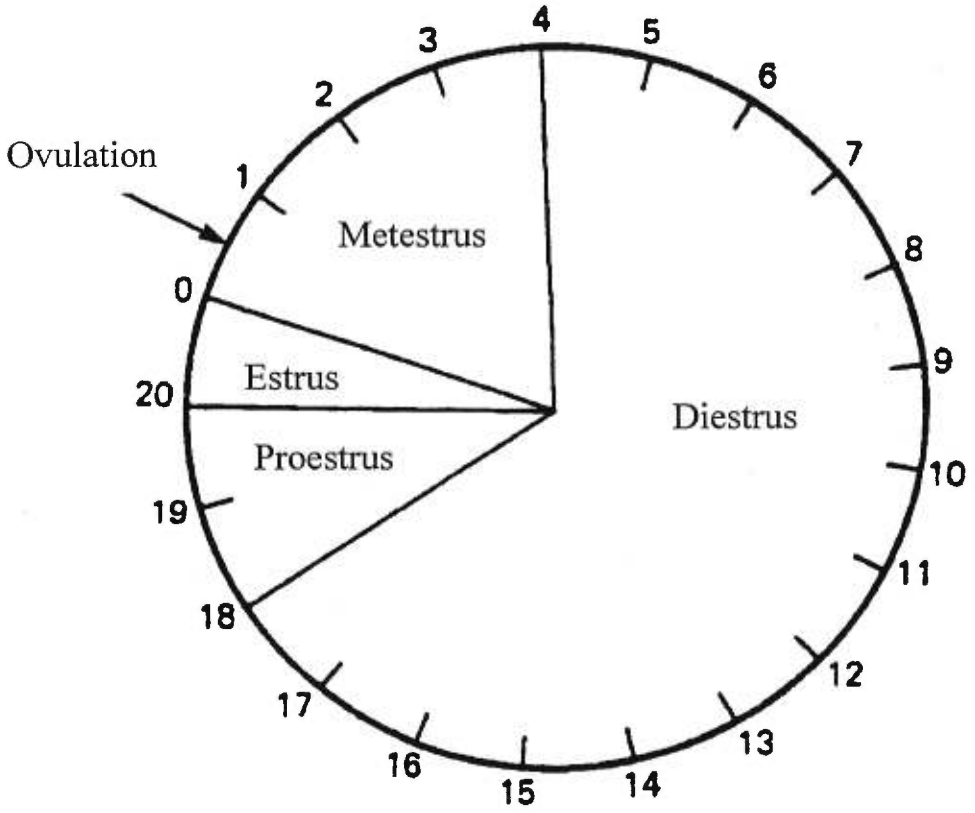
2.1. Estrous cycle

The cow is a polyestrous animal, once estrous cycles are established they continue indefinitely, unless interrupted by pregnancy. The average length of the estrous cycle in the cow is 21 days and is classically divided into four phases (Fig. 1): estrus (Day 0), the period of sexual receptivity; followed by metestrus (Days 1-4), the postovulatory period; diestrus (Days 5-18) corresponds to the luteal phase and pro-estrus (Days 18-20) is the period just prior to estrus. Estrus marks the beginning of each estrous cycle and lasts about 7 h (6-30 h) (Peters and Ball, 1987).

Various steroid hormones are secreted by the bovine ovary, the most important of which are the estrogens and P4. The principal biologically active estrogen is estradiol-17β (E2), which is produced by the granulosa cells of the follicle (Hay and Moor, 1978). Concentrations of E2 are low in peripheral plasma for most of the estrous cycle (Fig. 2). They rise during the four days before estrus reaching a peak on the day of, or the day before, standing estrus (Glencross and Pope, 1972; Glencross *et al.*, 1973). A secondary peak in E2 concentrations occurs on Day 6 of the cycle and appears to be related to the presence of a large follicle (Glencross *et al.*, 1973; Ireland and Roche, 1983). P4 is secreted by the cells of corpus luteum. Plasma P4 concentrations begin to rise from about Day 4 of the cycle, reaching a peak around Day 8 and remaining high until Day 17 (Fig. 2). Concentrations then decline to below 1 ng/ml before the next estrus and ovulation.

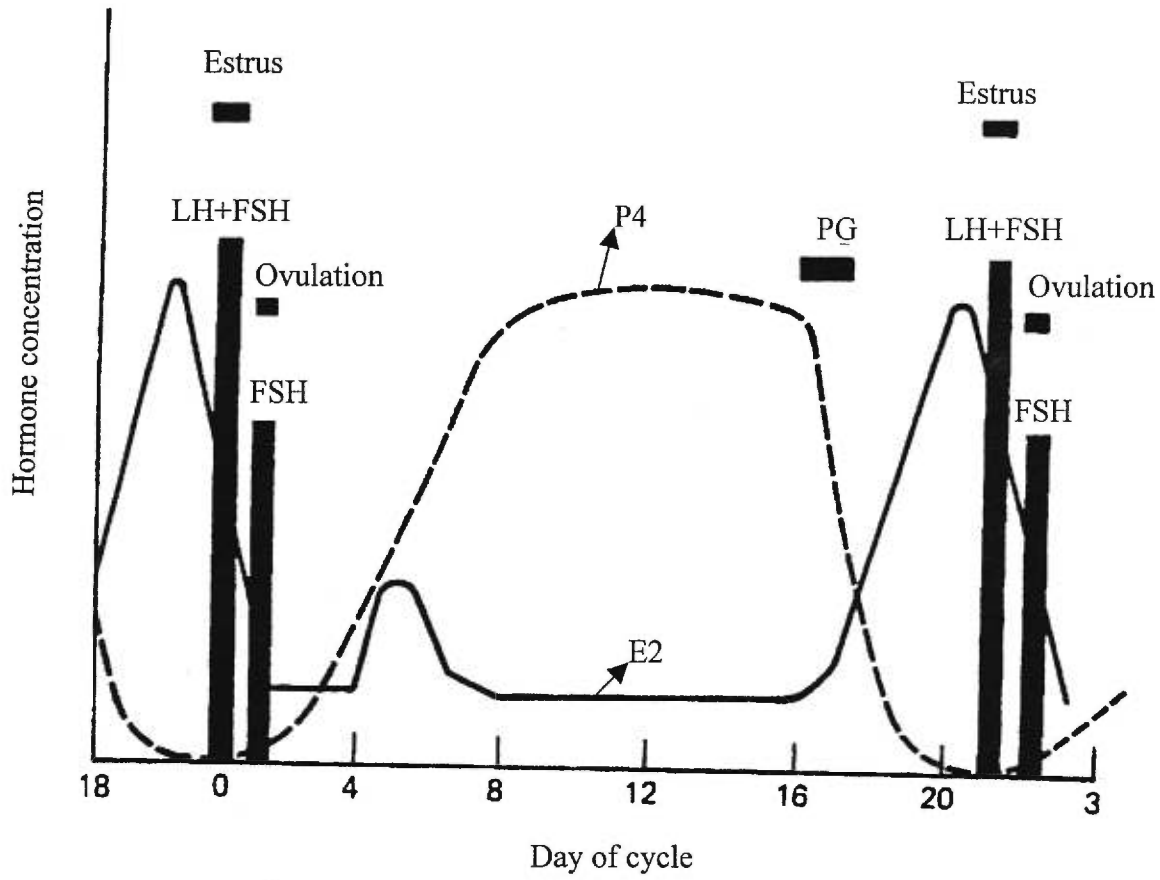
2.1.1. Ovulation and formation of the corpus luteum

Fig.1. The four stages of the estrous cycle in cow. Adapted from AR Peters and PJH Ball (1987)



Proestrus+Estrus=follicular phase
Metestrus+Diestrus=luteal phase

Fig. 2. Changes in blood plasma hormone concentrations during the bovine estrous cycle (schematic): ___ , estradiol; _ _ _ _ , progesterone. Adapted from AR Peters and PJH Ball (1987).

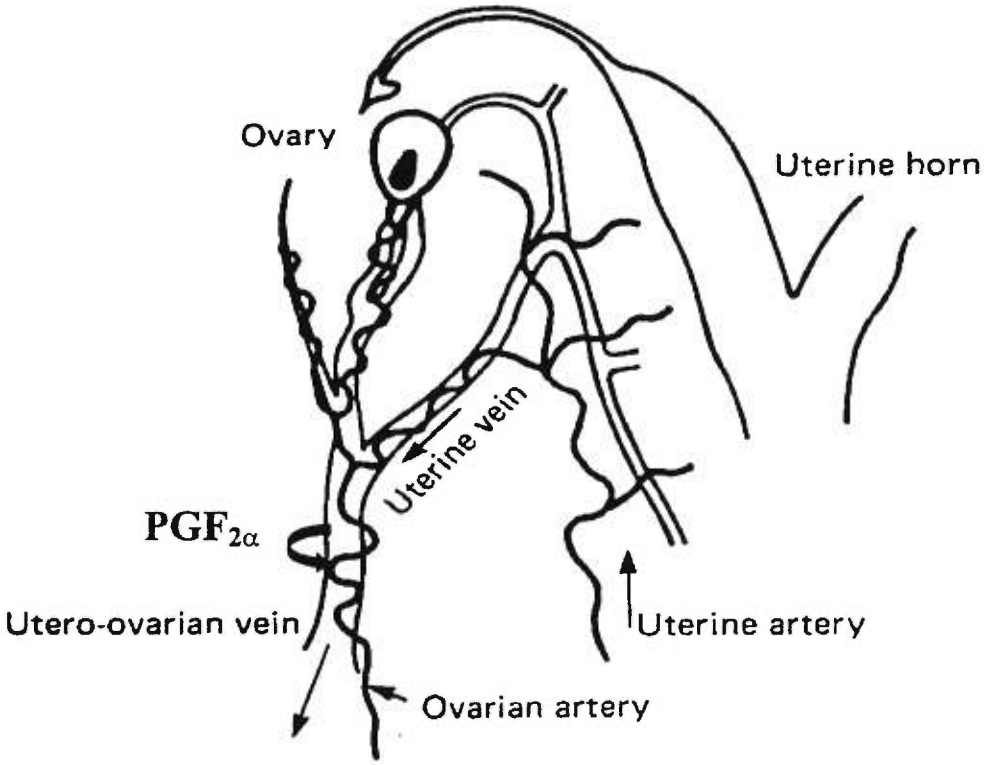

Estrus
Estrus
LH+FSH
Ovulation
FSH
P4
PG
LH+FSH
Ovulation
FSH
E2
Hormone concentration
18
0
4
8
12
16
20
3
Day of cycle

Cows ovulate spontaneously 12-15 h after the onset of estrus in response to an E2-induced discharge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. After ovulation and during metestrus, the cavity of the ovulated follicle is invaded by cells derived from the granulosa and theca interna layers of the follicle. These cells are large and termed luteal cells and are richly supplied with blood vessels. The structure usually protrudes from the surface of the ovary, is yellow-brown in colour and is known as the corpus luteum (CL). This process is termed luteinization (Schwall *et al.*, 1986). The CL begins to secrete P4 on about day 4, marking the beginning of diestrus, and reaches its maximum size and activity between days 7 and 14. The CL persists on the surface of the ovary until a few days before the next ovulation, when it begins to degenerate rapidly, a process known as luteolysis (Peters and Ball, 1987).

2.1.2. Luteolysis and luteolysin

Luteolysis is essential for the normal estrous cycle and is uterus-dependent in ruminants. Removal of the uterus (hysterectomy) results in the prolongation of the luteal phase (Wiltbank and Casida, 1956) and each uterine horn exerts its luteolytic effect on the ipsilateral corpus luteum only. This is because the uterus-derived $PGF_{2\alpha}$ was shown to be transferred to the ovary by a countercurrent exchange system. $PGF_{2\alpha}$ is released from the endometrium into the uterine vein, the ovarian artery is closely associated with the utero-ovarian vein and there is diffusion of $PGF_{2\alpha}$ from the uterine vein to the ovarian artery and hence to the CL (Fig. 3) (McCracken *et al.*, 1972; McCracken *et al.*, 1973). If the ovary and uterus are surgically separated, then the life span of the CL is prolonged (Hixon and Hansel, 1974). However, the identity of the luteolytic

Fig. 3. Utero-ovarian vasculature in the sheep. $PGF_{2\alpha}$ is believed to leave the uterus via the uterine vein and is transferred to ovarian arterial blood by diffusion through the walls of the utero-ovarian vein and ovarian artery. Adapted from AR Peters and PJH Ball (1987).


Ovary
Uterine horn
Uterine vein
$PGF_{2\alpha}$
Utero-ovarian vein
Uterine artery
Ovarian artery

factor was not known until the observation of the luteolytic effect of $PGF_{2\alpha}$ in rat by Pharriss and Wyngarden (1969). This was confirmed in sheep by the intra-arterial infusion of $PGF_{2\alpha}$, which caused rapid luteolysis (McCracken *et al.*, 1972). $PGF_{2\alpha}$ is released as a series of episodic surges on days 14 to 15 of the ovine estrous cycle and can be detected in utero-ovarian plasma (Thorburn *et al.*, 1973). This episodic release of $PGF_{2\alpha}$ was subsequently confirmed by the detection of 13,14-dihydro-15-keto-PG $F_{2\alpha}$ (PGFM) in the peripheral blood of sheep around the time of luteolysis (Peterson *et al.*, 1976). In view of the pulsatile nature of $PGF_{2\alpha}$ secretion from the uterus at luteolysis, pulsed infusion of $PGF_{2\alpha}$, when given directly into the arterial supply of ovary of the sheep, was more effective than a single dose in causing complete CL regression (Schramm *et al.*, 1983).

Uterine-derived $PGF_{2\alpha}$ binds to receptors on luteal cells and initiates intracellular events which terminate production of P4 and induce cell death. Luteolytic effects of $PGF_{2\alpha}$ have been attributed to a: (1) decrease in luteal blood flow (Nett *et al.*, 1976; Niswender *et al.*, 1976); (2) reduction in the number of LH receptors (Fletcher and Niswender, 1982); (3) direct cytotoxic effect on luteal cells (Niswender *et al.*, 1985); (4) decreased utilization of lipoprotein cholesterol for P4 synthesis, including a suppression of luteal cholesterol synthesis (Pate and Condon, 1989); (5) reduced activity of the steroidogenic enzymes (Caffrey *et al.*, 1979; Torday *et al.*, 1980); (6) change in lipid composition and increased membrane fluidity (Riley and Carlson, 1985).

2.1.3. Changes in endometrium

During the estrous cycle, major histological and biochemical changes in the uterus takes place. These changes are believed to be regulated by ovarian

steroid hormones. The most striking changes occur in the endometrium and its glands. During the follicular phase of the cycle the uterine glands are rather simple and straight, with few branchings. This appearance of the glands is typical of E2 stimulation, and it can be duplicated by the injection of E2 into castrated females. Histological sections through E2-stimulated uterine endometria show a multitude of spaces, which are the lumina of the simple, nearly unbranched glands (Nalbandov, 1976).

During the luteal phase, when P4 is acting on the uterus, the endometrium increases in thickness conspicuously. The glands grow rapidly in diameter and length, becoming extremely branched and convoluted. There is a cyclic sloughing off and regeneration of the uterine endometrium, a process that seems to be hormone-controlled, just as in primates. Endometrial destruction and regeneration in cows involve no bleeding, possibly because only the epithelial layer of the endometrium is involved, and it takes place late in the luteal phase and is completed by the onset of the follicular phase (Nalbandov, 1976).

2.2. Regulation of $PGF_{2\alpha}$ secretion in uterine endometrium

Uterine endometrium is composed of two major cell populations, the epithelium and the stroma. The epithelium secretes mainly $PGF_{2\alpha}$ while the stroma produces mainly PGE2 (Fortier *et al.*, 1988). The pulsatile release of $PGF_{2\alpha}$ from the endometrium is induced by oxytocin (OT) and this response is mediated by OTR. OTR number changes during the estrous cycle and is closely correlated to the profile of ovarian steroid hormones (E2 and P4). E2 and P4 are believed to control the sensitivity of the endometrium to OT stimulation by regulating the OTR numbers.

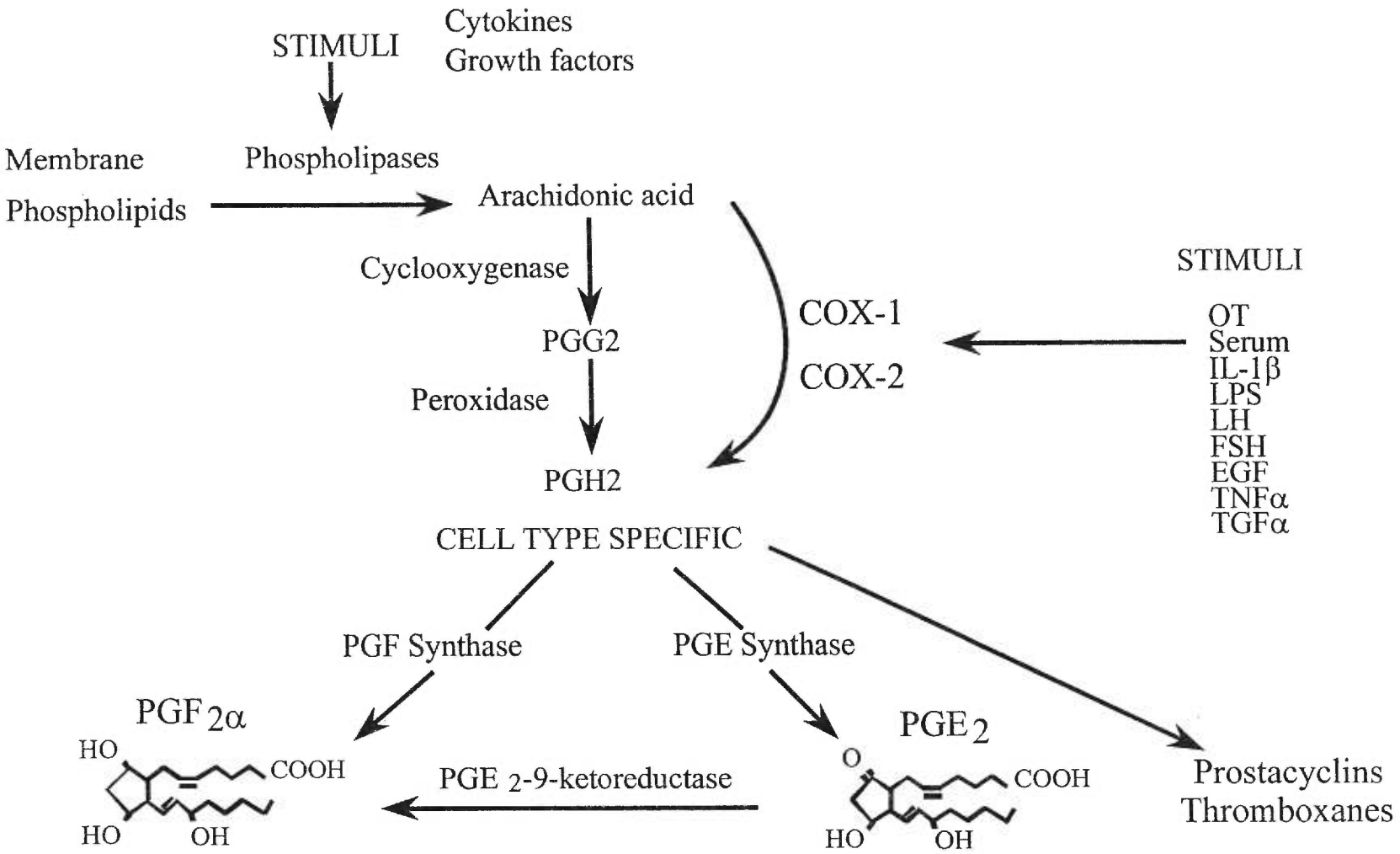
2.2.1. Synthesis of PGs

PGs are produced by virtually every nucleated cell of an organism (Heap *et al.*, 1985). These metabolites of arachidonic acid, first identified in seminal plasma, are involved in the regulation of several reproductive processes (Smith *et al.*, 1996). Arachidonic acid is liberated from the membrane bound phospholipid by phospholipase A2 (PLA2) (Fig. 4). Several phospholipases have been characterized, including cardiac and secretory PLA2. Phospholipases were originally considered to be the most important regulatory step in PG production (Williams and DuBois, 1996). Following liberation from phospholipid, free arachidonic acid is converted to PG G/H2 (PGH2), the precursor of PGs, via PG G/H synthase (PGHS). PGHS is also refered to as cyclooxygenase (COX). COX is the rate-limiting enzyme in the PG synthesis pathway. It is a bifunctional enzyme, catalyzing both the bisoxygenation of arachidonic acid to the hydroperoxy arachidonate metabolite PGG2 as well as catalyzing the peroxidative reduction of PG G2 to the endoperoxide PGH2 (Williams and DuBois, 1996).

Two isoforms of COX have been identified, COX-1 and COX-2. COX-1 was purified from bovine vesicular glands by Miyamoto *et al.* in 1976. COX-1 is a constitutively expressed enzyme and its mRNA and protein are present at relatively stable levels in many tissues. In 1989, Simmons *et al.* identified a second, inducible form of COX (COX-2). This COX isoform was independently identified by differential screening of a phorbol ester-stimulated Swiss-3T3 fibroblast cDNA library (Kujubu *et al.*, 1991). COX-2 expression is inducible by a wide range of extracellular and intracellular stimuli, including lipopolysaccharide (LPS) (O'Sullivan *et al.*, 1992), interleukin-1 (IL-1), tumor necrosis factor (TNF) (Jones *et al.*, 1993), serum (DeWitt and Meade, 1993),

Fig. 4. Arachidonate metabolism via cyclooxygenase pathway. Schematic representation of the regulation of arachidonic acid metabolism as it relates to cyclooxygenase enzymes. OT, oxytocin; IL-1β, interleukin-1β; LPS, lipopolysaccharide; LH, luteinizing hormone; FSH, follicle stimulating hormone; EGF, epidermal growth factor; TNFα, tumor necrosis factor-α; TGFα, transforming growth factor-α. Adapted from CS Williams and RN Dubois (1996).

Synthesis of Prostaglandins



epidermal growth factor (EGF) (Hamasaki *et al.*, 1993), transforming growth factor-α (TGF-α) (DuBois *et al.*, 1994), human chorionic gonadotropin (HCG) (Sirois, 1994), interferon-γ (IFN-γ) (Riese *et al.*, 1994), platelet activating factor (PAF) (Bazan *et al.*, 1994).

PGFS is the enzyme responsible for the conversion of PGH2 to $PGF_{2\alpha}$. A PGFS transcript from bovine lung was about 1.4 kb (Watanabe *et al.*, 1988). Another enzyme, PGE synthase (PGES) is responsible for the conversion of PGH2 to PGE2, and PGE2-9-ketoreductase can convert PGE2 to $PGF_{2\alpha}$.

2.2.1.1. Localization of cyclooxygenases

The human COX-1 locus maps to chromosome 9q32-q33.3. Fluorescence in situ hybridization localized human COX-2 to chromosome 1q25.2-q25.3 (Yokoyama and Tanabe, 1989). COX has been localized by immunocytochemistry to the endoplasmic reticulum with the dioxygenase activity oriented toward the cytoplasm (Merlie *et al.*, 1988). COX-1 functions primarily in the endoplasmic reticulum, whereas COX-2 activity is located in both the endoplasmic reticulum and the perinuclear envelope (Morita *et al.*, 1995). If COX-1 and COX-2 are located in separate subcellular compartments, then the distribution of their metabolites may also be partitioned. Eicosanoid products produced via COX-2 could be preferentially distributed to the nuclear compartment of the cell and therefore may modulate transcription of target genes (Williams and DuBois, 1996).

2.2.1.2. Genomic structure of cyclooxygenases

The genomic structure of both human (Yokoyama and Tanabe, 1989) and murine (Kraemer *et al.*, 1992) COX-1 is composed of 11 exons and 10 introns

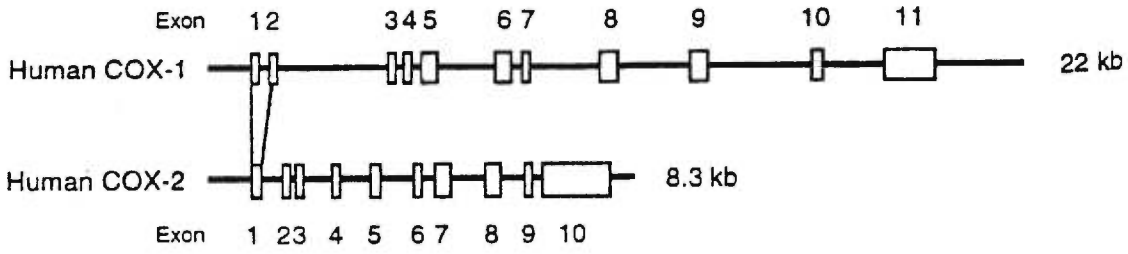
spanning 22.5 kb (Fig. 5). By comparison, the human COX-2 gene is only 8.3 kb in size. This reduction in size is primarily due to smaller introns. Most of the exons are conserved, with the exception of the absence of exon 2 in COX-2. The primary structures of these two isoenzymes were shown to be about 60% identical, as determined from deduced amino acid sequences of complementary DNAs (cDNAs) encoding COX-1 and/or COX-2 in sheep (DeWitt and Smith, 1988; Merlie *et al.*, 1988), chickens (Xie *et al.*, 1991), mice (DeWitt *et al.*, 1990; Kujubu *et al.*, 1991) and humans (Funk *et al.*, 1991; Hla and Neilson, 1992). The comparison of each COX isoform across species reveals a high degree of homology (~90%). Although the two isoforms are encoded by distinct genes located on different chromosomes (Funk *et al.*, 1991; Xie *et al.*, 1993), the structural and putative functional domains involved in enzyme catalysis are highly conserved (Kujubu *et al.*, 1991; Xie *et al.*, 1991; Hla and Neilson, 1992). However, the major difference between the two isoforms is their different patterns of expression and regulation in mammalian cells.

2.2.1.3. Expression of cyclooxygenase genes

The transcription of COX-1 and COX-2 yields mRNAs that are 2.7 and 4.5 kb in size, respectively. COX-1 mRNA encodes a 565-residue, 65-kDa protein containing a short signal peptide and four possible N-linked glycosylation sites. COX-2 is a 70-kDa protein and is about 75% homologous to the COX-1 protein. The cyclooxygenase and peroxidase regions are conserved between the two proteins (O'Banion *et al.*, 1992).

After mitogenic stimulation, the level of COX-2 mRNA rapidly increases within 30 min in a cycloheximide-independent fashion in human endothelial cells (DeWitt and Meade, 1993; Jones *et al.*, 1993) and remains elevated for 6-8 h

Fig. 5. Human cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) genomic structure. Adapted from CS Williams and RN DuBois (1996).


Exon 1 2 3 4 5 6 7 8 9 10 11
Human COX-1
22 kb
Human COX-2
8.3 kb
Exon 1 2 3 4 5 6 7 8 9 10

before rapidly declining to baseline levels within 24 h in rat intestinal epithelial cells (DuBois *et al.*, 1994). Additional stimulation results in no further COX-2 production, providing a classic example of immediate early or primary response gene activation (Nathans *et al.*, 1988). High levels of gonadotropins caused a dramatic, but transient, induction of COX-2 mRNA and protein in granulosa cells of rat (Wong and Richards, 1991; Sirois *et al.*, 1992a; Sirois and Richards, 1992b) and bovine (Sirois, 1994) preovulatory follicles *in vivo* and *in vitro*, but had no effect on COX-1 expression. The induction of COX-2 mRNA in bovine preovulatory follicles by gonadotropin takes much longer (18 h) (Sirois, 1994).

2.2.2. OT stimulation of the luteolytic release of $PGF_{2\alpha}$

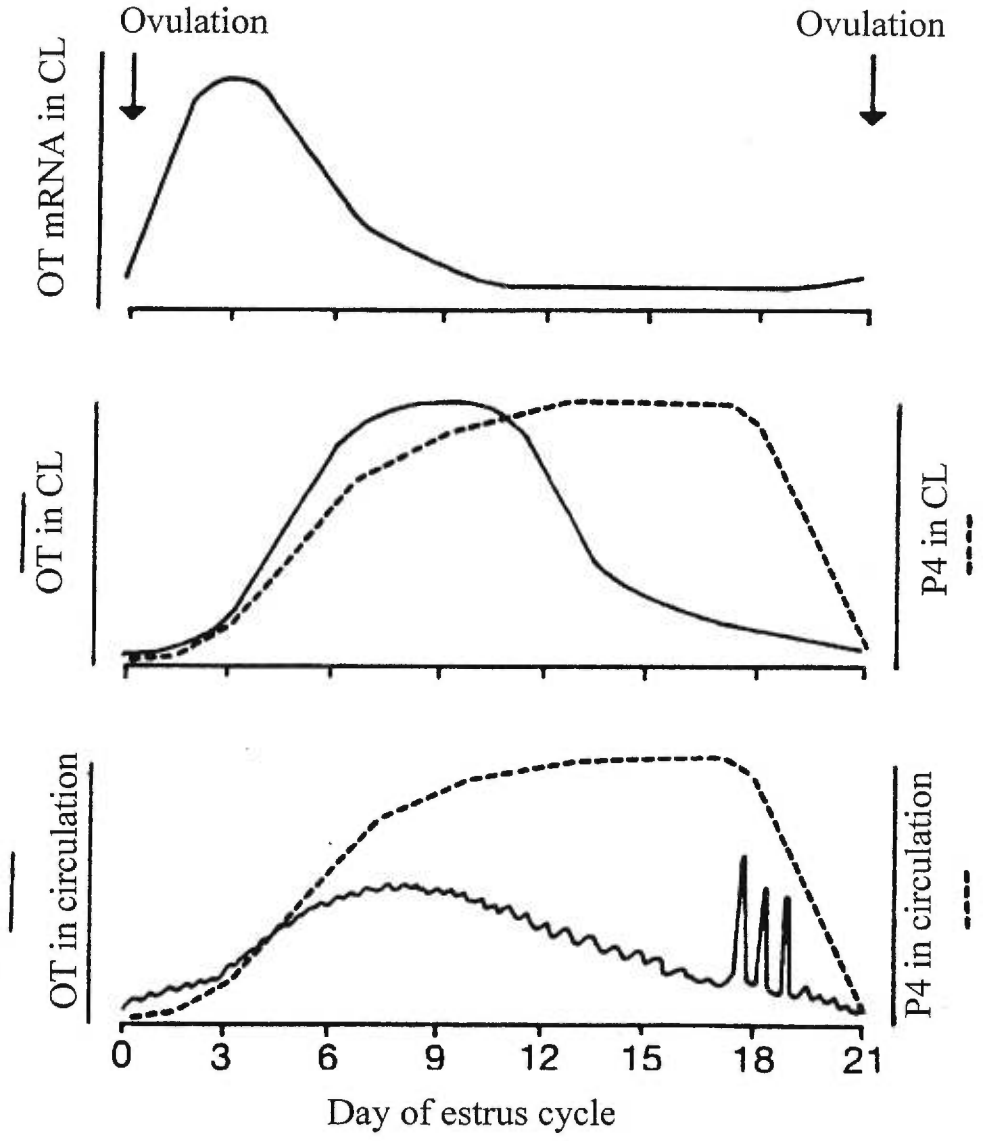
OT is the hormone responsible for the pulsatile release of $PGF_{2\alpha}$ and the induction of luteolysis. A role for OT in luteolysis was originally proposed by Armstrong and Hansel (1959) who showed that OT caused premature regression of the CL, this action no longer occurred if the uterus was removed (Armstrong and Hansel, 1959; Anderson *et al.*, 1965; Ginther *et al.*, 1967). OT stimulates $PGF_{2\alpha}$ production from the uterus in heifers (Lafrance and Goff, 1985; Schams *et al.*, 1985) and cultured bovine endometrial cells (Asselin *et al.*, 1997). In ruminants, pulses of OT occur concurrently with pulses of $PGF_{2\alpha}$ during luteolysis and most of this OT appears to be secreted from the CL (Walters and Schallenberger, 1984). In nonpregnant ewes, 96% of OT pulses occurred in association with a $PGF_{2\alpha}$ pulse, whereas in pregnant ewes 86% of OT pulses were not associated with a rise in $PGF_{2\alpha}$ (Hooper *et al.*, 1986). A similar correlation between pulses of OT and $PGF_{2\alpha}$ occurs during luteolysis in cattle. The most conclusive evidence for a role of OT in luteolysis is that treatment of ewes or goats with OT antagonist or antiserum against OT prevents or delays

luteolysis (Schams, 1983; Homeida and Khalafalla, 1987). The cellular mechanism by which OT stimulates the PGs secretion is up-regulation of COX-2 gene expression, rather than COX-1 and PLA2 mRNA (Asselin *et al.*, 1997).

OT is a nonapeptide (H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2) and is synthesized as part of larger precursor molecule in discrete populations of magnocellular neurons in the supraoptic and paraventricular nuclei of the hypothalamus (Watson *et al.*, 1982). The precursors are packaged into neurosecretory granules which are transported along the axons to the neurohypophysis, during which time processing occurs to produce OT plus its associated neurophysin (Wathes, 1989). However, the pituitary gland is not the only tissue that produces OT. Ovarian oxytocic activity was observed in extracts of corpus luteum in cow (Fields *et al.*, 1980; Fields *et al.*, 1983). The direct evidence for ovarian biosynthesis of OT was obtained from *in vitro* studies of bovine luteal cells. When incubated with ^{35}S-cysteine, these cultures produced labelled OT which co-eluted with synthetic OT on HPLC (Swann *et al.*, 1984; Wathes *et al.*, 1986).

The OT level in the ovary fluctuates during the estrous cycle (Fig. 6). OT is low in pre-ovulatory follicles (Wathes *et al.*, 1984; Kruip *et al.*, 1985; Schams *et al.*, 1985). Following ovulation there is a dramatic increase in the OT content of the young CL, with peak levels reached in the mid-luteal phase (days 8-12) (Wathes *et al.*, 1984; Fuchs, 1987). In contrast to the mid-cycle peak in OT content, measurements of OT mRNA show that transcription is maximal in the first few days of the cycle, peaking around day 3 and declining to very low levels from day 11 onwards (Ivell *et al.*, 1985). The lag between the peaks of mRNA and peptide must be caused by a delay in post-translational processing. Blood levels of OT follow the same trend as those in the CL, with a maximum in

Fig. 6. The alteration in the luteal content of oxytocin mRNA, oxytocin, and progesterone, and the blood levels of oxytocin and progesterone during the estrous cycle of the cow. Adapted from DC Wathes (1989).


Ovulation
Ovulation
OT mRNA in CL
OT in CL
P4 in CL
OT in circulation
P4 in circulation
0
3
6
9
12
15
18
21
Day of estrus cycle

the early- to mid-luteal phase (Schams, 1983; Wathes *et al.*, 1992). Both blood and tissue concentrations of OT drop before the P4 level starts to decline at luteolysis (Wathes *et al.*, 1984; Schams *et al.*, 1985). The secretion pattern of OT varies at different stages of the cycle. During the follicular phase, circulating levels are extremely low and only an occasional pulse can be detected (Walters and Schallenberger, 1984). These authers showed, as the luteal phase proceeds, OT levels fluctuate around an increasing baseline which is highest on about day 8. This overall secretion pattern is maintained, with a gradual decrease in baseline, until luteolysis begins on about day 18. At this stage, OT is released in distinct surges (each lasting about 2-3 h) with an interpulse interval of about 8 h (Schams *et al.*, 1985). The corpus luteum contains a finite store of OT, which cannot be replenished (Wathes and Lamming, 1995). In pregnant animals this is nearly all released by the fourth week of gestation (Parkinson *et al.*, 1992) so that after this time there are no further major episodes of OT secretion. Some release of OT may still occur from the neurohypophysis during pregnancy but, until the animal goes into labour, this is usually insufficient to stimulate uterine secretion of $PGF_{2\alpha}$.

A positive feedback loop exists between uterine $PGF_{2\alpha}$ and luteal OT. Luteal OT stimulates uterine secretion of $PGF_{2\alpha}$ and $PGF_{2\alpha}$ stimulates secretion of OT from the CL (Flint and Sheldrick, 1983; 1986). During spontaneous episodes of $PGF_{2\alpha}$ secretion, concentrations of $PGF_{2\alpha}$ in the utero-ovarian venous effluent increase before any increase in concentrations of OT is detectable (Moore *et al.*, 1986). Therefore, activation of the feedback loop begins on the uterine side of the loop. However, the stimulus that initiates secretion of $PGF_{2\alpha}$ is not known.

2.2.3. OT receptor and uterine sensitivity to OT stimulation

The possible role of uterine OTR in regulating the timing of luteolysis was suggested by McCracken *et al.* (1981) and it is now well established that OT binding to its receptor in the endometrium in the late luteal phase can trigger the release of $PGF_{2\alpha}$ (Flint *et al.*, 1990; Silvia *et al.*, 1991; Wathes and Hamon, 1993). Sensitivity of the uterus to circulating OT is determined by the concentration of the OTR on the cells in the uterine endometrium. Concentrations of OTR rise approximately 500-fold in the uterine endometrium at the time when luteal regression occurs (Roberts and McCracken, 1976; Sheldrick and Flint, 1985). Furthermore, premature induction of the OTR by administration of E2 results in premature luteolysis (Hixon and Flint, 1987), and inhibition of receptor expression by continuous administration of OT (which causes receptor downregulation) delays luteolysis (Flint and Sheldrick, 1985; Sheldrick and Flint, 1990). Since responsiveness of the uterus to OT is determined by the expression of the receptor, administered OT is seldom luteolytic in cattle, although it has been shown that OT administered shortly after estrus (when OTR concentrations remain high after the previous luteal regression) results in the shortening of the cycle in cattle (Armstrong and Hansel, 1959; Anderson *et al.*, 1965).

The time at which luteal regression occurs is determined principally by the time at which the OTR is generated in the uterus. Luteal OT concentrations are highest during the early mid-luteal phase, and decrease during the late luteal phase (Wathes and Denning Kendall, 1992). Conversely, the OTR numbers are very low at days 5-16, and increase at day 17, and reach the peak at estrus, then decrease at days 1-4 (Wathes and Lamming, 1995). This suggests that OT stimulation of $PGF_{2\alpha}$ at the time of luteolysis is regulated by uterine

OTR numbers. The use of radioactive labelled OT to localize these binding sites (Ayad *et al.*, 1991) and in situ hybridization to detect the location of OTR mRNA (Stevenson *et al.*, 1994) revealed that the initial minor increase in $PGF_{2\alpha}$, which occurred only in nonpregnant animals, was attributable exclusively to the appearance of receptors on the luminal epithelium. This change occurred before any measurable decline in circulating P4 concentrations, at a time when OT injection *in vivo* first leads to episodes of $PGF_{2\alpha}$ release (Roberts and McCracken, 1976; Fairclough *et al.*, 1984). Maximum concentrations of OTR are therefore not reached until luteolysis is complete and are not required to achieve either the initial luteolytic spikes or a high output of $PGF_{2\alpha}$. The small increase in the number of receptors on day 17 in cows occurs concomittantly with the start of luteolytic episodes of $PGF_{2\alpha}$ release (Mann and Lamming, 1994; Lamming and Mann, 1995). What causes the minor increase in OTR at the late stage of estrous cycle?

2.2.4. Regulation of OTR by steroids

The OTR expression is determined, at least in part, by circulating concentrations of steroid hormones (Vallet *et al.*, 1990; Beard *et al.*, 1991; Zhang *et al.*, 1992). Sheldrick and Flint (1985) showed that a marked increase in the concentration of endometrial OTR occurred simutaneously with the decline in plasma P4 concentration at luteolysis and the preovulatory increase in plasma E2 concentration. McCracken (1980) used an indirect OT-induced uterine $PGF_{2\alpha}$ response test to monitor OTR concentrations. A 12-day infusion of P4 and a 12-h infusion of E2 was needed before the uterus responded to OT challenge.

Treatment of ovariectomized ewes with P4 alone results in a rapid and complete loss of OTR (Wathes and Lamming, 1995). After about 10-12 days

some reappear even though P4 concentrations remain high (Vallet *et al.*, 1990; Fairclough and Lau, 1992). The receptors that develop in these circumstances are located exclusively on the luminal epithelium and the localization resembles that on day 14 of the natural cycle (Wathes and Lamming, 1995). Vallet *et al.* (1990) concluded that P4 initially depressed the OTR and that after long term treatment P4 caused a rise in the OTR.

2.2.5. Hormonal regulation of steroid receptors

The effect of steroids on OTR is mediated by steroid receptors and the bovine endometrium is a highly sensitive target organ for ovarian steroid hormones. The endometrial steroid receptor numbers determine the sensitivity of uterus to steroid stimulation and are themselves regulated by steroid hormones.

E2 receptor (ER) and P4 receptor (PR) concentrations change throughout the estrous cycle in response to changes in circulating steroid hormone concentrations (Boos *et al.*, 1996). PR and ER concentrations are very high during follicular phase, and low in the luteal phase. It is generally accepted that P4 and E2 exert opposing effects on steroid receptors in the uterus. E2 increase ER and PR number and P4 decreases PR and ER numbers (Vesanen *et al.*, 1988; Vesanen *et al.*, 1991).

McCracken *et al.* (1981) proposed that the duration of the luteal phase was determined by rising P4 concentrations in the early luteal phase downregulating its own receptor via a 'progesterone block' which lasted about 10 days. Loss of this inhibitory influence was thought to be the initiating factor in luteolysis, as it allowed OTR formation.

2.3. Actions of PGs in the uterus

2.3.1. Roles of PGs in implantation

PGs are involved in several aspects of implantation such as increase in vascular permeability (Kennedy, 1980; 1985) and changes in uterine blood flow (Burka, 1983) via their effect on cAMP (Kennedy, 1983). Blocking the synthesis of PGs with indomethacin during the first few days of pregnancy inhibits or delays implantation in mice (Saksena *et al.*, 1976), rats (Kennedy, 1977), hamsters (Evans and Kennedy, 1978), and rabbits (Hoffman, 1978), and the implantation sites appeared later and were smaller than in control animals and the embryonic development was retarded. Furthermore, the expected localized increases in vascular permability associated with implantation are blocked or delayed by indomethacin in rats (Kennedy, 1977) and rabbits (Hoffman, 1978).

The concentration of PGs increases in decidualizing tissue in normal pregnant animals, and this increase is blocked by indomethacin (Kennedy, 1977). Exogenous PGs increase vascular permeability in the uterine lumen. However, there has been some disagreement about which of the PGs is most effective. Kennedy (1979) found that PGE2 was effective in increasing vascular permeability of uterine lumen whereas $PGF_{2\alpha}$ was not. On the other hand, constant infusion of $PGF_{2\alpha}$ was as effective as PGE2 (Kennedy and Lukash, 1982). It has been shown that there are specific receptors for PGE2 in the stroma of rat endometrium (Kennedy, 1983) and that their concentration increases with P4. There are no receptors for $PGF_{2\alpha}$ in the rat endometrium (Martel *et al.*, 1985). It has been suggested that any effect of $PGF_{2\alpha}$ on decidual tissues is a result of its conversion to PGE2 or because $PGF_{2\alpha}$ cross-reacts with PGE2 receptors.

2.3.2. Roles of PGs in parturition

PGs play a central role in the initiation of parturition in all species studied (Mitchell and Flint, 1978; Kanda and Kuriyama, 1980; Teixeira *et al.*, 1994; Zhang *et al.*, 1996). Administration of PGs *in vivo* or *in vitro* markedly stimulate uterine contractility and enhance uterine sensitivity to other uterotonic agents (for example, oxytocin) (Crankshaw, 1995; Baguma-Nibasheka *et al.*, 1998) and results in abortion (Borten and Friedman, 1985) or labour in human (Maymon *et al.*, 1992). Treatment with PG synthase inhibitors (such as, aspirin and indomethacin) lengthens the gestation period, prolongs labor (Aiken, 1972) and arrests preterm labor induced with glucocorticoids in sheep (Mitchell and Flint, 1978). The sensitivity of the isolated longitudinal rat myometrium to exogenous PGs increases during the last stage of gestation (Kanda and Kuriyama, 1980). PG production by uterine and intrauterine tissues increases as pregnancy proceeds (Hirst *et al.*, 1995a). In sheep, PGs increased in maternal and fetal plasma and amniotic fluid before the increase in uterine mechanical activity during induced preterm labor (Olson *et al.*, 1985). The myometrium, endometrium, chorion, amnion and placenta are all potential sources of PG involved in parturition (Elger *et al.*, 1986).

Amnion membrane is a major source of PGs in the pregnant human uterus. The specific activity of COX, the enzyme catalyzing the committing step of prostanoid biosynthesis, in human amnion increases markedly with the onset of labor at term and further increases during term and preterm labor (Zakar *et al.*, 1994; 1996). COX-2, but not COX-1 mRNA levels increased following spontaneous parturition and were correlated with enzyme activity in human amnion (Cheng *et al.*, 1993; Hirst *et al.*, 1995b). Similarly, a selective increase in COX-2 expression and activity in chorion trophoblasts and mesenchymal cells with term labor onset was also observed (Mijovic *et al.*, 1997). The placenta is a

major site of PG production throughout pregnancy. The cellular localization of COX in placenta changes during ovine pregnancy, from predominantly maternal during the first half of gestation to undetectable and then to predominantly trophoblastic between Day 114 and term. Appearance of COX in the trophblast was considered to provide the trigger to the onset of parturition in sheep (Boshier *et al.*, 1991). The site of PG production during ovine pregnancy changes with a gestation-dependent manner. Furthermore, It has been shown that both COX-1 and COX-2 expression increased in the chorion laeve at term before labor, with COX-2 as the functionally prevalent isoform. This suggested the possibility that PGs originating in the fetal membranes promote the onset of normal labor (Mijovic *et al.*, 1998).

2.4. Maternal recognition of pregnancy

During early pregnancy, changes take place in response to the presence of a viable conceptus within the reproductive tract. These changes are assumed to result from the interaction of specific biochemical signals produced by the conceptus with receptors in the maternal system to create a uterine environment appropriate for continued embryonic growth and development. Short (1969) first used the term 'maternal recognition of pregnancy' to define this interchange of information during pregnancy. This dialogue can occur at various critical stages during pregnancy, but one critical process in ruminants involves paracrine regulation by the conceptus of the underlying endometrium that leads to an attenuation of luteolytic $PGF_{2\alpha}$ secretion. This antiluteolytic effect of the conceptus (Thatcher *et al.*, 1986) is the primary cause of maintenance of the corpus luteum in cattle.

2.4.1. Embryonic signal

2.4.1.1. Existence and identification

Early studies in the sheep by Moor and Rowson (1964) and in cow by Betteridge *et al.* (1978) demonstrated that the transfer of an embryo to the uterus of a synchronized non-pregnant recipient would prevent cyclic regression of the corpus luteum in the recipient even if transfered as late as day 13 of the cycle for sheep, day 16 for cow. Bovine blastocysts undergo a period of elongation, beginning around Day 14 of pregnancy. They appear to signal their presence to the mother at about Day 16, just after elongation is initiated (Betteridge *et al.*, 1978; Betteridge *et al.*, 1980). Cows from which conceptuses were removed after Day 17 had extended interestrous intervals compared to controls when conceptuses were removed at Day 13 (Northey and French, 1980). Later work indicated that the same effect could be achieved by infusing extracts or homogenates of embryos into the uterus (Moor, 1968; Martal *et al.*, 1979).

The signal produced by the conceptus was believed to be a protein because treatment of conceptuses with heat, or conceptus homogenates with proteases, destroyed activity (Rowson and Moor, 1967; Martal *et al.*, 1979). Martal *et al.* (1979) first purified a low molecular weight active factor from ovine conceptus homogenates, which was termed trophoblastin. Moreover, the active factor is present in Day 17-18 but not Day 12 conceptuses (Northey and French, 1980). Godkin *et al.* (1982) purified a protein that was released by conceptuses between Days 13 and 21 *in vitro*. Because the protein originated from the trophoblast and was the first discernible polypeptide secreted by conceptuses, it was designated ovine trophoblast protein-1 (oTP-1) (Godkin *et al.*, 1984b) and is presumably the same protein as trophoblastin. A single Day 16 embryo is estimated to produce from 100 to 500 μg of oTP-1 in a 24 h culture period *in vitro* (Ashworth and Bazer, 1989). A similar protein has been identified in

bovine trophoblasts (Bartol *et al.*, 1985), known as bovine trophoblast protein-1 (bTP-1) (Helmer *et al.*, 1987) and classified as bovine interferon-tau (bIFN-τ).

2.4.1.2. Characteristics

bTP-1 is a glycoprotein complex. The preprotein of bTP-1 contains a 23 amino acid signal sequence and a 172 amino acid sequence of the mature protein (Imakawa *et al.*, 1989). A potential site for N-glycosylation at Asn^{78} exists in bIFN-τ transcripts and seven isomers of two size classes (22 and 24 kDa) are present in the secreted proteins, which differ in isoelectric point. These isomers are glycosylation variants and N-linked glycoproteins, because deglycosylated products migrate as a single band (18 kDa) during electrophoresis. It was believed that these bTP-1 variants arise as a single translation product that undergoes differential post-translational glycosylations (Helmer *et al.*, 1988). However, the analysis of recombinant cDNA cloning appears to provide evidence that the various isoforms of bTP-1 produced by trophoblast tissue arise, in part, from translation of multiple mRNAs which are themselves the products of distinct genes (Roberts *et al.*, 1991). Base sequencing of several cDNAs corresponding to the mRNA for bTP-1 isolated from cDNA libaries representing day 18-19 bovine conceptus poly (A)+ mRNA that multiple mRNAs for bTP-1 exist. The mRNAs average about 1 kb in length. One apparently full-length cDNA clone consisted of 1035 bases up to the beginning of the poly (A) tail (Imakawa *et al.*, 1989). The pattern of bTP-1 expression varies considerably between conceptuses (Gross *et al.*, 1988b).

2.4.1.3. Structure of bIFN-τ genes and proteins

Like other Type I interferon genes, IFN-τ genes are intronless and have a 585 base pair open reading frame coding for a IFN-τ preprotein. bTP-1 was

initially identified as type I interferon following cDNA cloning and amino acid sequencing and later given the greek letter designation tau (τ). Bovine interferon-τ (bIFN-τ) and IFN omega (IFN-ω) genes were localized to chromosome 8 band 15 (Ryan *et al.*, 1993).

bTP-1 exhibits approximately 85% identity in cDNA sequence with bovine IFN-α_{II} in their protein-encoding regions and approximately 70% sequence identity in their 3'-untranslated regions (Imakawa *et al.*, 1987; Stewart *et al.*, 1987; Imakawa *et al.*, 1989). Within the coding region, oIFN-τ and bIFN-τ transcripts exhibit approximately 90% identity and their inferred amino acid sequences share about 80% identity. The 5' (76%) and 3' (92%) untranslated regions of oIFN-τ and bIFN-τ mRNAs are highly conserved and oIFN-τ and bIFN-τ genes share considerable identity in the 5' and 3' flanking regions (Imakawa *et al.*, 1989).

Reciprocal interspecies embryo transfer between sheep and goats demonstrated pregnancy recognition, but failure to maintain pregnancy beyond the period of placentation (Helmer *et al.*, 1987). Also, ovine trophoblastic vesicles extend the interestrous interval when placed in the uterine lumen of cattle (Heyman *et al.*, 1984). Purified recombinant oIFN-τ suppresses oxytocin-induced uterine $PGF_{2\alpha}$ production and extends interestrous interval in cattle (Meyer *et al.*, 1995) and goats (Newton *et al.*, 1996). Antiserum to oTP-1 immunoprecipitated all forms of bTP-1 (Helmer *et al.*, 1987).

The bTP-1 genes differ from the bovine IFN-α_{II} in the organization of the promoter regions upstream from the transcription start site (Roberts *et al.*, 1991). The IFN-τ gene promoters share high within and cross-species identity up to

position -400 (Leaman and Roberts, 1992). A consensus IFN-τ promoter contains GAAANN sequences and putative interferon response element (IRE-1) binding hexamers although the arrangement of these motifs differs from the viral response elements present in other Type I IFNs. Functionally, this is supported by the poor virus-inducibility of IFN-τ genes (Leaman *et al.*, 1992). Because IFN-τ promoter constructs are expressed in uninduced cells of trophoblast origin (JAR and BeWo cells), but not in nontrophoblast cells, it is postulated that trophoblast-cell-specific factors activate transcription of the IFN-τ genes via distal enhancer elements (Leaman *et al.*, 1994). Nephew *et al.* (1993) provided evidence for such an element in sequence data for four additional oIFN-τ genes. Only one of those genes (clone 010) was expressed at levels comparable to oIFN-τ during the pregnancy recognition period (Roberts *et al.*, 1992). Clone 010 had an AP-1-like regulatory element (ATGGGTCAGA) starting at -929 suggesting that factors which affect AP-1 enhancer activity, like granulocyte-macrophage colony-stimulating factor (GM-CSF), may influence oIFN-τ gene expression (Imakawa *et al.*, 1993). GM-CSF is expressed by ovine endometrium during early pregnancy and addition of GM-CSF to cultured ovine conceptuses resulted in an approximate doubling of antiviral activity (oIFN-τ) in the culture medium (Imakawa *et al.*, 1993). This suggests that uterine cytokines may influence pregnancy recognition signals and subsequent fetal/placental development. However, the other results show that IFN-τ secretion, total radiolabeled protein from either bovine (Day 16-18) and ovine (Day 17) conceptuses or *in vitro* produced bovine blastocysts at Day 7-8 are unresponsive to recombinant bovine GM-CSF (rbGM-CSF) (de Moraes *et al.*, 1997).

IFN-τ has multiple active sites. This special structure may be attributable to its unique functions, such as high antiviral and antiproliferative activity

without cytotoxicity. Four synthetic peptides corresponding to the 1-37, 62-92, 119-150, and 139-172 of oIFN-τ amino acid sequence inhibited oIFN-τ antiviral activity in a dose-dependent manner. Three of them, (62-92), (119-150), and (139-172) (Pontzer *et al.*, 1990) inhibited IFN-α antiviral activity. The amino-terminal peptide, (1-37), was not inhibitory (Pontzer *et al.*, 1994). These suggest that the internal and carboxy-terminal reactive domains of IFN-τ may interact with a common type I IFN site on the receptor, while the amino terminus interacts with a site that elicits activity unique to oIFN-τ (Li and Roberts, 1994). The antiproliferative activity of oIFN-τ was localized primarily to the broad carboxy-terminal region, with oIFN-τ (119-150) being the most effective inhibitor of oIFN-τ-induced reduction of cell proliferation. The amino-terminus peptide (1-37) of oIFN-τ also has antiluteolytic agonist activity (Pontzer *et al.*, 1994).

2.4.1.4. Time and localization of IFN-τ production

bIFN-τ is produced as early as Day 12 on the basis of antiviral activity in media from cultured bovine embryos (Betteridge *et al.*, 1988). But the major production of bIFN-τ appears to begin at Days 15-17 of gestation (Bartol *et al.*, 1985) and lasts at least through day 36 of pregnancy (Godkin *et al.*, 1988). The increase in expression of bIFN-τ mRNA on Day 15-16 occurs coincident with elongation of the blastocyst, a morphological change from a spherical to filamentous form and maternal recognition of pregnancy (Farin *et al.*, 1990).

The IFN-τ production is related to the rate at which bovine embryos reach the blastocyst stage and their cell number. Blastocysts derived from embryos that had reached the four or eight-cell stage 44 h after insemination produced significantly more IFN than embryos derived from two-cell embryos

(Kubisch *et al.*, 1998). bIFN-τ is produced in mononucleate cells of the trophectoderm (Lifsey *et al.*, 1989; Morgan *et al.*, 1993). This was further confirmed by the observation of Farin *et al.* (1990) in which the bTP-1 mRNA was localized exclusively in the trophectoderm of the bovine conceptus.

2.4.1.5. IFN-τ gene expression

Multiple genes encoding for IFN-τ in sheep are not equally expressed in trophectoderm and their encoded proteins have significantly different biological potency (Ealy *et al.*, 1998). The control of IFN-τ gene expression is very different from that of other type I IFN genes (Leaman *et al.*, 1992). Multiple regulatory elements are required to direct trophoblast IFN gene expression in trophectoderm. Trophoblast-specific expression depends upon distal as well as proximal promoter regulatory elements. The proximal promoter region (to position -126) directs basal expression, and a more distal promoter region (positions -280 to -400) acts as an enhancer (Leaman *et al.*, 1994).

The genes for TP-1 are inducible by virus but are expressed preferentially in trophoblast cells and are functionally distinct from IFN-ω genes. Although conceptuses express mRNA for IFN-α, IFN-ω, and TP-1, TP-1 constitutes greater than 99% of the IFN produced. In contrast, leukocytes produced predominantly IFN-α, although TP-1 mRNA is inducible by Sendai virus to very low levels. A single day 18 bovine conceptuse secretes approximately 10^5 units of IFN antiviral activity per hour in culture, amounts approximately 300 times higher than those produced by Sendai virus-induced leukocytes. Transfected bTP-1 genes are expressed in human choriocarcinoma (JAR) cells in the absence of any specific stimulus, whereas these cells do not secrete antiviral activity constitutively or after transfection with a bovine IFN-ω gene. The transfected

TP-1 gene is not expressed in nontrophoblast cells (mouse L929 and Chinese hamster ovary) (Cross and Roberts, 1991).

2.4.1.6. Type I interferon receptor

High affinity, low capacity binding of oIFN-τ to receptors in the endometrium was first reported by Godkin *et al.* (1984a). These receptors were assumed to mediate the complex physiological responses, including the marked changes in protein synthesis that occurred when the endometrium was first exposed to oIFN-τ during early pregnancy. These receptors have an apparent molecular weight of 100 kDa in ovine endometrial membrane preparations (Hansen *et al.*, 1989). The human type I IFN receptor is a 95-100 kDa transmembrane glycoprotein generated from a 2.7-kb mRNA encoding a 65-kDa protein with 15 potential asparagine-linked glycosylation sites (Roberts *et al.*, 1992b). Stewart *et al.* (1987) later showed that purified oIFN-τ displaced [^{125}I] huIFN-α from such receptors and thereby confirmed that the former was a type I IFN. IFN-α, -β, -ω and -τ act through the Type I IFN receptor, indicating that they share portions of the epitope involved in receptor binding. The dissociation constant (K_d) values of these receptors to oIFN-τ are in the range of 0.1×10^{-10} M to 0.4×10^{-10} M and these values seemed to vary slightly according to the stage of the estrous cycle (Knickerbocker and Niswender, 1989). In addition, some evidence has been provided for a group of lower affinity sites ($K_d = 10^{-10}$ M) (Hansen *et al.*, 1989) and may correspond to those first noted by Godkin *et al.* (1984a). However, since secreted oIFN-τ is a mixture of at least four isoforms, it is possible that the noted complexity of binding was the result of varying affinities of different subtypes for a single receptor, a phenomenon well known among the IFN-α (Yonehara *et al.*, 1983). Interactions between Type I endometrial receptors and oIFN-τ appear to differ from those for rbIFN-α_{II} or

rhuIFN-α since antiluteolytic activity is at least seven-fold greater for oIFN-τ (Plante *et al.*, 1988; Stewart *et al.*, 1989).

Two subunits, IFNAR1 and IFNAR2, of this receptor have been identified so far (Cutrone and Langer, 1997; Langer *et al.*, 1998). Although there is extensive inferred amino acid sequence similarity between bovine and ovine IFNAR1 (92% identity) and between bovine and ovine IFNAR2 (88% identity), they have diverged extensively from the human receptor subunits (approximately 67% and approximately 58% identity, respectively). Despite these differences in primary structure, the respective subunits from all three species are organized similarly in their extracellular and cytoplasmic regions, and the bovine and ovine subunits have each retained a number of polypeptide motifs implicated in signal transduction (Han *et al.*, 1997). These uterine receptors also appear not to be splice variants. The cloned ovine IFNAR1 subunit, for example, possesses the expected four extracellular SD100 domains of full-length bovine and huIFNAR1. Both subunits are expressed not only in endometrium, but in all other tissues examined except those of preimplantation conceptuses, which presumably cannot respond to the IFN-τ they produce (Han *et al.*, 1997).

The sheep and human Type I receptors have the same order of binding affinity ($K_d = 10^{-10}$ M), but binding capacity was considerably lower for human (6.0 fmol mg^{-1}) than for sheep (47-123 fmol mg^{-1}) endometrium (Russell *et al.*, 1993). Numbers of unoccupied receptors are similar for cyclic and pregnant ewes on Days 8 and 12, but decrease thereafter for pregnant ewes. Ovine endometrial IFN-τ receptor numbers vary cyclically and are modified by the actions of ovarian steroids (Knickerbocker and Niswender, 1989). Human endometrial membrane preparations from proliferative-phase tissue showed

very little specific binding in contrast to luteal-phase endometrium. Treatment of ovariectomized ewes with E2 increased receptor number in endometrial tissues compared with tissue from ovariectomized, P4 -treated, or (E2 + P4)-treated groups (Russell *et al.*, 1993).

2.4.1.7. Type I IFN receptor signal transduction system

Binding of IFN-τ to endometrium does not stimulate increases in cAMP, cGMP, or IP turnover (Vallet *et al.*, 1987; Mirando *et al.*, 1990b); however, IFN-τ is assumed to activate a signal transduction system similar, if not identical with that of IFN-α and other type I IFNs. Type I IFN appear to initiate their effects through activation of latent transcription factors that then bind to IFN response elements on selected target genes (Kerr and Stark, 1991; Williams, 1991).

The type I IFN transcription factor family includes IFN-stimulated gene factor-3 (ISGF3), interferon regulatory factor-1 (IRF-1), IRF-2, interferon consensus sequence binding protein (ICBSP), and lymphoid-specific IRF (LSIRF). The ICBSP and LSIRF are unique to cells of lymphoid lineage (Matsuyama *et al.*, 1995). In contrast, ISGF3, IRF-1 and IRF-2 represent a transcription factor network that regulates expression of IFN-inducible genes in several cell types (Darnell *et al.*, 1994). The ISGF3 transcription factor complex is composed of four proteins which are dissociated and normally located in the cytoplasm (Schindler *et al.*, 1992). The binding of a type I IFN to its receptor immediately activates latent tyrosine kinases, janus kinase-1 (Jak1) and tyrosine kinase-2 (Tyk2), which phosphorylate tyrosine residues of substrate proteins called STATs (signal transducers and activators of transcription), including STAT1 (p84), STAT1a (p91), and STAT2 (p113). These three phosphoproteins

then bind a fourth DNA-binding protein, p48, and the multimeric protein complex is transported to the nucleus (Darnell *et al.*, 1994). This ISGF3 transcription factor complex binds to an IFN-stimulated responsive element (ISRE) present in the promoter/enhancer region of IFN-responsive genes and increases rates of gene transcription (Darnell, 1996).

2.4.2. Bioactivities of IFN-τ

2.4.2.1. Antiviral activity of IFN-τ

Like the other known interferons, oTP-1 and bTP-1 can protect several cell types such as, bovine kidney epithelial cells (GBK-2) (Imakawa *et al.*, 1987); human A_{549} cells (Roberts *et al.*, 1989) known to possess type I receptors from lysis by a range of viruses, including the commonly employed vesicular stomatis virus (VSV) and encephalomyocarditis virus, with at least an equivalent potency to IFN-α. The activity of the preparation purified by Roberts *et al.* (1989) was greater than 10^7 relative units mg^{-1}. Its antiviral activity was thus as potent as those of other known IFN-α_1s, which range from 10^7 to 10^9 units mg^{-1} (Pestka and Langer, 1987). The IFN activity of oTP-1 could be neutralized by antiserum raised against total human leukocyte IFN, but not by antibodies raised against purified human IFN-α_1. Similarly, the total bovine conceptus secretion (Betteridge *et al.*, 1988) and purified bTP-1 (Godkin *et al.*, 1988) have also been proved to have antiviral activity. The purified preparation has a specific activity of about 10^7 units mg^{-1}.

oIFN-τ has antiviral activity similar to that of human IFN-α on the Madin-Darby bovine kidney (MDBK) cell line. However, IFN-τ was > 30-fold less toxic than IFN-α_1 at high concentrations (Pontzer *et al.*, 1991). Differential recognition of the type I IFN receptor by these two type I IFNs is responsible for

their disparate cytotoxicities (Subramaniam *et al.*, 1995). It had been demonstrated that IFN-α_1 had a higher binding affinity (K_d=4.45 x 10^{-11} M) than IFN-τ (K_d= 3.90 x 10^{-10} M) to type I IFN receptor on MDBK cells. Consistent with its higher binding affinity, IFN-α_1 was several fold more effective than IFN-τ in competitive binding against ^{125}I-IFN-τ to receptor on MDBK cells (Li and Roberts, 1994). However, maximal IFN antiviral activity required only fractional occupancy of receptors, whereas toxicity was associated with maximal receptor occupancy. Hence, IFN-α_1, with the higher binding affinity, was more toxic than IFN-τ (Subramaniam *et al.*, 1995).

2.4.2.2. Antiproliferative activity of IFN-τ

Both bTP-1 and oTP-1 inhibit proliferation of lymphocytes after exposure to mitogens (Roberts *et al.*, 1989) and mixed lymphocyte cultures (Skopets *et al.*, 1992). The IFN-τ can also markedly slow the proliferation of bovine kidney epithelial (Roberts *et al.*, 1989) and oviductal cells and the human amnionic line, WISH cells and inhibit both colony size and number (Pontzer *et al.*, 1991). oTP-1 was as effective as human and bovine IFN-αs and displays potent cross-species activity. Its activity was dose dependent, and inhibition of proliferation could be observed at concentrations as low as 1 unit/ml. Concentrations as high as 50,000 units/ml stopped proliferation, while viability was not impaired (Pontzer *et al.*, 1988). Cell cycle analysis revealed an increased proportion of cells in S phase and a corresponding decreased proportion of cells in G2/M after 48 h of oTP-1 treatment. Therefore, oTP-1 appears to inhibit progress of cells through S phase. oTP-1 antiproliferative effects can be observed as early as 12 h after the initiation of culture and are maintained through 6 days (Pontzer *et al.*, 1991).

IFNs are used in cancer therapy and have been found to be effective in the treatment of cancer such as myelogenous (Kasimir-Bauer *et al.*, 1998) and hairy cell leukemias (Lichtman *et al.*, 1998). A significant problem with the currently used IFNs is the undesirable side effect of toxicity, such as, pyrogenicity, vomiting, diarrhea, and malaise at high concentrations (Kasimir-Bauer *et al.*, 1998; Lichtman *et al.*, 1998). However, at high concentrations, oIFN-τ is far less cytotoxic than either bIFN-α or a huIFN-α preparation (Pontzer *et al.*, 1991). IFN-τ may be a useful therapeutic agent which can be used at higher doses, for longer periods of time, or as an alternative of IFN-α for treatment of viral diseases and cancers in veterinary and human medicine. Since IFN-τ exerts negative regulatory effects on ER and OTR gene transcription, it may be also a useful therapeutic agent for treatment of E2-dependent diseases of mammary and reproductive tissues (Bazer *et al.*, 1997).

Unlike other bovine cells tested previously (lymphocytes and oviductal cells), growth of bovine endometrial cells prepared from the endometrium of cows at Days 11-17 after estrus were not consistently inhibited by IFN-τ or IFN-α. Such reduced responsiveness of endometrial cells to the antiproliferative effects of type I interferons could allow for growth of the endometrium during the period of pregnancy when the conceptus produces IFN-τ (Davidson *et al.*, 1994).

2.4.2.3. Induction of endometrial proteins by IFN-τ

Although an inhibitory effect on gene expression has been suggested as a general feature of IFNs, the production of at least 11 proteins is increased by IFN-τ in the endometrium (Godkin *et al.*, 1984a; Dooley *et al.*, 1997; Hansen *et al.*, 1997;).

2.4.2.3.1. 2′,5′-oligoadenylate synthetase

2′,5′-oligoadenylate (2-5[A]) synthetase is involved in cell division and selective degradation of mRNA (Short and Fulton, 1987; Short *et al.*, 1991). In the IFN-induced antiviral state, this enzyme catalyzes the production of oligomers of adenine which activate a latent endoribonuclease to degrade invading viral RNA, as well as cellular RNA (Bazer *et al.*, 1997).

The IFN-τ is capable of up-regulating endometrial synthesis of 2-5[A] synthetase (Short *et al.*, 1991). Uterine cellular 2-5[A] synthetase changes during the bovine estrous cycle and early pregnancy. During the estrous cycle, 2-5[A] synthetase was greatest on Day 5 and declined approximately 10-fold by Day 15. Cellular content of 2-5[A] synthetase was similar among all three endometrial cell types. In the gravid horn of pregnant animals, presence of a conceptus significantly increased 2-5[A] synthetase in all endometrial cell types compared to levels on Days 15 and 18 of the estrous cycle. On Day 18, levels of 2-5[A] synthetase were 30-fold greater in epithelium (surface and glandular) from pregnant cows compared to that from cyclic cows (Schmitt *et al.*, 1993).

2.4.2.3.2. Acidic secretory protein

Both bIFN-α and IFN-τ, with about equal potency, markedly up-regulate a highly acidic 70,000 Mr secretory protein in both ovine endometrial explants (Godkin *et al.*, 1984a) and cultured uterine epithelial cells (Vallet *et al.*, 1987). This same protein has been observed to be a product of endometrium in contact with trophoblast only during the period of oIFN-τ production and to be induced when non-pregnant ewes are injected with bIFN-α (Godkin *et al.*, 1984a; Vallet *et al.*, 1987).

2.4.2.3.3. Granulocyte chemotactic protein-2

IFN-τ stimulates uterine secretion of an 8-kDa bovine granulocyte chemotactic protein-2 (bGCP-2) (Naivar *et al.*, 1995). bGCP-2 is an alpha-chemokine that acts primarily as a potent chemoattractant for granulocyte cells of the immune system (Proost *et al.*, 1993). bGCP-2 was not released by endometrium from day 14 nonpregnant cows, but was released in response to IFN-τ (Teixeira *et al.*, 1997). The regulation of bGCP-2 by IFN-τ may have important implications for cytokine networking in the uterus during pregnancy. Also, the regulation of inflammation and angiogenesis by bGCP-2 working together with other cytokines may be integral to establishing early pregnancy and implantation in the cow (Dooley *et al.*, 1997).

2.4.2.3.4. Ubiquitin cross-reactive protein

Bovine ubiquitin cross-reactive protein (boUCRP) is secreted by the endometrium from days 15 to 26 of pregnancy in response to IFN-τ (Hansen *et al.*, 1997). The transcription of the UCRP gene is transient during early pregnancy and regulated by the conceptus and IFN-τ (Austin *et al.*, 1996). A single UCRP transcript of approximately 700 bp can be induced in cultured endometrial cells by rbIFN-τ. The UCRP mRNA was not detected in endometrium as well as spleen, kidney, liver, corpus luteum or muscle on days 15, 17, 18 or 19 of the cyclic cow. However, it was detectable in endometrium from pregnant cows by day 15, reached its highest levels by day 17, remained elevated on days 18, 19 and 21, and then declined to amounts on day 26 that were not detectable (Hansen *et al.*, 1997). In response to rbIFN-τ, UCRP becomes conjugated to endometrial cytosolic proteins during early pregnancy. The

regulation of uterine proteins by UCRP may be integral to the maintenance of early pregnancy in ruminants (Johnson *et al.*, 1998).

2.4.2.4. Antiluteolytic effects of IFN-τ

Luteolysis in large ruminant species is prevented by inhibition of the pulsatile production or release of $PGF_{2\alpha}$ by the endometrium (McCracken *et al.*, 1972; Hixon and Hansel, 1974). Accumulated evidence shows that the trophoblast IFN-τ is the antiluteolytic agent. Intrauterine administration of either whole conceptus secretory proteins (CSP) or purified IFN-τ reduces the number and amplitude of luteolytic pulses of $PGF_{2\alpha}$ (Danet-Desnoyers *et al.*, 1994). Intrauterine infusion of bovine CSP in cyclic cows between days 15 and 21 increases the interestrous interval from 23-24 days to 30-39 days and inhibits uterine production of $PGF_{2\alpha}$ in response to exogenous E2 (Li and Roberts, 1994). Intrauterine injection of rbIFN-τ extended the lifespan of the corpus luteum and interestrous interval and abolished the OT-induced increase in PGFM (Meyer *et al.*, 1995). Recombinant ovine IFN-τ (roIFN-τ) inhibited OT-stimulated $PGF_{2\alpha}$ production in cultured bovine endometrial cells (Asselin *et al.*, 1997). The antiluteolytic effects of IFN-τ are assumed to be limited to the uterine endometrium because IFN-τ is not detectable in the uterine venous or lymphatic drainage (Lamming *et al.*, 1991).

Thatcher *et al* (1985) postulated that the antiluteolytic effect of the conceptus may be mediated by the induction of an intracellular molecule in endometrial tissue that inhibited the enzymatic conversion of arachidonic acid to $PGF_{2\alpha}$. An endometrial PG synthesis inhibitor (EPSI) was shown to be present in bovine endometrium and that its activity was enhanced during pregnancy (Gross *et al.*, 1988a; Danet-Desnoyers *et al.*, 1993). The EPSI presumably acted on

PGHS since both $PGF_{2\alpha}$ and PGE2 were inhibited. EPSI was originally thought to be a protein (Gross *et al.*, 1988a), but it is now known that EPSI is linoleic acid (Thatcher *et al.*, 1994), a fatty acid shown previously to inhibit PGHS activity (Elattar and Lin, 1989). Linoleic acid acts as a competitive inhibitor of PGHS when added to the bovine microsome assay (Thatcher *et al.*, 1994). Endometrial microsomes from pregnant cows had higher linoleic acid concentrations and lower arachidonic acid concentrations than did cyclic cows (Thatcher *et al.*, 1995). Consequently, the ratio of linoleic acid to arachidonic acid was higher in microsomes of pregnant cows.

2.4.2.5. Effects of IFN-τ on endometrial oxytocin receptor

The endometrial OTR expression determines the time at which luteolysis occurs in nonpregnant animals, and thereby determines the duration of the estrous cycle (Flint *et al.*, 1994). Inhibition of receptor expression by trophoblast IFN blocks the process of luteal regression and leads to continued P4 secretion and the successful growth of the conceptus. In ewes, OTR increases in the critical day 12-14 period preceding the onset of luteolysis but are downregulated during pregnancy (Roberts *et al.*, 1992a; Mirando *et al.*, 1993). Administration of conceptus secretory proteins or rbIFN-τ into the uterine lumen reduces OTR expression (Vallet and Lamming, 1991). The action of trophoblast IFN-τ is exerted locally (Lamming *et al.*, 1991). The OTR concentrations are low in the pregnant horns which have high concentrations of IFN-τ; whereas the reverse is true in nonpregnant uterine horns. Since both uterine horns are exposed to identical concentrations of circulating steroids, any extra-uterine effects are ruled out (Lamming *et al.*, 1995). During the luteolytic period endometrial expression of OTR is high, exogenous oIFN-τ cannot prevent binding of OT to its receptor, inhibit OT stimulation of endometrial

inositol phosphate metabolism, or inhibit oxytocin stimulation of endometrial PGF secretion (Bazer, 1992). Rather, the endometrium of cyclic ewes must be exposed to IFN-τ from Days 12 to 14 (i.e., 2-3 days before OTR is expected to increase), to block oxytocin-induced inositol phospholipid metabolism and $PGF_{2\alpha}$ secretion (Bazer, 1992). Intrauterine injections of IFN-τ on Days 11-15 prevent increases in endometrial ER and E2-dependent increases in OTR gene expression (Mirando *et al.*, 1993; Spencer *et al.*, 1995c), an effect which requires P4 (Ott *et al.*, 1992; Spencer *et al.*, 1996a). Thus, it appears that IFN-τ cannot modify OTR response to OT after OTR is formed but IFN-τ can block expression of OTR and luteolysis. Endometrial OTR expression was suppressed in ovariectomized ewes that received intrauterine injections of roIFN-τ from Days 11 to 15 regardless of steroid treatment (Spencer *et al.*, 1995c). The endometrium of cyclic ewes must be exposed to IFN-τ from days 11 or 12 to day 14, i.e., 2-3 days before OTR increase in endometrial lumenal epithelia of cyclic ewes, to block expression of OTR and OT induction of inositol phospholipid metabolism and luteolytic pulses of $PGF_{2\alpha}$ (Zhang *et al.*, 1991; Spencer *et al.*, 1996b).

The inhibitory effect of IFN-τ on OTR is transient. If endometrial tissue, which is unresponsive to oxytocin challenge *in vivo*, is removed from day 12, 14 or 16 pregnant ewes and treated instead *in vitro* following removal of the influence of the conceptus, then oxytocin can elicit a $PGF_{2\alpha}$ response after only 3 h in culture (Silvia and Raw, 1993). mRNA encoding the OTR is not detectable in the endometrium at this stage of pregnancy (Stevenson *et al.*, 1994), which implies that gene transcription and translation are activated immediately after removal of IFN-τ from the endometrial environment.

2.4.2.6. Effects of IFN-τ on steroid receptor expression

E2 and P4 play major roles in expression of the OTR gene in ovine endometrium (Matthews *et al.*, 1991; Beard and Lamming, 1994). Therefore, it is reasonable to anticipate that IFN-τ regulates endometrial ER and PR gene expression to control OTR formation.

Endometrial ER and PR gene expression is regulated in a tissue and cell-type-specific manner which differs between the estrous cycle and early pregnancy (Spencer *et al.*, 1996a). In pregnant ewes, endometrial PR mRNA levels decline between Days 10 to 12 and Day 16, whereas PR protein is low and does not change between Days 10 and 16. Endometrial ER mRNA and protein decreases from Days 10 to 16 in pregnant ewes but increase during this period in cyclic ewes (Spencer and Bazer, 1995). Immunoreactive ER are absent in luminal and superficial glandular epithelium of pregnant ewes; however, ER expression in deep glandular epithelium, stroma or myometrium is similar for Day 15 pregnant and cyclic ewes (Spencer *et al.*, 1995b; 1996a). Thus, products of the conceptus may either stablize endometrial PR and/or inhibit ER expression in the luminal epithelium and superficial glandular epithelium during pregnancy recognition (Ott *et al.*, 1993). Intrauterine infusion of ovine conceptus secretory proteins from Days 11 to 15 (Mirando *et al.*, 1993) or of roIFN-τ from Days 11 to 14 (Spencer *et al.*, 1995c) in cyclic ewes prevented the increase in ER mRNA and protein in the endometrium.

In summary, luteolysis and the prevention of luteolysis are two critical events in cow. At the late stage of luteal phase, the minor increase in OTR number enhances the sensitivity of uterine endometrium to OT stimulation and results in the episodic release of $PGF_{2\alpha}$. The CL is degenerated by $PGF_{2\alpha}$ pulse. The initiation of luteolysis process is regulated by steroid hormones. In the pregnant cow, IFN-τ which is produced by conceptus attenuates $PGF_{2\alpha}$ secretion

and prevents luteal regression. However, regulation of the post-receptor processes involved in the PGs synthesis by steroid and IFN-τ remain to be determined. Furthermore, the respective roles of different uterine endometrial cells in the initiation and prevention of luteolysis are not well understood.

We hypothesized that, 1) steroid hormones and interferon have differential effects on the different endometrial cell types; 2) steroid hormones, OT and IFN-τ act on the PG synthesis pathway, in particular by regulating COX-2 and PGFS expression.

The objectives of this study were, 1) to establish an appropriate primary endometrial cell culture system which can respond to steroid hormones, oxytocin and IFN-τ; 2) to examine the effect of steroid hormones on the receptors for E2 and P4; 3) to study the mechanisms by which the embryo modifies the ratio of $PGF_{2\alpha}$ to PGE2.

In order to achieve these goals, the following strategies were exployed; 1) maximized the purity of isolated cells, as measured by immunocytochemical methods, and measured the changes in proliferation, morphology and steroid hormone receptor number of these cells in response to steroid hormone treatment; 2) examined the effect of steroids and IFN-τ on the basal secretion of PGs, and on the expression of cyclooxygenase-2 (COX-2) and PG F synthase (PGFS) genes in cultured bovine endometrial cells; 3) investigated the effect of IFN-τ on OTR and OT-induced PG secretion, and on COX-2 and PGFS gene expression in bovine endometrial epithelial cells.

3. Articles

3.1. Article 1. Differential effects of oestradiol and progesterone on proliferation and morphology of cultured bovine uterine epithelial and stromal cells

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Differential effects of oestradiol and progesterone on proliferation and morphology of cultured bovine uterine epithelial and stromal cells

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The effect of oestrogen and progesterone on the proliferation of cultured bovine uterine epithelial and stromal cells was assessed. Epithelial and stromal cells recovered from cows at day 1 to day 3 of the oestrous cycle were cultured in RPMI medium supplemented with 5% steroid-free fetal calf serum for 4 and 8 days. The addition of progesterone to the culture medium altered the morphology of stromal cells. Oestradiol (0.1–10 nmol l^{-1}) and progesterone (50 nmol l^{-1}) significantly increased the total DNA (from 9.6 ± 0.96 to 25.6 ± 0.99 μg per well, $P < 0.001$) and protein content (from 76.6 ± 2.6 to 125.8 ± 2.6 μg per well, $P < 0.001$) and decreased the ratio of protein to DNA (from 8.0 ± 0.24 to 4.9 ± 0.24, $P < 0.01$) in stromal cells during the first 4 days. During the second 4 days, the relative percentages of increase in DNA content were not affected by steroids, indicating that the major effect of steroids on stromal cell proliferation was exerted during the first 4 days of incubation. The morphology of epithelial cells was not influenced by the addition of steroids. DNA content of epithelial cells was reduced by the addition of oestrogen (from 22.9 ± 2.1 to 15.0 ± 2.0 μg per well, $P < 0.01$), but not progesterone (from 22.9 ± 2.1 to 25.8 ± 2.0 μg per well, $P > 0.05$). Total protein content of epithelial cells was reduced by oestradiol by day 4 (from 111.0 ± 6.2 to 71.0 ± 6.2 μg per well, $P < 0.01$), but not by day 8 (from 305.0 ± 10.5 to 296.0 ± 10.5 μg per well, $P > 0.05$). Progesterone increased the total protein content (from 305.0 ± 10.5 to 366.0 ± 10.5 μg per well, $P < 0.01$). Oestradiol significantly enhanced the ratio of protein to DNA in epithelial cells at day 8 (from 10.1 ± 1.0 to 16.8 ± 1.0, $P < 0.01$). These results show that oestradiol and progesterone have different effects on the proliferation and morphology of epithelial and stromal cells *in vitro*.

Introduction

The endometrium undergoes major histological and biochemical changes during the oestrous cycle. These changes are induced by progesterone and oestradiol and are necessary to provide a suitable environment for embryo development in the pregnant animal. There are marked morphological changes in the endometrium, such as an increase in the thickness and growth of the glands during the oestrous cycle in ruminants (Nalbandov, 1976). Progesterone and oestradiol are also responsible for maintaining synchrony between the embryo and the endometrium (Wilmut *et al.*, 1985) and play an important role in regulating prostaglandin release at luteolysis (McCracken *et al.*, 1984; Lafrance and Goff, 1988). Understanding the mechanisms involved in the action of progesterone and oestradiol in these processes is difficult due to the complexity of the interactions involved.

Recent work in sheep has shown that growth of epithelial and stromal cells is correlated with systemic concentrations of progesterone and oestradiol (Johnson *et al.*, 1997). The proliferation and differentiation of uterine epithelial and stromal cells are controlled primarily by oestradiol and progesterone (Conti *et al.*, 1981; Irwin *et al.*, 1991; Watson *et al.*, 1994). However, the growth and differentiation responses to these hormones in the different endometrial cell types are not yet fully understood. Furthermore, the effect of oestradiol and progesterone on uterine endometrial proliferation observed *in vitro* is inconsistent and depends on experimental conditions, such as the presence of serum and the purity of the isolated cell population. Oestradiol, progesterone or their combination stimulate the growth of human endometrial cells (Pavlik and Katzenellenbogen, 1978) and isolated stromal cells (Irwin *et al.*, 1991) cultured in the presence of a steroid-free calf serum. In combination, but not separately, oestradiol and progesterone significantly increased [^{3}H]thymidine incorporation by human endometrial stromal cells in serum-free medium (Chegini *et al.*, 1992). A lack of effect by oestradiol and progesterone on the proliferation of endometrial, epithelial and stromal cells from rodents and humans has been reported (Iguchi *et al.*, 1983; Tomooka and McLachlan, 1986; Irwin *et al.*, 1989; Chegini *et al.*, 1992; Rossi *et al.*, 1992). It has been suggested that this lack of oestradiol may be due to the absence of growth factors,

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such as insulin (Van der Burg *et al.*, 1988), insulin-related polypeptides (Murphy *et al.*, 1987) and epidermal growth factor (Mukku and Stancel, 1985; Di Augustine *et al.*, 1988). These factors are considered to be potential mediators of oestrogen-induced proliferation. Inhibitory effects of oestrogen on the proliferation of guinea-pig (Alkhalaf *et al.*, 1991) and human (Marshburn *et al.*, 1994) endometrial glandular epithelium have also been reported.

We have previously shown that progesterone and oestradiol can alter prostaglandin secretion from epithelial and stromal cells isolated from bovine endometria (Bergeron and Goff, 1993; Asselin *et al.*, 1996). However, little information is available on the effect of these hormones on bovine endometrial cell proliferation. Although oestradiol is uterotropic *in vivo*, no effect of oestradiol has been observed on the growth of bovine endometrial cells *in vitro* (Tiemann *et al.*, 1994; Asselin and Fortier, 1996). The objective of the study reported here was to determine if oestradiol and progesterone are able to alter the DNA and protein content of endometrial stromal and epithelial cells grown *in vitro*.

Materials and Methods

Chemicals and reagents

Tissue culture medium (RPMI 1640), Hank's Buffered Saline Solution (HBSS, calcium and magnesium free), fetal calf serum (FCS), antibiotics and Trypan blue were purchased from Gibco (Grand Island, NY). Collagenase (Type II), trypsin (Type III, from bovine pancreas), DNase I (Type I, from bovine pancreas), gentamicin, calf thymus DNA, Hoechst No. 33258, BSA, oestradiol, progesterone and goat anti-mouse immunoglobulin G (IgG) conjugated to fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co. (St Louis, MO). Monoclonal antibodies to cytokeratin (No. 18) and fibronectin were obtained from Boehringer (Mannheim). Matri-gel was obtained from VWR Scientific (Ontario). Bio-Rad protein assay-dye reagent concentrate was obtained from Bio-Rad Laboratories (Mississauga, Ontario).

Preparation and culture of cells

Bovine uteri from cows at day 1 to day 3 of the oestrous cycle were collected at an abattoir and transported on ice to the laboratory. Days 1–3 were selected because the stage of oestrus can be determined accurately from slaughterhouse material owing to the presence of the corpus haemorrhagicum in the ovary and, thus, variability among uterine samples is reduced. The endometrial epithelial and stromal cells were separated by a modification of the procedure described by Fortier *et al.* (1988). Briefly, the two horns of the uterus were placed in sterile HBSS containing 10 µl antibiotics ml^{-1} (100 units of penicillin, 100 µg of streptomycin and 0.25 µg of amphotericin B). The myometrial layers were dissected from the two horns, and the remaining endometrial tissue was then everted to expose the epithelium. The everted horns were first digested for 2 h in HBSS with 0.3% (w/v) trypsin at room temperature to obtain epithelial cells. At the end of incubation, the digested horns were scraped slightly with forceps and washed twice in HBSS. The cells from the digestion and washings were pooled and FCS was added (10% final volume) to block the action of trypsin. The horns were then further digested to obtain stromal cells by incubating in HBSS with 0.064% (w/v) trypsin III, 0.064% (w/v) collagenase II and 0.032% (w/v) DNase I for 45 min at 37°C. FCS was added (10% final volume) to block the action of trypsin at the end of incubation.

Epithelial cells. Most of the epithelial cells were in clumps after trypsin digestion. Therefore, it was possible to separate them from single stromal cells by low-speed centrifugation (60 ***g*** for 5 min). The centrifugation pellet was then washed three more times with HBSS. The epithelial cells were purified further by suspension in 20 ml RPMI medium supplemented with 5% FCS and 50 µg ml^{-1} of gentamicin and plated onto 100 mm × 20 mm Petri dishes (Nunclon, Grand Island, NY) and incubated at 37°C with 5% CO_2, 95% air for 3 h. At the end of the incubation, any contaminating stromal cells that had adhered to the dish and the floating epithelial cells were collected. The cells were counted and their viability determined by Trypan blue exclusion. At this stage > 95% of cells were viable. The epithelial cells were then plated onto Matri-gel coated six-well plates at a concentration of 5×10^5 cells per well (Corning, NY). Since the epithelial cells took about 48 h to attach to the plates, they were cultured for 4 more days in RPMI-1640 medium containing 5% FCS that had been depleted of steroids by dextran–charcoal extraction at 37°C in humidified air (5% CO_2). The cells were then cultured for 4 or 8 days in the presence or absence of various concentrations of oestradiol and progesterone.

Stromal cells. The cell suspension of stromal cells was centrifuged at 60 ***g*** for 5 min to remove cell clumps and the supernatant was then centrifuged at 1000 ***g*** for 10 min. The pelleted stromal cells were washed twice with HBSS and plated onto six-well plates at a concentration of 2×10^6 cells per well and incubated for 3 h. The floating cells were washed away by gentle pipetting. The attached stromal cells were then cultured in RPMI-1640 medium containing 5% FCS, which was depleted of steroids by dextran–charcoal extraction, in the presence or absence of various concentrations of oestradiol and progesterone. At the time of plating, the viability of the stromal cells was > 95%.

Cell proliferation experiment

All cells were cultured in phenol red-free RPMI-1640 medium containing 5% FCS (depleted of steroids by dextran–charcoal extraction) in the presence or absence of steroids at 37°C in humidified air (5% CO_2). The charcoal extraction procedure decreased added [^{3}H]oestradiol (25 pg ml^{-1}) by at least 100-fold. The stromal and epithelial cells had reached about 60% confluency at the beginning of culture. Cells were cultured for 4 or 8 days in medium alone or in medium containing oestradiol (0.1, 1 or 10 nmol l^{-1}), progesterone (50 nmol l^{-1}) or oestradiol (1 nmol l^{-1}) plus progesterone (50 nmol l^{-1}). The medium was changed every 2 days. The 4- and 8-day culture periods presented in the results represent the time the cells were exposed to the hormone treatment.

Determination of DNA content

At the end of the culture period, the medium was removed and the cells were rinsed twice with HBSS and then detached with 1 mmol EDTA l^{-1} in HBSS and the use of a rubber scraper. Cells were pelleted by centrifuging at 1000 ***g*** for 5 min, and 100 µl of 0.2% (w/v) SDS in ETN buffer (10 mmol l^{-1} EDTA, 10 mmol l^{-1} Tris–HCl, 100 mmol l^{-1} NaCl, pH 7.0) was added to the pellet. The pellet was sonified 10 times for stromal cells and 15 times for epithelial cells using a Branson sonifier-450 (VWR Scientific, Ontario) at 10% power. The DNA content in 10 µl sonified cell suspension was determined using the bisbenzimide fluorescent dye method of Labarca and Paigen (1980). Calf thymus DNA was used as standard at concentrations of 4, 8, 16, 32, 64, 128 and 250 ng ml^{-1}. In some experiments ($n = 3$), the numbers of cells were counted after detachment from the culture dish, and a linear relationship between the number of cells and the DNA content was observed ($y = (35 + 179.8x)10^3$, $r^2 = 0.999$, where y is the number of cells and x is the DNA content (µg)).

Immunocytochemistry

Homogeneity of the cells was examined by indirect immunofluorescent staining for specific markers of epithelial cells (cytokeratin) and stromal cells (fibronectin). Cells were cultured in 24-well plates, washed with PBS and fixed using 2% formaldehyde in PBS for 15 min at room temperature. The cells were permeabilized by treatment with 0.5% Triton-X100 overnight, rinsed with PBS and then incubated for 1 h with either anticytokeratin or antifibronectin at a concentration of 10 µg ml^{-1}. Negative controls were incubated with bovine serum. The cells were washed with PBS and incubated with the second antibody, goat anti-mouse IgG conjugated to FITC, for 1 h at 37°C. The slides were examined with a Universel Zeiss epifluorescence microscope.

Measurement of total protein

Total protein was measured in 10 µl of sonified cell suspension using the Bradford method (Bio-Rad Laboratories). BSA was used as standard (0.0875 to 1.4 mg ml^{-1}).

Statistical analyses

Each treatment was carried out in triplicate using the cells from one uterus and each experiment was repeated with 6–10 different uteri. The effects of treatment on DNA content and on the ratio of total protein to DNA content of uterine cells were evaluated by least-squares analysis of variance. Treatments were analysed in multifactorial design (ANOVA) which included the main effects of experiments, cell type, duration of culture and hormone treatments, and the appropriate interactions. Simple linear contrasts were used to determine differences between individual means. All differences were considered significant at $P < 0.05$.

Results

Effect of oestradiol and progesterone on cell morphology

Clumps of epithelial cells attached to the culture surface as small colonies within 48 h, and were polygonal or spherical in shape. When incubation time was extended, the cells became a monolayer and exhibited cuboidal or columnar morphology characteristic of epithelial cells (Fig. 1a). Stromal cells presented a monolayer of flat, spindle- and fibroblast-like cells. The homogeneity of the cell populations was examined by immunocytochemistry. Epithelial cell contamination of stromal cells was about 3% and stromal cell contamination of epithelial cells was less than 1% (Fig. 1).

The effect of oestradiol and progesterone on the morphology of epithelial and stromal cells was evaluated after 4 days of culture. The general pattern of growth was observed using an inverted phase-contrast microscope. Neither oestradiol nor progesterone treatment resulted in a change in the appearance of the epithelial cells. The morphology of the stromal cells was not altered by oestradiol at the different concentrations used. However, progesterone (50 nmol l^{-1}) and the combination of progesterone (50 nmol l^{-1}) plus oestradiol (1 nmol l^{-1}) significantly changed the morphology of stromal cells (Fig. 2). Instead of the homogeneous distribution of cells seen in control or oestradiol-treated groups (Fig. 2a, b), the progesterone-treated cells migrated together to form many star-shaped colonies (Fig. 2c). The appearance of these colonies was more dense (Fig. 2d) in the oestradiol plus progesterone treatment group than in the progesterone alone treatment group.

Effect of oestradiol and progesterone on the DNA and protein content of stromal cells

Both oestradiol and progesterone significantly stimulated proliferation of stromal cells after incubation for 4 days ($P < 0.001$) (Fig. 3a). The effect of oestradiol was dose dependent with the maximum dose being between 1 and 10 nmol l^{-1}. Progesterone stimulated DNA content to a greater degree than oestradiol ($P < 0.001$) and the effect of progesterone was not modified by oestradiol, indicating no interaction between these two hormones.

The total protein content of stromal cells measured after 4 days of incubation followed the same pattern as the DNA content (Fig. 3b). The stimulating effect of oestradiol was dose dependent and there were no differences among the treatments of 10 nmol oestradiol l^{-1}, 50 nmol progesterone l^{-1} and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}. Oestradiol and progesterone significantly decreased the ratio of total protein to DNA content ($P < 0.05$, Fig. 3c). The ratio declined 19%, 29%, 25%, 36% and 39% for cells treated with 0.1, 1, 10 nmol oestradiol l^{-1}, 50 nmol progesterone l^{-1}, and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}, respectively.

The effects of hormone treatment and duration of culture on cell growth were highly significant ($P < 0.0001$), and there was an interaction between treatments and duration ($P < 0.01$) for DNA content, protein content, and protein to DNA ratio (Fig. 4). In medium alone, the DNA content of stromal cells increased 34.4% within the following 4 days ($P < 0.01$) (Fig. 4a). Although the absolute increases in DNA content (value at day 8 minus value at day 4) during the second 4 days of culture (5.4, 7.6, 6.2 µg per well, for 1 nmol oestradiol l^{-1}, 50 nmol progesterone l^{-1}, and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}, respectively) were higher than that

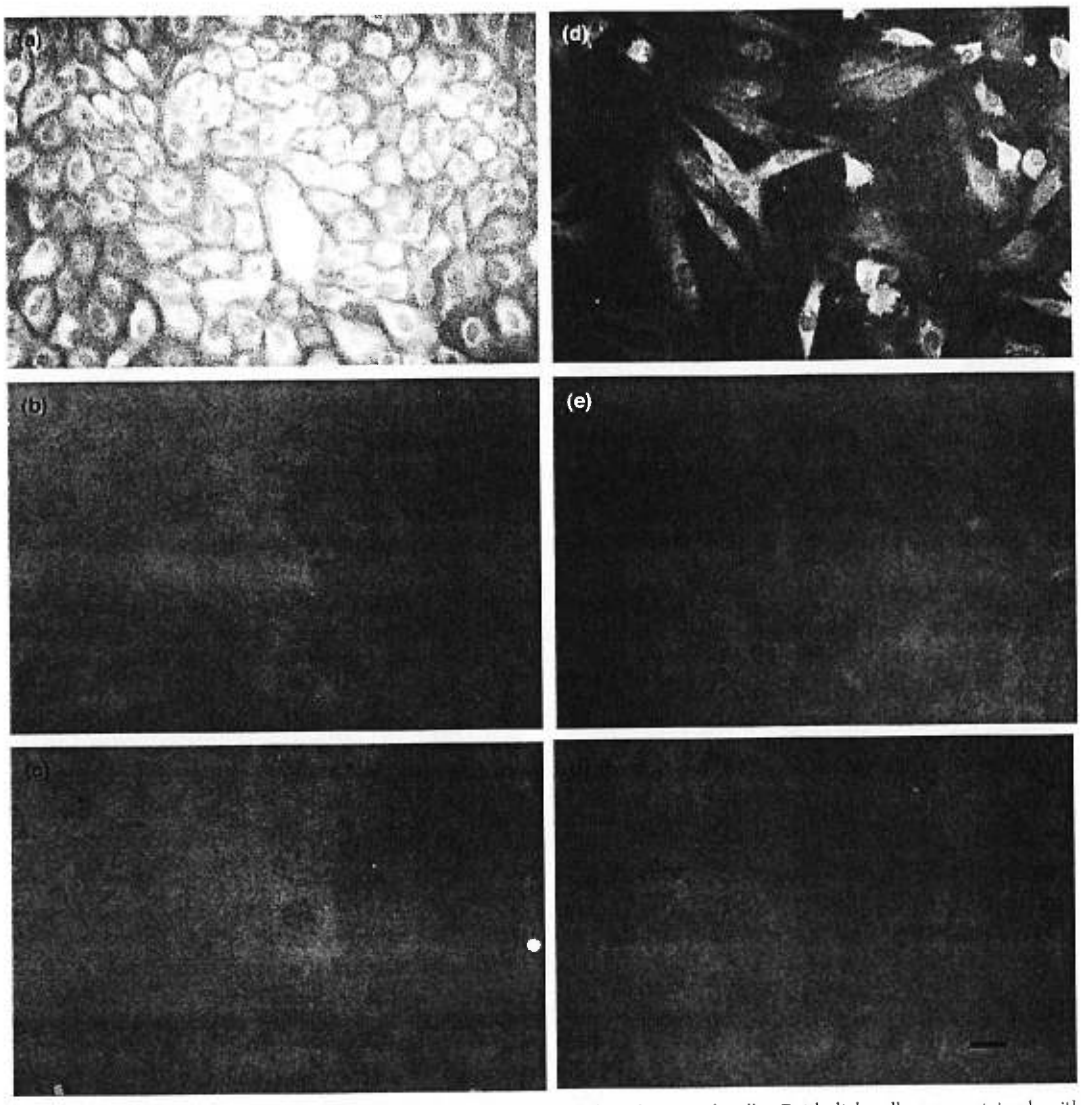

Fig. 1. Immunofluorescent staining of purified bovine epithelial and stromal cells. Epithelial cells were stained with anticytokeratin (a), but not with antifibronectin (b) or non-immune serum (c). Stromal cells were stained with antifibronectin (d), but not with anticytokeratin (e) or non-immune serum (f). Scale bar represents 25 μm.

of the control (3.3 μg per well), the relative increase in DNA (the increase in DNA between days 4 and 8 expressed as a percentage of the content at day 4) of the hormone-treated cells decreased when compared with the control (34.4%, 28.9%, 30.9% and 24.2%, for control, 1 nmol oestradiol l^{-1}, 50 nmol progesterone l^{-1}, and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}, respectively). This result indicated that DNA synthesis had slowed during the second 4 days of culture and that oestrogen and progesterone were less effective in stimulating DNA synthesis during this time. In contrast to the change in DNA content, oestradiol and progesterone increased both the absolute (44, 81, 88, 104 μg per well for control, 1 nmol oestradiol l^{-1}, 50 nmol progesterone l^{-1}, and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}, respectively) and the relative protein content in stromal cells (58%, 76%, 70%, and 82% for control, 1 nmol oestradiol l^{-1}, 50 nmol progesterone l^{-1}, and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}, respectively). At day 8, the ratios of total protein to DNA content increased significantly compared with those at day 4 (17.5, 36.8, 31.4, and 46.9 for control, 1 nmol oestradiol l^{-1}, 50 nmol progesterone l^{-1}, and 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}, respectively, Fig. 4c). Thus, the effect of hormone treatment on the protein content of the cells was enhanced with the extended incubation time.

Effect of oestradiol and progesterone on the DNA and protein content of epithelial cells

Treatment with oestradiol alone inhibited the proliferation of endometrial epithelial cells in a dose-dependent manner

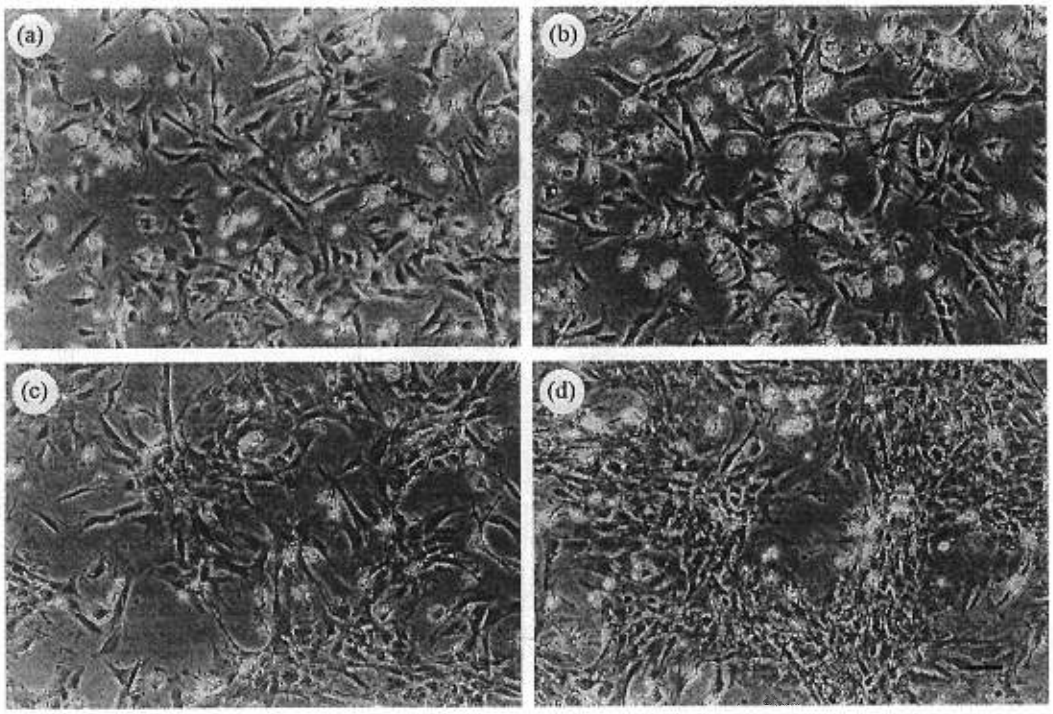

Fig. 2. Influence of oestradiol and progesterone on the morphology of bovine endometrial stromal cells. Stromal cells were cultured in RPMI medium supplemented with 5% steroid-free fetal calf serum (a) alone, or in presence of (b) 10 nmol oestradiol l^{-1}, (c) 50 nmol progesterone l^{-1}, or (d) 10 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1} for 4 days. The photographs were taken using an inverted phase contrast microscope. Scale bar represents 50 μm.

(Fig. 5a). When 0.1 nmol oestradiol l^{-1} was used, DNA content decreased by 11.8%, but was not statistically different from the control. However, when increased concentrations of oestradiol (1 and 10 nmol l^{-1}) were used, the inhibitory effect of oestradiol on epithelial proliferation was significant (27.9% and 34.5%, respectively, $P < 0.001$). In contrast, progesterone had no significant effect on the proliferation of epithelial cells, but did block the inhibitory effect of oestradiol on epithelial proliferation.

The changes in total protein content of endometrial epithelial cells measured at day 4 (Fig. 5b) were similar to those observed for the DNA content. The total protein contents for the various concentrations of oestradiol (0.1, 1, 10 nmol l^{-1}) were 15.9%, 28.8%, and 36% lower than that of the control ($P < 0.05$). Progesterone alone or progesterone plus oestradiol had no significant effect on total protein content.

The results of the ANOVA for DNA content, protein content and the ratio of protein to DNA showed that the effects of hormone treatments, duration of culture, and the interaction between treatments and duration were significant ($P < 0.01$) (Fig. 6). The DNA content did not change between days 4 and 8 in the epithelial cells treated with oestradiol (1 nmol l^{-1}) or progesterone (50 nmol l^{-1}) plus oestradiol (1 nmol l^{-1}) ($P > 0.05$) (Fig. 6a). Thus, there was no obvious cell proliferation in the presence of 1 nmol oestradiol l^{-1} during this time. However, the DNA content increased 46.7% in the control and 45.3% in the presence of progesterone alone (50 nmol l^{-1}) ($P < 0.01$). Also, after more prolonged culture (8 days), progesterone was not able to prevent the inhibitory effect of oestradiol on proliferation of epithelial cells. Therefore, the effect of oestradiol and progesterone on epithelial proliferation was time dependent.

After 8 days of incubation, total protein content in epithelial cells was increased by progesterone (50 nmol l^{-1}) and progesterone plus oestradiol (19.9%, 17.3%, respectively, over control, $P < 0.01$) and the inhibitory effect of oestradiol (1 nmol l^{-1}) was not observed (Fig. 6b). After 4 days of incubation there was no effect of treatment on the protein: DNA ratio (Fig. 6c). However, after 8 days of incubation, this ratio was increased significantly by the presence of oestradiol (1 nmol l^{-1}), either alone, or in combination with progesterone (Fig. 6c). This result indicates that even though oestradiol inhibited DNA synthesis in endometrial epithelial cells, it did not inhibit protein synthesis, and even stimulated protein synthesis with time.

Discussion

In the present report, a method is described for studying the effects of progesterone and oestradiol on the proliferation and morphology of bovine uterine endometrial epithelial and stromal cells using an *in vitro* primary cell culture system. The purification procedures used resulted in a homogeneous stromal cell population with very little epithelial cell

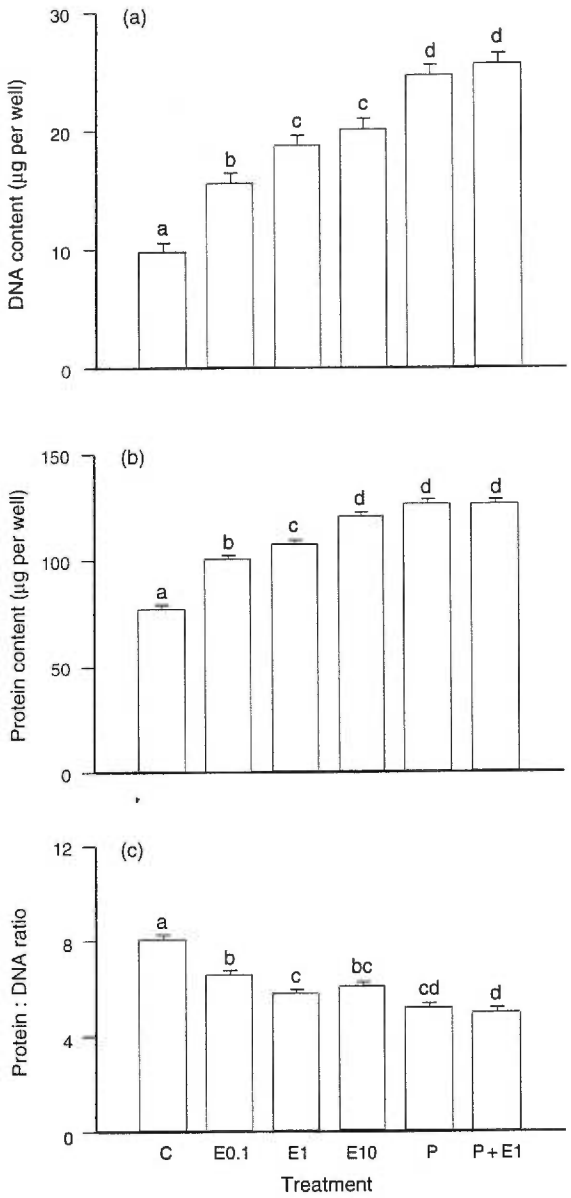

Fig. 3. Effects of oestradiol and progesterone on (a) DNA content, (b) protein content and (c) the ratio of protein to DNA in bovine endometrial stromal cells. Primary bovine endometrial stromal cells were cultured with RPMI medium supplemented with 5% steroid-free fetal calf serum in the absence (C) or presence of various doses of oestradiol (E0.1: 0.1 nmol l^{-1}; E1: 1 nmol l^{-1}; E10: 10 nmol l^{-1}) or progesterone (P: 50 nmol l^{-1}) or oestradiol plus progesterone (P + E1: 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}) for 4 days. Data represent least-square means ± SEM. Bars with different letters are significantly different ($P < 0.01$).

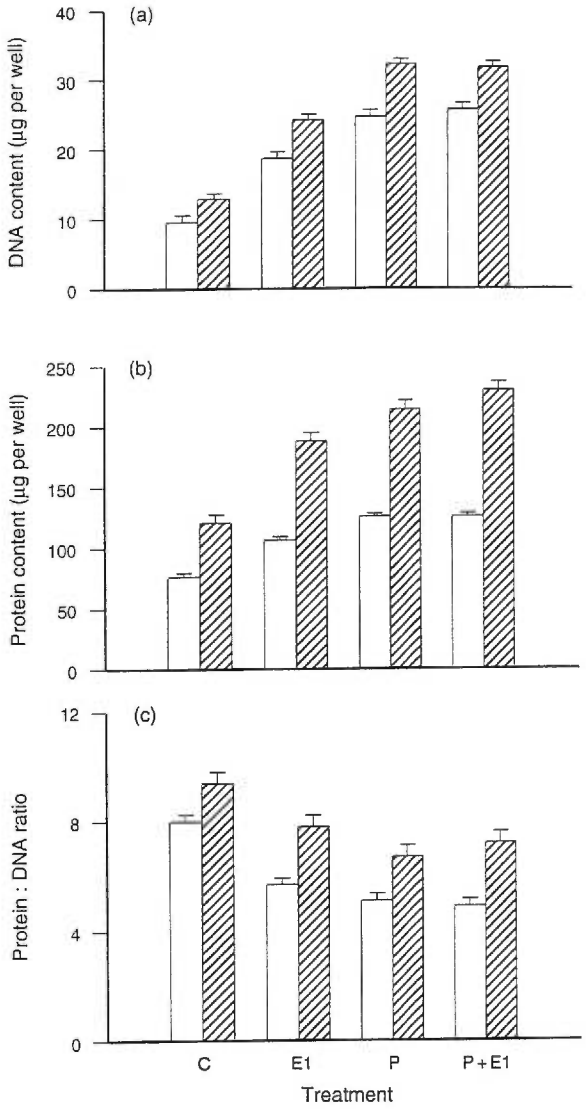

Fig. 4. Time-dependent effect of oestradiol and progesterone on (a) DNA content (b) protein content and (c) ratio of protein to DNA in bovine endometrial stromal cells. Primary bovine endometrial stromal cells were cultured with RPMI medium supplemented with 5% steroid-free fetal calf serum in the absence (C) or presence of either oestradiol (E1: 1 nmol l^{-1}), or progesterone (P: 50 nmol l^{-1}), or oestradiol plus progesterone (P + E1: 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}) for 4 days (□) or 8 days (▨). Data represent least-square means ± SEM.

contamination. Stromal cell contamination of the epithelial cells was approximately 3%. The effects of oestradiol on bovine endometrial cell proliferation have been shown for the first time using this system.

Oestradiol did not significantly change the morphology of either cell type. However, progesterone, either alone or in combination with oestradiol, altered the morphology of stromal, but not epithelial cells. Significant changes in the morphology of cultured uterine stromal cells, induced by the addition of ovarian steroids or progesterone, have been observed in humans (Gurpide and Holinka, 1987; Holinka, 1988), and it has been suggested that progestins may affect cytoskeletal proteins (Gurpide and Holinka, 1987).

Nuclear incorporation of [^{3}H]thymidine is a conventional and widely used measurement of DNA synthesis and cell proliferation. However, some discrepancies between

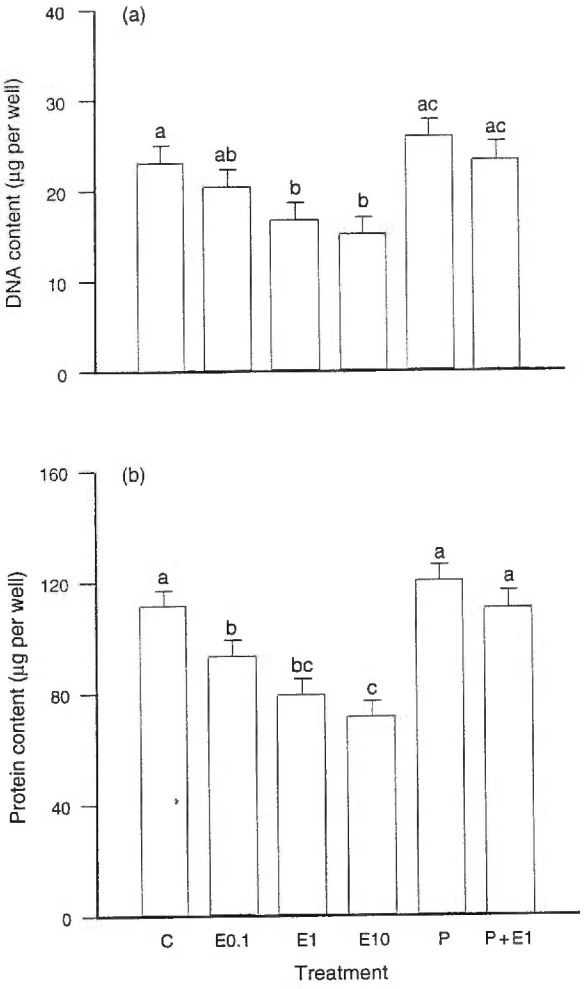

Fig. 5. Effects of oestradiol and progesterone on (a) DNA content and (b) protein content in bovine endometrial epithelial cells. Primary bovine endometrial epithelial cells were cultured with RPMI medium supplemented with 5% steroid-free fetal calf serum in the absence or presence of various doses of oestradiol (E0.1: 0.1 nmol l^{-1}; E1: 1 nmol l^{-1}; E10: 10 nmol l^{-1}) or progesterone (P: 50 nmol l^{-1}) or oestradiol plus progesterone (P + E1: 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}) for 4 days. Data represent least-square means ± SEM. Bars with different letters are significantly different ($P < 0.01$).

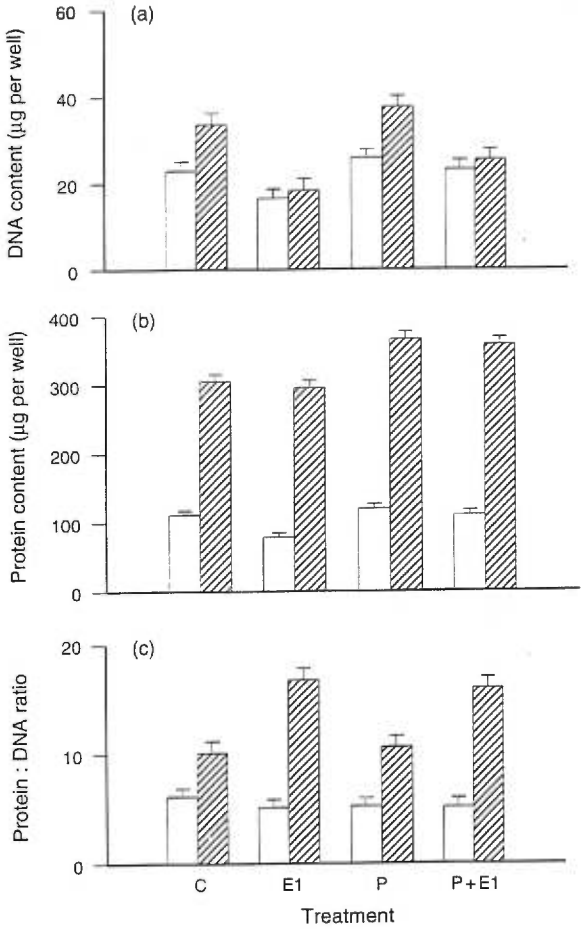

Fig. 6. Time-dependent effect of oestradiol and progesterone on (a) DNA content (b) protein content and (c) ratio of protein to DNA in bovine endometrial epithelial cells. Primary bovine endometrial epithelial cells were cultured with RPMI medium supplemented with 5% steroid-free fetal calf serum in the absence (C) or presence of either oestradiol (E1: 1 nmol l^{-1}), or progesterone (P: 50 nmol l^{-1}), or oestradiol plus progesterone (P + E1: 1 nmol oestradiol l^{-1} plus 50 nmol progesterone l^{-1}) for 4 days (□) or 8 days (▨). Data represent least-square means ± SEM.

[^{3}H]thymidine incorporation and true cell proliferation have been reported (Friedman *et al.*, 1981; Jozan *et al.*, 1983, 1985). Jozan *et al.* (1983) noted increased incorporation of [^{3}H]thymidine after oestradiol treatment, but no change in the number of cells, no increase of the size of nuclei, and no increase in DNA content. This discrepancy was also shown in primary cultures of rat hepatocytes that incorporated [^{3}H]thymidine but did not divide (Friedman *et al.*, 1981). The explanation for this discrepancy is that the addition of [^{3}H]thymidine during a short period of time could miss the S phase of DNA synthesis. If the incubation time was extended, there would be more time for the cell to degrade labelled thymidine and to spread the label in its general metabolism (Murat *et al.*, 1990). The direct measurement of DNA content used in the present study is highly correlated with cell number ($r^2 = 0.999$), which is consistent with a previous report (Inaba *et al.*, 1988), and therefore, should be a better indication of cell proliferation. However, even with the use of DNA and protein measurement, the possibility that treatment of the cells affects cell viability cannot be eliminated.

In vivo, oestradiol causes hypertrophy and hyperplasia of the endometrium and progesterone is thought to act on an oestradiol-primed uterus to stimulate growth of the endometrium. Progesterone is known to modify the action of oestrogen on the uterus by redirecting the proliferative response from the epithelium to the stroma. Martin and Finn (1969) reported that oestradiol stimulated the proliferation of the luminal epithelial cells, but not the stromal cells in mice uteri. However, after pretreatment with progesterone, oestradiol stimulated mitosis in the stroma, but not in the epithelium.

Recent results in sheep have shown that highest rates of cell proliferation occur at the beginning of the oestrous cycle, with proliferation being greater in the luminal epithelium and glands than in the stroma (Johnson *et al.*, 1997). In ovariectomized sheep, oestradiol stimulates proliferation in the epithelium and stroma, whereas progesterone predominantly stimulates the proliferation of epithelial cells (Johnson *et al.*, 1994).

In the present study, the proliferation of bovine endometrial stromal cells was stimulated by oestradiol and progesterone, either alone or in combination. Similar effects have been reported for oestradiol and progesterone on the proliferation of human endometrial stromal cells (Irwin *et al.*, 1991; Chegini *et al.*, 1992). The observed stimulation by oestradiol is also consistent with the effect of oestradiol on ovariectomized sheep (Johnson *et al.*, 1997). However, progesterone had little effect in the ovariectomized sheep in that study, whereas, in the present study, it stimulated stromal cell proliferation *in vitro*. Therefore, it is possible that the response to progesterone *in vivo* is modified by the epithelial cells. The present results differ from previously published data on the effects of steroids on bovine endometrial cells. Tiemann *et al.* (1994) found that oestradiol and progesterone were ineffective in stimulating [^{3}H]thymidine incorporation by epithelial and stromal cells in a serum-free medium. Asselin and Fortier (1996) recently reported that oestradiol had no effect on cell proliferation, while progesterone inhibited [^{3}H]thymidine incorporation in cultured bovine epithelial and stromal cells. There are three possible explanations for these differences: (1) as explained above, thymidine incorporation may give different results to DNA or protein measurement. (2) In the study of Tiemann *et al.* (1994), cells were isolated from the uterus at the mid-luteal phase, whereas in the present study, cells were isolated early in the cycle. Thus, the stage of the cycle at which the samples were taken could affect the response *in vitro*. There is evidence to support this suggestion. In sheep, proliferation of both the epithelial and stromal cells is greatest at the beginning of the cycle (Johnson *et al.*, 1997). Oestradiol stimulates the proliferation of stromal cells in immature rodents (Eide, 1975; Kirkland *et al.*, 1979; Pollard, 1990), but not in adult rats and mice (Quarmby and Korach, 1984; Pollard, 1990). Fleming and Gurpide (1982) also demonstrated that human endometrial stromal cells, obtained at different phases of the oestrous cycle, grow differently in culture. There is clear evidence of cyclical changes in the expression of steroid receptors in endometrial tissue (Bergqvist, 1991; Prentice *et al.*, 1992). Thus, the time that tissue samples are taken might result in differences in the expression of steroid and epidermal growth factor receptors (Mellor and Thomas, 1994) and alter the response *in vitro*. (3) Serum or growth factors may be necessary for oestradiol to exert its effect. The ineffectiveness of oestradiol on the proliferation of stromal cells (Fleming and Gurpide, 1982; Mellor and Thomas, 1994; Tiemann *et al.*, 1994; Asselin and Fortier, 1996) could be due to a lack of unknown serum factors or growth factors required for cultured cells to respond to oestrogen stimulation (Kano-Sueoka *et al.*, 1979; Page *et al.*, 1983; Irwin *et al.*, 1991; Tiemann *et al.*, 1994). The medium used in this study was supplemented with 5% FCS to provide the required serum factors or growth factors. The choice of 5% FCS was based on the consideration that higher concentrations of serum may mask any effects of treatment since cell proliferation increases as serum concentration in the medium increases.

Oestradiol and progesterone increased the total protein content of stromal cells. However, the ratios of protein to DNA were significantly decreased by treatment with oestradiol and progesterone for 4 days. It is possible that the cells became multi-nucleated (this was not examined). These results do agree with a previous study in rats (McCormack, 1980), which demonstrated that the ratios of protein to DNA in stromal cells remained unchanged for 12 h after an injection of oestradiol, and then decreased significantly by 24 h. In addition, in sheep, the ratio of protein to DNA decreases at mid-cycle suggesting an *in vivo* effect of steroid hormones (Johnson *et al.*, 1997).

Oestradiol alone inhibited proliferation in epithelial cells in a dose-dependent manner, and the addition of progesterone prevented this inhibitory effect of oestradiol. In mouse epithelial cells (Huet-Hudson *et al.*, 1989), c-*myc* induction and enhanced proliferation are oestradiol-dependent and in sheep, oestradiol stimulates the proliferation of the luminal epithelium (Johnson *et al.*, 1994). However, Marshburn *et al.* (1994) showed that DNA synthesis in cultured human endometrial epithelial glands was decreased by 40% after treatment with oestradiol (1×10^{-8} mol l^{-1}) for 5 days. A similar inhibitory effect of oestradiol on DNA synthesis was also observed by Alkhalaf *et al.* (1991) in the endometrial epithelial cells of guinea-pigs. The inhibitory effect of oestradiol on epithelial cell proliferation may be due to the lack of stromal–epithelial interaction. Stroma can mediate the hormonal responsiveness of epithelial cells in humans (Brenner *et al.*, 1990) and mice (Inaba *et al.*, 1988; Haslam and Counterman, 1991) and a paracrine interaction between stromal and epithelial cells may be required before an increase in proliferation in response to oestradiol will occur (Cunha *et al.*, 1983).

The ratios of protein to DNA of epithelial cells remained unchanged for 4 days after treatment with oestradiol and progesterone. However, oestradiol significantly increased the ratios in epithelial cells after 8 days of treatment to much higher values than in stromal cells. Smith *et al.* (1970) demonstrated that oestradiol enhances protein synthesis in the epithelial cells of adult mice, and that protein synthesis was higher in epithelial cells than in stromal cells. However, McCormack and Glasser (1980) showed that oestradiol administration did not significantly change the ratios of protein to DNA in rat epithelial cells.

In conclusion, progesterone altered the morphology of stromal cells, but not of epithelial cells. The present study demonstrates for the first time that both oestradiol and progesterone can enhance the proliferation of bovine stromal cells. Oestradiol inhibited the growth of epithelial cells in a dose-dependent manner, while progesterone appeared to have no effect on the proliferation of epithelial cells. Oestradiol and progesterone decreased the ratios of protein to DNA in stromal cells, whereas oestradiol enhanced the ratios in epithelial cells. Stromal cells were more responsive to steroids than epithelial cells. It is possible that an interaction between stromal and epithelial cells is necessary for the normal response of epithelial cells *in vivo*.

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References

Alkhalaf M, Mahfoudi A, Propper AY and Adessi GL (1991) Additive effect of oestradiol-17β and serum on synthesis of deoxyribonucleic acid in guinea-pig endometrial cells in culture *Journal of Reproduction and Fertility* **93** 295–302

Asselin E and Fortier MA (1996) Influence of thrombin on proliferation and prostaglandin production in cultured bovine endometrial cells *Journal of Cellular Physiology* **168** 600–607

Asselin E, Goff AK, Bergeron H and Fortier MA (1996) Influence of sex steroids on the production of prostaglandins F2α and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium *Biology of Reproduction* **54** 371–379

Bergeron H and Goff AK (1993) Effects of progesterone and estradiol on oxytocin stimulation of prostaglandin F2α release by stromal and epithelial cells of bovine endometrium *Biology of Reproduction* **48** (Supplement 1) 226 (Abstract)

Bergqvist A (1991) Steroid receptors in endometriosis. In *Modern Approaches to Endometriosis* pp 33–55. Eds EJ Thomas and J Rock. Kluwer Academic Publishers, Dordrecht, Holland

Brenner RM, West NB and McClellen MC (1990) Oestrogen and progestin receptors in the reproductive tract of male and female primates *Biology of Reproduction* **42** 11–19

Chegini N, Rossi MJ and Masterson BJ (1992) Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and EGF and PDGF β-receptors in human endometrial tissue: localization and *in vitro* action *Endocrinology* **130** 2373–2385

Conti CJ, Gimenez-Conti IB, Zerbe GO and Gerschenson LE (1981) Differential effects of estradiol-17β and progesterone on the proliferation of glandular and luminal cells of rabbit uterine epithelium *Biology of Reproduction* **24** 643–648

Cunha GR, Chung JM, Taguchi D and Fujii H (1983) Hormone-induced morphogenesis and growth: role of mesenchymal–epithelial interation *Recent Progress in Hormone Research* **39** 559–598

Di Augustine RP, Petrusz P, Bell GI, Brown CF, Korach KS, McLachlan JA and Teng CT (1988) Influence of estrogens on mouse uterine epidermal growth factor precursor protein and messenger ribonucleic acid *Endocrinology* **122** 2355–2363

Eide A (1975) The effect of oestradiol on the DNA synthesis in neonatal mouse uterus and cervix *Cell Tissue Research* **156** 551

Fleming H and Gurpide E (1982) Growth characteristics of primary cultures of stromal cells from human endometrium *Journal of Steroid Biochemistry* **16** 717–720

Fortier MA, Guibault LA and Grasso F (1988) Specific properties of epithelial and stromal cells from the endometrium of cows *Journal of Reproduction and Fertility* **83** 239–248

Friedman DL, Claaus TH, Pilkis SJ and Pine GE (1981) Hormonal regulation of DNA synthesis in primary culture of adult rat hepatocytes *Experimental Cell Research* **135** 283–290

Gurpide E and Holinka CF (1987) Proliferative potential and polymorphism of human endometrial stromal cells *Gynecological Endocrinology* **1** 71–81

Haslam SZ and Counterman LJ (1991) Mammary stroma modulates hormonal responsiveness of mammary epithelium *in vivo* in the mouse *Endocrinology* **129** 2017–2023

Holinka CF (1988) Growth and hormonal responsiveness of human endometrial stromal cells in culture *Human cell* **1** 207–217

Huet-Hudson YM, Andrews GK and Dey SK (1989) Cell type-specific localization of *c-myc* protein in the mouse uterus: modulation by steroid hormones and analysis of preimplantation period *Endocrinology* **125** 1683–1690

Iguchi T, Uchima FDA, Ostrander PL and Bern HA (1983) Growth of normal mouse vaginal epithelial cells in and on collagen gels *Proceedings of the National Academy of Sciences of USA* **80** 3743–3747

Inaba T, Wiest WG, Strickler RC and Mori J (1988) Augmentation of the response of mouse uterine epithelial cells to estradiol by uterine stroma *Endocrinology* **123** 1253–1258

Irwin JC, Kirk D, King RJB, Quigley MM and Gwatkin RBL (1989) Hormonal regulation of human endometrial stromal cells in culture: an *in vitro* model for decidualization *Fertility and Sterility* **52** 761–768

Irwin JC, Utian WH and Eckert RL (1991) Sex steroids and growth factors differentially regulate the growth and differentiation of cultured human endometrial stromal cells *Endocrinology* **129** 2385–2392

Johnson DA, Redmer DA and Reynolds LP (1994) Histological evaluation of cell proliferation in the uterus of intact and steroid-treated ovariectomized (OVX) ewes *Journal of Animal Science* **72** (Supplement 2) 74 (Abstract)

Johnson ML, Radmer DA and Reynolds LP (1997) Uterine growth, cell proliferation, and c-*fos* proto-oncogene expression throughout the estrous cycle in ewes *Biology of Reproduction* **56** 393–401

Jozan S, Mirouze A, Gay AG, Marques B and David JF (1983) Estradiol and ^{3}H-thymidine incorporation in cultured human cancer cells (breast–endometrium) *Bulletin de L'Association des Anatomistes* **67** 193–197

Jozan S, Gay G, Marques B, Mirouze A and David JF (1985) Oestradiol is effective in stimulating ^{3}H-thymidine incorporation but not on proliferation of breast cancer cultured cells *Cell and Tissue Kinetics* **18** 457–464

Kano-Sueoka T, Errick JE and Cohen DM (1979). Effects of hormones and a novel mammary growth factor on a rat-mammary carcinoma in culture. In *Hormone and Cell Culture* pp 449–512. Eds GH Sato and R Ross. Cold Spring Harbor, New York

Kirkland JL, LaPointe L, Justin E and Stancel GM (1979) Effect of estrogen on mitosis in individual cell types of the immature rat uterus *Biology of Reproduction* **21** 269

Labarca C and Paigen K (1980) A simple, rapid and sensitive DNA assay procedure *Analytical Biochemistry* **102** 344–352

Lafrance M and Goff AK (1988) Effects of progesterone and oestradiol-17β on oxytocin-induced release of prostaglandin F2α *Journal of Reproduction and Fertility* **82** 429–436

McCormack SA and Glasser SR (1980) Differential response of individual uterine cell types from immature rats treated with estradiol *Endocrinology* **106** 1634–1649

McCracken JA, Schramm W and Okulicz WC (1984) Hormone receptor control of pulsatile secretion of PGF2α from the ovine uterus during luteolysis and its abrogation in early pregnancy *Animal Reproduction Science* **7** 31–55

Marshburn PB, Arici AM and Casey ML (1994) Expression of transforming growth factor-β1 messenger ribonucleic acid and the modulation of deoxyribonucleic acid synthesis by transforming growth factor-β1 in human endometrial cells *American Journal of Obstetrics and Gynecology* **170** 1152–1158

Martin L and Finn CA (1969) Duration of progesterone treatment required for a stromal response to oestradiol-17β in the uterus of the mouse *Journal of Endocrinology* **44** 279–280

Mellor SJ and Thomas EJ (1994) The actions of estradiol and epidermal growth factor in endometrial and endometriotic stroma *in vitro*. *Fertility and Sterility* **62** 507–513

Mukku LJ and Stancel GM (1985) Regulation of epidermal growth factor receptor by estrogen *Journal of Biological Chemistry* **260** 9820–9824

Murat JC, Gamet L, Cazenave Y and Trocheris V (1990) Questions about the use of ^{3}H-thymidine incorporation as a reliable method to estimate cell proliferation rate *Biochemical Journal* **270** 563–564

Murphy LJ, Murphy LC and Friesen HG (1987) Estrogen induces insulin-like growth factor 1 expression in the rat uterus Molecular *Endocrinology* **1** 445–450

Nalbandov AV (1976) Reproductive physiology of mammals and birds. In *Reproductive Physiology of Mammals and Birds* pp 126–156. Ed. SG Salisbury. WH Freeman and Company, San Francisco

Page MJ, Field JK, Everett NP and Green CD (1983) Serum regulation of the estrogen responsiveness of the human breast cancer cell line MCF-7 *Cancer Research* **43** 1244–1250

Pavlik EJ and Katzenellenbogen BS (1978) Human endometrial cells in primary tissue culture: estrogen interactions and modulation of cell proliferation *Journal of Clinical Endocrinology and Metabolism* **47** 333–344

Pollard JW (1990) Regulation of polypeptide growth factor synthesis and growth factor-related gene expression in the rat and mouse uterus before and after implantation *Journal of Reproduction and Fertility* **88** 721–731

Prentice A, Randall BJ, Weddell A, McGill A, Henry L and Horne CHW (1992) Ovarian steroid receptor expression in endometriosis and in two potential parent epithelia: endometrium and peritoneal mesothelium *Human Reproduction* **7** 1318–1325

Quarmby VE and Korach KS (1984) The influence of 17β-estradiol on patterns of cell division in the uterus *Endocrinology* **114** 694–702

Rossi MJ, Chegini N and Masterson BJ (1992) Presence of epidermal growth factor, platelet-derived growth factor, and their receptors in human myometrial tissue and smooth muscle cells: their action in smooth muscle cells *in vitro*. *Endocrinology* **30** 1716–1727

Smith JA, Martin L, King RJB and Vértes M (1970) Effects of oestradiol-17β and progesterone on total and nuclear-protein synthesis in epithelial and stromal tissues of the mouse uterus, and of progesterone on the ability of these tissues to bind oestradiol-17β Biochemical Journal **119** 773–784

Tiemann U, Schneider F, Tuchscherer A and Becker F (1994) Influence of epidermal growth factor and steroids on the proliferation of cultured uterine cells in bovine treated with different gonadotropins *Reproduction in Domestic Animals* **29** 513–524

Tomooka DRP and McLachlan JA (1986) Proliferation of mouse uterine epithelial cells *in vitro*. *Endocrinology* **118** 1011–1018

Van der Burg GR, Blankenstein MA, Delaat SW and Van Zoelen EJJ (1988) Mitogenic stimulation of human breast cancer cells in a growth defined medium *Journal of Cell Physiology* **134** 101–108

Watson H, Franks S and Bonney RC (1994) Regulation of epidermal growth factor receptor (EGFR) by oestradiol and progesterone in human endometrial stromal cells *Journal of Endocrinology* **140** 231

Wilmut I, Sales DI and Ashworth CJ (1985) The influence of variation in embryo stage and maternal hormone profiles on embryo survival in farm animals *Theriogenology* **23** 107–119

3.2. Article 2. Hormonal regulation of oestrogen and progesterone receptors in cultured bovine endometrial cells

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Hormonal regulation of oestrogen and progesterone receptors in cultured bovine endometrial cells

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Summary

Changes in progesterone (P4) and/or oestradiol-17β (E2) receptor numbers in the endometrium are thought to play a role in inducing luteolysis. To help understand the mechanisms involved, the effect of E2 and P4 on the regulation of their receptors in cultured bovine uterine epithelial and stromal cells was examined. Cells were obtained from cows at day 1 to 3 of the oestrous cycle and were cultured for 4 or 8 days in medium alone (RPMI medium + 5% charcoal-dextran stripped new-born calf serum) or with E2, P4 or E2+P4. At the end of culture, receptor binding was measured by saturation analysis. Specific binding of both [^{3}H] ORG 2058 and [^{3}H] oestradiol to epithelial and stromal cells exhibited high affinities (K_d = 1.1 x 10^{-9} M, K_d = 6 x 10^{-10} M respectively, for P4 receptor (PR); K_d = 5.5 x 10^{-9} M, K_d = 7 x 10^{-10} M respectively, for E2 receptor (ER)). In the stromal cells, E2 (0.1-10 nM) increased E2 receptor number (from 0.21 ± 0.06 to 0.70 ± 0.058 fmol/ μg DNA) and PR numbers (from 1.4 ± 0.83 to 6.6 ± 0.70 fmol/μg DNA) after 4 days' culture ($p<0.01$) in a dose-dependent manner. After culture for 8 days, the stimulatory effect of E2 increased. P4 (50 nM) alone had no effect on either ER or PR numbers ($p>0.05$), however, it did inhibit the stimulatory effect of E2. In epithelial cells, the lower doses of E2 used (0.1, 1 nM) stimulated the PR number ($p=0.05$) after 4 days' culture, whereas, the highest dose of E2 used (10 nM), P4 (50 nM) alone and P4 (50 nM) plus E2 (1 nM) had no effect. After culture for 8 days, the stimulatory effect of E2 diminished. In contrast to the PR, ER numbers increased with the increase of E2 concentrations ($p<0.01$). These data show that PR numbers were higher in the stromal than in epithelial cells, whereas ER numbers were higher in epithelial cells than in stromal cells. E2 upregulates its own receptor and increases PR in

both cell types *in vitro*, P4 alone had little effect but inhibited the effects of E2 on PR.

Introduction

The bovine endometrium is a highly sensitive target organ for ovarian steroid hormones (Boos *et al.*, 1996). In ruminants, the uterine epithelium, stroma and myometrium all contain receptors for progesterone (PR) and oestrogen (ER) (Wathes and Hamon, 1993; Boos *et al.*, 1996). The endometrial receptor number determines the sensitivity of uterus to steroid stimulation and are themselves regulated by steroid hormones. It was shown that concentrations of ER and PR change throughout the oestrous cycle in response to changes in circulating steroid hormone concentrations (Boos *et al.*, 1996). The highest ER and PR concentrations were observed at postpartum anoestrus, oestrous and during the follicular phase of the oestrous cycle, when plasma progesterone concentrations were low (Vesanen *et al.*, 1988; Vesanan *et al.*, 1991). During the luteal phase of the oestrous cycle, when progesterone production by the corpus luteum increases, ER and PR concentrations decline in the endometrium (Findlay *et al.*, 1982; Zelinski *et al.*, 1982; Vesanen *et al.*, 1988; Vesanan *et al.*, 1991; Salamonsen *et al.*, 1992; Boos *et al.*, 1996). It is generally accepted that progesterone and oestradiol exert opposing effects on steroid receptors in the uterus. Oestradiol increases ER and PR concentrations and progesterone decreases both ER and PR (Katzenellenbogen, 1980; Leavitt *et al.*, 1983; Clark *et al.*, 1985).

Timing and initiation of luteolysis are critical for the normal oestrous cycle in ruminant animals (Lamming and Mann, 1995). Changes in PR and/or ER number are thought to play a role in luteolysis in sheep and cattle

(McCracken *et al.*, 1984; Spencer *et al.*, 1995; Wathes and Lamming, 1995). The number of uterine ER and PR may influence uterine prostaglandin F2α (PGF2α) secretion, and thus the lifespan of the corpus luteum during the oestrous cycle (Zollers *et al.*, 1993), and early pregnancy (Spencer and Bazer, 1995). The length of the luteal phase is controlled by the time of initiation and length of exposure to progesterone. Early administration of progesterone shortens the interoestrous interval in the ewe and cow, and removal of progesterone stimulation using a PR antagonist delays luteolysis in sheep (Woody and Ginther, 1968; Ginther, 1970; Ottobre *et al.*, 1980; Garrett *et al.*, 1988). Due to the inhibitory effect of progesterone on PR and ER, extended exposure of the uterus to progesterone will down-regulate PR and then desensitize the uterus to progesterone. As the effect of progesterone declines, ER numbers increase in the presence of oestrogen and thus increase the sensitivity of uterus to oestrogen. It was also shown that oxytocin receptor (OTR) number in the endometrium is regulated by progesterone and oestrogen; progesterone inhibits OTR and oestrogen enhances OTR (Hixon and Flint, 1987; Spencer *et al.*, 1995; Spencer *et al.*, 1996). Therefore, down-regulation of PR increased OTR and sensitivity of endometrium to oxytocin stimulation which induced luteolytic pusatile release of PGF2α (McCracken *et al.*, 1984).

The various cell types present in the bovine endometrium exhibit significantly different patterns of oestrogen and progesterone receptor immunoreactivities during the oestrous cycle (Boos *et al.*, 1996). In sheep, ER distribution varies according to the cell type, tissue compartment (caruncular or intercaruncular endometrium) and location of the particular cell type within that compartment (Salamonsen *et al.*, 1992; Spencer *et al.*, 1996). This suggests that different cell types within the uterus respond in different manners to identical hormonal stimuli (Boos *et al.*, 1996).

Modulation of progesterone and oestrogen receptors by steroid hormones in different endometrial cell populations is not completely understood owing to the complexity of the interactions involved. In this study, we used endometrial stromal and epithelial cells in primary culture to investigate hormonal regulation of ER and PR in the bovine uterus.

Materials and Methods

Chemicals and reagents

Tissue culture medium (RPMI 1640), Hank's Buffered Saline Solution (HBSS, calcium and magnesium free), new-born calf serum (NBCS), antibiotics and trypan blue were purchased from GIBCO (Grand Island, NY, USA). Collagenase (Type II), trypsin (Type III, from bovine pancreas), DNase I (Type I, from bovine pancreas), Gentamicin, calf thymus DNA, Hoechst NO. 33258, bovine serum albumin (BSA), 17β-oestradiol and progesterone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of oestradiol and progesterone were prepared by dissolving the steroids in ethanol. Matri-gel was obtained from VWR Canlab (Ontario, Canada). Bio-Rad protein assay-dye reagent concentrate was obtained from Bio-Rad Laboratories (CA, USA).

(2, 4, 6, 7-^{3}H) oestradiol (83.0 Ci/mmol), (^{3}H) ORG 2058 (16α-Ethyl-21-hydroxy-19-nor (6,7-^{3}H) pregn-4-ene-3,20-dione; 49.0 Ci/mmol), and unlabeled ORG were purchased from Amersham Life Science (Oakville, Ontario, Canada). Diethylstilbesterol was obtained from ICN Biochemicals Inc. (Aurora, Ohio, USA).

Preparation and culture of cells

Uteri from cows at days 1 to 3 of the oestrous cycle (ovaries with a corpus haemorrhagicum) were collected at the slaughterhouse and transported on ice

to the laboratory. Uteri collected at days 1-3 of the cycle were used in these experiments for two reasons. First, because of the presence of a corpus haemorrhagicum the stage of the cycle could be estimated accurately and thus variability reduce between experiments. Second, the tissue was taken before it was exposed to elevated levels of P4 and should be useful to examine the long term effects of P4 *in vitro*. Endometrial epithelial and stromal cells were separated by a modification of the procedure described by Fortier *et al.* (1988). Briefly, the two uterine horns were placed in sterile HBSS containing 100 units penicillin, 100 μg streptomycin and 0.25 μg amphotericin ml^{-1}. The myometrial layers were dissected, and each uterine horns was then everted to expose the epithelium. The everted horns were first digested for 2 hr in HBSS with 0.3% (w/v) trypsin at room temperature to obtain epithelial cells. At the end of incubation, the digested horns were scraped lightly with forceps, washed twice in HBSS and then further digested to obtain stromal cells by incubating in HBSS with 0.064% (w/v) trypsin III, 0.064% (w/v) collagenase II and 0.032% (w/v) DNase I for 45 min at 37°C. Immediately after each cell suspension was collected, 10% NBCS was added to inhibit the trypsin. For epithelial cells, the cell suspension was centrifuged at 60 g for 5 min and then the pellet was washed 3 times with HBSS. For further purification, the epithelial cell pellet was suspended in 20 ml RPMI medium supplemented with 5% NBCS and 50 μg ml^{-1} of gentamicin, plated onto 100 x 20 mm Nunclon petri dishes (Grand Island, NY, USA) and then incubated at 37°C with 5% CO_2, 95% air for 3 hr. At the end of incubation, contaminating stromal cells adhered to the dish and the floating epithelial cells were collected. After cell counting and viability determination by trypan-blue exclusion, 2.5×10^6 cells per dish were plated onto matri-gel coated 100 X 20 mm dishes. Since the epithelial cells took about 48 hr to attach to the plates they were cultured 4 more days at 37°C in humidified air (5% CO_2) in

RPMI-1640 medium containing 5% NBCS that was depleted of steroids by dextran-charcoal extraction. The cells were then cultured for 4 or 8 days in presence or absence of various concentrations of oestrogen and progesterone. Medium was changed every two days.

For stromal cells, the cell suspension was centrifuged at 60 g for 5 min to remove clumps of cells and then the supernatant was centrifuged at 1000 g for 10 min. The pelleted cells were washed twice with HBSS. The stromal cell suspension was plated onto dishes at a concentration of 1×10^7 cells per dish and after a 3 hr incubation, the floating cells were washed away by gentle pipeting. The attached stromal cells were then cultured in RPMI-1640 medium containing 5% NBCS, which was depleted of steroids by dextran-charcoal extraction, in presence or absence of various concentrations of oestrogen and progesterone at 37°C in humidified air (5% CO_2) for 4 or 8 days. Medium was changed every two days.

The homogeneity of the cell populations was examined by immunocytochemistry. Epithelial cell contamination of stromal cells was about 3% and stromal cell contamination of epithelial cells was less than 1% (Xiao and Goff, 1998). The cells were approximately 40% confluent at the time of treatment and were 70-80% and 100% confluent on days 4 and 8, respectively.

Progesterone and oestrogen receptor assays

After treated with different hormonal regimens for 4 or 8 days, uterine cells were rinsed with HBSS and detached from the dish with HBSS containing 1 mM EDTA and gentle scraping with a rubber scraper. The cells were pelleted at 500 g for 5 min and resuspended in 250 µl of HED buffer (20 mM Hepes, 1.5 mM EDTA, 0.25 mM dithiothreitol, pH 7.4). Five µl of cell suspension was taken for DNA measurement. The rest of the cell suspension was sonicated and

pelleted at 15,000 g for 10 min. The supernatant was collected and the pellet treated twice with 150 µl extraction buffer (0.6 M KCl in HED) on ice for 20 min. This treatment extracted any remaining nuclear materials. At the end of the extraction, the cell debris was separated by centrifugation at 15,000 g for 10 min. The supernatant was combined with the initial supernatant collected after sonication and was used for oestrogen and progesterone receptor measurements. The protein concentration was determined by the method of Bradford (1976) (Bio-Rad Laboratories, CA, USA). DNA content was measured by the bisbenzimide fluorescent dye method of Labarca and Paigen (1980). Calf thymus DNA was used as standard at concentrations of 4, 8, 16, 32, 64, 128, 250 ng ml^{-1}.

Saturation analysis of oestradiol and progesterone receptors in primary cultures of uterine endometrial cells was performed in the presence of 1 nM oestradiol for 4 days. The supernatants were incubated with 0.5-20 nM [^{3}H] ORG 2058 or 0.5-10 nM [^{3}H] oestradiol for PR and ER measurement, respectively. The binding affinities of [^{3}H] ORG 2058 and [^{3}H] oestradiol for cell supernatants were determined using the least squares curve fitting program LIGAND (Munson and Rodbard, 1980). ORG 2058 was used for measurement of PR because of its low nonspecific binding and, unlike P4, it does not bind to the corticosteroid receptor. The data are presented as saturation and Scatchard curves (Scatchard, 1949).

To determine the effect of steroid treatment on PR number, supernatants (0.8-1.0 mg ml^{-1} of total protein) were incubated with 10 nM [^{3}H] ORG 2058 in HED buffer and with or without a 200-fold excess of unlabelled ORG 2058 at 4°C for 2.5-4 hr to measure total and nonspecific binding, respectively. For the ER number assay, cell supernatants were incubated with 5 nM [^{3}H] oestradiol in HED buffer with or without a 200-fold excess of unlabelled diethylstilbestrol

at 4°C for 16 hr, and then incubated at 30°C for 3 hr. After incubation, bound and free steroid were separated by adsorption with dextran-coated charcoal (DCC; 0.5% charcoal, 0.05% dextran in HED buffer) for 15 min at 4°C as described by Leake and Habib (1987). Specific binding (total minus nonspecific binding) values are expressed as femtomole per μg DNA.

Statistical analysis

Each treatment was carried out in triplicate using the cells from one uterus and each experiment was repeated with 6 different uteri. The data were log transformed where necessary to eliminate heterogeneity of variance. For each cell type, regression analysis was used to describe the effect of dose of oestradiol on receptor number, and a 2 x 2 x 2 factorial design (ANOVA) was used to analyze the effects of oestradiol, progesterone and duration of culture. The ANOVA included the main effects of duration of culture, progesterone treatment, oestradiol treatment and all interactions. Since uterus was nested within an experiment it was included as a random variable in the F-test for the effect of experiment. Simple effect comparisons to determine differences between individual means were performed using preplanned orthogonal contrasts. A probability of $p<0.05$ was considered to be statistically significant. The data were analyzed using the computer program JMP (SAS Institute Inc., NC, USA).

Results

Characterization of oestradiol and progesterone receptor binding in endometrial cells

Results show that specific binding of [^{3}H] ORG 2058 and [^{3}H] oestradiol by epithelial and stromal cells from bovine uterine endometrium collected at

days 1-3 of the oestrous cycle was saturable and exhibited high affinities (Figs. 1-2). The PR were saturated at 5 nM and 10 nM for epithelial and stromal cells, respectively (Fig. 1A, 1C). The disassociation constants (K_d) are 1.1 nM for epithelial cells (Fig. 1B) and 0.6 nM for stromal cells (Fig. 1D). The ER were saturated at 5 nM for both epithelial and stromal cells (Fig. 2A, 2C). The disassociation constants (K_d) are 5.5 nM for epithelial cells (Fig. 2B) and 0.7 nM for stromal cells (Fig. 2D).

Effect of oestradiol and progesterone on PR number in stromal cells

Oestradiol stimulated PR number in stromal cells after 4 days' incubation in a linear dose-dependent manner ($p<0.05$, Rsquare=0.12), with the maximum effect at 10 nM (Fig. 3A). Fig. 3B shows the time-dependent effect of oestrogen and progesterone on the PR number in stromal cells. Results of ANOVA showed effects of oestradiol (1nM) and duration of culture on PR numbers in stromal cells ($p<0.01$). There was a duration x oestradiol interaction ($p<0.05$) showing that the response to oestradiol increased with time, and a progesterone x oestradiol interaction ($p<0.001$) showing that progesterone alone had no effect but inhibited the oestradiol stimulation of the PR.

Effect of oestradiol and progesterone on PR number in epithelial cells

Oestradiol stimulated PR number in epithelial cells after 4 days (Fig. 4A). Regression analysis revealed that the best fit regression line was cubic ($p<0.05$, RSquare = 0.3). The lowest concentration of oestradiol used (0.1 nM) was the most effective (167% higher than control). Progesterone alone had no effect on PR number in cultured epithelial cells (Fig. 4B). ANOVA showed an effect of oestradiol ($p=0.05$) and an oestradiol x progesterone interaction ($P<0.05$). Progesterone blocked the oestradiol induced increase in PR number at

4 days of culture, and there was no effect of either progesterone or oestradiol after 8 days of culture.

Effect of oestradiol and progesterone on ER number in stromal cells

Oestradiol stimulated ER number in stromal cells after 4 days ($p<0.01$) (Fig. 5A). The effect of oestradiol was dose-dependent, an regression analysis showed that the best fit regression line was quadratic ($p<0.001$, Rsquare=0.22). The maximum response was seen at 1 nM after which the response declined. Fig. 5B shows the time-dependent effect of oestradiol and progesterone on ER number in stromal cells. Progesterone alone had no effect on ER number. Effects of oestradiol treatment and duration of culture were detected ($p<0.001$), and there was a significant progesterone x oestradiol interaction ($p<0.05$) and a duration x progesterone x oestradiol interaction was suggested ($p=0.08$). ER number increased markedly in the stromal cells between days 4 and 8 of culture ($p<0.001$). Progesterone had an inhibitory effect on the stimulation of ER number by oestradiol after 4 days ($p<0.05$), but not 8 days, of culture.

Effect of oestradiol and progesterone on ER number in epithelial cells

Oestradiol stimulated ER number in epithelial cells in a dose-dependent manner after 4 days (Fig. 6A). Regression analysis showed that the best fit regression line was linear ($p<0.005$, Rsquare=0.51). The low concentration of oestradiol (0.1 nM) had no effect on ER number in epithelial cells ($p>0.05$). ER numbers in epithelial cells did not respond to progesterone (50nM) (Fig. 6B) and there was no change in the response to oestradiol between 4 and 8 days of culture.

Discussion

The mechanisms involved in the timing of luteolysis in cattle are not completely understood. Changes in steroid receptors are considered to play an important role in this process (Meyer *et al.*, 1988; Zollers *et al.*, 1993). To investigate the mechanisms involved in luteolysis at the cellular level, it is necessary to have a culture system in which the cells respond in a fashion similar to that observed *in vivo* (Katzenellenbogen, 1980; Leavitt *et al.*, 1983; Clark *et al.*, 1985) . In this study, we used a primary cell culture system to investigate effects of steroid hormones on oestrogen and progesterone receptor concentrations in epithelial and stromal cells of the bovine endometrium.

Low-speed cell supernatants were used for receptor measurements in this study instead of ultra-speed supernatants because, in preliminary experiments no differences were observed in the receptor contents of the supernatants separated by ultra-speed or low-speed centrifugations. This was also demonstrated by Leake and Habib (Leake and Habib, 1987). Our results show that both progesterone and oestrogen receptors in cultured bovine endometrial cells are of high affinity (K_d = 1.1 nM and 0.6 nM respectively for PR of epithelial and stromal cells; K_d = 5.5 nM and 0.7 nM respectively for ER of epithelial and stromal cells). These are in the same range as the affinities of the ER and PR in endometrial tissue reported by others. The K_d of ER in bovine endometrial tissue at different stages of oestrous cycle range from 0.26 to 2.5 nM and that of PR from 0.14 to 0.17 nM (Vesanen *et al.*, 1988). In another study, the K_d of endometrial cytosolic PR in cattle was 2.6 nM (Meyer *et al.*, 1988). In cultured uterine epithelial cells of immature rats, the K_ds of cytosolic and nuclear ER were 0.56 and 0.27 nM respectively; and those of stromal cells were 0.42 and 0.32 nM for cytosolic and nuclear ER, respectively (McCormack and Glasser, 1980). Thus, in our primary culture system ER and PR are detectable

and their affinities approximate the values reported previously in endometrial tissues.

Presently there are two known forms of the PR and ER. The PR forms A and B are present in human endometrium (Tseng and Zhu, 1997). The ER exists in the classical α form and the newly discovered β form (Kuiper *et al.*, 1996). The ERβ is present in the bovine endometrium (Rosenfeld *et al.*, 1998) and might be involved in uterine function. Both forms of the ER and PR are therefore likely to be present in the cell preparations used in the present study, however, no attempt has been made to distinguish which of the receptor forms change in response to the steroid treatment of the cells.

The basal level of PR was higher in stromal cells than in epithelial cells. This agrees with what has been observed in the endometrium during the early luteal phase of the cow (Boos *et al.*, 1996). In contrast, the basal level of ER was higher in epithelial cells than in stromal cells, whereas in cattle and ovariectomized sheep (Ing and Tornesi, 1997), ER was higher in the stromal cells. This may be related to the stage of the cycle from which the uterus came since receptor numbers change during the oestrous cycle (Wathes and Hamon, 1993; Boos *et al.*, 1996). Also, luminal and glandular epithelial cells were not separated in the present study and it is possible that there are differences between these two cell types.

Progesterone and oestradiol receptor concentrations in the endometrium are highest at oestrous and during the follicular phase of the oestrous cycle when endogenous oestradiol is high (Findlay *et al.*, 1982; Zelinski *et al.*, 1982; Vesanen *et al.*, 1988; Vesanan *et al.*, 1991; Salamonsen *et al.*, 1992; Boos *et al.*, 1996). This is presumably due to an up-regulation of these receptors by oestradiol. In ovariectomized ewes, oestradiol treatment increased PR and ER concentrations in both epithelial and stromal cells (Wathes *et al.*, 1996; Ing and

Tornesi, 1997). Oestradiol also induced synthesis of endometrial progesterone receptors *in vitro* and *in vivo* in rats (Kassis *et al.*, 1984) and sheep (Zelinski *et al.*, 1980). Results from the present study, that oestradiol increased PR and ER concentrations in a dose- and time-dependent manner in both cell types, agree with the *in vivo* observations. Although E2 was shown to upregulate PR in several cell culture systems, we believe this is the first evidence of an upregulation of the ER by E2 *in vitro*. Furthermore, the effect of E2 was more pronounced in stromal cells than in epithelial cells. This is similar to data from ovariectomized ewes, where E2 treatment causes a greater increase in ER in the stroma than in the luminal epithelium and that the response in stromal cells appeared earlier and was more prolonged than in the other cell types (Ing and Tornesi, 1997). E2 stimulation of PR and ER concentrations also increased with time in the stromal cells, whereas it diminished during extended culture in the epithelial cells. This is consistant with the observation of Inaba *et al* (1988) that PR levels in the epithelial cells decreased to low levels between 4 and 8 days of oestradiol treatment, while those in stromal cells remained high. This could mean that E2 stimulation of receptor number differs between the two cell types.

Opposite to the stimulatory action of oestradiol, P4 reportedly inhibits both ER and PR (Katzenellenbogen, 1980; Leavitt *et al.*, 1983; Clark *et al.*, 1985; Sumida *et al.*, 1988). It is probably this inhibitory effect of progesterone that is responsible for the decline in endometrial receptors by the mid-luteal phase in cows (Meyer *et al.*, 1988) and ewes (Miller *et al.*, 1977). From our study, it would appear that P4 treatment is unable to inhibit either PR or ER below the level seen in the untreated isolated cells. It is possible that the concentration of P4 used (close to the highest concentration observed in peripheral plasma) is not enough to exert an inhibition *in vitro* or that an interaction between the different cell types is necessary for a normal response. Interestingly, differences were

observed between ovariectomized steroid-treated ewes and normal animals where P4 treatment did not reduce ER concentrations in ovariectomized ewes as seen in cyclic ewes (Wathes *et al.*, 1996). It was suggested that factors from the ovary other than progesterone and oestradiol are involved in ER regulation. Thus, other factors (such as growth factors and cytokins) might play a role in receptor regulation in the isolated cells.

Although P4 alone did not inhibit receptor numbers, it did inhibit the stimulatory effect of E2 on PR in both cell types and ER in stromal cells. This agrees with the observations *in vivo* that, in ovariectomized ewes, progesterone inhibits the increase in PR induced by E2 treatment (Stone *et al.*, 1978; Wathes *et al.*, 1996). Even though the concentration of E2 used in the present study was much higher than peripheral plasma concentrations, the effect of P4 is still apparent and thus the ratio of P4 to E2 is probably not important. This agrees with the previous report by Sumida and Pasqualini (Sumida and Pasqualini, 1979) in the fetal guinea pig, showing that even a large excess of oestradiol was not able to increase the number of progesterone receptors as long as progesterone was present. It is not clear why progesterone did not inhibit the E2 stimulation of the ER in the epithelial cells. There could be a difference in receptor regulation in the two cell types or down regulation of the ER in the epithelial cells might be mediated by the stromal cells as suggested by Wathes *et al* (1996).

In conclusion, we report for the first time the effect of steroid hormones on PR and ER concentrations in primary cell culture of bovine uterine endometrial cells. Oestradiol increased the PR and ER numbers in both cell types. Progesterone alone had no effect on ER and PR number, but inhibited the E2-induced increase in PR in both cell types. Our results tend to support the notion that an interaction between cell types is necessary for the normal

function of epithelial cells. Therefore, this isolated cell model system should be very useful to study the effect of steroid hormones and cell type interactions on endometrial function in the cow.

Acknowledgement

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References

Boos A, Meyer W, Schwarz R and Grunert E (1996) Immunohistochemical assessment of oestrogen receptor and progesterone receptor distribution in biopsy samples of the bovine endometrium collected throughout the oestrous cycle *Animal Reproduction science* **44** 11-21

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Analytical Biochemistry* **72** 248-254

Clark JH, Scrader WT and O'Malley BW (1985) Mechanisms of steroid hormone action. In: *Textbook of Endocrinology* pp33-75 Eds JD Witson and DW Fortec. Saunders: Philadelphia

Findlay JK, Clarke IJ, Swaney J, Colvin N and Doughton B (1982) Oestrogen receptors and protein synthesis in caruncular and intercaruncular endometrium of sheep before implantation *Journal of Reproduction and Fertility* **64** 329-339

Fortier MA, Guibault LA and Grasso F (1988) Specific properties of epithelial and stromal cells from the endometrium of cows *Journal of Reproduction and Fertility* **83** 239-248

Garrett JE, Geisert RD, Zavy MT, Gries LK, Wettemann RP and Buchanan DS (1988) Effect of exogenous progesterone on prostaglandin F2α release and the interestrous interval in the bovine *Prostaglandins* **36** 85-96

Ginther OJ (1970) Effect of progesterone on length of estrous cycle in cattle *American Journal of Veterinary Research* **31** 493-496

Hixon JE and Flint APF (1987) Effects of a luteolytic dose of oestradiol benzoate on uterine oxytocin receptor concentrations, phosphoinositide turnover and prostaglandin F-2α secretion in sheep *Journal of Reproduction and Fertility* **79** 457-467

Inaba T, Wiest WG, Strickler RC and Mori J (1988) Augmentation of the response of mouse uterine epithelial cells to estradiol by uterine stroma *Endocrinology* **123** 1253-1258

Ing NH and Tornesi MB (1997) Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells *Biology of Reproduction* **56** 1205-1215

Kassis JA, Sakai D, Walent JH and Gorski J (1984) Primary cultures of estrogen-responsive cells from rat uteri: induction of progesterone receptors and a secreted protein *Endocrinology* 1558-1565

Katzenellenbogen BS (1980) Dynamics of steroid hormone receptor action *Annual review of physiology* **42** 17-35

Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson J- (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary *Proceedings of the National Academy of Sciences USA* **93** 5925-5930.

Labarca C and Paigen K (1980) A simple, rapid and sensitive DNA assay procedure *Analytical biochemistry* **102** 344-352

Lamming G and Mann GE (1995) Control of endometrial oxytocin receptors and prostaglandin F2α production in cows by ptrogesterone and oestradiol *Journal of Reproduction and Fertility* **103** 69-73

Leake R and Habib F (1987) Steroid hormone receptors: Assays and characterization. In: *Steroid hormones: a practical approach* p69 Eds B Green and RE Leake. IRL press Ltd.

Leavitt WW, MacDonald RG and Okulicz WC (1983) Hormonal regulation of estrogen and progesterone receptor systems. In: *Biochemical actions of hormones* pp323-356 ED G Litwack. Academic Press: New York

McCormack SA and Glasser SR (1980) Differential response of individual uterine cell types from immature rats treated with estradiol *Endocrinology* **106** 1634-1649

McCracken JA, Schramm W and Okulicz WC (1984) Hormone receptor control of pulsatile secretion of PGF2α from the ovine uterus during luteolysis and its abrogation in early pregnancy *Animal Reproduction Science* **7** 31-55

Meyer HH, Mittermeier T and Schams D (1988) Dynamics of oxytocin, estrogen and progestin receptors in the bovine endometrium during the estrous cycle *Acta Endocrinologica* **118** 96-104

Miller BG, Murphey L and Stone GM (1977) Hormone receptor levels and hormone, RNA and protein metabolism in the genital tract during the oestrous cycle of the ewe *Journal of Endocrinology* **73** 91-98

Munson PJ and Rodbard D (1980) LIGAND: a versatile computerized approach for the characterization of ligand binding systems *Analytical Biochemistry* **107** 220-239

Ottobre JS, Lewis GS, Thayne WV and Inskeep EK (1980) Mechanism by which progesterone shortens the estrous cycle of the ewe *Biology of Reproduction* **23** 1046-1053

Rosenfeld CS, Yuan X, Manikkam M, Calder MD, Garverick HA and Lubahn DB (1998) Sequencing, cloning, and localisation of bovine estrogen receptor-beta (ERβ) in the reproductive tract of the cow *Biology of Reproduction* **58** 120

Salamonsen LA, Cherny RA and Findlay JK (1992) Studies *in vitro* of effects of steroid hormones and the blastocust on endometrial function in the sheep *Reproduction, Fertility and Development* **4** 275-281

Scatchard G (1949) The attraction of proteins for small molecules and ions *Annals of New York Acadamy Sciences* **51** 660-672

Spencer TE, Becker WC, George P, Mirande MA, Ogle TF and Bazer FW (1995) Ovine interferon-τ regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone *Biology of reproduction* **53** 732-745

Spencer TE and Bazer FW (1995) Temporal and spatial regulation of uterine receptors for oestrogen and progesterone during the estrous cycle and early pregnancy in ewes *Biology of Reproduction* **53** 1527-1544

Spencer TE, Mirando MA, Mayes JS, Watson GH, Ott TL and Bazer FW (1996) Effects of interferon-τ and progesterone on oestrogen-stimulated expression of receptors for oestrogen, progesterone and oxytocin in the endometrium of ovariectomized ewes *Reproduction, Fertility and Development* **8** 843-853

Stone GM, Murphey L and Miller BG (1978) Hormone receptor levels and metabolic activity in the uterus of the ewe: regulation by oestradiol and progesterone *Australian Journal of Biology Science* **31** 395-403

Sumida C and Pasqualini JR (1979) Determinaton of cytosol and nuclear estradiol-binding sites in fetal pig uterus by [^{3}H] estradiol exchange *Endocrinology* **105** 406-413

Sumida C, Lecerf F and Pasqualini JR (1988) Control of progesterone receptors in fetal uterine cells in culture: effects of estradiol, progestins, antiestrogens, and growth factors *Endocrinology* **122** 3-11

Tseng L and Zhu HH (1997) Regulation of progesterone receptor messenger ribonucleic acid by progestin in human endometrial stromal cells ***Biology of Reproduction*** **57** 1360-1366

Vesanan M, Isomaa V, Alanko M and Vihko R (1991) Bovine uterine, cervical and ovarian estrogen and progesterone receptor concentrations *Animal Reproduction science* **26** 61-71

Vesanen M, Isomaa V and Vihko R (1988) Cytosol estrogen and progesterone peceptors in bovine endometrium after uterine involution postpartum and in the estrous cycle *Animal Reproduction Science* **17** 9-20

Wathes D and Lamming GE (1995) The oxytocin receptor, luteolysis and the maintenance of pregnancy *Journal of Reproduction and Fertility* **49** (Suppl) 53-67

Wathes DC and Hamon M (1993) Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe *Journal of Endocrinology* **138** 479-491

Wathes DC, Mann GE, Payne JJ, Riley PR, Stevenson KR and Lamming GE (1996) Regulation of oxytocin, oestradiol and progesterone receptor concentrations in different uterine regions by oestradiol, progesterone and oxytocin in ovariectomized ewes *Journal of Endocrinology* **151** 375-393

Woody CO and Ginther OJ (1968) Effect of exogenous progesterone on corpora lutea in unilaterally hysterectomized heifers *Journal of Animal Science* **27** 1387-1390

Xiao CW and Goff AK (1998) Differential effects of oestradiol and progesterone on proliferation and morphology of cultured bovine uterine epithelial and stromal cells *Journal of Reproduction and Fertility* **112** 315-324

Zelinski MB, Hirota NA, Keenan EJ and Stormshak F (1980) Influence of exogenous estradiol-17β on endometrial progesterone and estrogen receptors during the luteal phase of the ovine estrous cycle *Biology of Reproduction* **23** 743-751

Zelinski MB, Noel B, Weber DW and Stomshak F (1982) Characterization of cytoplasmic progesterone receptors in the bovine endometrium during proestrus and diestrus *Journal of Animal Science* **55** 376-383

Zollers WG, Garverick HA, Smith MF and Moffatt RJ (1993) Concentrations of progesterone and oxytocin receptors in endometrium of postpartum cows expected to have a short or normal oestrous cycle *Journal of Reproduction and Fertility* **97** 329-337

Fig. 1. Saturation analysis of specifically bound [^{3}H] ORG 2058 to primary cultures of bovine uterine cells. Cells were cultured in RPMI 1640 containing 5% NBCS in the presence of 1 nM oestradiol for 4 days. The whole cell extracts were treated with 0.5-20 x 10^{-9} M [^{3}H] ORG 2058 with or without 200-fold molar excess of unlabeled ORG 2058. Total binding (□), nonspecific binding (◆), and specific binding (■) by epithelial cells (A) and stromal cells (C) are presented as saturation curves. Corresponding Scatchard plots of specific binding are presented in B and D. B/F, Bound to free ratio.

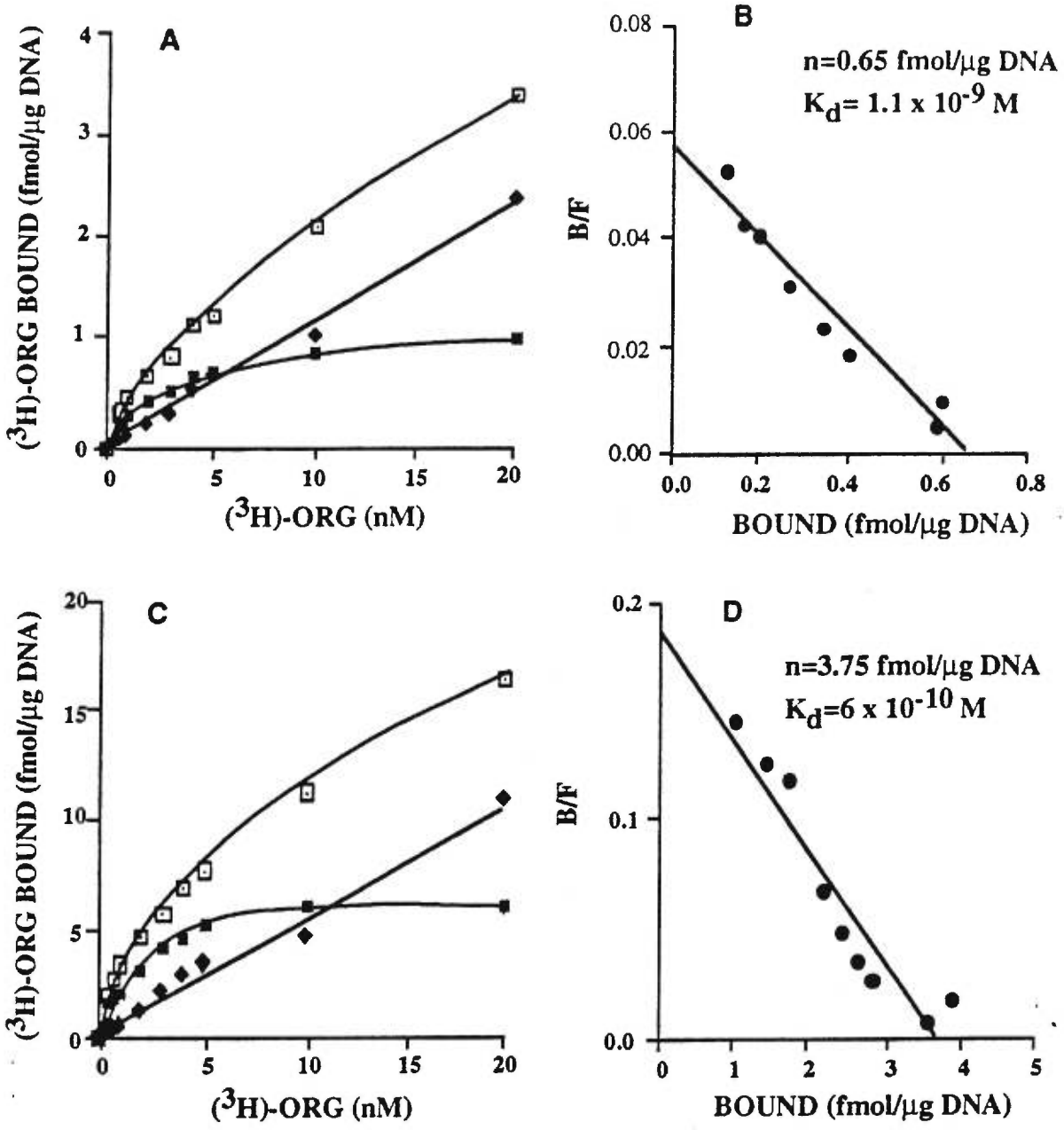

A
(3H)-ORG BOUND (fmol/µg DNA)
(3H)-ORG (nM)
B
n=0.65 fmol/µg DNA
Kd= 1.1 x 10-9 M
B/F
BOUND (fmol/µg DNA)
C
(3H)-ORG BOUND (fmol/µg DNA)
(3H)-ORG (nM)
D
n=3.75 fmol/µg DNA
Kd=6 x 10-10 M
B/F
BOUND (fmol/µg DNA)

Fig. 2. Saturation analysis of specifically bound [^{3}H] oestradiol to primary cultures of bovine uterine cells. Cells were cultured in RPMI 1640 containing 5% NBCS in the presence of 1 nM oestradiol for 4 days. The whole cell extracts were treated with 0.5-10 x 10^{-9} M [^{3}H] oestradiol with or without 200-fold molar excess of unlabeled diethylstilbesterol. Total binding (□), nonspecific binding (◆), and specific binding (■) by epithelial cells (A) and stromal cells (C) are presented as saturation curves. Corresponding Scatchard plots of specific binding are presented in B and D. B/F, Bound to free ratio.

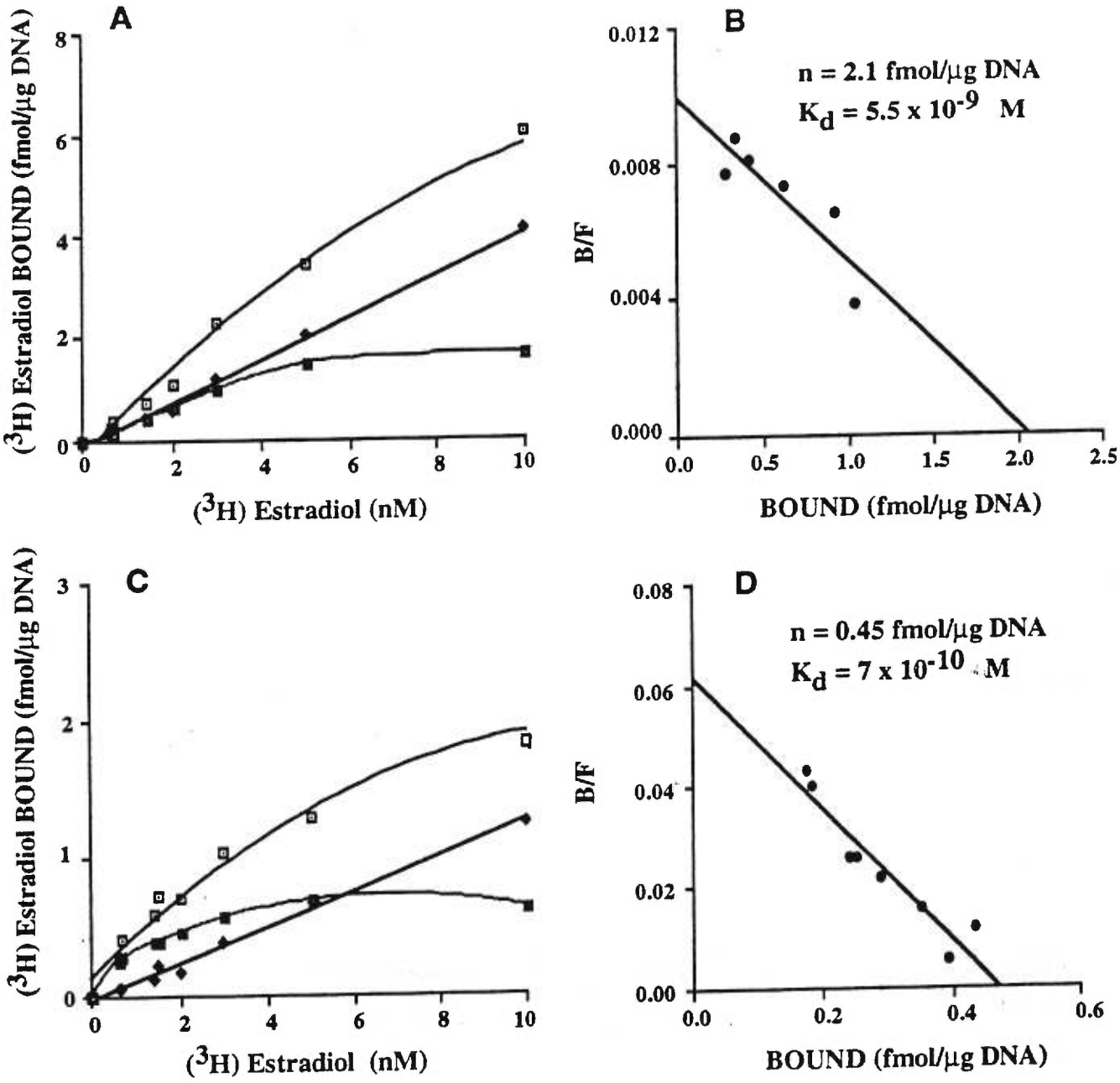
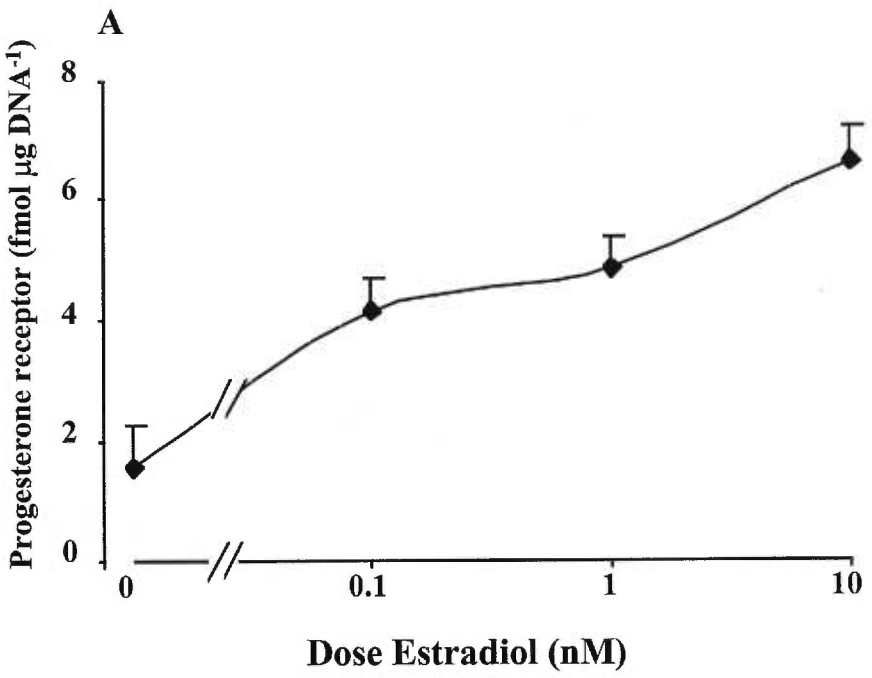

A
(3H) Estradiol BOUND (fmol/µg DNA)
(3H) Estradiol (nM)
B
n = 2.1 fmol/µg DNA
Kd = 5.5 x 10-9 M
B/F
BOUND (fmol/µg DNA)
C
(3H) Estradiol BOUND (fmol/µg DNA)
(3H) Estradiol (nM)
D
n = 0.45 fmol/µg DNA
Kd = 7 x 10-10 M
B/F
BOUND (fmol/µg DNA)

Fig. 3. Time and dose-dependent effect of oestradiol and progesterone on progesterone receptor number in bovine endometrial stromal cells. (A) Primary stromal cells were cultured in RPMI 1640 containing 5% NBCS in the presence or absence of various doses of oestradiol (0.1, 1, and 10 nM) for 4 days. (B) Primary stromal cells were cultured in RPMI 1640 containing 5% NBCS in the presence or absence of 1 nM oestradiol (E1), or 50 nM progesterone (P), or 50 nM progesterone plus 1 nM oestradiol (P+E1) for 4 days (hatched bars) or 8 days (shaded bars). Data represent least-square means ± SE, the minimum detection limit is 0.11 fmol $\mu g\ DNA^{-1}$.


A
Progesterone receptor (fmol µg DNA-1)
8
6
4
2
0
0
0.1
1
10
Dose Estradiol (nM)

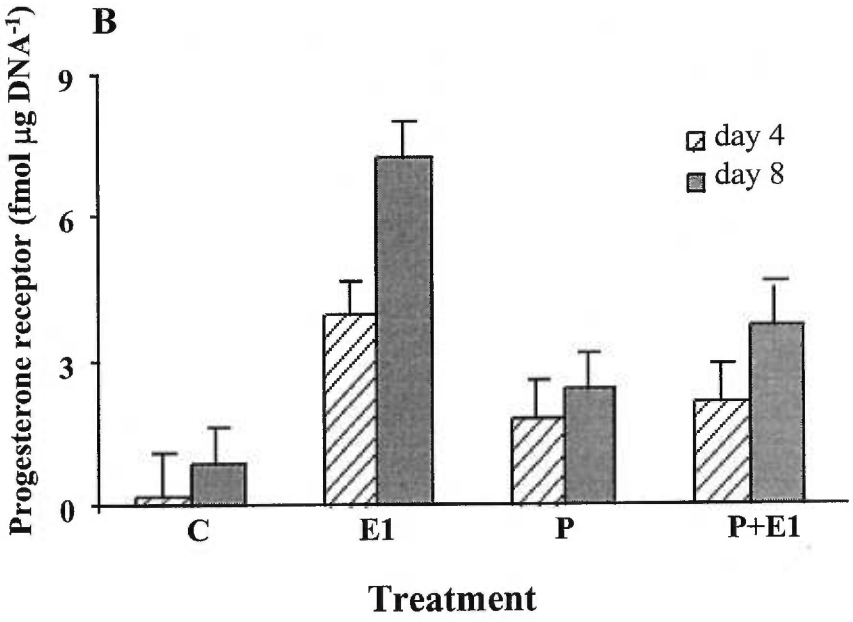
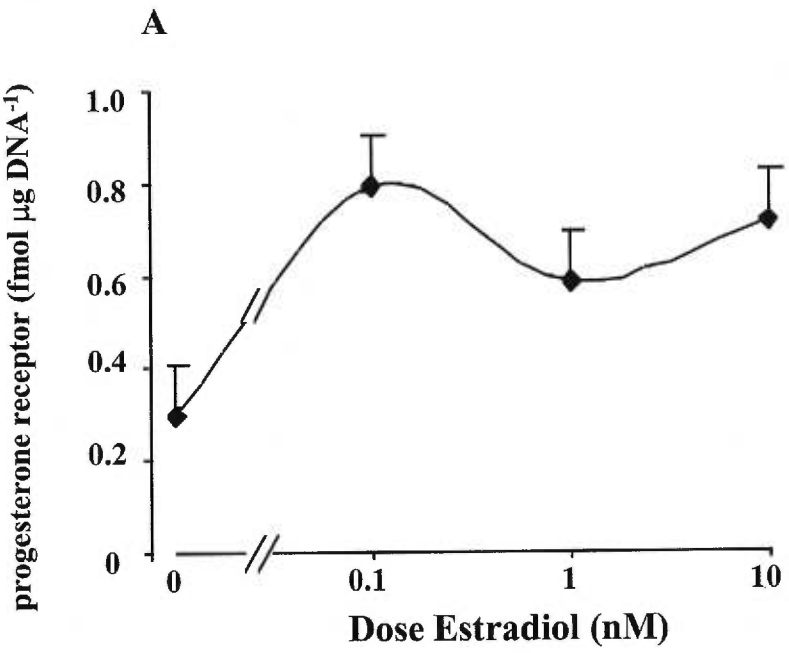

B
Progesterone receptor (fmol µg DNA-1)
9
6
3
0
day 4
day 8
C
E1
P
P+E1
Treatment

Fig. 4. Time and dose-dependent effect of oestradiol and progesterone on progesterone receptor number in bovine endometrial epithelial cells. (A) Primary epithelial cells were cultured in RPMI 1640 containing 5% NBCS in the presence or absence of various doses of oestradiol (0.1, 1, and 10 nM) for 4 days. (B) Primary epithelial cells were cultured in RPMI 1640 containing 5% NBCS in the presence or absence of 1 nM oestradiol (E1), or 50 nM progesterone (P), or 50 nM progesterone plus 1 nM oestradiol (P+E1) for 4 days (hatched bars) or 8 days (shaded bars). Data represent least-square means ± SE, the minimum detection limit is 0.11 fmol $\mu g\ DNA^{-1}$.


A
progesterone receptor (fmol µg DNA-1)
1.0
0.8
0.6
0.4
0.2
0
0
0.1
1
10
Dose Estradiol (nM)

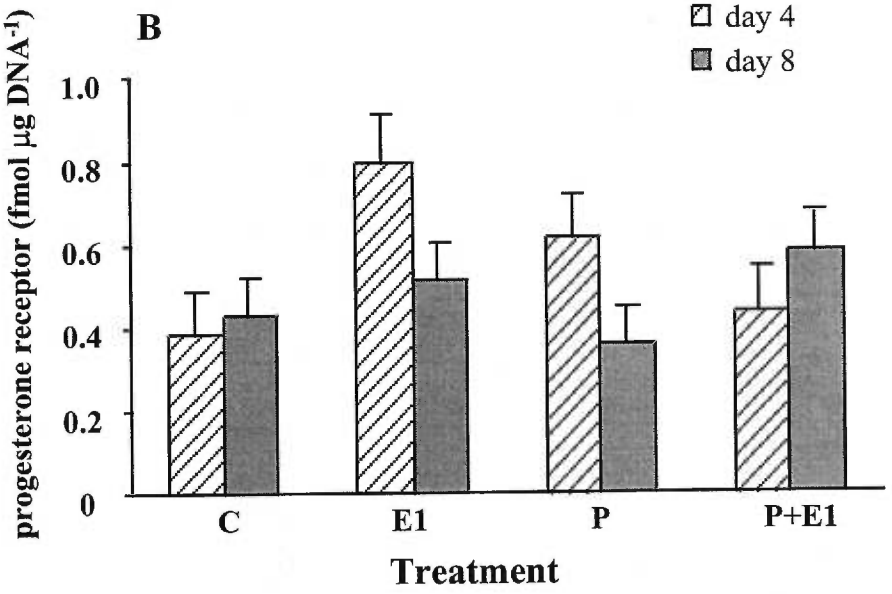

B
day 4
day 8
progesterone receptor (fmol µg DNA-1)
1.0
0.8
0.6
0.4
0.2
0
C
E1
P
P+E1
Treatment

Fig. 5. Time and dose-dependent effect of oestradiol and progesterone on oestrogen receptor number in bovine endometrial stromal cells. (A) Primary stromal cells were cultured in RPMI 1640 containing 5% NBCS in the presence or absence of various doses of oestradiol (0.1, 1, and 10 nM) for 4 days. (B) Primary stromal cells were cultured in RPMI 1640 containing 5% NBCS in the presence or absence of 1 nM oestradiol (E1), or 50 nM progesterone (P), or 50 nM progesterone plus 1 nM oestradiol (P+E1) for 4 days (hatched bars) or 8 days (shaded bars). The receptor number is presented on a logarithmic scale in figure B. Data represent least-square means ± SE, the minimum detection limit is 0.06 fmol μg DNA^{-1}.

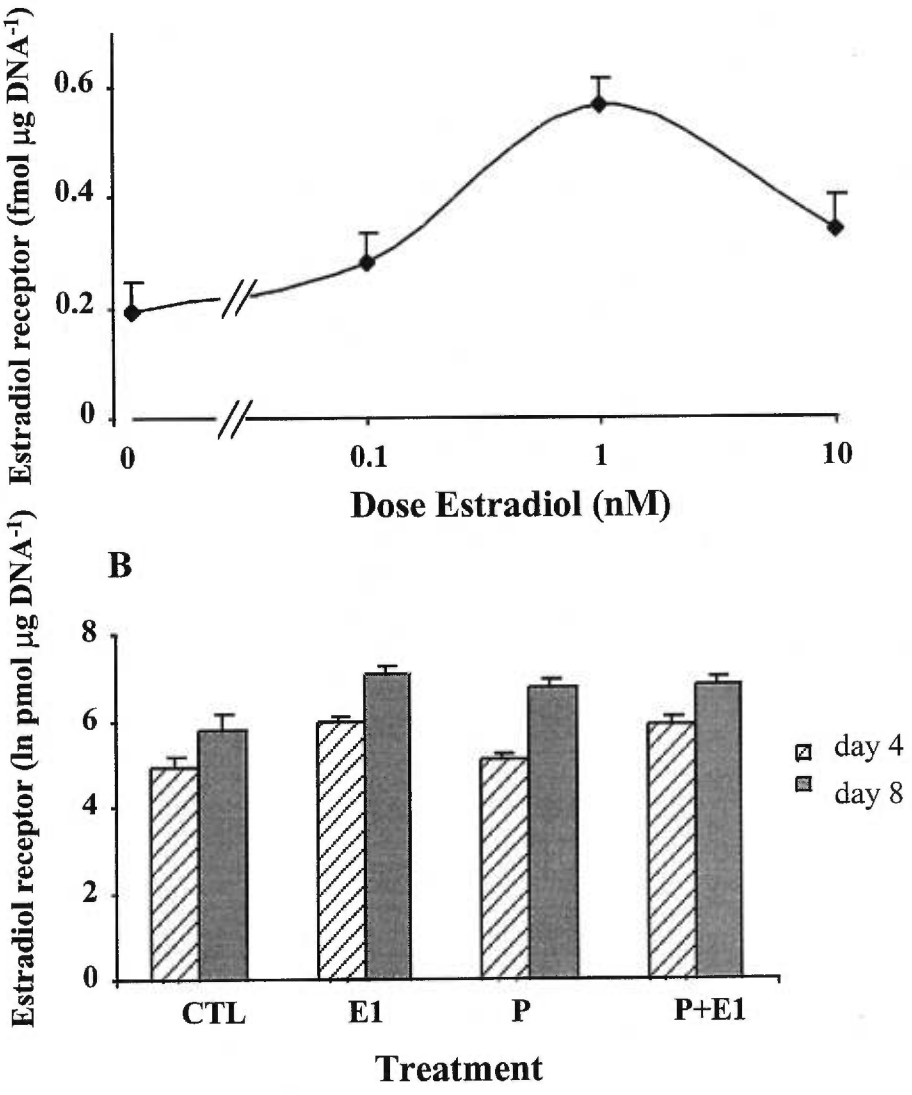
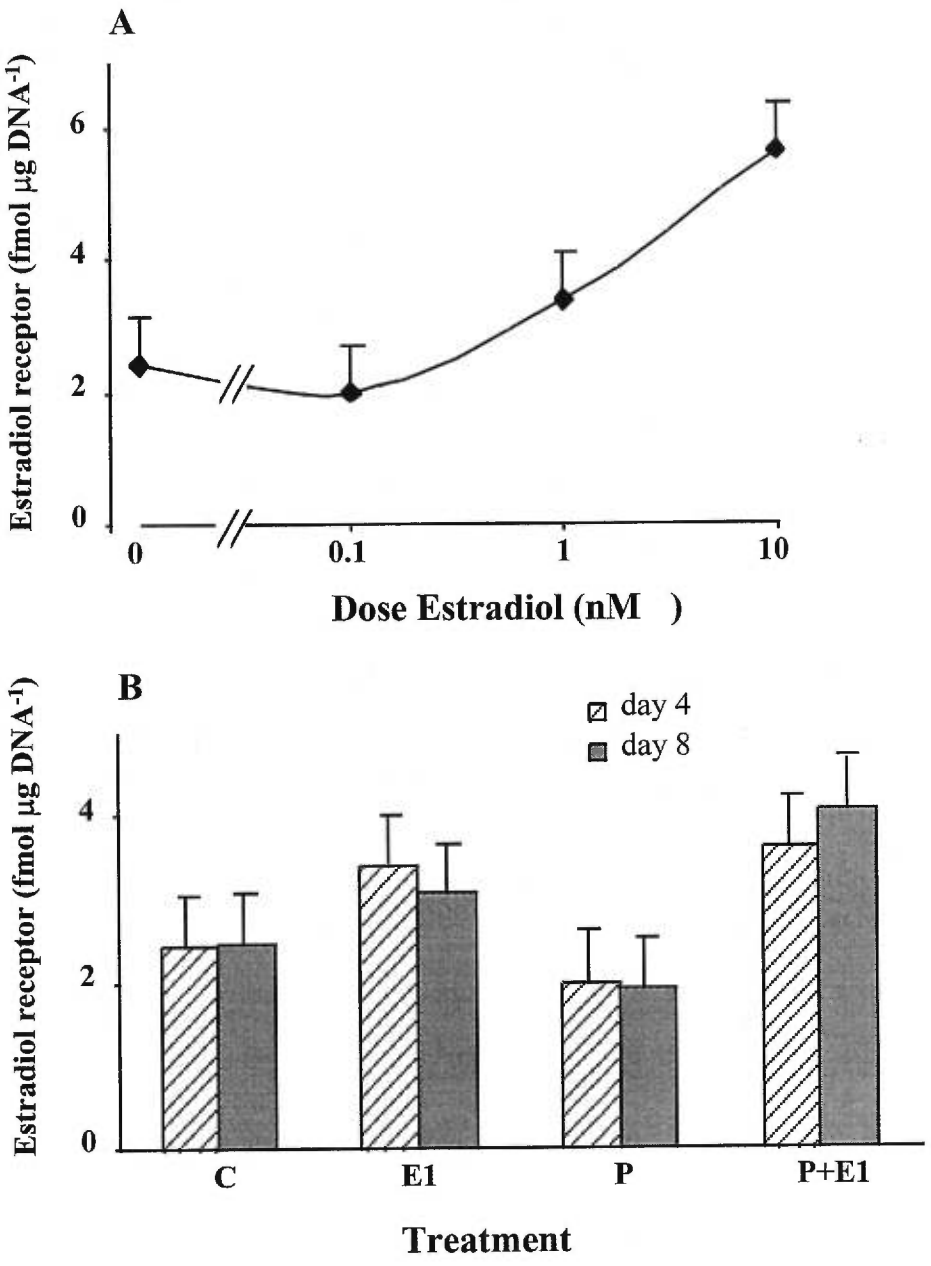

Estradiol receptor (fmol µg DNA-1)
0.6
0.4
0.2
0
0
0.1
1
10
Dose Estradiol (nM)
B
Estradiol receptor (ln pmol µg DNA-1)
8
6
4
2
0
CTL
E1
P
P+E1
Treatment
day 4
day 8

Fig. 6. Time and dose-dependent effect of oestradiol and progesterone on oestrogen receptor number in bovine endometrial epithelial cells. (A) Primary epithelial cells were cultured in RPMI 1640 containing 5% NBCS in the presence or absence of various doses of oestradiol (0.1, 1, and 10 nM) for 4 days. (B) Primary epithelial cells were cultured in RPMI 1640 containing 5% NBCS in the presence or absence of 1 nM oestradiol (E1), or 50 nM progesterone (P), or 50 nM progesterone plus 1 nM oestradiol (P+E1) for 4 days (hatched bars) or 8 days (shaded bars). Data represent least-square means ± SE, the minimum detection limit is 0.06 fmol $\mu g\ DNA^{-1}$.


A
Estradiol receptor (fmol µg DNA-1)
6
4
2
0
0
0.1
1
10
Dose Estradiol (nM)
B
day 4
day 8
Estradiol receptor (fmol µg DNA-1)
4
2
0
C
E1
P
P+E1
Treatment

3.3. Article 3. Regulation of cyclooxygenase-2 and prostaglandin F synthase gene expression by steroid hormones and interferon-τ in bovine endometrial cells

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Regulation of Cyclooxygenase-2 and Prostaglandin F Synthase Gene Expression by Steroid Hormones and Interferon-τ in Bovine Endometrial Cells*

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ABSTRACT

Estradiol (E2) and progesterone are responsible for regulating PG synthesis in the endometrium during the estrous cycle and interferon-τ (IFN-τ) alters PG synthesis during early pregnancy in ruminants. In this study, we examined the effects of these steroid hormones and recombinant bovine IFN-τ(rbIFN-τ) on PG production and on cyclooxygenase-2 (COX-2) and PG F (PGF) synthase (PGFS) gene expression in isolated endometrial cells. E2 decreased both $PGF_{2\alpha}$ and PG E_2 (PGE_2) whereas progesterone increased $PGF_{2\alpha}$ secretion in epithelial cells. Steroid hormones had no effect on PG production in stromal cells. rbIFN-τ attenuated both $PGF_{2\alpha}$ and PGE_2 production in epithelial cells and enhanced their production, and the ratio of PGE_2 to $PGF_{2\alpha}$, in stromal cells. Northern blot analysis showed that E2 and rbIFN-τ decreased COX-2 messenger RNA (mRNA) levels in epithelial cells. Conversely, rbIFN-τ increased COX-2 mRNA in stromal cells. Furthermore, rbIFN-τ decreased PGFS mRNA in both cell types and this was associated with the increase in $PGE_2/PGF_{2\alpha}$ ratio. These results show that the regulation of PG synthesis by steroid hormones is different in endometrial epithelial and stromal cells *in vitro*. The attenuation of $PGF_{2\alpha}$ secretion from epithelial cells and increased PGE_2 production in stromal cells by rbIFN-τ are modulated by steroid hormones. (*Endocrinology* **139:** 2293–2299, 1998)

PG $F_{2\alpha}$ ($PGF_{2\alpha}$) and PG E_2 (PGE_2) are major secretory products of the uterine endometrium in ruminants (1). $PGF_{2\alpha}$, primarily secreted from the surface epithelium of the uterus, is the luteolytic agent in livestock (2–5). PGE_2 protects the corpus luteum (CL) from spontaneous regression (6) and may play a role in pregnancy maintenance. In ovariectomized guinea-pig, rat, and sheep (1), uterine $PGF_{2\alpha}$ concentrations are low and are restored to normal after treatment with progesterone (P4) followed by estrogen. In cultured bovine uterine endometrial epithelial cells, but not stromal cells, the basal production of $PGF_{2\alpha}$ and PGE_2 was reduced by estradiol (E2) and increased by P4 (7). The control of PGE_2 secretion might differ from that of $PGF_{2\alpha}$, because PGE_2 secretion from the endometrium does not show the same cyclical changes as $PGF_{2\alpha}$ (1). Ovarian steroids play an important role in the regulation of endometrial PG synthesis, but the sites and mechanisms of action have not been determined (1).

Interferon-τ (IFN-τ) is produced by the trophoblast tissue, between days 15–24 of gestation (8), and prevents luteolysis by suppressing endometrial $PGF_{2\alpha}$ secretion. Intrauterine infusion of recombinant bovine IFN-α (rbIFN-α) prolonged the luteal lifespan in cows (9). Administration of IFN-τ attenuated the episodic release of uterine $PGF_{2\alpha}$ by down-regulation of estrogen receptor and oxytocin receptor expression (10). *In vitro*, natural bovine IFN-τ significantly diminished basal $PGF_{2\alpha}$ (but not PGE_2) secretion by bovine uterine endometrial explants (11, 12). rbIFN-τ significantly diminished both basal $PGF_{2\alpha}$ and PGE_2 secretion by bovine endometrial epithelial cells (12–15). Natural ovine IFN-τ diminished both basal $PGF_{2\alpha}$ and PGE_2 secretion by ovine endometrial epithelial cells (16).

Cyclooxygenase (COX) is the key rate-limiting enzyme responsible for the conversion of arachidonic acid to PGG_2 and PGH_2, the precursor for $PGF_{2\alpha}$, PGE_2, and other members of PG family (17). Two isoforms of COX (COX-1 and COX-2) have been identified in mammalian cells. COX-1 is a constitutively expressed enzyme; COX-2 is highly induced by various inducers, such as phorbol esters, mitogens, cytokines, and serum (18–20). PG F synthase (PGFS) was first discovered in rabbit liver by Wong (21). The purified PGFS from bovine lung catalyzed the reduction of PGH_2 to $PGF_{2\alpha}$, PGD_2 to a stereoisomer of $PGF_{2\alpha}$ (9α, 11β-PGF_2) (22). A PGFS transcript from bovine lung was about 1.4 kb (23). However, the uterine endometrial expression and regulation of PGFS have not been reported.

The regulation of COX-2 and PGFS expression by steroids and IFN-τ are not well understood. Arslan and Zingg (20) found that both IL-1β and TNFα induced $PGF_{2\alpha}$ release and COX-2 messenger RNA (mRNA) expression in rat uterine stromal cells *in vitro*. A recent study in sheep (24) showed that COX-1 protein was expressed at steady-state levels in the endometrium during the estrous cycle and during comparable stages of pregnancy. In contrast, COX-2 protein was highly and transiently expressed from days 12–15 of the estrous cycle and declined, thereafter, to undetectable levels. Endometrium from early pregnant ewes showed a similar pattern of COX-2 expression, although there was a slower

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decrease beyond day 15. P4 induced endometrial COX-2, and E2 slightly increased COX-2 expression, but only after P4 priming (24).

rbIFN-τ has no significant effect on COX-1 mRNA expression (2.8 kb mRNA) in bovine endometrial epithelial cells (14) and ovine endometrial COX-1 mRNA is not regulated by the conceptus (17). However, the effects of IFN-τ on the inducible COX-2 mRNA have not been reported.

The objective of this study was to investigate the effect of steroid hormone and IFN-τ on COX-2 and PGFS gene expression in different cell types of bovine uterine endometrium. It was hypothesized that steroid treatment of the cells would modify the effect of IFN-τ on PG synthesis and on COX-2 and PGFS expression.

Materials and Methods

Chemicals and reagents

Tissue culture medium (RPMI 1640), HBSS (calcium and magnesium free), FCS, antibiotics, and trypan-blue were purchased from GIBCO (Grand Island, NY). Collagenase (Type II), trypsin (Type III, from bovine pancreas), deoxyribonuclease I (Type I, from bovine pancreas), Gentamicin, calf thymus DNA, Hoechst NO. 33258, 17β-E2, P4, lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5) and E-TOXATE kit were purchased from Sigma Chemical Co. (St. Louis, MO). Matrigel was obtained from VWR Scientific (Ontario, Canada). rbIFN-τ was generously provided by Dr. R. M. Roberts (25).

Preparation and culture of cells

Bovine uteri from cows, at days 1–3 of the estrous cycle (ovaries containing a corpus hemorrhagicum), were collected at the slaughterhouse and transported, on ice, to the laboratory. Days 1–3 were selected because the stage of the estrous cycle can be accurately determined from slaughterhouse material, because of the presence of the corpus hemorrhagicum. This should decrease variability between uteri. The endometrial epithelial and stromal cells were separated by a modification of the procedure described by Fortier *et al.* (26). Briefly, the two horns of the uteri were placed in sterile HBSS containing 100 U penicillin, 100 μg streptomycin, and 0.25 μg amphotericin per milliliter. The myometrial layers were dissected from the two horns, and the horns were then inverted to expose the epithelium. The inverted horns were first digested for 2 h in HBSS with 0.3% trypsin at room temperature. At the end of incubation, the digested horns were scraped slightly with forceps and then washed twice in HBSS and further digested in HBSS with 0.064% trypsin III, 0.064% collagenase II, and 0.032% deoxyribonuclease I for 45 min at 37 C, to obtain the stromal cells. Once the cell suspension was collected, 10% FCS was added to block the action of trypsin. The cell suspension was centrifuged at 60 × *g* for 5 min. The pellets were washed three more times with HBSS. The supernatants were pooled and centrifuged at 1000 × *g* for 10 min and washed twice with HBSS. Because most of the epithelial cells are in the form of clumps after trypsin digestion, it is possible to separate them from single stromal cells by low-speed centrifugation (60 × *g* for 5 min). The pellet was then suspended in 20 ml RPMI medium, containing 50 μg/ml gentamicin, and plated onto 100 × 20 mm Nunclon petri dishes (GIBCO, Grand Island, NY) and incubated at 37 C with 5% CO_2-95% air for 3 h to allow for attachment of contaminating stromal cells. At the end of incubation, the floating cells were collected. After cell counting and viability determination by trypan-blue exclusion, cells were plated onto matri-gel-coated 100 × 20 mm Nunclon petri dishes. The stromal cell suspension was plated onto 100 × 20 mm Nunclon petri dishes for 3 h. The floating contaminating epithelial cells were washed away by gentle pipetting, and the medium was replaced. At the time of plating, the viability of both cell types was greater than 95%.

Hormone treatment

After confluence (about 7 days), cells were incubated in RPMI medium supplemented with 5% dextran-charcoal treated FCS (DC-FCS) in the presence or absence of E2 (10 nM), P4 (50 nM), or the combination of E2 (10 nM) and P4 (50 nM) for 4 days. Each group of cells was then incubated for a further 48 h with the same steroid regimen and in either the presence or absence of rbIFN-τ (10 and 100 ng/ml). At the end of the culture, medium was collected for PG measurement, and the cells were lysed with guanidinium isothiocyanate and stored at −70 C for RNA isolation. Ten microliters of cell lysate were taken for DNA measurement. DNA content was determined by the bisbenzimide fluorescent dye method of Labarca and Paigen (27).

Isolation of total RNA and Northern blot analysis

Total RNA was isolated from the cultured cells by centrifugation through a density gradient of 5.7 M cesium chloride. Forty micrograms of total RNA was denatured at 56 C for 15 min, electrophoresed in 1.2% agarose gel, and passively transferred to Hybond nylon membranes by capillary blotting. The nylon membranes were UV-cross-linked for 30 sec at 150 mJ in a UV chamber (Bio-Rad GS Gene Linker; Bio-Rad Labs, Richmond, CA) and prehybridized for 4–6 h in hybridization buffer at 55 C. Blots were hybridized with the appropriate ^{32}P-labeled probes (1 × 10^6 cpm/ml) for 16 h at 55 C; washed in 2 × saline sodium citrate, 0.1% SDS at 60 C for 15–20 min; and then, successively, in 2 × saline sodium citrate, 0.1% SDS at 55 C for 15–20 min twice. Autoradiography was performed with Kodak XAR-5 (Mandel Scientific Company Ltd., Guelph, Ontario, Canada) and double intensifying screen at −70 C for various exposure times. For rehybridization with a different probe, blots were boiled for 3 min in diethyl pyrocarbonate (DEPC)-treated H_2O, containing 0.1% SDS, and exposed to film overnight to check completeness of probe removal. Autographic bands were scanned using Foto/Analyst (Fotodyne Inc., New Berlin, WI), and the intensity of the autographic bands were quantitated by the NIH-image program. The amount of mRNA loaded was normalized using 28S mRNA.

Probes

Mouse cyclooxygenase-2, bovine PGFS, and human 28S complementary DNA (cDNA) inserts were used as probes to detect COX-2, PGFS, and 28S gene expression in cultured bovine uterine endometrial cells. The probes included a mouse COX-2 cDNA (28), previously validated with bovine tissues (29). A bovine PGFS cDNA probe was generated by RT-PCR. Five micrograms of RNA, extracted from granulosa cells of bovine preovulatory follicles (29), were reversed transcribed using avian myeloblastosis virus RT (Pharmacia Biotech, Montréal, Canada) and oligodeoxythymidine primers. For the PCR reaction, homologous primers were designed from the published bovine PGHS sequence lung form (23). The sense 25-mer primer 5′-TTAATGATGGCCACTTCATTC-CTGT-3′ corresponded to region from +29 to +53 bp from the start codon, and the antisense 25-mer primer 5′-GAGTCAGTTCAAAGT-CAAACACCTG-3′ was from +841 to +865 bp of the bovine PGFS lung form (23). The expected 837-bp PCR product was subcloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA), and its identity was confirmed by DNA sequencing using the T7 Sequencing Kit (Pharmacia), which employs the Sanger dideoxy nucleotide chain termination method (30).

RIA of $PGF_{2\alpha}$ and PGE_2

Concentrations of $PGF_{2\alpha}$ were measured in 100-μl aliquots of culture medium after 10-fold or 100-fold dilution with assay buffer. Serial dilutions of medium samples (n = 3) were parallel to the standard curve. The antibody was purchased from Cayman Chemical Co. (Ann Arbor, MI); its cross-reactivity against PGFM, 6-keto $PGF_{1\alpha}$, PGD_2, PGE_2, and AA was 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, and the intra- and interassay coefficients of variation were 9.2 and 12.3%, respectively.

Concentrations of PGE_2 were measured directly in 10- or 100-μl aliquots of culture medium. The antiserum was purchased from Assay Designs Inc. (Ann Arbor, MI); its cross-reactivity against PGE_1, $PGF_{1\alpha}$, $PGF_{2\alpha}$, and 6-keto $PGF_{1\alpha}$ was 70, 1.4, 0.7, and 0.6%, respectively. The sensitivity of the assay was 40 pg/ml, and the intra- and interassay coefficients of variation were 6.3 and 8.6%, respectively.

Endotoxin assay

The Limulus amebocyte lysate assay was used to measure the endotoxin concentration in all reagents used in this experiment. The protocol was provided by Sigma. The endotoxin contents of all reagents, including rbIFN-τ, are lower than the detectable level by this method (<0.1 ng/ml).

Statistical analysis

Each treatment was carried out using the cells from one uterus, and each experiment was repeated with four different uteri. Effects of treatment on $PGF_{2\alpha}$, PGE_2 production, and COX-2 and PGFS expression of uterine cells were evaluated by least-squares ANOVA. Treatments were analyzed in multifactorial design (ANOVA), which included the main effects of experiments, cell type, and hormone treatments. Simple contrasts were used to determine differences between individual means. A probability of $P < 0.05$ was considered to be statistically significant.

Results

LPS stimulates PG production by endometrial cells

LPS is a strong stimulator of PGs, especially PGE_2, in many types of cells. To determine whether endometrial cells respond to LPS stimulation, PGE_2 and $PGF_{2\alpha}$ content was measured in the culture medium after incubating epithelial and stromal cells with various doses of LPS for 24 h. Both epithelial and stromal cells responded to LPS in a dose-dependent fashion (Fig. 1). In the epithelial cells, LPS had a much greater effect on PGE_2 than on $PGF_{2\alpha}$ production (Fig. 1a). LPS markedly increased the ratio of PGE_2 to $PGF_{2\alpha}$ in epithelial cells. In the stromal cells, LPS had a biphasic effect on $PGF_{2\alpha}$ secretion (Fig. 1b). The stimulation of $PGF_{2\alpha}$ and PGE_2 was similar, except at the 100-ng/ml dose.

Effects of steroid hormones and IFN-τ on PG production

In epithelial cells, the basal concentrations of $PGF_{2\alpha}$ and PGE_2 in the medium from the untreated control cells were 11.6 ± 1.5 and 2.8 ± 0.65 ng/μg DNA, respectively. E2, alone or in the presence of P4, significantly reduced $PGF_{2\alpha}$ and PGE_2 secretion ($P < 0.01$; Fig. 2, a and b). P4 alone increased $PGF_{2\alpha}$ ($P < 0.02$), but not PGE_2, secretion. E2, either alone or in the presence of P4, decreased the ratio of PGE_2 to $PGF_{2\alpha}$ ($P < 0.01$; Fig. 2c). rbIFN-τ diminished $PGF_{2\alpha}$ production ($P < 0.01$) at both concentrations in the control, E2, and P4-treated groups; however, no effect of IFN was observed when the cells were treated with E2 and P4 together. rbIFN-τ decreased PGE_2 secretion when the cells were not treated with steroids. In the presence of P4, rbIFN-τ increased PGE_2 production; and in the presence of E2 or E2+P4, rbIFN-τ had no effect on PGE_2. IFN had no effect on the PGE/PGF ratio when the cells were treated with E2 or P4+E2. However, in the control and P4-treated cells, IFN significantly increased the PGE/PGF ratio ($P < 0.01$).

In stromal cells, the basal concentrations of $PGF_{2\alpha}$ and PGE_2 in the medium from the control cells were 0.64 ± 0.08

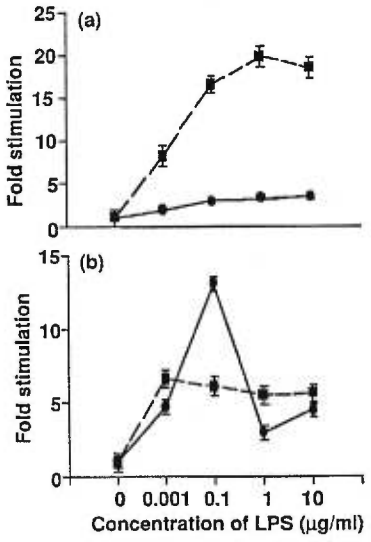

FIG. 1. LPS stimulation of PG secretion by bovine endometrial cells. Confluent primary epithelial (a) and stromal (b) cells were cultured in RPMI 1640 containing 5% DC-FCS, in the presence or absence of various doses of LPS (0.001–10 μg/ml), for 24 h. Culture media were collected for $PGF_{2\alpha}$ (●) and PGE_2 (■) measurement by RIA. Data are normalized to the DNA contents of respective wells and presented as fold stimulation of the medium control.

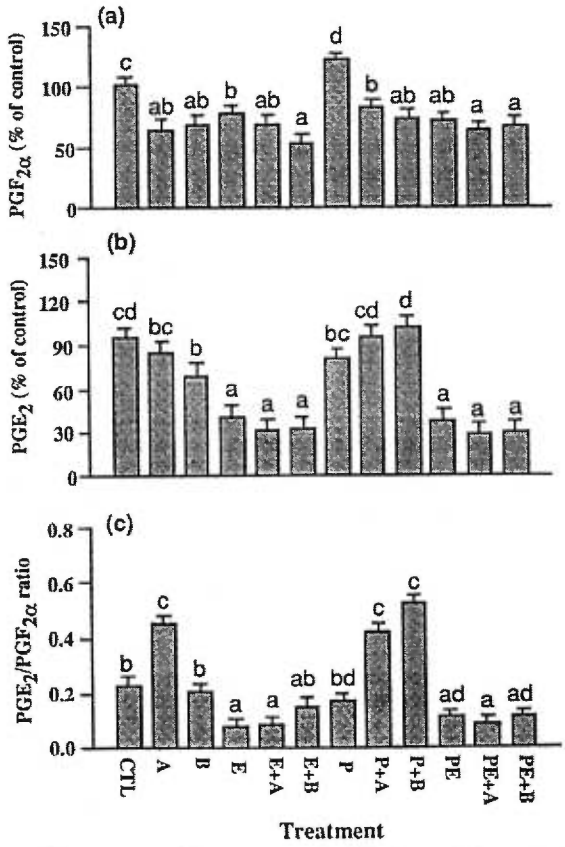

FIG. 2. Effect of steroid hormones and rbIFN-τ on PG production in epithelial cells. Confluent epithelial cells were incubated in RPMI medium supplemented with 5% DC-FCS, in the presence or absence of E2 (E, 10 nM), P4 (P, 50 nM), or their combination (PE), for 4 days. The medium was replaced with fresh RPMI plus 5% steroid-free FCS, in the presence or absence of rbIFN-τ (A, 10 ng/ml; B, 100 ng/ml), E, E + rbIFN-τ, P, P + rbIFN-τ, PE, or PE + rbIFN-τ. The concentrations of E2 and P4 are the same as above. Cells were incubated for another 48 h, and the culture medium was collected for $PGF_{2\alpha}$ (a) and PGE_2 (b) measurement by RIA. Data are normalized to the DNA contents of the respective wells. The ratio of PGE_2 to $PGF_{2\alpha}$ was calculated (c). The *bars* with different *letters* are statistically different ($P < 0.05$).

and 7.6 ± 1.7 ng/μg DNA, respectively. Steroid hormones had no significant effect on either $PGF_{2\alpha}$ or PGE_2 secretion (Fig. 3, a and b). The higher concentration (100 ng/ml) of rbIFN-τ increased $PGF_{2\alpha}$ production in the control and E2 groups (Fig. 3a) and PGE_2 secretion in all the groups (Fig. 3b). The ratio of PGE_2 to $PGF_{2\alpha}$ was increased significantly by rbIFN-τ in all groups, except the cells treated with E2 alone (Fig. 3c).

Effect of steroid hormones and IFN-τ on COX-2 mRNA levels

COX-2 is an inducible rate-limiting enzyme for the conversion of arachidonic acid to PGG_2 and PGH_2, the precursor for $PGF_{2\alpha}$ and PGE_2. To determine whether the regulation of PG production in endometrial cells by steroids and rbIFN-τ was correlated with COX-2 gene expression, COX-2 mRNA levels were measured by Northern blot analysis. A 4.1-kb transcript was detected by the mouse COX-2 cDNA probe in both cell types (Fig. 4, a and c). In epithelial cells (Fig. 4b), E2, alone and in combination with P4, decreased COX-2 mRNA ($P < 0.01$), whereas P4 alone had no effect. IFN-τ attenuated COX-2 mRNA in a dose-dependent manner in the control and P4-treated cells ($P < 0.01$). However, in the presence of E2, IFN-τ had no effect on COX-2 expression.

In stromal cells (Fig. 4d), steroid hormones had no significant effect on the amount of COX-2 mRNA. IFN-τ significantly increased COX-2 mRNA in a dose-dependent manner in all groups ($P < 0.01$); however, the addition of E2 markedly decreased the induction of COX-2 gene expression by P4 ($P < 0.01$).

Effect of steroid hormones and IFN-τ on PGFS mRNA levels

PG F synthase (PGFS) is responsible for the reduction of PGH_2 to $PGF_{2\alpha}$ and PGD_2 to 9α, 11β-PGF_2 (a stereoisomer of

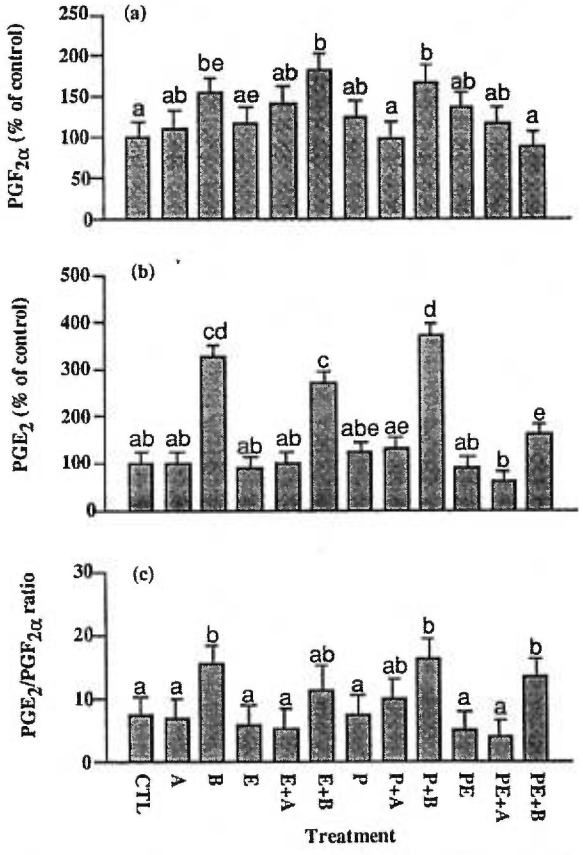

FIG. 3. Effect of steroid hormones and rbIFN-τ on PG production in stromal cells. Confluent stromal cells were incubated in RPMI medium supplemented with 5% DC-FCS, in the presence or absence of E2 (E, 10 nM), P4 (P, 50 nM), or their combination (PE), for 4 days. The medium was replaced with fresh RPMI plus 5% steroid-free FCS, in the presence or absence of rbIFN-τ (A, 10 ng/ml; B, 100 ng/ml), E, E + rbIFN-τ, P, P + rbIFN-τ, PE, or PE + rbIFN-τ. The concentrations of E2 and P4 are the same as above. Cells were incubated for another 48 h, and the culture medium was collected for $PGF_{2\alpha}$ (a) and PGE_2 (b) measurement by RIA. Data are normalized to the DNA contents of the respective well. The ratio of PGE_2 to $PGF_{2\alpha}$ was calculated (c). Different *superscript letters* indicate significant differences ($P < 0.05$).

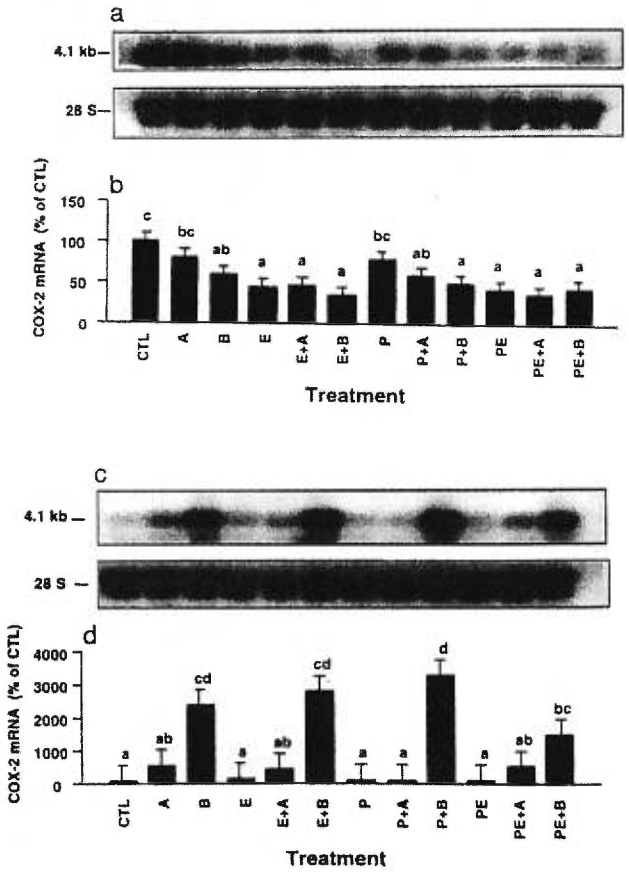

FIG. 4. Northern blot analysis of COX-2 mRNA in endometrial cells. RNA was isolated from epithelial cells (a) and stromal cells (c) after hormone and rbIFN-τ treatment, as described in Fig. 3. A representative sample is shown, when 40 μg total RNA per lane was loaded and blots were hybridized with ^{32}P-labeled mouse COX-2 cDNA probe. The stripped blots were rehybridized with human 28S cDNA probe as a loading control. Autographic bands were scanned by a densitometer and normalized to their 28S values for epithelial (b) and stromal (d) cells. Different *superscript letters* indicate significant differences ($P < 0.05$).

$PGF_{2\alpha}$). To determine whether the attenuation of $PGF_{2\alpha}$ secretion in epithelial cells, and the increase in ratio of PGE_2 to $PGF_{2\alpha}$ in stromal cells, induced by rbIFN-τ is correlated with PGFS expression in these cells, the changes in PGFS mRNA levels induced by steroids and rbIFN-τ was determined by Northern blot analysis. A 1.4-kb transcript was detected by a bovine PGFS probe (Fig. 5, a and c). In epithelial cells (Fig. 5b), steroid hormones alone had no significant effect on PGFS expression. However, rbIFN-τ decreased PGFS mRNA in a dose-dependent manner in all steroid treatment groups ($P < 0.01$).

In stromal cells (Fig. 5d), neither E2 nor P4 had any effect on PGFS mRNA levels; however, the presence of both E2 and P4 seemed to up-regulate PGFS mRNA, compared with the addition of P4 alone ($P < 0.05$). rbIFN-τ, alone or in presence of steroid hormones, decreased PGFS expression ($P < 0.01$).

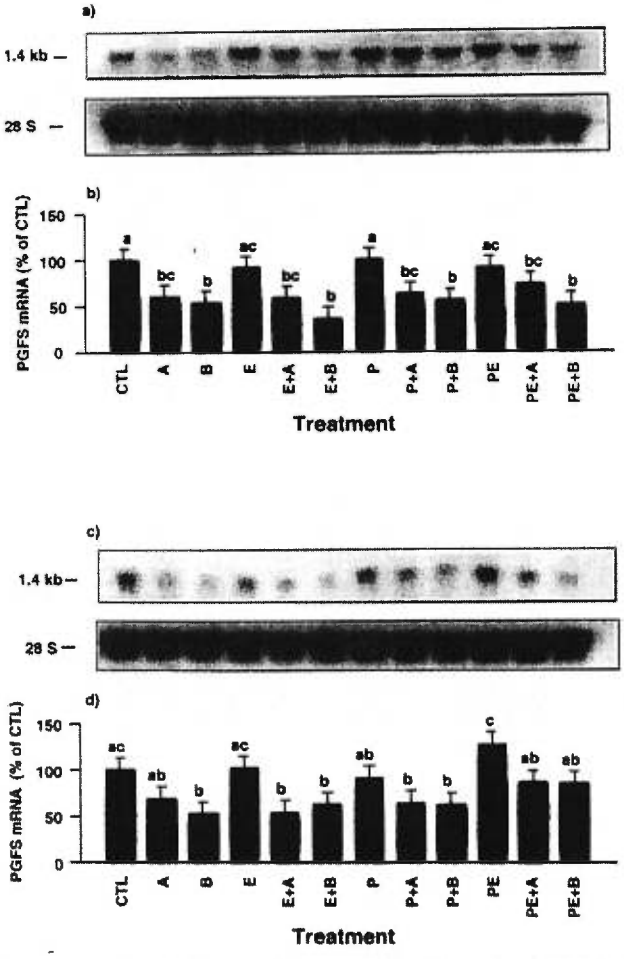

FIG. 5. Northern blot analysis of PGFS in endometrial cells. The same blots used in Fig. 4 for COX-2 hybridization of epithelial (a) and stromal (c) cells were stripped and hybridized with ^{32}P-labeled PGFS probe. The densitometric values of PGFS for epithelial (b) and stromal (d) cells were normalized to their 28S values. The *columns* represent the mean ± SEM of four replicates. Different *superscript letters* indicate significant differences ($P < 0.05$).

Discussion

$PGF_{2\alpha}$ is the luteolysin, secreted from the endometrium, that is responsible for the regression of the CL at the end of the estrous cycle in ruminants. IFN is the embryonic factor responsible for preventing the secretion of $PGF_{2\alpha}$ and, possibly, increasing the secretion of PGE_2, a luteotrophic factor. Although it is known that both P4 and E2 are essential for the induction of luteolysis, and that one action of IFN is to suppress the action of E2 by down-regulating its receptor (10), the exact mechanism of action of these hormones is still not completely understood.

The present study is the first to simultaneously examine the effects of steroid hormones and IFN on PG secretion and on COX-2 and PGF synthase gene expression in isolated endometrial cells. E2 significantly decreased $PGF_{2\alpha}$ and PGE_2 production, whereas P4 increased $PGF_{2\alpha}$, but not PGE_2, secretion in epithelial cells. E2 and P4 had no effect on $PGF_{2\alpha}$ and PGE_2 secretion by stromal cells. This is generally consistent with the previous report by Asselin *et al.* (7). *In vivo*, P4 increases uterine secretion of $PGF_{2\alpha}$ (31). E2 is generally thought to stimulate PG synthesis, because administration of E2 to cows at midcycle (32) or to P4-primed ovariectomized cows (33) stimulates $PGF_{2\alpha}$ secretion. At the present time, it is not clear why E2 decreases $PGF_{2\alpha}$ secretion and prevents the P4-induced stimulation in isolated cells *in vitro* but stimulates secretion *in vivo*.

The decrease in $PGF_{2\alpha}$ secretion induced by E2 *in vitro* is probably caused by decreased COX-2 enzyme activity, given that it is associated with a decrease in COX-2, but not PGFS, mRNA. P4 did not increase either COX-2 or PGFS mRNA, although it increased $PGF_{2\alpha}$ production; thus, the effect of P4 on $PGF_{2\alpha}$ production is probably not at the level of the gene expression of these enzymes. This was also suggested by Smith *et al.* (34). P4 may, however, act at the level of translation of COX-2, because Charpigny *et al.* (24) have shown that ovine endometrial COX-2 protein was highly induced by P4. Steroid hormone treatment does not affect the amount of the COX-1 protein (24), and it is unlikely that P4 acts via this constitutively expressed enzyme.

rbIFN-τ attenuated the secretion of both $PGF_{2\alpha}$ and PGE_2 from epithelial cells. This agrees with previous reports (12–16, 35). The decrease in PG secretion, brought about by rbIFN-τ, was probably caused by the decrease in COX-2 mRNA. In the presence of P4, rbIFN-τ enhanced the secretion of PGE_2 and thus increased the $PGE_2/PGF_{2\alpha}$ ratio. This might be explained by the inhibition of PGFS expression by rbIFN-τ, whereby more of the precursor for PG synthesis will be available for synthesis of PGE_2. In contrast to its inhibitory effect on $PGF_{2\alpha}$ secretion in epithelial cells, rbIFN-τ markedly enhanced $PGF_{2\alpha}$ and PGE_2 production in stromal cells. IFN had a greater effect on PGE_2 than on $PGF_{2\alpha}$, resulting in a net increase in the $PGE_2/PGF_{2\alpha}$ ratio. The increase in PG secretion was associated with an increase in COX-2 expression, and the decrease in PGFS mRNA by rbIFN-τ may be responsible for the increased $PGE_2/PGF_{2\alpha}$ ratio. The stimulation of PGE_2 by rbTP-1 (rbIFN-τ) has been previously reported in bovine endometrial stromal cells (13) (36). Because PGE_2 is considered to be a luteoprotective agent (37) and stromal cells are a predominant cell population in the endometrium,

this induction of PGE_2 by rbIFN-τ may play an important role in the maintenance of the CL.

Our results, and those of others (12–16, 35), differ from those reported by Asselin *et al.* (36), who showed that rbIFN-τ and recombinant ovine IFN-τ stimulated both $PGF_{2\alpha}$ and PGE_2 in cultured bovine uterine epithelial cells. One possible explanation of the different results is LPS contamination of the IFN-τ, because gram-negative bacteria, including *Escherichia coli* used to produce recombinant IFN-τ, contain LPS in their cell membrane. LPS is a strong stimulator of PG synthesis (especially PGE_2) in many types of cells, such as human monocytes (38), gingival fibroblasts (39), neutrophils (40), rat peritoneal macrophages (41), and blood-derived macrophages of red deer (42). The results presented in this study show that LPS is also a powerful stimulator of PG synthesis in endometrial epithelial and stromal cells. LPS increased PGE_2 secretion more than $PGF_{2\alpha}$ in the epithelial cells, whereas the stimulation of $PGF_{2\alpha}$ and PGE_2 were similar (except at 0.1 μg/ml) in stromal cells. In our study, all the reagents and the rbIFN-τ were assayed for endotoxin, and the content was lower than the limit of detection (<0.1 ng/ml) of the Limulus amebocyte lysate assay. It might be important to screen recombinant products for endotoxin content when used to investigate PG synthesis.

In conclusion, this study showed differential effects of E2, P4, and rbIFN-τ in the regulation of PG production, and COX-2 and PGFS gene expression in cultured bovine endometrial cells. Our results show that E2 inhibited $PGF_{2\alpha}$ and PGE_2 production by down-regulating COX-2 expression in epithelial cells. P4 increased $PGF_{2\alpha}$ secretion but did not up-regulate COX-2. rbIFN-τ attenuated $PGF_{2\alpha}$ and PGE_2 in epithelial cells and enhanced $PGF_{2\alpha}$ and PGE_2 in stromal cells by down- and up-regulating COX-2 mRNA, respectively. The changes in the ratio of PGE_2 to $PGF_{2\alpha}$, brought about by rbIFN-τ, are associated with a decrease in PGFS mRNA abundance.

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References

1. **Salamonsen LA, Findlay JK** 1990 Regulation of endometrial prostaglandins during the menstrual cycle and in early pregnancy. Reprod Fertil Dev 2:443–457
2. **Goding J** 1972 The demonstration that $PGF_{2\alpha}$ is the uterine luteolysin in the ewe. J Reprod Fertil 38:261–272
3. **McCracken J, Carlson JC, Glew ME, Goding JR, Baird DT, Green K, Samuelsson B** 1972 Prostaglandin $F_{2\alpha}$ identified as a luteolytic hormone in sheep. Nature New Biol 238:129–134
4. **McCracken J, Barcikowski B, Carlson JC, Green K, Samuelsson B** 1973 The physiological role of prostaglandin $F_{2\alpha}$ in corpus luteum regression. Adv Biosci 9:599–624
5. **Bazèr FW, Ott TL, Spencer TE** 1994 Pregnancy recognition in ruminants, pigs and horses: signals from the trophoblast. Theriogenology 41:79–94
6. **Magness R, Huie JM, Weenes CW** 1981 Effect of chronic ipsilateral or contralateral intrauterine infusion of prostaglandin E_2 (PGE_2) on luteal function of unilaterally ovariectomized ewes. Prostaglandins Med 6:389–401
7. **Asselin E, Goff AK, Bergeron H, Fortier MA** 1996 Influence of sex steroids on the production of prostaglandins $F_{2\alpha}$ and E_2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium. Biol Reprod 54:371–379
8. **Bartol FF, Roberts RM, Bazer FW, Lewis GS, Godkin JD, Thatcher WW** 1985 Characterization of proteins produced *in vitro* by periattachment bovine conceptuses. Biol Reprod 32:681–693
9. **Plante C, Hansen PJ, Thatcher WW** 1988 Prolongation of luteal lifespan in cows by intrauterine infusion of recombinant bovine α-interferon. Endocrinology 122:2342–2344
10. **Spencer TE, Becker WC, George P, Mirande MA, Ogle TF, Bazer FW** 1995 Ovine interferon-τ regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. Biol Reprod 53:732–745
11. **Helmer SD, Gross TS, Newton GK, Hanson PJ, Thatcher WW** 1989 Bovine trophoblast protein-1 complex alters endometrial protein and prostaglandin secretion and induces an intracellular inhibitor of prostaglandin synthesis *in vitro*. J Reprod Fertil 87:421–430
12. **Godkin JD, Smith SE, Johnson RD, Doré JJE** 1997 The role of trophoblast interferons in the maintenance of early pregnancy in ruminants. Am J Reprod Immunol 37:137–143
13. **Smith SE, Godkin JD** 1992 Characterization of isolated bovine uterine glandular epithelial and stromal cells. Biol Reprod [Suppl 1] 46:488
14. **Smith SE, Doré JJE, Godkin JD** 1993 Effects of recombinant bovine trophoblast protein-1 on the regulation of gene expression and prostaglandin $F_{2\alpha}$ release by isolated bovine uterine glandular epithelial and stromal cells. Biol Reprod [Suppl 1] 48:404
15. **Danet-Desnoyers G, Wetzels C, Thatcher WW** 1994 Natural and recombinant bovine interferon-τ regulate basal and oxytocin-induced secretion of prostaglandins $F_{2\alpha}$ and E_2 by epithelial cells and stromal cells in the endometrium. Reprod Fertil Dev 6:193–202
16. **Salamonsen LA, Stuchberry SJ, O'Grady CM, Godkin JD, Findlay JK** 1988 Interferon-α mimics effects of ovine trophoblast protein-1 on prostaglandin and protein secretion by ovine endometrial cells *in vitro*. J Endocrinol 117:R1–R2
17. **Salamonsen LA, Hampton AL, Clements JA, Findlay JK** 1991 Regulation of gene expression and cellular localization of prostaglandin synthase by oestrogen and progesterone in the ovine uterus. J Reprod Fertil 92:393–406
18. **Kujubu DA, Fletcher BS, Varnum BC, Lim RW, Herschman HR** 1991 TIS10, a phorbol ester tumor promoter-inducible mRNA from swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 266:12866–12872
19. **O'Banion MK, Sadowski HB, Winn V, Young DA** 1991 A serum- and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. J Biol Chem 266:23261–23267
20. **Arslan A, Zingg HH** 1996 Regulation of COX-2 gene expression in rat uterus *in vivo* and *in vitro*. Prostaglandins 52:463–481
21. **Wong PYK** 1981 Purification and partial characterization of prostaglandin D_2 11-keto reductase in rabbit liver. Biochim Biophys Acta 659:169–178
22. **Liston TE, Roberts LJ** 1985 Transformation of prostaglandin D_2 to 9 alpha, 11 beta-(15S)-trihydroxyprosta-(5Z, 13E)-dien-1-oic acid (9 alpha, 11 beta-prostaglandin F2): a unique biologically active prostaglandin produced enzymatically *in vivo* in humans. Proc Natl Acad Sci USA 82:6030–6034
23. **Watanabe K, Fujii Y, Nakayama K, Ohkubo H, Kuramitsu S, Kagamiyama H, Nakanishi S, Hayaishi O** 1988 Structural similarity of bovine lung prostaglandin F synthase to lens ϵ-crystallin of the European common frog. Proc Natl Acad Sci USA 85:11–15
24. **Charpigny G, Reinaud P, Tamby JP, Créminon C, Martal J, Maclouf J, Guillomot M** 1997 Expression of cyclooxygenase-1 and -2 in ovine endometrium during the estrous cycle and early pregnancy. Endocrinology 138:2163–2171
25. **Roberts RM, Cross JC, Leaman DW** 1992 Interferons as hormones of pregnancy. Endocr Rev 13:432–452
26. **Fortier MA, Guibault LA, Grasso F** 1988 Specific properties of epithelial and stromal cells from the endometrium of cows. J Reprod Fertil 83:239–248
27. **Labarca C, Paigen K** 1980 A simple, rapid and sensitive DNA assay procedure. Anal Biochem 102:344–352
28. **Simmons D, Xie W, Chipman JG, Evett JE** 1992 Multiple cyclooxygenase: cloning of a mitogen-inducible form. In: Martyn-Bailey J (ed) Prostaglandins, Leukotrines, Lipoxins and PAF. Plenum Press, New York, pp 67–78
29. **Sirois J** 1994 Induction of prostaglandin endoperoxide synthase-2 by human chorionic gonadotropin in bovine preovulatory follicles *in vivo*. Endocrinology 135:841–848
30. **Sanger F, Nicklen S, Coulson AR** 1977 DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
31. **Scaramuzzi RJ, Baird DT, Boyle HP, Land RB, Wheeler AG** 1977 The secretion of prostaglandin F from the autotransplanted uterus of the ewe. J Reprod Fertil 49:157–160
32. **Thatcher WW, Bartol FF, Knickerbocker JJ, Curl JS, Wolfenson D, Bazer FW, Roberts RM** 1984 Maternal recognition of pregnancy in cattle [Review]. J Dairy Sci 67:2797–2811
33. **Lafrance M, Goff AK** 1988 Effects of progesterone and estradiol-17β on oxytocin-induced release of prostaglandin F2α. J Reprod Fertil 82:429–436
34. **Smith SK, Abel MH, Baird DT** 1984 Effects of 17β-estradiol and progesterone on the levels of prostaglandins $F_{2\alpha}$ and E in human endometrium. Prostaglandins 27:591–597
35. **Salamonsen LA, Manikhot J, Healy DL, Findlay JK** 1989 Ovine trophoblast

protein-1 and human interferon alpha reduce prostaglandin synthesis by ovine endometrial cells. Prostaglandins 38:289–307

36. **Asselin E, Bazer FW, Fortier MA** 1997 Recombinant ovine and bovine interferons tau regulate prostaglandin production and oxytocin response in cultured bovine endometrial cells. Biol Reprod 56:402–408
37. **Bazer FW, Thatcher WW, Hansen PJ, Mirando MA, Ott TL, Plante C** 1991 Physiological mechanisms of pregnancy recognition in ruminants [Review]. J Reprod Fertil [Suppl] 43:39–47
38. **Patrignani P, Santini G, Panara MR, Sciulli MG, Greco A, Rotondo MT, di Giamberardino M, Maclouf J, Ciabattoni G, Patrono C** 1996 Induction of prostaglandin endoperoxide synthase-2 in human monocytes associated with cyclo-oxygenase-dependent F2-isoprostane formation. Br J Pharmacol 118:1285–1293
39. **Takiguchi H, Yamaguchi M, Mochizuki K, Abiko Y** 1996 Effect of *in vitro* aging on Campylobacter rectus lipopolysaccharide-stimulated PGE_2 release from human gingival fibroblasts. Oral Dis 2:202–209
40. **Niiro H, Otsuka T, Izuhara K, Yamaoka K, Ohshima K, Tanabe T, Hara S, Nemoto Y, Tanaka Y, Nakashima H, Niho Y** 1997 Regulation by interleukin-10 and interleukin-4 of cyclooxygenase-2 expression in human neutrophils. Blood 89:1621–1628
41. **Nakatsugi S, Sugimoto N, Furukawa M** 1996 Effects of non-steroidal anti-inflammatory drugs on prostaglandin E_2 production by cyclooxygenase-2 from endogenous and exogenous arachidonic acid in rat peritoneal macrophages stimulated with lipopolysaccharide. Prostaglandins Leukot Essent Fatty Acids 55:451–457
42. **Cross M, Slobbe LJ, Buchan GS, Griffin JF** 1996 *In vitro* responses of cervine macrophages to bacterial stimulants. Vet Immunol Immunopathol 53:249–256

3.4. Article 4. Downregulation of oxytocin-induced COX-2 and prostaglandin F synthase expression by interferon-τ in bovine endometrial cells

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Downregulation of oxytocin-induced COX-2 and prostaglandin F synthase expression by interferon-τ in bovine endometrial cells[1]

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Short title : IFN-τ downregulates OT-induced COX-2 and PGF synthase

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Abstract

Oxytocin (OT) is responsible for the episodic release of luteolytic prostaglandin (PG) $F_{2\alpha}$ from the uterus in ruminants. The attenuation of OT-stimulated uterine $PGF_{2\alpha}$ secretion by interferon-τ (IFN-τ) is essential for prevention of luteolysis during pregnancy in cows. To better understand the mechanisms involved, the effect of recombinant bovine IFN-τ (rbIFN-τ) on OT-induced PG production, cyclooxygenase-2 (COX-2) and PGF synthase (PGFS) expression in cultured endometrial epithelial cells was investigated. Cells were obtained from cows at days 1-3 of the estrous cycle and cultured to confluence in RPMI medium supplemented with 5% steroid-free fetal calf serum. The cells were then incubated in the presence or absence of either 100 ng/ml OT or OT plus 100 ng/ml rbIFN-τ for 3, 6, 12 and 24 h. OT significantly increased $PGF_{2\alpha}$ and PGE_2 secretion at all time points ($p<0.01$), while rbIFN-τ inhibited the OT-induced PG production and reduced OT receptor binding in a time-dependent manner. 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 ($p<0.01$). OT also caused an increase in COX-2 protein, which peaked at 12 h (11-fold increase), as measured by Western blot. Addition of rbIFN-τ suppressed the induction of COX-2 mRNA (89%, $p<0.01$) and COX-2 protein (50%, $p<0.01$) by OT. OT also increased PGFS mRNA, and this stimulation was attenuated by rbIFN-τ ($p<0.01$). To ensure that the decrease in COX-2 was not solely due to down-regulation of the OT receptor, cells were stimulated with a phorbol ester (PMA) in the presence and absence of rbIFN-τ. The results showed that rbIFN-τ also decreased PMA-stimulated PG production and COX-2 protein. It can be concluded that rbIFN-τ inhibition of OT-stimulated PG production is due to downregulation of OT receptor, COX-2 and PGFS.

Introduction

Prostaglandin (PG) $F_{2\alpha}$, primarily secreted from the surface epithelium of the uterus, is the luteolytic agent in ruminants [1-3] and oxytocin (OT) is responsible for its episodic release [4-7]. OT stimulates secretion of $PGF_{2\alpha}$ from the endometrium [8-12] and the uterine OT receptor concentrations determine the sensitivity of endometrium to OT stimulation [6, 9, 13].

The attenuation of pulsatile secretion of $PGF_{2\alpha}$ from the uterus is essential for the prevention of luteolysis during pregnancy in cows. The trophoblast secretes a protein, interferon-τ (IFN-τ), between days 15 to 24 of gestation [14] that prevents luteolysis by suppressing endometrial $PGF_{2\alpha}$ secretion. Conceptus secretory proteins (CSP) suppress OT-induced $PGF_{2\alpha}$ production [15, 16], also treatment with natural or recombinant bovine IFN-τ (rbIFN-τ) reduces the OT-induced secretion of $PGF_{2\alpha}$ and PGE_2 by bovine endometrial epithelial cells *in vitro* [8, 17, 18]. The suppression of OT-induced $PGF_{2\alpha}$ by IFN-τ is generally thought to be due to a decrease in OT receptor number. It has been demonstrated that the endometrial OT receptor concentration on ovine uterine endometrium is reduced in pregnant animals [19-21], and that intrauterine infusion of rbIFN-τ attenuates OT binding to the endometrium in cattle [22] and sheep [23].

Although IFN-τ reduces OT receptor number in the endometrium, this is probably not the only effect the embryo has on PG secretion during early pregnancy. An inhibitor of PG synthesis is induced in the endometrium of pregnant cows [24-26]. This inhibitor has recently been identified as linoleic acid [27] and is thought to compete with arachidonic acid for the active site on the cyclooxygenase (COX) enzyme, which is the key rate-limiting enzyme responsible for the conversion of arachidonic acid to PGG_2 and PGH_2. These

compounds are the precursors for $PGF_{2\alpha}$, PGE_2 and other members of the prostaglandin family [28]. Two isoforms of COX (COX-1 and COX-2) have been identified in mammalian cells. COX-1 is a constitutively expressed enzyme, COX-2 is induced by various substances, such as phorbol esters, mitogens, cytokines and serum [29-31], and by OT in the uterus [11, 18, 32].

The inhibitory effect of IFN-τ on OT-induced $PGF_{2\alpha}$ is well established, but the molecular mechanisms involved have not yet been completely elucidated. One mechanism by which IFN inhibits the OT-induced $PGF_{2\alpha}$ synthesis is by reducing estradiol receptor number and thus preventing an estrogen-induced increase in OT receptor number [33, 34]. However, other mechanisms such as the production of an inhibitor of PG synthesis [27, 35] may also be involved. Furthermore, since treatment of ovariectomized cows with progesterone alone results in an increase in response to OT [36, 37], some induction of OT receptor may be independent of estradiol action. If this is the case, and IFN suppresses estradiol-induced OT receptor, then IFN may also have other effects on PG synthesis to ensure that luteolysis does not occur. Since OT increases COX-2 mRNA *in vivo* [11] and *in vitro* [18], the objectives of the present study were to determine 1) the effect of IFN-τ on OT-induced COX-2 and 2) the effect of OT and IFN-τ on PGFS, the enzyme that converts PGH2 to $PGF_{2\alpha}$. Since IFN-τ decreases OT receptor number *in vivo*, the effect of IFN-τ on PMA-stimulated PG synthesis was also examined to ensure that effects of IFN-τ were not solely due to changes in OT binding.

Materials and Methods

Chemicals and reagents:

Tissue culture medium (RPMI 1640), Hank's Buffered Saline Solution (HBSS, calcium and magnesium free), fetal calf serum (FCS), antibiotics and trypan blue were purchased from GIBCO (Grand Island, NY, USA). Collagenase (Type II), trypsin (Type III, from bovine pancreas), DNase I (Type I, from bovine pancreas), Gentamicin, calf thymus DNA, Hoechst NO. 33258, phorbol 12-myristate 13-acetate (PMA) and E-TOXATE kit were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Matrigel was obtained from VWR Scientific (Mississauga, Ontario, Canada). Oxytocin was purchased from Vetroquinol (Joliette, Quebec, Canada). Recombinant bovine interferon-τ was generously provided by Dr. R.M. Roberts [38]. The polyclonal rabbit COX-2 antibody (MF243) was kindly provided by Drs. Jilly F. Evant and Stacia Kargman (Merck Frosst Center for Therapeutic Research).

Preparation and culture of cells

Uteri from cows at days 1 to 3 of the estrous cycle were collected at the slaughterhouse and transported on ice to the laboratory. 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 endometrial epithelial and stromal cells were separated as described previously [39, 40]. Briefly, the two horns of each uterus were washed with sterile HBSS containing 100 units penicillin, 100 μg streptomycin and 0.25 μg amphotericin per ml. The myometrial layers were dissected away and the horns were then everted to expose the epithelium. The everted horns were digested for 2 h in HBSS with 0.3% trypsin at room temperature. At the end of incubation, the digested horns were scraped gently with forceps and then washed twice in HBSS. The scrapings and washings were combined with the digested cells and then FCS was added to a final concentration of 10% to block the action of

trypsin. The cell suspension was centrifuged at 60 x g for 5 min. The pellet was washed three more times with HBSS. Since most epithelial cells are in clumps after trypsin digestion, it is possible to separate them from completely dispersed stromal cells by this low-speed centrifugation (60 x g for 5 min). The pellet was then suspended in 20 ml RPMI medium containing 50 μg/ml gentamicin and plated onto 100 x 20 mm Nunclon petri dishes (GIBCO, Grand Island, NY) and incubated at 37 C in 5% CO_2, 95% air for 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 onto matri-gel coated 100 x 20 mm Nunclon petri dishes. At the time of plating, the cell viability was greater than 95%.

Treatment of cells

After reaching confluence, usually at about 7 d, cells were incubated in RPMI medium supplemented with 5% dextran-charcoal treated FCS (DC-FCS) in presence or absence of 100 ng/ml rbIFN-τ, 100 ng/ml OT, or OT plus rbIFN-τ for 3, 6, 12, and 24 h. In a second experiment, cells were incubated in the presence or absence of PMA (100 ng/ml) or PMA plus rbIFN-τ (100 ng/ml) for 12 h. At the end of the culture, medium was collected for PG measurement. The cells were either lysed with guanidinium isothiocyanate and stored at -70 C for RNA isolation, or scraped from the dish and suspended in 5 ml HBSS and then pelleted by centrifugation at 1000 x g for 5 min at 4 C. The cell pellets were stored at -70 C for Western blotting. For the oxytocin binding experiment, the confluent epithelial cells were incubated in RPMI medium supplemented with 5% dextran-charcoal treated FCS (DC-FCS) in presence or absence of 100 ng/ml rbIFN-τ for 3, 6, 12, and 24 h. The doses of OT and IFN-τ used in these

experiments were those that gave the maximum response in preliminary dose-response studies (data not shown). The concentration of IFN-τ in the uterine lumen is not known and the dose of IFN-τ used is in the same range as in previously published studies [8].DNA content was determined by the bisbenzimide fluorescent dye method of Labarca and Paigen [41].

Isolation of total RNA and Northern blot analysis

Total RNA was isolated from the cultured cells by centrifugation through a density gradient of 5.7 M cesium chloride. Twenty-five micrograms of total RNA were denatured at 56 C for 15 min, electrophoresed in 1.2% agarose gel and passively transferred to Hybond nylon membranes by capillary blotting. The nylon membranes were UV-cross-linked for 30 sec at 150 mj in a UV chamber (Bio-Rad GS Gene Linker; Bio-Rad Labs, Richmond, CA) and prehybridized for 4-6 h in hybridization buffer at 55 C. Blots were hybridized with the appropriate ^{32}P-labeled probes (1 X 10^6 cpm/ml) for 16 h at 55 C, washed in 2 X saline-sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) at 60 C for 15-20 min and then successively in 2 X SSC, 0.1% SDS at 55 C for 15-20 min twice. Autoradiography was performed with Kodak XAR-5 (Mandel Scientific Company Ltd., Guelph, Ontario, Canada) and double intensifying screen at -70 C for various exposure times. For rehybridization with a different probe, blots were boiled for 3 min in DEPC-treated H_2O containing 0.1% SDS and exposed to film overnight to check for completeness of the removal of the probe. Autoradiographic bands were scanned using Foto/Analyst (Fotodyne Inc., New Berlin, WI) and the intensity of the bands was quantitated by the NIH-Image program. The amount of mRNA loaded was normalized using 28S mRNA.

Probes

Mouse COX-2, bovine PGFS and human 28S cDNAs were used as probes to detect COX-2, PGFS and 28S gene expression in cultured bovine endometrial epithelial cells. A mouse COX-2 cDNA [42] was previously validated for use with bovine tissues [43]. A bovine PGFS cDNA probe was generated by RT-PCR. Five micrograms of RNA extracted from granulosa cells of bovine preovulatory follicles [43] were reversed-transcribed using AMV (Avian Myeloblastosis Virus) reverse transcriptase (Pharmacia Biotech, Montreal, Canada) and oligodeoxythymidine primers. For the PCR reaction, homologous primers were designed from the published bovine PGFS sequence lung form [44]. The sense 25-mer primer 5'-TTAATGATGGCCACTTCATTCCTGT-3' corresponded to region from +29 to +53 bp from the start codon, and the antisense 25-mer primer 5'-GAGTCAGTTCAAAGTCAAACACCTG-3' was from +841 to +865 bp of the bovine PGFS lung form [44]. The expected 837-bp PCR product was subcloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA), and its identity was confirmed by DNA sequencing using the T7 Sequencing Kit (Pharmacia), which employs the dideoxy nucleotide chain termination method [45].

Cell extracts and Western analysis of COX-2 protein

Solubilized cell extracts were prepared as previously described [43]. Briefly, cells were homogenized on ice in 500 µl TED homogenization buffer (50 mM Tris, 10 mM EDTA, 1 mM DEDTC, pH 8.0) supplemented with 2 mM octyl glucoside, and centrifuged at 30,000 x g for 1 h at 4 C. The crude pellets containing membranes, nuclei and mitochondria were sonicated (5 sec/cycle; 4 cycles) in 200 µl TED sonication buffer (20 mM Tris, 50 mM EDTA, 0.1 mM

DEDTC, pH 8.0) containing 32 mM octyl glucoside. The sonicates were centrifuged at 13,000 x g for 25 min at 4 C. The supernatants (solubilized cell extracts) were stored at -70 C until Western analysis. The protein concentration was determined using the Bio-Rad Protein Assay.

Fifty μg of cell extracts were separated by 10% one-dimensional SDS-PAGE, and electrophoretically transferred onto nitrocellulose membrane. The membranes were incubated with COX-2 antibody MF 243 and ^{125}I-labeled protein A was used to visualize immunopositive proteins as described previously [43]. Autoradiographic bands were scanned by Foto/Analyst (Fotodyne Inc., New Berlin, WI) and the intensity of the bands was quantitated by the NIH-Image program.

OT receptor and binding assay

OT binding was measured using whole cells. At the end of the culture, the medium was aspirated and 100 μl of 10 nM ^{3}H-OT either with (non-specific binding) or without (total binding) a 200-fold excess of unlabelled OT in binding buffer (25 mM Tris-HCl, pH 7.6; 0.1% BSA (w/v); 1 mM $MnCl_2$) [46] was added to each of three wells for each treatment. For saturation analysis of oxytocin receptor, epithelial cells were incubated with 0.15-15 nM [^{3}H]-OT. The binding affinity of [^{3}H]-OT for cell supernatants was determined using the least squares curve fitting program LIGAND [47]. Plates were incubated for 20 min at room temperature and then washed three times with 1 ml of saline. Solubilization solution (250 μl, 0.5% Triton-X 100, 1 M NaOH) was added into each well and incubated overnight at 37 C. The solubilized cells were neutralized with 62.5 μl of 4 M HCl and then counted in 4 ml of scintillant liquid. Specific binding (total minus nonspecific binding) values were expressed as DPM per μg DNA.

Radioimmunoassay of $PGF_{2\alpha}$ and PGE_2

Concentrations of $PGF_{2\alpha}$ were measured in 100 µl aliquots of culture medium after 10-fold or 100-fold dilution with assay buffer. Serial dilutions of medium samples (n = 3) were parallel to the standard curve. The antibody was purchased from Cayman Chemical Co., Ann Arbor, MI; its cross-reactivities against 13, 14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM), 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2 and AA 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 and the intra- and inter-assay coefficients of variation were 9.2 and 12.3% respectively.

Concentrations of PGE_2 were measured directly in 10 or 100 µl aliquots of culture medium. The antiserum was purchased from Assay designs Inc. (Ann Arbor, MI); its cross-reactivity against PGE_1, $PGF_{1\alpha}$, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$ was 70, 1.4, 0.7 and 0.6% respectively. The sensitivity of the assay was 40 pg/ml and the intra- and inter-assay coefficients of variation were 6.3 and 8.6% respectively.

Endotoxin assay

The Limulus amebocyte lysate assay was used to measure the endotoxin concentration in all reagents used in this experiment, according to the protocol provided by Sigma. The endotoxin content of all reagents, including rbIFN-τ was lower than the minimum detectable level by this method (< 0.1 ng/ml).

Statistical analysis

Each experiment was carried out using the cells from one uterus and was repeated with 4 different uteri collected at different times from the slaughterhouse. Effects of treatment on $PGF_{2\alpha}$ and PGE_2 production, OT

receptor number and COX-2 and PGFS expression in uterine cells were evaluated by least-squares analysis of variance. The data for $PGF_{2\alpha}$ concentrations were transformed to logarithms to eliminate heterogeneity of variance. The effect of treatment was analyzed using a 2-way factorial design, which included the main effects of hormone treatment (control, OT and OT plus rbIFN-τ) and incubation time, and the treatment x time interaction. Since uterus was nested within an experiment it was included as a random variable in the F-test for the effect of experiment. If a significant treatment or treatment x time interaction was found, further analysis was performed using OT and OT + IFN-τ as treatments to isolate effects of IFN. Simple effect comparisons were performed to determine differences between individual means. A probability of $p<0.05$ was considered to be statistically significant. The data were analyzed using the computer program, JMP (SAS Institute Inc., NC, USA).

Results

Effect of IFN-τ on OT-induced prostaglandin secretion

The basal secretion of $PGF_{2\alpha}$ and PGE_2 by untreated epithelial cells did not change with time (treatment x time interaction, $p<0.05$). OT stimulated secretion of both $PGF_{2\alpha}$ and PGE_2, an effect which increased with time ($p<0.01$) (Figs. 1a and b). The increase in PGE_2 was maximum at 12 h whereas $PGF_{2\alpha}$ continued to increase at 24 h. Addition of rbIFN-τ inhibited the OT-induced increase in $PGF_{2\alpha}$ ($p=0.02$) and PGE_2 ($p<0.01$). OT and OT plus rbIFN-τ decreased the $PGE_2/PGF_{2\alpha}$ ratio at 24 h ($p<0.05$), but not before (Fig. 1c).

Effect of IFN-τ on OT-induced COX-2

Unstimulated levels of COX-2 mRNA did not change with time (treatment x time interaction, $p<0.01$). OT significantly upregulated COX-2 mRNA (Fig. 2, $p<0.001$) at all time points measured. The OT-induced COX-2 mRNA steady-state level was highest at 3 h, and then decreased with time. rbIFN-τ dramatically reduced the OT-induced COX-2 mRNA levels at 3 and 6 h ($p<0.01$) but had no significant effect at 12 and 24 h.

The specific COX-2 antibody used bound to two COX-2 protein bands with molecular weights of approximately 74,000 M_r and 62,000 M_r, respectively (Fig.3a). The smaller M_r band has previously been observed in rat [48], sheep [49] and bovine [43], and is believed to be a proteolytic fragment [43]. The concentration of COX-2 did not change with time in the untreated cells. OT markedly increased COX-2 protein at all time points (3.8, 6.2, 10.2, and 5.2 fold at 3, 6, 12, and 24 h, respectively. Fig. 3, $p<0.001$) with the maximum stimulation at 12 h. Addition of rbIFN-τ suppressed the induction of COX-2 protein by OT ($p<0.001$) at all times, reducing its abundance by 42, 56, 52, and 47% at 3, 6, 12, and 24 h, respectively.

Effect of IFN-τ on OT-induced PGFS mRNA levels

A 1.4 kb transcript hybridized with the bovine PGFS cDNA probe (Fig. 4). The basal and stimulated levels of PGFS decreased with time ($P<0.05$). OT upregulated PGFS mRNA ($p<0.001$) at all times and rbIFN-τ significantly inhibited this induction ($p<0.01$), reducing PGFS mRNA by 32, 29.5, 12.7, and 24.1% at 3, 6, 12, and 24 h, respectively.

Effect of IFN-τ on OT binding

Results shown in Figure 5 indicated that specific binding of [^{3}H]-OT by epithelial cells was saturable and exhibited high affinity. The disassociation constant (K_d) is 4.9 nM . To determine if rbIFN-τ altered OT binding to the cells *in vitro*, confluent epithelial cells were treated with 100 ng/ml rbIFN-τ for 3, 6, 12, and 24 h, and OT binding was measured using ^{3}H-OT. The results show that rbIFN-τ significantly decreased OT binding to the cells as early as 3 h after treatment ($p<0.01$, Fig. 6).

Effect of IFN-τ on PMA-stimulated $PGF_{2\alpha}$ production

The effect of rbIFN-τ on PMA-stimulated prostaglandin synthesis was examined to determine whether the observed decrease in OT-induced prostaglandin secretion by rbIFN-τ was due solely to a decrease in OT binding to the cells. PMA stimulated $PGF_{2\alpha}$ production by the epithelial cells and this stimulation was reduced by rbIFN-τ ($p<0.01$) (Fig. 7). rbIFN-τ also inhibited the PMA stimulation of COX-2 protein in the epithelial cells ($p<0.01$) (Fig. 8).

Discussion

Although it is well known that IFN-τ from the embryo inhibits OT-induced secretion of $PGF_{2\alpha}$ from the endometrium, the exact mechanisms involved are not completely understood. It is widely accepted that IFN-τ decreases OT receptor number in the endometrium, resulting in a decrease in the response to OT in pregnant animals. In the cow there is also an increase in an inhibitor of prostaglandin synthesis in pregnant animals [35], which suggests that IFN-τ has actions on the endometrium other than decreasing OT receptor. In this study we investigated the possibility that IFN-τ inhibits the OT-stimulated COX-2. Since it is difficult to dissociate an effect of IFN-τ on OT

receptor-mediated events and post-receptor effects *in vivo*, an *in vitro* model was used to examine the effect of rbIFN-τ on the induction of PG secretion, COX-2 mRNA and protein and PGFS mRNA by OT. In the epithelial cell preparation used, OT significantly stimulated PG production an effect which was attenuated by rbIFN-τ. This is in agreement with previous reports [8, 15, 17, 18, 50] and reflects the events that occur *in vivo*. The cells were prepared from uteri taken at day 3 of the cycle, a time when the uterus is still responsive to OT, and thus should respond in a similar fashion to the endometrium *in vivo* at the time of luteolysis. It is possible that the cells differentiate during the 7-day culture, but since these cells respond to OT and IFN-τ in a similar fashion to that observed *in vivo* we believe the results are representative of the situation *in vivo*.

The *in vitro* stimulation of $PGF_{2\alpha}$ in bovine endometrial epithelial cells by OT is associated with an increase in COX-2 mRNA at 24 h after OT treatment [18], but changes in COX-2 mRNA, COX-2 protein and PGFS over time have not been previously documented. Our results show a rapid induction in COX-2 mRNA by OT, which was maximal by 3 h and then decreased over time. The maximal OT-induced COX-2 protein appeared later than the mRNA, with the lag period of 3-9 h suggesting that the latter is the product of translation of the former. OT also upregulated PGFS expression which could explain, at least in part, the difference in the production of $PGF_{2\alpha}$ and PGE_2. Production of PGE_2 by the epithelial cells plateaued at 12 h, resulting in a significant decrease in the ratio of PGE_2 to $PGF_{2\alpha}$ at 24 h. An increase in PGFS would be expected to result in an increase in the conversion of the precursor, PGH_2, to $PGF_{2\alpha}$ at the expense of PGE_2.

In vivo studies in sheep also show that OT induces a rapid increase in both serum PGFM and endometrial COX-2 mRNA, peaking at 25 min post

injection [11]. Thus, the *in vivo* and *in vitro* data show that both COX-2 mRNA and protein are rapidly and transiently expressed in endometrial tissue. This is consistent with COX-2 being an immediate early gene in the endometrial epithelium, as in many other tissues [51]. In cattle, pulses of $PGF_{2\alpha}$ occur every 6-8 h during luteolysis [52]. Since COX-2 protein concentration peaks about 12 h after administration of OT, it is possible that OT is responsible not only for the immediate stimulation of $PGF_{2\alpha}$ during a luteolytic episode, but may also increase COX-2 and PGFS and, thereby, the capacity of the uterus to synthesize $PGF_{2\alpha}$ as luteolysis proceeds. There is an increase in COX around the time of luteolysis in sheep [53] but whether or not OT is involved in its induction remains to be established. Charpigny et al (1997) [53], did not detect any difference between COX-2 levels in cycling and pregnant sheep, suggesting that OT is not involved. However, in their study, COX-2 protein was measured in whole endometrium. Therefore, since IFN-τ stimulates COX-2 in the stromal but inhibits COX-2 in the epithelial cells [40], it is possible that changes in COX-2 in the epithelial cells were masked.

Interferon-τ is responsible for inhibiting $PGF_{2\alpha}$ secretion in pregnant ruminants and one of its actions is to decrease OT receptor number in the endometrium. Rates of OT receptor gene transcription are two-fold lower in the endometrium of day 15 cyclic ewes receiving intrauterine injections of recombinant ovine IFN-τ than in control ewes [34]. Intrauterine infusion of rbIFN-τ also attenuates OT binding to the endometrium in the cow [22] and sheep [23]. Suppression of the estradiol upregulation of OT receptor may not, however, be the only mechanism involved. OT is able to induce release of $PGF_{2\alpha}$ in pregnant cows and ewes [54], albeit much lower than that observed in the cyclic animals [50]. This suggests that functional OT receptors are present in the pregnant uterus. It is likely, therefore, that IFN-τ decreases $PGF_{2\alpha}$ secretion in

the endometrium of the pregnant cow by actions other than the reduction of OT receptors. One such mechanism is the production of an inhibitor of PG synthesis, as occurs in the endometrium of the pregnant cow [35]. This inhibitor has recently been identified as linoleic acid, which competes with arachidonic acid for the active site of the COX enzyme [27]. Our results show that rbIFN-τ also inhibits the OT-induced increase in COX-2 and PGFS mRNA. Together the data suggest that rbIFN-τ has several effects on the endometrium that result in decreased $PGF_{2\alpha}$ secretion in the pregnant animal and thus the maintenance of the corpus luteum (CL).

Since OT and IFN-τ were added at the same time, it is unlikely that the observed effect of IFN-τ was due to a change in OT receptor. However, rbIFN-τ did decrease OT binding by the epithelial cells. To ensure that the rbIFN-τ inhibition of the OT-induced COX-2 was not solely due to a decrease in OT receptor, the effect of rbIFN-τ on PMA-induced $PGF_{2\alpha}$ secretion was examined. OT acts via the protein kinase C intracellular pathway. Thus, activating protein kinase C with PMA bypasses the interaction of OT with its receptor. PMA markedly stimulated $PGF_{2\alpha}$ secretion by epithelial cells, which is consistent with previous reports in cows [55, 56] and pigs [57]. PMA stimulated PG synthesis in the endometrial epithelial cells by increasing COX-2 protein, consistent with its effect in other cell types. The effect of PMA on $PGF_{2\alpha}$ synthesis and COX-2 protein was significantly reduced when the cells were treated with rbIFN-τ. These results indicate that the inhibitory effect of IFN-τ on OT-induced $PGF_{2\alpha}$ secretion is not only due to the decrease in OT receptor, but also to downregulation of COX-2. This is consistent with our previous demonstration that rbIFN-τ decreases basal $PGF_{2\alpha}$ secretion by downregulation of both COX-2 and PGFS mRNA [40].

In conclusion, the present study shows that OT stimulates PGFS as well as COX-2 in endometrial epithelial cells and that rbIFN-τ inhibits this OT-induced COX-2 and PGFS. These data suggest that during pregnancy rbIFN-τ inhibits OT-induced $PGF_{2\alpha}$ secretion from the endometrium not only by downregulating OT receptor, but also by decreasing COX-2 and PGFS via a mechanism independent of changes in OT receptor.

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REFERENCES

1. McCracken JA, Carlson JC, Glew ME, Goding JR, Baird DT, Green K, Samuelsson B. Prostaglandin $F_{2\alpha}$ identified as a luteolytic hormone in sheep. Nature New Biol 1972; 238:129-134.
2. McCracken JA, Barcikowski B, Carlson JC, Green K, Samuelsson B. The physiological role of prostaglandin $F_{2\alpha}$ in corpus luteum regression. Adv Biosci 1973; 9:599-624.
3. Bazer FW, Ott TL, Spencer TE. Pregnancy recognition in ruminants, pigs and horses: signals from the trophoblast. Theriogenology 1994; 41:79-94.
4. Fairclough RJ, Moore LG, McGowan LT, Peterson AJ, Smith JF, Tervit HR, Watkins WB. Temporal relationship between plasma concentrations of

13,14-dihydro-15-keto-prostaglandin F and neurophysin I/II around luteolysis in sheep. Prostaglandins 1980; 20:199-208.

5. Sheldrick EL, Mitchell MD, Flint APF. Delayed luteal regression in ewes immunized against oxytocin. J Reprod Fertil 1980; 59:37-42.
6. McCracken JA, Schramm W, Okulicz WC. Hormone receptor control of pulsatile secretion of $PGF_{2\alpha}$ from the ovine uterus during luteolysis and its abrogation in early pregnancy. Anim Reprod Sci 1984; 7:31-55.
7. Homeida AM, Khalafalla AE. Effects of oxytocin-antagonist injections on luteal regression in the goat. Br J Pharmacol 1987; 90:281-284.
8. Danet-Desnoyers G, Wetzels C, Thatcher WW. Natural and recombinant bovine interferon-τ regulate basal and oxytocin-induced secretion of prostaglandins $F_{2\alpha}$ and E_2 by epithelial cells and stromal cells in the endometrium. Reprod Fertil Dev 1994; 6:193-202.
9. Roberts JS, McCracken JA, Gavagan JE, Soloff MS. Oxytocin-stimulated release of prostaglandin F2alpha from ovine endometrium *in vitro*: correlation with estrous cycle and oxytocin-receptor binding. Endocrinology 1976; 99:1107-1114.
10. Silvia WJ, Homanics GE. Role of phospholipase C in mediating oxytocin-induced release of prostaglandin F2 alpha from ovine endometrial tissue. Prostaglandins 1988; 35:535-548.
11. Burns PD, Tsai SJ, Wiltbank MC, Hayes SH, Graf GA, Silvia WJ. Effect of oxytocin on concentrations of prostaglandin H synthase-2 mRNA in ovine endometrial tissue *in vivo*. Endocrinology 1997; 138:5637-5640.
12. Burns PD, Graf GA, Hayes SH, Silvia WJ. Cellular mechanisms by which oxytocin stimulates uterine PGF2 alpha sythesis in bovine endometrium: roles of phospholipases C and A2. Dom Anim Endocrinol 1997; 14:181-191.
13. Jenner LJ, Parkinson TJ, Lamming GE. Uterine oxytocin receptors in cyclic and pregnant cows. J Reprod Fertil 1991; 91:49-58.

14. Bartol FF, Roberts RM, Bazer FW, Lewis GS, Godkin JD, Thatcher WW. Characterization of proteins produced *in vitro* by periattachment bovine conceptuses. Biol Reprod 1985; 32:681-693.
15. Fincher KB, Bazer FW, Hansen PJ, Thatcher WW, Roberts RM. Proteins secreted by the sheep conceptus suppress induction of uterine prostaglandin F-2α release by oestradiol and oxytocin. J Reprod Fertil 1986; 76:425-433.
16. Vallet JL, Gross TS, Fliss MF, Bazer FW. Effects of pregnancy, oxytocin, ovine trophoblast protein-1 and their interactions on endometrial production of prostaglandin F2 alpha *in vitro* in perifusion chambers. Prostaglandins 1989; 38:113-124.
17. Godkin JD, Smith SE, Johnson RD, Dore JJE. The role of trophoblast interferons in the maintenance of early pregnancy in ruminants. Am J Reprod Immunol 1997; 37:137-143.
18. Asselin E, Drolet P, Fortier MA. Cellular mechanisms involved during oxytocin-induced prostaglandin $F_{2\alpha}$ production in endometrial epithelial cells *in vitro*: Role of cyclooxygenase-2. Endocrinology 1997; 138:4798-4805.
19. Vallet JL, Lamming GE. Ovine conceptus secretory proteins and bovine recombinant interferon α-1 decrease endometrial oxytocin receptor concentrations in cyclic and progesterone-treated ovariectomized ewes. J Endocrinol 1991; 131:475-482.
20. Ayad VJ, Parkinson TJ, Matthews EL,Wild ML. Effects of pregnancy and systemic or intrauterine oxytocin infusion on the distribution of endometrial oxytocin receptors in the ewe: an autoradiographic study. J Endocrinol 1993; 137:423-431.
21. Lamming GE, Wathes DC, Flint APF, Payne JH, Stevenson KR, Vallet JL. Local action of trophoblast interferons in suppression of the development of oxytocin and oestradiol receptors in ovine endometrium. J Reprod Fertil 1995; 105:165-175.

22. Meyer MD, Desnoyers GD, Oldick B, Thatcher WW, Drost M, Schalue TK, Roberts RM. Treatment with recombinant bovine interferon-τ in utero attenuates secretion of prostaglandin F from cultured endometrial epithelial cells. J Dairy Sci 1996; 79:1375-1384.
23. Mirando MA, Harney JP, Zhou Y, Ogle TF, Ott TL, Moffatt RJ, Bazer FW. Changes in progesterone and oestrogen receptor mRNA and protein and oxytocin receptors in endometrium of ewes after intrauterine injection of ovine trophoblast interferon. J Mol Endocrinol 1993; 10:185-192.
24. Thatcher WW, Knickerbocker JJ, Bartol FF, Bazer FW, Roberts RM, Drost M. Maternal recognition of pregnancy in relation to the survival of transferred embryos: endocrine aspects. Theriogenology 1985; 23:129-143.
25. Gross TS, Thatcher WW, Hansen PJ, Helmer SD. Presence of an intracellular endometrial inhibitor of prostaglandin synthesis during early pregnancy in the cow. Prostaglandins 1988; 35:359-378.
26. Danet-Desnoyers G, Johnson JW, O'Keefe SF, Thatcher WW. Characterization of a bovine endometrial prostaglandin synthesis inhibitor (EOSI). Biol Reprod 1993; 48 (Suppl 1):115 (Abstract).
27. Thatcher WW, Staples CR, Danet-Desnoyers G, Oldick B, Schmitt E-P. Embryo health and mortality in sheep and cattle. J Anim Sci 1994; 72:16-30.
28. Salamonsen LA, Hampton AL, Clements JA, Findlay JK. Regulation of gene expression and cellular localization of prostaglandin synthase by oestrogen and progesterone in the ovine uterus. J Reprod Fertil 1991; 92:393-406.
29. Kujubu DA, Fletcher BS, Varnum BC, Lim RW, Herschman HR. TIS10, a phorbol ester tumor promoter-inducible mRNA from swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 1991; 266:12866-12872.

30. O'Banion MK, Sadowski HB, Winn V, Young DA. A serum- and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. J Biol Chem 1991; 266:23261-23267.
31. Arslan A, Zingg HH. Regulation of COX-2 gene expression in rat uterus *in vivo* and *in vitro*. Prostaglandins 1996; 52:463-481.
32. Shemesh M, Dombrovski L, Gurevich M, Shore LS, Fuchs AR, Fields MJ. Regulation of bovine cervical secretion of prostaglandins and synthesis of cyclooxygenase by oxytocin. Reprod Fertil Dev 1997; 9:525-530.
33. Spencer TE, Mirando MA, Mayes JS, Watson GH, Ott TL, Bazer FW. Effects of interferon-τ and progesterone on oestrogen-stimulated expression of receptors for oestrogen, progesterone and oxytocin in the endometrium of ovariectomized ewes. Reprod Fertil Dev 1996; 8:843-853.
34. Spencer TE, Bazer FW. Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. Endocrinology 1996; 137:1144-1147.
35. Basu S, Kindahl H. Prostaglandin biosynthesis and its regulation in the bovine endometrium: A comparison between nonpregnant and pregnant status. Theriogenology 1987; 28:175-193.
36. Lafrance M, Goff AK. Effects of progesterone and estradiol-17β on oxytocin-induced release of prostaglandin F2α. J Reprod Fertil 1988; 82:429-436.
37. Lamming GE, Mann GE. Control of endometrial oxytocin receptors and prostaglandin $F_{2\alpha}$ production in cows by progesterone and oestradiol. J Reprod Fertil 1995; 103:69-73.
38. Roberts RM, Cross JC, Leaman DW. Interferons as hormones of pregnancy. Endocr Rev 1992; 13:432-452.
39. Xiao CW, Goff AK. Differential effects of oestradiol and progesterone on proliferation and morphology of cultured bovine uterine epithelial and stromal cells. J Reprod Fertil 1998; 112:315-324.

40. Xiao CW, Liu JM, Sirois J, Goff AK. Regulation of cyclooxygenase-2 and prostaglandin F synthase gene expression by steroid hormones and interferon-tau in bovine endometrial cells. Endocrinology 1998; 139:2293-2299.
41. Labarca C, Paigen K. A simple, rapid and sensitive DNA assay procedure. Anal Biochem 1980; 102:344-352.
42. Simmons DL, Xie W, Chipman JG, Evett JE. Multiple cyclooxygenases: cloning of a mitogen-inducible form. In: J Martyn Bailey (ed.), Prostaglandins, leukotrines, lipoxins and PAF, New York, NY: Plenum Press; 1992: 67-78.
43. Sirois J. Induction of prostaglandin endoperoxide synthase-2 by human chorionic gonadotropin in bovine preovulatory follicles *in vivo*. Endocrinology 1994; 135:841-848.
44. Watanabe K, Fujii Y, Nakayama K, Ohkubo H, Kuramitsu S, Kagamiyama H, Nakanishi S, Hayaishi O. Structural similarity of bovine lung prostaglandin F synthase to lens ε-crystallin of the European common frog. Proc Natl Acad Sci USA 1988; 85:11-15.
45. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 1977; 74:5463-5467.
46. Wathes DC, Mann GE, Payne JJ, Riley PR, Stevenson KR, Lamming GE. Regulation of oxytocin, oestradiol and progesterone receptor concentrations in different uterine regions by oestradiol, progesterone and oxytocin in ovariectomized ewes. J Endocrinol 1996; 151:375-393.
47. Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for the characterization of ligand binding systems. Anal Biochem 1980; 107:220-239.
48. Sirois J, Richards JS. Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human

chorionic gonadotropin in granulosa cells of rat preovulatory follicles. J Biol Chem 1992; 267:6382-6388.

49. Wimsatt J, Nathanielsz PWN, Sirois J. Induction of prostaglandin endoperoxide synthase isoform-2 in ovine cotyledonary tissues during late gestation. Endocrinology 1993; 133:1068-1076.
50. Lafrance M, Goff AK. Effect of pregnancy on oxytocin-induced release of prostaglandin F2 alpha in heifers. Biol Reprod 1985; 33:1113-1119.
51. Smith WL, Garavito RM, Dewitt DL. Prostaglandin endoperoxide H synthases (Cyclooxygenases)-1 and -2. J Biol Chem 1996; 271:33157-33160.
52. Kindahl H, Lindell JO, Edqvist LE. Release of prostaglandin $F_{2\alpha}$ during the oestrous cycle. Acta Vet Scand 1981; 77:143-158.
53. Charpigny G, Reinaud P, Tamby JP, Crèminon C, Martal J, Maclouf J, Guillomot M. Expression of cyclooxygenase-1 and -2 in ovine endometrium during the estrous cycle and early pregnancy. Endocrinology 1997; 138:2163-2171.
54. Burgess KM, Ralph MM, Jenkin G, Thorburn GD. Effect of oxytocin and estradiol on uterine prostaglandin release in nonpregnant and early-pregnant ewes. Biol Reprod 1990; 42:822-833.
55. Lafrance M, Goff AK. Control of bovine uterine prostaglandin $F_{2\alpha}$ release *in vitro*. Biol Reprod 1990; 42:288-293.
56. Kim JJ, Fortier MA. Cell type specificity and protein kinase C dependency on the stimulation of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ production by oxytocin and platelet-activating factor in bovine endometrial cells. J Reprod Fertil 1995; 103:239-247.
57. Tysseling KA, Uzumcu M, Hoagland TA, Crain RC, Mirando MA. The role of phosphoinositide-derived second messengers in oxytocin-stimulated prostaglandin F2 alpha release from endometrium of pigs. Dom Anim Endocrinol 1996, 13:411-420

Fig. 1. Effect of OT and rbIFN-τ on prostaglandin production in epithelial cells. Confluent epithelial cells were incubated in RPMI medium supplemented with 5% DC-FCS, control (CTL, open bar), with 100 ng/ml OT (solid bar) or with OT plus 100 ng/ml rbIFN-τ (hatched bar) for 3, 6, 12, and 24 h. The culture medium was collected for $PGF_{2\alpha}$ (a) and PGE_2 (b) measurement by RIA. Data are normalized to the DNA content of the respective wells. The ratio of PGE_2 to $PGF_{2\alpha}$ was calculated (c). The data are presented as the least-square means ± SEM (n=4). There was a significant inhibitory effect of rbIFN-τ on the OT-induced increase in both $PGF_{2\alpha}$ ($p=0.02$) and PGE_2 ($p<0.01$). OT and OT + rbIFN-τ decreased the $PGE_2/PGF_{2\alpha}$ ratio at 12 and 24 h ($p<0.05$) but not before.

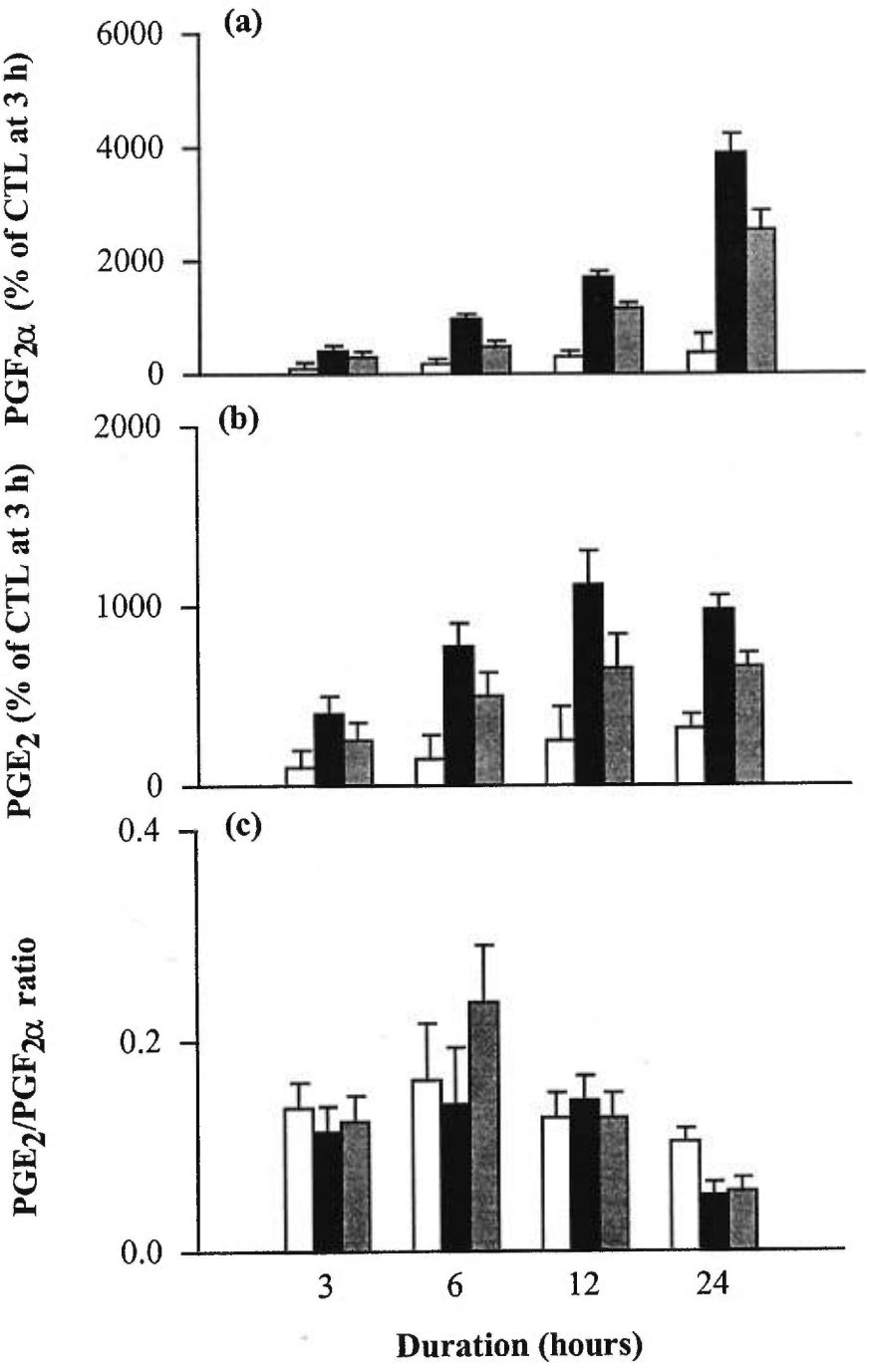

(a)
6000
4000
2000
0
PGF2α (% of CTL at 3 h)
(b)
2000
1000
0
PGE2 (% of CTL at 3 h)
(c)
0.4
0.2
0.0
PGE2/PGF2α ratio
3
6
12
24
Duration (hours)

Fig. 2. Northern blot analysis of COX-2 mRNA in epithelial cells. RNA was isolated from epithelial cells. A representative sample is shown (a), when 25 μg of total RNA per lane was loaded and blots were hybridized with ^{32}P-labeled mouse COX-2 cDNA probe. The stripped blots were rehybridized with human 28S cDNA probe as a loading control. Autoradiographic bands were scanned by a densitometer and normalized to their 28S values (b). Open bars, solid bars, and hatched bars represent Control, OT, and OT plus rbINF-τ, respectively. Data are expressed as the least-square means ± SEM (n=4). rbIFN-τ reduced the OT-induced COX-2 mRNA levels at 3 and 6 h ($p<0.001$) but not later.

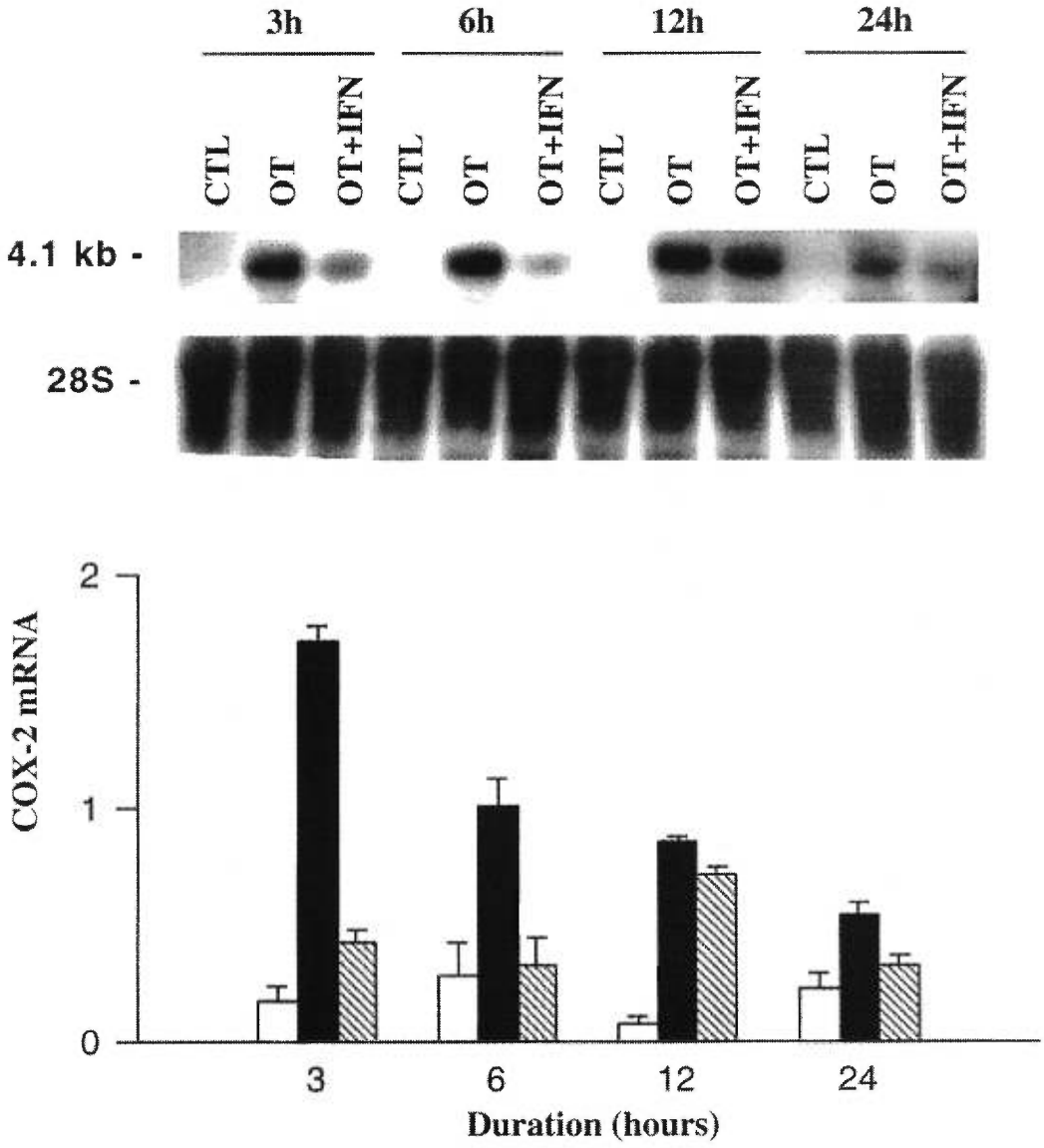
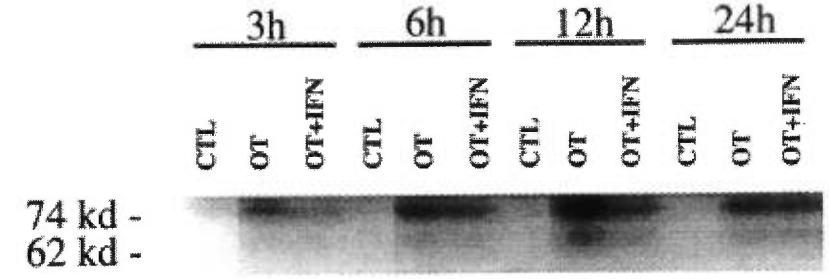

3h
6h
12h
24h
CTL
OT
OT+IFN
4.1 kb -
28S -
COX-2 mRNA
2
1
0
3
6
12
24
Duration (hours)

Fig. 3. Western blot analysis of COX-2 in epithelial cells. Solubilized cell extracts were prepared from epithelial cells after OT and rbIFN-τ treatment as described in Fig. 1. Fifty μg of protein per lane was loaded and the membranes were incubated with COX-2 antibody MF 243 and ^{125}I-labeled protein A was used to visualize immunopositive proteins (a). Autoradiographic bands were scanned by a densitometer and quantified (b). Open bars, solid bars, and hatched bars represent Control, OT, and OT plus rbINF-τ, respectively. Data are expressed as the least-square means ± SEM (n=4). rbIFN-τ suppressed the induction of COX-2 protein by OT at all time points ($p<0.001$).

a)


3h
6h
12h
24h
CTL
OT
OT+IFN
74 kd -
62 kd -

b)

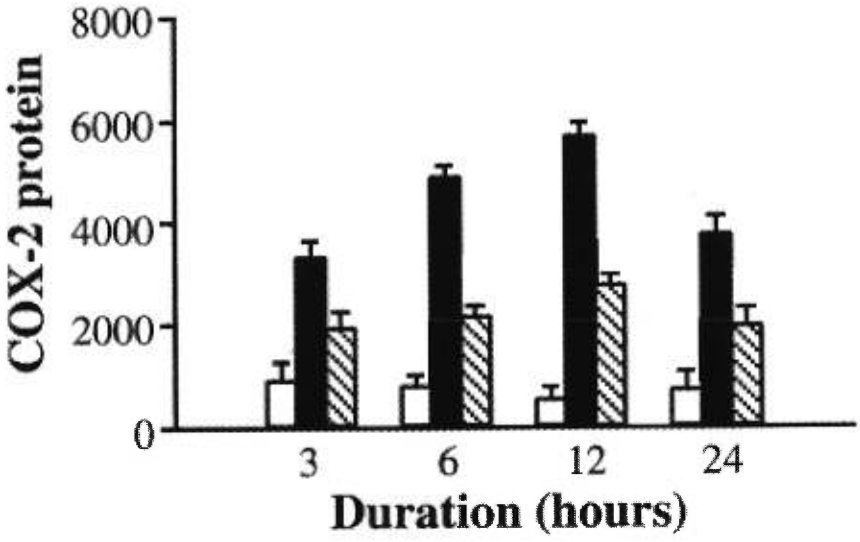

COX-2 protein
8000
6000
4000
2000
0
3
6
12
24
Duration (hours)

Fig. 4. Northern blot analysis of PGFS in epithelial cells. The same blots used in Fig. 2. for COX-2 hybridization were stripped and hybridized with ^{32}P-labeled PGFS probe (a). The densitometric values of PGFS were normalized to their 28S values (b). Open bars, solid bars, and hatched bars represent Control, OT, and OT plus rbINF-τ, respectively. Data are expressed as the least-square means ± SEM (n=4). rbIFN-τ inhibited the OT-induced PGFS mRNA at all time points ($p<0.01$).

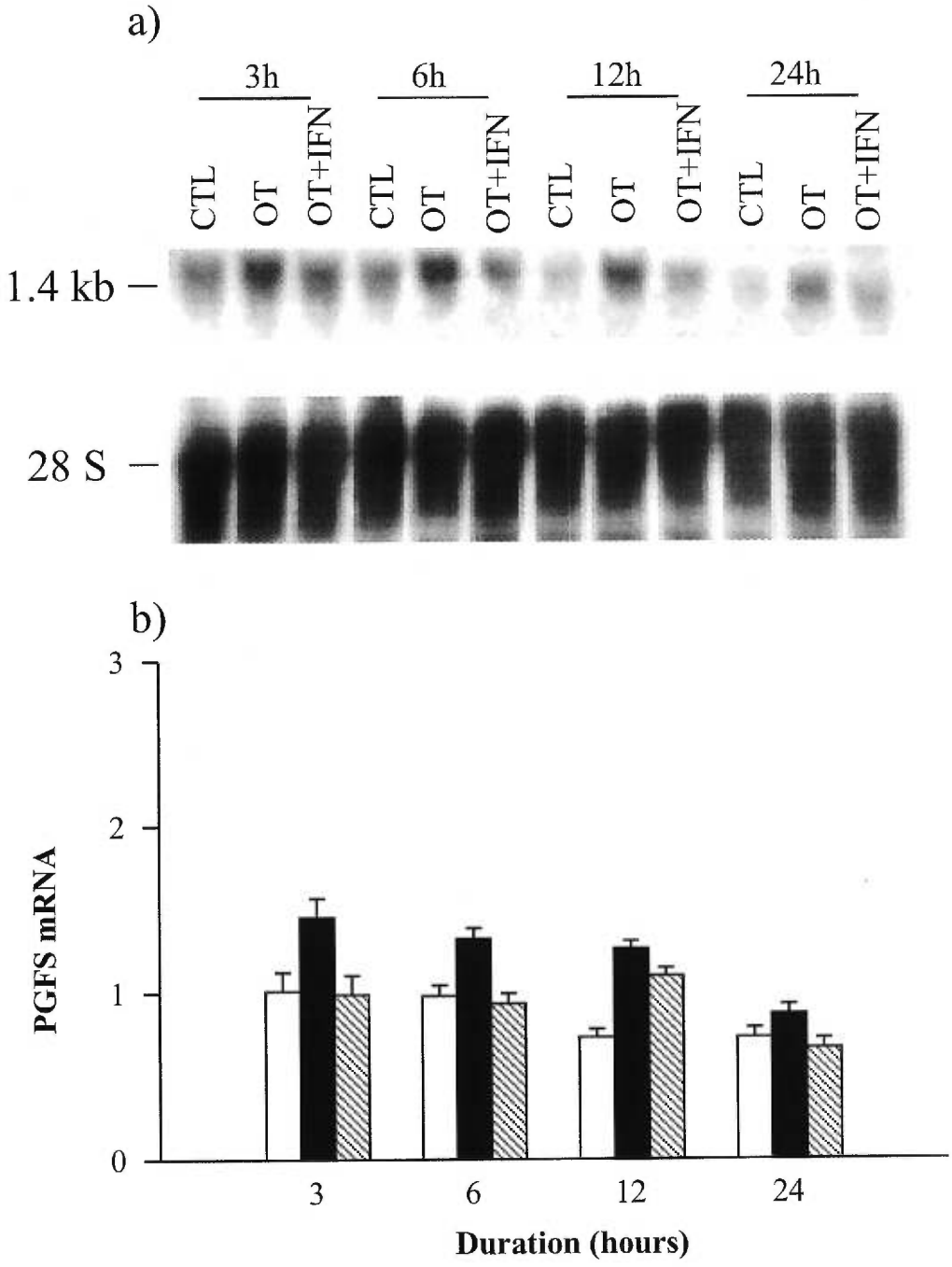

a)
3h
6h
12h
24h
CTL
OT
OT+IFN
CTL
OT
OT+IFN
CTL
OT
OT+IFN
CTL
OT
OT+IFN
1.4 kb
28 S
b)
PGFS mRNA
0
1
2
3
3
6
12
24
Duration (hours)

Fig. 5. Saturation analysis of specifically bound [^{3}H]-oxytocin to primary cultures of bovine endometrial epithelial cells. Cells were cultured to confluence in RPMI 1640 containing 5% DC-FCS. The cells were then incubated with 0.15-15 nM [^{3}H]-OT with or without 200-fold molar excess of unlabeled oxytocin. a) Total binding (O), nonspecific binding (•), and specific binding (∇) by epithelial cells are presented as saturation curves. b) The corresponding Scatchard plot of specific binding is presented. B/F, bound to free ratio.

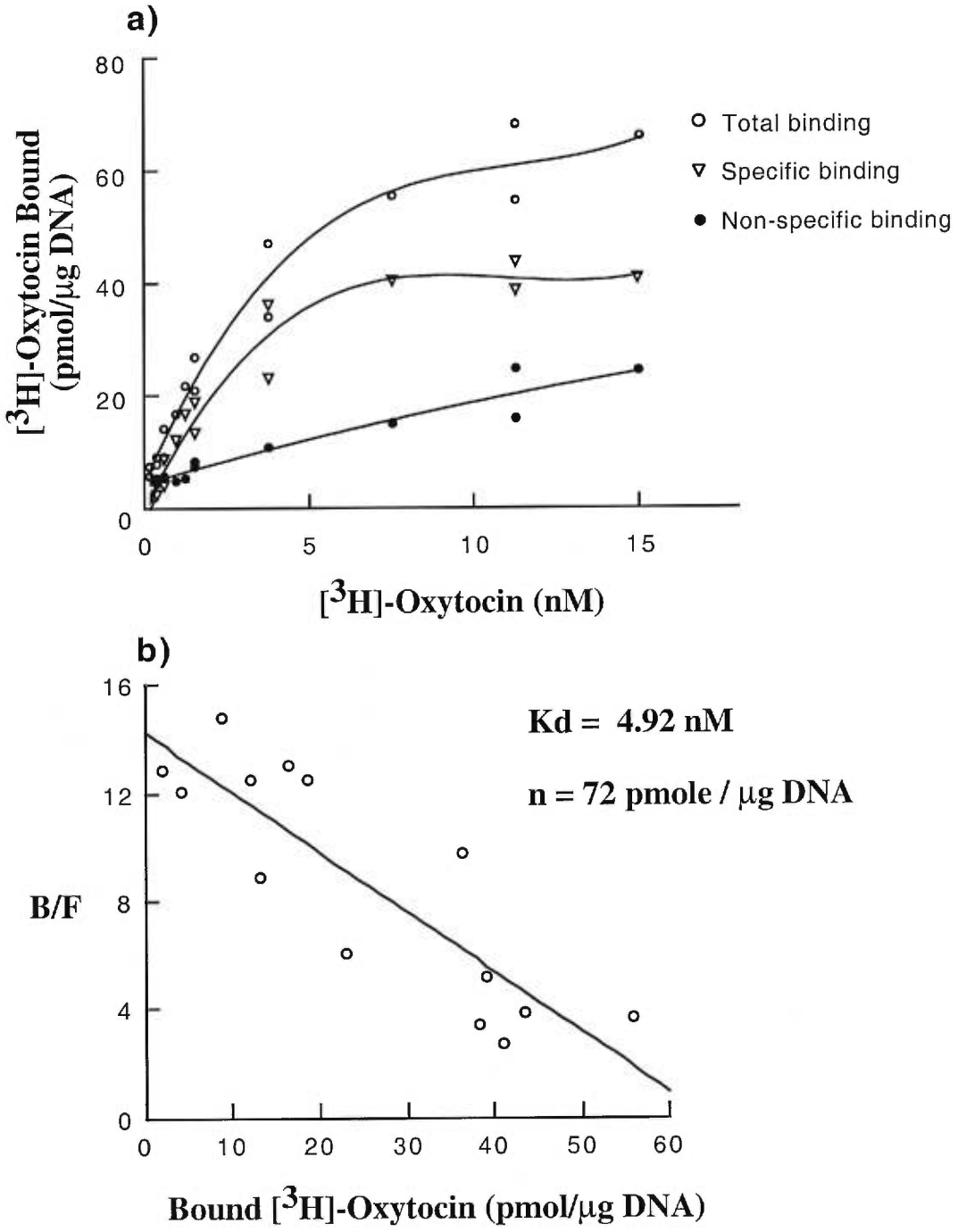

a)
80
60
40
20
0
0
5
10
15
[3H]-Oxytocin Bound (pmol/μg DNA)
[3H]-Oxytocin (nM)
Total binding
Specific binding
Non-specific binding
b)
16
12
8
4
0
0
10
20
30
40
50
60
B/F
Bound [3H]-Oxytocin (pmol/μg DNA)
Kd = 4.92 nM
n = 72 pmole / μg DNA

Fig. 6. Effect of rbIFN-τ on OT receptor number in epithelial cells. Confluent epithelial cells were incubated in RPMI medium supplemented with 5% DC-FCS in presence or absence of 100 ng/ml rbIFN-τ for 3, 6, 12, and 24 h. At the end of the culture, the whole cells were incubated with 100 μl of 10 nM ^{3}H-OT in the presence or absence of 200-fold molar excess of unlabelled OT in the binding buffer to measure the total and non-specific binding. Data are presented as specific binding, normalized to the respective DNA content. Data are expressed as the least-square means ± SEM (n=4) and ** represents significant difference between medium control and rbIFN-τ treatment within the same time point ($p<0.01$).

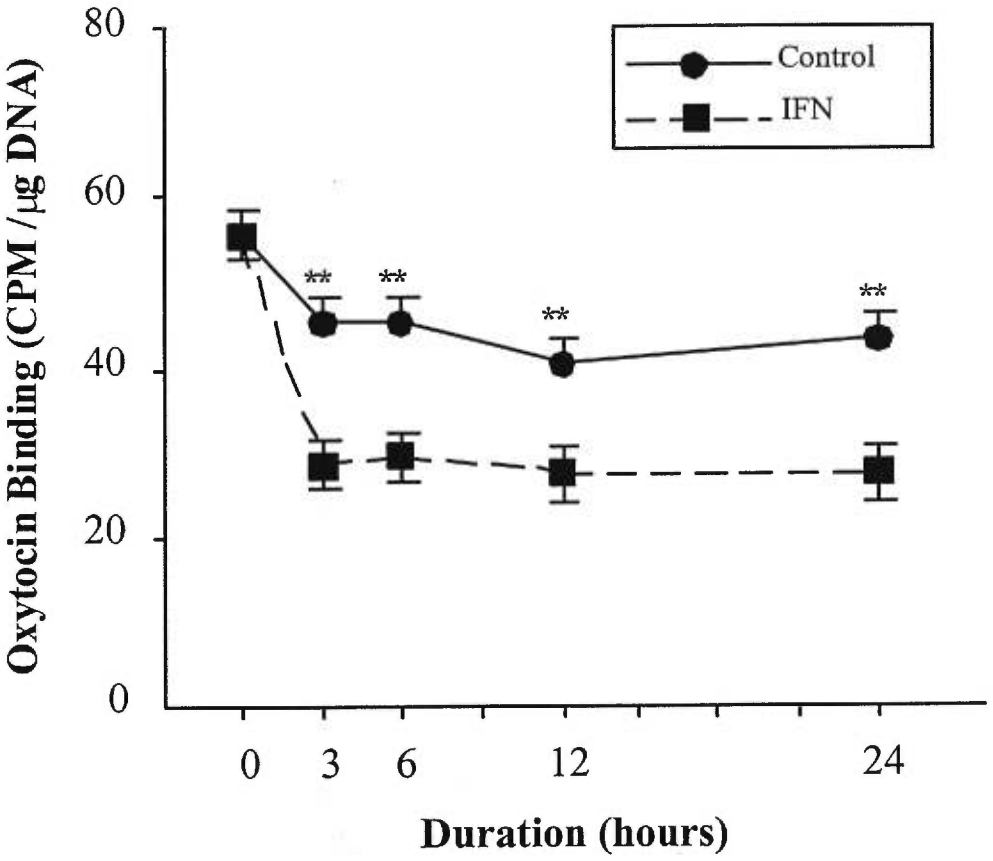

80
60
40
20
0
Oxytocin Binding (CPM /μg DNA)
Control
IFN
**
**
**
**
0
3
6
12
24
Duration (hours)

Fig. 7. Effect of rbIFN-τ on PMA-induced $PGF_{2\alpha}$ in epithelial cells. Confluent epithelial cells were incubated in RPMI medium supplemented with 5% DC-FCS in absence (CTL) or presence of 100 ng/ml PMA or PMA plus rbIFN-τ for 12 h. The culture medium was collected for $PGF_{2\alpha}$ measurement by RIA. Data are normalized to the DNA contents of the respective wells. Data are presented as the least-square means ± SEM (n=4). rbIFN-τ decreased the PMA-stimulated $PGF_{2\alpha}$ production ($p<0.01$).

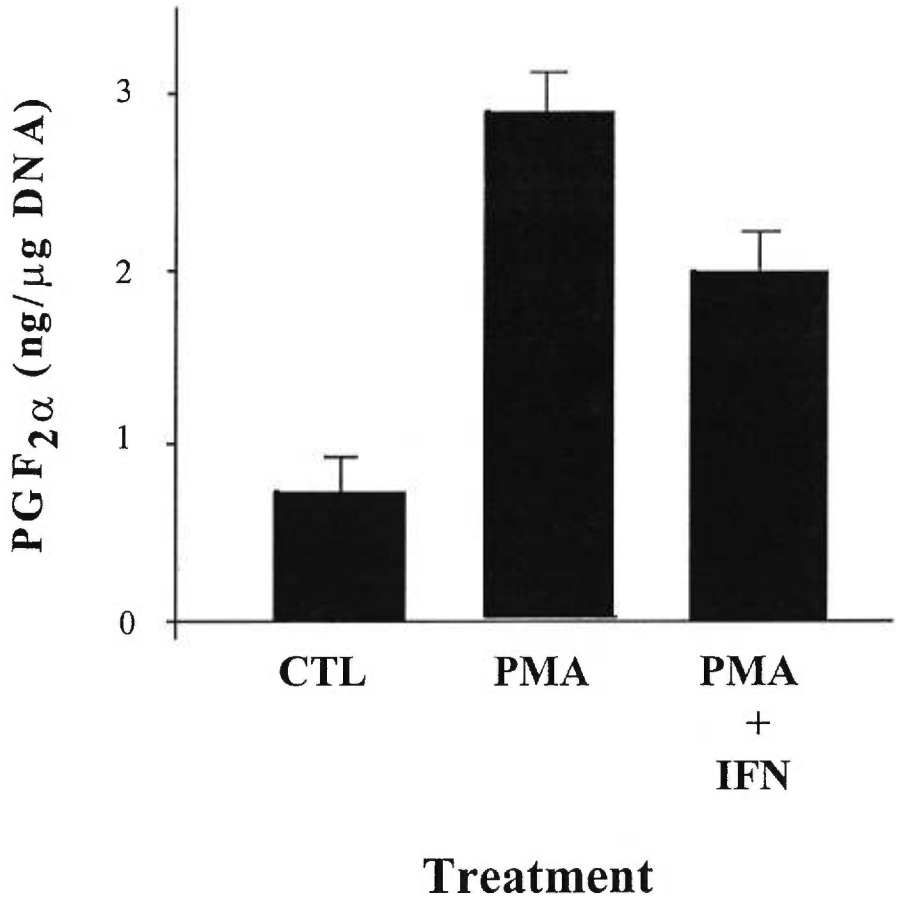
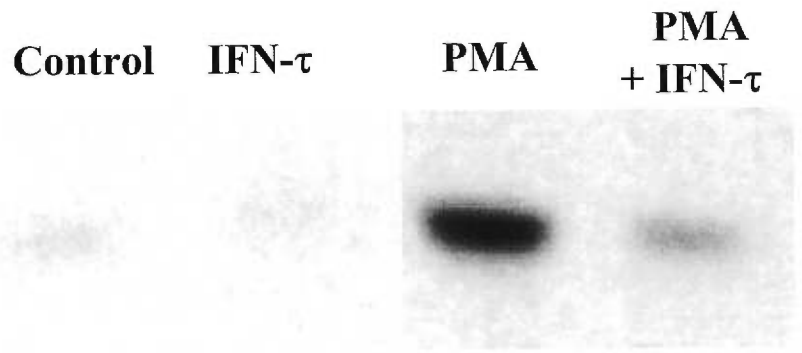
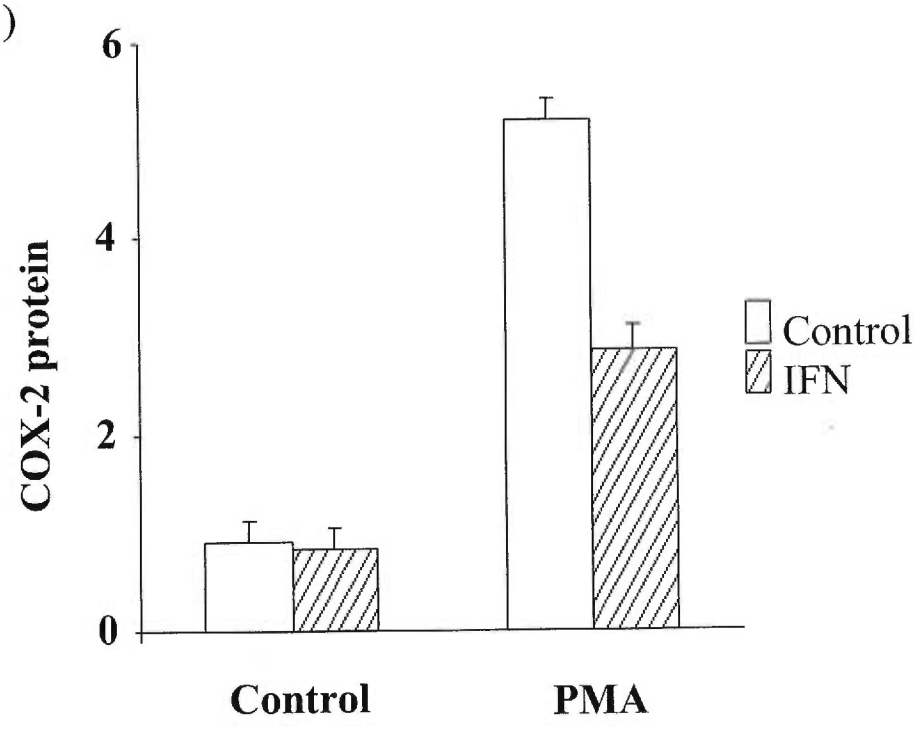

PGF2α (ng/μg DNA)
3
2
1
0
CTL
PMA
PMA + IFN
Treatment

Fig. 8. Effect of rbIFN-τ on PMA-induced COX-2 protein. Solubilized cell extracts were prepared from epithelial cells after treatment for 12 h with PMA and rbIFN-τ. Fifty μg of protein per lane was loaded and the membranes were incubated with COX-2 antibody MF 243 and ^{125}I-labeled protein A was used to visualize immunopositive proteins (a). Autoradiographic bands were scanned by a densitometer and quantified (b). Data are expressed as the least-square means ± SEM (n=3). rbIFN-τ suppressed the induction of COX-2 protein by PMA ($p<0.01$).

a)


Control
IFN-τ
PMA
PMA
+ IFN-τ

b)


6
4
2
0
COX-2 protein
Control
IFN
Control
PMA

4. General Discussion

Ovariectomized and hysterectomized animals are very useful in *in vivo* experimental systems to elucidate the mechanisms involved in many aspects of reproductive physiology which are related to the ovary and uterus. With the establishment of *in situ* hybridization and immunocytochemical techiques, the expression of specific genes and proteins can be localized within tissues. However, due to the interaction between the factor being tested and other unknown factors *in vivo* or the interaction between cell types, it is difficult to determine the direct effect on specific cell types. This can make it difficult to determine the exact mechanism of action of hormones or other mediators. In this circumstance, *in vitro* systems are very helpful for the further study at the cellular and molecular level. *In vitro* primary cell culture systems have various advantages: 1) they can be used to study the functions and responses of one single cell type to individual factors (such as, growth factors, steroid hormones, and cytokines) or interactions between different cell types under defined conditions; 2) responsiveness of a cell to a given stimulus can be studied in culture for prolonged periods, a problem with tissue perfusion, explant, or *in vivo* studies; 3) It is possible to identify the para- and autocrine factors involved in control of endometrial function; 4) The cell numbers in an experimental sample can be easily varied up or down, according to the requirements, and good reproducibility can be achieved between replicates (Findlay *et al.*, 1989). There are also disadvantages of using *in vitro* systems, these are that, 1) they fail to define the activity of the organ as a whole; 2) due to the culture conditions there may be a lack of, or a change in, the responsiveness of cells in culture such that their functional character is different when compared with the cells in whole tissue (Sirbasku, 1980).

The purification of isolated primary cells has been a problem since the beginning of use of primary cell culture. The impurity of isolated cells influences the results, especially when the tested agent has opposite effects on different cell types. To establish an optimal system, high homogenity of isolated cell populations is the key. In these studies, the use of low-speed centrifugation to separate the epithelial clumps from the individual stromal cells, and a further purification procedure to allow the stromal cells to attach during a short term incubation resulted in highly homogenous cell populations.

This study shows for the first time that different types of bovine endometrial cells respond differently to E2 and P4, it also confirms that OT and IFN-τ have different effects in these different cell types. Our results show that E2 has differential effects on proliferation, PR number and PG secretion in different endometrial cell types *in vitro*. E2 increased the proliferation and PR number in stromal cells, while the higher concentrations of E2 inhibits proliferation and has no effect on PR number in epithelial cells. The absence of a stimulatory effect of E2 on epithelial proliferation might be due to the lack of interactions between stromal and epithelial cells (Cunha *et al.*, 1983; Inaba *et al.*, 1988; Haslam and Counterman, 1991). E2 is thought to act through ER to regulate uterine epithelial cell growth, proliferation, differentiation and secretory protein production. However, it has been shown that E2-induced uterine epithelial proliferation is a paracrine event mediated by ER-positive stroma in mouse; epithelial ER is neither necessary nor sufficient for E2-induced uterine mitogenesis in the absence of ER-positive stroma in mouse (Cooke *et al.*, 1997). In contrast, the production of E2-dependent epithelial secretory proteins, such as lactoferrin (LF) and increase in expression of their mRNA requires both stromal and epithelial ERα (Setiawan *et al.*, 1997). Recent evidence, however, shows that another non-ER mediated signaling pathway exists for estrogen

action. The expression of the E2-responsive gene, LF, in the uterus is independent of the classical ER because it was altered by E2 in ER 'knock-out' mice (Das *et al.*, 1997).

This study shows that E2 increases ER number in both cell types, and although P4 alone has no effect on ER, it inhibits the stimulation of E2 on ER in stromal cells. However, it is not clear why P4 did not inhibit the E2 stimulation of the ER in the epithelial cells. There could be a difference in receptor regulation in the two cell types or down regulation of the ER in the epithelial cells might be mediated by the stromal cells as suggested by Wathes *et al.* (1996). Changes in steroid receptors are considered to play an important role in the timing of luteolysis in cattle (Meyer *et al.*, 1988; Zollers *et al.*, 1993). It was suggested that high concentration of P4 during the luteal phase inhibits both ER and PR (Katzenellenbogen, 1980; Leavitt *et al.*, 1983; Clark *et al.*, 1985; Sumida *et al.*, 1988). The complete inhibition of P4 on PR takes about 10 days. Thereafter, the inhibition of P4 on ER will be released, and E2 increases ER and OTR. This will initiate the pulsatile release of luteolytic PGF2α (McCracken *et al.*, 1984). The pulse is amplified by a positive feedback loop existing between the uterus and ovary (Flint and Sheldrick, 1983; Flint and Sheldrick, 1986). It has been shown that the increase in ER mRNA concentrations induced by E2 is via a posttranscriptional increase in ER mRNA stability rather than an increase in ER gene transcription in ewes. E2 can increase the half-life of ER mRNA from 7.5 h to greater than 24 h (Ing and Bhattacharyya, 1997). Since P4 acts via its receptor to suppress the ER throughout most the luteal phase, work is needed to examine the effect of prolonged exposure of the cells to P4 on PR and ER levels. Regulation of the PR is probably the key to the timing of luteolysis. The use of this *in vitro* system could be very useful to study the molecular mechanisms

involved in the regulation of the PR that would be difficult to be carried out *in vivo*.

E2 is generally thought to stimulate PG synthesis because administration of E2 to cows at mid-cycle (Thatcher *et al.*, 1984), or to P4 primed ovariectomized cows (Lafrance and Goff, 1988), stimulates PGF2α secretion. However, our results show that treatment with E2 inhibits PG secretion and COX-2 gene expression in epithelial cells. Therefore, E2 may have a dual function in luteolysis, inhibiting PG synthesis, but increasing OTR number and thus the sensitivity of uterus to OT. We have preliminary data that show that the treatment of epithelial cells with E2 increases their sensitivity to OT. P4 also increases PG secretion from these cells (Fig. 2, article 3). Thus, in the *in vivo* situation, P4 increases PG synthesis in the epithelial cells. The main action of E2, even though it might decrease the PG synthetic capacity of the endometrium, is to increase the sensitivity of the tissue to OT. Further studies are needed using the cell culture system to examine in more detail the effect of long term treatment of the cells with P4 on their ability to respond to E2 and OT.

The prevention of luteolysis is essential for the maintenance of pregnancy. The primary signal of pregnancy, IFN-τ, secreted by the trophoblast, is the antiluteolytic agent. Our results further support this notion and begin to unravel the mechanisms by which IFN-τ regulates PG secretion at the cellular and molecular levels. IFN-τ might have a dual function in the prevention of luteolysis. IFN-τ stimulates PGE2 secretion by the stromal cells and inhibits the basal and OT-induced PGF2α secretion by epithelial cells. PGE2 is believed to be luteoprotective (Bazer *et al.*, 1991) and PGF2α is luteolytic. The actions of IFN-τ on PG production by endometrial cells were positively correlated with the changes in COX-2 gene expression. Treatment of the cells with IFN-τ also

increased the PGE2/PGF2α ratio, this change in the ratio of PGE2 to PGF2α is at least partially due to the inhibition of PGFS expression by IFN-τ.

IFN is believed to inhibit PGF2α secretion in pregnant animals by preventing the increase in OTR (Mirando *et al.*, 1993; Stevenson *et al.*, 1994; Spencer *et al.*, 1996a). Our results show that rbIFN-τ not only decreases OTR but also inhibits the OT-induced PG secretion and COX-2 mRNA and protein in endometrial epithelial cells. This inhibition of PG production does not appear to be a result of the decrease in only OTR number, but also by some other post-receptor mechanisms, since IFN-τ also inhibts the basal secretion of PGs, steady state levels of COX-2 and PGFS mRNA. OT acts via stimulation of the protein Kinase C (PKC) intracellular pathway and PKC can be activated by PMA. Furthermore, the OTR can be bypassed by treating the cells with PMA, which stimulates PKC and thus PG secretion. The inhibition of PMA-induced PG secretion by IFN-τ in epithelial cells supports our hypothesis that IFN-τ acts on some post-OTR steps. However, further studies are necessary to examine the effect of IFN-τ on PMA stimulated COX-2 mRNA and protein levels.

This study provides new data that elucidates the possible functions of steroid hormones, OT and IFN-τ as well as the different type of endometrial cells, in luteolysis and the recognition of pregnancy. However, a few important aspects remain to be investigated. First, the interaction between stromal cells and epithelial cells is still not well understood. Recent evidence indicates that the stroma mediates receptor-negative epithelial cell functions via hormone-induced growth factors, such as keratinocyte growth factor (KGF or FGF-7) and hepatocyte growth factor (HGF). KGF is produced by stromal cells in response to P4, and its receptor appears to be expressed exclusively on epithelial cells (Alarid *et al.*, 1994; Rubin *et al.*, 1995). HGF which is induced by E2 in stromal cells binds to its high-affinity receptor to induce mitogenesis, cell motility and

morphogenesis in renal epithelial cells (Cantley *et al.*, 1994). However, these factors have not been identified and characterized in ovine (Spencer *et al.*, 1996b). Whether similar factors exist in bovine endometrial stromal cells to mediate epithelial cell function is still unknown.

Secondly, the possible mediators and mechanism of action of IFN-τ needs to be further clarified. It has been shown that IFN-τ induces an increase in an inhibitor of PG synthesis in the endometrium, which has been identified as linoleic acid (Danet-Desnoyers *et al.*, 1993; Thatcher *et al.*, 1994) and competes with arachidonic acid to inhibit PG production. The question is, which aspect of IFN-τ actions, the availability of arachidonic acid (substrate of COX), inhibition of OTR, or inhibition of post-OTR mechanisms, is the more important for the prevention of $PGF_{2\alpha}$ secretion. It has been found that the ratio of linoleic acid to arachidonic acid was higher in microsomes of pregnant cows (Thatcher *et al.*, 1995) and linoleic acid acts as a competitive inhibitor to reduce the availability of arachidonic acid for the PG synthesis and the PGHS activity in terms of conversion rate of arachidonic acid to PGs (Elattar and Lin, 1989). However, our results show that IFN-τ also acts on COX-2, PGFS gene expression. Therefore, it remains to be investigated if linoleic acid mediates the effect of IFN-τ on these gene expression.

The intracellular mediators of IFN-τ action also need further investigation. One mediator of the action of IFN-τ could be nitric oxide (NO). NO may directly affect COX activity. COX is a Fe^{++} containing enzyme and since NO binds with Fe^{++}, it would be possible for NO to up- or down-regulate the activity of COX. NO mediates the secretion of PGs during parturition. Our preliminary results also show that NO increases PG secretion in stromal cells, and a NO synthase (NOS) inhibitor reduces the IFN-τ-stimulating release of PGs

by stromal cells. This suggests that NO can mediate the action of IFN-τ in stromal cells.

Thirdly, our results show that IFN-τ induced PGE2 secretion by stromal cells *in vitro*. Antiluteolytic effects of oIFN-τ are assumed to be limited to the uterine lumen since there is no evidence that it is released into either the uterine venous or lymphatic drainage (Bazer, 1992). Furthermore, oIFN-τ does not prevent increased expression of ER by cells of the stroma and deep glandular epithelium. However, intrauterine injections of roIFN-τ induce expression of Mx mRNA throughout the entire uterine wall (epithelium, stroma, and myometrium) (Ott *et al.*, 1995). Therefore, IFN-τ may stimulate epithelial cells to produce mediators that act as paracrine signals to activate gene expression in stroma. Alternatively, IFN-τ may have access to the entire uterine wall through an as yet undefined mechanism (Spencer *et al.*, 1996b). Our studies have shown that IFN-τ can have direct effects on the stromal cells and thus it would appear that IFN-τ could affect the different cell types within the endometrium *in vivo*.

5. General Conclusion

The objective of this work was to characterize the action of E2, P4, OT and IFN-τ on the individual cell types of the endometrium, in order to begin establish the mechanisms involved in the initiation of luteolysis and maintenance of pregnancy. We have shown that:

1) P4 altered the morphology of stromal cells, but not of epithelial cells. Both E2 and P4 enhance the proliferation of stromal cells. E2 inhibited the growth of epithelial cells, while, P4 appears to have no effect on epithelial cell proliferation. E2 increased the PR and ER numbers in both cell types. P4 inhibited the E2-induced increase in PR in both cell types, but did not affect

the basal levels of ER and PR. An interaction between stromal and epithelial cells may be necessary for the normal function of the epithelial cells *in vivo*.

2) E2, P4 and rbIFN-τ have differential effects in the regulation of PG production, COX-2 and PGFS gene expression. E2 inhibited $PGF_{2\alpha}$ and PGE2 production by downregulating COX-2 expression in epithelial cells. P4 increased $PGF_{2\alpha}$ secretion but did not upregulate COX-2. rbIFN-τ attenuated $PGF_{2\alpha}$ and PGE2 in epithelial cells and enhanced $PGF_{2\alpha}$ and PGE2 in stromal cells by down- and up-regulating COX-2 mRNA, respectively. The changes in the ratio of PGE2 to $PGF_{2\alpha}$ are associated with a decrease in PGFS transcription induced by rbIFN-τ. rbIFN-τ inhibits OT-induced $PGF_{2\alpha}$ secretion by downregulating OTR, COX-2 and PGFS and the decrease in COX-2 is not solely due to a decrease in OTR.
3) In general, IFN-τ has a dual function in the prevention of luteal regression. It stimulates the stromal cells to secret PGE2, which is believed to be luteoprotective agent and may also play a role in implantation. Meanwhile, IFN-τ inhibits the basal and OT-induced $PGF_{2\alpha}$ (luteolysin) secretion.

The effect of E2, P4, OT and IFN-τ all differ between the epithelial cells and stromal cells. The regulation of luteolysis is complex, involving several hormones and changes in uterine sensitivity to these hormones over time. This regulation appears to be further complicated by the fact that the different cell types react differently to the hormones involved. It remains to be established exactly how both of these cell types are involved in luteolysis and its prevention during early pregnancy.

6. General References

Aiken JW (1972) Aspirin and indomethacin prolong parturition in rats: evidence that prostaglandins contribute to expulsion of foetus *Nature* **240** 21-25

Alarid ET, Rubin JS, Young P, Chedid M, Ron D, Aaronson SA, Cunha GR (1994) Keratinocyte growth factor functions in epithelial induction during seminal vesicle development *Proc Natl Acad Sci USA* **91** 1074-1078

Anderson LL, Bowerman AM, Melampy RM (1965) Oxytocin on ovarian function in cycling and hysterectomized heifers *J Anim Sci* **24** 965-968

Armstrong DT, Hansel W (1959) Alteration of the bovine oestrous cycle with oxytocin *J Dairy Sci* **42** 533-542

Ashworth CJ, Bazer FW (1989) Changes in ovine conceptus and endometrial function following asynchronous embryo transfer or administration of progesterone *Biol Reprod* **40** 425-433

Asselin E, Drolet P, Fortier MA (1997) Cellular mechanisms involved during oxytocin-induced prostaglandin $F_{2\alpha}$ production in endometrial epithelial cells *in vitro*: Role of cyclooxygenase-2 *Endocrinology* **138** 4798-4805

Austin KJ, Ward SK, Teixeira MG, Dean VC, Moore DW, Hansen TR (1996) Ubiquitin cross-reactive protein is released by the bovine uterus in response to interferon during early pregnancy *Biol Reprod* **54** 600-606

Ayad VJ, Guldenaar SEF, Wathes DC (1991) Characterization and localization of oxytocin receptors in the uterus and oviduct of the non-pregnant ewe using an iodinated receptor antagonist *J Endocrinol* **128** 187-195

Baguma-Nibasheka M, Wentworth RA, Green LR, Jenkins SL, Nathanielsz PW (1998) Differences in the in vitro sensitivity of ovine myometrium and mesometrium to oxytocin and prostaglandins E2 and F2alpha *Biol Reprod* **58** 73-78

Bartol FF, Roberts RM, Bazer FW, Lewis GS, Godkin JD, Thatcher WW (1985) Characterization of proteins produced *in vitro* by periattachment bovine conceptuses *Biol Reprod* **32** 681-693

Bazan NG, Fletcher BS, Herschman HR, Mukherjee PK (1994) Platelet-activating factor and retinoic acid synergistically activate the inducible prostaglandin synthase gene *Proc Natl Acad Sci USA* **91** 5252-5256

Bazer FW (1992) Mediators of maternal recognition of pregnancy in mammals *Proc Soc Exp Biol Med* **199** 373-384

Bazer FW, Spencer TE, Ott TL (1997) Interferon tau: a novel pregnancy recognition signal. [Review] *Am J Reprod Immunol* **37** 412-420

Bazer FW, Thatcher WW, Hansen PJ, Mirando MA, Ott TL, Plante C (1991) Physiological mechanisms of pregnancy recognition in ruminants. [Review] *J Reprod Fertil* **43 (Suppl.)** 39-47

Beard AP, Lamming GE (1994) Oestradiol concentration and the development of the uterine oxytocin receptor and oxytocin-induced PGF2α release in ewes *J Reprod Fertil* **100** 469-475

Beard AP, Hunter MG, Vallet JL, Lamming GE (1991) The quantitative control of oxytocin-induced uterine $PGF_{2\alpha}$ release by progesterone and oestradiol in the ewe *J Reprod Fertil* Abst Series 8, 6

Betteridge KJ, Derbyshire JB, Rorie RW, Scodvas JM, Johnson WH (1988) Interferon activity released by bovine embryos and trophoblastic tissue *in vitro* *J Reprod Fertil* Abst Series 1, 21

Betteridge KJ, Eaglesome MD, Randall GCB, Mitchell D (1980) Collection, description and transfer of embryos from cattle 10-16 days afetr estrous *J Reprod Fertil* **59** 205-216

Betteridge KJ, Eaglesome ND, Randall GCB, Mitchell D, Lugden EA (1978) Maternal progesterone levels as evidence of luteotrophic or antiluteolytic effects on embryos transferred to heifers 12-17 days after estrous *Theriogenology* 9 86-93

Boos A, Meyer W, Schwarz R, Grunert E (1996) Immunohistochemical assessment of oestrogen receptor and progesterone receptor distribution in biopsy samples of the bovine endometrium collected throughout the oestrous cycle *Anim Reprod Sci* **44** 11-21

Borten M, Friedman EA (1985) Early pregnancy interruption with a single PGF2 alpha 15-methyl-analogue vaginal suppository *J Reprod Med* **30** 741-744

Boshier DP, Jacobs RA, Han VK, Smith W, Riley SC, Challis JR (1991) Immunohistochemical localization of prostaglandin H synthase in the sheep placenta from early pregnancy to term *Biol Reprod* **45** 322-327

Burka JF (1983) Arachidonic acid metabolites and smooth muscle. In Biochemistry of Smooth Muscle, vol. III, PP. 141-168. Ed. NL Stephens. CRC Press, Boca Raton

Caffrey JL, Fletcher PW, Diekman MA, O'Callaghan PL, Niswender GD (1979) The activity of ovine luteal cholesterol esterase during several experimental conditions *Biol Reprod* **21** 601-608

Cantley LG, Barros EJ, Gandhi M, Rauchmann M, Nigam SK (1994) Regulation of mitogenesis, motogenesis and tubulogenesis by hepatocyte growth factor in renal collecting duct cells *Am J Physiol* **267** F271-F280

Cheng L, Kelly RW, Thong KJ, Hume R, Baird DT (1993) The effects of mifepristone (RU486) on prostaglandin dehydrogenase in decidual and chorionic tissue in early pregnancy *Human Reprod* **8** 705-709

Clark JH, Scrader WT, O'Malley BW (1985). *Mechanisms of steroid hormone action*. Philadelphia, Saunders.

Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, Taylor J, Lubahn DB, Cunha GR (1997) Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium *Proc Natl Acad Sci USA* **94** 6535-6540

Crankshaw D (1995) Effects of the isoprostane, 8-epi-prostaglandin F2 alpha, on the contractility of the human myometrium *in vitro Eur J Pharmacol* **285** 151-158

Cross JC, Roberts RM (1991) Constitutive and trophoblast-specific expression of a class of bovine interferon genes *Proc Natl Acad Sci USA* **88** 3817-3821

Cunha GR, Chung JM, Taguchi D, Fujii H (1983) Hormone-induced morphogenesis and growth: Role of mesenchymal-epithelial interation *Recent Prog Horm Res* **39** 559-598

Cutrone EC, Langer JA (1997) Contributions of cloned type I interferon receptor subunits to differential ligand binding *FEBS Lett* **404** 197-202

Danet-Desnoyers G, Johnson JW, O'Keefe SF, Thatcher WW (1993) Characterization of a bovine endometrial prostaglandin synthesis inhibitor (EOSI) *Biol Reprod* **48 (Suppl. 1)** 115 (Abstract)

Danet-Desnoyers G, Wetzels C, Thatcher WW (1994) Natural and recombinant bovine interferon-τ regulate basal and oxytocin-induced secretion of prostaglandins $F_{2\alpha}$ and E2 by epithelial cells and stromal cells in the endometrium *Reprod Fertil Dev* **6** 193-202

Darnell JE Jr (1996) The JAK-STAT pathway: summary of initial studies and recent advances *Recent Prog Horm Res* **51** 391-403

Darnell JE Jr, Kerr IM, Stark GR (1994) JAK-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins *Science* **264** 1415-1421

Das SK, Taylor JA, Paria BC, Dey SK, Lubahn (1997) Estrogenic responses in estrogen receptor (ER)-deficient mice reveal a novel non-ER mediated signaling pathway *Biol Reprod* **56 (Suppl. 1)** 151 (Abstract)

Davidson JA, Betts GJ, Tiemann U, Kamwanja LA, Monterroso VH, Hansen PJ (1994) Effects of interferon-τ and interferon-α on proliferation of bovine endometrial cells *Biol Reprod* **51** 700-705

de Moraes AA, Davidson JA, Fleming JG, Bazer FW, Edwards JL, Betts JG, Hansen PJ (1997) Lack of effect of granulocyte-macrophage colony-stimulating factor on secretion of interferon-tau, other proteins, and prostaglandin E2 by the bovine and ovine conceptus *Domest Anim Endocrinol* **14** 193-197

DeWitt DL, El-Haarith EA, Kraemer SA, Andrews MJ, Yao EF, Armstrong RL, Smith WL (1990) The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthase *J Biol Chem* **265** 5192-5198

DeWitt DL, Meade EA (1993) Serum and glucocorticoid regulation of gene transcription and expression of prostaglandin H synthase-1 and prostaglandin H synthase-2 isozymes *Arch Biochem Biophys* **306** 94-102

DeWitt DL, Smith WL (1988) Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from complementary DNA sequence *Proc Natl Acad Sci USA* **85** 1412-1416

Dooley VD, Johnson GA, Francis BR, Hansen TR (1997) Bovine granulocyte chemotactic protein-2 is secreted by the endometrium in response to interferon-tau (IFN-tau) *Endocrine* **6** 31-37

DuBois RN, Awad J, Morrow J, Roberts LJ, Bishop PR (1994) Regulation of eicosanoid production and mitogenesis in rat intestinal epithelial cells by transforming growth factor-α and phorbol ester *J Clin Invest* **93** 493-498

Ealy AD, Green JA, Alexenko AP, Keisler DH, Roberts RM (1998) Different ovine interferon-tau genes are not expressed identically and their protein products display different activities *Biol Reprod* **58** 566-573

Elattar TMA, Lin HS (1989) Comparison of the inhibitory effect of polyunsaturated fatty acids on prostaglandin synthesis I oral squamous carcinoma cells *Prostaglandins Leukotrienes and Essent Fatty Acids* **38** 119-125

Elger W, Beier S, Chwalisz K, Fahnrich M, Hasan SH, Henderson D, Neef G, Rohde R (1986) Studies on the mechanisms of action of progesterone antagonists *J Steroid Biochem* **25** 835-845

Evans CA, Kennedy TG (1978) The importance of prostaglandin synthesis for the initiation of blastocyst implantation in the hamster *J Reprod Fertil* **54** 255-261

Fairclough RJ, Lau TM (1992) Hormonal control of concentrations of endometrial oxytocin receptors in the ewe *Reprod Fertil Dev* **4** 313-320

Fairclough RJ, Moore LG, Peterson AJ, Watkins WB (1984) Effect of oxytocin on plasma concentration of 13,14-dihydro-15-keto prostaglandin F and the oxytocin-associated neurophysin during the estrous cycle and early pregnancy in the ewe *Biol Reprod* **21** 845-856

Farin CE, Imakama K, Hansen TR, Mcdonnell JJ, Murphy CN, Farin PW, Roberts RM (1990) Expression of trophoblastic interferon genes in sheep and cattle *Biol Reprod* **43** 210-218

Fields MJ, Fields PA, Castro-Hernandez A, Larkin LH (1980) Evidence for relaxin in corpora lutea of late pregnant cows *Endocrinology* **107** 869-876

Fields PA, Eldridge RK, Fuchs AR, Roberts RF, Fields MJ (1983) Human placental and bovine corpora luteal oxytocin *Endocrinology* **112** 1544-1546

Findlay JK, Chery RA., and Salamonsen LA. (1989) The use of isolated cells to study endometrial function *in vitro* *Oxford Reviews of Reprod Biol* **12** 181-223

Fletcher PW, Niswender GD (1982) Effect of PGF_{2_α} on progesterone secretion and adenylate cyclase activity in ovine luteal tissue *Prostaglandins* **20** 803-818

Flint APF, Lamming GE, Stewart HJ, Abayasekara DR (1994) The role of the endometrial oxytocin receptor in determining the length of the sterile oestrous cycle and ensuring maintenance of luteal function in early pregnancy in ruminants *Philos Trans R Soc Lond B Biol Sci* **344** 291-304

Flint APF, Sheldrick EL (1986) Ovarian oxytocin and the maternal recognition of pregnancy *J Reprod Fertil* **76** 831-839

Flint APF, Sheldrick EL (1985) Continuous infusion of oxytocin prevents induction of uterine oxytocin receptor and blocks luteal regression in cyclic ewes *J Reprod Fertil* **75** 623-631

Flint APF, Sheldrick EL (1983) Evidence for a systemic role for ovarian oxytocin in luteal regression in sheep *J Reprod Fertil* **67** 215-225

Flint APF, Sheldrick EL, McCann TJ, Jones DSC (1990) Luteal oxytocin: characteristics and control of synchronous episodes of oxytocin and $PGF_{2\alpha}$ secretion at luteolysis in ruminants *Domest Anim Endocrinol* **7** 111-124

Fortier MA, Guibault LA, Grasso F (1988) Specific properties of epithelial and stromal cells from the endometrium of cows *J Reprod Fertil* **83** 239-248

Fuchs AR (1987) Prostaglandin F2 alpha and oxytocin interactions in ovarian and uterine function *J Steroid Biochem* **27** 1073-1080

Funk CD, Funk LB, Kennedy ME, Pong AS, Fitzgerald GA (1991) Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression and gene chromosomal assignment *FASEB J* **5** 2304-2312

Ginther OJ, Woody CO, Mahajan S, Janakiraman K, Casida LE (1967) Effect of oxytocin administration on the oestrous cycle of unilaterally hysterectomised heifers *J Reprod Fertil* **14** 225-229

Glencross RG, Munro IB, Senior BE, Pope GS (1973) Concentrations of oestradiol-17 beta, oestrone and progesterone in jugular venous plasma of cows during the oestrous cycle and in early pregnancy *Acta Endocrinologica, Copenhagan* **73** 374-384

Glencross RG, Pope GS (1972) Concentration of oestrogens in jugular-venous plasma of cows during the oestrus cycle. *Biochem J* **128** 81P

Godkin JD, Bazer FW, Moffatt J, Sessions F, Roberts RM (1982) Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts on day 13-21 *J Reprod Fertil* **65** 141-150

Godkin JD, Bazer FW, Roberts RM (1984a) Ovine trophoblast protein 1, an early secreted blastocyst protein, binds specifically to uterine endometrium and affects protein synthesis *Endocrinology* **114** 120-130

Godkin JD, Bazer FW, Thatcher WW, Roberst RM (1984b) Proteins released by cultured Day 15-16 conceptuses prolong luteal maintenance when introduced into the uterine lumen of cyclic ewes *J Reprod Fertil* **71** 57-64

Godkin JD, Lifsey BJ, Gillespie BE (1988) Characterization of bovine conceptus proteins produced during the peri- and postattachment periods of early pregnency *Biol Reprod* **38** 703-711

Gross TS, Plante C, Thatcher WW, Hansen PJ, Helmer SD, Putney DJ (1988b) Secretory proteins of the bovine conceptus alter endometrial prostaglandin and protein secretion *in vitro* *Biol Reprod* **39** 977-987

Gross TS, Thatcher WW, Hansen PJ, Helmer SD (1988a) Presence of an intracellular endometrial inhibitor of prostaglandin synthesis during early pregnancy in the cow *Prostaglandins* **35** 359-378

Hamasaki Y, Kitzler J, Hardman R, Nettesheim P, Eling TE (1993) Phorbol ester and epidermal growth factor enhance the expression of two inducible prostaglandin H sythase genes in rat tracheal epithelial cells *Arch Biochem Biophys* **304** 226-234

Han CS, Mathialagan N, Klemann SW, Roberts RM (1997) Molecular cloning of ovine and bovine type I interferon receptor subunits from uteri, and endometrial expression of messenger ribonucleic acid for ovine receptors during the estrous cycle and pregnancy *Endocrinology* **138** 4757-4767

Hansen TR, Austin KJ, Johnson GA (1997) Transient ubiquitin cross-reactive protein gene expression in the bovine endometrium *Endocrinology* **138** 5079-5082

Hansen TR, Kazemi M, Keisler DH, Malathy PV, Imakawa K, Roberts RM (1989) Complex binding of the embryonic interferon, ovine trophoblastprotein-1, to endometrial receptors *J Interferon Res* **9** 215-225

Haslam SZ, Counterman LJ (1991) Mammary stroma modulates hormonal responsiveness of mammary epithelium *in vivo* in the mouse *Endocrinology* **129** 2017-2023

Hay MF, Moor RM (1978) *Control of Ovulation*. London, Butterworths.

Heap RB, Fleet IR, Hamon M (1985) Prostaglandin $F_{2\alpha}$ is transferred from the uterus to the ovary in the sheep by lymphatic and blood vascular pathway *J Reprod Fertil* **74** 645-656

Helmer SD, Hansen PJ, Anthony RV, Thatcher WW, Bazer FW, Roberts RM (1987) Identification of bovine trophoblast protein-1, a secretory protein immunologically related to ovine trophoblast protein-1 *J Reprod Fertil* **79** 83-91

Helmer SD, Hansen PJ, Thatcher WW (1988) Differential glycosylation of the components of the bovine trophoblastprotein-1 complex *Mol Cell Endocrinol* **58** 103-107

Heyman Y, Camous S, Fevre J, Meziou W, Martal J (1984) Maintenance of the corpus luteum after uterine transfer of trophoblastic vesicles to cyclic cows and ewes *J Reprod Fertil* **70** 533-540

Hirst JJ, Teixeira FJ, Zakar T, Olson DM (1995a) Prostaglandin H synthase-2 expression increases in human gestational tissues with spontaneous labour onset. *Reprod Fertil Dev* **7** 633-637

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

Hixon JE, Flint APF (1987) Effects of a luteolytic dose of oestradiol benzoate on uterine oxytocin receptor concentrations, phosphoinositide turnover and prostaglandin $F\text{-}_{2\alpha}$ secretion in sheep *J Reprod Fertil* **79** 457-467

Hixon JE, Hansel W (1974) Evidence for preferential transfer of prostaglandin F2alpha to the ovarian artery following intrauterine administration in cattle *Biol Reprod* **11** 543-552

Hla T, Neilson K (1992) Human cyclooxygenase-2 cDNA *Proc Natl Acad Sci USA* **89** 7384-7388

Hoffman LH (1978) Antifertility effects of indomethacin during early pregnancy in the rabbit *Biol Reprod* **18** 148-153

Homeida AM, Khalafalla AE (1987) Effects of oxytocin-antagonist injections on luteal regression in the goat *Br J Pharmacol* **90** 281-284

Hooper SB, Watkins WB, Thorburn GD (1986) Oxytocin. oxytocin-associated neurophysin, and prostaglandin $F_{2\alpha}$ concentrations in the utero-ovarian vein of pregnant and nonpregnant sheep *Endocrinology* **119** 2590-2597

Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM (1987) Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm *Nature* **330** 377-379

Imakawa K, Hansen TR, Malathy PV, Anthony RV, Polites HG, Marotti KR, Roberts RM (1989) Molecular cloning and characterization of complementary deoxyribonucleic acids corresponding to bovine trophoblast protein-1: a comparison with ovine trophoblast protein-1 and bovine interferon-alpha II *Mol Endocrinol* **3** 127-139

Imakawa K, Helmer SD, Nephew KP, Meka CSR, Christenson RK (1993) A novel role for GM-CSF: Enhancement of pregnancy specific interferon production, ovine trophoblast protein-1 *Endocrinology* **132** 1869-1871

Inaba T, Wiest WG, Strickler RC, Mori J (1988) Augmentation of the response of mouse uterine epithelial cells to estradiol by uterine stroma *Endocrinology* **123** 1253-1258

Ing NH, Bhattacharyya S (1997) Estradiol stabilizes estrogen receptor messenger RNA in sheep endometrium *Biol Reprod* **56 (Suppl. 1)** 151 (Abstract)

Ireland JJ, Roche JF (1983) Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins *Endocrinology* **112** 150-156

Ivell R, Brackett KH, Fields MY, Richter D (1985) Ovulation triggers oxytocin gene expression in the bovine ovary *FEBS Lett* **190** 263-267

Johnson GA, Austin KJ, Vankirk EA, Hansen TR (1998) Pregnancy and interferon-tau induce conjugation of bovine ubiquitin cross-reactive protein to cytosolic uterine proteins *Biol Reprod* **58** 898-904

Jones DA, Carlton DP, McIntyre TM, Zimmerman GA, Prescott SM (1993) Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines *J Biol Chem* **268** 9049-9054

Kanda S, Kuriyama H (1980) Specific features of smooth muscle cells recorded from the placental region of the myometrium of pregnant rats *J Physiol* **299** 127-144

Kasimir-Bauer S, Ottinger H, Wuttke M, Stellberg W, Grosse-Wilde H, Seeber S, Scheulen ME (1998) Chronic myelogenous leukemia: effect of interferon-alpha treatment on phagocytic activity and capacity of circulating neutrophils *Leuk Res* **22** 115-117

Katzenellenbogen BS (1980) Dynamics of steroid hormone receptor action *Ann Rev Physiol* **42** 17-35

Kennedy TG (1985) Evidence for the involvement of prostaglandins throughout the decidual cell reaction in the rat *Biol Reprod* **33** 140-146

Kennedy TG (1983) Prostaglandin E2, adenosine 3': 5'-cyclic monophosphate and changes in endometrial vascular permeability in rat uteri sensitized for the decidual cell reaction *Biol Reprod* **29** 1069-1076

Kennedy TG (1980) Prostaglandins and the endometrial vascular permeability changes preceding blastocyst implantation and decidualization *Prog Reprod Biol* **7** 234-243

Kennedy TG (1979) Prostaglandins and increased endometrial vascular permeability resulting from the appliaction of an artificial stimulus to the uterus of the rat sensitized for the decidual cell reaction *Biol Reprod* **20** 560-566

Kennedy TG (1977) Evidence for a role for prostaglandins in the initiation of blastocyst implantation in the rat *Biol Reprod* **16** 286-291

Kennedy TG, Lukash LA (1982) Induction of decidualization in rats by the intruterine infusion of prostaglandins *Biol Reprod* **27** 253-260

Kennedy TG, Martel D, Psychoyos A (1983) Endometrial prostaglandin E2 binding: Characterization in rats sensitized for decidual cell reaction and changes during pseudopregnancy *Biol Reprod* **29** 556-564

Kerr IM, Stark GR (1991) Minireview: The control of interferon-inducible gene expression *FEBS Lett* **285** 194

Knickerbocker JJ, Niswender GD (1989) Characterization of endometrial receptors for ovine trophoblast protein-1 during the estrous cycle and early pregnancy in sheep *Biol Reprod* **40** 361-369

Kraemer S, Meade E, DeWitt D (1992) Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5' flanking regulatory sequence *Arch Biochem Biophys* **293** 391-400

Kruip TAM, Vullings HGB, Schams D, Jonis J, Klarenbeek A (1985) Immunocytochemical demonstration of oxytocin in bovine ovarian tissues *Acta Endocrinologica, Copenhagan* **109** 537-542

Kubisch HM, Larson MA, Roberts RM (1998) Relationship between age of blastocyt formation and interferon-tau secretion by *in vitro*-derived bovine embryos *Mol Reprod Dev* **49** 254-260

Kujubu DA, Fletcher BS, Varnum BC, Lim RW, and Herschman HR (1991) TIS10, a phorbol ester tumor promoter-inducible mRNA from swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue *J Biol Chem* **266** 12866-12872

Lafrance M, Goff AK (1988) Effects of progesterone and estradiol-17β on oxytocin-induced release of prostaglandin $F_{2\alpha}$ *J Reprod Fertil* **82** 429-436

Lafrance M, Goff AK (1985) Effect of pregnancy on oxytocin-induced release of prostaglandin F2 alpha in heifers *Biol Reprod* **33** 1113-1119

Lamming GE, Flint APF, Vallet JL (1991) Trophoblast interferons act locally to suppress the uterine oxytocin receptor in sheep *J Reprod Fertil* **Abst Series 8** Abst 38

Lamming GE, Mann GE (1995) Control of endometrial oxytocin receptors and prostaglandin $F_{2\alpha}$ production in cows by ptrogesterone and oestradiol *J Reprod Fertil* **103** 69-73

Lamming GE, Wathes DC, Flint AP, Payne JH, Stevenson KR, Vallet JL (1995) Local action of trophoblast interferons in suppression of the development of oxytocin and oestradiol receptors in ovine endometrium *J Reprod Fertil* **105** 165-75

Langer JA, Yang J, Carmillo P, Ling LE (1998) Bovine type I interferon receptor protein BoIFNAR-1 has high-affinity and broad specificity for human type I interferons *FEBS Lett* **421** 131-135

Leaman DW, Cross JC, Roberts R (1994) Multiple regulatory elements are required to direct trophoblast interferon gene expression in choriocarcinoma cells and trophectoderm *Mol Endocrinol* **8** 456-468

Leaman DW, Cross JC, Roberts RM (1992) Genes for the trophoblast interferons and their distribution among mammals *Reprod Fertil Dev* **4** 349-353

Leaman DW, Roberts RM (1992) Genes for the trophoblast interferons in sheep, goat, and musk ox and distribution of related genes among mammals *J Interferon Res* **12** 1-11

Leavitt WW, MacDonald RG, Okulicz WC (1983) *Hormonal regulation of estrogen and progesterone receptor systems*. New York, Academic Press.

Li J, Roberts RM (1994) Interferon-τ and interferon-a interact with the same receptors in bovine endometrium *J Biol Chem* **269** 13544-13550

Lichtman SM, Wasil T, Ahmad M, Brody J (1998) Development of essential thrombocythemia in a patient treated with interferon alfa and pentoststin for hairy cell leukemia *Leuk Lymphoma* **28** 423-427

Lifsey BJ Jr, Baumbach GA, Godkin JD (1989) Isolation, characterization and immunocytochemical localization of bovine trophoblast protein-1 *Biol Reprod* **40** 343-352

Mann GE, Lamming GE (1994) Use of repeated biopsies to monitor endometrial oxytocin receptors in cows *Vet Record* **135** 403-405

Martel D, Kennedy TG, Monier MN, Psychoyos A (1985) Failure to detect specific binding sites for prostaglandin $F_{2\alpha}$ in membrane preparations from rat endometrium *J Reprod Fertil* **75** 265-274

Martal J, Lacroix MC, Loudes C, Saunier M, Wintenberger-Torres S (1979) Trophoblastin, an antiluteolytic protein present in early pregnancy in sheep *J Reprod Fertil* **56** 63-73

Matthews EL, Ayad VJ, Wathes DC (1991) The effect of estradiol-17β on oxytocin receptor concentrations in the reproductive tract of the anoestrous ewe *J Reprod Fertil* **Abstract Series 8** 81

Matsuyama T, Grossman A, Mittrucker H-W, Siderovski DP, Kiefer F, Kawakami T, Richardson CD, Taniguchi T, Yoshinaga SK, Mak TW (1995) Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE) *Nucleic Acids Res* **23** 2127-2136

Maymon R, Haimovich L, Shulman A, Pomeranz M, Holtzinger M, Bahary C (1992) Third-trimester uterine rupture after prostaglandin E2 use for labor induction *J Reprod Med* **37** 449-452

McCracken JA (1980) Hormone receptor control of pulsatile secretion of $PGF_{2\alpha}$ by the ovine uterus *Adv Prosta Thromb Res* **8** 1329-1344

McCracken JA, Barcikowski B, Carlson JC, Green K, and Samuelsson B (1973) The physiological role of prostaglandin $F_{2\alpha}$ in corpus luteum regression *Adv Biosci* **9** 599-624

McCracken JA, Carlson JC, Glew ME, Goding JR, Baird DT, Green K, Samuelsson B (1972) Prostaglandin F_{2_α} identified as a luteolytic hormone in sheep *Nature New Biol* **238** 129-134

McCracken JA, Schramm W, Barcikowski B, Wilson L (1981) The identification of prostaglandin F_{2_α} as a uterine luteolytic hormone and the hormonal control of its stnthesis *Acta Veterinaria Scandinavica* **71 (Suppl)** 71-88

McCracken JA, Schramm W, Okulicz WC (1984) Hormone receptor control of pulsatile secretion of PGF_{2_α} from the ovine uterus during luteolysis and its abrogation in early pregnancy *Anim Reprod Sci* **7** 31-55

Merlie JP, Fagan D, Mudd J, Needleman P (1988) Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase) *J Biol Chem* **263** 3550-3553

Meyer HH, Mittermeier T, Schams D (1988) Dynamics of oxytocin, estrogen and progestin receptors in the bovine endometrium during the estrous cycle *Acta Endocrinologica* **118** 96-104

Meyer MD, Hansen PJ, Thatcher WW, Drost M, Badinga L, Roberts RM, Li J, Ott TL, Bazer FW (1995) Extension of corpus luteum lifespan and reduction of uterine secretion of prostaglandin F2 alpha of cows in response to recombinant interferon-tau *J Dairy Sci* **78** 1921-1931

Mijovic JE, Zakar T, Nairn TK, Olson DM (1998) Prostaglandin endoperoxide H synthase (PGHS) activity and PGHS-1 and -2 messenger ribonucleic acid abundance in human chorion throughout gestation and with preterm labor *J Clin Endocrinol Metab* **831**358-1367

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

Mirando MA, Harney JP, Zhou Y, Ogle TF, Ott TL, Moffatt RJ, Bazer FW (1993) Changes in progesterone and oestrogen receptor mRNA and protein

and oxytocin receptors in endometrium of ewes after intrauterine injection of ovine trophoblast interferon *J Mol Endocrinol* **10** 185-192

Mirando MA, Ott TL, Harney JP, Bazer FW (1990a) Ovine trophoblast protein-one inhibits development of endometrial responsiveness to oxytocin in ewes *Biol Reprod* **43** 1070-1078

Mirando MA, Ott TL, Vallet JL, Davis M, Bazer FW (1990b) Oxytocin-stimulated inositol phosphate turnover in endometrium of ewes is influenced by stage of the estrous cycle, pregnancy, and intrauterine infusion of ovine conceptus secretory proteins *Biol Reprod* **42** 98-105

Mitchell MD, Flint APF (1978) Use of meclofenamic acid to investigate the role of prostaglandin biosynthesis during induced parturition in sheep *J Endocrinol* **76** 101-109

Miyamoto T, Ogino N, Yamamoto S, Hayaishi O (1976) Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes *J Biol Chem* **251** 2629-2636

Moor RM (1968) Effect of embryo on corpus luteum function *J Anim Sci* **27 (Suppl. 1)** 97-118

Moor RM, Rowson LEA (1964) Influence of the embryo and uterus on luteal function in the sheep *Nature* **201** 522-523

Moore LG, Choy VJ, Elliot RL, Watkins WB (1986) Evidence for the pulsatile release of $PGF_{2\alpha}$ inducing the release of ovarian oxytocin during luteolysis in the ewe *J Reprod Fertil* **76** 159-166

Morgan G, Wooding FB, Godkin JD (1993) Localization of bovine trophoblast protein-1 in the cow blastocyst during implantation: an immunological cryoultrastructural study *Placenta* **14** 641-649

Morita LM, Schindler MK, Regier JC, Otta TH, DeWitt DL, Smith WL (1995) Different intracellular localization for prostaglandin endoperoxide H synthase-1 and -2 *J Biol Chem* **270** 10902-10908

Naivar KA, Ward SK, Austin KJ, Moore DW, Hansen TR (1995) Secretion of bovine uterine proteins in response to type I IFNs *Biol Reprod* **52** 848-854

Nalbandov AV (1976) *Reproductive physiology of mammals and birds.* San Francisco, USA, W. H. Freeman and Company.

Nathans D, Lau LF, Christy B, Hartzell S, Nakabeppu Y, Ryder K (1988) Genomic response to growth factors *Cold Spring Harbor Symp Quant Biol* **53** 893-900

Nephew KP, Whaley AE, Christenson RK, Imakawa K (1993) Differential expression of distinct mRNAs for ovine trophoblast protein-1 and related sheep type I interferons *Biol Reprod* **48** 768-778

Nett TM, McClellan MC, Niswender GD (1976) Effects of prostaglandins on the ovine corpus luteum: blood flow, secretion of progesterone and morphology *Biol Reprod* **15** 66-78

Newton GR, Ott TL, Woldesenbe S, Shelton AH Bazer FW (1996) Biochemical and immunological properties of related small ruminant trophoblast interferons *Theriogenology* **46** 703-716

Niswender GD, Reimers TJ, Diekman MA, Nett TM (1976) Blood flow: A mediator of ovarian function *Biol Reprod* **14** 64-81

Niswender GD, Roess DA, Sawyer HR, Silvia WJ, Barisas BG (1985) Differences in the lateral mobility of receptors for luteinizing hormone (LH) in the luteal plasma membrane when occupied by ovine LH versus human chorionic gonadotropin *Endocrinology* **116** 164-169

Northey DL, French LR (1980) Effect of embryo removal and intrauterine infusion of embryonic homogenates on the lifespan of the bovine corpus luteum *J Anim Sci* **50** 298-302

O'Banion MK, Winn VD, Young DA (1992) cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase *Proc Natl Acad Sci USA* **89** 4888-4892

Olson DM, Lye SJ, Skinner K, Challis JR (1985) Prostanoid concentrations in maternal/fetal plasma and amniotic fluid and intrauterine tissue prostanoid output in relation to myometrial contractility during the onset of adrenocorticotropin-induced preterm labor in sheep *Endocrinology* **116** 389-397

O'Sullivan MG, Chilton FH, Huggins EM, McCall CE (1992) Lipopolysaccharide priming of alveolar macrophages for enhanced synthesis of prostanoids involves induction of a novel prostaglandin H synthase *J Biol Chem* **267** 14547-14550

Ott TL, Mirando MA, Davis MA, Bazer FW (1992) Effects of ovine conceptus secretory proteins and progesterone on oxytocin-stimulated endometrial production of prostaglandin and turnover of inositol phosphate in ovariectomized ewes *J Reprod Fertil* **95** 19-29

Ott TL, Wiley AA, Spencer TE, Bartol FF, Bazer FW (1995) Uterine expression of interferon-induced Mx in cyclic and pregnant ewes *Biol Reprod* **52 (Suppl 1)** 143

Ott TL, Zhou Y, Mirando MA, Stevens C, Harney JP, Ogle TF, Bazer FW (1993) Changes in progesterone and oestrogen receptor mRNA and protein during maternal recognition of pregnancy and luteolysis in ewes *J Mol Endocrinol* **10** 171-183

Parkinson TJ, Wathes DC, Jenner LJ, Lamming GE (1992) Plasma and luteal concentrations of oxytocin in cyclic and early-pregnant cattle *J Reprod Fertil* **94** 161-167

Pate JL, Condon WA (1989) Regulation of steroidogenesis and cholesterol synthesis by prostaglandin $F_{2\alpha}$ and lipoproteins in bovine luteal cells *J Reprod Fertil* **87** 439-446

Pestka AJ, Langer JA, (1987) Interferons and their actions *Annu Rev Biochem* **56** 727-777

Peters AR, Ball PJH (1987). The ovarian cycle. In: *Reproduction in Cattle.* A. Peters, Ball PJH. England, Butterworth & Co. Ltd: 20-40.

Peterson AJ, Tervit HR, Fairclough RJ, Hawick PG, Smith JF (1976) Jugular levels of 13,14-dihydro-15-keto-prostaglandin F and progesterone around luteolysis and early pregnancy in ewes *Prostaglandins* **12** 551-558

Pharriss BB, Wyngarden LJ (1969) The effect of prostaglandin $F_{2\alpha}$ on the progestogen content of ovaries from pseudopregnant rats *Proc Soc Exptl Biol Med* **130** 92-94

Plante C, Hansen PJ, Thatcher WW (1988) Prolongation of luteal lifespan in cows by intrauterine infusion of recombinant bovine α-interferon *Endocrinology* **122** 2342-2344

Pontzer CH, Bazer FW, Johnson HM (1991) Antiproliferative activity of a pregnancy recognition hormone, ovine trophoblast protein-1 *Can Res* **51** 5304-5307

Pontzer CH, Ott TL, Bazer FW, Johnson HM (1994) Structure/function studies with interferon tau: evidence for multiple active sites *J Interferon Res* **14** 133-141

Pontzer CH, Ott TL, Bazer FW, Johnson HM (1990) Localization of an antiviral site on the pregnancy recognition hormone, ovine trophoblast protein 1 *Proc Natl Acad Sci USA* **87** 5945-5949

Pontzer CH, Torres BA, Vallet JL, Bazer FW, Johson HM (1988) Antiviral activity of the pregnancy recognition hormone ovine trophoblast protein-1 *Biochem Biophys Res Commun* **152** 801

Proost P, Wuyts A, Conings R, Lenaerts JP, Billiau A, Opdenakker G, Van Damme J (1993) Human and bovine granulocyte chemotactic protein-2: complete amino acid sequence and functional characterization as chemokines *Biochemistry* **32** 10170-10177

Riese J, Hoff T, Nordhoff A, DeWitt DL, Resch K, Kaever V (1994) Transient expression of prostaglandin endoperoxide synthase-2 during mouse macrophage activation *J Leukocyte Biol* **55** 476-482

Riley JCM, Carlson JC (1985) Calcium-regulated plasma membrane rigidification during corpus luteum regression in the rat *Biol Reprod* **32** 77-82

Roberts JS, McCracken JA (1976) Does prostaglandin F_{2_α} released from the uterus by oxytocin mediate the oxytocic action of oxytocin? *Biol Reprod* **15** 457-463

Roberts JS, McCracken JA, Gavagan JE, Soloff MS (1976) Oxytocin-stimulated release of prostaglandin F2alpha from ovine endometrium *in vitro*: correlation with estrous cycle and oxytocin-receptor binding *Endocrinology* **99** 1107-1114

Roberts RM, Cross JC, Leaman DW (1992b) Interferons as hormones of pregnancy *Endocrine Rev* **13** 432-452

Roberts RM, Imakawa, K, Niwano Y, Kazemi M, Malathy PV, Hansen TR, Glass AA, Kronenberg LH (1989) Interferon production by the preimplantation sheep embryo *J Interferon Res* **9** 178-187

Roberts RM, Klemann SW, Leaman DW, Bixby JA, Cross JC, Farin CE, Imakawa K, Hansen TR (1991) The polypeptides and genes for ovine and bovine trophoblast protein-1 *J Reprod Fertil* **43 (Suppl)** 3-12

Roberts RM, Leaman DW, Cross JC (1992a) Role of interferons in maternal recognition of pregnancy in ruminants *Proc Soc Exp Biol Med* **200** 7-18

Rowson LE, Moor RM (1967) The influence of embryonic tissue homogenate infused into the uterus, on the life-span of the corpus luteum in the sheep *J Reprod Fertil* **13** 511-516

Rubin JS, Bottaro DP, Chedid M, Miki T, Ron D, Cheon H-G, Taylor WG, Fortney E, Sakata H, Finch PW, LaRochelle WJ (1995) Keratinocyte growth factor *Cell Biol Int* **19** 399-411

Russell DL, Manalo GG Jr, Findlay JK, Salamonsen LA (1993) Binding sites for interferons on ovine and human endometrial membranes *Reprod Fertil Dev* **5** 219-227

Ryan AM, Gallagher DS, Womack JE (1993) Somatic cell mapping of omega and trophoblast interferon genes tobovine syntenic group U18 and in situ localization to chromosome 8 *Cyt Cell Genet* **63** 6-10

Saksena SK, Lau IF, Chang MC (1976) Relationship between oestrogen, prostaglandin $F_{2\alpha}$ and histamine in delayed implantation in the mouse *Acta Endocrinol* **81** 801-807

Schams D (1983) Oxytocin determination by radioimmunoassay III. Improvement to subpicrogram sensitivity and application to blood levels in cyclic cattle *Acta Endocrinologica, Copenhagen* **103** 180-183

Schams D, Kruip TAM, Koll R (1985) Oxytocin determination in steroid producing tissues and *in vitro* production in ovarian follicles *Acta Endocrinologica, Copenhagen* **109** 530-536

Schindler C, Shuai K, Prezioso VR, Darnell JE Jr (1992) Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor *Science* **257** 809-813

Schmitt RA, Geisert RD, Zavy MT, Short EC (1993) Uterine celluler changes in 2', 5'-Oligoadenylate Synthetase during the Bovine Estrous Cycle and Early Pregnancy *Biol Reprod* **48** 460-466

Schramm W, Bovaird L, Glew ME, McCracken JA (1983) Corpus luteum regression induced by ultra-low pulses of prostaglandin F2α *Prostaglandins* **26** 347-364

Schwall RH, Sawyer HR, Niswender GD (1986) Differential regulation by LH and prostaglandins of steroidogenesis in small and large luteal cells of the ewe *J Reprod Fertil* **76** 821-829

Setiawan T, Buchanan D, Taylor JA, Young P, Lubahn DB, Cunha GR, Cooke PS (1997) Role of stromal and epithelial estrogen receptors (ER) in uterine epithelial secretory protein production *Biol Reprod* **56 (Suppl 1)** 83 (Abstract)

Sheldrick EL, Flint APF (1990) Effect of continuous infusion of oxytocin on prostaglandin F2α secretion and luteolysis in the cyclic ewe *Reprod Fertil Dev* **2** 89-99

Sheldrick EL, Flint APF (1985) Endocrine control of uterine oxytocin receptors in the ewe *J Endocrinol* **106** 249-258

Short EC, Fulton RW (1987) Induction and measurement of 2',5'-oligoadenylate synthetase in madin-darby bovine kidney cells and cattle *JClin Microb* **25** 1735-1740

Short EC, Geisert RD, Helmer SD, Zavy MT, Fulton RW (1991) Expression of antiviral activity and induction of 2',5'-oligoadenylate synthetas by concetory proteins enriched in bovine trophoblast protein-1 *Biol Reprod* **44** 261-268

Short RV (1969). Implantation of the maternal recognition of pregnancy. In: *Foetal Anatomy*, Wolstenholme GEW, O'Connor M (eds), Ciba Foundation Symposium, London: J&A Churchill Ltd., pp. 2-26.

Silvia WJ, Lewis GS, McCracken JA, Thatcher WW, Wilson L (1991) Hormonal regulation of uterine secretion of prostaglandin F2α *Biol Reprod* **45** 655-663

Silvia WJ, Raw RE (1993) Activity of phospholipase C and release of PGF2α by endometrial tissue fron ewes during the oestrous cycle and early pregnancy *J Reprod Fertil* **97** 529-537

Simmons DL, Levy DB, Yannoni Y, Erikson RL (1989) Identification of a phorbol ester-repressible v-*src*-inducible gene *Proc Natl Acad Sci USA* **86** 1178-1182

Sirbasku DA (1980) Estromedins: uterine-derived growth factors for estrogen-responsive tumour cells. *Control mechanism in animal cells.* L Jimenez de Asua (ed) New York, Raven Press: pp. 293-298.

Sirois J (1994) Induction of prostaglandin endoperoxide synthase-2 by human chorionic gonadotropin in bovine preovulatory follicles *in vivo* *Endocrinology* **135** 841-848

Sirois J, Richards JS (1992b) Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles *J Biol Chem* **267** 6382-6388

Sirois J, Simmons DL, Richards JS (1992a) Hormonal regulation of messenger ribonucccleic acid encoding a novel isoform of prostaglandin endoperoxide H synthase in rat preovulatory follicles *J Biol Chem* **267** 11586-11592

Skopets B, Li J, Thatcher WW, Roberts RM, Hansen PJ (1992) Inhibition of lymphocyte proliferation by bovine trophoblast protein-1 (type I trophoblast interferon) and bovine interferon-α1 *Vet Immunol Immunopathol* **34** 81-96

Smith WL, Garavito RM, DeWitt DL (1996) Prostaglandin endoperoxide H synthases (Cyclooxygenases)-1 and -2 *J Biol Chem* **271** 33157-33160

Spencer TE, Bazer FW (1996) Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium *Endocrinology* **137** 1144-1147

Spencer TE, Bazer FW (1995) Temporal and spatial regulation of uterine receptors for oestrogen and progesterone during the estrous cycle and early pregnancy in ewes *Biol Reprod* **53** 1527-1544

Spencer TE, Becker WC, George P, MirandO MA, Ogle TF, Bazer FW (1995a) Ovine interferon-τ regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone *Biol Reprod* **53** 732-745

Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW (1995b) Ovine interferon-tau (IFN-τ) inhibits estrogen receptor up-regulation and estrogen-induced luteolysis in cyclic ewes *Endocrinology* **136** 4932-4944

Spencer TE, Mirando MA, Mayes JS, Watson GH, Ott TL, Bazer FW (1996a) Effects of interferon-τ and progesterone on oestrogen-stimulated expression

of receptors for oestrogen, progesterone and oxytocin in the endometrium of ovariectomized ewes *Reprod Fertil Dev* **8** 843-853

Spencer TE, Ott TL, Bazer FW (1996b) tau-Interferon: pregnancy recognition signal in ruminants *Pro Soc Exp Biol Med* **213** 215-229

Spencer TE, Ing NH, Ott TL, Mayes JS, Becker WC, Watson GH, Mirando MA, Brazer FW (1995c) Intrauterine injection of ovine interferon-tau alters oestrogen receptor and oxytocin receptor expression in the endometrium of cyclic ewes *J Mol Endocrinol* **15** 203-220

Stevenson KR, Riley PR, Stewart HJ, Flint AP, Wathes DC (1994) Localization of oxytocin receptor mRNA in the ovine uterus during the oestrous cycle and early pregnancy *J Mol Endocrinol* **12** 93-105

Stewart HJ, McCann SH, Barker PJ, Lee KE, Lamming GE, Flint APF (1987) Interferon sequence homology and receptor binding activity of ovine trophoblast and luteolytic protein *J Endocrinol* **115** R13-15

Stewart HJ, McCann, SH, Lamming GE, Flint APF (1989) Evidence for a role for interferon in the maternal recognition of pregnency *J Reprod Fertil* **37** 91-99

Subramaniam PS, Khan SA, Pontzer CH, Johnson HM (1995) Differential recognition of the type I interferon receptor by interferons tau and alpha is responsible for their disparate cytotoxicities *Proc Natl Acad Sci USA* **92** 12270-12274

Sumida C, Lecerf F, Pasqualini JR (1988) Control of progesterone receptors in fetal uterine cells in culture: effects of estradiol, progestins, antiestrogens, and growth factors *Endocrinology* **122** 3-11

Swann RW, O'shaughnessy PJ, Birkett SD, Wathes DC, Porter DG, Pickering BT (1984) Biosynthesis of oxytocin in the corpus luteum *FEBS Lett* **174** 262-266

Teixeira FJ, Zakar T, Hirst JJ, Guo F, Sadowsky DW, Machin G, Demianczuk N, Resch B, Olson DM (1994) Prostaglandin endoperoxide-H synthase

(PGHS) activity and immunoreactive PGHS-1 and PGHS-2 levels in human amnion throughout gestation, at term, and during labor *J Clin Endocrinol Metab* **78** 1396-1402

Teixeira MG, Austin KJ, Perry DJ, Dooley VD, Johnson GA, Francis BR, Hansen TR (1997) Bovine granulocyte chemotactic protein-2 is secreted by the endometrium in response to interferon-tau (IFN-tau) *Endocrine* 1997 **6** 31-37

Thatcher WW, Bartol FF, Knickerbocker JJ, Curl JS, Wolfenson D, Bazer FW, Roberts RM (1984) Maternal recognition of pregnancy in cattle. [Review] *J Dairy Sci* **67** 2797-2811

Thatcher WW, Bazer FW, Sharp DC, Roberts RM (1986) Interrelationships between uterus and conceptus to maintain corpus luteum function in early pregnancy: sheep, cattle, pigs and horses. [Review] *J Anim Sci* **62 (Suppl 2)** 25-46

Thatcher WW, Knickerbocker JJ, Bartol FF, Bazer FW, Roberts RM, Drost M (1985) Maternal recognition of pregnancy in relation to the survival of transferred embryos: endocrine aspects *Theriogenology* **23** 129-143

Thatcher WW, Meyer MD, Danet-Desnoyers G (1995) Maternal recognition of pregnancy *J Reprod Fertil* **49 (Suppl)** 15-28

Thatcher WW, Staples CR, Danet-Desnoyers G, Oldick B, Schmitt E-P (1994) Embryo health and mortality in sheep and cattle *J Anim Sci* **72** 16-30

Thorburn GD, Cox RI, Currie WB, Restall BJ, Schneider W (1973) Prostaglandin F and progesterone concentrations in the utero-ovarian venous plasma of the ewe during the oestrous cycle and early pregnancy *J Reprod Fertil* **18 (Suppl)** 151-158

Torday JS, Jefcoate GR, First NL (1980) Effect of prostaglandin F2α on steroidogenesis by porcine corpora lutea *J Reprod Fertil* **58** 301-310

Vallet JL, Bazer FW, Fliss MF, Thatcher WW (1988) Effect of ovine conceptus secretory proteins and purified ovine trophoblast protein-1 on interoestrous

interval and plasma concentrations of prostaglandins F-2 alpha and E and of 13,14-dihydro-15-keto prostaglandin F-2 alpha in cyclic ewes *J Reprod Fertil* **84** 493-504

Vallet JL, Bazer FW, Roberts RM (1987) The Effect of ovine trophoblast protein-one on endometrial protein secretion and cyclic nucleotides *Biol Reprod* **37** 1307-1316

Vallet JL, Lamming GE (1991) Ovine conceptus secretory proteins and bovine recombinant interferon α-1 decrease endometrial oxytocin receptor concentrations in cyclic and progesterone-treated ovariectomized ewes *J Endocrinol* **131** 475-482

Vallet JL, Lamming GE, Batten M (1990) Control of endometrial oxytocin receptor and uterine response to oxytocin by progesterone and oestradiol in the ewe *J Reprod Fertil* **90** 625-634

Vesanan M, Isomaa V, Alanko M, Vihko R (1991) Bovine uterine, cervical and ovarian estrogen and progesterone receptor concentrations *Anim Reprod Sci* **26** 61-71

Vesanen M, Isomaa V, Vihko R (1988) Cytosol estrogen and progesterone peceptors in bovine endometrium after uterine involution postpartum and in the estrous cycle *Anim Reprod Sci* **17** 9-20

Walters DL, Schallenberger E (1984) Pulsatile secretion of gonadotrophins, ovarian steroids and ovarian oxytocin during the periovulatory phase of the oestrous cycle in the cow *J Reprod Fertil* **71** 503-512

Watanabe K, Fujii Y, Nakayama K, Ohkubo H, Kuramitsu S, Kagamiyama H, Nakanishi S, Hayaishi O (1988) Structural similarity of bovine lung prostaglandin F synthase to lens ε-crystallin of the European common frog *Proc Natl Acad Sci USA* **85** 11-15

Wathes DC (1989) Oxytocin and vasopressin in the gonads. New York, Oxford University Press.

Wathes DC, Denning Kendall PA (1992) Control of synthesis and secretion of ovarian oxytocin in ruminants *J Reprod Fertil* **45 (Suppl)** 39-52

Wathes DC, Hamon M (1993) Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe *J Endocrinol* **138** 479-491

Wathes DC, Lamming GE (1995) The oxytocin receptor, luteolysis and the maintenance of pregnancy *J Reprod Fertil* **49(Suppl)** 53-67

Wathes DC, Mann GE, Payne JJ, Riley PR, Stevenson KR, Lamming GE (1996) Regulation of oxytocin, oestradiol and progesterone receptor concentrations in different uterine regions by oestradiol, progesterone and oxytocin in ovariectomized ewes *J Endocrinol* **151** 375-393

Wathes DC, Swann RW, Pickering BT (1984) Variation in oxytocin, vasopressin and neurophysin concentrations in the bovine ovary during the oestrous cycle and pregnancy *J Reprod Fertil* **71** 551-557

Wathes DC, Swann RW, Porter DG, Pickering BT (1986) Oxytocin as an ovarian hormone *Current Topics in Neuroendocrinol* **6** 129-152

Watson SJ, Akil H, Fischli W, Goldstein A, Zimmerman E, Nilaver G, van wimersma Griedanus TB (1982) Dynorphin and vasopressin: common localization in magnocellular neurons *Science* **216** 85-87

Williams BRG (1991) Transcriptional regulation of interferon-stimulated genes *Eur J Biochem* **200** 1

Williams CS, DuBois RN (1996) Prostaglandin endoperoxide synthase: Why two isoforms? *Am J Physiol* **270** (Gastrointest Liver Physiol 33) G393-G400

Wiltbank JN, Casida LE (1956) Alteration of ovarian activity by hysterectomy *J Anim Sci* 15 134-140

Wong WYL, Richards JS (1991) Evidence for two antigenically distinct molecular weight variants of prostaglandin H synthase in the rat ovary *Mol Endocrinol* **5** 1269-1279

Xie W, Chipman JG, Robertson DL, Erikson RL, Simmons DL (1991) Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing *Proc Natl Acad Sci USA* **88** 2692-2696

Xie W, Merrill JR, Bradshaw WS, Simmons DL (1993) Structural determination and promoter analysis of the chicken mitogen-inducible prostaglandin G/H synthase gene and genetic mapping of the murine homology *Arch Biochem Biophys* **300** 247-252

Yokoyama C, Tanabe T (1989) Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme *Biochem Biophys Res Commun* **165** 888-894

Yonehara S, Yonehara-Takahashi M, Ishi A, Nagata S (1983) Different binding of human interferon-a-1 and a-2 to common receptors on human and bovine cells *J Biol Chem* **258** 9046

Zakar T, Olson DM, Teixeira FJ, Hirst JJ (1996) Regulation of prostaglandin endoperoxide H2 synthase in term human gestational tissues *Acta Physiol Hung* **84** 109-118

Zakar T, Teixeira FJ, Hirst JJ, Guo F, MacLeod EA, Olson DM (1994) Regulation of prostaglandin endoperoxide H synthase by glucocorticoids and activators of protein kinase C in the human amnion *J Reprod Fertil* **100** 43-50

Zarco L, Stabenfeldt GH, Basu S, Bradford GE, Kindahl H (1988) Modification of prostaglandin F-2α synthesis and release in the ewe during the initial establishment of pregnancy *J Reprod Fertil* **83** 527-536

Zhang J, Weston PG, Hixon JE (1992) Role of progesterone and oestradiol in the regulation of uterine oxytocin receptors in ewes *J Reprod Fertil* **94** 395-404

Zhang J, Weston PG, Hixon JE (1991) Influence of estradiol on the secretion of oxytocin and prostaglandin F2α during luteolysis in the ewe *Biol Reprod* **45** 395-403

Zhang Q, Wu WX, Brenna JT, Nathanielsz PW (1996) The expression of cytosolic phospholipase A2 and prostaglandin endoperoxide synthase in

ovine maternal uterine and fetal tissues during late gestation and labor *Endocrinology* **137** 4010-4017

Zollers WG, Garverick HA, Smith MF, Moffatt RJ (1993) Concentrations of progesterone and oxytocin receptors in endometrium of postpartum cows expected to have a short or normal oestrous cycle *J Reprod Fertil* **97** 329-337