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REGULATION OF PROSTAGLANDIN E_2 (PGE_2) AND $PGF_{2\alpha}$ PRODUCTION BY OXYTOCIN AND INTERFERON-τ IN BOVINE ENDOMETRIAL CELL LINES

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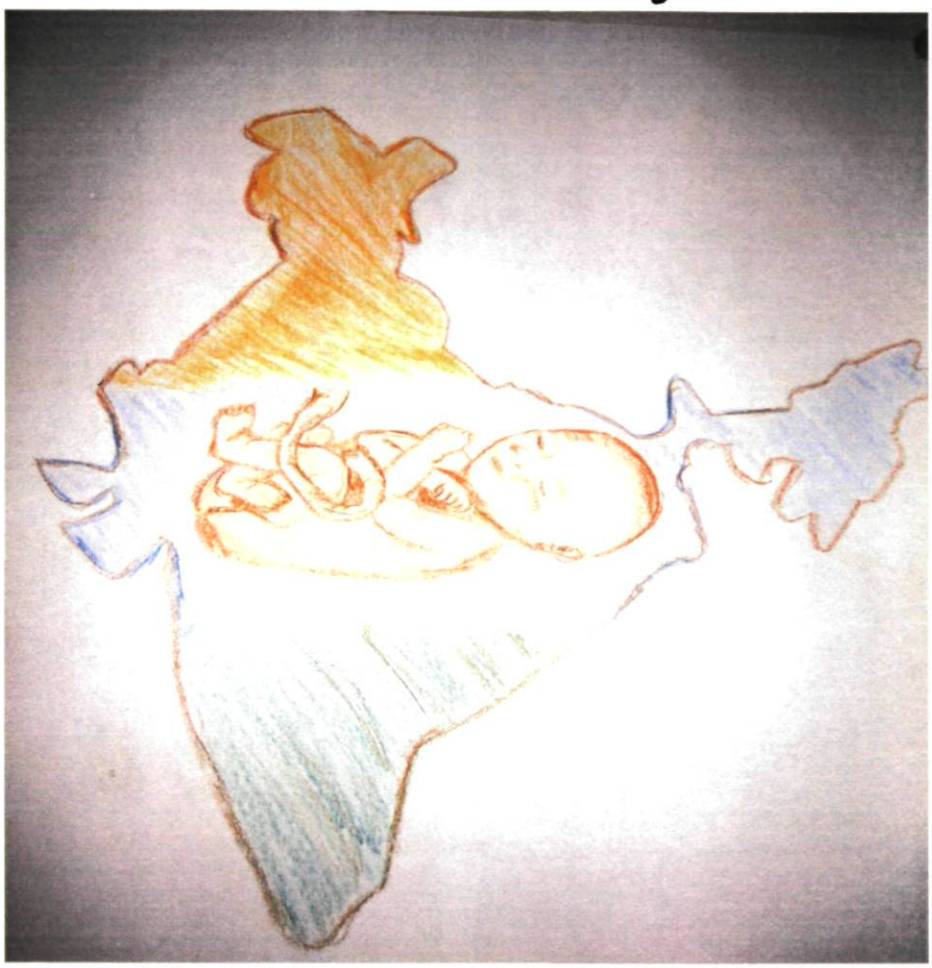
Dedicated to...

The Sentiments of



Motherly... *Love*

Mother-Land... *India*

Mother-Tongue... *Tamil*

Résumé

Chez les ruminants, la prostaglandine lutéolytique $F_{2\alpha}$ ($PGF_{2\alpha}$) est produite dans l'endomètre par les cellules endométriales épithéliales en réponse à l'oxytocine (OT). Par ailleurs, la prostaglandine potentiellement lutéoprotective et pro-nidation PGE_2 est stimulée dans les cellules stromales de l'endomètre en réponse au signal d'origine embryonnaire interféron-τ (IFNτ). Afin de pousser plus loin les études *in vitro* amorcées avec des cultures primaires, nous avons établi des lignées stables de cellules endométriales de l'endomètre bovin. Les cellules épithéliales (bEEL) et stromales caronculaires (CSC) ont servi à l'étude de la signalisation et de la régulation transcriptionnelle responsable de la production des prostaglandines (PGs). Des études phénotypiques et fonctionnelles ont révélé qu'autant les cellules bEEL que les CSC ont retenu les caractéristiques distinctives des cultures primaires. Les cellules bEEL s'avèrent « un modèle antilutéolique *in vitro* » typique car l'IFNτ y inhibe la production de $PGF_{2\alpha}$ induite par l'OT en 3-6 h. De plus, l'effet inhibiteur de l'IFNτ n'est pas médié par la diminution du récepteur à l'oxytocine ou l'expression de la cycloxygénase-2 (COX2) tel que stipulé chez le mouton. Les résultats suggèrent plutôt que l'IFNτ peut interférer dans l'axe de signalisation de la production de $PGF_{2\alpha}$ induite par l'OT. De ce fait, nous avons étudié le mécanisme de transduction du signal de $PGF_{2\alpha}$ induit par l'OT dans les cellules bEEL. Le relachement de $PGF_{2\alpha}$ induit par l'OT est dépendant de l'activation de Ras par la régulation extracellulaire du signal du module kinase ½ (ERK1/2). La transactivation d'EGFR, ainsi que l'activation de c-Src et PI3K sont requis pour l'activation de RAS.

En résumé, les résultats suggèrent que la sous-unité activé $G\alpha_i\beta\gamma$ serait impliquée dans la production de $PGF_{2\alpha}$ induite par l'OT. À l'instar de ce qui a été observé in vivo, l'IFNτ à concentration élevée stimule préférentiellement la production de PGE_2 dans les cellules CSC alors qu'il induit COX2 de manière concentration-dépendante dans les deux types de cellules bEEL et CSC. Il est donc tentant de spéculer que la reconnaissance maternelle de la grossesse chez les ruminants est un phénomène physiologique transitoire d'inflammation régulé par l'effet paracrine de l'IFNτ induisant le PGE_2 stromal.

Abstract

In ruminants, the luteolytic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is produced by endometrial epithelial cells in response to oxytocin (OT) whereas its luteoprotective and pro-nidatory counterpart PGE_2 is released by endometrial stromal cells in response to the embryonic signal interferon-τ (IFNτ). We have established stable bovine endometrial epithelial (bEEL) and caruncular stromal (CSC) cell lines to study the signalling and transcripitional mechanisms underlying the regulation of PGs production. Phenotypical and functional studies revealed that both bEEL and CSC retain the cardinal characters of endometrial cells in primary culture. Epithelial bEEL cells constitute an ideal '*in vitro* antiluteolytic model' where IFNτ was found to inhibit OT induced $PGF_{2\alpha}$ production within 3-6 h. Since the inhibitory effect of IFNτ is not mediated by either down-regulation of OT receptor or cycloxygenase-2 (COX2) as hypothesized in ewes, we propose that IFNτ may disrupt the signalling axis of OT induced $PGF_{2\alpha}$ production. Signalling studies with kinase inhibitors showed OT induced production in bEEL involved Ras-dependent activation of extracellular signal regulated kinase ½ (ERK1/2) with phosphoinositide 3 kinase (PI3K), c-Src tyrosine kinases and transactivation of epidermal growth factor receptor (EGFR) as intermediates of activated $G\alpha_i\beta\gamma$. Interestingly, high concentrations of IFNτ stimulated PGE_2 production from the CSC cells. Collectively, the results suggest that IFNτ inhibits $PGF_{2\alpha}$ secretion by epithelial cells by hampering the OT signalling pathway and stimulating PGE_2 production in stromal cells.

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Preface

For the past two decades, primary cultures of the bovine endometrial epithelial and stromal cells have been used to study the effects of oxytocin (OT), sex steroids and interferon-tau (IFNτ) on prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) production. Developing a primary culture is laborious, time-consuming and suffers from the risks of microbial contamination and hindered by limited lifespan of resulting cells. Thus, the basic idea of the project was to establish stable bovine endometrial epithelial and stromal cell lines that retain the properties of primary cultures.

We could easily establish immortalized bovine endometrial stromal cell lines from the caruncular and intercaruncular regions using SV40 Large T Antigen (TAG). A detailed characterization of our best caruncular stromal cell line (CSC) indicated that it is of mesenchymal origin and produces more PGE_2 than $PGF_{2\alpha}$, a key feature of primary stromal cells. Treatment of CSC with phorbol ester and increasing concentrations of IFNt indicated that IFNt stimulated COX2 expression and PGE_2 production especially at higher concentrations ($\geq 2\mu gml^{-1}$). Since endometrial stroma expresses most of the IFN stimulated genes (ISG) (Bazer and Spencer 2006), CSC may be a good model to study their transcriptional regulation.

We made many unfruitful attempts to introduce immortalizing genes (SV40 TAG and human telomerase-hTERT) using different gene delivery techniques such as electroporation and lentiviral infection into epithelial cells in primary culture. However, we were fortuitous to get a spontaneously transformed bovine endometrial epithelial cell line (bEEL) following an attempt to introduce hTERT gene using lentiviral vector. The resulting bEEL cells expressed epithelial cell specific cytokeratin and OT responsiveness. Using this system, we have shown that IFNτ inhibited OT induced $PGF_{2\alpha}$ production through a mechanism not involving down-regulation of COX2 or OT receptor. Signalling studies indicated that OT induced $PGF_{2\alpha}$ production was linked to Ras-dependent activation of the extracellular signal regulated kinase ½ (ERK1/2) module through phosphoinositide-3-kinase (PI3K)/c-Src tyrosine kinase/epidermal growth factor (EGFR) receptor tyrosine kinase. In summary, bEEL cells appear as a good *in vitro* model to investigate OT mediated $PGF_{2\alpha}$ production and the antiluteolytic effects of IFNτ.

The authors of Chapter II are: Krishnaswamy N (did experiments and drafted the paper), Danyod G (contributed in the doing th experiments), Chapdelaine P (preparation of constructs, imparted skills on basic experimentation, discussions) and Fortier MA (supervisor of this article and thesis).

The authors of Chapter III are: Krishnaswamy N (did experiments and drafted the paper), Chapdelaine P (preparation of constructs, imparted skills on basic experimentation, discussions), Tremblay JP (Collaborator) and Fortier MA (supervisor of this article and thesis).

The authors of Chapter IV are: Krishnaswamy N (did experiments and drafted the paper), Chapdelaine P (data analysis, interpretation of western images, discussions) Taniguchi H (checked the reproducibility of the experiments in Fig. 1, 2, and 5, writing the article), Kauffenstein G (Calcium mobilization experiments, correcting the article), Danyod G (checked the reproducibility of the observation in ig. 1, 2, and 5) Chakravarti A (checked the reproducibility of the experiments in Fig. 3, 4 and calcium mobilization) and Fortier MA (supervisor of this article and thesis).

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List of Abbreviations

AA	arachidonic acid;
AKR1B5	aldoketoreductase 1 B5
ATP	Adenosine triphosphate
bEEL	bovine endometrial epithelial cells
BEND	bovine endometrial cells
COX	cyclooxygenase
cPGES	cytosolic PGE synthase
cPLA2	cytosolic phospholipase A2
CSC	caruncular stromal cells
DAG	diacyl glycerol
EGFR	epidermal growth factor receptor
ERK1/2	extracellular signal regulated kinase ½
ERα	estrogen receptor-α
E_2	estradiol
GPCR	guanine nucleotide-protein coupled receptor
IBMX	isobutyl methyl xanthine
ICSC	intercaruncular stromal cells
IFN	interferon
IFN-τ	interferon-τ
IP3	inositol 1,4, 5 triphosphate
ISG	interferon stimulated genes
JAK	janus kinase
MAPK	mitogen-actived protein kinase
mPGES	microsomal PGE synthase
MRP	maternal recognition of pregnancy
OT	oxytocin

OTR	oxytocin receptor
PG	prostaglandin
PGFM	$PGF_{2\alpha}$ metabolite
PI3K	phosphoinositide-3-kinase
PKC	protein kinase C
PLC	phospholipase C
PLA_2	phospholipase A_2
PMA	phorbol myristate acetate
PPADS	pyridoxal-phospate-6-azophenyl-2',4'-disulfonate
PR	progesterone receptor
PTx	pertussis toxin
P_4	progesterone
RB2	reactive blue 2
ROD	relative optical density
STAT	signal transducer and activator of transcription
SV40	simian virus 40
TAG	T antigen
TNFα	tumor necrosis factor-α

Chapter I

General Introduction

1.1 Prostaglandins

Prostaglandins (PGs) were initially believed to be secreted by the prostate gland and were isolated from the seminal fluid in 1935 by the Swedish physiologist, von Euler. They are produced by almost all nucleated cells of the body except lymphocytes. PGs are derivatives of 20-carbon fatty acids and those produced from arachidonic acid (AA) are termed series-2, of which prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are the central players involved in reproduction. PGs share a common initial biosynthetic pathway whereby hydrolysis of cell-membrane phospholipids, by phospholipase A_2 (PLA_2) liberates AA in response to the diverse physiological and pathological stimuli (Murakami, Nakatani et al. 1997). Upon release, AA is converted into unstable endoperoxide intermediates, PGG_2 and PGH_2 by the action of PG synthase H2/cyclooxygenase (COX) in a rate limiting reaction. Three isoforms of COX have been identified to date of which, the constitutively expressed COX-1 and inducible COX-2 are well studied (Smith, Garavito et al. 1996). The oxygenated intermediate PGH_2 is in turn metabolized by cell-specific terminal synthases and isomerases to release PGE_2 and $PGF_{2\alpha}$ (Fig. 1.1).

Prostaglandins (PG) are best known as paracrine lipid mediators of pain and inflammation and non-steroidal anti-inflammatory drugs (NSAID), which inhibit PG biosynthesis, are the mainstay of pain management. However, they equally regulate the entire gamut of physiologically important inflammatory functions. For instance, PGE_2 is involved in ovulation, implantation and cervical ripening, whereas $PGF_{2\alpha}$ regulates luteolysis, parturition and postpartum involution of the uterus in the female mammals (Weems, Weems et al. 2006).

1.2 The bovine estrous cycle and its endocrine control

The length of estrous cycle in the cattle is 21±3 days that can be divided conventionally into four phases: estrus, metestrus, diestrus and proestrus. Estrus is defined as the period when the female is receptive to the male and will stand for mating. It is characterized by

high levels of estradiol (E_2) and lasts for 12 to 18 hours in the cow. During metestrus, E2 precipitously drops due to ovulation and corpus luteum (CL) is formed; this phase lasts for about 3 days. Luteal phase, also called diestrus, is characterized by the high circulating levels of progesterone (P_4) produced by the growing CL. It starts about day 5 of the cycle, when an increase in blood concentration of progesterone can first be detected, and ends with beginning of regression of the CL on day 16 to 17. Proestrus is characterized by decreasing levels of P_4 and increasing levels of E_2 due to the rapid follicular growth. Morphological changes in the ovary are shown in Fig. 1.2.

FSH is secreted in small pulses and each pulse recruits the growth of a cohort of follicles and E_2 secretion. LH stimulates P_4 production from the CL. High levels of circulating P_4 exert a negative feedback on the LH pulses; thereby preventing the ovulation of the growing follicles during luteal phase. The lifespan of CL, governing the length of estrous cycle, is dependent on the pulsatile secretion of $PGF_{2\alpha}$ from the endometrium that causes luteolysis. An abrupt drop in circulating P_4 due to $PGF_{2\alpha}$ mediated luteolysis removes the block on LH; thus allowing E_2 from the Graafian follicle to induce preovulatory LH surge (Inskeep 1995). The endocrine profile of the estrous cycle is presented in Fig.1.3.

1.3 Paradigm of $PGF_{2\alpha}$ regulation in ruminants

It is indisputable that the pulsatile secretion of luteolytic $PGF_{2\alpha}$ from the ruminant endometrium is central to the control of the estrous cycle. Initial studies in the ewes showed that mechanical stimulation of the uterus during the early and late but not middle of the cycle stimulated $PGF_{2\alpha}$ production from the uterus. Because the mechanical stimulation of the uterus can activate OT release through Ferguson reflex, it was thought that OT might be responsible for $PGF_{2\alpha}$ secretion. In fact, infusion of OT at physiological concentrations evoked $PGF_{2\alpha}$ secretion during the early and late estrous cycle. Analysis of the OT receptors in the ovine uterus revealed the abundance of OTR in the endometrium rather than the myometrium was correlated with the ability of endometrium to produce $PGF_{2\alpha}$ in response to OT *in vitro*. Further, it was shown that E_2 potentiated the OT induced $PGF_{2\alpha}$ thus suggesting further synergism following P_4 priming (McCracken, Custer et al. 1999).

It has been shown that neurohypophyseal oxytocin (OT) initiates luteolysis by stimulating $PGF_{2\alpha}$ secretion from the endometrium and it is believed to be under the control of a central OT pulse generator. Injections of $PGF_{2\alpha}$ analogues induced an acute rise in the levels of OT and were found to be of luteal origin. Later, it was shown that the CL indeed released OT thus generating an amplification loop (Silvia, Lewis et al. 1991). In the endometrium, OT receptors (OTR) are expressed only in epithelial cells and up-regulated around day 13-15 (late luteal phase) of the estrous cycle (Robinson, Mann et al. 2001). It is hypothesized that OTR expression is upregulated by estrogen. In turn, the development of estrogen receptor α (ERα) in epithelial cells is blocked by progesterone during early luteal phase; this inhibition is overcome by the loss of progesterone receptors (PR) in epithelial cells by auto down-regulation during the late luteal phase in the ewes (Spencer and Bazer 2002). However, ERα is highly expressed during the mid-luteal phase in the bovine endometrium indicating species specific mechanisms (Kimmins and MacLaren 2001; Robinson, Mann et al. 2001). Since PR is absent in epithelial cells during early pregnancy, progesterone regulation of epithelial cell function is likely to be mediated by PR positive stromal cells (Robinson, Mann et al. 2001; Spencer and Bazer 2004).

1.4 Signalling pathway of OT induced PG production

Oxytocin signals through its cognate OT receptor (OTR) that belongs to the large family of membrane bound heptahelical guanine nucleotide-binding protein (G-protein) coupled receptors (GPCR) (Gimpl and Fahrenholz 2001). The heterotrimeric G proteins consist of α-, β- and γ-subunits. The α-subunit, which is classified into $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ based on amino acid identity, dissociates from the tightly bound βγ subunit following the exchange of GTP for GDP by the agonist-activated receptor (Goldsmith and Dhanasekaran 2007). In the bovine and ovine endometrial epithelial cells and human and rabbit amniotic cells, OT induced PG production is functionally coupled to $G\alpha_q$ subunit that activates phospholipase Cβ (PLC) (Flint, Leat et al. 1986; Moore, Dubyak et al. 1988; Asselin, Drolet et al. 1998; Jeng, Liebenthal et al. 2000). Activated PLCβ catalyzes phophatidyl inositol 4,5-biphosphate (PIP2) to produce second messangers such as inositol 1,4,5-triphosphate (IP3) and 1,2-diacyl glycerol (DAG), which are involved in the release of

intracellular Ca^{2+} and activation of protein kinase C (PKC), respectively. In Chinese hamster ovary (CHO) cells stably transfected with rat OTR, PKC activates p42/44 mitogen activated protein kinase (MAPK) or extracellular-signal regulated kinase ½ (ERK1/2) pathway to induce PGE_2 production (Strakova, Copland et al. 1998) (Fig. 1.4). Apart from $G\alpha_q$, pertussis toxin (PTx) sensitive $G\alpha_i$ also inhibits OT induced ERK1/2 activation in human and rat myometrial and CHO-OTR cells (Ohmichi, Koike et al. 1995; Strakova and Soloff 1997), whereas PTx had no effect on OT induced $PGF_{2\alpha}$ in the ovine endometrial explants (Burns, Mendes et al. 2001). It has also been reported that OT induced ERK1/2 phosphorlylation is mediated through the transactivation of epidermal growth factor receptor (EGFR) tyrosine kinase by the $G\beta\gamma$ subunit in the myometrial and COSM6-OTR cells (Zhong, Yang et al. 2003). The OT mediated $PGF_{2\alpha}$ production in the bovine endometrium is assumed to follow that of the ewe, but it has not been studied in detail.

1.5 Maternal recognition of pregnancy in ruminants

Following a fertile estrus, the embryo of a eutherian mammal follows a programmed path of cell cleavage and development until the blastocyst is formed. Implantation is the process by which the blastocyst attaches to the endometrium. Preimplantation development of the conceptus is more extensive in the ruminants where the hatched blastocyst continues to expand for several days of early pregnancy. In fact, recognition of pregnancy precedes implantation in ruminants. The spherical expansion is mainly limited to the trophectoderm and around when the spherical blastocyst exceeds 15 cm in length, it initiates implantation at caruncles, which are specialized non-glandular areas of the endometrium. During the process of preimplantation elongation, the conceptus secretes a wide variety of factors into the uterine lumen. They are collectively called 'conceptus secreted proteins' and were shown to inhibit endometrial $PGF_{2\alpha}$ release. Later, a low molecular secretory protein (18-24 KDa) was purified from cultured ovine conceptus and was termed ovine trophoblastic protein-1 (oTP-1) (Godkin, Bazer et al. 1982). Shortly thereafter, bovine trophoblastic protein-1 (bTP-1) was isolated. Infusion of oTP-1 into the uteri of non-pregnant ewes resulted in an extension of luteal lifespan comparable to that observed with total conceptus secretory products and crippled the pulsatile release of $PGF_{2\alpha}$ from the endometrium. These

observations and the fact that oTP-1 production was limited to the critical window of maternal recognition of pregnancy pointed at this peptide as the antiluteolytic embryonic signal. Molecular cloning of oTP-1 and bTP-1 later revealed that they belong to the type I family of interferons and according to their trophoblast origin, was termed trophoblastic interferons (IFNτ).

Trophoblastic cells of the elongating bovine conceptus secrete massive quantities of interferon-τ (IFNτ) on day 17-19 that coincides with the critical window of maternal recognition of pregnancy (MRP) (Roberts, Cross et al. 1992). It is the single largest protein elaborated by the bovine and ovine conceptus during the peri-implantation period. It acts on the endometrium by paracrine mechanism to rescue the CL from luteolysis by inhibiting the release of pulsatile secretions of $PGF_{2\alpha}$ and is considered to be the maternal recognition signal. In fact, one of the consistent observations is that intrauterine administration of recombinant IFNτ extends the estrous cycle length in ruminants and is used as a model to study the antiluteolytic effects of IFNτ (Roberts, Cross et al. 1992; Chen, Green et al. 2006) (Fig. 1.5). The widely accepted model of antiluteolytic activity of IFNτ is based on studies in ewes. According to this model, IFNτ causes transcriptional repression of ERα during early pregnancy in the ovine endometrium.. This blocks the up-regulation of COX2 and OTR during the luteolytic window (Fig.1.6).

IFNτ belongs to the family of Type-I interferon; it signals through the JAK-STAT pathway and induces the expression of IFN stimulated genes (ISG) (Fig. 1.7). Binding of IFNτ to its cognate IFNAR receptor cross activates the Janus protein tyrosine kinases, which then phosphorylate the latent cytoplasmic signal transducers and activators of transcription 1 and 2 (STAT-1 and 2). The tyrosine phosphorylation of STATs leads to the formation of two transcriptional activator complexes, IFNα activated factor and IFN stimulted gene factor 3 (ISGF3). These transcriptional activator complexes bind to the corresponding cis-binding elements in the DNA like gamma activated factor (GAF) and IFN stimulated response element (ISRE) to stimulate ISGs. Most of the ISGs are present in the endometrial stroma and deep glands. These include: signal transducer and activator of transcription (Stat) 1 and 2 (Stewart, Johnson et al. 2001), ISG17 (Hansen, Austin et al. 1997; Johnson, Burghardt et

al. 1999), β2-microglobulin (Vallet, Barker et al. 1991), IFN regulatory factor one (IRF-1) (Spencer, Ott et al. 1998), Mx protein (Ott, Yin et al. 1998), granulocyte chemotactic protein-2 (Teixeira, Austin et al. 1997) and 2',5'-oligoadenylate synthetase (OAS) (Schmitt, Geisert et al. 1993). In addition to the cardinal JAK-STAT pathway, Type-I IFNs also induce STAT-1 independent genes (Kim, Choi et al. 2003).

1.6 Effect of IFNτ on COX2 expression and PGE_2 and $PGF_{2\alpha}$ production

It has been proposed that IFNτ inhibits $PGF_{2\alpha}$ release from the endometrial epithelial cells either by directly blocking COX2 or indirectly by preventing the up-regulation of OTR through the transcriptional repression of ERα. However, we and others have shown that COX2 is up-regulated during the luteolytic window and maternal recognition of pregnancy in both sheep and cow (Charpigny, Reinaud et al. 1997; Kim, Choi et al. 2003; Emond, MacLaren et al. 2004; Guzeloglu, Bilby et al. 2004) with higher levels of $PGF_{2\alpha}$ or its inactive metabolite during early pregnancy (Peterson, Tervit et al. 1976; Zarco, Stabenfeldt et al. 1988; Zarco, Stabenfeldt et al. 1988). Altered ratio of PGE_2 to $PGF_{2\alpha}$ may explain increased COX2, since higher concentrations of IFNτ stimulates PGE_2 in primary epithelial and BEND cell (Asselin, Bazer et al. 1997; Binelli, Guzeloglu et al. 2000). Thus, IFNτ may either suppress the expression of COX2 possibly through IFN stimulatory response elements (ISRE) present in the COX-2 promoter to inhibit luteolytic $PGF_{2\alpha}$ (Pru, Rueda et al. 2001) or induce COX-2 to produce luteoprotective/luteotropic PGE_2 (Henderson, Scaramuzzi et al. 1977; Magness, Huie et al. 1981; Weems, Hoyer et al. 1985), which may act through the stromal EP2 receptors that is up-regulated during early pregnancy (Arosh, Banu et al. 2004). It has been shown that IFNτ stimulates COX2 expression through p38 MAPK pathway in BEND and myometrial cells (Doualla-Bell and Koromilas 2001; Guzeloglu, Subramaniam et al. 2004). Thus, unraveling the IFNτ transduction pathways in relation to PGE_2 and $PGF_{2\alpha}$ production from the epithelial and stromal cells may help to evolve a unifying molecular mechanism governing MRP in ruminants.

1.7 Importance of stable endometrial cell lines in bovine

Over the last 15 years, our laboratory has established primary cultures of endometrial epithelial and stromal cells as a model to study PG regulation in bovine. Despite the fact that primary culture is closest to the *in vivo* settings, stable cell lines are preferred for the following reasons: Availability of apparently healthy genitalia from day 1-5 of estrous cycle on a given day is random. They are highly vulnerable for bacterial contamination since abattoir is the most common source of tissues. Primary culture activities are suspended during the summer due to a high incidence of bacterial contamination and uterine pathologies (Erb and Martin 1980). Developing a primary culture is laborious, time and resource consuming and the success is often unpredictable. Separation of the epithelial cells from those of stromal cells is difficult; thus, primary culture may not be homogenous in terms of cell type. Contamination by other cell types increases the variability in response to the treatment. Above all, cells in primary culture have limited lifespan and die because of replicative senescence. At present, only one bovine endometrial epithelial cell line (BEND) derived out of spontaneous mutation, is available as a model to investigate the mechanisms regulating PG production (Staggs, Austin et al. 1998; Binelli, Guzeloglu et al. 2000). However, BEND cells do not respond to OT, produce more PGE_2 than $PGF_{2\alpha}$ in response to phorbol ester treatment (Parent, Chapdelaine et al. 2002; Guzeloglu, Michel et al. 2004), a feature typical of stromal cells (Fortier, Guilbault et al. 1988; Asselin, Goff et al. 1996) and have a mixed phenotype expressing epithelial and stromal cell markers (Binelli, Subramaniam et al. 2001). In addition low passage endometrial epithelial cells (Cell applications, Inc. Catalog no. B932-05, San Diego, CA) are also commercially available to investigate the mechanisms regulating PG production in the bovine. The low passage cells are OT responsive but their replicative senescence status is oblivious. In recent years, immortal endometrial epithelial and stromal cell lines have been established in the ovine and swine (Zhang, Paria et al. 1991; Johnson, Burghardt et al. 1999) and used to study cell-specific signalling and transcriptional mechanisms (Spencer and Bazer 1996; Stewart, Johnson et al. 2001). To date, information on OT responsiveness of the ovine endometrial epithelial cell is lacking.

1.8 Rationale and hypothesis of the project

Oxytocin is the trigger for the luteolytic pulses of $PGF_{2\alpha}$ from the endometrium and IFNτ, the maternal recognition signal in ruminants counteracts to prevent the release of $PGF_{2\alpha}$; however, the mechanisms by which it regulates COX2, the luteolytic $PGF_{2\alpha}$ in the epithelial cells and luteoprotective/luteotropic PGE_2 in the stromal cells is not well understood. And, there are no satisfactory endometrial cell lines available in the bovine to explore OT and/or IFNτ regulated signalling and transcriptional pathways influencing PG production.

General objectives

1. To generate stable *in vitro* cell lines by immortalizing the endometrial epithelial and stromal cells.
2. To validate the cell lines by phenotypic and functional studies.

Specific objectives

The cell lines will be used to

1. Study the effect of OT and IFNτ on PGE_2 and $PGF_{2\alpha}$ production in the bovine endometrial epithelial cells
2. Study the interaction of IFNτ with OT and other PG stimulators like phorbol ester in the bovine endometrial stromal cells.
3. Study define the signal transduction pathway of OT induced $PGF_{2\alpha}$ production.

Figure 1.1: Biosynthetic pathway of eicosanoids. Source: http://www.pinnaclefitness-online.com/Fitness-research/prostaglandin_%20pathway.JPG)

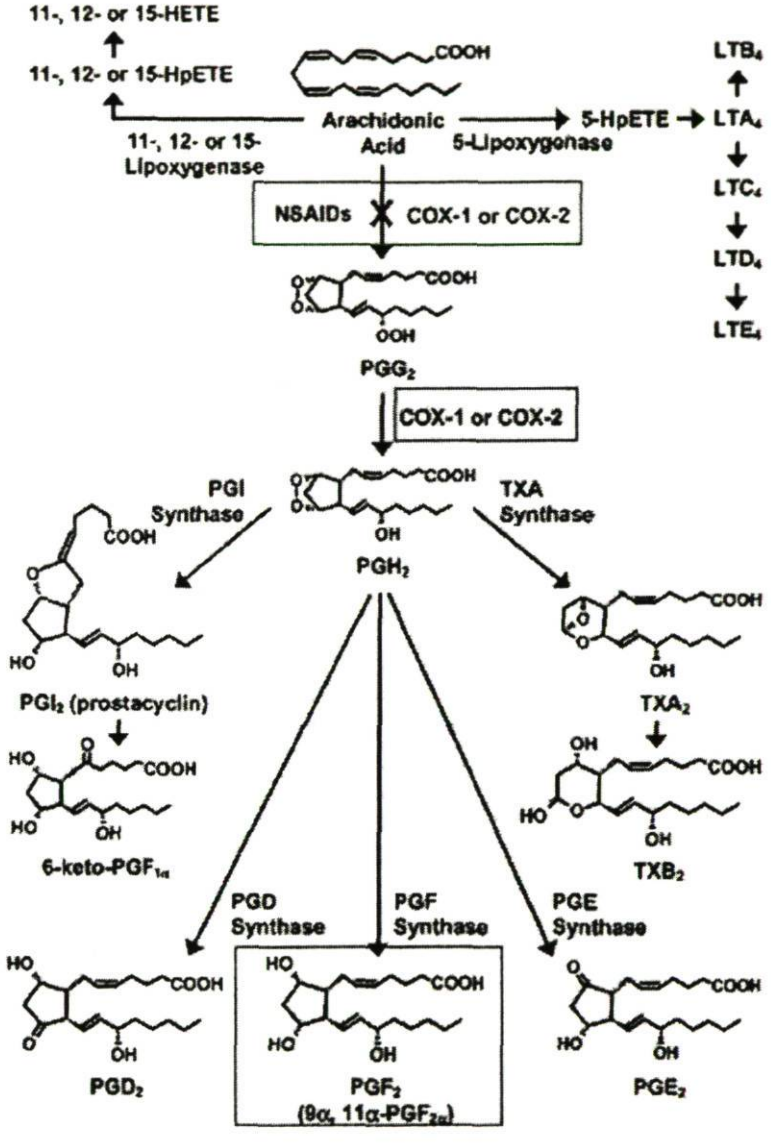


Figure 1.2: Ovarian changes during the estrous cycle in the bovine. (Source: Whitter, JC (1993) http://extension.missouri.edu/publications/DisplayPub.aspx?P=G2015)

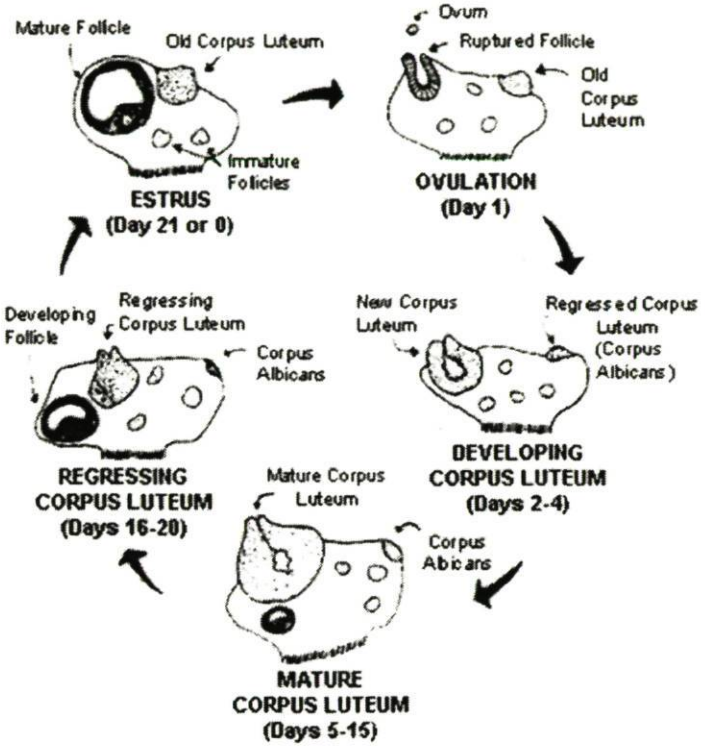


Figure 1.3: Endocrine changes during the estrous cycle in the bovine. (Source: Whitter, JC (1993) http://extension.missouri.edu/publications/DisplayPub.aspx?P=G2015)

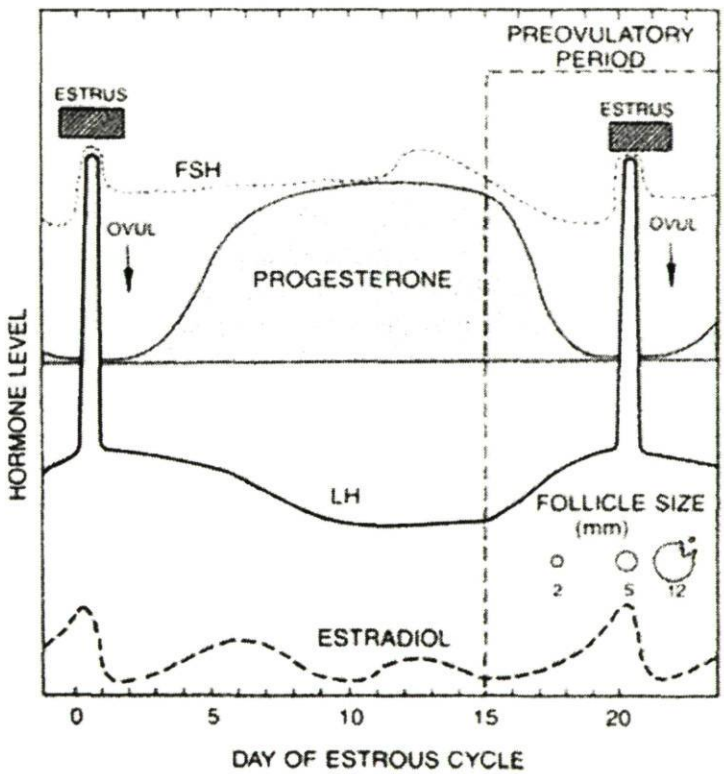
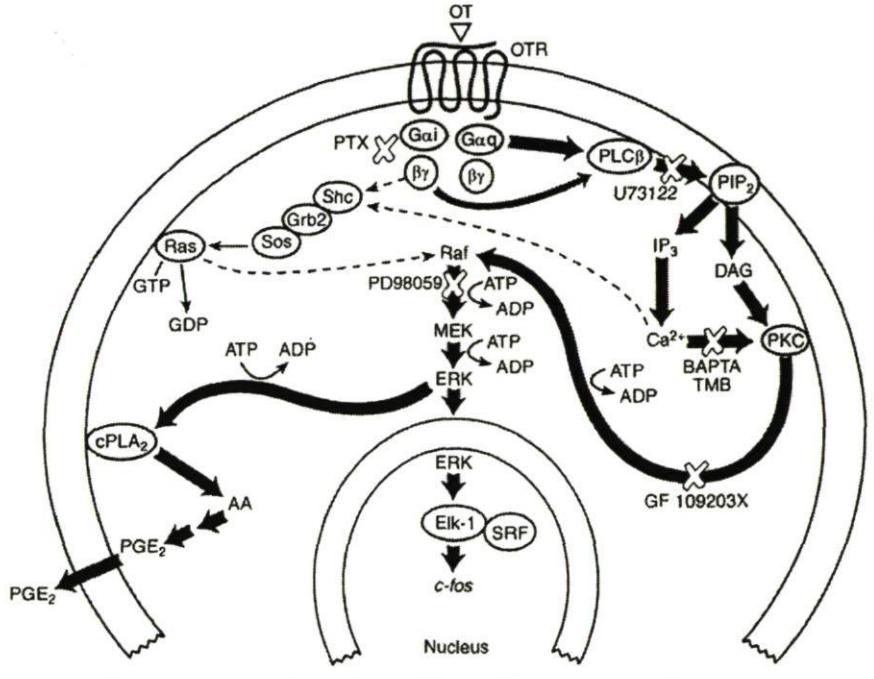


Figure 1.4: Signal transduction of OT induced PGE_2 production in Chinese hamster ovarian cells stably transfected with rat OT receptor (Strakova, Copland et al. 1998).



Strakova, Z. et al. Am J Physiol Endocrinol Metab 274: E634-E641 1998

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Figure 1.5: Intrauterine infusion of different truncated products of IFNτ extends the length of the estrous cycle in the ovine (Roberts, Cross et al. 1992). (p3 represents recombinant ov IFNτ variant and p3 Trn6 is its truncated counterpart)

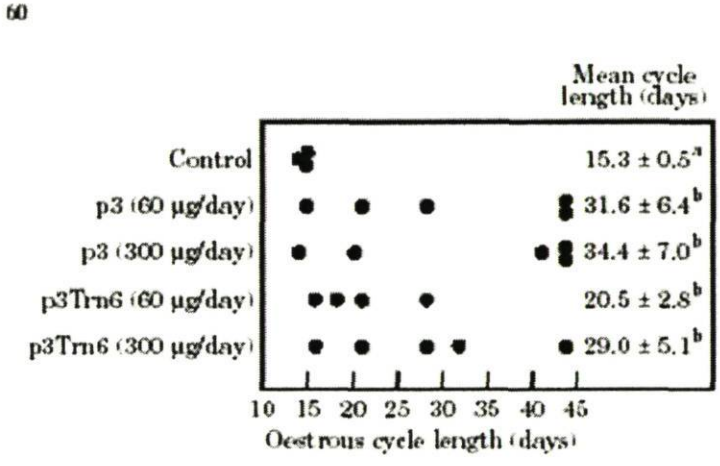


Figure 1.6: A hypothetical model explaining the antiluteolytic effect of IFNτ in the ovine (Spencer and Bazer 2004).

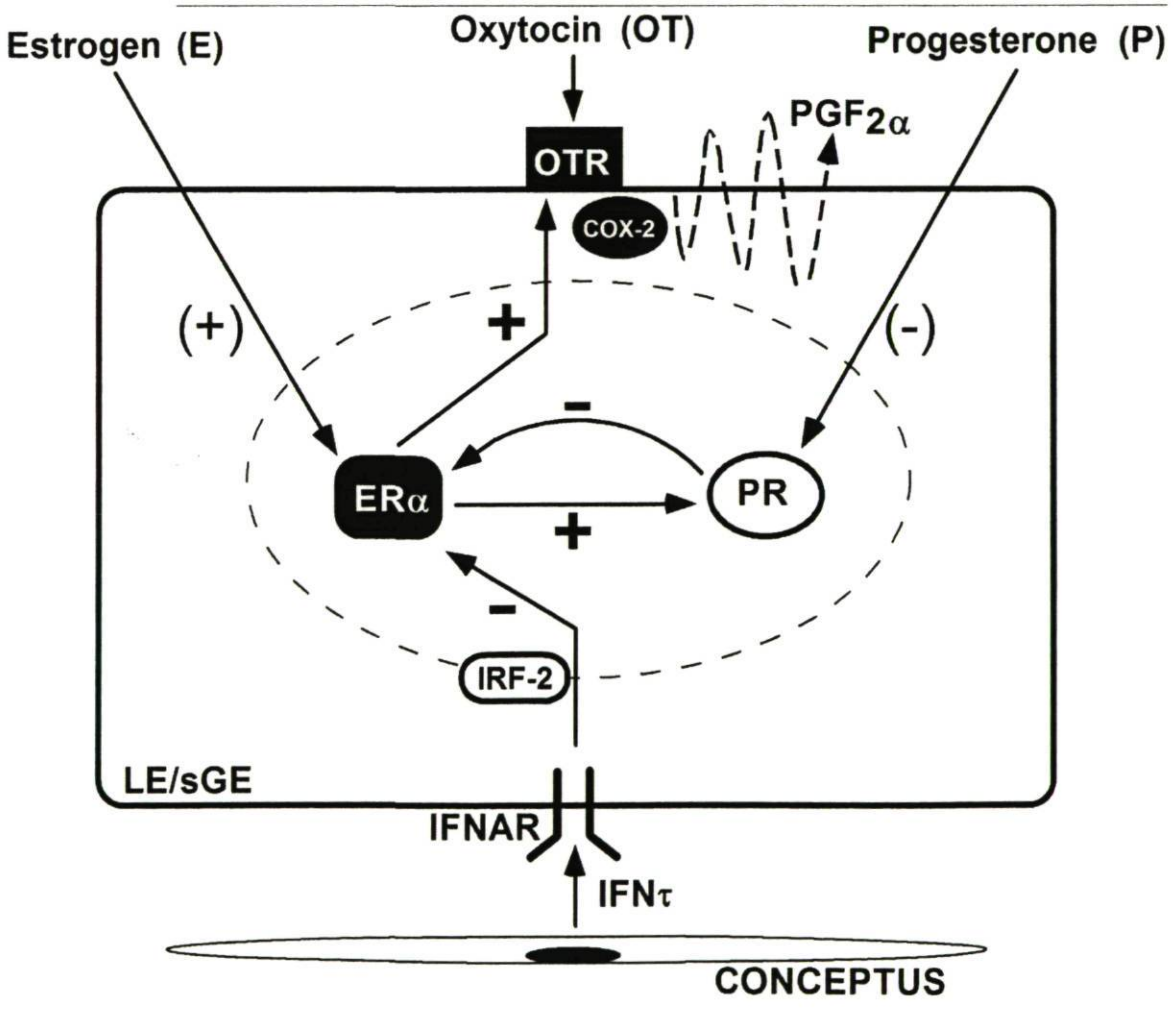
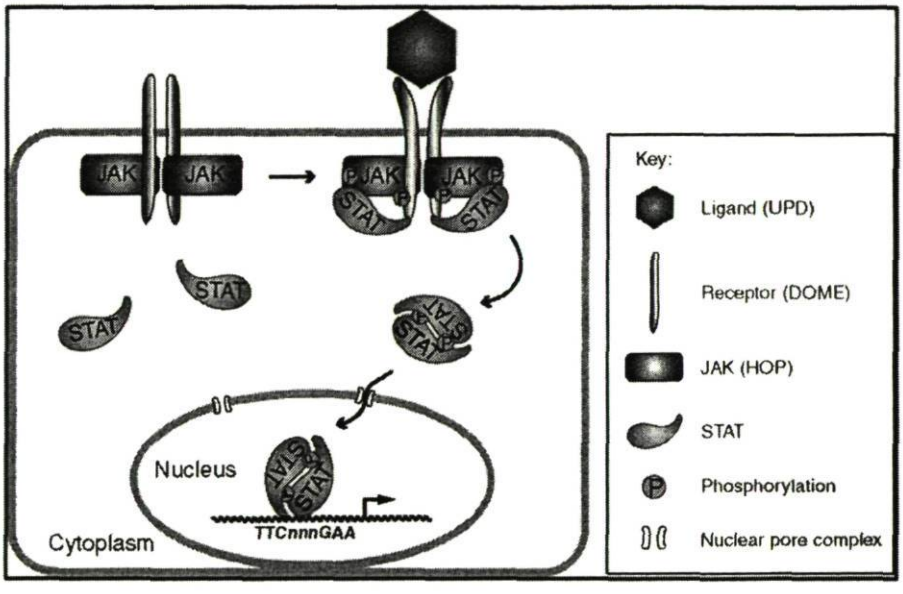


Figure 1.7: Signaling pathway of Type-I IFNs including IFNτ (Arbouzova and Zeidler 2006). UPD (ligand indicates IFNτ).
(Used with permission)



CHAPTER II

OXYTOCIN RECEPTOR DOWNREGULATION IS NOT NECESSARY FOR INHIBITION OF OXYTOCIN INDUCED $PGF_{2\alpha}$ PRODUCTION BY INTERFERON-τ IN A BOVINE ENDOMETRIAL EPITHELIAL CELL LINE

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Oxytocin receptor downregulation is not necessary for inhibition of oxytocin induced $PGF_{2\alpha}$ production by interferon-τ in a bovine endometrial epithelial cell line.

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Résumé

L'interféron-τ (IFNτ) est le signal embryonnaire responsable de la reconnaissance de la gestation chez les ruminants. On croit que l'action primaire d'IFNτ est médiée par l'inhibition de la prostaglandine F2α (PGF2α) libérée des cellules endométriales épithéliales en réponse à l'ocytocine (OT). Notre hypothèse de travail est que l'effet antilutéolytique d'IFNτ implique aussi la modulation de production de PG en aval du récepteur OT (OTR) et/ou de la cyclooxygenase 2 (COX2). Il n'y a actuellement aucune lignée de cellules endométriales sensibles à l'OT disponible pour étudier les mécanismes moléculaires sous-tendant nos hypothèses. En conséquence, nous avons d'abord établi une lignée de cellules épithéliales bovines immortalisées (bEEL) possédant la réponse à l'OT. Ces cellules sont cytokératine positives, expriment les récepteurs de stéroïdes et produisent plus de PGF2α que de PGE2. Les cellules bEEL répondent à l'OT en augmentant l'expression de COX2 et la production PGF2α de façon proportionnelle au temps et à la dose. IFNτ (20 ngml-1) inhibe significativement la production PGF2α induite par OT, mais étonnamment, l'effet n'est pas médié par la diminution d'OTR ou de COX2. Plutôt IFNτ augmente COX2 tout en diminuant la production de PG de façon dose dépendante. Cela suggère que l'expression de COX2 n'est pas une cible primaire pour l'effet antilutéolytique d'IFNτ. Parce qu'IFNτ inhibe la production de PGF2α stimulée par OT en moins de 3 heures, le mécanisme implique probablement une interférence directe au niveau des signaux de transduction de l'OT en aval d'OTR. En résumé, les cellules bEEL offrent un modèle unique pour étudier in vitro les mécanismes moléculaires et cellulaires sous-tendant la réponse et les interactions entre OT et IFNτ en lien avec la lutéolyse et la reconnaissance de la gestation chez le bovin.

(*Endocrinology* 150: 897–905, 2009)

Abstract

Interferon-τ (IFNτ) is the embryonic signal responsible for pregnancy recognition in ruminants. The primary action of IFNτ is believed to be mediated through inhibition of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released from the endometrial epithelial cells in response to oxytocin (OT). Our working hypothesis is that the antiluteolytic effect of IFNτ also involves modulation of PG production downstream of OT receptor (OTR) and/or cyclooxygenase 2 (COX2). There is currently no OT sensitive endometrial cell line to study the molecular mechanisms underlying our hypotheses. Accordingly, we have first established an immortalized bovine endometrial epithelial cell line (bEEL) exhibiting OT response. These cells are cytokeratin positive, express steroid receptors and exhibit preferential production of $PGF_{2\alpha}$ over PGE_2. The bEEL cells are highly sensitive to OT showing time and dose dependent increases in COX2 expression and $PGF_{2\alpha}$ production. Interestingly, IFNτ (20 $ngml^{-1}$) significantly inhibits OT induced $PGF_{2\alpha}$ production, but surprisingly, the effect is not mediated through down-regulation of either OTR or COX2. Rather, IFNτ up regulates COX2 in a time and dose dependent manner, while decreasing PG production. This suggests that COX2 expression is not a primary target for the antiluteolytic effect of IFNτ. Because IFNτ inhibits OT stimulated $PGF_{2\alpha}$ production within 3 hours, the mechanism likely involves a direct interference at the level of the OT signaling pathway in addition to the down regulation of OTR observed *in vivo*. In summary, bEEL cells offer a unique *in vitro* model for investigating the molecular and cellular mechanisms underlying OT and IFNτ response in relation with luteolysis and recognition of pregnancy in the bovine.

Introduction

In ruminants, complex interactions between endometrial cells and the embryo determine the fate of the ovarian corpus luteum toward recognition of pregnancy or return to a new estrous cycle. Paracrine interactions between the trophoblast and endometrial epithelial cells are essential for recognition and establishment of pregnancy (Spencer and Bazer 2004) as illustrated clearly in the uterine gland knock out model in the ewe (Gray, Taylor et al. 2001).

At the end of the luteal phase, the release of luteolytic pulses of $PGF_{2\alpha}$ by endometrial epithelial cells, in response to oxytocin (OT) of pituitary and ovarian origin, constitutes the default response. Trophoblastic interferon (IFNτ) is the embryonic signal released as a paracrine factor to prevent luteolysis and effect recognition of pregnancy. It has been proposed that IFNτ inhibits $PGF_{2\alpha}$ production in epithelial cells by preventing the up-regulation of estrogen receptor-α (ERα) and OTR through activating the transcriptional repressor, IFN-regulatory factor-2 (IRF2) (Spencer and Bazer 1996; Spencer, Johnson et al. 2007). In absence of OTR, OT is not able to induce the release of luteolytic $PGF_{2\alpha}$ by endometrial epithelial cells. This mechanism requires the presence of a viable embryo and of IFNτ for at least 3 days prior to the release of OT. However, *in vivo* observations derived from embryo transfer in the cow and *in vitro* experiments suggest that alternate mechanisms are also present to allow IFNτ to effect recognition of pregnancy within a much shorter period. In the cow, it is possible to obtain viable term pregnancies following transfer of blastocysts up to day 16, a mere 12 h before the onset of luteolysis (Betteridge, Eaglesome et al. 1980). In primary endometrial cell cultures, we and others have observed that IFNτ could block OT stimulated $PGF_{2\alpha}$ production in a time and dose dependent manner. It has been demonstrated that OT stimulation of $PGF_{2\alpha}$ production involves stimulation of COX2 expression; a limiting step in the production of PGs (Asselin, Drolet et al. 1997). However, COX2 is upregulated during early pregnancy both in the ewe (Charpigny, Reinaud et al. 1997; Kim, Choi et al. 2003) and cow (Guzeloglu, Bilby et al. 2004) and higher levels of $PGF_{2\alpha}$/PGFM are found in early pregnant compared to cyclic ewes (Zarco, Stabenfeldt et al. 1988; Zarco, Stabenfeldt et al. 1988). Intrauterine administration of IFNτ (Emond, MacLaren et al. 2004) as well as treatment of primary

endometrial cell cultures with IFNτ both upregulate COX2 (Asselin, Lacroix et al. 1997). Thus, the apparently conflicting observations of decreased $PGF_{2\alpha}$ production with increased COX2 during the implantation window suggest that IFNτ may modulate functional coupling of terminal PG synthases with COX2 rather than down regulating it. We have demonstrated that OT induces $PGF_{2\alpha}$ production in primary cultures of bovine endometrial epithelial cells (Asselin, Drolet et al. 1998). However, the precise endocrine and paracrine regulation of OTR expression in endometrial epithelial cells is not well understood (Gimpl and Fahrenholz 2001). It has also been proposed that IFNτ could favor establishment of pregnancy by promoting the production of PGE_2 relative to $PGF_{2\alpha}$ (Arosh, Banu et al. 2004).

The inhibitory effect of IFNτ on OT induced $PGF_{2\alpha}$ production has been reproduced *in vitro* (Tithof, Roberts et al. 2007). It has also been shown that IFNτ down regulates OTR at transcriptional level in bovine primary culture (Horn, Bathgate et al. 1998) and activates STAT1 & 2 in immortalized ovine luminal epithelial cells (Stewart, Johnson et al. 2001). Investigation of the mechanistic link between IFNτ si gnaling, OTR regulation, COX2 expression and $PGF_{2\alpha}$ in endometrial epithelial cells should provide a better understanding of recognition and establishment of pregnancy in ruminants.

We have been the first to implement the use of primary bovine endometrial cell cultures as a model to study the regulation of PG synthesis in relation with endometrial function (Fortier, Guilbault et al. 1988; Asselin, Goff et al. 1996; Parent, Chapdelaine et al. 2002). However, it is becoming increasingly difficult to have a constant supply of good quality primary cultures especially during summer. More importantly, cells in primary culture have limited lifespan and die because of replicative senescence. Therefore, a stable *in vitro* culture system of endometrial epithelial cells would represent an ideal model to study the signaling pathways and transcriptional mechanisms controlling COX2 expression and $PGF_{2\alpha}$ secretion. In fact, bovine endometrial epithelial cells have been proposed as a model to study the OTR regulation (Horn, Bathgate et al. 1998). At present, low passage endometrial epithelial cells, (Cell applications, Inc. Catalog no. B932-05, San Diego, CA) and bovine endometrial cells (BEND) are commercially available to investigate the mechanisms regulating PG production in the bovine. The low passage cells are OT

responsive but exhibit the variability and limited lifespan characteristic of primary cultures; BEND cells are stable, but lack OT responsiveness while exhibiting a mixed phenotype (Asselin, Goff et al. 1996; Binelli, Guzeloglu et al. 2000). Immortalized cell lines of luminal and glandular epithelial cells have been generated in the ewe, but data on OT responsiveness was not reported (Johnson, Burghardt et al. 1999; Wang, Johnson et al. 2000). We present here, a newly derived endometrial epithelial cell line that we used to study the molecular and cellular mechanisms involved in the interaction between OT and IFNτ on the regulation of PG production.

Materials and Methods

Generation of bovine endometrial epithelial (bEEL) cells

Primary epithelial cell cultures were established as described previously (Asselin, Goff et al. 1996) and infected with a replication defective lentiviral vector expressing human telomerase and puromycin resistance genes. After antibiotic treatment, 15 clones were selected and seeded. Because of an apparent growth arrest of all clones, we elected to harvest all the cells and plate them together to establish growth threshold concentration. Two weeks later, the culture reached confluency and the fastest growing clone was isolated by repeated passage. After 40 population doublings (PD), cells reached homogeneity but surprisingly did not express the telomerase transgene. We conclude that the resulting bovine endometrial epithelial cells (bEEL) represent a serendipitous cell line that evolved spontaneously or in response to telomerase without integration of the transgene. We have then conducted a thorough characterization to confirm the stability of the cell line and correspondence with the lineage sought for.

Immunofluorescence Analysis

bEEL cells were cultured on Lab Tek 4-well chamber slides (Nalge Nunc International Rochester, NY, USA) until confluency and fixed in -20°C methanol for 10 min for immunostaining with cytokeratin (Sigma) and vimentin (in house Ab) as described previously (Chapdelaine, Kang et al. 2006).

RT-PCR and real-time PCR

Total RNA (1μg), extracted from the cell line, was reverse-transcribed using random primer and Superscript III RT (Invitrogen Life Technologies Inc., Burlington, Ontario,

Canada). To demonstrate ERα and PR in the cell line, the following specific sets of primers were used: For ERα, the sense and antisense primers were 5′ATGACCCTACCAGACCTTTCAGT3′ and 5′ATTTGAGGCACACAAACTCTTC3′, respectively. Similarly, for progesterone receptor (PR), the forward and reverse primers were 5′ATTGTTGATAAAATCCGCAGAAA3′ and 5′GAGGTATCAGGTTTGCTGTTGTC 3′, respectively. ERα primers were deduced from Accession No. NM_001001443, while PR specific primers were designed from accession no. AY656812. Real-time PCR for OTR was done in a light cycler (Roche Diagnostics, Canada) with SYBR (Qiagen, Canada) with forward (AAGATGACCTTCATCGTCGTG) and reverse (CGTGAAGAGCATGTAGATCCAG) primers derived from AF101724 and 18S rRNA served as internal standard.

Enzyme Immunoassay of $PGF_{2\alpha}$

$PGF_{2\alpha}$ was assayed by competitive elisa using acetylcholinesterase –linked tracers (Cayman, Ann Arbor, MI, USA) as described previously (Asselin, Goff et al. 1996).

Western Blot analysis

An aliquot of 15μg protein was loaded in each lane, resolved on 12.5% SDS-PAGE and electro-transferred onto 0.45 μm nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada). The membranes were blocked in 5% (w/v) non-fat dried milk in phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST) for 1 h at room temperature and incubated overnight at 4°C with respective primary antibodies. The primary antibody dilutions were as follows: 1/3000 for anti-COX2 (Merck 241) (Kindly provided by Dr S Kargman, Merck Frosst Montreal); 1/1000 for STAT1 (BD Biosciences, Canada) and anti pS727 STAT1 (Upstate biotechnology, NY); 1/500 for OTR (Santa Cruz Biotechnology) and 1/5000 for βactin (Sigma). After three washes of 10 min each in PBS, the membranes were incubated for 1 h at room temperature with appropriate secondary antibody. The membranes were washed three times in PBST. The membranes were treated for 1 min with enhanced chemilumunescent substrate (Perkin-Elmer) and exposed to Bio-Max film. Relative optical density (ROD) of immunoblots from three replicates of each experimental protocol was quantitated by densitometry (Alpha imager).

Experimental protocols

To minimize a possible influence of cell generation and for better homogeneity, bEEL cells used for all the experiments were within 30-40 PD. Cells from frozen aliquots were grown to confluency, trypsinized and seeded @ 4 $X10^4$ $cellsml^{-1}$ in 24-well plates so that they reach confluency about 96 h later. The culture medium was then replaced and supplemented with 10% steroid free medium and cells incubated overnight. Cells were then treated according to specific protocols in serum free medium for 24 h unless indicated otherwise and culture medium was harvested and stored at -20°C until PG assay. Cell lysates preparation, protein extraction and estimation were done as described previously (Chapdelaine, Vignola et al. 2001). Cells were grown in 6-well plates for RT-PCR and real time PCR experiments. All experiments were replicated three times and each treatment was run in quadruplicate unless indicated otherwise.

Preliminary experiments indicated that OT, phorbol ester, TNFα and lipopolysaccharide stimulated both PGE_2 and $PGF_{2\alpha}$ production in the bEEL cells; however, the ratio of $PGF_{2\alpha}$ to PGE_2 was always in favor of the former; thus, we limit the presentation of our data to $PGF_{2\alpha}$ production. Western analysis of cPLA2 and terminal synthases revealed positive and constant expression without apparent regulation whereas COX2 was strongly regulated. Accordingly, we have elected to show only COX2 to represent the modulation of PG biosynthesis.

Following these pilot studies, we aimed at determination of optimal concentrations of OT and IFNτ with respect to $PGF_{2\alpha}$ production and COX2 and/or STAT1 expression in bEEL cells. We have then studied the time dependent $PGF_{2\alpha}$ production and relevant enzyme expression over 24 h period with optimal OT (10^{-7}M) and IFNτ (20 $ngml^{-1}$) doses. Lastly, the effect of IFNτ (20$ngml^{-1}$) on OT response was assessed at the level of $PGF_{2\alpha}$ production and COX2, STAT1 and OTR expression at 24 h. Then, the effect of co-incubation of OT and IFNτ (20$ngml^{-1}$) on $PGF_{2\alpha}$ production was studied at 3, 6, and 12h.

Results

Morphology, phenotype, growth and culture characteristics

Under phase contrast microscopy, confluent bEEL cells present honey comb morphology (4X) and a polygonal shape (100 or 200X) which are typical of primary epithelial cells. Immunofluorescence analysis confirmed that bEEL cells were positive for cytokeratin and

negative for vimentin; in clear contrast with endometrial stromal cells expressing an opposite expression profile (Fig.1). Expression of ERα and PR was demonstrated by RT-PCR (results not shown). bEEL cells could be grown past confluency without apparent signs of senescence for a minimum of three weeks thus allowing prolonged treatment with agents such as sex steroids to mimic the hormonal changes of the estrous cycle.

Effect of OT on PG production

Oxytocin induced a dose dependent increase in $PGF_{2\alpha}$ production that reached significant levels at concentrations as low as 10^{-9}M ($P<0.05$, Fig. 2A). The dose response is progressive and suggests the presence of OTR functionally coupled with PG production. OT also induced COX2 expression consistently across all concentrations tested (Fig. 2 B&C). Based on these response characteristics, we selected 10^{-7}M as the optimal OT concentration for further studies. $PGF_{2\alpha}$ production was stimulated in a time dependent manner by OT (10^{-7}M) and significant levels were reached as early as 3 h post-treatment. Similarly, COX2 expression was stimulated in a time dependent manner and significant at 6 h post treatment ($P<0.05$, Fig. 3). As a negative control, we have performed the same dose response experiment using a stromal cell line and found no OT response at any level (results not shown).

Effect of IFNτ on PG production

Interferon-τ induced a dose dependent increase in $PGF_{2\alpha}$ production that reached significant levels at concentrations in the high physiological range ($P<0.05$). However, the level of stimulation (3 fold) is much lower than that achieved in response to OT (50 folds) (Fig. 4A). Surprisingly, COX2 expression is up regulated by IFNτ at concentrations as low as 20 $ngml^{-1}$, but by contrast with $PGF_{2\alpha}$ production, the expression level is similar to that reached in response to OT (Fig. 5 B&C). Phosphorylation of STAT1 and upregulation of total STAT1 confirms that the typical Jak-Stat transduction system used by IFNτ is stimulated and functional in bEEL. Because of the particular response pattern to IFNτ, the 20$ngml^{-1}$ dose where COX2 expression but not PG production is increased was selected for the time dependent response. As could be expected, $PGF_{2\alpha}$ production by IFNτ does not differ significantly from the basal production (Fig. 5A). However, IFNτ treatment significantly upregulated COX2 and STAT1 as compared to the control ($P<0.05$,Fig. 5C).

Effect of IFNτ on OT response

Interferon-τ (20ngml^{-1}) significantly blocks OT induced $PGF_{2\alpha}$ production in bEEL cells at 24 h ($P<0.05$, Fig. 6A). The same pattern of response albeit at a much reduced level of PG production is observed and significant as early as 3 h after the onset of treatment and also at 6 and 12 h ($P<0.05$, Fig. 7). Western and densitometric analyses show that IFNτ does not alter the ability of OT to stimulate COX2 expression. Similarly, OT does not influence STAT-1 expression or phosphorylation nor does it affect the stimulation induced by IFNτ on these parameters (Fig. 6 B&C). Inhibition of OT stimulation of $PGF_{2\alpha}$ production is not accompanied by any effect at the OTR expression either at the messenger or the protein level (Fig. 6 B, C and D). The effect of IFNτ on OT response was tested at higher concentrations of IFNτ and the results were exactly the same (data not shown).

Discussion

We were able to generate a stable endometrial epithelial cell line from bovine endometrium with morphological and functional properties very similar to primary cultures. Unprecedentedly, this cell line expresses OTR functionally coupled with the regulation of $PGF_{2\alpha}$ production. To our knowledge, this is the first report of OT responsiveness in a stable ruminant endometrial cell line. These bEEL cells also respond to IFNτ thus allowing studying the molecular mechanisms underlying the actions of these important regulators of endometrial function at the time of maternal recognition of pregnancy. Indeed, OT and IFNτ interact to modulate the production of the luteolytic factor $PGF_{2\alpha}$ at the time of recognition of pregnancy. The currently accepted mechanism underlying recognition of pregnancy based on inhibition of increased pulsatile release of $PGF_{2\alpha}$ in response to OT is challenged by *in vivo* and *in vitro* observations. In ruminants, upregulation of COX2 during the luteolytic window (Charpigny, Reinaud et al. 1997; Arosh, Parent et al. 2002) together with increased OTR expression in epithelial cells during the late luteal phase support the view that OT initiates $PGF_{2\alpha}$ production and triggers luteolysis (Mann and Lamming 1995; Robinson, Mann et al. 2001). However, COX2, which is most often associated with increased production of PGs remains high in presence of a viable embryo in cows and ewes and the same is observed *in vivo* and *in vitro* in response to IFNτ (Asselin, Drolet et al. 1997; Kim, Choi et al. 2003). We have proposed that IFNτ mediated recognition of

pregnancy may be implemented by a reduction in the production of the luteolytic $PGF_{2\alpha}$ by epithelial cells relative to the putative luteotrophic PGE_2 by stromal cells (Parent, Chapdelaine et al. 2002). In the present report, using bEEL cells as an *in vitro* model, we demonstrate a direct upregulation of COX2 following OT stimulation as was previously shown in primary epithelial cells (Asselin, Drolet et al. 1997). A significant increase in $PGF_{2\alpha}$ as early as 3 h post-OT treatment ($P<0.05$, Fig. 3) and progressive dose dependent increase at concentrations up to 10^{-5}M (Fig. 2) suggest functional expression of OTR in bEEL cells. Spontaneous expression of OTR *in vitro* contrasts with estrogen driven upregulation *in vivo*, but as proposed previously (Fuchs, Drolet et al. 1998), it suggests that the expression may be under repressor control *in vivo*. Primary cultures of bovine endometrial epithelial cells have been shown to represent an ideal model to investigate OTR regulation (Horn, Bathgate et al. 1998) and now bEEL cells present the additional convenience of a stable cell line expressing OTR.

We also demonstrate time and dose dependent stimulation of COX2 and $PGF_{2\alpha}$ production in response to IFNτ. In addition, phosphorylation of STAT1, which is detectable after 24 h and upregulation of total STAT1, confirms the IFNτ responsiveness of the bEEL cells (Fig. 4&5). This is in agreement with the results on the immortal ovine endometrial luminal cells (Stewart, Johnson et al. 2001). Co-treatment of bEEL with OT and IFNτ indicates that OT does not affect the stimulatory effect of IFNτ on STAT1 (Fig. 7). However, IFNτ reduces by 50% the production of $PGF_{2\alpha}$ in response to OT while it does not influence COX2 expression. This combined with the observation that IFNτ induces COX2 expression as much as OT while being much less potent to stimulate $PGF_{2\alpha}$ production suggests that it may act on the coupling of COX2 with terminal synthases. At the molecular level in bEEL, COX2 and STAT1 can be considered as markers of OT and IFNτ responses, respectively. The observation that IFNτ could significantly block OT induced $PGF_{2\alpha}$ secretion is consistent with the antiluteolytic effects *in vivo* (Fincher, Bazer et al. 1986; Meyer, Hansen et al. 1995) and *in vitro* (Asselin, Drolet et al. 1997; Tithof, Roberts et al. 2007). Since the co-incubation of OT and IFNτ suppresses $PGF_{2\alpha}$ production significantly as early as 3-6 h ($P<0.05$) and OTR expression is not modulated even after 24 h post treatment, it is likely not mediated through down regulation of OTR. This is also supported by the observation

that pretreatment of bEEL cells with IFNτ ($20ngml^{-1}$) for 48 h before OT challenge does not increase the rate of inhibition (data not shown). It has been reported that COX2 is upregulated in the endometrium during early pregnancy in the ewe (Charpigny, Reinaud et al. 1997; Kim, Choi et al. 2003), cow (Guzeloglu, Bilby et al. 2004) and also following intrauterine infusions (Emond, MacLaren et al. 2004). Taken together, these results bring out two interpretations: First, IFNτ may mediate its antiluteolytic effect by uncoupling COX2 and $PGF_{2\alpha}$ synthase or interfere with OT signaling rather than down regulating COX2 or OTR. Second, COX2 appears as an IFNτ responsive gene that may be required not only for OT mediated luteolysis but also for IFNτ mediated recognition of pregnancy in the bovine. In summary, bEEL cell is a good *in vitro* model to investigate the mechanisms associated with the inhibition of OT induced $PGF_{2\alpha}$ production by IFNτ.

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Figure 2.1: Characterization of cytoskeletal proteins expressed in bEEL cell. Subconfluent bEEL cells were stained with cytokeratin and vimentin antibodies detected with a fluorescent secondary antibody. Left panels represent phase-contrast illumination, center and right panels show cytokeratin and vimentin fluorescence, respectively (Magnification 100X). Note the characteristic expression patterns of epithelial and stromal cells.

Figure 1

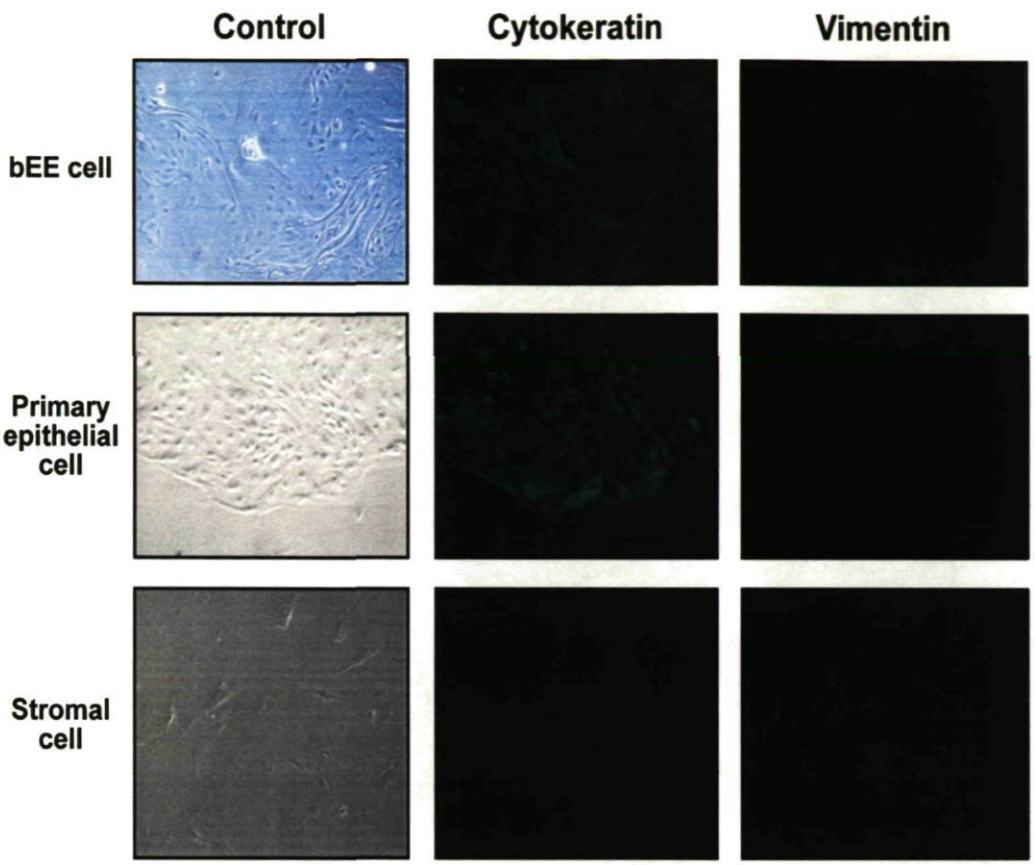

Control
Cytokeratin
Vimentin
bEE cell
Primary epithelial cell
Stromal cell

Figure 2.2: Effect of increasing concentrations of OT on COX2 and $PGF_{2\alpha}$ production in bEEL cell.

bEEL cells were treated with increasing concentrations of OT (10^{-9} to 10^{-5}M) for 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). A) $PGF_{2\alpha}$ production. B) Representative immunoblots of COX2 and βActin. Lanes 1-6 indicate different concentrations of OT; 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}M, respectively. C) Relative optical density (ROD) values are ratio between COX2 and βActin.

Figure 2

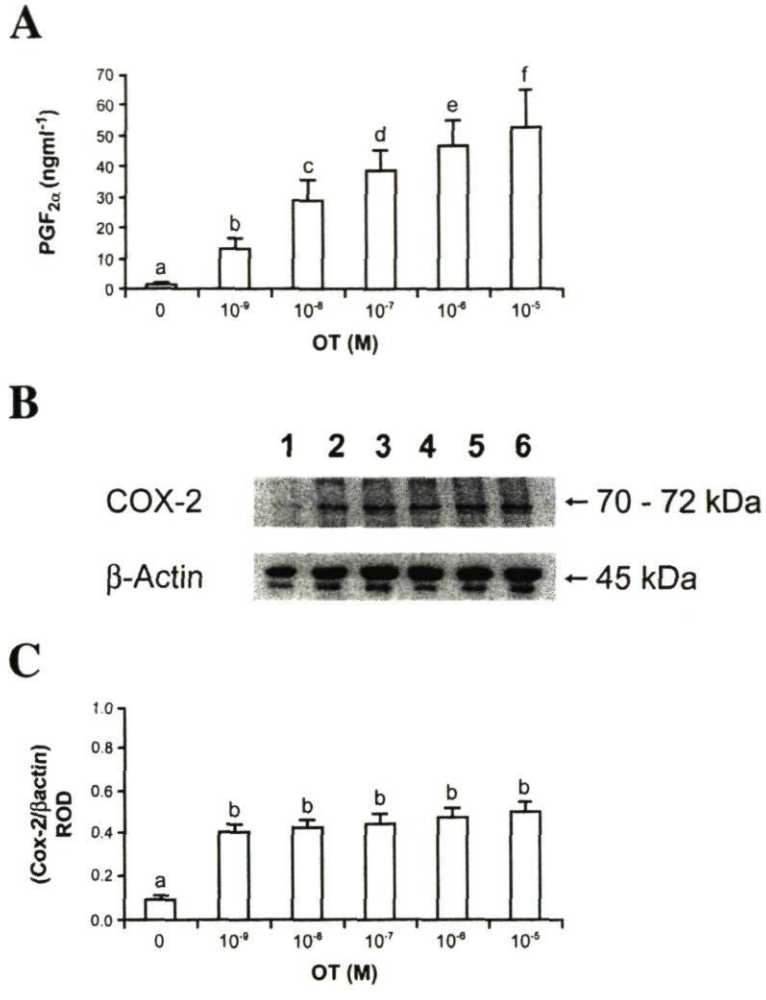

A
PGF2α (ngml-1)
70
60
50
40
30
20
10
0
a
b
c
d
e
f
0
10-9
10-8
10-7
10-6
10-5
OT (M)
B
1 2 3 4 5 6
COX-2
← 70 - 72 kDa
β-Actin
← 45 kDa
C
(Cox-2/βactin) ROD
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
b
b
b
0
10-9
10-8
10-7
10-6
10-5
OT (M)

Figure 2.3: Effect of OT (10^{-7}M) on COX2 and $PGF_{2\alpha}$ production in bEEL cells over a period of 24 hours.

bEEL cells were stimulated with OT (10^{-7}M) for 0, 3, 6, 12, 18 and 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). A) $PGF_{2\alpha}$ production. B) Representative immunoblots of COX2 and βActin. Lanes 1-6 represent time intervals; 0, 3, 6, 12, 18 and 24 h, respectively. C) Relative optical density (ROD) values are ratio between COX2 and βActin.

Figure 3

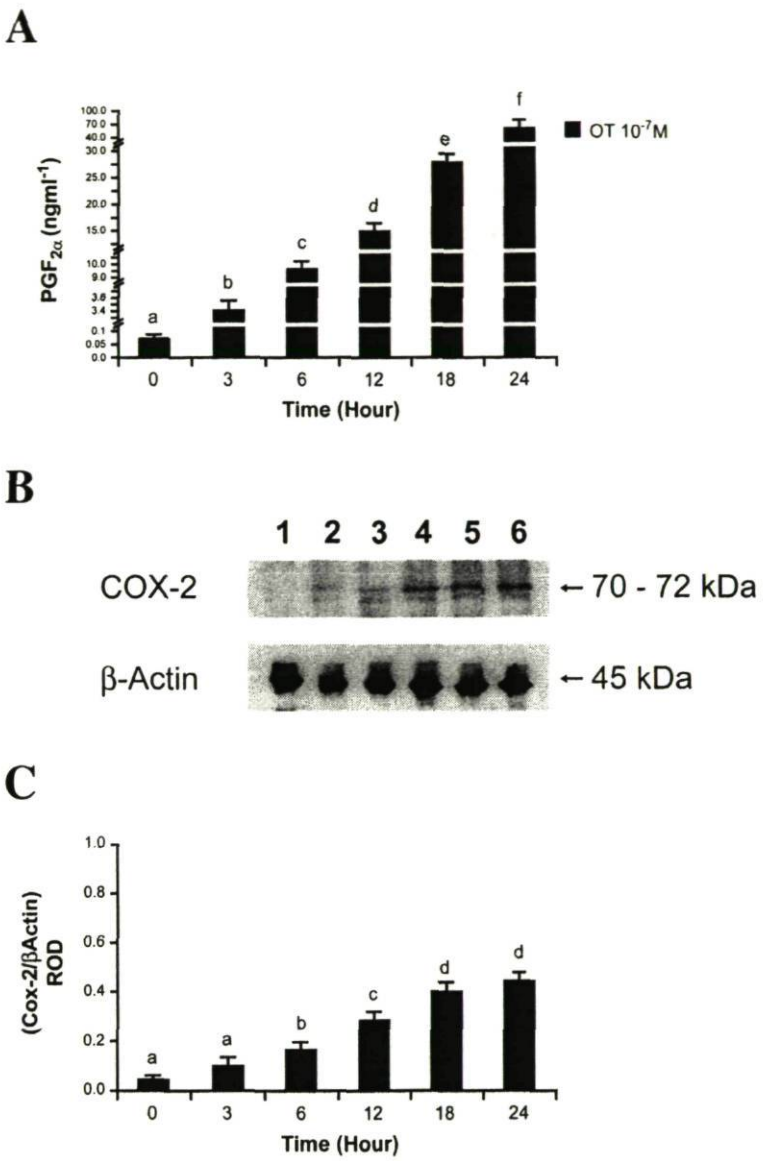
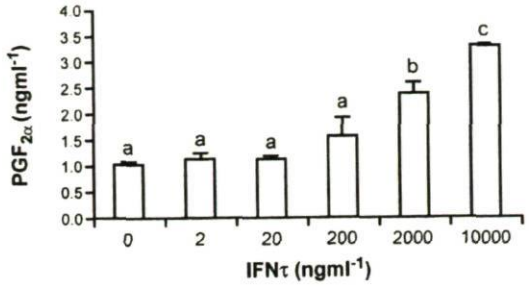

A
PGF2α (ngml-1)
OT 10-7M
a
b
c
d
e
f
0
3
6
12
18
24
Time (Hour)
B
1 2 3 4 5 6
COX-2
← 70 - 72 kDa
β-Actin
← 45 kDa
C
(Cox-2/βActin) ROD
Time (Hour)

Figure 2.4: Effect of different concentrations of IFNτ on COX2, pSTAT1 and STAT1 and $PGF_{2\alpha}$ production in bEEL cells.

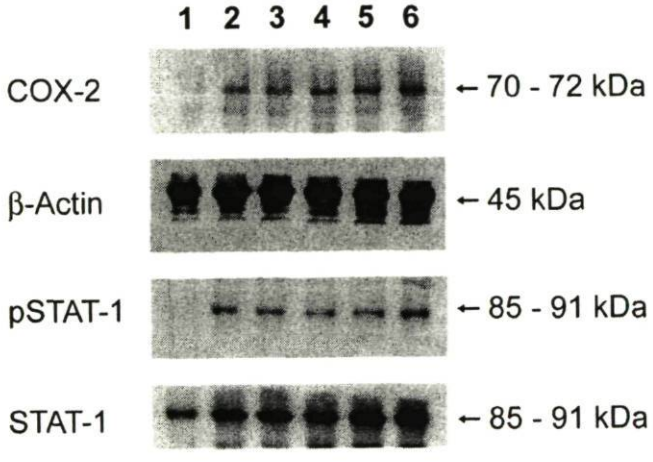
bEEL were treated with increasing concentrations of IFNτ (0-10000 $ngml^{-1}$) for 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent the Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). A) $PGF_{2\alpha}$ production. B) Representative immunoblots of COX2, βActin, phosphorylated and total STAT1. Lanes 1-6 represent different concentrations of IFNτ; 0, 2, 20, 200, 2000 and 10000 $ngml^{-1}$, respectively. C) ROD values are ratio between COX2/βActin and phosphorylated to total STAT1.

Figure 4

A


PGF2α (ngml-1)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
a
b
c
0
2
20
200
2000
10000
IFNτ (ngml-1)

B


1 2 3 4 5 6
COX-2
← 70 - 72 kDa
β-Actin
← 45 kDa
pSTAT-1
← 85 - 91 kDa
STAT-1
← 85 - 91 kDa

C

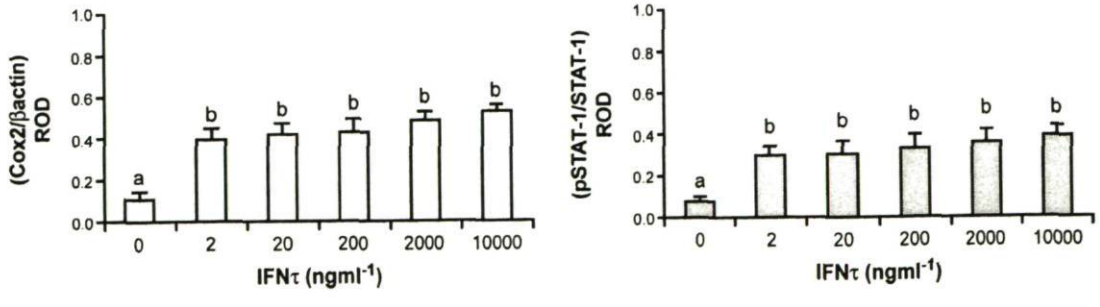

(Cox2/βactin) ROD
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
b
b
b
0
2
20
200
2000
10000
IFNτ (ngml-1)
(pSTAT-1/STAT-1) ROD
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
b
b
b
0
2
20
200
2000
10000
IFNτ (ngml-1)

Figure 2.5: Effect of IFNτ (20 $ngml^{-1}$) on time dependent expression of COX2, pSTAT1 and STAT1 and $PGF_{2\alpha}$ production in bEEL cells.

bEEL cells were treated or not with IFNτ (20 $ngml^{-1}$) for 0, 6, 12, and 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). A) $PGF_{2\alpha}$ production. B) Representative immunoblots of COX2, βActin, phosphorylated and total STAT1. Lanes 1-4 represent time intervals; 0, 6, 12 and 24 h, respectively. Left panel shows basal expression while right shows the effect of IFNτ (20 $ngml^{-1}$). C) ROD values are ratio between COX2/βActin and phosphorylated to total STAT1.

Figure 5

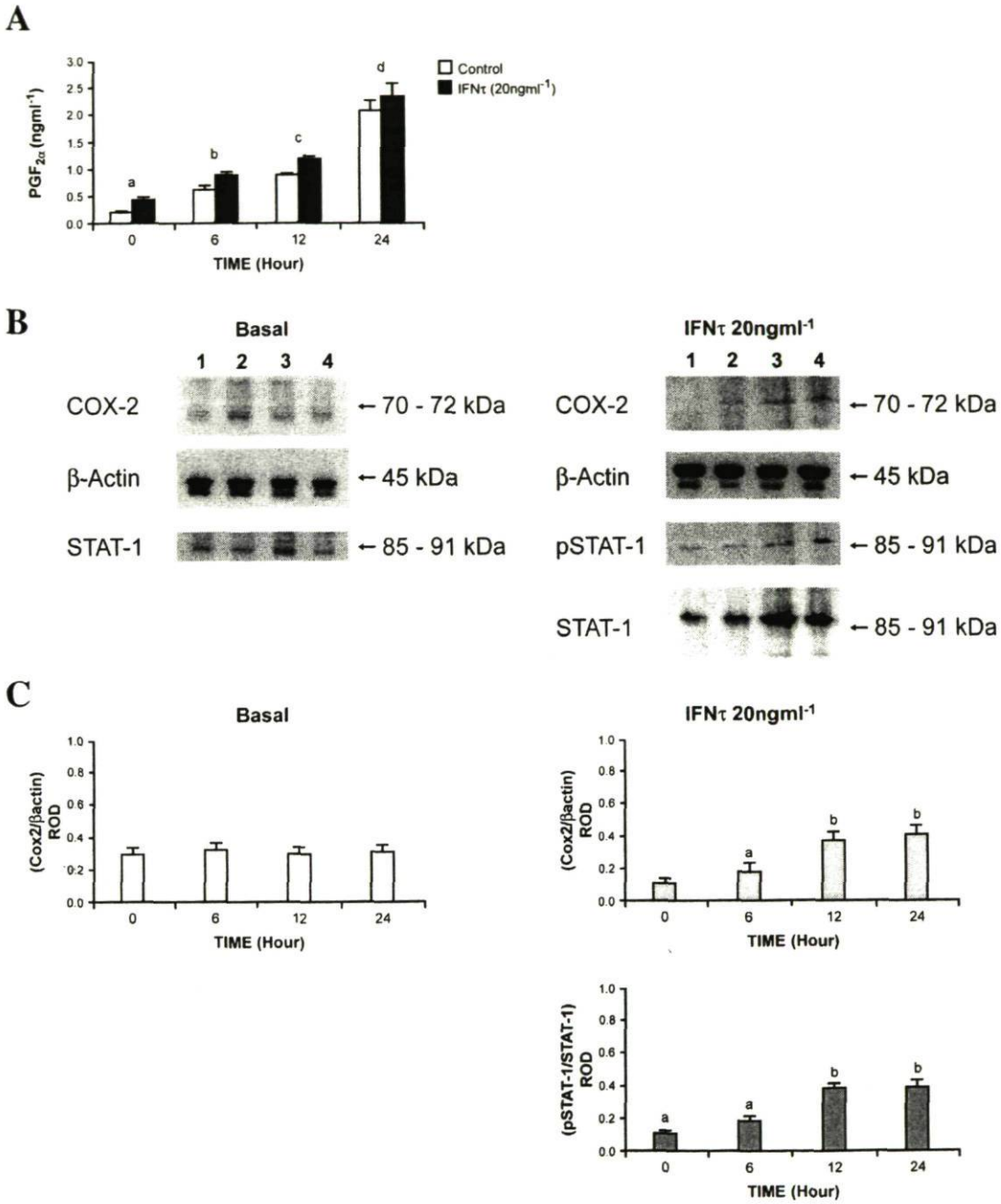

A
Control
IFNτ (20ngml-1)
PGF2α (ngml-1)
TIME (Hour)
B
Basal
IFNτ 20ngml-1
1 2 3 4
COX-2
β-Actin
STAT-1
pSTAT-1
← 70 - 72 kDa
← 45 kDa
← 85 - 91 kDa
C
(Cox2/βactin) ROD
(pSTAT-1/STAT-1) ROD
TIME (Hour)

Figure 2.6: Interaction between OT (10^{-7}M) and IFNτ (20 ngml^{-1}) in bEEL cell.

bEEL cells were treated simultaneously with OT (10^{-7}M) and IFNτ (20 ngml^{-1}) for 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). A) $PGF_{2\alpha}$ production. B) Representative immunoblots of COX2, OTR, βActin, phosphorylated and total STAT1. Lanes 1-4 represent; 1) Control 2) OT 10^{-7}M 3) OT 10^{-7}M+IFNτ 20 ngml^{-1} 4) IFNτ 20 ngml^{-1} C) ROD values are ratio between COX2/βActin, OTR/βActin and phosphorylated to total STAT1. D) Real-time PCR based quantitation of OTR transcripts. Values represent ratio of OTR/18S rRNA.

Figure 6

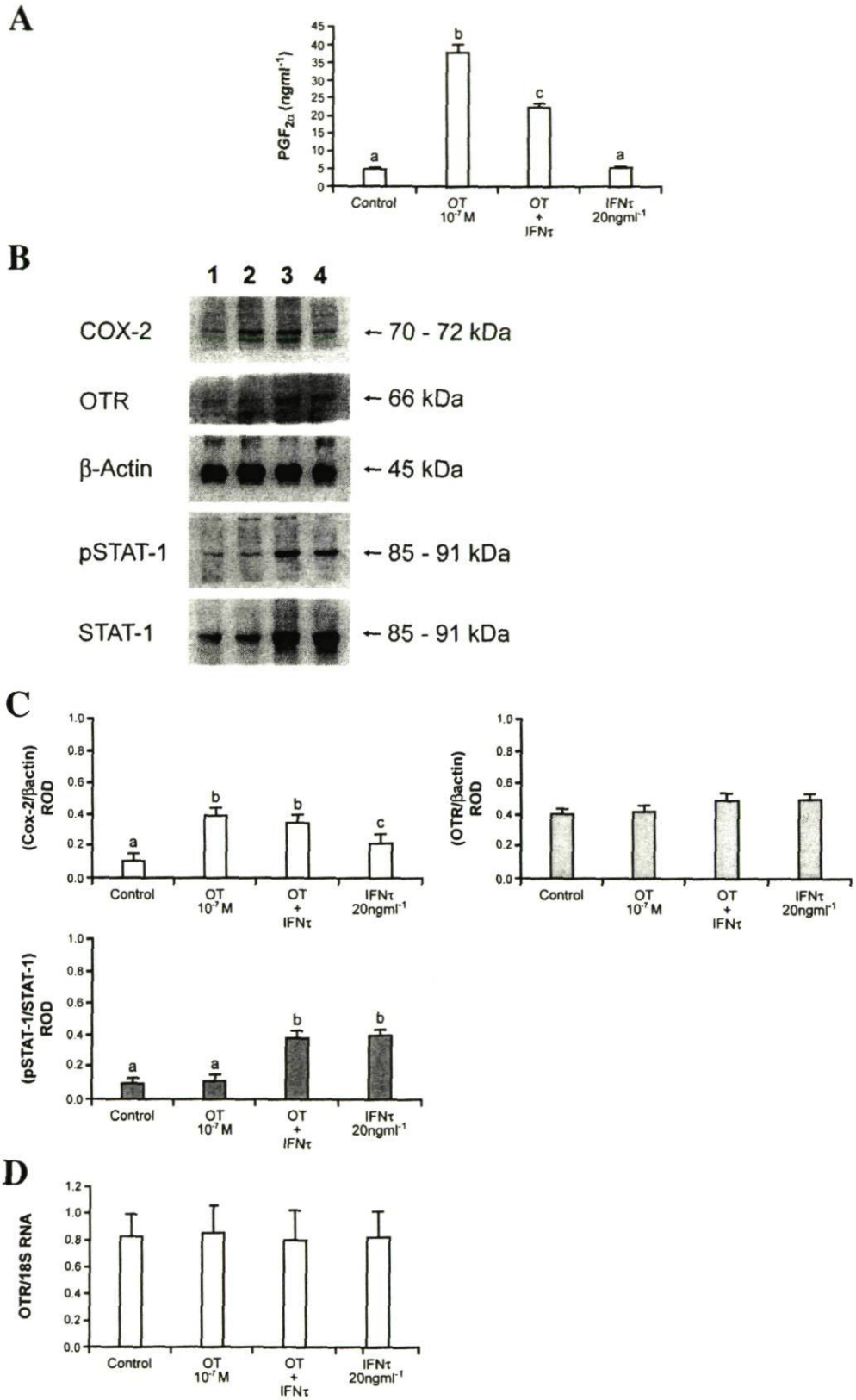
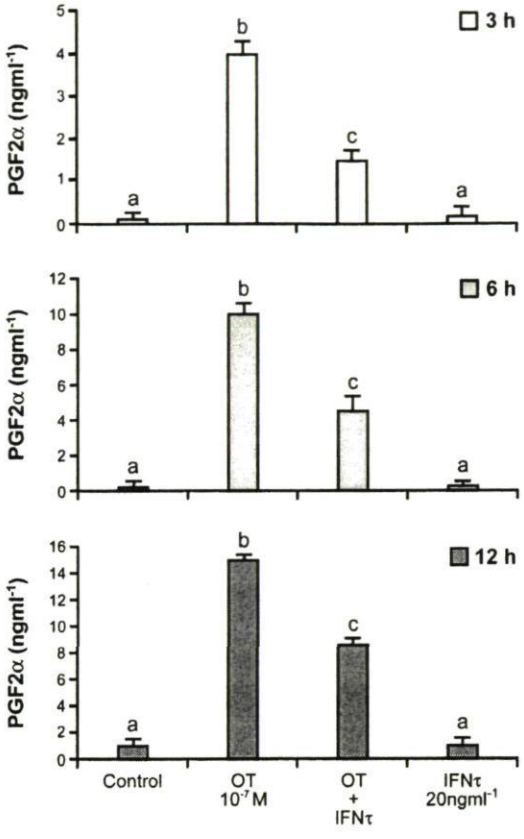

A
PGF2α (ngml-1)
Control
OT 10-7 M
OT + IFNτ
IFNτ 20ngml-1
B
1 2 3 4
COX-2
← 70 - 72 kDa
OTR
← 66 kDa
β-Actin
← 45 kDa
pSTAT-1
← 85 - 91 kDa
STAT-1
← 85 - 91 kDa
C
(Cox-2/βactin) ROD
(OTR/βactin) ROD
(pSTAT-1/STAT-1) ROD
D
OTR/18S RNA

Figure 2.7: Coincubation of OT (10^{-7}M) and IFNτ (20 $ngml^{-1}$) on $PGF_{2\alpha}$ production at 3, 6 and 12 h in bEEL cells.

bEEL cells were treated simultaneously with OT (10^{-7}M) and IFNτ (20 $ngml^{-1}$) for 3 to 12 h. $PGF_{2\alpha}$ was measured in the culture medium. Values represent $PGF_{2\alpha}$ production as Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$).

Figure 7


3 h
6 h
12 h
PGF2α (ngml-1)
a
b
c
Control
OT 10-7 M
OT + IFNτ
IFNτ 20ngml-1

Chapter III

OXYTOCIN (OT) INDUCED PROSTAGLANDIN $F_{2\alpha}$ ($PGF_{2\alpha}$) PRODUCTION INVOLVES Ras-DEPENDENT ACTIVATION OF EXTRACELLULAR SIGNAL REGULATED KINASE 1/2 (ERK1/2) BY $G\alpha_i\beta\gamma$ SUBUNIT IN BOVINE ENDOMETRIAL EPITHELIAL CELLS (bEEL)

Submitted to Endocrinology for publication (ENDO-09-0727 on June 23, 2009)

Oxytocin (OT) Induced Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) Production Involves Ras-Dependent Activation Of Extracellular Signal Regulated Kinase 1/2 (ERK1/2) By $G\alpha_i\beta\gamma$ Subunit In Bovine Endometrial Epithelial Cells (bEEL)

Narayanan Krishnaswamy, Pierre Chapdelaine, Hiroaki Taniguchi, Gilles Kauffenstein, Arpita Chakravarti, Ghislain Danyod and Michel A. Fortier

Unité d'Ontogénie et Reproduction, Centre Hospitalier Universitaire de Québec, Centre Hospitalier de l'Université Laval, Centre de Recherche en Biologie de la Reproduction (N.K., P.C., H.T. and G.D.), Centre de Recherche en Rhumatologie et Immunologie (G.K. and A.C.) and Département d'Obstétrique et Gynécologie, Université Laval (M.A.F.), Québec, G1V 4G2, Canada

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Running title: Signaling pathway of OT induced $PGF_{2\alpha}$ in the bovine

Key words: $PGF_{2\alpha}$, oxytocin signaling, luteolysis

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Reprint requests to corresponding author

Disclosure statement: The authors of this manuscript have nothing to declare.

Résumé

L'Ocytocine (OT) déclenche la libération pulsatile de la prostaglandine F2α ($PGF_{2\alpha}$) par les cellules endométriales épithéliales induisant ainsi la lutéolyse chez les ruminants. Nous avons proposé que le signal embryonnaire interféron-τ (IFNτ) exerce son effet antilutéolytique en perturbant le système de signalisation de l'OT. En conséquence, nous avons essayé de définir le sentier régulant la production de $PGF_{2\alpha}$ en réponse à l'OT en utilisant des inhibiteurs de kinases et la lignée de cellules épithéliales bovines (bEEL) développée récemment. OT (500nM) a rapidement activé ERK1/2 et la production de $PGF_{2\alpha}$ via l'axe Ras-Raf1-MEK-ERK1/2. Bien que la mobilisation du Ca^{++} intracellulaire en réponse à OT indique un couplage fonctionnel avec Gαq, la libération de $PGF_{2\alpha}$ était indépendante de la protéine kinase C (PKC) ou du sentier ERK1/2 Ca^{++} dépendant. Le degré et l'étendue d'inhibition par suramin et la toxine pertussis (PTx) indiquent que les sous-unités de protéine G participent au moins en partie à la production de $PGF_{2\alpha}$ induite par OT. L'activation du sentier de la protéine kinase A (PKA) avec l'AMPc a montré une faible synergie avec OT alors que l'activation de Gαi avec mastoparan induit une augmentation modeste de la production de $PGF_{2\alpha}$. L'inhibition de stimulation de l'OT par différents inhibiteurs de tyrosine kinases suggère une participation active de phosphoinositide-3-kinase (PI3K), c-Src et du récepteur du facteur de croissance épidermique (EGFR). L'inhibition de la sous-unité Gβγ n'a pas réduit la production de $PGF_{2\alpha}$ induite par OT alors que son activation a un effet stimulateur faible en comparaison d'OT. En résumé, les résultats suggèrent que la stimulation de production de $PGF_{2\alpha}$ en réponse à OT dans les cellules bEEL est couplée à une proteine G sensible à PTx et emprunte la voie de signalisation PI3K/c-Src/EGFR tyrosine kinase pour activer le phospho -relais Ras-dépendant du module ERK1/2.

Abstract

Oxytocin (OT) triggers the luteolytic pulses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrial epithelial cells in ruminants. We have proposed that the embryonic signal, interferon-τ (IFNτ) exerts its antiluteolytic effect by disrupting the OT signaling axis. Accordingly, we have attempted to define the signaling pathway of OT induced $PGF_{2\alpha}$ production in bovine endometrial epithelial cell line (bEEL) using kinase inhibitors. OT (500nM) rapidly activated ERK1/2 and $PGF_{2\alpha}$ production through the Ras-Raf1-MEK-ERK1/2 axis. Even though intracellular Ca^{2+} mobilization by OT indicated functional $G\alpha_q$, $PGF_{2\alpha}$ release was independent of the protein kinase C (PKC) or Ca^{2+} mediated ERK1/2 pathway. The degree and magnitude of inhibition by suramin and pertussis toxin (PTx) indicated that OT induced $PGF_{2\alpha}$ production was partly coupled to G-protein subunits. Activating the protein kinase A (PKA) pathway with cAMP mimetics showed a weak synergism with OT while activation of $G\alpha_i$ with mastoparan showed a modest increase in $PGF_{2\alpha}$ production. Inhibition of OT stimulation by different tyrosine kinases inhibitors suggested the involvement of phosphoinositide-3-kinase (PI3K), c-Src and epidermal growth factor receptor (EGFR). Inhibition of the Gβγ subunit did not reduce OT induced $PGF_{2\alpha}$ production and activation produced a weak increase in $PGF_{2\alpha}$ as compared to OT. In summary, the results suggest that OT induced $PGF_{2\alpha}$ production in bEEL cells is coupled to PTx sensitive $G\alpha_i\beta\gamma$ that signals through PI3K/c-Src/EGFR tyrosine kinase to activate Ras-dependent phospho-relay of the ERK1/2 module.

Introduction

Prostaglandins (PG), best known as lipid mediators of pain and inflammation, equally regulate the entire range of physiologically important inflammatory functions including ovulation, luteolysis, implantation, cervical ripening, parturition and postpartum involution of the uterus in

the female mammals (Weems, Weems et al. 2006). In ruminants, oxytocin (OT) triggers the pulsatile release of $PGF_{2\alpha}$ from the endometrium at the end of an infertile estrous cycle to induce luteolysis (McCracken, Custer et al. 1999; Spencer and Bazer 2004). OT receptor (OTR), which is upregulated during the late luteal phase and at estrus, is hypothesized to be downregulated during the early pregnancy by the conceptus derived trophoblastic interferon-τ (IFNτ) (Spencer and Bazer 2004). However, our *prima facie* evidence suggests an alternative hypothesis whereby IFNτ impairs the signaling axis of OT induced $PGF_{2\alpha}$ to exert its antiluteolytic effect in bovine endometrial epithelial cells (bEEL) (Krishnaswamy, Danyod et al. 2009). Understanding the signal transduction pathway of OT induced $PGF_{2\alpha}$ production will provide insight into the mechanisms underlying maternal recognition of pregnancy in ruminants.

OT signals through its cognate receptor that belongs to the large family of membrane bound heptahelical guanine nucleotide-binding protein (G-protein) coupled receptors (GPCR) (Gimpl and Fahrenholz 2001). In the ovine endometrium, OT stimulated the release of inositol 1, 4, 5-triphosphate (IP3) and diacyl glycerol (DAG) presumably through the activation of phospholipase C (PLC) (Flint, Leat et al. 1986) and DAG induced $PGF_{2\alpha}$ production (Silvia, Lee et al. 1994). In the bovine, OT stimulated the release of IP3 (Asselin, Drolet et al. 1997) and Ca^{2+} was essential for $PGF_{2\alpha}$ release (Burns, Hayes et al. 1998). OT was shown to induce PGE_2 production through a PLCβ–DAG-PKC- ERK1/2 (protein kinase C; extracellular signal regulated kinase 1/2) pathway in an OTR over expression model (Strakova, Copland et al. 1998). In addition, OT activated ERK1/2 (also called p42/44 mitogen activated protein kinase [MAPK]) pathway in the ovine endometrium (Burns, Mendes et al. 2001) and human breast Hs578T cells (Copland, Jeng et al. 1999). In general, OT mediated ERK1/2 activation is believed to be mediated through $G\alpha_q$ (Gimpl and Fahrenholz 2001). However, while pertussis toxin (PTx)

sensitive $G\alpha_i$ is involved in ERK1/2 activation in human and rat myometrial (Ohmichi, Koike et al. 1995; Strakova and Soloff 1997) and CHO-OTR cells (Strakova, Copland et al. 1998), PTx had no effect on OT induced $PGF_{2\alpha}$ in ovine endometrial explants (Burns, Mendes et al. 2001) or OT neurons of lactating rat (Wang and Hatton 2007).

In addition to the $G\alpha$ based paradigms, several GPCRs activate ERK1/2 pathway through tyrosine kinase /Ras dependent activation of $\beta\gamma$ subunits dissociating from $G\alpha_i$ (Goldsmith and Dhanasekaran 2007; May and Hill 2008; Smrcka 2008). Transactivation of epidermal growth factor receptor (EGFR) tyrosine kinase, following OT induced ERK1/2 phosphorylation in PHM1 and COSM6-OTR cells (Zhong, Yang et al. 2003), and suckling induced burst firing of supraoptical OT neurons appear to be mediated principally through $G\beta\gamma$ subunits (Wang and Hatton 2007). Thus, the available evidence suggests that $G\alpha_q$, PTx sensitive $G\alpha_{i/o}$ and $G\beta\gamma$ subunits are functionally linked to OT induced ERK phosphorylation depending on cell type, and the major intracellular events are release of Ca^{2+}, activation of PKC and transactivation of EGFR. Here, we provide evidence that OTR is coupled to PTx sensitive $G\alpha_i\beta\gamma$ in bEEL cells and OT induced $PGF_{2\alpha}$ production involves Ras-dependent activation of ERK1/2 pathway with phosphoinositide 3 kinase (PI3K), c-Src and EGFR as intermediates that link the ligand-activated $G\alpha_i\beta\gamma$ with Ras.

Material and Methods

Materials- RPMI 1640 media was purchased from Invitrogen, Canada; fetal bovine serum, HBSS, penicillin and streptomycin were from Multicell, Wisent Inc., Canada; 6 and 24-well format culture plates and other culture-wares like flasks, filters and pipettes were from Sarstedt, Canada. Biochemicals like ATP, 8Br cAMP, forskolin, GW5074, isobutyl methyl xanthine (IBMX), OT, pyridoxal-phospate-6-azophenyl-2',4'-disulfonate (PPADS) and suramin were

sourced from Sigma, Canada; AG1478, GF109203x, LY294002, mastoparan, mSIRK, munamycin, PD98059 and PP2 were from Calbiochem, Canada; BAPTA-AM, fura-2 AM and gallein was from Tocris Bioscience, Canada. Reactive blue 2 (RB2) was from ICN Biochemicals, USA. PTx was from List Biological Laboratories, Campbell, CA, USA. Protein marker and nitrocellulose membrane were from Bio-Rad, Canada; western enhanced chemiluminescent kit was from Perkin-Elmer; Biomax X ray film from Kodak Corporation, USA; laboratory chemicals like glycine, SDS, Tris, Tween-20, glycerol, dimethyl sulfoxide (DMSO), sodium and potassium phosphates and sodium chloride were from Fisher chemicals, Canada; Elisa plates were from Nunc Corporation, rabbit anti-sheep antibody (Ab) was from Jackson ImmunoResearch Laboratories, Inc., USA; $PGF_{2\alpha}$ Ab and acetyl choline linked tracer were from Cayman chemicals, USA.

Cell culture- Freeze-thawed bEEL cells were seeded in 75 cm^2 culture flasks containing RPMI 1640 supplemented with FBS (10%) and streptopenicillin (1%) and the media was replaced every 48 h. Confluent cultures were trypsinized and seeded in 24-well format culture plates (for experiments involving $PGF_{2\alpha}$ assay) or 6-well plates (for western blotting experiments) at $4X10^4$ cells/ml so that confluency was seen by 96 h. On day 5, cells were exposed to fresh steroid-free medium overnight for treatment on next day.

Treatment protocol- Serum free RPMI 1640 served as the solvent for the biochemicals. On day 6, each and preincubated for 1 h with vehicle (DMSO or ethanol or dicholoromethane) or inhibitor/activator. After aspirating the media, the cells were treated in the presence or absence of OT (500 nM) and/or inhibitor/activator for 6 h. Supernatant was harvested and stored at -20°C until assay for $PGF_{2\alpha}$. However, for the experiments shown in Figs. 3B, 4E and 6B, cells were treated with OT (500 nM) or ATP (100 μM), mastoparan (1 μM) or mSIRK (5 μM) without preincubation. For immuno-blotting (6-well dish), cells were serum starved for 3 h before

preincubation in order to minimize background ERK1/2 phosphorylation caused by the serum growth factors and then treated with or without OT for 3 min. The stimulation was stopped by the addition of ice-cold PBS. Cell lysate preparation, protein extraction and estimation were done as described previously (Chapdelaine, Vignola et al. 2001).

Enzyme immunoassay- $PGF_{2\alpha}$ was assayed by custom-made competitive ELISA as described previously (Asselin, Goff et al. 1996).

Western blotting- An aliquot of 20 µg protein was loaded in each lane, resolved in 10% SDS-PAGE and electrotransferred onto 0.45 µm nitrocellulose membrane. The membrane was blocked in 5% (w/v) non-fat dry milk resuspended in PBS containing 0.05% Tween-20 for 1 h and incubated overnight at 4°C in phospho-ERK1/2 Ab (Cell signaling, USA; 1/1000 dilution in 5% BSA-PBS-Tween-20). The membrane was washed for 10 min in PBS-Tween for 3 times and incubated with goat anti-rabbit (1/10000) Ab for 1h. After 3 washes of each 10 min, the membranes were exposed to enhanced chemiluminescent substrate. The membrane was stripped and reprobed for total ERK1/2 (Santa Cruz Biotechnology, USA; 1/1000 dilution). Relative optical density (ROD) of the immunoblots from three different experiments was quantitated using Alpha imager 2000 (Alpha Innotech Coportation, USA).

Intracellular Ca^{2+} mobilization- Intracellular Ca^{2+} mobilization was done as reported elsewhere (Ambler and Taylor 1986). Briefly, bEEL cells were seeded and grown as described above onto rectangular glass cover slips (10 mm X 20 mm) in a 6-well culture plate. Confluent cover slips were washed briefly in HBSS; incubated with Ca^{2+} sensitive fluorescent dye, Fura-2 AM (2.5 µM) for 45 min at room temperature in the dark. After three washes in HBSS, two cover slips were placed back to back into a 1-cm2 quartz cuvette fitted with a nonreactive support stage containing 3 ml HBSS using a holder. Cover slips fitted snugly into a horizontal diagonal slot in the stage and the bathing medium was permanently stirred with a magnetic bar to allow fast

agonists mixing. Fluorescence was measured using a spectroflurometer (Fluorolog, Jobin Yvon, Horiba, USA). Cells were excited alternately at 340 and 380 nm, and the signal emitted at 510 nm was collected. Peak excitation of Ca^{2+}-bound Fura-2 was at 340 nm while it was 380 nm for free-Fura-2. Results were expressed as 340/380 ratio. Basal tracings were recorded for 45 sec before the addition of OT (500 nM) or ATP (100 μM).

Statistical analysis- Completely randomized design with equal sample size was used for the experiments, where treatment with inhibitors/activator represented the main effect. Since a maximum of 6 experimental units (replicates) can be allotted per group for 4 treatments in a 24-well format culture, each experiment was repeated thrice except for PTx which was repeated twice. The resulting data on $PGF_{2\alpha}$ ($ngml^{-1}$) was expressed as mean±SEM (n=18) and used for data analysis. The group mean of different treatments was tested by one-variable ANOVA using GraphPad Prism 5. If the null hypothesis was rejected, Tukey's multiple comparison was used as a post-hoc test to find the critical difference between pairs of treatment means. Densitometric data on ERK1/2 expression was analyzed by one-way ANOVA using orthogonal contrast (control Vs different time points). In all the experiments, confidence level was set at 95% to determine the significance of difference ($P<0.05$).

Results

Role of intracellular Ca^{2+}, PKC, Raf-1 and MEK on OT mediated $PGF_{2\alpha}$ production in bEEL cells. Because OT stimulates intracellular Ca^{2+} release in most cell systems studied so far, this was studied first in bEEL cells. As expected, Ca^{2+} mobilization could be observed following addition of OT (500 nM). ATP (100 μM), a standard stimulator of Ca^{2+} release in other models was used as a positive control (Fig 1A) (Mori, Hosomi et al. 1997). Unexpectedly, chelating the intracellular Ca^{2+} with BAPTA-AM (5 μM) did not significantly reduce OT induced $PGF_{2\alpha}$ production in bEEL cells (Fig. 1B, $P>0.05$). We then sought to explore the other arm of the

phospholipase C (PLC) pathway using PMA as a surrogate of DAG to activate protein kinase C (PKC). As expected, pretreatment with the pan-PKC inhibitor, GF109203x (2.4 μM) significantly reduced PMA induced $PGF_{2\alpha}$ production (Fig. 1D, $P<0.05$); however, it did not significantly affect OT induced $PGF_{2\alpha}$ production in bEEL cells (Fig. 1C, $P>0.05$). While GF109203x inhibited PMA induced $PGF_{2\alpha}$ production, it is worth mentioning that alone, it stimulated $PGF_{2\alpha}$ production almost 3 folds (Fig. 1 C&D, $P<0.05$). Since Raf-1 (also called C-Raf) is the immediate downstream target of PKC and since OT has also been shown to activate it in other cell systems, bEEL cells were pretreated with the Raf-1 inhibitor GW5074 (1 μM) before stimulation with OT (500 nM) or PMA (10 nM). Under those conditions, GW5074 practically abolished OT or PMA induced $PGF_{2\alpha}$ production (Fig. 1E&F, $P<0.05$). Involvement of Raf-1 in OT induced $PGF_{2\alpha}$ production suggested that its phosphorylation target MEK could be involved and indeed, pretreatment with the MEK inhibitor PD98059 (10 μM) reduced OT or PMA induced $PGF_{2\alpha}$ production by 80% (Fig. 1G&H, $P<0.05$).

Demonstration of ERK1/2 phosphorylation by OT and involvement of Ras protein on OT induced $PGF_{2\alpha}$ production in bEEL cells. Since Raf-1 and MEK mediated OT induced $PGF_{2\alpha}$ production in bEEL cells, we studied the phosphorylation of ERK1/2 at different time points for 30 min following OT stimulation. Fig. 2A shows that it is significant and maximal after 5 min and stable up to 30 min ($P<0.05$). Moreover, in a preliminary experiment, we observed that OT induced ERK1/2 phosphorylation was still evident 3 h post stimulation (result not shown). Since OT mediated activation of ERK1/2 pathway did not involve PKC, we investigated the role of the small G protein, Ras on OT induced $PGF_{2\alpha}$ production in bEEL cells. Pretreatment of bEEL cells with the Ras inhibitor munamycin (2 μM) decreased OT induced $PGF_{2\alpha}$ production by 70% (Fig. 2B, $P<0.05$); however, it also had an intrinsic $PGF_{2\alpha}$ stimulating ability (Fig. 2B, $P<0.05$).

Role of G protein subunits on OT induced $PGF_{2\alpha}$ production in bEEL cells. It is well known that OT mediates its effect through its cognate GPCR. To understand the role of the G protein subunits on OT induced $PGF_{2\alpha}$ production, we uncoupled OTR from the underlying subunits by pretreating bEEL cells with suramin (100 μM). Because bEEL cells express functional G protein coupled nucleotide receptors as evidenced by ATP (100 μM) induced Ca^{2+} mobilization (Fig. 1A), non-specific antagonists of P2 nucleotide receptors like PPADS (100 μM) and RB2 (100 μM) were included to rule out the possibility of OT induced ATP release was responsible for $PGF_{2\alpha}$ production through the nucleotide receptor. Suramin inhibited OT induced $PGF_{2\alpha}$ production by 50% (Fig. 3A, $P<0.05$) whereas PPADS and RB2 did not affect OT induced $PGF_{2\alpha}$ production (Fig. 3A, $P>0.05$). Interestingly, $PGF_{2\alpha}$ production was stimulated by ATP, albeit at a reduced level compared to OT (Fig. 3B, $P<0.05$).The results indicate that OT induced $PGF_{2\alpha}$ production is not mediated through ATP release and OTR is coupled to the underlying G proteins in bEEL cells.

Involvement of $G\alpha_{i/o}$ subunit on OT induced $PGF_{2\alpha}$ production in bEEL cells. In order to understand the role of the Gα subunit on OT induced $PGF_{2\alpha}$ production, we pretreated bEEL cells with PTx that catalyzes ADP-ribosylation of Gα subunit thereby preventing the dissociation of α- and βγ- subunit from the ligand-activated receptor. Based on PTx susceptibility, $G\alpha_i$ is classified as sensitive whereas other α- subtype such as $G\alpha_q$ and $G\alpha_{12}$ are insensitive. Pretreatment of bEEL cells with PTx (100 $ngml^{-1}$) reduced OT induced $PGF_{2\alpha}$ production by 50-60% suggesting that the $G\alpha_i$ subunit mediates the response ($P<0.05$; Fig. 4A). One of the consequences of $G\alpha_i$ inhibition is elevated intracellular cAMP. Accordingly, intracellular cAMP was increased in bEEL cells by addition of cAMP mimetics such as the cell-permeable analogue-8Br cAMP (0.5 mM), the diterpenoid adenylate cyclase activator-forskolin (10 μM) and phosphodiesterase

inhibitor-IBMX (50 μM). Contrary to the expected inhibition, elevated intracellular cAMP revealed a weak synergism with OT induced (Fig. 4 B, C & D, $P<0.05$) while not affecting basal $PGF_{2\alpha}$ production (Fig. 4 B, C & D; $P>0.05$). Since elevating cAMP did not reproduce the inhibitory effect of PTx, we treated bEEL cells with mastoparan (1 μM), which is known to directly activate the PTx sensitive $G\alpha_{i/o}$ subunit. Mastoparan (1 μM) significantly stimulated $PGF_{2\alpha}$ production in bEEL cells (Fig. 4E; $P<0.05$). However, it was not as potent as OT in stimulating $PGF_{2\alpha}$ production.

Role of receptor and non-receptor tyrosine kinases on OT induced $PGF_{2\alpha}$ production in bEEL cells. The above results do not fully explain the OT signaling mechanism associated with induced $PGF_{2\alpha}$ production. Because OT was shown to transactivate the epidermal growth factor receptor (EGFR) tyrosine kinases in other systems, this was tested in bEEL cells (Zhong, Yang et al. 2003). Following pretreatment of cells with the EGFR inhibitor tyrphostin (10 μM, also known as AG1478) OT induced $PGF_{2\alpha}$ production was reduced by at least 80% (Fig. 5A; $P<0.05$). Then, we sought to study the role of non-receptor tyrosine kinase, c-Src which is often involved in the activation of EGFR. Pretreatment with Src family of tyrosine kinase inhibitor, PP2 (10 μM, also known as AG1879) significantly reduced OT induced $PGF_{2\alpha}$ output (Fig. 5B; $P<0.05$). Since phosphoinositide 3 kinase (PI3K) is known to activate c-Src family of tyrosine kinases, we inhibited it with the reversible inhibitor, LY294002 (25 μM) and found that it significantly inhibited OT induced $PGF_{2\alpha}$ production in bEEL cells (Fig. 5C; $P<0.05$).

Role of Gβγ subunit on OT induced $PGF_{2\alpha}$production in bEEL cells. Involvement of PI3K/c-Src/EGFR kinases on OT induced $PGF_{2\alpha}$ production suggested that the βγ subunit dissociated from the PTx sensitive $G\alpha_{i/o}$ may also contribute for $PGF_{2\alpha}$ production. Pretreatment of bEEL cells with the Gβγ inhibitor, gallein (10 μM) did not reduce OT induced $PGF_{2\alpha}$ production

significantly ($P>0.05$; Fig. 6A) and the Gβγ activating peptide, mSIRK (5 μM) produced a significant increase in $PGF_{2\alpha}$ production ($P<0.05$; Fig.6 B) but much weaker than OT.

Discussion

The cellular and molecular mechanism of luteolysis and antiluteolysis in ruminants has been investigated using endometrial explants, primary culture, and immortalized cells. While the major limitation of explants and primary cultures is heterogeneity in cell types and OT response, the first bovine endometrial cell line developed Bovine endometrial cell (BEND) lacked OT response and phorbol ester was used as a surrogate, assuming that PKC was an immediate downstream mediator (Binelli, Guzeloglu et al. 2000). Similarly, OT responsiveness of one ovine endometrial epithelial cell line was not described (Johnson, Burghardt et al. 1999). In other words, lack of an OT sensitive endometrial cell line partly hampered the understanding of the biochemical and molecular targets of OT and IFNτ. Using bEEL cells, we have recently shown that IFNτ inhibited OT induced $PGF_{2\alpha}$ production in 3-6 h suggesting that it may impair the intracellular effector(s) of OT mediated signal in the bovine (Krishnaswamy, Danyod et al. 2009). This observation prompted us to dissect the signaling pathway of OT induced $PGF_{2\alpha}$ production through a kinase inhibitor approach.

Release of intracellular Ca^{2+} in bEEL cells by OT (Fig. 1A) is in agreement with our observations in primary bovine endometrial epithelial cells (Asselin, Drolet et al. 1997). It has been shown that OT stimulated IP3 release from the ovine endometrial explants suggesting the hydrolysis of phosphoinositides by PLC (Flint, Leat et al. 1986) and Ca^{2+} was reported to be essential for $PGF_{2\alpha}$ production in the bovine (Burns, Hayes et al. 1998). However, observations with BAPTA-AM suggest that intracellular Ca^{2+} release is not essential for OT induced $PGF_{2\alpha}$ production in bEEL cells (Fig. 1B, $P>0.05$). Then, we sought to explore the role of PKC in OT induced $PGF_{2\alpha}$

production for the following reasons: DAG, which activates PKC, has been proposed to mediate OT induced $PGF_{2\alpha}$ production in the ovine (Flint, Leat et al. 1986; Silvia, Lee et al. 1994). Second, PMA, a direct activator of PKC, is used as a surrogate for OT in BEND cells (Binelli, Guzeloglu et al. 2000). Surprisingly, the general PKC inhibitor, GF109203x did not reduce OT induced $PGF_{2\alpha}$ production in bEEL cells (Fig. 1C $P>0.05$), whereas PMA induced $PGF_{2\alpha}$ production was inhibited significantly (Fig. 1D, $P<0.05$). Since ERK1/2 activation by PKC was shown to mediate OT induced PGE_2 production in CHO cells overexpressing OTR (Strakova, Copland et al. 1998), we inhibited Raf-1 and MEK known to induce ERK1/2 phosphorylation. Inhibition of PMA induced $PGF_{2\alpha}$ production by GW5074 and PD98059 in bEEL cells (Fig. 1F&H, $P<0.05$) is in agreement with observations in BEND cells (Pru, Rueda et al. 2001) and inhibition of OT induced $PGF_{2\alpha}$ production by PD98059 concurs with observations in the ovine (Burns, Mendes et al. 2001). The results described above exclude intracellular Ca^{2+} and PKC are necessary for OT induced $PGF_{2\alpha}$ production in bEEL cells, thus implying that $G\alpha_q$ subunit is not the major effector of OT action.

Rapid phosphorylation of ERK1/2 following OT stimulation (Fig. 2A, $P<0.05$) validated the observations with Raf-1 and MEK inhibitors in bEEL cells and is in agreement with the observations in cells from the ovine endometrium (Burns, Mendes et al. 2001), human mammary gland (Copland, Jeng et al. 1999), human and rabbit amnion (Moore, Dubyak et al. 1988; Jeng, Liebenthal et al. 2000), as well as OTR-COSM6 and PHM1 (Zhong, Yang et al. 2003) and CHO-OTR (Strakova, Copland et al. 1998) cell lines. Then, we inhibited Ras protein for two reasons: First, PKC was not involved in OT induced $PGF_{2\alpha}$ production in bEEL cells. Second, Ras is known to activate ERK1/2 pathway in some of the GPCRs (Pace, Faure et al. 1995). Inhibition by munamycin suggested that OT induced $PGF_{2\alpha}$ production is Ras-dependent and PKC independent in bEEL cells (Fig. 2B, $P<0.05$). It has been reported that the activation of Ras leads

to the recruitment of Raf family of serine/threonine kinases to the cell membrane before phosphorylation of ERK1/2 (Marais, Light et al. 1997). To the best of our knowledge, this is the first observation demonstrating the activation of Ras-Raf1-MEK-ERK1/2 pathway during OT induced $PGF_{2\alpha}$ production.

OTR belongs to the family of membrane bound GPCRs (Gimpl and Fahrenholz 2001). In the classical GPCR mediated signaling, agonist activated receptors catalyze the exchange of the bound GDP to GTP from the α- subunit and this process is inhibited by suramin (Huang, Dehaven et al. 1990). Accordingly, treatment of bEEL cells with suramin (100 μM) inhibited OT induced $PGF_{2\alpha}$ production thus suggesting that OTR is coupled to G proteins ($P<0.05$; Fig 3A). Inhibition of OT induced $PGF_{2\alpha}$ production by suramin was only 50% as compared to 80% achieved by Ras, Raf-1 and MEK inhibitors. This may be due to a reduction in the binding affinity of agonists for GPCR (Huang, Dehaven et al. 1990) or GPCR independent mechanism may also contribute, in part, to the overall $PGF_{2\alpha}$ production following OT treatment. Both OT and ATP generated Ca^{2+} oscillations in bEEL cells (Fig. 1A) and stimulated $PGF_{2\alpha}$ production (Fig. 3B, $P<0.05$). It is known that nucleotide receptors (P2) are a subtype of GPCR. Therefore, it was possible to argue that OT might stimulate the release of ATP which, in turn, contributes to the total $PGF_{2\alpha}$ production and the inhibitory effect of suramin might also be through inhibition of P2 receptors. It was reported that ATP and other nucleotides stimulated $PGF_{2\alpha}$ production through P2 receptors from the guinea-pig endometrium (Aitken, Poyser et al. 2001). We show here that the non-specific blockers of P2 receptors PPADS and RB2 do not affect OT induced $PGF_{2\alpha}$ production thus ruling out the involvement of ATP in mediating OT induced $PGF_{2\alpha}$ production. Collectively, these results indicate that OT induced $PGF_{2\alpha}$ production is coupled to G proteins.

Inhibition of OT induced $PGF_{2\alpha}$ production by suramin prompted us to identify the specific subunit of G proteins and we used PTx because it irreversibly inhibits the $G\alpha_i$ subtype. OT induced $PGF_{2\alpha}$ production was inhibited significantly by PTx (Fig. 4A, $P<0.05$), which is in agreement with its effect on PGE_2 production in cells over expressing OTR (Strakova, Copland et al. 1998). However, OT induced $PGF_{2\alpha}$ production was shown not to be coupled to the PTx sensitive $G\alpha$ subunit in the ovine endometrium (Burns, Mendes et al. 2001). Further, a PTx sensitive $G\alpha_i$ component has been identified in the myometrial OTR of human (Phaneuf, Europe-Finner et al. 1993) and rat (Strakova and Soloff 1997). It is known that $G\alpha_i$ activates ERK1/2 pathway through its inhibitory effect on adenylate cyclase and PKA (Goldsmith and Dhanasekaran 2007). As PTx treatment leads to the accumulation of intracellular cAMP by removing the inhibitory effect of $G\alpha_i$ on adenylate cyclase activity, we used three different means to elevate intracellular cAMP to activate PKA - by substituting the cell permeable analogue (8 Br cAMP), activating adenylate cyclase by forskolin and inhibiting phosphodiesterase mediated catabolism of cAMP by IBMX. Surprisingly, none of the condition inhibited OT induced $PGF_{2\alpha}$ production. On the contrary, cAMP mimetics showed a weak synergism on OT induced $PGF_{2\alpha}$ production (Fig. 4 B, C & D; $P<0.05$), which may be due to the stimulation of PG production by PKA-dependent and ERK1/2 independent mechanism. Since elevating cAMP is an indirect way of mimicking $G\alpha_i$ activation, we treated bEEL cells with mastoparan (1 μM), which is known to directly activate PTx sensitive $G\alpha_{i/o}$ subunit (Higashijima, Uzu et al. 1988). Mastoparan produced a significant, but modest increase in $PGF_{2\alpha}$ production as compared to OT (Fig. 4E; $P<0.05$). The observations with PTx and mastoparan suggest that OT induced $PGF_{2\alpha}$ production in bEEL cells is partly mediated by PTx sensitive $G\alpha_{i/o,}$ but not through its well known inhibitory effect on adenylate cyclase. Because of the fact that both suramin and PTx partially reduced (50-60%) OT

induced $PGF_{2\alpha}$ production (Fig. 3A and 4A), whereas MEK and Raf-1 inhibitors (PD98059 and GW5074) practically abolished PG output (Fig. 1E and 1G), it is possible that GPCR independent mechanisms may contribute, in part, to the total $PGF_{2\alpha}$ production by OT (Blumer, Smrcka et al. 2007).

Many GPCR agonists induce receptor transactivation, a process of concerted signaling events that culminate in the ectodomain shedding of the cell surface proteins which acts as ligand for receptor tyrosine kinases like EGFR (Ohtsu, Dempsey et al. 2006). Inhibition of EGFR with tyrphostin nearly abolished OT induced $PGF_{2\alpha}$ production (Fig. 5A; $P<0.05$). Recently, transactivation of EGFR was shown to be essential for ERK1/2 phosphorylation by OT in myometrial and OTR-COSM6 cells (Zhong, Yang et al. 2003). Because c-Src and PI3K are proposed to activate EGFR (May and Hill 2008), we inhibited the kinases and found profound decrease in OT induced $PGF_{2\alpha}$ production (Fig. 5 B & C; $P<0.05$). It has been shown that Ras activation requires phosphorylation by different tyrosine kinases including c-Src for activation of the ERK1/2 pathway (Marais, Light et al. 1997) and PI3K for the formation of adaptor protein complex (Shc-Grb-Sos) to activate the ERK1/2 pathway in COS7 cells (Hawes, Luttrell et al. 1996). Similarly, PI3K and c-Src activation of the ERK1/2 pathway by platelet derived growth factor (PDGF) involves PTx sensitive Gα-protein (Conway, Rakhit et al. 1999). To the best of our knowledge, this is the first observation that demonstrates a role of EGFR, c-Src and PI3K in OT induced $PGF_{2\alpha}$ production.

Ras-dependent activation of ERK1/2 pathway has been shown to be mediated by Gβγ subunits derived from $G\alpha_i$ (Faure, Voyno-Yasenetskaya et al. 1994; van Biesen, Hawes et al. 1995). Since OT induced $PGF_{2\alpha}$ production in bEEL cells is Ras dependent and also has a PTx sensitive $G\alpha_i$, we inhibited the Gβγ subunit with gallein (10 μM) and found that it did not significantly decrease

OT induced $PGF_{2\alpha}$ production in bEEL cells ($P>0.05$; Fig. 6A). It has been reported that Gβγ–dependent chemotaxis in neutrophils was inhibited by gallein (Lehmann, Seneviratne et al. 2008). Activation of Gβγ activating peptide, mSIRK (5 μM) produced a weak, but significant increase in $PGF_{2\alpha}$ production as compared to OT ($P<0.05$; Fig.6 B). Thus, it appears that the Gβγ subunit does not contribute significantly to OT induced $PGF_{2\alpha}$ production in bEEL cells.

In summary, OT induced $PGF_{2\alpha}$ production in bEEL cell appears to be coupled to $G\alpha_i\beta\gamma$ subunits that activates ERK1/2 pathway through a Ras-dependent mechanism. Further, EGFR, c-Src and PI3K are essential intermediates required for the activation of Ras (Fig. 7). It is interesting to note that this pathway involves the activation of the classical proto-oncogenes such as Ras, c-Src and PI3K.

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Abbreviations

bEEL, bovine endometrial epithelial cell; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal regulated kinase ½; GPCR, G-protein coupled receptor; MAPK, mitogen-actived protein kinase; OT, oxytocin; OTR, oxytocin receptor; PI3K, phosphoinositide-3-kinase; PKC, protein kinase C; PLC, phospholipase C; PTx, pertussis toxin.

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Figure 3.1: Role of intracellular Ca^{2+}, PKC, Raf-1 and MEK on OT mediated $PGF_{2\alpha}$ production in bEEL cells. A. Cover slip preparation of bEEL cells was incubated with Fura-2 AM (2.5 μM) for 45 min. Then, stimulated with OT (500 nM) and the Ca^{2+} mobilizations were recorded for 3-4 min in a spectrofluorometer. ATP (100 μM) served as positive control. **B.** Effect of BAPTA-AM (5 μM) on OT induced $PGF_{2\alpha}$ production. **C and D**. Effect of GF109203x (2.4 μM) on OT (500 nM) or PMA (10 nM) induced $PGF_{2\alpha}$ production. **E and F.** Effect of GW5074 (1 μM) on OT (500 nM) or PMA (10 nM) induced $PGF_{2\alpha}$ production. **G and H.** Effect of PD98059 (10 μM) on OT (500 nM) or PMA (10 nM) induced $PGF_{2\alpha}$ production. **B through H.** Confluent bEEL cells were pre-incubated with DMSO or appropriate inhibitor for 1 h and co-incubated with OT (500 nM) or PMA (10 nM) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM (18) of three different experiments run in hexaplicate. Data was analyzed by one-way ANOVA with Tukey's multiple comparison as *post-hoc* test to find the critical difference between pairs of treatment means. Bars with different superscripts differ significantly ($P < 0.05$).

Figure 1

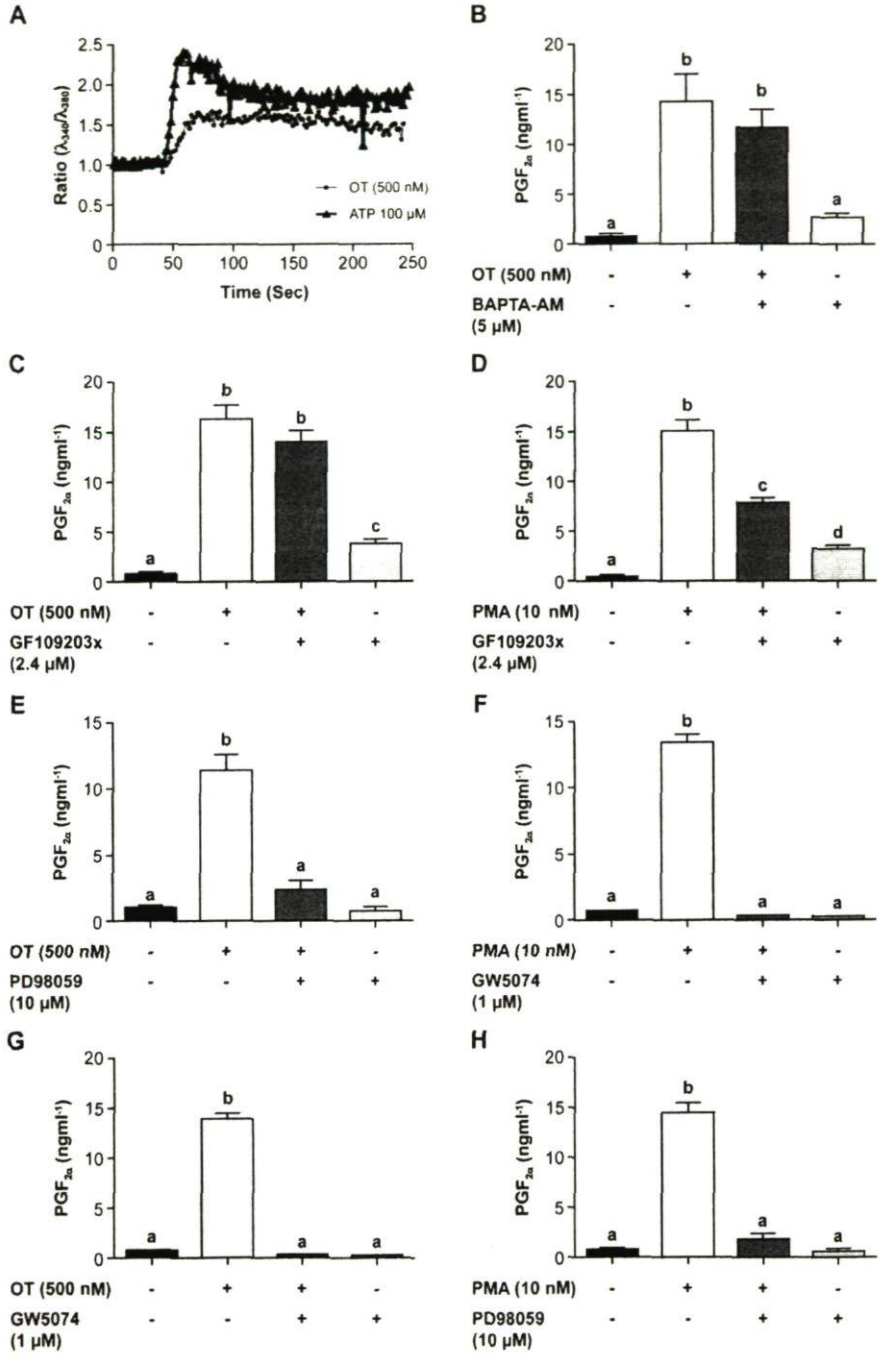

A
Ratio ($\lambda_{340}/\lambda_{380}$)
Time (Sec)
OT (500 nM)
ATP 100 µM
B
$PGF_{2\alpha}$ (ngml^{-1})
OT (500 nM)
BAPTA-AM (5 µM)
C
$PGF_{2\alpha}$ (ngml^{-1})
OT (500 nM)
GF109203x (2.4 µM)
D
$PGF_{2\alpha}$ (ngml^{-1})
PMA (10 nM)
GF109203x (2.4 µM)
E
$PGF_{2\alpha}$ (ngml^{-1})
OT (500 nM)
PD98059 (10 µM)
F
$PGF_{2\alpha}$ (ngml^{-1})
PMA (10 nM)
GW5074 (1 µM)
G
$PGF_{2\alpha}$ (ngml^{-1})
OT (500 nM)
GW5074 (1 µM)
H
$PGF_{2\alpha}$ (ngml^{-1})
PMA (10 nM)
PD98059 (10 µM)

Figure 3.2: Demonstration of ERK1/2 phosphorylation by OT and involvement of Ras protein on OT induced $PGF_{2\alpha}$ production in bEEL cells. A. Confluent bEEL cells were serum starved for 3 h and stimulated with OT (500 nM) for 5, 10, 15, 20 and 30 min. Protein extracted from the cell lysate was probed for phospho or total ERK1/2. Representative immunoblots of phospho and total ERK1/2 are shown. Relative optical density (ROD) values are densitometric ratio of phosphorylated to total ERK1/2 quantified from three different experiments. Data was analyzed by one-way ANOVA with orthogonal contrasts. Bars with * indicate a significant difference from the control ($P<0.05$). **B.** Confluent bEEL cells were pre-incubated with dichloromethane (vehicle) or Ras inhibitor (munamycin 2 μM) for 1 h and co-incubated in the presence or absence of OT (500 nM) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM (18) of three different experiments run in hexaplicate. Data was analyzed by one-way ANOVA with Tukey's multiple comparison as *post-hoc* test to find the critical difference between pairs of treatment means. Bars with different superscripts differ significantly ($P < 0.05$).

Figure 2

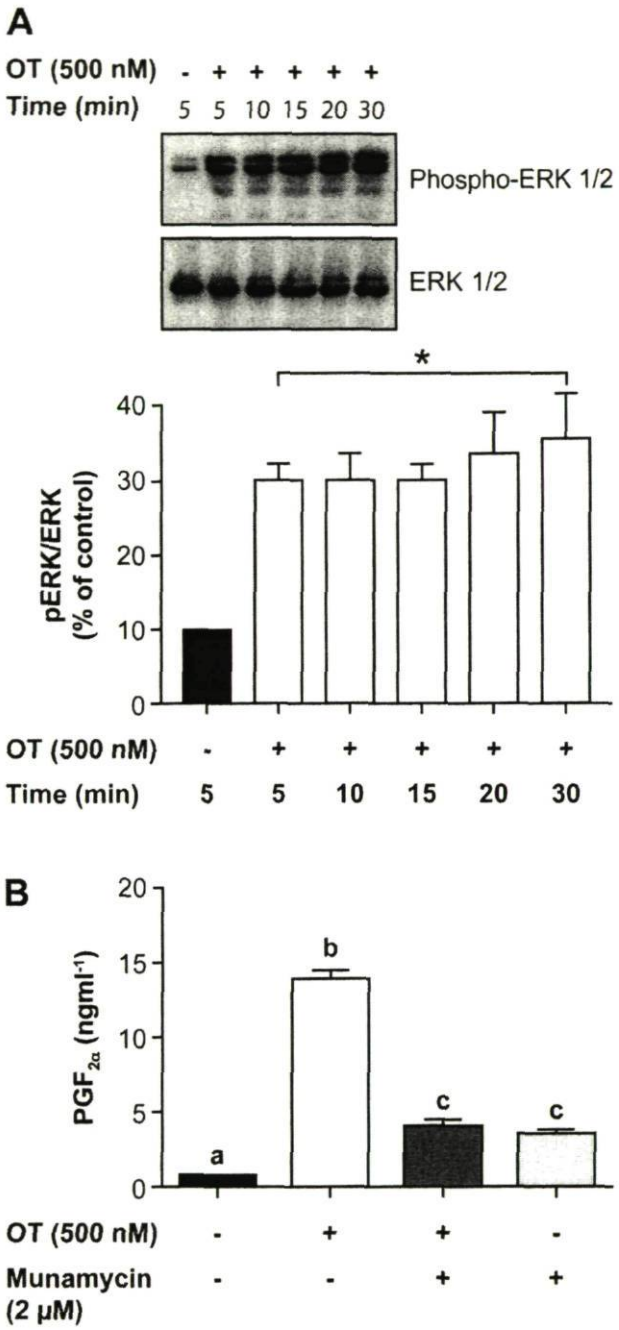

A
OT (500 nM) - + + + + +
Time (min) 5 5 10 15 20 30
Phospho-ERK 1/2
ERK 1/2
*
pERK/ERK (% of control)
40
30
20
10
0
OT (500 nM) - + + + + +
Time (min) 5 5 10 15 20 30
B
PGF2α (ngml-1)
20
15
10
5
0
a
b
c
c
OT (500 nM) - + + -
Munamycin (2 μM) - - + +

Figure 3.3: Role of G protein subunits on OT induced $PGF_{2\alpha}$ production in bEEL cells. A. Confluent bEEL cells were pre-incubated with endotoxin free water or suramin (100 μM), pyridoxal phosphate azophenyl disulfonate (PPADS 100 μM) and reactive blue 2 (RB2 100 μM) for 1 h and co-incubated the presence or absence of OT (500 nM) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM (18) of three different experiments run in hexaplicate. Data was analyzed by one-way ANOVA with Tukey's multiple comparison as *post-hoc* test to find the critical difference between pairs of treatment means. Bars with different superscripts differ significantly ($P < 0.05$). **B.** Confluent bEEL cells were treated with OT (500 nM) or ATP (100 μM) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM (18) of three different experiments run in hexaplicate. Data was analyzed as mentioned in Fig. 3A.

Figure 3

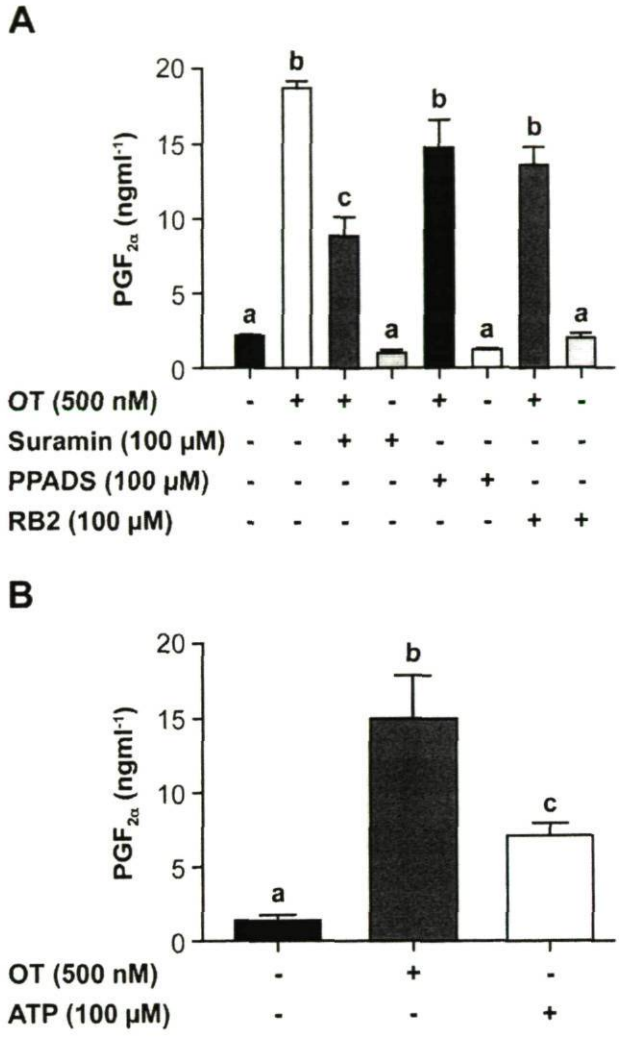

A
20
15
10
5
0
PGF2α (ngml-1)
a
b
c
a
b
a
b
a
OT (500 nM) - + + - + - + -
Suramin (100 µM) - - + + - - - -
PPADS (100 µM) - - - - + + - -
RB2 (100 µM) - - - - - - + +
B
20
15
10
5
0
PGF2α (ngml-1)
a
b
c
OT (500 nM) - + -
ATP (100 µM) - - +

Figure 3.4: Involvement of $G\alpha_{i/o}$ subunit on OT induced $PGF_{2\alpha}$ production in bEEL cells. **A.** Effect of pertussis toxin (PTx, 100 $ngml^{-1}$) on OT induced $PGF_{2\alpha}$ production. Confluent bEEL cells were pre-incubated with endoxin free water or PTx (100 $ngml^{-1}$) for 1 h and co-incubated with or without OT (500 nM) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM of two experiments run in hexaplicate. Data was analyzed by one way ANOVA with Tukey's *post-hoc* test to find the critical difference between pairs of treatment means. Bars with different superscripts differ significantly ($P < 0.05$). **B through D.** Effect of 8Br cAMP (0.5 mM), forskolin (10 μM) or IBMX (50 μM) on OT induced $PGF_{2\alpha}$ production. Confluent bEEL cells were pre-incubated with DMSO or appropriate inhibitor/activator for 1 h and co-incubated in the presence or absence of OT (500 nM) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM of three experiments run in hexaplicate. Data was analyzed as mentioned Fig. 4A. **E.** Activation of $G\alpha_{i/o}$ by mastoparan on $PGF_{2\alpha}$ production. Confluent bEEL cells were treated with OT (500 nM) or mastoparan (1 μM) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM (18) of three different experiments run in hexaplicate. Data was analyzed as mentioned in Fig. 4A.

Figure 4

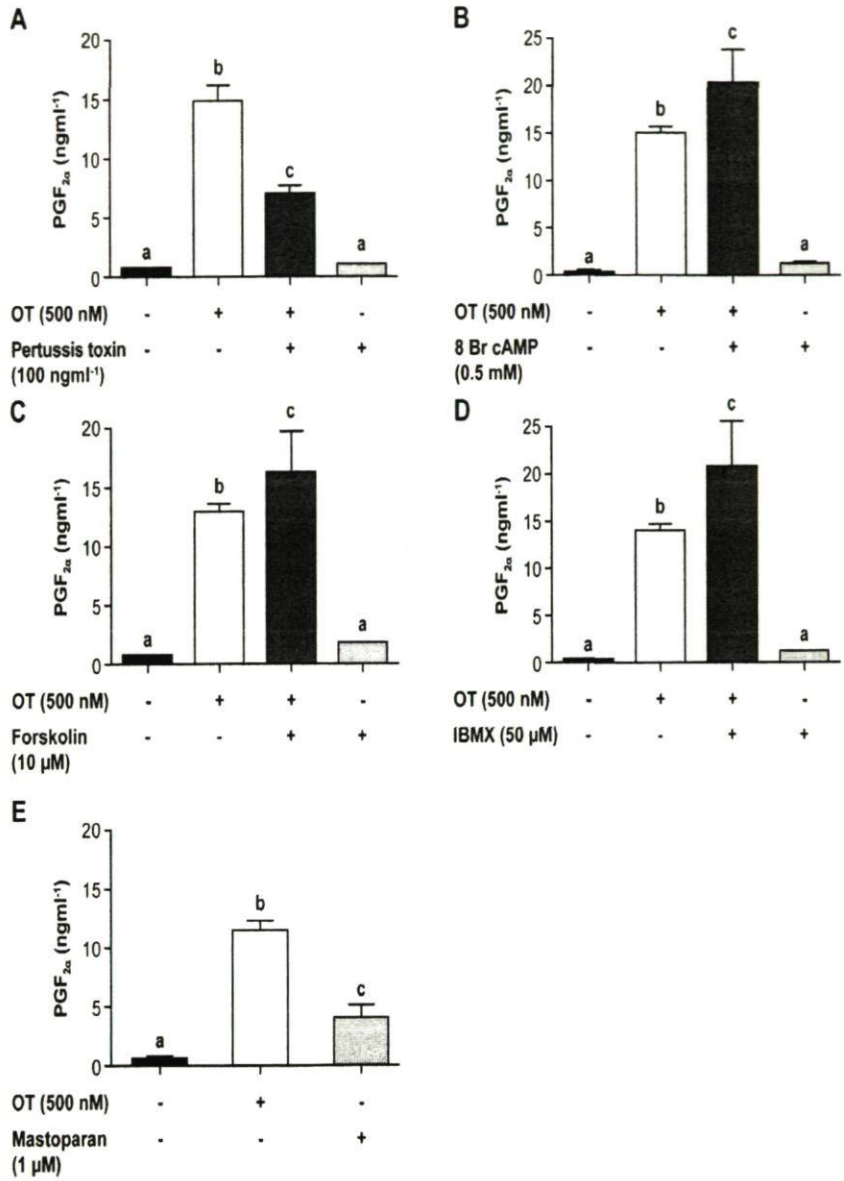

A
B
C
D
E
PGF2α (ngml-1)
OT (500 nM)
Pertussis toxin (100 ngml-1)
8 Br cAMP (0.5 mM)
Forskolin (10 µM)
IBMX (50 µM)
Mastoparan (1 µM)
a
b
c

Figure 3.5: Role of receptor and non-receptor tyrosine kinases on OT induced $PGF_{2\alpha}$ production in bEEL cells. A. Effect of AG1478 (10 μM) on OT induced $PGF_{2\alpha}$ production. **B.** Effect of PP2 (10 μM) on OT induced $PGF_{2\alpha}$ production. **C.** Effect of LY294002 (25 μM) on OT induced $PGF_{2\alpha}$ production. In the above experiments, bEEL cells were pre-incubated with vehicle (DMSO) or respective inhibitors for 1 h before co-incubating with OT for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM (18) of three experiments run in hexaplicate. Data was analyzed by one way ANOVA with Tukey's *post-hoc* test to find the critical difference between pairs of treatment means. Bars with different superscripts differ significantly ($P < 0.05$).

Figure 5

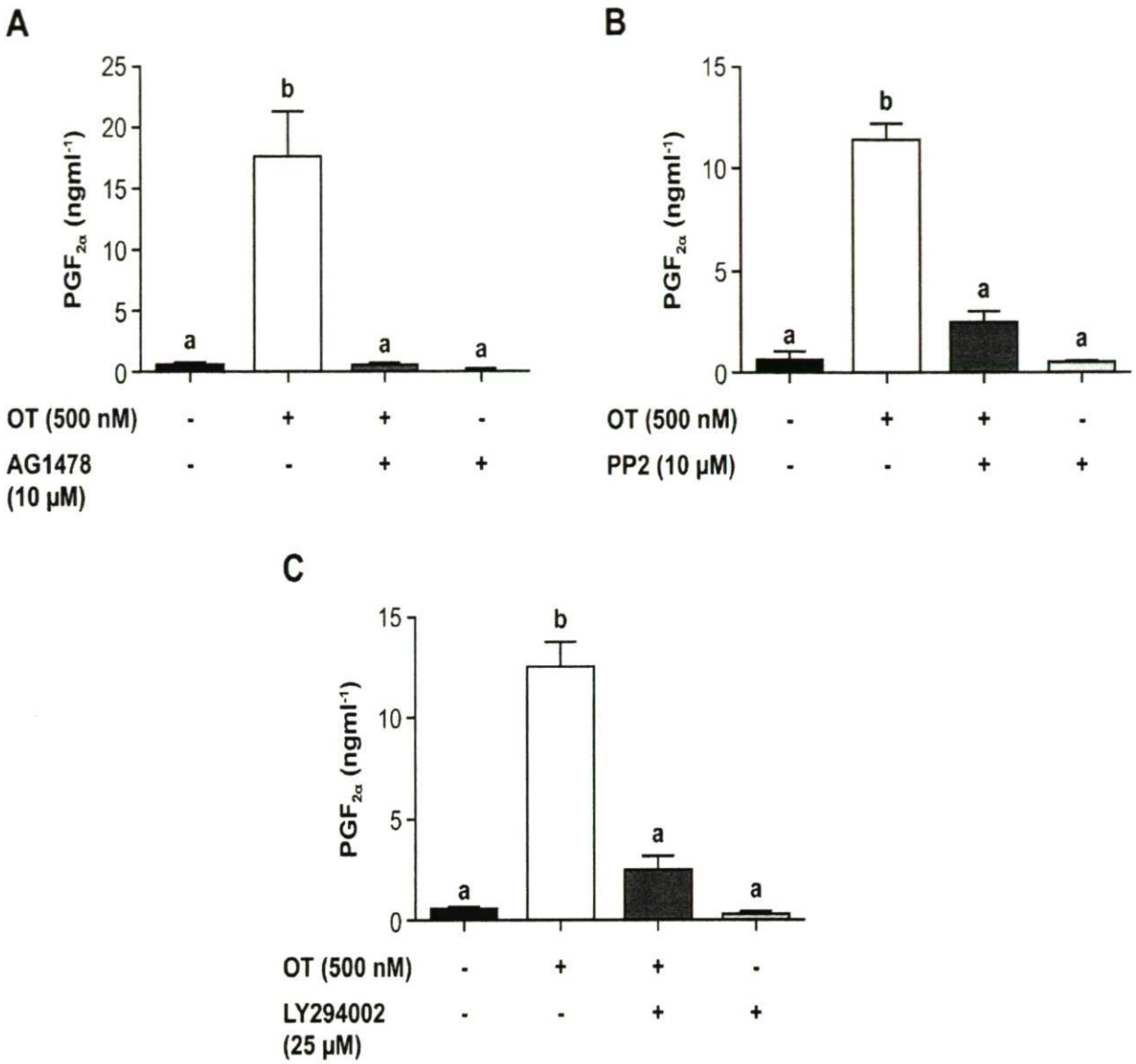

A
25
20
15
10
5
0
PGF2α (ngml-1)
b
a
a
a
OT (500 nM) - + + -
AG1478 (10 μM) - - + +
B
15
10
5
0
PGF2α (ngml-1)
b
a
a
a
OT (500 nM) - + + -
PP2 (10 μM) - - + +
C
15
10
5
0
PGF2α (ngml-1)
b
a
a
a
OT (500 nM) - + + -
LY294002 (25 μM) - - + +

Figure 3.6: Role of Gβγ subunit on OT induced $PGF_{2\alpha}$ production in bEEL cells. A. Confluent bEEL cells were pre-incubated with DMSO or Gβγ inhibitor, gallein (10 μM) for 1 h before co-incubating with OT for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM (18) of three experiments run in hexaplicate. Data was analyzed by one way ANOVA with Tukey's *post-hoc* test to find the critical difference between pairs of treatment means. Bars with different superscripts differ significantly ($P < 0.05$). **B.** Confluent bEEL cells were treated with OT (500 nM) or Gβγ activating peptide, mSIRK (5 μM) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium and the values represent mean ± SEM (18) of three experiments run in hexaplicate. Data was analyzed as mention in Fig. 6A.

Figure 6

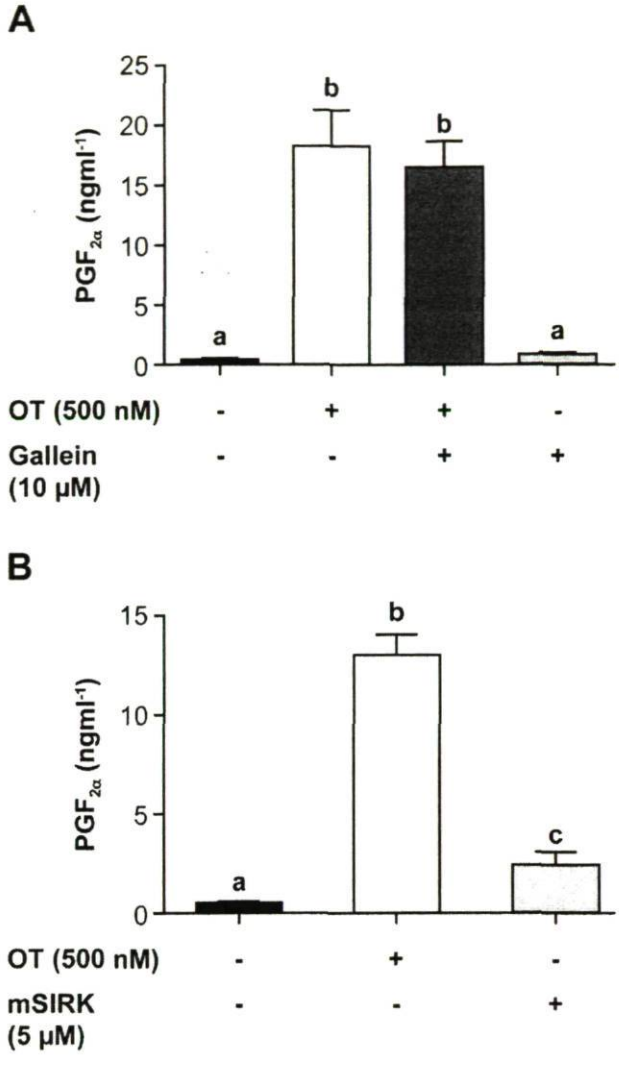
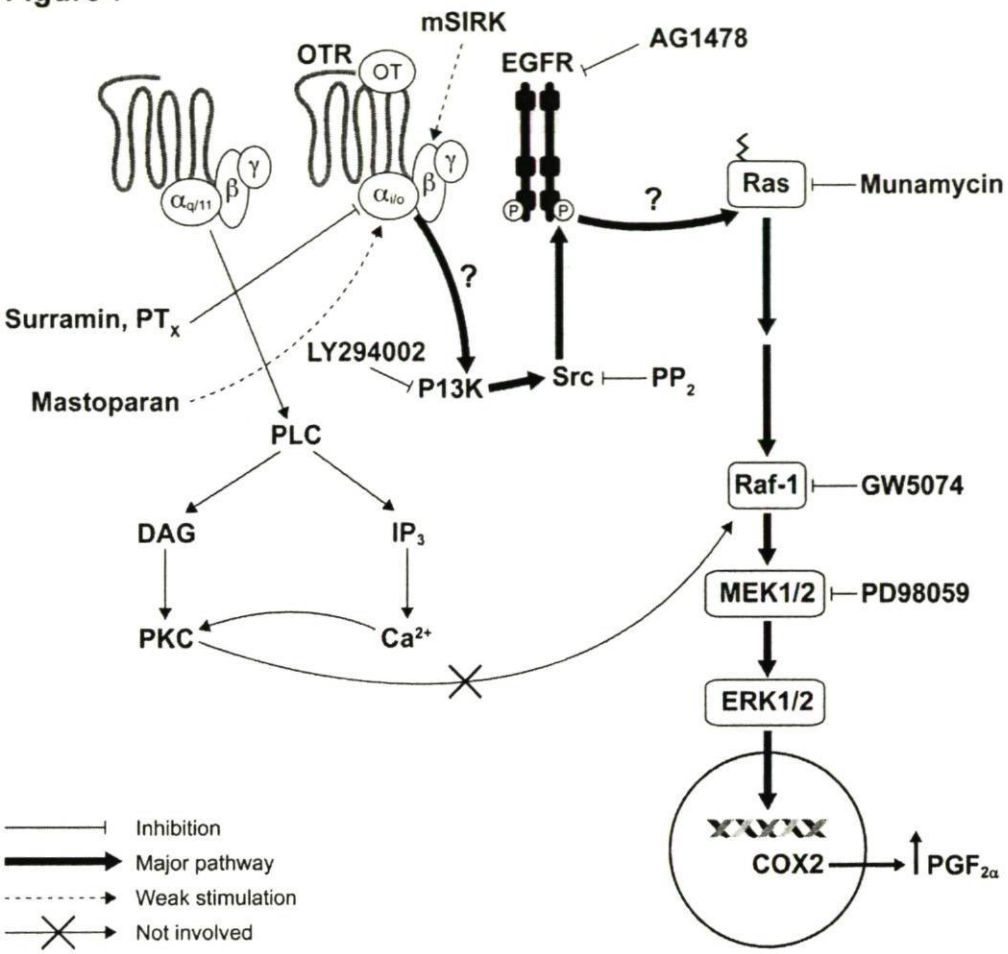

A
25
20
15
10
5
0
PGF2α (ngml-1)
b
b
a
a
OT (500 nM) - + + -
Gallein - - + +
(10 μM)
B
15
10
5
0
PGF2α (ngml-1)
b
a
c
OT (500 nM) - + -
mSIRK - - +
(5 μM)

Figure 3.7: A proposed signaling pathway of OT induced $PGF_{2\alpha}$ production in bEEL cells (Bold arrow). Binding of OT to its cognate receptor dissociates the pertussis toxin (PTx) sensitive $G\alpha_i\beta\gamma$ to activate Ras-Raf1-MEK-ERK1/2 axis. The $G\alpha_i$ does not mediate its effect by inhibiting adenylate cyclase. Phosphorylation of PI3K, c-Src and transactivation of EGFR are the early signaling events, perhaps required for the assembly of adaptor protein complex to activate Ras. In this system, $G\alpha_q$ activates Ca^{2+} mobilization presumably through phospholipase C. But, OT induced $PGF_{2\alpha}$ production is independent of PKC and Ca^{2+} (Thin arrow).

Figure 7


mSIRK
AG1478
OTR
OT
EGFR
Ras
Munamycin
$\alpha_{q/11}$
β
γ
$\alpha_{i/o}$
?
Surramin, PT_x
LY294002
P13K
Src
PP_2
Mastoparan
PLC
Raf-1
GW5074
DAG
IP_3
MEK1/2
PD98059
PKC
Ca^{2+}
ERK1/2
COX2
$PGF_{2\alpha}$
Inhibition
Major pathway
Weak stimulation
Not involved

CHAPTER IV

DEVELOPMENT AND CHARACTERIZATION OF A SIMIAN VIRUS 40 IMMORTALIZED BOVINE ENDOMETRIAL STROMAL CELL LINE

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DEVELOPMENT AND CHARACTERIZATION OF A SIMIAN VIRUS 40 IMMORTALIZED BOVINE ENDOMETRIAL STROMAL CELL LINE

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Running title: IFNτ regulation of PGs in bovine endometrial stromal cells

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Résumé

Chez les ruminants l'interferon-τ (IFNτ) est le signal maternel de reconnaissance qui inhibe la production de prostaglandine (PG) F2α par les cellules endométriales épithéliales et stimule les gènes sensibles à l'interféron dans les cellules stromales. Ces dernières agissent comme intermédiaires dans l'action de la progestérone sur les cellules épithéliales durant la gestation. Notre hypothèse de travail est que l'IFNτ agit comme médiateur qui permet la production de PGE2 par les cellules endométriales stromales tout en supprimant la production de PGF2α par les cellules épithéliales. Dans ce rapport, nous documentons l'immortalisation et la caractérisation fonctionnelle d'une lignée cellulaire dérivée des cellules stromales de la région caronculaire de l'endomètre bovin [cellules stromales caronculaires (CSC)]. Les cellules primaires stromales ont été immortalisées par nucléofection avec le virus simien SV40 à antigène T large (simian virus 40 large T antigen) et l'intégrase. La lignée cellulaire résultante CSC exprime la vimentine, les récepteurs à l'estrogène et la progestérone, propriétés spécifiques aux cellules stromales, et est propice aux transfections transitoires. La production de PGE2 basale ou stimulée est supérieure à PGF2α et est associée à l'expression de la cyclooxygénase (COX) 2. Le phorbol-myristate-acétate (PMA) et l' IFNτ augmentent COX2 et la production de PG de façon dose-dépendante. Lorsqu'ajouté ensemble de faibles concentrations d'IFNτ inhibent l'expression de COX2 induite par le PMA; tandis que cette inhibition est perdue en utilisant de plus forte concentrations. L'expression du transducteur de signal et activateur de transcription 1 (STAT1) est induit par l'IFNτ à toutes les concentrations étudiées mais n'es pas modulée par PMA. Parce que STAT1 ne s'exprime pas selon la réponse biphasique de l'IFNτ, nous avons analysé la voie de signalisation de MAPK p38 en utilisant un inhibiteur sélectif SB203580. L'inhibition de la voie de signalisation MAPK p 38 abolit l'action de l'IFNτ sur la production de PG. En résumé, les cellules CSC semblent un bon modèle pour l'étude des cellules stromales en lien avec les mécanismes d'action de l'IFNτ et la production des prostaglandines chez le bovin.

(*Endocrinology* 150: 485–491, 2009)

Abstract

In ruminants, Interferon-τ (IFNτ) is the maternal recognition signal inhibiting prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production by endometrial epithelial cells and stimulating IFN stimulated genes (ISGs) in the stroma. Stromal cells mediate the action of progesterone on epithelial cells during pregnancy. Our working hypothesis is that IFNτ acts as a molecular switch that turns on PGE_2 production in endometrial stromal cells while suppressing $PGF_{2\alpha}$ production from epithelial cells. In this report, we document immortalization and functional characterization of a bovine stromal cell line from the caruncular region of the endometrium (CSC). Primary stromal cells were immortalized by nucleofection with SV40 Large T antigen (SV40 TAG) and integrase. The resulting cell line CSC, expresses stromal cell specific vimentin, estrogen and progesterone receptors and is amenable for transient transfection. Basal and stimulated production of PGE_2 is higher than $PGF_{2\alpha}$ and associated with cyclooxygenase 2 (COX2) expression. Phorbol myristate acetate (PMA) and IFNτ up-regulate COX2 and PG production in a dose-dependent manner. When added together, low concentrations of IFNτ inhibit PMA-induced COX2 expression; whereas this inhibition is lost at high concentrations. Expression of signal transducer and activator of transcription 1 (STAT1) is induced by IFNτ at all concentrations studied, but is not modulated by PMA. Since expression of STAT1 does not exhibit the biphasic response to IFNτ, we investigated the p38 mitogen activated protein kinase (p38 MAPK) pathway using the selective inhibitor SB203580. Inhibition of the p38 MAPK pathway abolishes IFNτ action on PG production. In summary, CSC appears as a good stromal cell model for investigating the molecular mechanisms related to IFNτ action and PG production in the bovine.

Introduction

Epithelial and stromal cells of the endometrium are the site of recognition of pregnancy. In ruminants, epithelial cells are the target of oxytocin (OT) to generate luteolytic pulses of $PGF_{2\alpha}$ (McCracken, Custer et al. 1999). However, spatio-temporal expression of oxytocin receptor (OTR) is co-regulated with that of estrogen and progesterone receptors (ERα and PR) and may involve paracrine interactions between epithelial and stromal cells (Wathes and Hamon 1993; Spencer and Bazer 2002; Cunha, Cooke et al. 2004). In addition, $PGF_{2\alpha}$ of stromal origin, secreted in response to tumor necrosis factor α (TNFα) may contribute to the initiation of luteolysis (Okuda, Kasahara et al. 2004).

Interferon-τ (IFNτ) is the maternal recognition signal in ruminants. Apart from inhibiting $PGF_{2\alpha}$ pulses of epithelial origin, IFNτ stimulates a set of genes (IFN stimulated genes-ISG) in the endometrial stroma (Bazer and Spencer 2006). Using bovine primary stromal cell cultures we have shown that IFNτ stimulates the production of PGE_2 (Parent, Chapdelaine et al. 2002). Generation of stable *in vitro* endometrial culture systems appears as the logical next step for investigating the complex signaling pathways and transcriptional mechanisms regulated by IFNτ in the bovine. At present, a spontaneously derived bovine endometrial epithelial cell line, BEND, is used as a model to investigate the mechanisms regulating PG production, but it expresses both epithelial and stromal cell markers suggesting a mixed phenotype (Binelli, Subramaniam et al. 2001). Immortalized cell lines of luminal and glandular epithelial cells and stromal cells have been generated and characterized in sheep (Johnson, Burghardt et al. 1999) but no bovine stromal cells are available. In this report, we document the generation of a stromal cell line and show its utility in studying the regulation of PG biosynthesis in response to the embryonic signal IFNτ.

Materials and Methods

Immortalization and clone selection

Primary stromal cell cultures were prepared as described previously with minor modifications (Asselin, Goff et al. 1996). Stromal cells were transfected by nucleofection with a plasmid expressing SV40 TAG and a gene resistant to aminoglycoside G418 (neomycin) and an other plasmid coding for bacteriophage phiC31 integrase (Quenneville, Chapdelaine et al. 2007). The CMV promoter of the SV40 TAG transgene was flanked by an attB sequence to improve plasmid integration into the genome. Sub-confluent cultures of caruncular and intercaruncular stromal cells were trypsinized and resuspended in serum free media and 5 μg plasmid DNA coding for

integrase and 5μg vector DNA containing SV40 Large TAG were added to $1X10^6$ cells and nucleofected using the T16 program. Nucleofection efficiency was 60% as assessed by green fluorescence protein. After 3 days, the cells were trypsinized and cultured in 150x20 mm petri plates for 7 days in presence G418 (200μg/ml) to select resistant colonies. A total of 33 clones (7 caruncular and 26 intercaruncular) were picked using O-ring and clonally propagated in separate T-25 flasks up to 10 passages (P10). We then selected one caruncular (CSC; clone #: CAR7) and one intercaruncular (ICSC; clone #: ICAR6) stromal clone according to basal and TNFα (6nM) induced PGE_2 and $PGF_{2\alpha}$ production, growth rate and stromal morphology and passed the cell lines until P50.

Immunofluorescence Analysis

CSC and ICSC were cultured on Lab Tek 4-chamber slides (Nalge Nunc International Rochester, NY, USA) and analyzed for expression of cytokeratin (Sigma), vimentin (in house Ab) and SV40 Large T Ag (Oncogene Research Products, San Diego) by immunofluorescence as described previously (Chapdelaine, Kang et al. 2006). Lipofectamine mediated transfection of green fluorescent protein was done in CSC as per the manufacturer's instructions (Invitrogen Life Technologies Inc., Canada).

Experimental protocols

The caruncular stromal cell line (CSC) was selected for the subsequent studies because it exhibited optimal growth rate and a PG production profile representative of all clones tested including those from intercaruncular areas (ICSC). Typically, CSC cultures were initiated from a frozen aliquot and grown to confluency in a T75 flask for 60-72 h at 37°C and 5% CO_2. The monolayer was trypsinized, extended with RPMI-1640 supplemented with 10% fetal bovine serum, divided into two equal volumes and centrifuged at 1500 rpm for 10 min. One fraction was frozen and stored at -150°C for future experiments. The other fraction was diluted at $4\ X10^4$ cells/ml and seeded in 24-well plates. Confluent cultures were exposed to steroid free medium overnight before treatment. At the end of the experiment, culture medium was harvested and stored at -20°C until analysis for PG. Protein extraction and estimation was done as described previously (Chapdelaine, Vignola et al. 2001). All experiments were replicated three times and each treatment was run in quadruplicate unless indicated otherwise. For functional characterization, cells were treated with IFNτ (10μg/ml), lipopolysaccharide (LPS, 10 ng/ml), phorbol myristate acetate (PMA 10 nM), OT (500 nM), TNFα (6 nM) and COX2 inhibitor NS-398 (1μM). The concentrations used were based on previously published conditions (Parent,

Chapdelaine et al. 2002). PGE_2 and $PGF_{2\alpha}$ production by CSC cells was then associated with expression levels of key enzymes of the biosynthetic cascade. Experiment-2 aimed at comparing PG biosynthesis following treatment with increasing concentrations of IFNτ and PMA from 0.02 to 20 μg/ml and 1 pM to 100 nM, respectively and their interactions on COX2 and STAT1 genes in CSC cells. In experiment-3, the involvement of p38 mitogen activated kinase (p38 MAPK) was tested by treating CSC with high concentration of IFNτ (10μg/ml) in presence or not of the selective inhibitor of p38 MAPK, SB203580 (1μM).

Enzyme Immunoassays (EIAs) of PGE_2 and $PGF_{2\alpha}$

Prostaglandins were assayed by competitive EIA using acetylcholinesterase –linked PG tracers (Cayman, Ann Arbor, MI, USA) as described previously using rabbit anti-PGE_2 (kindly provided by Dr TG Kennedy) and sheep anti- $PGF_{2\alpha}$ (Bio-Quant, Ann Arbor, MI, USA).(Asselin, Goff et al. 1996).

Western Blot analysis

An aliquot of 10μg protein was loaded in each lane, resolved on 12.5% SDS-PAGE and electro-transferred onto 0.45 μm nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada). However, for detection of cytosolic phospholipase A2 (cPLA2), 7% gel was used and for mPGES-1, 0.2 μm nitrocellulose membrane was used. The membranes were blocked in 5% (w/v) non-fat dried milk in PBS containing 0.05% Tween-20 (PBST) for 1 h at room temperature and incubated overnight at 4°C with respective primary antibodies. The primary antibody dilutions were as follows: 1/500 for anti cPLA2 (Santa-Cruz Biotechnology); 1/3000 for anti-COX1 and anti-COX2 (kindly provided by Dr S Kargman, Merck Frosst Montreal, Canada); 1/250 for anti-mPGES-1, (Cayman, Canada); 1/500 for mPGES-2 and cPGES and 1/2000 for anti-AKR1B5, a polyclonal serum raised in our laboratory using recombinant protein; 1/1000 for STAT1 (BD Biosciences, Canada) and anti pS727 STAT1 (Upstate biotechnology, NY); 1/1000 for phosphorylated and unphosphorylated p38 antibodies (Upstate biotechnology, NY) and 1/5000 for βactin (Sigma). After three washes of 10 min each in PBS, the membranes were incubated for 1h at RT with appropriate secondary antibody. The membranes were washed three times in PBST; treated for 1 min with enhanced chemiluminescent substrate (Perkin-Elmer) and exposed to Bio-Max film. Relative optical density (ROD) of three different immunoblots from each experiment was quantitated by densitometry (Alpha imager).

RT-PCR

Total RNA was extracted using TRIzol (Invitrogen Life Technologies Inc., Burlington, Ontario, Canada); reverse-transcribed with Superscript II RT (Invitrogen Life Technologies Inc., Burlington, Ontario, Canada). To demonstrate ERα and PR in the cell line, the following specific sets of primers were used: For ERα, the sense and antisense primers were 5′ATGACCCTACCAGACCTTTCAGT3′ and 5′ATTTGAGGCACACAAACTCTTC3′, respectively. Similarly, for PR, the forward and reverse primers were 5′ATTGTTGATAAAATCCGCAGAAA3′ and 5′GAGGTATCAGGTTTGCTGTTGTC 3′, respectively. ERα primers were deduced from Accession No. NM_001001443, while PR specific primers were designed based on the accession no. AY656812.

Statistical Analysis

Randomized block design (RBD) was used in all the experiments with treatment as the main factor and plate as random effect. The resulting data on PG production was transformed into fold stimulation (except Fig. 2) by dividing each observation by the mean of control. Statview program was used for analyzing the transformed data. The group mean of different treatments was tested by two-way ANOVA with Fisher`s PLSD as the post-hoc test to find the critical difference between pair of treatment means. Confidence level was set at 95% ($P<0.05$) to determine statistical significance. Data are presented as the Mean ± SEM.

Results

Establishment of bovine endometrial stromal cell lines and phenotype characterization

The selected stromal cell line could be propagated until at least P50 while maintaining all phenotypic characteristics of primary cultures and expression of ERα and PR mRNA (Fig. 1C). CSC and ICSC expressed SV40 TAG, mesenchyme specific vimentin but were negative for the epithelial specific cytokeratin (Fig.1A&B). The cell line is also amenable for transient transfection. Average basal and TNFα stimulated PGE_2 and $PGF_{2\alpha}$ production of all bovine endometrial stromal clones from either caruncles (n=7) or intercaruncles (n=26) are shown in Fig.2A. PGE_2 production was higher ($P<0.05$) than $PGF_{2\alpha}$ under basal and TNFα stimulated conditions (Fig.2A) which was abolished in presence of the COX-2 inhibitor NS-398 (Fig.2B). PGE_2 production was also significantly higher in the caruncular clones ($P<0.05$).

Regulation of PG biosynthesis and expression of corresponding genes

In the caruncular cell line CSC, PGE_2 and $PGF_{2\alpha}$ production increased significantly following stimulation with IFNτ, TNFα , LPS and PMA ($P< 0.05$), but not with OT (Fig. 3A). Note that in

Fig. 3 and following figures, the scale used for PGE_2 is higher than that of $PGF_{2\alpha}$. Analysis of the enzymes in the PGE_2 and $PGF_{2\alpha}$ biosynthetic pathway indicated that increased PG production was mostly coupled with increased COX2 and cPLA2 expression. Terminal synthases, although all expressed at a significant level, did not exhibit any modulation (Fig. 3B). Increased expression of COX2 together with inhibition of TNFα stimulation by NS398 (Fig. 2B) confirms COX2 as a rate-limiting step.

CSC as a model for functional studies

As was reported for primary stromal and BEND cells, PMA induced COX2 expression and PGE_2 and $PGF_{2\alpha}$ production in a dose-dependent manner in CSC (Fig. 4). Similarly, IFNτ increased ($P<0.05$) PGE_2 and $PGF_{2\alpha}$ production as well as COX2 expression (Fig.5 A and B) in a dose dependent manner. Expression of COX2 closely parallels PG production (Fig 5C). Treatment with IFNτ stimulated STAT1 phosphorylation, and total STAT1 expression (Fig 5 B&C). IFNτ was able to inhibit PMA stimulated PG production at low concentrations but this response disappeared at higher concentrations (Fig. 6A). The same effect was observed at the level of COX2 expression (Fig. 6 B&C). However, PMA had no effect on STAT1 expression or on its stimulation by IFNτ (Fig. 6 B&C). When opposite conditions were tested, the effect of increasing concentrations of PMA stimulated further PG production and COX2 expression (Fig. 7 A, B & C) but did not impact on IFNτ induced STAT1 expression (Fig. 7 C). Since the dose-dependent biphasic effect of IFNτ on COX2 was not observed at the level of STAT1 in CSC, we hypothesized that MAPK known to regulate COX2 expression in the myometrium (Doualla-Bell and Koromilas 2001), could be involved. Accordingly, IFNτ stimulated COX2 expression and PG production was significantly blocked by the selective inhibitor SB203580 (Fig. 8 A,B&C) suggesting a contribution of the p38 MAPK pathway in IFNτ mediated signaling.

Discussion

The presence of SV40 TAG in CSC and ICSC and repeated passage without apparent senescence confirmed the permanent status of the selected cell lines. Vimentin positive and cytokeratin negative features support the stromal phenotype and mesenchymal origin of the cell lines. TNFα was used to stimulate PG production during initial screening of the stromal clones because this cytokine has been ascribed a role in the initiation of luteolysis (Miyamoto, Skarzynski et al. 2000). Both caruncular and intercaruncular clones produced PGE_2 levels 10-30 folds higher than $PGF_{2\alpha}$, a feature distinguishing endometrial stromal from epithelial cells (Asselin, Goff et al.

1996). Basal and TNFα stimulated PGE_2 production were significantly higher in the caruncular than the intercaruncular stromal clones, a finding supported by our previous observations (Asselin, Drolet et al. 1998). Increased PG production in response to IFNτ, TNFα, LPS and PMA and non-responsiveness to OT observed in this study is in agreement with our previous observations on primary stromal cell cultures (Asselin, Goff et al. 1996; Parent, Chapdelaine et al. 2002). When we analyzed the biosynthetic enzymes, we found that only COX2 and cPLA2 are regulated in parallel with PG production. This was confirmed using NS-398, a COX2 specific inhibitor that blocked TNFα stimulated PG production. These observations are also in agreement with what was observed by us in primary stromal cells for COX2 (Parent, Chapdelaine et al. 2002) and by others in BEND cells for cPLA2 (Binelli, Guzeloglu et al. 2000). Under the present experimental conditions, we have found that PGE and PGF terminal synthases are expressed, but not modulated. Since mPGES1 is known to be associated with COX2 in situations of increased PGE_2 production, constant expression of the former with induced expression of the latter appears to be sufficient to explain our observations in CSC. Taken together, these results show that the PG enzymatic machinery is intact and functional in CSC where COX2 appears as the rate-limiting enzyme. Since CSC is also amenable for transfection, this cell line can be used in combination with gene silencing experiments with siRNA to confirm the contribution of the different members of the PG biosynthetic cascade. Stromal cell specific phenotype, presence of ERα and PR, preferential production of PGE_2 over $PGF_{2\alpha}$ and responsiveness to known PG production inducers validate CSC as a *bona fide* stromal cell model to study the molecular aspects of *in vitro* regulation of PG production in bovine.

Using this validated model, we studied the regulation of COX2 and PG production in response to PMA and IFNτ and their interactions, and attempted to correlate it with the expression of STAT1 involved in IFNτ mediated signaling (Johnson, Burghardt et al. 1999; Binelli, Subramaniam et al. 2001). Phorbol ester was chosen since it is used as a surrogate for OT response in BEND cells. It is clear that both PMA and IFNτ induce COX2 expression and PGE_2 and $PGF_{2\alpha}$ production in a dose-dependent manner. However, IFNτ alone is able to phosphorylate and up-regulate STAT1. Phosphorylation of STAT1, which is still detectable after 24 hours in CSC is comparable with the persistent tyrosine phosphorylation of STAT1 observed in ovine endometrial epithelial cell line and may be involved in sustained effects of IFNτ (Stewart, Johnson et al. 2001). Interaction studies showed that PMA- induced COX2 expression can be inhibited by low but not high

concentrations of IFNτ. Second, PMA does not affect IFNτ induced up-regulation of STAT1. Since the apparent biphasic effect of IFNτ on COX2 could not be correlated at the level of STAT1, we chose to probe the p38 MAPK pathway known to be involved in other systems. Interestingly, the p38 MAPK inhibitor SB203580 blocked the effect of IFNτ on COX2 expression and PG production. This result is supported by the observation that IFNτ confers transcriptional stability to COX2 in bovine myometrial cells through p38 MAPK (Doualla-Bell and Koromilas 2001).

The biphasic effect or dose-dependent dichotomy of IFNτ on COX2 expression may throw some light on the up-regulation of COX2 observed during the maternal recognition window in ruminants (Charpigny, Reinaud et al. 1997; Kim, Choi et al. 2003; Guzeloglu, Bilby et al. 2004) as well as following intrauterine infusions of IFNτ (Emond, MacLaren et al. 2004). Given that copious production of IFNτ occurs during recognition of pregnancy (Ashworth and Bazer 1989) and since ISGs are mainly present in the endometrial stroma (Bazer and Spencer 2006), it is possible that the up-regulation of COX2 by high concentrations of IFNτ reflects the *in vivo* conditions. COX2 mediates inflammation and tumorigenesis (Trifan and Hla 2003), and is traditionally viewed as pathological, but it is also necessary for normal female reproductive function (Thomson, Telfer et al. 1999; Sirois, Sayasith et al. 2004). In humans and rodents, implantation is associated with elevated levels of PGE_2 by the decidualizing stromal cells (Dey, Lim et al. 2004). Though the implantation is superficial and its onset is relatively late in ruminants as compared to human, it is associated with up-regulation of COX2 (Charpigny, Reinaud et al. 1997; Kim, Choi et al. 2003; Emond, MacLaren et al. 2004; Guzeloglu, Bilby et al. 2004). Our preliminary results with CSC suggest that IFNτ may influence PGE_2 and COX2 through the p38 MAPK pathway to mediate its pro-gestation effects in the endometrial stroma. In this respect, CSC may serve as an ideal model for investigating the paradigm of counteraction of the luteolytic $PGF_{2\alpha}$ and the immunomodulatory PGE_2, at the time of maternal recognition of pregnancy.

Acknowledgements

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Abbreviations

AA, arachidonic acid; AKR1B5, aldoketoreductase 1 B5; BEND, bovine endometrial cell; COX, cyclooxygenase; cPGES, cytosolic PGE synthase; cPLA2, cytosolic phospholipase A2; CSC, caruncular stromal cell; ERα, estrogen receptor-α; ICSC, intercaruncular stromal cell; IFN, interferon regulatory factor; IFN-τ, interferon-τ; ISG, interferon stimulated genes; JAK, janus kinase; mPGES, microsomal PGE synthase; MAPK, mitogen activated protein kinase; OT, oxytocin; OTR, oxytocin receptor; PG, prostaglandin; PGFM, $PGF_{2\alpha}$ metabolite; PMA, phorbol myristate acetate; PR, progesterone receptor; ROD, relative optical density; STAT, signal transducer and activator of transcription; SV40 large TAG, simian virus 40 Large T antigen; TNFα, tumor necrosis factor-α.

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Figure 4.1: A) Characterization of cytoskeletal proteins expressed in the selected caruncular stromal cell line CSC. Subconfluent endometrial cells were stained with cytokeratin and vimentin antibodies and detected with a fluorescent secondary antibody. Left panels represent phase-contrast illumination, while center and right panels show cytokeratin and vimentin fluorescence, respectively (Magnification 100X).

B) Integration of SV-40 Large T antigen within the genome of bovine caruncular and intercaruncular stromal clones. 1. Demonstration by immunofluroescence (100X) 2. Detection of SV40 TAG by immunoblot. Lanes 1-3 represent primary stromal cells, CSC and ICSC.

C) Demonstration of estrogen and progesterone receptors in CSC by RT-PCR. Lanes 1-3 represent primary stromal cells, CSC and ICSC.

Figure 1

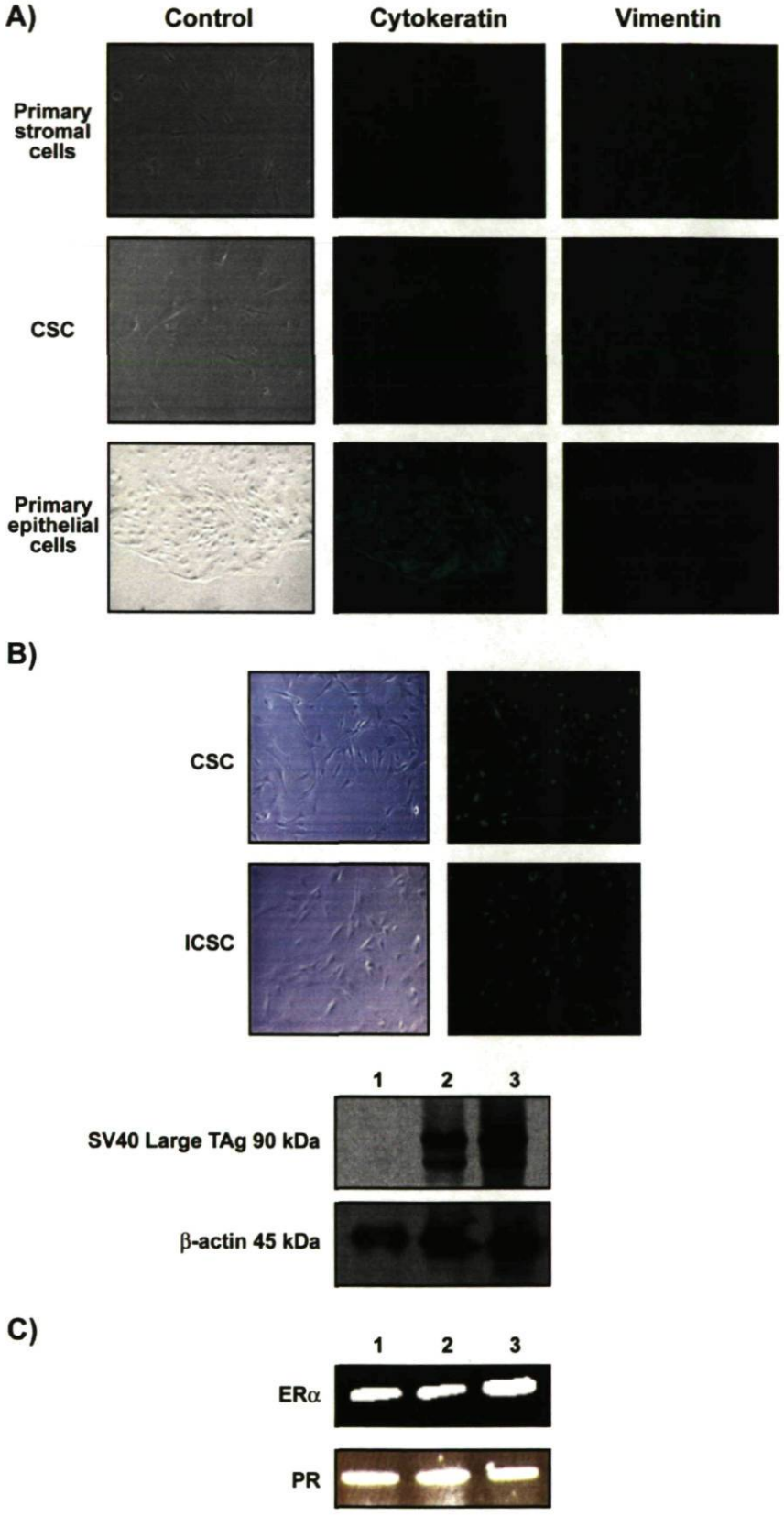

A)
Control
Cytokeratin
Vimentin
Primary stromal cells
CSC
Primary epithelial cells
B)
CSC
ICSC
1
2
3
SV40 Large TAg 90 kDa
β-actin 45 kDa
C)
1
2
3
ERα
PR

Figure 4.2: Prostaglandin E_2 and $F_{2\alpha}$ production in immortalized endometrial stromal cells. A) Immortalized cell lines from all caruncular (CAR, n=7) and intercaruncular (ICAR, n=26) clones were grown to confluency and stimulated or not with TNFα 6nM for 24h. Results are the Mean ± SEM of PG levels from all clones. B) Effect of NS398, a COX2 inhibitor, on TNFα induced PG production in CSC. Bars with different superscripts differ significantly ($P< 0.05$).

Figure 2

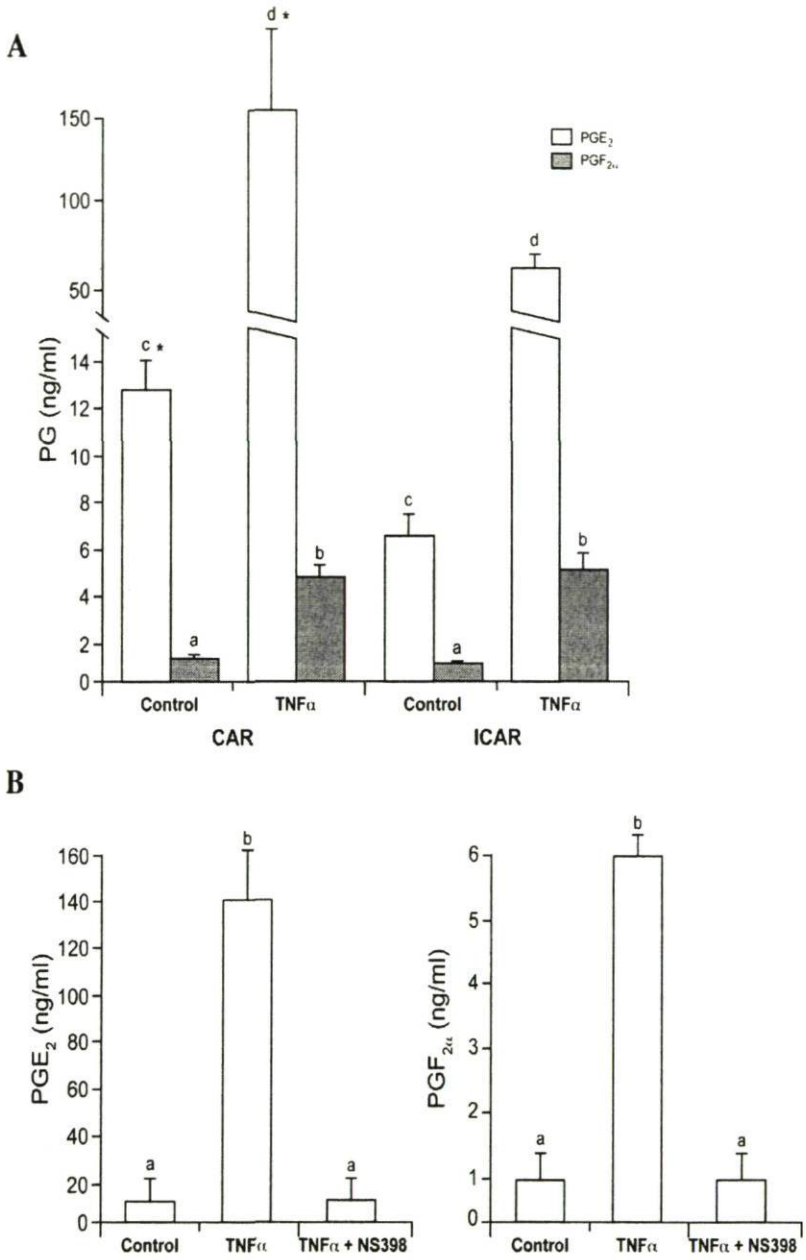
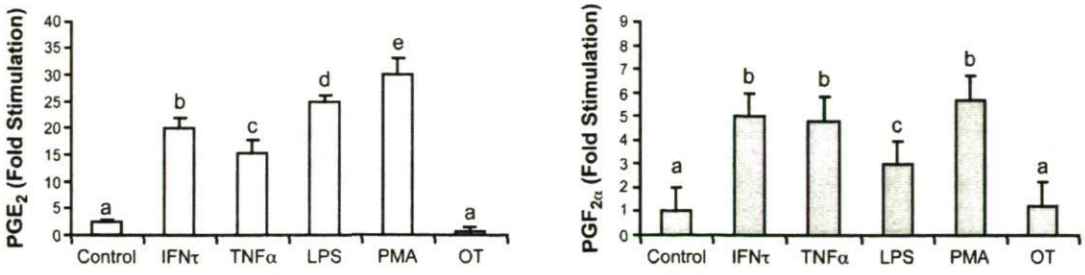

A
PG (ng/ml)
PGE2
PGF2α
Control
TNFα
CAR
ICAR
B
PGE2 (ng/ml)
PGF2α (ng/ml)
Control
TNFα
TNFα + NS398

Figure 4.3: Effect of IFNτ, TNFα, LPS, PMA and OT on PG production in CSC.

A) PGE_2 and $PGF_{2\alpha}$ production. Values represent the Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). B) Representative immunoblots of cPLA2, COX 1&2, AKR1B5, mPGES 1&2, cPGES and βActin. Lanes 1-6 refer to control, IFNτ 10μg/ml, TNFα 6 nM, LPS 0.01μg/ml, PMA10nM and OT 0.5μM, respectively.

Figure 3

A


PGE2 (Fold Stimulation)
40
35
30
25
20
15
10
5
0
a
b
c
d
e
a
Control
IFNτ
TNFα
LPS
PMA
OT
PGF2α (Fold Stimulation)
9
8
7
6
5
4
3
2
1
0
a
b
b
c
b
a
Control
IFNτ
TNFα
LPS
PMA
OT

B

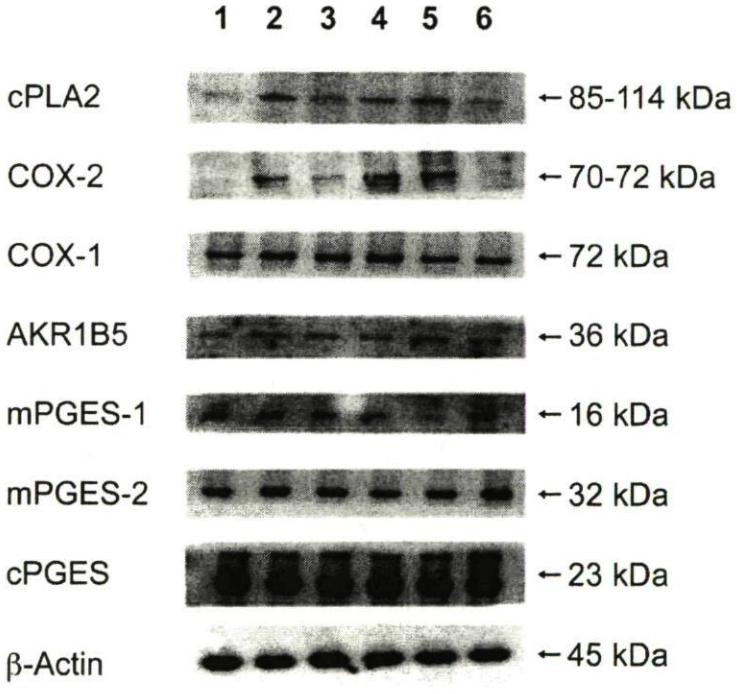
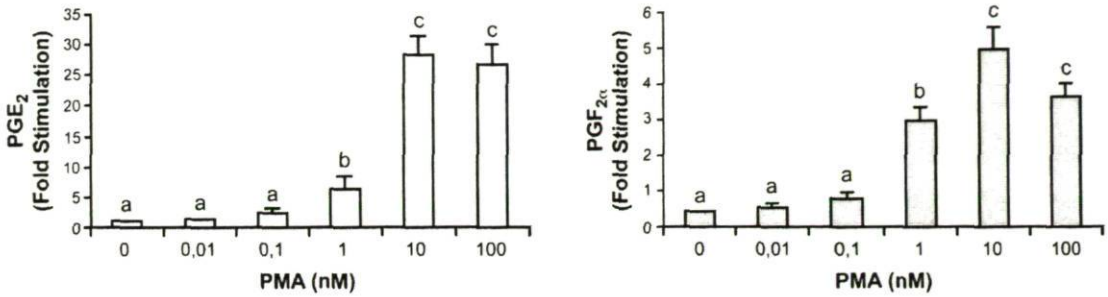

1 2 3 4 5 6
cPLA2
← 85-114 kDa
COX-2
← 70-72 kDa
COX-1
← 72 kDa
AKR1B5
← 36 kDa
mPGES-1
← 16 kDa
mPGES-2
← 32 kDa
cPGES
← 23 kDa
β-Actin
← 45 kDa

Figure 4.4: Effect of PMA on PG production and COX2 expression in CSC.

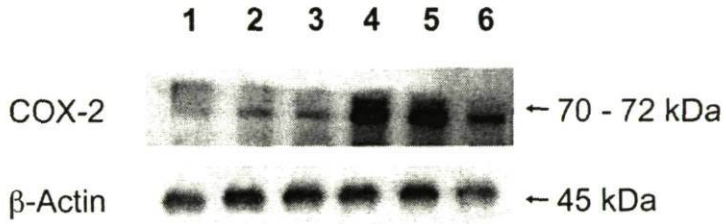
CSC were treated with increasing concentrations of PMA (0-100 nM) for 24 h and PGs were measured in the culture medium. Values represent the Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). A) PGE_2 and $PGF_{2\alpha}$ production. B) Representative immunoblots of COX2 and βActin. Numbers 1-6 indicate different concentrations of PMA; 0, 0.01, 0.1, 1.0, 10 & 100 nM, respectively. C) Relative optical density (ROD) values are ratio between COX2 and βActin.

Figure 4

A


PGE2 (Fold Stimulation)
35
30
25
20
15
10
5
0
a a a b c c
0 0,01 0,1 1 10 100
PMA (nM)
PGF2α (Fold Stimulation)
6
5
4
3
2
1
0
a a a b c c
0 0,01 0,1 1 10 100
PMA (nM)

B


1 2 3 4 5 6
COX-2
← 70 - 72 kDa
β-Actin
← 45 kDa

C

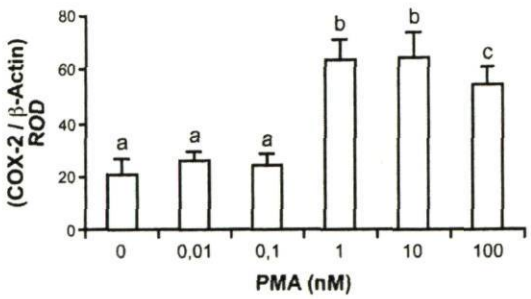

(COX-2 / β-Actin) ROD
80
60
40
20
0
a a a b b c
0 0,01 0,1 1 10 100
PMA (nM)

Figure 4.5: Effect of IFNτ on PG production and COX2 expression in CSC.

CSC were treated with increasing concentrations of IFNτ (0-20μg/ml) for 24 h and PGs were measured in the culture medium. Values represent the Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). Lanes 1-6 on B and X-axis of C indicate different concentrations of IFNτ; 0, 0.02, 0.2, 2.0, 10 and 20 μg/ml, respectively. A) PGE_2 and $PGF_{2\alpha}$ production. B) Representative immunoblots of COX2, phosphorylated and unphosphorylated STAT1 and βActin. C) ROD values are ratio between COX2/βActin and phosphorylated to total STAT1.

Figure 5

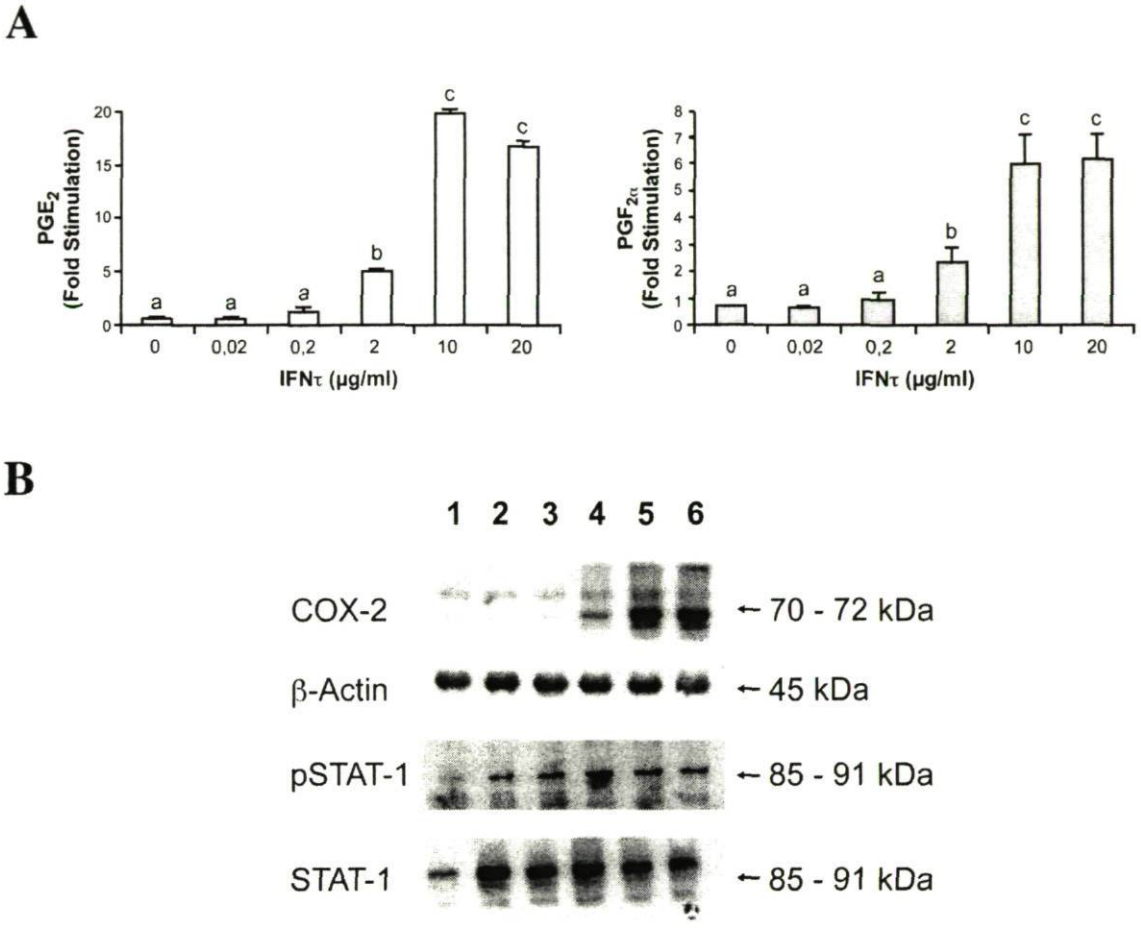
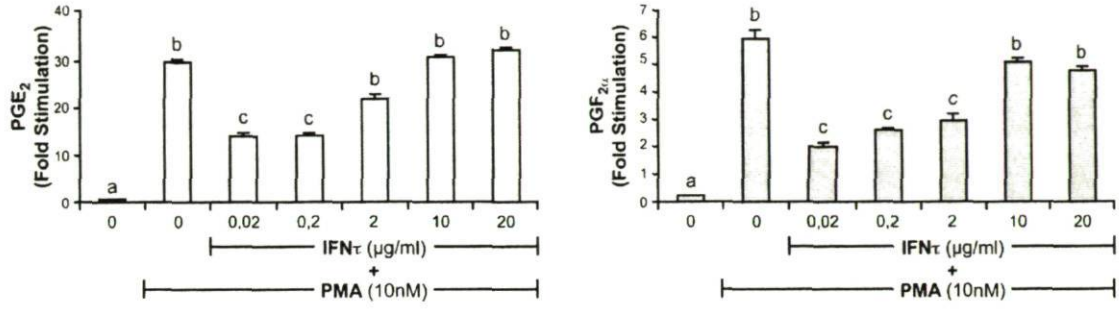

A
PGE2 (Fold Stimulation)
IFNτ (µg/ml)
0
0,02
0,2
2
10
20
PGF2α (Fold Stimulation)
B
1 2 3 4 5 6
COX-2
← 70 - 72 kDa
β-Actin
← 45 kDa
pSTAT-1
← 85 - 91 kDa
STAT-1
← 85 - 91 kDa
C
(COX-2 / β-Actin) ROD
(pSTAT-1/STAT-1) ROD

Figure 4.6: Interaction between PMA and IFNτ in CSC.

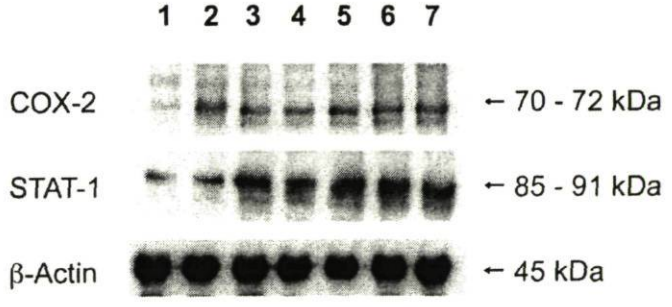
CSCs were treated with PMA 10 nM and various concentrations of IFNτ (0-20μg/ml) for 24 h and PGs were measured in the culture medium. Values represent the Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). Numbers on B and X-axis of C indicate: 1) Control 2) PMA 10nM 3) PMA 10nM+IFNτ 0.02μg/ml 4) PMA 10nM+IFNτ 0.2μg/ml 5) PMA 10nM+IFNτ 2.0μg/ml 6) PMA 10nM+IFNτ 10μg/ml 7) PMA 10nM+IFNτ 20μg/ml. A) PGE_2 and $PGF_{2\alpha}$ production. B) Representative immunoblots of COX2, STAT1 and βActin. C) ROD values are ratio between COX2, STAT1 and βActin.

Figure 6

A


PGE2 (Fold Stimulation)
PGF2α (Fold Stimulation)
0
0
0,02
0,2
2
10
20
IFNτ (µg/ml)
+
PMA (10nM)

B


1 2 3 4 5 6 7
COX-2
← 70 - 72 kDa
STAT-1
← 85 - 91 kDa
β-Actin
← 45 kDa

C

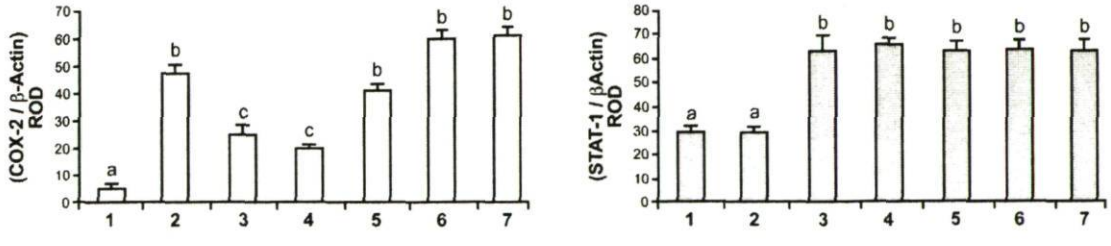
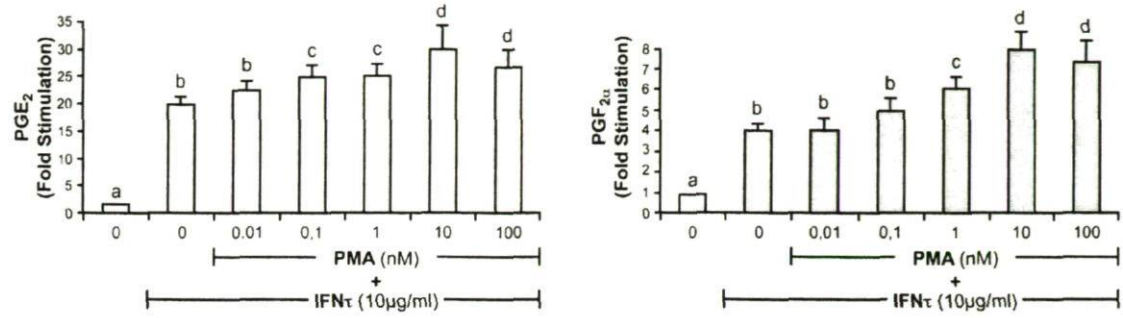

(COX-2 / β-Actin) ROD
(STAT-1 / βActin) ROD
1 2 3 4 5 6 7

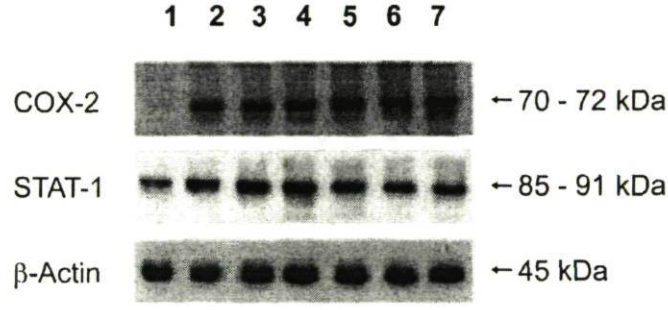
Figure 4.7: Interaction between IFNτ and increasing concentrations of PMA in CSC. CSC were treated with IFNτ 10μg/ml and various concentrations of PMA (0.01-100 nM) for 24 h and PGs were measured in the culture medium. Values represent the Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). Numbers on B and X-axis of C indicate: 1) Control 2) IFNτ 10μg/ml 3) IFNτ 10μgml+PMA 0.01nM 4) IFNτ 10μgml+PMA 0.1nM 5)IFNτ 10μgml+PMA 1.0nM 6) IFNτ 10μgml+PMA 10nM 7) IFNτ 10μgml+PMA 20nM. A) PGE_2 and $PGF_{2\alpha}$ production. B) Representative immunoblots of COX2, STAT1 and βActin. C) ROD values are ratio between COX2 or STAT1 and βActin.

Figure 7

A


PGE2 (Fold Stimulation)
PGF2α (Fold Stimulation)
PMA (nM)
+
IFNτ (10µg/ml)
0
0
0,01
0,1
1
10
100

B


1 2 3 4 5 6 7
COX-2
STAT-1
β-Actin
←70 - 72 kDa
←85 - 91 kDa
←45 kDa

C

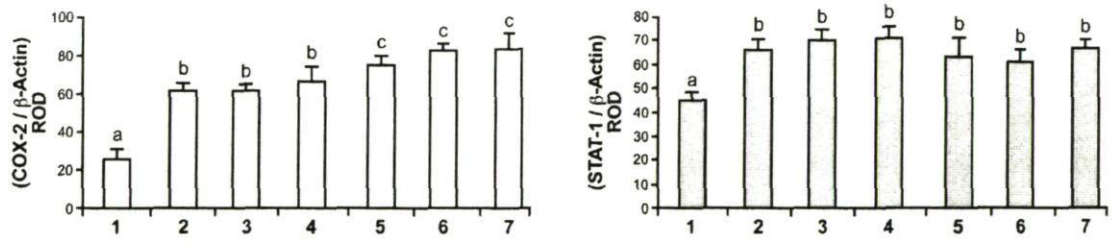
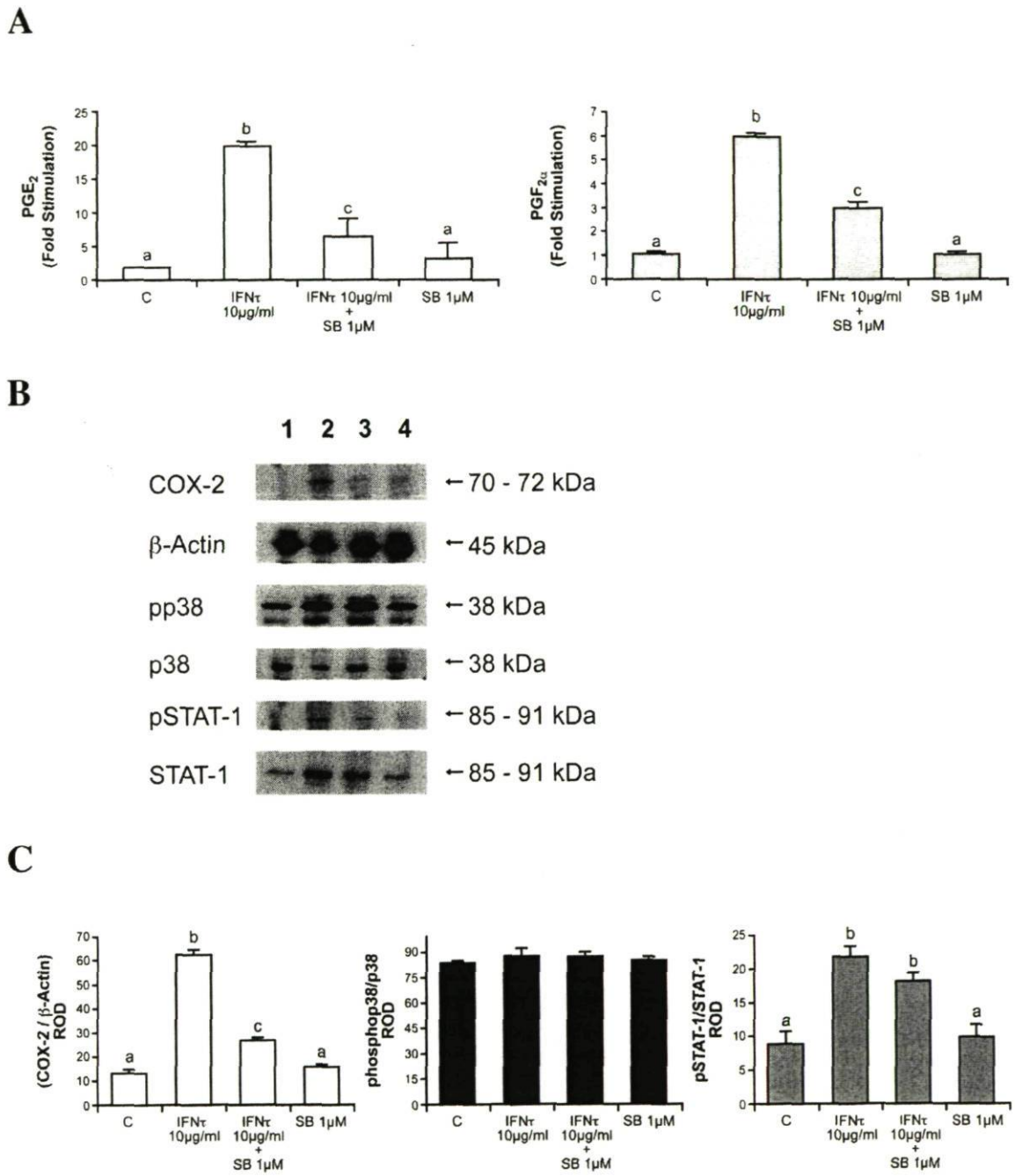

(COX-2 / β-Actin) ROD
(STAT-1 / β-Actin) ROD
1
2
3
4
5
6
7

Figure 4.8: Effect of the p38 MAPK inhibitor, SB203580 (1μM) on IFNτ (10 μg/ml) induced PG production in CSC.

CSC were treated with IFNτ 10μg/ml in presence or absence of SB203580 (1μM) for 24 h.A) PGE_2 and $PGF_{2\alpha}$ production. Values represent the Mean ± SEM of three different experiments run in quadruplicate. Bars with different superscripts differ significantly ($P<0.05$). B) Representative immunoblots of COX2, βActin, phosphorylated and unphosphorylated p38 MAPK and STAT1. Lanes 1-4 indicate control, IFNτ 10μg/ml, IFNτ 10μg/ml + SB203580 1μM and SB203580 1μM, respectively. C) ROD values are ratio between COX2/βActin and phosphorylated to total p38MAPK and STAT1.

Figure 8


A
PGE2 (Fold Stimulation)
PGF2α (Fold Stimulation)
C
IFNτ 10μg/ml
IFNτ 10μg/ml + SB 1μM
SB 1μM
B
1 2 3 4
COX-2
β-Actin
pp38
p38
pSTAT-1
STAT-1
←70 - 72 kDa
←45 kDa
←38 kDa
←38 kDa
←85 - 91 kDa
←85 - 91 kDa
C
(COX-2 / β-Actin) ROD
phosphop38/p38 ROD
pSTAT-1/STAT-1 ROD

Chapter V

General Discussion

Over the last 15 years, in spite of intrinsic limitations, our laboratory has established bovine endometrial primary cell cultures as a model to study the regulation of PG synthesis in relation with recognition of pregnancy (Fortier, Guilbault et al. 1988; Asselin, Goff et al. 1996; Parent, Villeneuve et al. 2003). In general, primary cell cultures are highly vulnerable to bacterial contamination and the incidence is prohibitively high during the summer thus forcing us to keep the primary culture operations in abeyance. Uterine pathologies are also more prevalent during summer (Erb and Martin, 1980). It is difficult to get a constant supply of healthy genital tracts from the abattoir. It is difficult to have a precise estimate of seeding concentration at the time of pre-plating, because epithelial cells usually appear as sheets or glands following trypsin digestion. It takes about 7-10 days to establish a primary culture. Above all, cells in primary culture have limited lifespan and die because of replicative senescence. Inspite of all the oddities, we have established the production profiles of PGE_2 and $PGF_{2\alpha}$ following the treatment with OT, estrogen and progesterone and studied the expression of COX-2 (Asselin, Goff et al. 1996; Asselin, Lacroix et al. 1997). Accordingly, the basic objectives of the present work was to establish stable *in vitro* culture systems of endometrial epithelial and stromal cells by generating cell lines and test their ability to mimic the primary culture responses. The specific objective was to study cellular mechanisms behind the interaction between OT and IFNτ in relation to PGE_2 and $PGF_{2\alpha}$ production.

Immortalization of primary bovine endometrial epithelial cells was difficult. Our experiments with electroporation and lentiviral infection of primary epithelial cells suggest that the cell membrane has some 'barrier'. We got bEEL cells following a lentiviral infection without the integration of the immortal gene. These cells retained all the cardinal characters of primary endometrial epithelial cells. Though OT stimulation induced $PGF_{2\alpha}$ as well as PGE_2 production, the ratio of $PGF_{2\alpha}/PGE_2$ was always higher than CSC. Functional studies with OT and IFNτ showed three interesting observations: First, IFNτ upregulated COX2 expression in a time and concentration dependent manner that was not accompanied by an increase in PG production. Second, a significant inhibition of OT

induced $PGF_{2\alpha}$ production by IFNτ (20 ng/ml) was not mediated through the downregulation of COX2 or OTR in bEEL cells. Third, IFNτ (20 ng/ml) could inhibit OT induced $PGF_{2\alpha}$ production within 3-6 h. Collectively, these observations differed from the current hypothesis of OTR down-regulation by IFNτ (Spencer and Bazer 2004) and indicated that IFNτ, in part, exerts its antiluteolytic effect by disrupting OT signaling pathway perhaps through limiting the supply of arachidonic acid or uncoupling COX2 from terminal synthase.

Accordingly, we studied the signaling pathway of OT induced $PGF_{2\alpha}$ production in bEEL cells using kinase inhibitor approach and got interesting results. First, Ras-dependent and PKC independent activation of ERK1/2 pathway was involved in OT induced $PGF_{2\alpha}$ production suggesting the involvement of $G\alpha_i$ but not $G\alpha_q$ subunit. Second, $G\alpha_i$ mediated its effect through a mechanism that did not involve its classical inhibitory effect on adenylate cyclase. Third, transactivation of EGFR, c-Src tyrosine kinase and PI3K were involved in OT induced $PGF_{2\alpha}$production. Fourth, the $G\beta\gamma$ subunit was not involved in OT induced $PGF_{2\alpha}$ production. Identifying OT sensitive isoforms of PLA2 and the effect of OT on PGF synthase are the prerequisites for identifying the site of action of IFNτ.

Immortalization of bovine endometrial stromal cells with SV-40 Large T antigen (SV40 TAG) was easier than that of epithelial cells. Stromal cells from the caruncular and non-caruncular regions were used for two reasons: First, we have shown previously that the PG production was superior in the caruncular than the intercaruncular region (Asselin, Drolet et al. 1998). Second, caruncles are the unique sites of placental attachment in the bovine and are non-glandular as compared to the intercaruncular region. SV-40 TAG served as an immortalizing gene because we used it for establishing human endometrial epithelial and stromal cell lines (Chapdelaine, Kang et al. 2006). Based on the initial screening of the clones, we selected the best caruncular stromal cell (CSC) clone in terms of PG production ability and replication vigor. Stellate or spindle shaped morphology, vimentin positivity, presence of estrogen and progesterone receptor transcripts, lack of OT response, IFNτ responsiveness and high levels of basal and stimulated (PMA, IFNτ, LPS, TNFα) PGE_2 and low $PGF_{2\alpha}$ production validated that CSC is a *bona fide* representative of primary bovine

endometrial stromal cells. As shown previously(Asselin, Lacroix et al. 1997), functional studies with CSC showed that IFNτ upregulated COX2 expression and stimulated PGE_2 production especially at concentrations above 2 μg/ml. We have shown that this was mediated by p38 MAPK pathway. However, at concentrations less than 200 ng/ml, it inhibited PMA induced COX2 expression and PGE_2 production.

In summary, we were successful in establishing bovine endometrial epithelial (bEEL) and stromal (CSC) cell lines that reliably represented primary endometrial cells. Induced (OT or PMA) PG production ($F_{2\alpha}$ or E_2) from either cell type (bEEL or CSC) was inhibited by IFNτ (20 ng/ml) and this was not through COX2 downregulation. Rather, IFNτ upregulated COX2 expression in both cell lines. We draw the following conclusions based on the studies:

Conclusions:

1. bEEL cell is a good *in vitro* model to identify the molecular targets of OT and/or IFNτ. The antiluteolytic effect of IFNτ was not mediated through the down regulation of either COX-2 or OTR. Rather, it appears that IFNτ may disrupt some kinases (MEK1 or ERK1/2) or transcriptional factors downstream of C-Raf (also called as Raf-1) in the ERK1/2 pathway by which OT stimulates $PGF_{2\alpha}$ production in bEEL cells.

2. Ras-dependent and PKC independent activation of ERK1/2 (p42/44 MAPK) pathway was involved in OT-induced $PGF_{2\alpha}$ production in bEEL cells. Binding of OT to its cognate GPCR led to the transactivation of EGFR and this process might be the prerequisite for the activation of Ras protein. Similarly, phosphorylation of PI3K and c-Src tyrosine kinases was unequivocally involved in OT induced $PGF_{2\alpha}$ production and appears to be necessary for the transactivation of EGFR. And, OT induced $PGF_{2\alpha}$ production seems to be coupled to PTx sensitive $G\alpha_i\beta\gamma$.

3. The stromal cell line, CSC retained the cardinal characters of the primary bovine endometrial stromal cells. IFNτ stimulated COX2 expression and PGE_2 production in a dose-dependent manner. Phorbol ester induced PG production was inhibited by

low concentrations (< 200 $ngml^{-1}$) and this inhibition was lost at high concentrations (> 200 $ngml^{-1}$).

4. IFNτ, at concentrations less than 200 μgml^{-1} inhibited induced (OT or PMA) PG production in both cell lines; however at high concentrations, it up-regulated COX-2 and increased PGE_2 production, especially in CSC. Thus, the net effect of IFNτ on the endometrium may be to decrease the $PGF_{2\alpha}$ to PGE_2 ratio.

Future prospects:

Identifying the molecular target of IFNτ, by which it reduces OT induced PG production will, in part, explain the mechanism of pregnancy recognition in the bovine. Theoretically, bEEL cells open up a range of interesting avenues for research, which are briefly outlined.

1. Arginine vasopressin (antidiuretic hormone) is a congener of OT that acts through its cognate GPCR. It is worth testing whether vasopressin could produce an oxytocic response (implying receptor promiscuity) in bEEL cells.

2. Endometritis due to Gram negative bacteria is one of the leading causes of the delay in calving- to-conception interval and accounts for economic loss in the bovine. Treatment of bEEL cells with endotoxin (lipopolysaccharide, LPS) may give insight into the signaling mechanism of endotoxin induced injury to bEEL cells. It may also explain the elevated PGE_2 and $F_{2\alpha}$ levels in cows with retained CL due to postpartum metritis/pyometra.

3. Since IFN is a viral induced gene and LPS represents endotoxic shock caused by Gram negative bacteria, the interaction between IFN and LPS offers a crude *in vitro* model of primary viral infection that is superimposed by secondary bacterial. This model suits to the clinical settings in the bovine since many of the epitheliotropic viruses like bovine viral diarrhea virus (BVD) and infectious bovine rhinotrecheaitis virus (IBR) infect the endometrial epithelial cells that is superimposed by E.coli, the common cause of postpartum and subclinical endometritis.

4. bEEL cells express the transcripts of E_2 and P_4 receptor and the preliminary experiments showed that the cells are responsive to co-treatment with OT and IFNτ after 4 weeks of culture. It would be interesting to study the effects of E_2 and P_4 priming on OT and/or IFNτ in terms of PG production.

5. Intrauterine deposition of embryos into the ipsilateral horn is a common commercial practice in any of the embryo transfer programs. Coculture of embryo with bEEL cells may serve as a model to study the embryo-epithelial interaction. It is worth investigating the effect of IFNτ treatment on embryo quality.

6. The feasibility of establishing '*in vitro* endometrium' using bEEL and CSC may be attempted in combination with different co-culture techniques as this will enable to study the paracrine interactions.

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Appendix 1

Oxytocin Receptor Down-Regulation Is Not Necessary for Reducing Oxytocin-Induced Prostaglandin $F_{2\alpha}$ Accumulation by Interferon-τ in a Bovine Endometrial Epithelial Cell Line

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Interferon-τ (IFNτ) is the embryonic signal responsible for pregnancy recognition in ruminants. The primary action of IFNτ is believed to be mediated through inhibition of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released from the endometrial epithelial cells in response to oxytocin (OT). Our working hypothesis was that the antiluteolytic effect of IFNτ also involved modulation of PG production downstream of OT receptor (OTR) and/or cyclooxygenase 2 (COX2). There is currently no OT-sensitive endometrial cell line to study the molecular mechanisms underlying our hypotheses. Therefore, we established an immortalized bovine endometrial epithelial cell line (bEEL) exhibiting OT response. These cells were cytokeratin positive, expressed steroid receptors, and exhibited preferential accumulation of $PGF_{2\alpha}$ over PGE_2. The bEEL cells were highly sensitive to OT, showing time- and concentration-dependent increase in COX2 transcript and protein and $PGF_{2\alpha}$ accumulation. Interestingly, IFNτ (20 ng/ml) significantly reduced OT-induced $PGF_{2\alpha}$ accumulation, but surprisingly, the effect was not mediated through down-regulation of either OTR or COX2. Rather, IFNτ up-regulated COX2 in a time- and concentration-dependent manner while decreasing OT-induced PG accumulation. This suggests that COX2 is not a primary target for the antiluteolytic effect of IFNτ. Because IFNτ reduced OT-stimulated $PGF_{2\alpha}$ accumulation within 3 h, the mechanism likely involves a direct interference at the level of the OT signaling or transcription in addition to the down-regulation of OTR observed *in vivo*. In summary, bEEL cells offer a unique *in vitro* model for investigating the cellular and molecular mechanisms underlying OT and IFNτ response in relation with luteolysis and recognition of pregnancy in the bovine. (***Endocrinology* 150: 897–905, 2009)**

In ruminants, complex interactions between endometrial cells and the embryo determine the fate of the ovarian corpus luteum toward recognition of pregnancy or return to a new estrous cycle. Paracrine interactions between the trophoblast and endometrial epithelial cells are essential for recognition and establishment of pregnancy (1) as illustrated in the endometrial gland knockout model in the ewe (2).

At the end of the luteal phase, release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by the endometrial epithelial cells in response to oxytocin (OT) of pituitary and ovarian origin causes luteolysis. Trophoblastic interferon (IFNτ) is the embryonic signal released as a paracrine factor to prevent luteolysis and effect recognition of pregnancy. It has been proposed that IFNτ inhibits $PGF_{2\alpha}$ production in epithelial cells by preventing the up-regulation of estrogen receptor-α (ERα) and OT receptor (OTR) through activating the transcriptional repressor IFN-regulatory factor-2 (IRF2) (3, 4). However, *in vivo* observations derived from embryo transfer in the cow and *in vitro* experiments suggest that alternate mechanisms are also present to allow IFNτ to effect recognition of pregnancy within a much shorter period. In the cow, it is possible to obtain viable term pregnancies after transfer of blastocysts up to d 16, a mere 12 h before the onset of luteolysis (5). In primary endometrial cell cultures (6) and low-passage endometrial epithelial cells (7), IFNτ reduced OT-induced $PGF_{2\alpha}$

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Abbreviations: bEEL, Bovine endometrial epithelial cell line; COX2, cyclooxygenase 2; ERα, estrogen receptor-α; IFNτ, interferon-τ; OT, oxytocin; OTR, OT receptor; PD, population doubling; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PR, progesterone receptor; ROD, relative OD; STAT1, signal transducer and activator of transcription-1.

accumulation. It has been demonstrated that OT stimulation of $PGF_{2\alpha}$ production involves stimulation of cyclooxygenase 2 (COX2) expression (8). However, COX2 is also up-regulated during early pregnancy both in the ewe (9, 10) and cow (11), and higher levels of $PGF_{2\alpha}$/PGFM are found in early pregnant compared with cyclic ewes (12, 13). Intrauterine administration of IFNτ (14) as well as treatment of primary endometrial cell cultures with IFNτ both up-regulate COX2 (15). Thus, the apparently conflicting observations of decreased $PGF_{2\alpha}$ production with increased COX2 during the implantation window suggest that IFNτ may modulate functional coupling of terminal PG synthases with COX2 rather than down regulating it. We have demonstrated that OT induces $PGF_{2\alpha}$ accumulation in primary cultures of bovine endometrial epithelial cells (16). However, the precise endocrine and paracrine regulation of OTR expression in endometrial epithelial cells is not well understood (17). It has also been proposed that IFNτ could favor establishment of pregnancy by promoting the production of PGE_2 relative to $PGF_{2\alpha}$ (18).

The inhibitory effect of IFNτ on OT-induced $PGF_{2\alpha}$ production has been reproduced *in vitro* (7). It has also been shown that IFNτ down-regulates OTR at the transcriptional level in bovine primary endometrial epithelial cultures (19) and activates signal transducer and activator of transcription-1 (STAT1) and -2 in immortalized ovine luminal epithelial cells (20). Investigation of the mechanistic link between IFNτ signaling, OTR regulation, COX2 expression, and $PGF_{2\alpha}$ in endometrial epithelial cells should provide a better understanding of recognition and establishment of pregnancy in ruminants.

We have been the first to implement the use of primary bovine endometrial cell cultures as a model to study the regulation of PG synthesis in relation with endometrial function (21–23). However, it is becoming increasingly difficult to have a constant supply of good quality primary cultures especially during summer. More importantly, cells in primary culture have a limited lifespan and die because of replicative senescence. Therefore, a stable *in vitro* culture system of endometrial epithelial cells would represent an ideal model to study the signaling pathways and transcriptional mechanisms controlling COX2 expression and $PGF_{2\alpha}$ secretion. In fact, bovine endometrial epithelial cells have been proposed as a model to study the OTR regulation (19). At present, low-passage endometrial epithelial cells (catalog item B932-05; Cell Applications, Inc. San Diego, CA) and bovine endometrial cells are commercially available to investigate the mechanisms regulating PG production in the bovine. The low-passage cells are OT responsive but exhibit the variability and limited lifespan characteristic of primary cultures; bovine endometrial cells are stable but lack OT responsiveness while exhibiting a mixed phenotype (22, 24). Immortalized cell lines of luminal and glandular epithelial cells have been generated in the ewe, but data on OT responsiveness were not reported (25, 26). We present here a novel bovine endometrial epithelial cell line (bEEL) that we used to study the molecular and cellular mechanisms involved in the interaction between OT and IFNτ on the regulation of PG production.

Materials and Methods

Generation of bEEL

Primary epithelial cell cultures were established as described previously (22) and infected with a replication-defective lentiviral vector expressing human telomerase and puromycin resistance genes. After antibiotic treatment, 15 clones were selected and seeded. Because of an apparent growth arrest of all clones, we elected to harvest all the cells and plate them together to establish a growth threshold concentration. Two weeks later, the culture reached confluency, and the fastest growing clone was isolated by repeated passage. Under a stable seeding density of 30% and generation time of 4 d, cells reached homogeneity at around 25 population doublings (PD) and maintained a stable phenotype at least up to 50 PD. Presence of telomerase transgene was tested at different PD from 25–40, and surprisingly, it could not be detected. We conclude that the resulting bovine endometrial epithelial cells (bEEL) represent a serendipitous cell line that evolved spontaneously or in response to telomerase without integration of the transgene. We have then conducted a thorough characterization to confirm the stability of the cell line and correspondence with the lineage sought for. Frozen stock bEEL cells are kept at 30 PD, and all experiments were conducted between 30 and 40 PD.

RT-PCR and real-time PCR

Total RNA (1 μg), extracted from the cell line, was reverse-transcribed using random primer and Superscript III RT (Invitrogen Life Technologies Inc., Burlington, Ontario, Canada). To demonstrate ERα and progesterone receptor (PR) in the cell line, the following specific sets of primers were used: for ERα, sense and antisense primers 5′-ATGACCCTACCAGACCTTTCAGT-3′ and 5′-ATTTGAGGCACACAAACTCTTC-3′, respectively, and for PR, forward and reverse primers 5′-ATTGTTGATAAAATCCGCAGAAA-3′ and 5′-GAGGTATCAGGTTTGCTGTTGTC-3′, respectively. ERα primers were deduced from accession no. NM_001001443, whereas PR-specific primers were designed from accession no. AY656812. Real-time PCR was done in a light cycler (Roche Diagnostics, Laval, Quebec, Canada) with SYBR (QIAGEN, Mississauga, Ontario, Canada) using forward (AAGATGACCTTCATCGTCGTG) and reverse (CGTGAAGAGCATGTAGATCCAG) primers derived from accession no. AF101724 for OTR, forward (CCAGAGCTCTTCCTCCTGTG) and reverse (GGCAAAGAATGCAAACATCA) primers derived from accession no NM_174445 for COX2, and forward (GTAACCCGTTGAACCCCATT) and reverse (CCATCCAATCGGTAGTAGCG) primers derived from accession no. M10098 used for 18S rRNA serving as the internal standard.

Enzyme immunoassay of $PGF_{2\alpha}$

$PGF_{2\alpha}$ was assayed by competitive ELISA using acetylcholinesterase-linked tracers (Cayman, Ann Arbor, MI) as described previously (22).

Western blot analysis

An aliquot of 15 μg protein was loaded in each lane, resolved on 12.5% SDS-PAGE, and electrotransferred onto 0.45-μm nitrocellulose membrane (Bio-Rad, Mississauga, Ontario, Canada). The membranes were blocked in 5% (wt/vol) nonfat dried milk in PBS containing 0.05% Tween 20 for 1 h at room temperature and incubated overnight at 4 C with respective primary antibodies. The primary antibody dilutions were as follows: 1/3000 for anti-COX2 (Merck 241) (kindly provided by Dr. S. Kargman, Merck Frosst Montreal), 1/1000 for STAT1 (BD Biosciences, Mississauga, Ontario, Canada) and anti-pS727 STAT1 (Upstate Biotechnology, Lake Placid, NY), 1/500 for OTR (Santa Cruz Biotechnology, Santa Cruz, CA), and 1/5000 for β-actin (Sigma, Oakville, Ontario, Canada). After three washes of 10 min each in PBS, the membranes were incubated for 1 h at room temperature with appropriate secondary antibody. The membranes were washed three times in PBS containing 0.05% Tween 20. The membranes were treated for 1 min with enhanced chemilumunescent substrate (PerkinElmer Life and An-

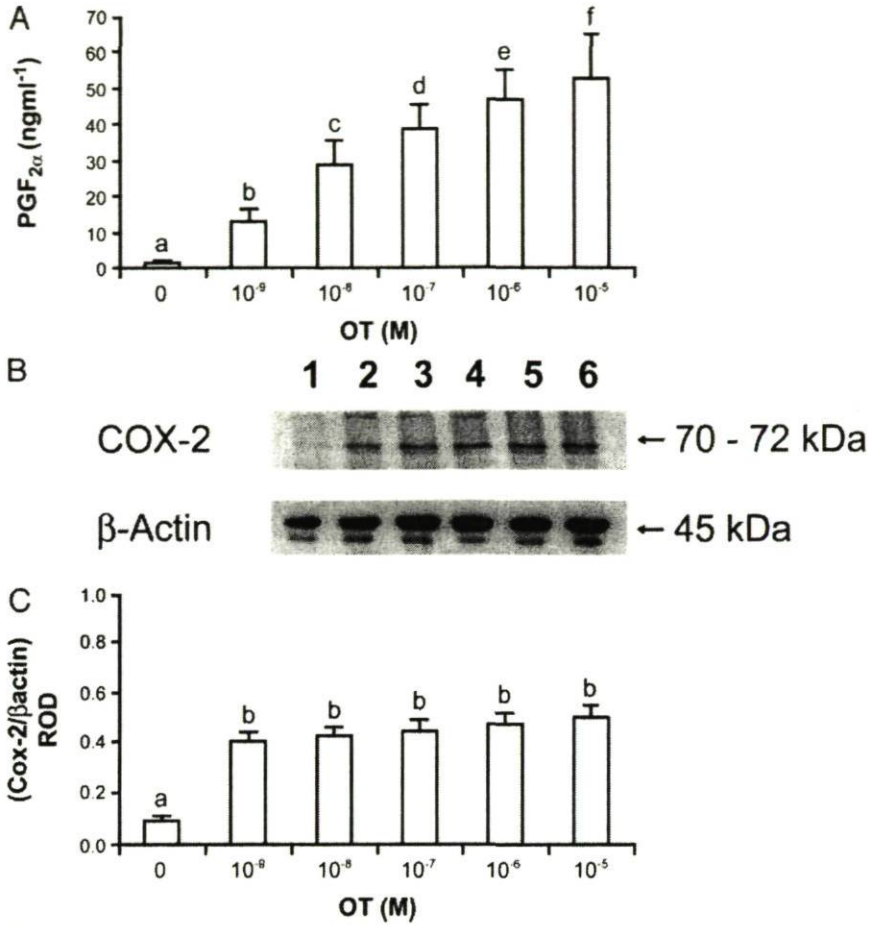

FIG. 1. Effect of increasing concentrations of OT on COX2 and $PGF_{2\alpha}$ accumulation in bEEL cell. bEEL cells were treated with increasing concentrations of OT (10^{-9} to 10^{-5} M) for 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent mean ± SEM of three different experiments run in quadruplicate. *Bars* with different *superscripts* differ significantly ($P < 0.05$). A, $PGF_{2\alpha}$ accumulation; B, representative immunoblots of COX2 and β-actin, with lanes 1–6 representing different concentrations of OT: 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M, respectively; C, ROD values are ratios between COX2 and β-actin.

alytical Sciences, Inc., Waltham, MA) and exposed to Bio-Max film (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA). Relative OD (ROD) of immunoblots from three replicates of each experimental protocol was quantitated by densitometry (α-imager).

Experimental protocols

To minimize a possible influence of cell generation and for better homogeneity, bEEL cells used for all the experiments were within 30–40 PD. Cells from frozen aliquots were grown to confluency in RPMI 1640 (Invitrogen) supplemented with 10% decomplemented fetal bovine serum and 1% streptomycin/penicillin, trypsinized and seeded at 4×10^4 cells/ml in 24-well plates. Medium was replaced 24 and 72 h after seeding. On d 5, the confluent cells were exposed to steroid-free medium and incubated overnight. Cells were then treated according to specific protocols in serum-free medium for 24 h unless indicated otherwise, and culture medium was harvested and stored at −20 C until PG assay. Cell lysates preparation, protein extraction, and estimation were done as described previously (27). Cells were grown in six-well plates for RT-PCR and real-time PCR experiments. All experiments were replicated three times, and each treatment was run in quadruplicate unless indicated otherwise.

Preliminary experiments indicated that OT, phorbol ester, TNFα, and lipopolysaccharide stimulated both PGE_2 and $PGF_{2\alpha}$ accumulation in the bEEL cells; however, the ratio of $PGF_{2\alpha}$ to PGE_2 was always in favor of the former; thus, we limit the presentation of our data to $PGF_{2\alpha}$ accumulation. Western analysis of cytosolic phospholipase A2 and terminal synthases revealed positive and constant expression without apparent regulation, whereas COX2 was strongly regulated. Accordingly, we have elected to show only COX2 to represent the modulation of PG biosynthesis.

After these pilot studies, we aimed to determine optimal concentrations of OT and IFNτ with respect to $PGF_{2\alpha}$ accumulation and COX2 and/or STAT1 protein in bEEL cells. We then studied the time-dependent $PGF_{2\alpha}$ accumulation and relevant enzyme expression over 24 h with optimal OT (10^{-7} M) and IFNτ (20 ng/ml) concentrations. Lastly, the effect of IFNτ (20 ng/ml) on OT response was assessed at the level of $PGF_{2\alpha}$ accumulation and COX2, STAT1, and OTR protein at 24 h. Then, the effect of coincubation of OT and IFNτ (20 ng/ml) on $PGF_{2\alpha}$ accumulation was studied at 3, 6, and 12 h.

Statistical analysis

Randomized block design was used in all the experiments with treatment as the fixed factor (main effect) and plate as random factor (interaction effect). The resulting data on PG accumulation (nanograms per milliliter) was expressed as mean ± SEM ($n = 12$) unless specified otherwise. GraphPad Prism version 4 was used for data analysis. The group mean of different treatments was tested by two-variable ANOVA with Bonferroni *post hoc* test to find the critical difference between pairs of treatment means. However, for PG accumulation data on Fig. 4, we used the Statview program offering the flexibility to enter the data of 2 (basal and IFNτ) × 4 (time 0, 6, 12, or 24 h) × 3 (plate) factorial designs.

Results

Morphology, phenotype, growth, and culture characteristics

Under phase-contrast microscopy, confluent bEEL cells present honeycomb morphology (4X) and a polygonal shape (100 or 200X), which are typical of primary epithelial cells. Immunofluorescence analysis confirmed that bEEL cells were positive for cytokeratin and negative for vimentin; in clear contrast with endometrial stromal cells expressing an opposite expression profile. Expression of ERα and PR was demonstrated by RT-PCR (results not shown). bEEL cells could be grown past confluency without apparent signs of senescence for at least 3 wk, thus allowing prolonged treatment with agents such as sex steroids to mimic the hormonal changes of the estrous cycle.

Effect of OT on PG accumulation

Oxytocin induced a concentration-dependent increase in $PGF_{2\alpha}$ accumulation that reached significant levels at 10^{-9} M, the lowest concentration tested ($P < 0.05$, Fig. 1A). The concentration response was progressive, suggesting that OTR was functionally coupled with PG production. OT also increased COX2 protein consistently across all concentrations tested (Fig.

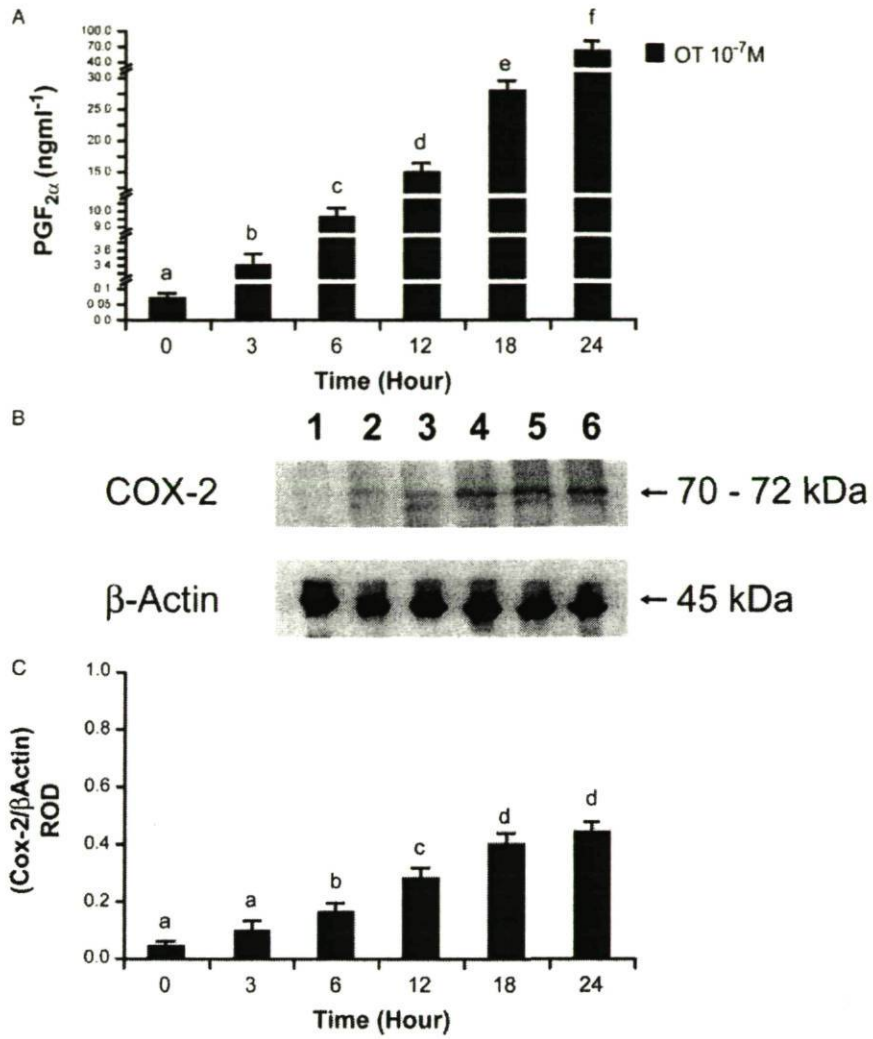

FIG. 2. Effect of OT (10^{-7} M) on COX2 and $PGF_{2\alpha}$ accumulation in bEEL cells over a period of 24 h. bEEL cells were stimulated with OT (10^{-7} M) for 0, 3, 6, 12, 18, or 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent mean ± SEM of three different experiments run in quadruplicate. *Bars with different superscripts* differ significantly ($P < 0.05$). A, $PGF_{2\alpha}$ accumulation; B, representative immunoblots of COX2 and β-actin, with lanes 1–6 representing time intervals 0, 3, 6, 12, 18, or 24 h, respectively; C, ROD values are ratios between COX2 and β-actin.

1, B and C). Based on these response characteristics, we selected 10^{-7} M as the optimal OT concentration for further studies. $PGF_{2\alpha}$ accumulation was stimulated in a time-dependent manner by OT (10^{-7} M) with significantly increased levels at 3 h, the earliest time tested. Similarly, COX2 protein was stimulated in a time-dependent manner and significant at 6 h after treatment ($P < 0.05$, Fig. 2). COX2 mRNA expression followed a similar pattern with significant stimulation at 3 h, reaching a maximum between 6 and 12 h, and then slightly declining at 24 h (results not shown). As a negative control, we have performed the same concentration response experiment using a stromal cell line and found no OT response at any level (results not shown).

Effect of IFNτ on PG accumulation

IFNτ induced a concentration-dependent increase in $PGF_{2\alpha}$ accumulation that reached significant levels at concentrations in the high physiological range ($P < 0.05$). However, the level of stimulation (3-fold) was much lower than that achieved in response to OT (50-fold) (Fig. 3A). Surprisingly, COX2 protein was up-regulated by IFNτ at concentrations as low as 2 ng/ml, but by contrast with $PGF_{2\alpha}$ accumulation, the protein level was similar to that reached in response to OT (Fig. 3, B and C). Phosphorylation of STAT1 and up-regulation of total STAT1 confirmed that the typical Jak-Stat transduction system used by IFNτ was intact and functional in bEEL. Because of the particular response pattern to IFNτ, the 20 ng/ml concentration where COX2 protein but not PG accumulation was increased was selected for the time-dependent response. As was expected, $PGF_{2\alpha}$ accumulation by IFNτ did not differ significantly from the basal production (Fig. 4A). However, IFNτ treatment significantly up-regulated COX2 and STAT1 protein compared with the control ($P < 0.05$, Fig. 4, B and C). The pattern of COX2 mRNA expression was identical to that of protein (results not shown).

Effect of IFNτ on OT response

IFNτ (20 ng/ml) significantly reduced OT-induced $PGF_{2\alpha}$ accumulation in bEEL cells at 24 h ($P < 0.05$, Fig. 5A). The same pattern of response albeit at a much reduced level of PG accumulation was observed and significant as early as 3 h after the onset of treatment and also at 6 and 12 h ($P < 0.05$, Fig. 6). Western and densitometric analyses showed that IFNτ did not alter the ability of OT to stimulate COX2 protein. Similarly, OT did not influence STAT-1 protein or phosphorylation nor did it affect the stimulation induced by IFNτ on these parameters (Fig. 5, B and C). The reduction in OT-induced accumulation of $PGF_{2\alpha}$ was not accompanied by any effect at the OTR expression either at the messenger or the protein level (Fig. 5, B–D). The effect of IFNτ on OT response was tested at higher concentrations of IFNτ, and the results were exactly the same (data not shown).

Discussion

We were able to generate a stable endometrial epithelial cell line from bovine endometrium with morphological and functional properties comparable to primary cultures. This cell line expresses OTR functionally coupled with the regulation of $PGF_{2\alpha}$ production. To our knowledge, this is the first report of OT responsiveness in a stable ruminant endometrial cell line. These bEEL cells also respond to IFNτ, thus allowing study of the molecular mechanisms underlying the actions of these important regulators of endometrial function at the time of maternal rec-

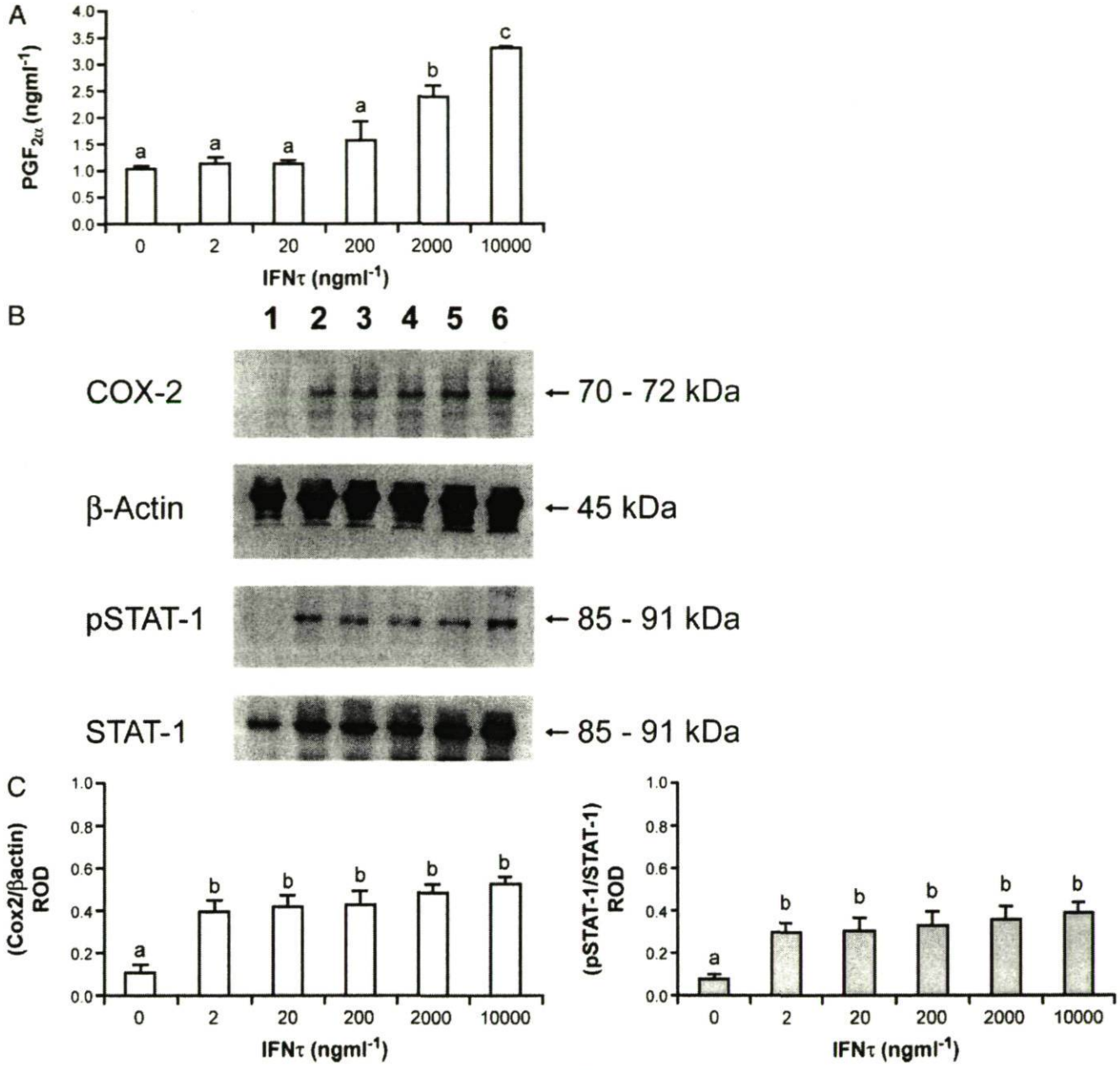

FIG. 3. Effect of different concentrations of IFNτ on COX2, pSTAT1, and STAT1, and $PGF_{2\alpha}$ accumulation in bEEL cells. bEEL cells were treated with increasing concentrations of IFNτ (0–10,000 ng/ml) for 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent the mean ± SEM of three different experiments run in quadruplicate. *Bars with different superscripts* differ significantly ($P < 0.05$). A, $PGF_{2\alpha}$ accumulation; B, representative immunoblots of COX2, β-actin, and phosphorylated and total STAT1, with lanes 1–6 representing different concentrations of IFNτ: 0, 2, 20, 200, 2000, and 10,000 ng/ml, respectively; C, ROD values are ratios between COX2/β-actin and phosphorylated to total STAT1.

ognition of pregnancy. Indeed, OT and IFNτ interact to modulate the production of the luteolytic factor $PGF_{2\alpha}$ at the time of recognition of pregnancy. The currently accepted mechanism underlying recognition of pregnancy based on inhibition of increased pulsatile release of $PGF_{2\alpha}$ in response to OT is challenged by *in vivo* and *in vitro* observations. In ruminants, up-regulation of COX2 during the luteolytic window (10, 28) together with increased OTR expression in epithelial cells during the late luteal phase support the view that OT initiates $PGF_{2\alpha}$ production and triggers luteolysis (29, 30). However, COX2, which is most often associated with increased production of PG, remains high in presence of a viable embryo in cows and ewes, and the same is observed *in vivo* and *in vitro* in response to IFNτ (8, 9). We have proposed that IFNτ-mediated recognition of pregnancy may be implemented by a reduction in the production of the luteolytic $PGF_{2\alpha}$ by epithelial cells relative to PGE_2 by stromal cells (23). In the present report, using bEEL cells as an *in vitro* model, we have demonstrated a direct up-regulation of COX2 after OT stimulation as was previously shown in primary epithelial cells (8). A significant increase in $PGF_{2\alpha}$ by 3 h after OT treatment ($P < 0.05$, Fig. 2) and progressive concentration-dependent increase at concentrations up to 10^{-5} M (Fig. 1) suggest functional expression of OTR in bEEL cells. Spontaneous expression of OTR *in vitro* contrasts with estrogen-driven up-regulation *in*

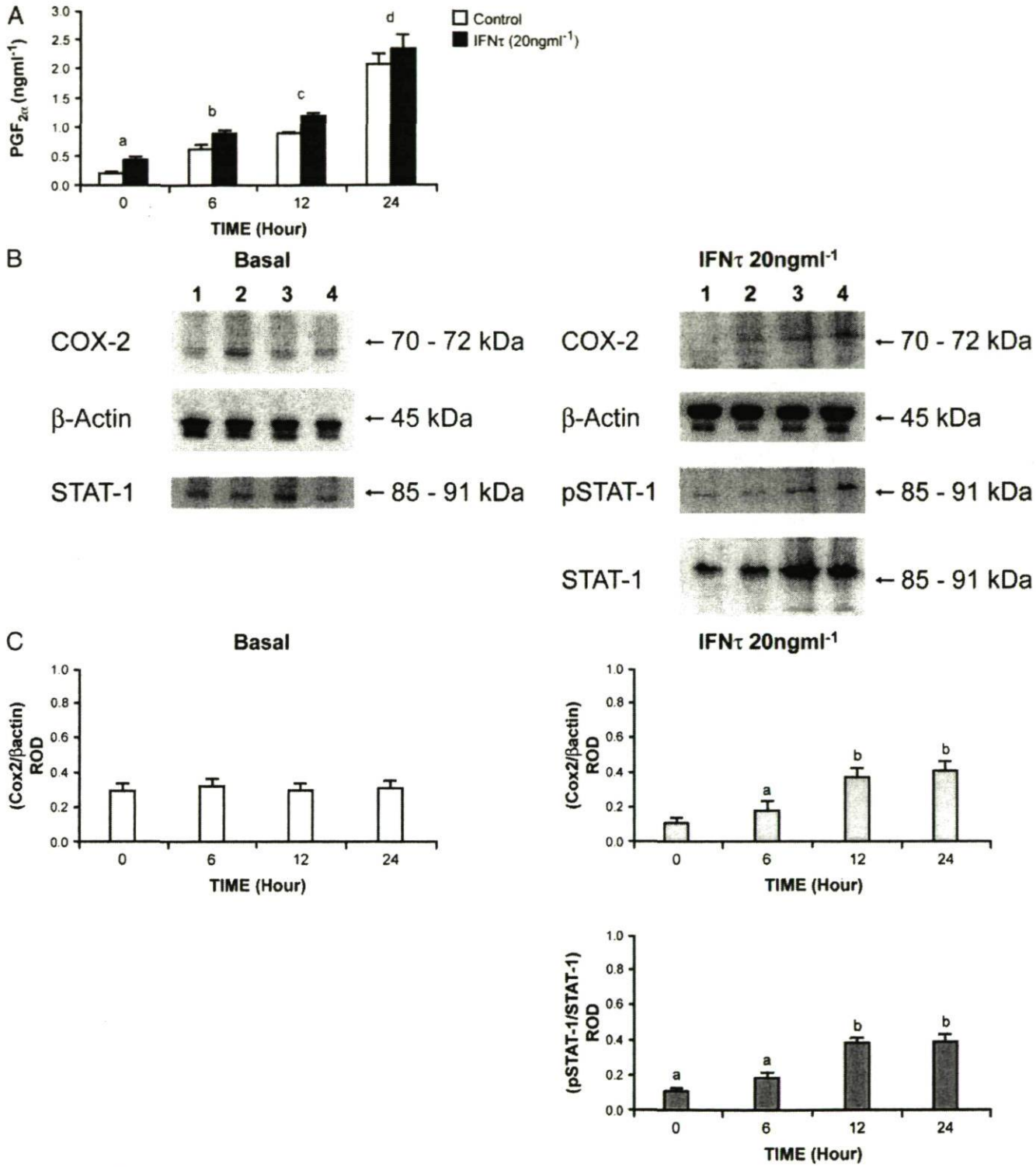

FIG. 4. Effect of IFNτ (20 ng/ml) on time-dependent expression of COX2, pSTAT1, and STAT1 and $PGF_{2\alpha}$ accumulation in bEEL cells. bEEL cells were treated or not with IFNτ (20 ng/ml) for 0, 6, 12, or 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent mean ± SEM of three different experiments run in quadruplicate. *Bars with different superscripts* differ significantly ($P < 0.05$). A, $PGF_{2\alpha}$ accumulation; B, representative immunoblots of COX2, β-actin, and phosphorylated and total STAT1, with lanes 1–4 representing time intervals 0, 6, 12, or 24 h, respectively, *left panel* showing basal expression, and *right panel* showing the effect of IFNτ (20 ng/ml); C, ROD values are ratios between COX2/β-actin and phosphorylated to total STAT1.

vivo, but as proposed previously (19, 31), it suggests that the expression may be under repressor control *in vivo*. Primary cultures of bovine endometrial epithelial cells have been shown to represent an ideal model to investigate OTR regulation (19), and now bEEL cells present the additional convenience of a stable cell line expressing OTR.

We have also demonstrated time- and concentration-dependent stimulation of COX2 and $PGF_{2\alpha}$ accumulation in response

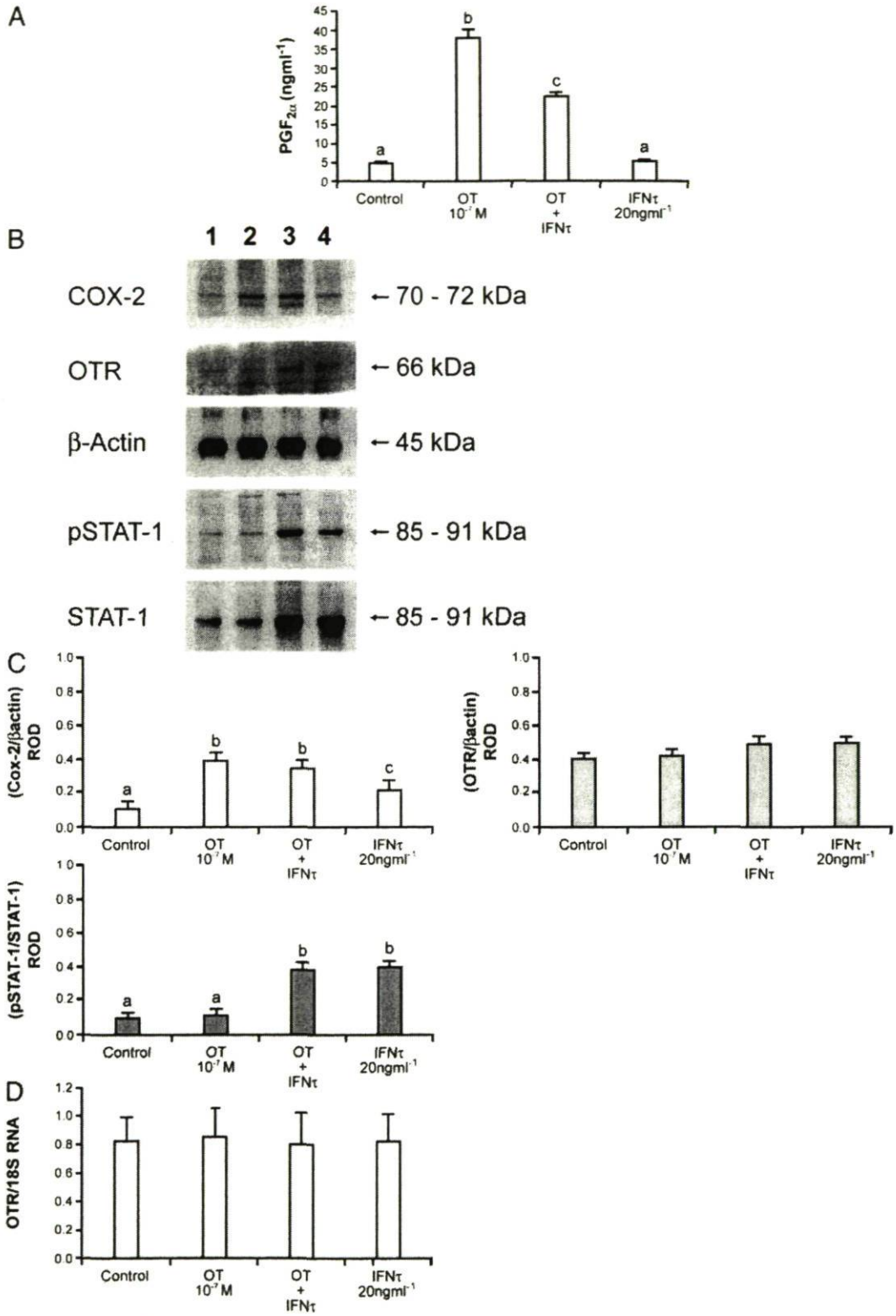

FIG. 5. Interaction between OT (10^{-7} M) and IFNτ (20 ng/ml) in bEEL cells. bEEL cells were treated simultaneously with OT (10^{-7} M) and IFNτ (20 ng/ml) for 24 h. $PGF_{2\alpha}$ was measured in the culture medium and cell lysates used for protein analysis. Values represent mean ± SEM of three different experiments run in quadruplicate. *Bars with different superscripts* differ significantly ($P < 0.05$). A, $PGF_{2\alpha}$ accumulation; B, representative immunoblots of COX2, OTR, β-actin, and phosphorylated and total STAT1, with lanes 1–4 representing 1) control, 2) OT 10^{-7} M, 3) OT 10^{-7} M+IFNτ 20 ng/ml, and 4) IFNτ 20 ng/ml; C, ROD values are ratios between COX2/β-actin, OTR/β-actin, and phosphorylated to total STAT1; D, real-time PCR-based quantitation of OTR transcripts. Values represent ratio of OTR/18S rRNA.

to IFNτ. In addition, phosphorylation of STAT1, which was detectable after 24 h, and up-regulation of total STAT1, confirmed the IFNτ responsiveness of the bEEL cells (Figs. 4 and 5). This is in agreement with the results on the immortal ovine endometrial luminal cells (20). Cotreatment of bEEL with OT and IFNτ indicated that OT did not affect the stimulatory effect of

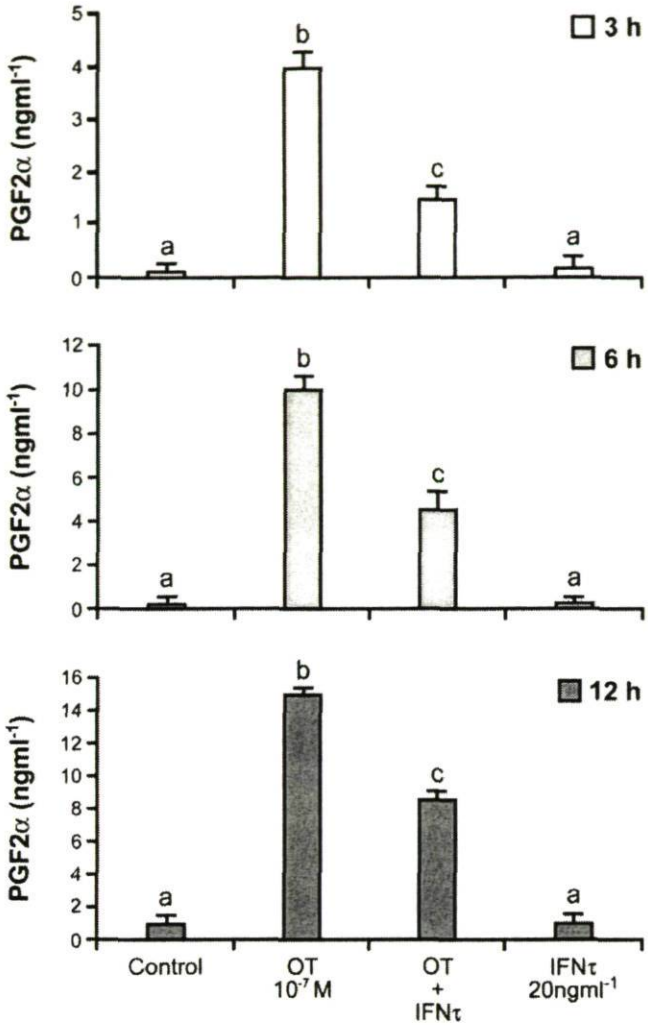

FIG. 6. Effect of OT (10^{-7} M) and IFNτ (20 ng/ml) cotreatment on $PGF_{2\alpha}$ accumulation at 3, 6, or 12 h in bEEL cells. bEEL cells were treated simultaneously with OT (10^{-7} M) and IFNτ (20 ng/ml) for 3–12 h. $PGF_{2\alpha}$ was measured in the culture medium. Values represent $PGF_{2\alpha}$ accumulation as mean ± SEM of three different experiments run in quadruplicate. *Bars with different superscripts* differ significantly ($P < 0.05$).

IFNτ on STAT1 (Fig. 5). However, IFNτ reduced by 50% the accumulation of $PGF_{2\alpha}$ in response to OT, whereas it did not influence COX2 protein. This combined with the observation that IFNτ increased COX2 protein as much as OT while being much less potent to stimulate $PGF_{2\alpha}$ accumulation suggests that it may act on the coupling of COX2 with terminal synthases. At the molecular level in bEEL, COX2 and STAT1 serve as markers of OT and IFNτ responses, respectively. The observation that IFNτ significantly reduced OT-induced $PGF_{2\alpha}$ secretion is consistent with the antiluteolytic effects *in vivo* (32, 33) and *in vitro* (7, 8). Because the coincubation of OT and IFNτ suppressed $PGF_{2\alpha}$ accumulation significantly in 3–6 h ($P < 0.05$), and OTR expression was not modulated even after 24 h after treatment, it is likely not mediated through down-regulation of OTR. This is also supported by the observation that pretreatment of bEEL cells with IFNτ (20 ng/ml) for 48 h before OT challenge did not increase the rate of inhibition (data not shown). It is also unlikely that the effect of IFNτ on OT-induced PG accumulation in bEEL cells is mediated by increased 15-hydroxy-PG dehydrogenase activity. Indeed, although we have shown that 15-hydroxy-PG dehydrogenase is expressed and regulated in bovine endometrium *in vivo* (34), it is not expressed in cultured endometrial cells (unpublished observations). We have confirmed that primary endometrial cells do not catabolize PGE_2 and $PGF_{2\alpha}$ into PGEM and PGFM *in vitro* (35). It has been reported that COX2 is up-regulated in the endometrium during early pregnancy in the ewe (9, 10) and cow (11) and also after intrauterine infusions of IFNτ (14). Taken together, these results bring out two interpretations. First, IFNτ may mediate an immediate antiluteolytic effect by uncoupling COX2 and $PGF_{2\alpha}$ synthase or interfere with OT signaling and transcription not requiring down-regulation of COX2 or OTR. Such a mechanism would explain that successful embryo transfer is possible a mere 12 h before the onset of luteolysis in cattle (5). Second, COX2 appears as an IFNτ-responsive gene that may be required not only for OT-mediated luteolysis but also for IFNτ-mediated recognition of pregnancy in the bovine. In summary, bEEL is a good *in vitro* model to investigate the mechanisms associated with the inhibition of OT-induced $PGF_{2\alpha}$ production by IFNτ.

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Disclosure Statement: The authors of this manuscript have nothing to declare.

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Appendix 2

Development and Characterization of a Simian Virus 40 Immortalized Bovine Endometrial Stromal Cell Line

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In ruminants, interferon-τ (IFNτ) is the maternal recognition signal inhibiting prostaglandin (PG) $F_{2\alpha}$ production by endometrial epithelial cells and stimulating interferon-stimulated genes in the stroma. Stromal cells mediate the action of progesterone on epithelial cells during pregnancy. Our working hypothesis is that IFNτ acts as a molecular switch that turns on PGE_2 production in endometrial stromal cells while suppressing $PGF_{2\alpha}$ production from epithelial cells. In this report we document immortalization and functional characterization of a bovine stromal cell line from the caruncular region of the endometrium [caruncular stromal cell (CSC)]. Primary stromal cells were immortalized by nucleofection with simian virus 40 large T antigen and integrase. The resulting cell line, CSC, expresses stromal cell-specific vimentin, estrogen, and progesterone receptors, and is amenable for transient transfection. Basal and stimulated production of PGE_2 is higher than $PGF_{2\alpha}$ and associated with cyclooxygenase (COX) 2 expression. Phorbol myristate acetate (PMA) and IFNτ up-regulate COX2 and PG production in a dose-dependent manner. When added together, low concentrations of IFNτ inhibit PMA-induced COX2 expression; whereas this inhibition is lost at high concentrations. Expression of signal transducer and activator of transcription 1 is induced by IFNτ at all concentrations studied but is not modulated by PMA. Because expression of signal transducer and activator of transcription 1 does not exhibit the biphasic response to IFNτ, we investigated the p38 MAPK pathway using the selective inhibitor SB203580. Inhibition of the p38 MAPK pathway abolishes IFNτ action on PG production. In summary, CSC appears as a good stromal cell model for investigating the molecular mechanisms related to IFNτ action and PG production in the bovine. **(*Endocrinology* 150: 485–491, 2009)**

Epithelial and stromal cells of the endometrium are the site of recognition of pregnancy. In ruminants, epithelial cells are the target of oxytocin (OT) to generate luteolytic pulses of prostaglandin (PG) $F_{2\alpha}$ (1). However, spatiotemporal expression of oxytocin receptor is coregulated with that of estrogen and progesterone receptors [ERs (ERα) and PRs, respectively], and may involve paracrine interactions between epithelial and stromal cells (2–4). In addition, $PGF_{2\alpha}$ of stromal origin, secreted in response to TNFα, may contribute to the initiation of luteolysis (5).

Interferon-τ (IFNτ) is the maternal recognition signal in ruminants. Apart from inhibiting $PGF_{2\alpha}$ pulses of epithelial origin, IFNτ stimulates a set of genes (interferon-stimulated genes) in the endometrial stroma (6). Using bovine primary stromal cell cultures, we have shown that IFNτ stimulates the production of PGE_2 (7). Generation of stable *in vitro* endometrial culture sys-

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Abbreviations: AKR1B5, Aldoketoreductase 1 B5; BEND, bovine endometrial cell; COX, cyclooxygenase; cPGES, cytosolic prostaglandin E synthase; cPLA2, cytosolic phospholipase A2; CSC, caruncular stromal cell; ER, estrogen receptor; ICSC, intercaruncular stromal cell; IFNτ, interferon-τ; LPS, lipopolysaccharide; mPGES, microsomal prostaglandin E synthase; OT, oxytocin; PG, prostaglandin; PMA, phorbol myristate acetate; PR, progesterone receptor; ROD, relative OD; STAT, signal transducer and activator of transcription; SV40 TAG, simian virus 40 large T antigen.

tems appears as the logical next step for investigating the complex signaling pathways and transcriptional mechanisms regulated by IFNτ in the bovine. At present, a spontaneously derived bovine endometrial epithelial cell line, bovine endometrial cell (BEND), is used as a model to investigate the mechanisms regulating PG production, but it expresses both epithelial and stromal cell markers, suggesting a mixed phenotype (8). Immortalized cell lines of luminal and glandular epithelial cells and stromal cells have been generated and characterized in sheep (9), but no bovine stromal cells are available. In this report we document the generation of a stromal cell line and show its utility in studying the regulation of PG biosynthesis in response to the embryonic signal IFNτ.

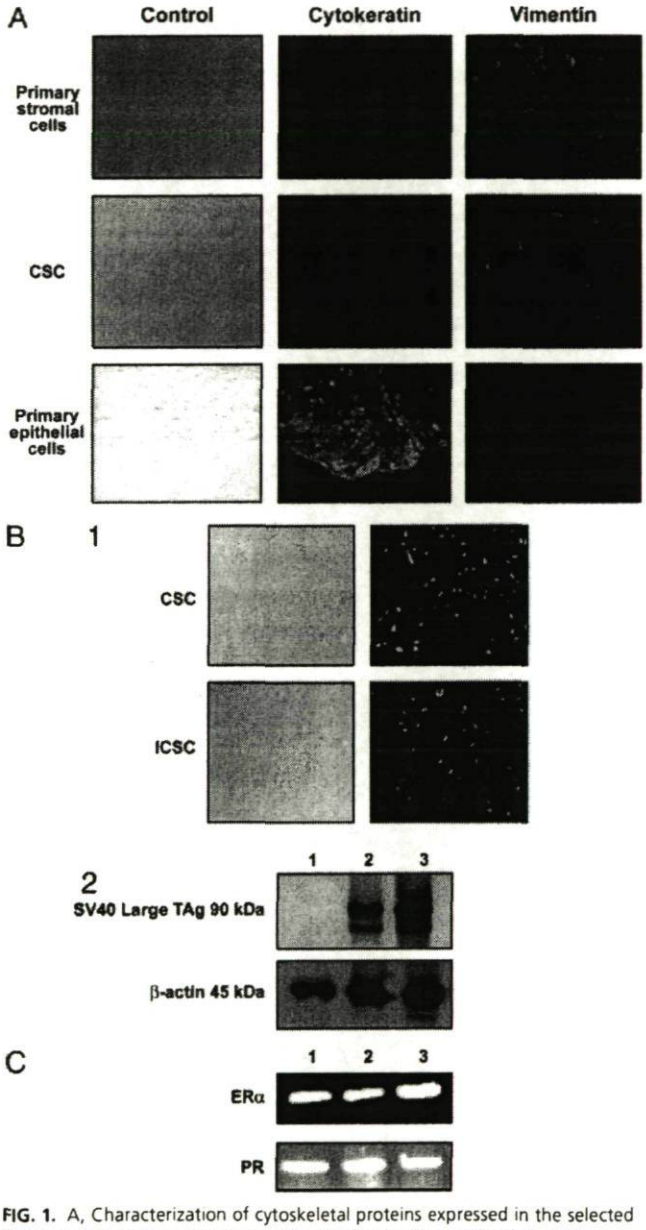

FIG. 1. A, Characterization of cytoskeletal proteins expressed in the selected CSC line. Subconfluent endometrial cells were stained with cytokeratin and vimentin antibodies, and detected with a fluorescent secondary antibody. *Left panels* represent phase-contrast illumination, whereas *center and right panels* show cytokeratin and vimentin fluorescence, respectively (magnification, ×100). B, Integration of SV40 TAG within the genome of bovine caruncular and intercaruncular stromal clones. 1, Demonstration by immunofluorescence (magnification, ×100) 2, Detection of SV40 TAG by immunoblot. Lanes 1–3 represent primary stromal cells, CSC and ICSC. C, Demonstration of ERs and PRs in CSCs by RT-PCR. Lanes 1–3 represent primary stromal cells, CSC and ICSC.

Materials and Methods

Immortalization and clone selection

Primary stromal cell cultures were prepared as described previously with minor modifications (10). Stromal cells were transfected by nucleofection with a plasmid expressing simian virus 40 large T antigen (SV40 TAG) and a gene resistant to aminoglycoside G418 (neomycin) and another plasmid coding for bacteriophage phiC31 integrase (11). The cytomegalovirus promoter of the SV40 TAG transgene was flanked by an attB sequence to improve plasmid integration into the genome. Subconfluent cultures of caruncular and intercaruncular stromal cells (ICSCs) were trypsinized and resuspended in serum free media, and 5 μg plasmid DNA coding for integrase and 5 μg vector DNA containing SV40 TAG were added to 1×10^6 cells and nucleofected using the T16 program. Nucleofection efficiency was 60% as assessed by green fluorescence protein. After 3 d, the cells were trypsinized and cultured in 150 × 20-mm petri plates for 7 d in presence G418 (200 μg/ml) to select resistant colonies. A total of 33 clones (seven caruncular and 26 intercaruncular) was picked using O-ring and clonally propagated in separate T-25 flasks up to 10 passages (P10). We then selected one caruncular (CSC) (clone

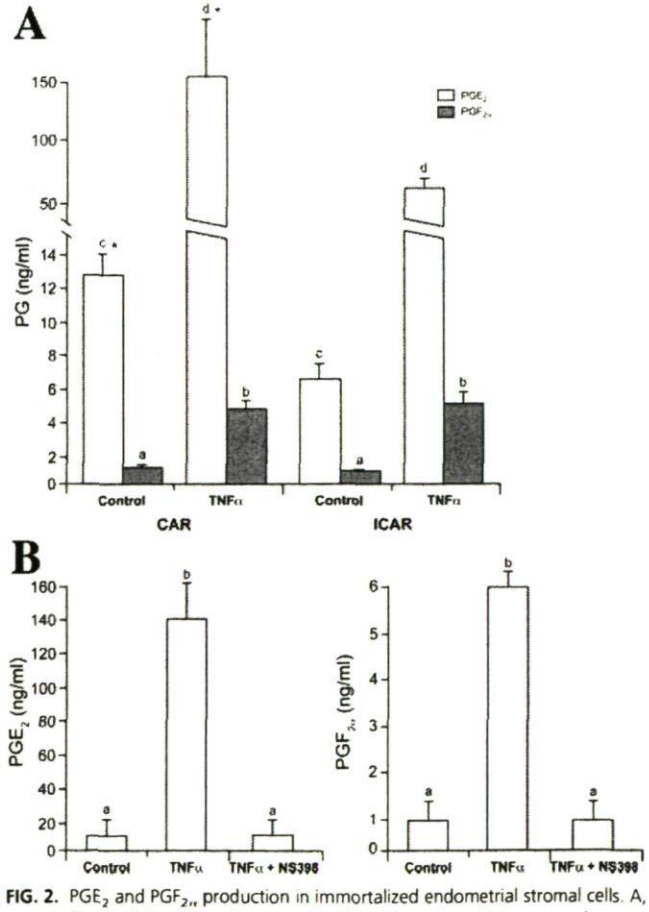

FIG. 2. PGE_2 and $PGF_{2\alpha}$ production in immortalized endometrial stromal cells. A, Immortalized cell lines from all caruncular (CAR) (n = 7) and intercaruncular (ICAR) (n = 26) clones were grown to confluency and stimulated or not with TNFα 6 nM for 24 h. Results are the mean ± SEM of PG levels from all clones. B, Effect of NS398, a COX2 inhibitor, on TNFα-induced PG production in CSCs. *Bars* with different *superscripts* differ significantly ($P < 0.05$).

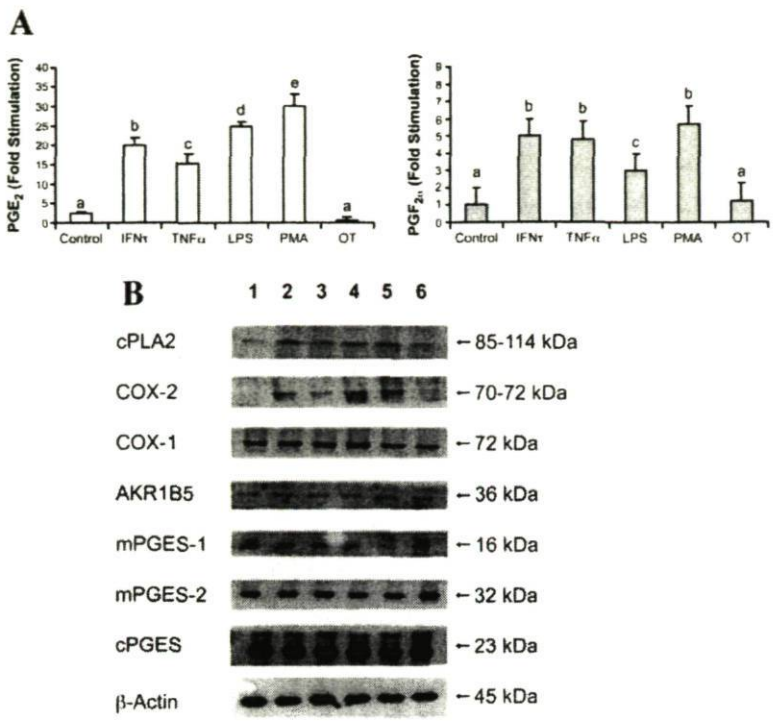

FIG. 3. Effect of IFNτ, TNFα, LPS, PMA, and OT on PG production in CSCs. A, PGE_2 and $PGF_{2\alpha}$ production. Values represent the mean ± SEM of three different experiments run in quadruplicate. *Bars* with different *superscripts* differ significantly ($P < 0.05$). B, Representative immunoblots of cPLA2, COX 1 and 2, AKR1B5, mPGES 1 and 2, cPGES, and β-actin. Lanes 1–6 refer to control, IFNτ 10 μg/ml, TNFα 6 nM, LPS 0.01 μg/ml, PMA10 nM, and OT 0.5 μM, respectively.

no. CAR7) and one intercaruncular (ICSC) (clone no. ICAR6) stromal cell clone according to basal and TNFα (6 nM) induced PGE_2 and $PGF_{2\alpha}$ production, growth rate, and stromal morphology, and passed the cell lines until P50.

Immunofluorescence analysis

CSCs and ICSCs were cultured on Lab Tek 4-chamber slides (Nalge Nunc Intl., Rochester, NY) and analyzed for expression of cytokeratin (Sigma-Aldrich Corp., St. Louis, MO), vimentin (in house antibody), and SV40 TAG (Oncogene Research Products, San Diego, CA) by immunofluorescence as described previously (12). Lipofectamine-mediated transfection of green fluorescent protein was done in CSCs as per the manufacturer's instructions (Invitrogen Life Technologies Inc., Burlington, Ontario, Canada).

Experimental protocols

The CSC line was selected for the subsequent studies because it exhibited optimal growth rate and a PG production profile representative of all clones tested, including those from intercaruncular areas (ICSC). Typically, CSC cultures were initiated from a frozen aliquot and grown to confluency in a T75 flask for 60–72 h at 37 C and 5% CO_2. The monolayer was trypsinized, extended with RPMI 1640 supplemented with 10% fetal bovine serum, divided into two equal volumes, and centrifuged at 1500 rpm for 10 min. One fraction was frozen and stored at −150 C for future experiments. The other fraction was diluted at 4×10^4 cells per ml and seeded in 24-well plates. Confluent cultures were exposed to steroid free medium overnight before treatment. At the end of the experiment, culture medium was harvested and stored at −20 C until analysis for PG. Protein extraction and estimation were done as described previously (13). All experiments were replicated three times, and each treatment was run in quadruplicate unless indicated otherwise. For functional characterization, cells were treated with IFNτ (10 μg/ml), lipopolysaccharide (LPS) (10 ng/ml), phorbol myristate acetate (PMA) (10 nM), OT (500 nM), TNFα (6 nM), and cyclooxygenase (COX) 2 inhibitor NS-398 (1 μM). The concentrations used were based on previously published conditions (7). PGE_2 and $PGF_{2\alpha}$ production by CSCs was then associated with expression levels of key enzymes of the biosynthetic cascade. Experiment 2 aimed at comparing PG biosynthesis after treatment with increasing concentrations of IFNτ and PMA from 0.02–20 μg/ml and 1 pM to 100 nM, respectively, and their interactions on COX2 and signal transducer and activator of transcription (STAT) 1 genes in CSCs. In experiment 3 the involvement of p38 MAPK was tested by treating CSCs with high concentration of IFNτ (10 μg/ml) in the presence or not of the selective inhibitor of p38 MAPK, SB203580 (1 μM).

Enzyme immunoassays of PGE_2 and $PGF_{2\alpha}$

PGs were assayed by competitive enzyme immunoassay using acetylcholinesterase-linked PG tracers (Cayman Chemical Co., Ann Arbor, MI) as described previously using rabbit anti-PGE_2 (kindly provided by Dr. T. G. Kennedy, University of Western Ontario, London, Ontario, Canada) and sheep anti- $PGF_{2\alpha}$ (Bio-Quant, Ann Arbor, MI) (10).

Western blot analysis

An aliquot of 10 μg protein was loaded in each lane, resolved on 12.5% SDS-PAGE, and electrotransferred onto 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories, Inc., Mississauga, Ontario, Canada). However, for detection of cytosolic phospholipase A2 (cPLA2), 7% gel was used, and for microsomal prostaglandin E synthase (mPGES)-1, 0.2 μm nitrocellulose membrane was used. The membranes were blocked in 5% (wt/vol) nonfat dried milk in PBS containing 0.05% Tween 20 for 1 h at room temperature and incubated overnight at 4 C with respective primary antibodies. The primary antibody dilutions were as follows: 1:500 for anti-cPLA2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); 1:3000 for anti-COX1 and anti-COX2 (kindly provided by Dr. S. Kargman,

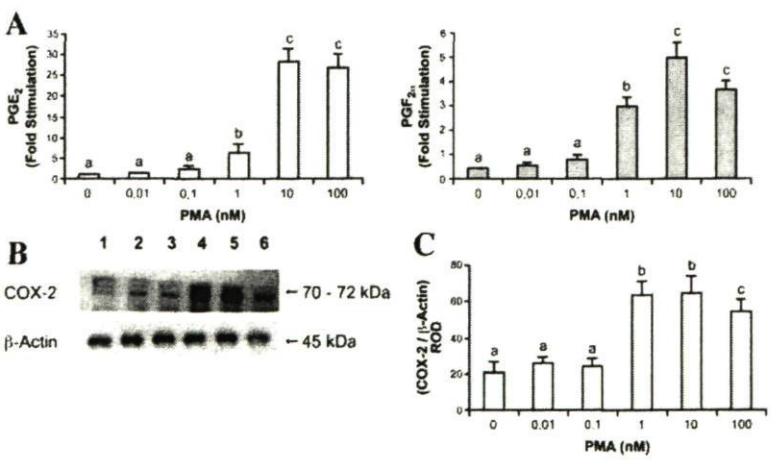

FIG. 4. Effect of PMA on PG production and COX2 expression in CSCs. CSCs were treated with increasing concentrations of PMA (0–100 nM) for 24 h, and PGs were measured in the culture medium. Values represent the mean ± SEM of three different experiments run in quadruplicate. *Bars with different superscripts* differ significantly ($P < 0.05$). A, PGE_2 and $PGF_{2\alpha}$ production. B, Representative immunoblots of COX2 and β-actin. Numbers 1–6 indicate different concentrations of PMA: 0, 0.01, 0.1, 1.0, 10, and 100 nM, respectively. C, ROD values are the ratio between COX2 and β-actin.

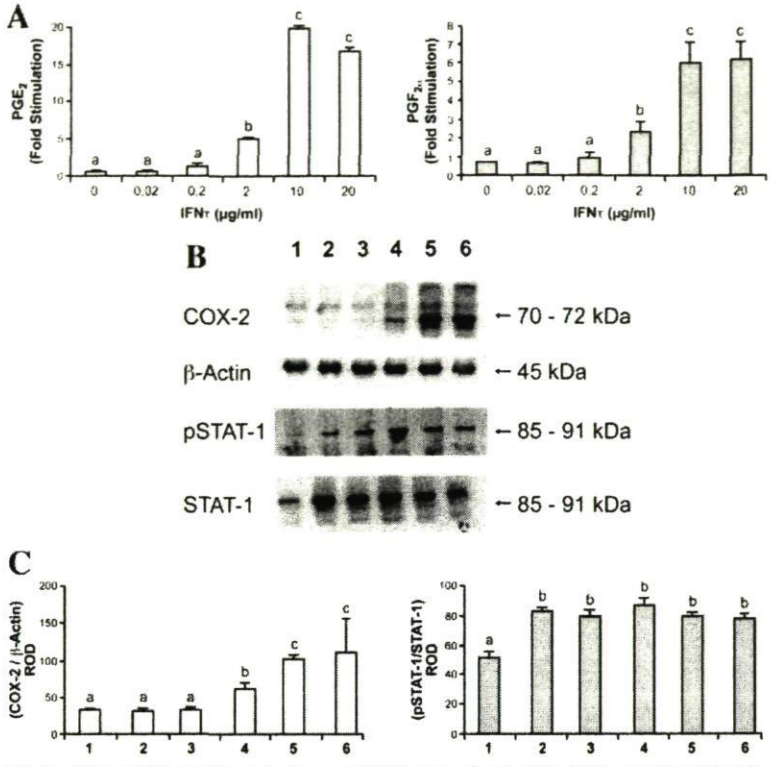

FIG. 5. Effect of IFNτ on PG production and COX2 expression in CSCs. CSCs were treated with increasing concentrations of IFNτ (0–20 μg/ml) for 24 h, and PGs were measured in the culture medium. Values represent the mean ± SEM of three different experiments run in quadruplicate. *Bars with different superscripts* differ significantly ($P < 0.05$). Lanes 1–6 on B and the x-axis of C indicate different concentrations of IFNτ: 0, 0.02, 0.2, 2.0, 10, and 20 μg/ml, respectively. A, PGE_2 and $PGF_{2\alpha}$ production. B, Representative immunoblots of COX2, phosphorylated and unphosphorylated STAT1 and β-actin. C, ROD values are the ratio between COX2/β-actin and phosphorylated to total STAT1.

Merck Frosst Canada Ltd., Kirkland, Quebec, Canada); 1:250 for anti-mPGES-1 (Cedarlane, Burlington, Ontario, Canada); 1:500 for mPGES-2 and cytosolic prostaglandin E synthase (cPGES), and 1:2000 for anti-aldoketoreductase 1 B5 (AKR1B5), a polyclonal serum raised in our laboratory using recombinant protein; 1:1000 for STAT1 (BD Biosciences, Mississauga, Ontario, Canada) and anti-pS727 STAT1 (Upstate Biotechnology Inc., Lake Placid, NY); 1:1000 for phosphorylated and unphosphorylated p38 antibodies (Upstate Biotechnology); and 1:5000 for β-actin (Sigma-Aldrich). After three washes of 10 min each in PBS, the membranes were incubated for 1 h at room temperature with appropriate secondary antibody. The membranes were washed three times in PBS containing 0.05% Tween 20, treated for 1 min with enhanced chemiluminescent substrate (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA), and exposed to Bio-Max film (PerkinElmer Life and Analytical Sciences). Relative OD (ROD) of three different immunoblots from each experiment was quantitated by densitometry (Alpha imager; Fisher Scientific Co., Ottawa, Ontario).

RT-PCR

Total RNA was extracted using TRIzol (Invitrogen Life Technologies), reverse transcribed with Superscript II RT (Invitrogen Life Technologies). To demonstrate ERα and PR in the cell line, the following specific sets of primers were used. For ERα, the sense and antisense primers were 5′-ATGACCCTACCAGACCTTTCAGT-3′ and 5′-ATTTGAGGCACACAAACTCTTC-3′, respectively. Similarly, for PR, the forward and reverse primers were 5′-ATTGTTGATAAAATCCGCAGAAA-3′ and 5′-GAGGTATCAGGTTTGCTGTTGTC-3′, respectively. ERα primers were deduced from accession no. NM_001001443, whereas PR specific primers were designed based on the accession no. AY656812.

Statistical analysis

Randomized block design was used in all the experiments with treatment as the main factor and plate as random effect. The resulting data on PG production were transformed into fold stimulation (except See Fig. 2) by dividing each observation by the mean of control. The StatView program (SAS Institute Inc., Cary, NC) was used for analyzing the transformed data. The group mean of different treatments was tested by two-way ANOVA with Fisher's projected least significant difference as the *post hoc* test to find the critical difference between pairs of treatment means. The confidence level was set at 95% ($P < 0.05$) to determine statistical significance. Data are presented as the mean ± SEM.

Results

Establishment of bovine endometrial stromal cell lines and phenotype characterization

The selected stromal cell line could be propagated until at least P50 while maintaining all phenotypical characteristics of primary cultures and expression of ERα and PR mRNA (Fig. 1C). CSCs and ICSCs expressed SV40 TAG, mesenchyme-specific vimentin but were negative for the epithelial-specific cytokeratin (Fig. 1, A and B). The cell line is also amenable for transient transfection. Average basal and TNFα-stimulated PGE_2 and $PGF_{2\alpha}$ production of all bovine endometrial stromal clones from either caruncles (n = 7) or intercaruncles (n = 26) is shown in Fig. 2A. PGE_2 production was higher ($P < 0.05$) than $PGF_{2\alpha}$ under basal and TNFα-stimulated conditions (Fig. 2A), which was abolished in the presence of the COX-2 inhibitor NS-398 (Fig. 2B). PGE_2 production was also significantly higher in the caruncular clones ($P < 0.05$).

Regulation of PG biosynthesis and expression of corresponding genes

In the caruncular cell line CSC, PGE_2, and $PGF_{2\alpha}$ production increased significantly after stimulation with IFNτ, TNFα, LPS, and PMA ($P < 0.05$), but not with OT (Fig. 3A). Note that in Fig. 3 and the following figures, the scale used for PGE_2 is higher than that of $PGF_{2\alpha}$. Analysis of the enzymes in the PGE_2 and $PGF_{2\alpha}$ biosynthetic pathway indicated that increased PG production was mostly coupled with increased COX2 and cPLA2 expression. Terminal synthases, although all expressed at a significant level, did not exhibit any modulation (Fig. 3B). Increased expression of COX2 together with inhibition of TNFα stimulation by NS398 (Fig. 2B) confirms COX2 as a rate-limiting step.

CSC as a model for functional studies

As was reported for primary stromal and BEND cells, PMA induced COX2 expression and PGE_2 and $PGF_{2\alpha}$ production in a dose-dependent manner in CSCs (Fig. 4). Similarly, IFNτ in-

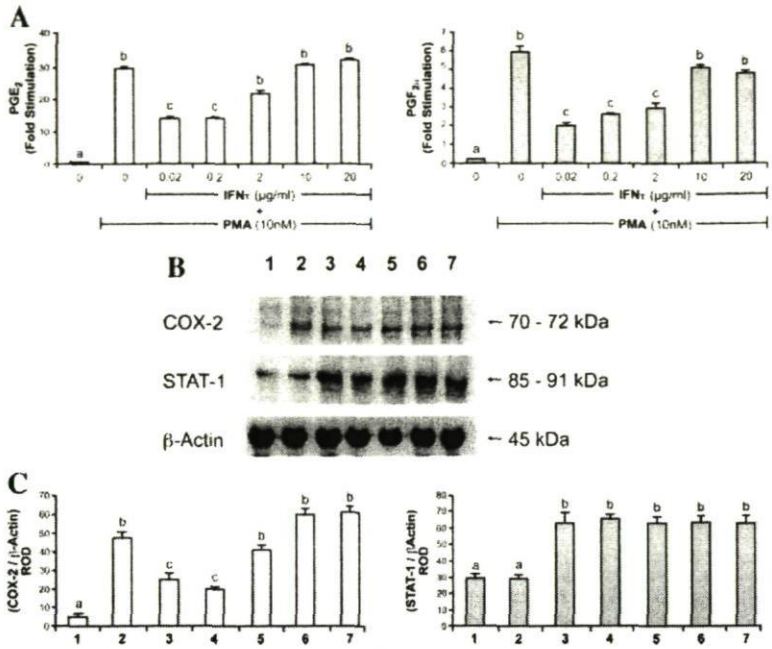

FIG. 6. Interaction between PMA and IFNτ in CSCs. CSCs were treated with PMA 10 nM and various concentrations of IFNτ (0–20 μg/ml) for 24 h, and PGs were measured in the culture medium. Values represent the mean ± SEM of three different experiments run in quadruplicate. *Bars with different superscripts* differ significantly ($P < 0.05$). Numbers on B and the x-axis of C indicate: control (1); PMA 10 nM (2); PMA 10 nM plus IFNτ 0.02 μg/ml (3); PMA 10 nM plus IFNτ 0.2 μg/ml (4); PMA 10 nM plus IFNτ 2.0 μg/ml (5); PMA 10 nM plus IFNτ 10 μg/ml (6); and PMA 10 nM plus IFNτ 20 μg/ml (7). A, PGE_2 and $PGF_{2\alpha}$ production. B, Representative immunoblots of COX2, STAT1, and β-actin. C, ROD values are the ratio between COX2, STAT1, and β-actin.

creased ($P < 0.05$) PGE_2 and $PGF_{2\alpha}$ production, as well as COX2 expression (Fig. 5, A and B) in a dose-dependent manner. Expression of COX2 closely parallels PG production (Fig. 5C).

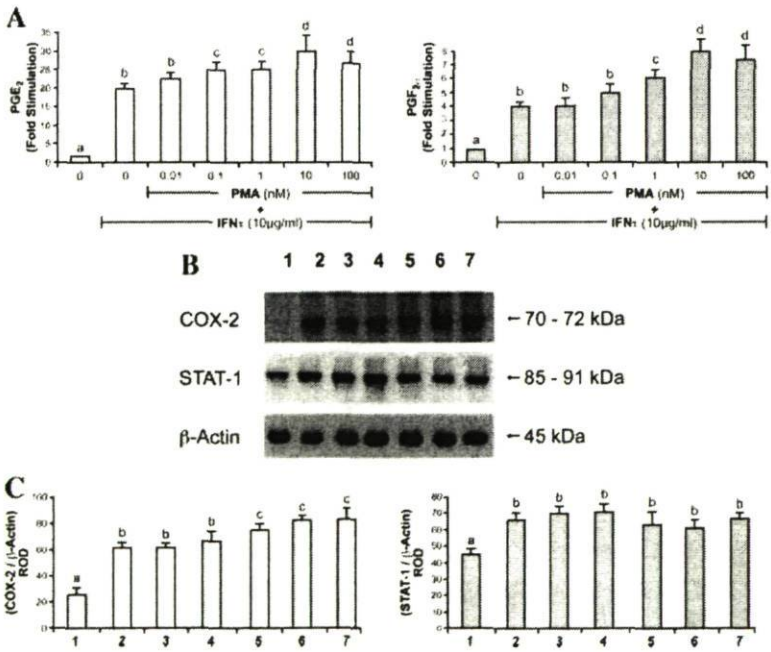

FIG. 7. Interaction between IFNτ and PMA in CSCs. CSCs were treated with IFNτ 10 μg/ml and various concentrations of PMA (0.01–100 nM) for 24 h, and PGs were measured in the culture medium. Values represent the mean ± SEM of three different experiments run in quadruplicate. *Bars* with different *superscripts* differ significantly ($P < 0.05$). Numbers on B and the x-axis of C indicate: control (1); IFNτ 10 μg/ml (2); IFNτ 10 μg/ml plus PMA 0.01 nM (3); IFNτ 10 μg/ml plus PMA 0.1 nM (4); IFNτ 10 μg/ml plus PMA 1.0 nM (5); IFNτ 10 μg/ml plus PMA 10 nM (6); and IFNτ 10 μg/ml plus PMA 20 nM (7). A, PGE_2 and $PGF_{2\alpha}$ production. B, Representative immunoblots of COX2, STAT1, and β-actin. C, ROD values are the ratio between COX2 or STAT1 and β-actin.

Treatment with IFNτ stimulated STAT1 phosphorylation, and total STAT1 expression (Fig. 5, B and C). IFNτ was able to inhibit PMA-stimulated PG production at low concentrations, but this response disappeared at higher concentrations (Fig. 6A). The same effect was observed at the level of COX2 expression (Fig. 6, B and C). However, PMA had no effect on STAT1 expression or on its stimulation by IFNτ. When opposite conditions were tested, the effect of increasing concentrations of PMA stimulated further PG production and COX2 expression (Fig. 7) but did not impact on IFNτ-induced STAT1 expression (Fig. 7C). Because a dose-dependent biphasic effect of IFNτ on COX2 was not observed at the level of STAT1 in CSC, we hypothesized that MAPK could be involved as demonstrated for COX2 expression in the myometrium (14). Accordingly, IFNτ stimulated phosphorylation of p38 and inhibition of the p38 MAPK pathway with SB203580 reduced COX2 expression and PG production (Fig. 8). These results support a contribution of the p38 MAPK pathway in endometrial IFNτ signaling.

Discussion

The presence of SV40 TAG in CSCs and ICSCs and repeated passage without apparent senescence confirmed the permanent status of the selected cell lines. Vimentin-positive and cytokeratin-negative features support the stromal phenotype and mesenchymal origin of the cell lines. TNFα was used to stimulate PG production during initial screening of the stromal clones because this cytokine has been ascribed a role in the initiation of luteolysis (15). Both caruncular and intercaruncular clones produced PGE_2 levels 10- to 30-fold higher than $PGF_{2\alpha}$, a feature distinguishing endometrial stromal from epithelial cells (10). Basal and TNFα-stimulated PGE_2 production was significantly higher in the caruncular than the intercaruncular stromal clones, as we reported previously (16). Increased PG production in response to IFNτ, TNFα, LPS, and PMA, and nonresponsiveness to OT observed in this study is in agreement with our previous observations on primary stromal cell cultures (7, 10). Among biosynthetic enzymes, only COX2 and cPLA2 are regulated in parallel with PG production. This was confirmed using NS-398, a COX2-specific inhibitor that blocked TNFα-stimulated PG production. These observations are also in agreement with what was observed by us in primary stromal cells for COX2 (7) and by others in BEND cells for cPLA2 (17). Although increased prostaglandin E synthase expression was seen at the transcriptional level previously (7), we report here that PGE and PGF terminal synthases are expressed but not modulated at the protein level. A possible explanation is that prostaglandin E

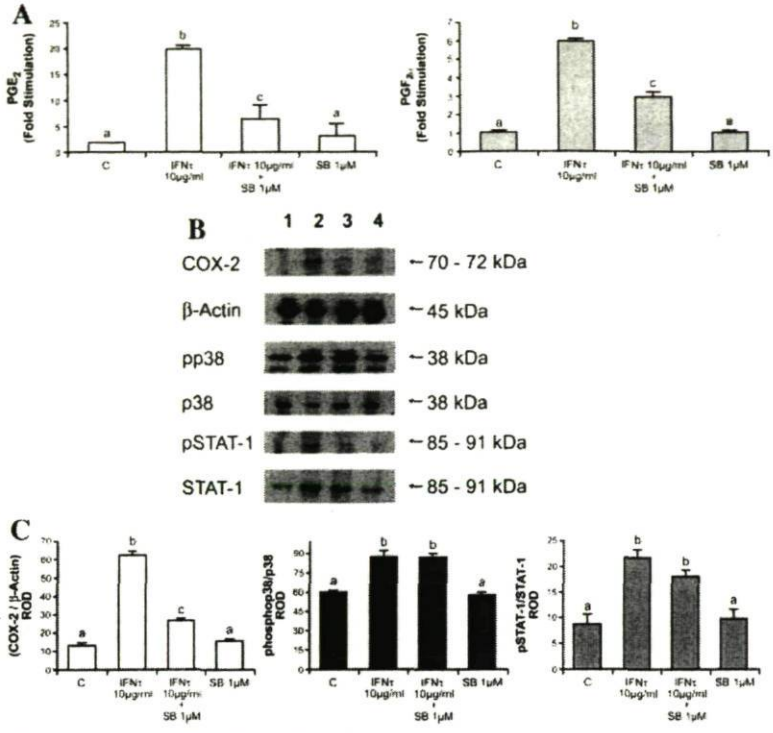

FIG. 8. Effect of the p38 MAPK inhibitor, SB203580, (1 μM) on IFNτ (10 μg/ml) induced PG production in CSCs. CSCs were treated with IFNτ 10 μg/ml in the presence or absence of SB203580 (1 μM) for 24 h. A, PGE_2 and $PGF_{2\alpha}$ production. Values represent the mean ± SEM of three different experiments run in quadruplicate. *Bars with different superscripts* differ significantly ($P < 0.05$). B, Representative immunoblots of COX2, β-actin, phosphorylated and unphosphorylated p38 MAPK and STAT1. Lanes 1–4 indicate control, IFNτ 10 μg/ml, IFNτ 10 μg/ml plus SB203580 1 μM, and SB203580 1 μM, respectively. C, ROD values are the ratio between COX2/β-actin and phosphorylated to total p38MAPK and STAT1.

synthase expression level is higher in CSCs than it was in primary stromal cells. Because mPGES1 is known to be associated with COX2 in situations of increased PGE_2 production, constant expression of the former with induced expression of the latter appears to be sufficient to explain our observations in CSCs. Together, these results show that the PG enzymatic machinery is intact and functional in CSCs where COX2 appears as the rate-limiting enzyme. Because the CSC is also amenable for transfection, this cell line can be used in combination with gene-silencing experiments with small interfering RNA to confirm the contribution of the different members of the PG biosynthetic cascade. Stromal cell-specific phenotype, the presence of ERα and PR, preferential production of PGE_2 over $PGF_{2\alpha}$, and responsiveness to known PG production inducers validate CSC as a *bona fide* stromal cell model to study the molecular aspects of *in vitro* regulation of PG production in bovine.

Using this validated model, we studied the regulation of COX2 and PG production in response to PMA and IFNτ and their interactions, and attempted to correlate it with the expression of STAT1 involved in IFNτ-mediated signaling (8, 9). Phorbol ester was chosen because it is used as a surrogate for OT response in BEND cells. It is clear that both PMA and IFNτ induce COX2 expression and PGE_2 and $PGF_{2\alpha}$ production in a dose-dependent manner. However, IFNτ alone is able to phosphorylate and up-regulate STAT1. Phosphorylation of STAT1, which is still detectable after 24 h in CSCs, is comparable with the persistent tyrosine phosphorylation of STAT1 observed in the ovine endometrial epithelial cell line and may be involved in sustained effects of IFNτ (18). Interaction studies showed that PMA- induced COX2 expression can be inhibited by low but not high concentrations of IFNτ. Second, PMA does not affect IFNτ-induced up-regulation of STAT1. Because the apparent biphasic effect of IFNτ on COX2 could not be correlated at the level of STAT1, we chose to probe the p38 MAPK pathway known to be involved in other systems. Interestingly, the p38 MAPK inhibitor SB203580 blocked the effect of IFNτ on COX2 expression and PG production. This result is supported by the observation that IFNτ confers transcriptional stability to COX2 in bovine myometrial cells through p38 MAPK (14).

The biphasic effect or dose-dependent dichotomy of IFNτ on COX2 expression may throw some light on the up-regulation of COX2 observed during the maternal recognition window in ruminants (19–21) as well as after intrauterine infusions of IFNτ (22). Given that copious production of IFNτ occurs during recognition of pregnancy (23) and because interferon-stimulated genes are mainly present in the endometrial stroma (6), it is possible that the up-regulation of COX2 by high concentrations of IFNτ reflects the *in vivo* conditions (24). COX2 mediates inflammation and tumorigenesis (25), and is traditionally viewed as pathological, but it is also necessary for normal female reproductive function (26, 27). In humans and rodents, implantation is associated with elevated levels of PGE_2 by the decidualizing stromal cells (28). Although the implantation is superficial and its onset is relatively late in ruminants compared with human, it is associated with up-regulation of COX2 (19–22). Our preliminary results with CSCs suggest that IFNτ may influence PGE_2 and COX2 through the p38 MAPK pathway to mediate its pro-gestation effects in the endometrial stroma. In this respect, the CSC may serve as an ideal model for investigating the paradigm of counteraction of the luteolytic $PGF_{2\alpha}$ and the immunomodulatory PGE_2, at the time of maternal recognition of pregnancy.

Acknowledgments

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Disclosure Statement: The authors have nothing to declare.

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Appendix 3

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S. BOUCHER-KOVALIK, P. CHAPDELAINE J.A.

A POSTGENOMIC INTEGRATRED VIEW OF PROSTAGLANDINS IN REPRODUCTION: IMPLICATIONS FOR OTHER BODY SYSTEMS.

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Prostaglandins are primary mediators of pain and are involved in pathological conditions such as hypertension, cancer and inflammation but are also needed for normal function of the female reproductive system. This may hold true for other systems because long term use of selective COX-2 inhibitors such as VIOXX and BEXTRA was associated with heart failure, leading to their withdrawal. A thorough study of the contribution of prostaglandins in the regulation of normal body function is clearly needed. A major drawback of the current therapeutic strategies aiming at controlling PGs is that they aim at early steps of biosynthesis thus blocking all PGs, good and bad. However, PGs often work as opposing dyads such as PGI2-TXA2 in the vascular system and PGF2α-PGE2 in the female reproductive system. The paradigm thus appears as effecting selective synthesis, transport and action of individual PG isoforms. In this respect, the female reproductive system appears as an ideal study model. Data from human and animal genome projects allowed identifying the corresponding members of the biosynthetic and signal transduction components of the PG system in different animal species. Of particular interest was that PG terminal synthase shared similarities or identity with enzymes previously known for steroid or sugar metabolism and free radical detoxification. We present here an integrated view of PG action based on observations in the female reproductive system, but with potential strategic implications for cardiovascular and metabolic complications.

Key words: *Prostaglandis, female reproduction, fertility, aldose reductase, menstrual disorders, ischemia*

Prostaglandin biosynthesis

Prostaglandins are notorious mediators of pain, fever, inflammation and hypertension, and their production has been a target for pharmacological therapy with non steroidal anti-inflammatory drugs (NSAIDs) for more than a century. PGs are produced by all nucleated cells of the body and act locally in a paracrine or autocrine fashion. The first limiting step in the generation of eicosanoids is the liberation of arachidonic acid from membrane phospholipids by phospholipases and the most relevant for the production of PGs from arachidonic acid is cPLA2α (1). Arachidonate can then be sequentially transformed into leukotrienes, not covered in the present review, and different active prostanoids (*Fig. 1*). PGH2, the common precursor of all PGs is generated from arachidonic acid (AA) by prostaglandin synthase (PGHS or COX). There are two isoforms encoded by distinct genes (2) the constitutive isoform, COX-1, is widely expressed in a variety of tissues and cells, whereas the inducible form, COX-2, is regulated by factors such as: cytokines or tumour promoters (3). A splice variant of COX-1

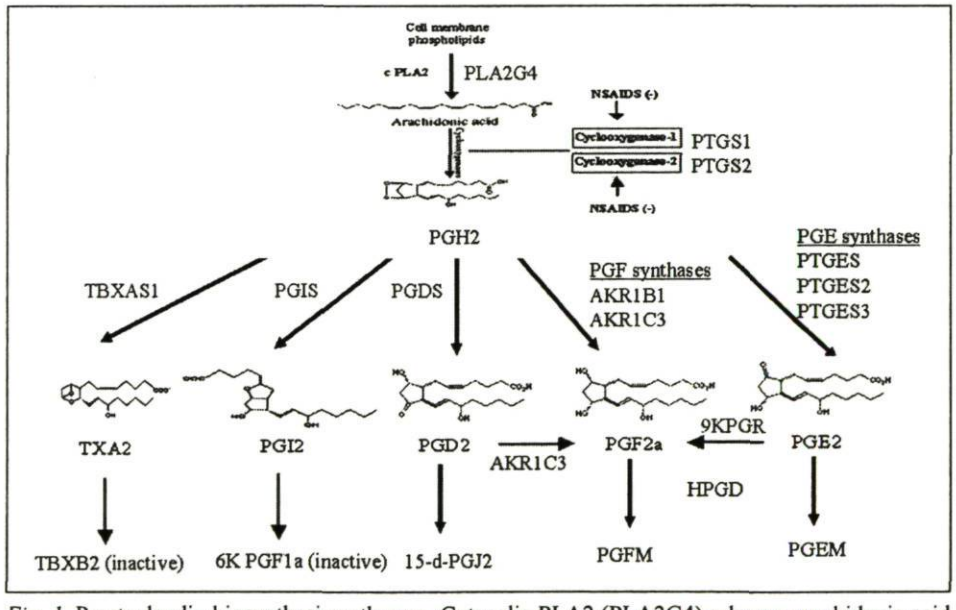

Fig. 1. Prostaglandin biosynthesis pathways. Cytosolic PLA2 (PLA2G4) releases arachidonic acid (AA) from membrane phospholipids and COX enzymes (PTGS1, PTGS2) convert it to PGG2 and PGH2, the common precursor for all PGs. PGH2 is then converted into one of the active PG by specific terminal synthases such as PGE synthases (PTGES, PTGES2, PTGES3), PGF synthases (AKR1B1, AKR1C3), PGD synthase (PGDS), PGI synthase (PGIS) and Thromboxane synthase (TXA1S). PGE2 and PGF2a are inactivated into PGEM and PGFM by HPGD (15-PGDH), PGD2 converts spontaneously into active PGJ2 whereas unstable PGI2 and TXA2 convert into inactive 6K-PGF1α and TXB2.

was referred to as COX-3, but its contribution to physiological or pathological conditions remains speculative. COX-1 is constitutively expressed in most tissues and responsible for housekeeping functions and immediate response to levels of AA above 10 μM. COX-2 is regulated by factors such as cytokines or tumour promoters and supports sustained production of PGs from relatively low levels of AA (below 2.5 μM) (4). PGH2 produced by COXs is the common precursor for generation of primary PGs including PGE2, PGF2α, PGD2, PGI2 and TxA2 by cell-specific isomerases and synthases such as PGES, PGFS, PGDS, PGIS and TXAS, respectively.

The physiological importance of prostaglandins has been confirmed in the mouse where targeted disruption of COX-1 (5) or COX-2 genes (6) resulted in severe nephropathy or reduced reproductive efficiency in homozygous null mice. In fact, female COX-2 null mice suffered from multiple failures in reproductive processes (7). Other studies have shown that COX-2 expressed in COX-1

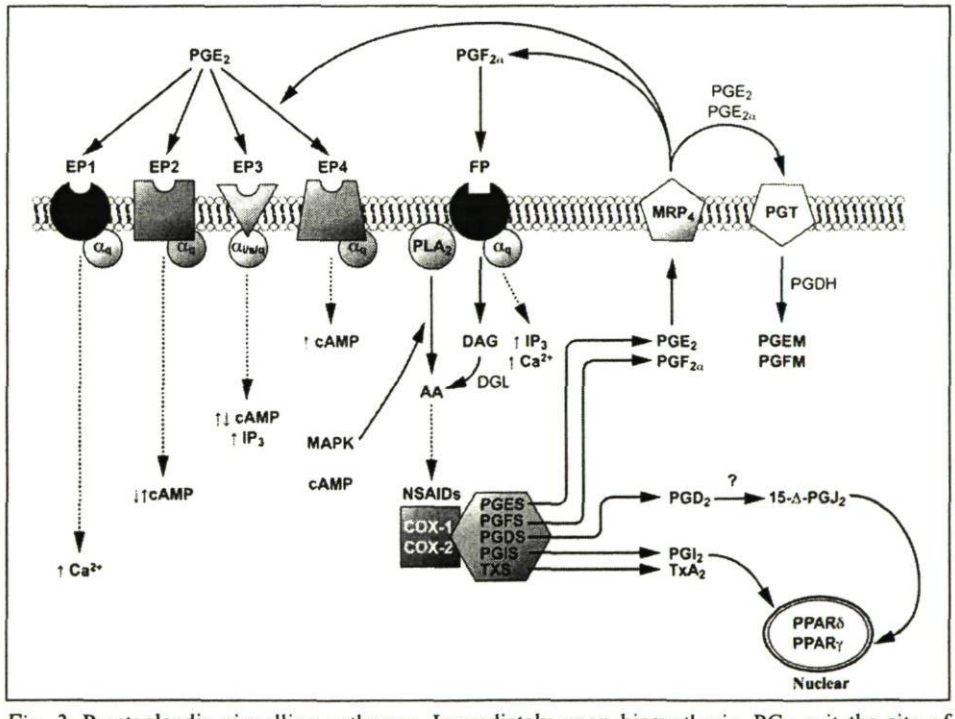

Fig. 2. Prostaglandin signalling pathways. Immediately upon biosynthesis, PGs exit the site of production passively or through constitutively expressed facilitated transport (MRP4) and either bind to specific membrane receptors in an autocrine or paracrine manner. PGE2 and PGF2α can travel across successive cell layers through PGT or enter target cells to act on nuclear receptors or be inactivated by 15-PGDH. The membrane DP, EP1-4, FP, IP and TP receptors are coupled to diverse G protein and second messengers as illustrated. PGJ2, the spontaneous active metabolite of PGD2 is the physiological ligand for the nuclear receptor PPARγ whereas PGI2 binds to PPARδ.

deficient mice was able to compensate at least partly (8). COX-1 -/- and COX-2-/- double knockouts induced death early after birth of the pups suggesting that PGs might be more important for survival than initially anticipated (9, 10).

Pharmacological control of PG biosynthesis is more than a century old. Indeed, Aspirin (ASA) was the first non steroidal anti-inflammatory drug (NSAID) commercialized. ASA shares with newer drugs like ibuprophen (ADVIL) the ability to inhibit non-selectively COX-1 and COX-2 activities (11). More recently, new inhibitors like NS-398 and SC-560 have been shown to specifically block either COX-2 (12) or COX-1 (13) opening the field for the development of more specific NSAIDs such as CELEBREX and VIOXX. However, severe side effects of COX-2 inhibition such as heart failure (14) and infertility (15) lead to the widely publicized withdrawal of VIOXX from the market. Total blockade of all PGs by NSAIDs provides a quick relieve of symptoms but unfortunately deprives from a physiological cure (16). In this respect, targeted action at the level of terminal synthases such as PGES and PGFS responsible for the selective production of PGE2 and PGF2α appears as promising and important to explore (17).

Prostaglandin signal transduction

The different prostaglandins exert a wide array of different or even opposite actions mediated by specific receptors sometimes taking multiple isoforms for a single prostaglandin (*Fig. 2*) (18, 19). PGs represent a class of local regulators with complementary or opposing actions depending on the type of PG or the receptor signalling their action. PGF2α acts through FP receptors coupled to Gq, PLC and Ca++ release whereas PGE2 acts through 4 classes of receptors, EP1 coupled to Gi and calcium channels, EP2 and EP4 coupled to Gs and cAMP generation, and EP3 for which there are 8 splice variants in the human coupled principally to the inhibitory Gi system (20). Considerable efforts were made to develop selective agonists and antagonists of PG receptors over the last 30 years, but most treatments aiming at controlling PG action are still based on systemic COX inhibition (21).

Peroxisome Proliferators-Activated Receptors (PPAR α,γ,δ) have been proposed as nuclear receptors for PGD_2 and PGI_2 (22). Recently EP2 and EP4 have been identified in the nuclear envelope suggesting the presence of functional nuclear receptors for PGE_2 (23). However, limited information is available on the putative actions of nuclear receptors.

Prostaglandins and reproduction

Apart from sex steroids, prostaglandins are probably the most important regulators of female reproductive function (ovulation, uterine receptivity, implantation and parturition) and associated pathologies (24). Reproductive tissues express different classes of prostaglandin receptors (25). Among the

different PGs, PGE2 and PGF2α are the main prostanoids produced in the human (26, 27) and bovine (28) endometrium. The physiological importance of PGs in reproduction has been confirmed in the mouse where targeted disruption of COX-1 or COX-2 genes reduced reproductive efficiency (5-7). Null mutation for cPLA2 a PG biosynthesis enzyme upstream of COX-2, also leads to an infertile phenotype (29). At the receptor level, deletion of the PGF2α receptor (FP) showed that it is necessary for parturition in the mouse (30) whereas EP2 receptors null mutants exhibit ovulation and peri-implantation problems (31, 32).

In the reproductive system, PGF2α and PGE_2 often exhibit opposite actions (*Fig. 3*) (33). The endometrial release of PGF2α in response to oxytocin is the initial signal triggering luteolysis in animals and ovarian PGF2α contributes to the luteolytic process in primates including humans (34). In presence of a viable embryo, the default luteolytic signal is counteracted by an antiluteolytic or a

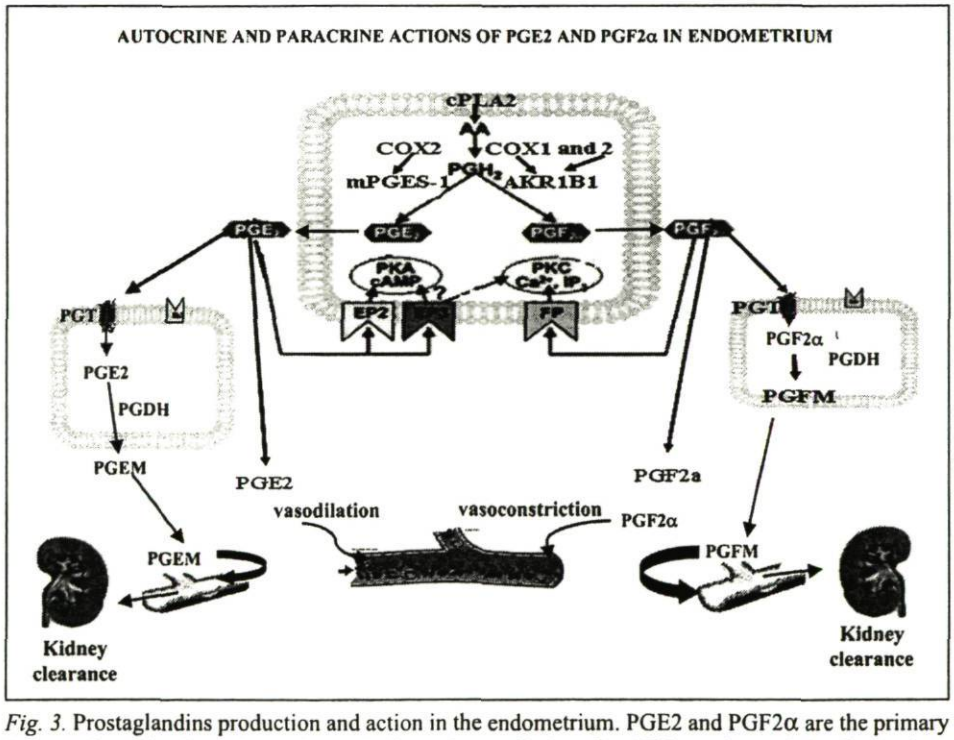

Fig. 3. Prostaglandins production and action in the endometrium. PGE2 and PGF2α are the primary PGs produced in the endometrium of all species studied so far. While endometrial cells produce both, epithelial cells preferentially release PGF2α and stromal cells PGE2. PGs can then act on the neighbouring cells to regulate endometrial function or travel across cells and tissues to reach the ovary and exert a luteolytic or luteotrophic effect to regulate progesterone production. When native PGs enter the vascular system, PGF2a exerts a TXA2 like contractile response whereas PGE2 induces a prostacyclin-like relaxation response. Native PGs are catabolised in the lung and metabolites cleared in the kidney.

luteotrophic signal or a combination of both to maintain the production of progesterone. PGF2α is also a potent constrictor of the myometrium and uterine blood vessels (21). By contrast, PGE2 is vasodilator able to exert a strong luteotrophic action in human (35). Prostaglandins, especially PGE2, are produced by early embryos and we have found that PGES (36) is increased at the time of maximal uterine receptivity. Similar observations in the mouse, suggest that PGE2 contribution to this process is well conserved among species (37, 38). At the time of implantation or recognition of pregnancy, PGE2 induces a local alteration in growth factors secretion and nutrients and increases vascular permeability (39, 40). PGE2 is a potent immunomodulator mediating the local maturation/differentiation processes (41) and inhibiting the lytic activities of both NK and lymphokine activated killer (LAK) cells (42) around the time of implantation in the endometrium. Consistent with the roles attributed to PGs, the treatment of pregnant females with NSAIDs inhibits implantation or at the very least reduces pregnancy rates (15). In humans, PGs interact with cytokines and PRL to regulate decidualization and with angiogenic and coagulation factors to regulate menstruation (43). During the menstrual cycle, the concentration of PGF2α is apparently higher than PGE2 during the secretory phase whereas levels of both PGs are low during the proliferative phase. The concentrations of PGE2 remain low whereas PGF2α goes higher during menstruation and lower during the implantation window (44). Our data and a recent reviews concur to state that across species, PGF2α and PGE2 are universally important in the regulation of endometrial function (43, 45).

SELECTIVE PGF2α AND PGE2 RELEASE

It is widely acknowledged that PGs play a critical role in reproductive processes. The expression of rate limiting enzymes such as phospholipase A2 (cPLA2α) and prostaglandin synthases 1 and 2 (PGHS-1/-2) also called cyclooxygenases (COX) regulates the rate of production of PGs as a group, but other mechanisms are needed for selective production of specific PGs. It is increasingly evident that the physiological action of PGs is regulated at multiple levels not only quantitatively, but also qualitatively by selective biosynthesis, expression of specific receptor subtypes, and specialized transport across cell membranes and compartments.

Very little has been done to identify the biosynthetic pathways leading to the formation of specific prostaglandins. Initially it was thought that a single type of PG was produced by distinct subsets of cells (46, 47). Our results with primary cultures now confirmed with clonal cell lines (48) demonstrated that endometrial cells can produce more than one PG. Therefore, conditions leading to the generation of a particular PG vary and must be set within individual cells.

Selective PGF2α production

PGF2α can be produced from three distinct pathways (*Fig. 1*) but most likely through reduction of PGH2 by 9, 11-endoperoxyde reduction referred to as PGFS activity. Several PGFS have been identified; three were isolated in the bovine: lung type prostaglandin F synthase (PGFS1) (49), lung type PGFS found in liver (PGFS2) (50) and liver type PGFS, also called dihydrodiol dehydrogenase 3 (DDBX) (51, 52). Others were identified respectively in human (AKR1C3) (53), sheep (54), Trypanosoma brucei (a protozoa) (55) and recently the porcine endometrium (56). All recognised mammal PGFSs belong to the aldoketoreductase 1C family, and are generally associated with hydroxysteroid dehydrogenase (HSD) activity. In the bovine endometrium we have shown that none of the presumed functional PGFS was expressed under any condition while we identified AKR1B5, an old enzyme with a new function, as a functional PGFS (28). We have studied the characteristics of various PGFS isoforms in relation with PGF2α production (*Fig. 4*). We found that aldoketoreductase 1B5 (AKR1B5) was the most likely PGFS involved in the production of PGF2a in bovine endometrium at the time of luteolysis (28). Interestingly, with its 20αHSD activity, this enzyme can also inactivate progesterone, another factor regulating endometrial function (*Fig. 5*). The human equivalent of the bovine AKR1B5 is AKR1B1 belonging to the AKR superfamily composed of 140 members divided into 15 families (57). AKR1B1 is one of 13 human AKRs catalyzing reactions on a broad and overlapping list of substrates making it difficult to find natural substrates and specific functions for any of these enzymes. AKR1B1 also known as the aldose reductase is highly expressed in the placenta for glucose metabolism and in the eye and kidney for osmotic regulation (58). We have accumulated several lines of evidence supporting the hypothesis that AKR1B1 is a functional PGFS in the human endometrium, but we are currently the only group exploring this avenue. We have studied AKR1B1 and demonstrated its association with PGF2α production in human endometrial cell lines (48) and in decidualized stromal cells (59). In a cell free system, purified AKR1B1 recombinant protein is able to produce PGF2α from PGH2. Endometrial cell lines transiently transfected with an expression vector coding for AKR1B1 exhibit increased ability to release PGF2α. In contrast, when AKR1B1 expression is knockdown with specific siRNAs, PGF2α production is decreased. We have found that the other potential PGF synthase (AKR1C3) is also expressed in endometrial cell cultures but its contribution to PGF2α production remains to be determined.

Selective PGE2 production

Three forms of PGE synthase (PGES) have been characterized so far (*Fig. 4*). Microsomal PGES-1 (mPGES-1) was the first identified and reported as inducible by agents such as cytokines and LPS (60). This enzyme is often coupled with COX-2 for delayed and sustained production of PGE2 (61). We have

described previously the regulation of mPGES-1 expression during the bovine oestrous cycle and its association with COX-2 (36). A cytosolic PGES (cPGES), identical to p23, a ubiquitous chaperone protein weakly bound to the steroid hormone receptor/hsp90 complex, was characterized and found coupled to COX-1 for immediate production of PGE2 (62). Enzymatic activity from a third PGES, microsomal PGES-2 (mPGES-2), was purified from bovine heart and cloning of homologous human and monkey sequences was done (63). This PGES is associated with both isoforms of COX with a slight preference for COX-2 (64) and we have documented its expression in the endometrium during the oestrous cycle (65) while we have cloned and sequenced the other two PGES from the macaque endometrium (66). In the bovine endometrium, all three PGES are expressed during the oestrous cycle with mPGES-1 dominating around ovulation. In cell cultures only mPGES-1 was found to increase in parallel with COX-2 when PGE2 production was stimulated with various factors (65).

In the human endometrium, the three known PGES, mPGES-1, mPGES-2 and cPGES are expressed during the menstrual cycle together with COX-1 and COX-2. At the mRNA level, mPGES-1 is expressed maximally during menses, mPGES-2 during the secretory phase and cPGES is expressed at a constant level. We have shown that mPGES-1 protein expression was stimulated following decidualization of stromal cells *in vitro* (59). In human endometrial cell lines, mPGES-1 mRNA and protein expression are highly stimulated by IL-1β and associated with PGE2 production (48). Accordingly, knockdown of mPGES-1 with a specific siRNAs decreased mRNA, protein and associated PGE2 production. It is worth noting that mPGES-1 appears to mediate most effects following stimulation of PGE2 production, but although mice with null mutation

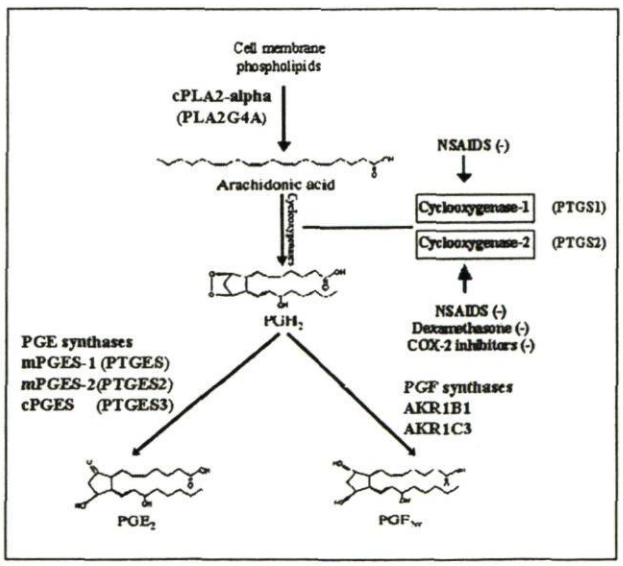

Fig. 4. Selective production of PGE2 and PGF2α. Following release of arachidonic acid (AA) from plasma membrane phospholipids primarily through cytosolic Phospholipase A2 (cPLA2), conversion into PGH2, the common precursor for all PGs, occurs through PGH synthase or COX, for which there are two isoforms COX-1 and COX-2 encoded by 2 genes, PTGS1 and PTGS2. PGH2 can then be converted into active PG by terminal synthases. We present two putative PGFS, AKR1B1 and AKR1C3 for PGF2α and three PGES, mPGES-1, mPGES-2 and cPGES for PGE2.

for this gene are insensitive to LPS and NFκB they do not exhibit the fertility problems found for COX-2, EP2 or cPLA2 knockout. Therefore, the mPGES-2 and cPGES are either able to compensate or are solicited as contributors for PGE2 production through an NFκB independent mechanism (67).

Physical association (team up) of PG biosynthetic enzymes

Biosynthesis of a specific PG requires simultaneous expression of the different members of the biosynthetic cascade (*Fig. 1, Fig. 4*). However, this does not rule out simultaneous expression of more than one terminal synthase. Therefore selective production may also involve functional association (compartmentalization) of complementary enzymes. This may include linking, association around scaffold proteins or grouping on a common structure. To date, no specific scaffolds involving PG biosynthesis have been identified. Terminal synthases are necessary to produce a specific PG but spatiotemporal association with upstream phospholipase and COXs is necessary to access the rate limiting precursors AA and PGH2. Functional associations between terminal synthases

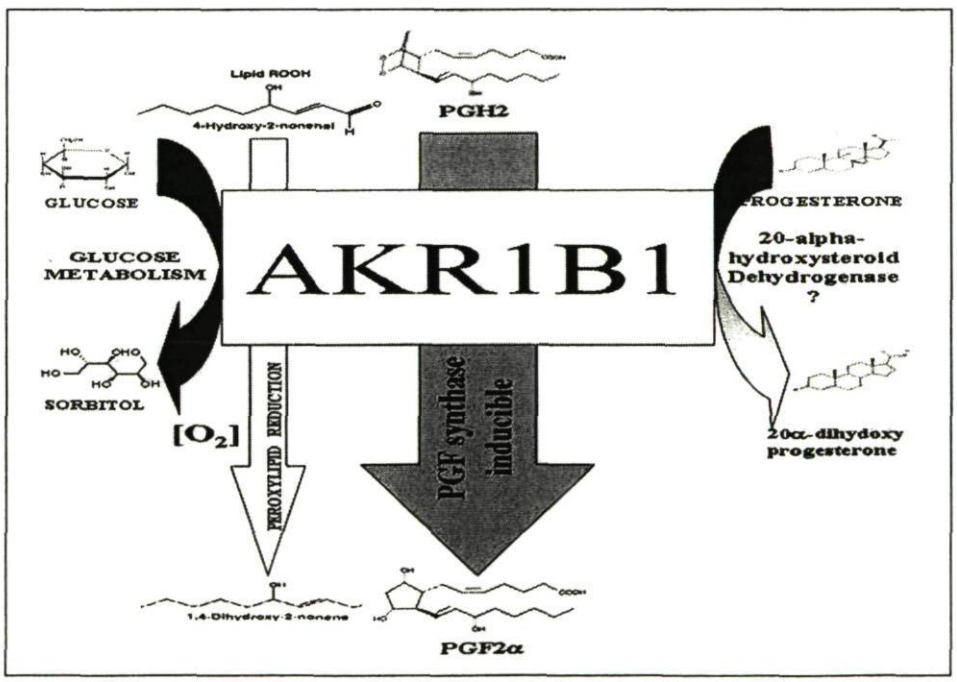

Fig. 5. Multiple enzyme activities of AKR1B1. AKR1B1 was first associated with conversion of glucose into sorbitol and accordingly named aldose reductase. The corresponding bovine AKR1B5 was first identified as a progesterone processing enzyme with 20α HSD activity. While both AKR1B5 and AKR1B1 express the latter activities, we have demonstrated that in both species, the primary activity of these enzymes is PGFS.

and upstream COXs have been described almost exclusively for the different PGES using transfected cell lines. Microsomal PGES-1 is often coupled with COX-2 for delayed and sustained production of PGE2 initiated by cytokines or LPS through an NFκB mediated mechanism (61). mPGES-2 is associated with both isoforms of COX with a slight preference for COX-2 (64) and cPGES is coupled to COX-1 for immediate release of PGE2 (62). There is no data available to link any PGFS with upstream enzymes of the PGF2α cascade at the functional or the transcription level (67), but our data suggest functional association of AKR1B1 with COX-2 (48) and potential association with COX-1. A study on AKR1C3 in transfected HEK-293 cells suggested preferential association with COX-1 (68). In bovine epithelial cells PGF2α production is stimulated preferentially by oxytocin through a PLC-PKC mediated pathway (69) and PGE2 with interferon (IFNt) (70) potentially through a NFκB mediated mechanism. In contrast, both PGs are increased simultaneously under all conditions tested so far in human endometrial cells (48, 71). There appear to be a trend for preferential stimulation of PGF2α and AKR1B1 in response to IL-1β in human endometrial cells, but significant increases in PGE2 and mPGES-1 are also observed.

Because the selective production of one PG such as PGE2 requires synchronous expression of at least 3 enzymes and because endometrial remodelling involves coordinate expression of multiple genes favouring cell proliferation and angiogenesis, key regulatory factors are likely to liberate transcription factors acting on a cassette of complementary genes. Some groups working on the involvement of PGs in cancer and inflammation have described important regulation by transcription factors such as TonE/NFAT5 (NFκB), NRF-2 and EGR-1, but very little has been done on reproductive tissues or non pathologic conditions such as pregnancy or menstruation. Our results showing a time and dose dependent increase in PGF2α associated with a parallel increase in AKR1B1 mRNA in response to IL-1β suggested transcriptional regulation of the AKR1B1 gene. Accordingly, we have cloned a 4.5 kb AKR1B1 promoter in the basic pGL3 vector coupled with the luciferase reporter gene. This construct is strongly activated by IL-1β. Progressive 5'deletions allowed to identify an IL-1β sensitive region located at -1177 to -1047. In the mouse AKR1B3 gene, homologous to the human AKR1B1, an Nrf2 binding motif is regulated by the antioxidant response element (ARE) present in the Multiple Stress Response Region (MSRR) (72). We have identified the corresponding putative trans-acting factors TonE/NFAT5, AP1, Nrf2 and NFκB in the AKR1B1 promoter. In the mouse, knocking out NFAT5 (TonE) leads to down-regulation of the AKR1B3 gene and poor embryo survival (73), disrupting the Nrf2 gene leads to a normal and fertile phenotype under controlled environment, but extreme susceptibility to oxidative stress, characteristic of PG biosynthesis (74). Interestingly, using constructs coupling different MSRR fragments of the AKR1B1 promoter with the SV40 pGL3-promoter, we have identified two AREs as important factors mediating the effect of IL-1β, potentially through Nrf2, in human endometrial

cells. In addition, mutations in the osmotic response element ORE (TONE) of the same MSSR fragment lead to decreased promoter activity following IL-1β stimulation. It was reported that NFκB was able to bind ORE of the AKR1B1 gene in human liver and lens cells treated with TNFα (75) whereas involvement of Nrf2 was shown in the regulation of TXA2 synthase an important vasoactive PG in platelets (76). Finally, numerous constructs and mouse mutation models used to characterize the NFκB system point to genes associated with AA metabolism as important targets (77). As observed for AKR1B1, PGE2 production and mPGES-1 mRNA exhibit a parallel increase in response to IL-1β suggesting transcriptional regulation of this gene. We have cloned a 4.2 kb promoter of the mPGES-1 gene and progressive deletion constructs showed that the -1059 +52 region conferred IL1B response whereas position -3096 to -2796 is associated with repressive activity. These are the first data describing promoter activity of a relatively long (4.2 kb) fragment for the human mPGES-1 gene. We hypothesize that Egr-1, an inducible zinc finger protein that recognizes the GC-rich consensus DNA sequence 5'-GCG(T/G)GGGCG-3'box present at the proximal promoter region -119/-112 and -108/-101 of the mPGES-1 gene is a functional transcription factor in endometrial cells. The same regions were found essential for the expression the mPGES-1 gene in osteoblasts and macrophage-like cells (78). IL-1β has also been reported to repress type II collagen gene in a chondrocyte cell line through Egr-1 (79) while it activates the Tissue Factor gene through Sp1 in Hela cells (80). Egr1 k/o mice have an infertile phenotype originating from lack of functional LH thus making it impossible to estimate its contribution on other aspects of endometrial function (81).

The proximal (1kb) promoters of cPLA2 and COX-2 genes contain several regions with putative cis-elements for NFκB (82). In human lens cells, NFκB proteins p50 and p65 interact with the ORE (osmotic response element) complex of the AKR1B1 promoter (75) corresponding precisely to the MSRR region mediating the effect of IL-1β in our endometrial cell lines. We believe that increased PGF2α production in response to IL-1β in endometrial cells is somewhat related to oxidative stress for which NFκB is considered a sensor (83). Interestingly, Egr-1 (also called Zif-268 or Krox-24) which is likely involved in the regulation of mPGES-1 is also identified as an oxidative stress-early inducible transcription factor when human lens epithelial cells are exposed to H2O2 (84). These data suggest that interactions between transcription factors and binding elements on the promoters of PG synthases, and especially ORE and ARE in the case of AKR1B1 and Egr1 for mPGES-1 provide a functional mean to achieve selective production of specific PGs.

Selective output of PGF2α and PGE2, local transport and catabolism

Prostaglandin transport: PGs predominate as charged anions and in spite of their lipid nature, diffuse poorly through plasma membranes. The mechanisms

responsible for the transport of newly synthesized PGs out of producing cells, either by simple diffusion (85), or a PG efflux transporter (86), are still in dispute. It has been shown that though anions cross the cell membrane by simple diffusion, the estimated flow rate would be too low for maintaining a biological function. Therefore, passive diffusion of PG into cells appears to be poor and is thought to be mediated by carriers (*Fig. 2*) such as prostaglandin transporter (PGT) (85). PGT was the first cloned PG transporter (87) and is a 12-transmembrane protein with a broad tissue expression. It is a functional uptake-carrier with high affinity for PGE2, PGF2α and PGD2 (85). PGT mRNA is expressed in reproductive tissues such as testis, ovary, and uterus (87, 88). PGT belongs to the super family of 12-transmembrane Organic Anion Transporting Polypeptide (OATP). It has been proposed that PGT mediates both the efflux of newly synthesised PGs to effect their biological actions through their cell surface receptors, and influx of PGs from the extra cellular milieu for their inactivation or action through specific nuclear receptors. PGT was found to be expressed preferentially in cell membranes of tissues capable of producing more PGs. Interestingly, PGT and cell surface PG receptors have comparable affinities for their substrates (85). Other members of the same transporter family such as CFTR are involved with efflux function and another member, MRP4 (86), has been proposed as a functional efflux carrier for PGs.

Our group has cloned bovine PGT (89) and characterized PGT as a key player in the action of PGs in the bovine reproductive system (89-92). Recently, we have shown the expression of PGT in the human endometrium (93). The co-expression of PGT and PGDH in a single cell type is believed to be associated with PG catabolism (94) whereas expression of PGT alone may favour transport of PGs across adjacent cells and tissues and mediate paracrine action of PGs (90-92). We have found that decidualization influenced the expression of hPGT and the distribution of PGF2a and PGE2 in the intra and extra-cellular compartments (59). We have also studied the expression of different members of the MRP and OATP transporters in the bovine endometrium and found that the former are preferentially expressed during the early part and the latter in the late part of the oestrous cycle. *In vitro*, the expression of both transporters was found to be modulated in parallel with PG biosynthesis in response to oxytocin and interferon.

PG catabolism: The first step for biological inactivation of PGs is effected by 15-PGDH (95) (96) and further catabolism by 15-13PGR generates the PGF2α and PGE2 metabolites PGFM and PGEM. There are two types of 15-PGDH, but only type I PGDH is associated with peripheral metabolism of PGs. We have found that endometrial 15-PGDH was modulated during the bovine oestrous cycle suggesting that local catabolism could exert a regulatory mechanism in the endometrium. In the mouse, it has been found that in preparation for parturition a peak of PGF2α is associated with an increase of COX-1 and PGF synthase and a decrease of 15-PGDH while cPLA2 and COX-2 are unaffected (97). In studies focusing on neoplasia pathways the COX-2-dependent production of PGE2 is associated with

tumorgenesis and this effect is exacerbated when 15-PGDH expression is reduced (98). In parallel, it has been shown that cytokines like IL-1β or TNF-α are able to reduce significantly 15-PGDH activity at the mRNA level and that the ratio of PGFM/ PGF2α is decreased significantly by steroid hormones (progesterone and dexamethasone) in trophoblast cells in culture (99). These results suggest that net PG production is regulated locally by a complex process involving both synthetic and catabolic enzymes. Preliminary results in the human endometrium indicate that the 15-PGDH protein is present in glandular epithelial cells during the early and mid secretory phases. Because treatment with PG biosynthesis blockers (NSAIDs) is efficient to treat many pathological conditions, we may assume that reduced peripheral catabolism may contribute to some of the disorders observed (100).

DIET AND PROSTAGLANDIN PRODUCTION

Free fatty acid composition and PGFnα and PGEn production

Prostaglandins are derived from eicosanoic (C20) fatty acids, and the trend to rely increasingly more on sn-6 PUFA yielding series 2 PGs potentially exacerbates adverse inflammatory and cardio-vascular conditions. In humans, the relative proportion of PUFA in body tissues depends on diet composition. In women, increased release of AA before menstruation is at the origin of increased PGF2α and PGE2 production considered responsible for dysmenorrhea (43, 101) and pre-menstrual syndrome (102). Recent circumstantial evidences suggest that altering even slightly (20%) the fat content of diet towards PUFA favouring series 1 or series 3 PGs can have a significant positive impact on health (*Fig. 6*). The biologically active series 2 prostaglandins are derived from arachidonic acid (AA) yielding the well known PGF2α and PGE2. Series 3 prostaglandins can be formed from eicosapentaenoic acid (EPA, 20:5n-3) the major fish oil omega 3, which gives rise to PGE3 and PGF3α. Series 1 prostaglandins are derived from dihomogamma linolenic acid (DGLA, 20:3n-6) which gives rise to PGH1, PGE1 and PGF1α. The biological activity of series 1 and 3 PGs vary among species and between tissues. Manipulation of the dietary intake of PUFAs in a variety of species and models was shown to impact on follicular development, ovulation, corpus luteum function, maternal recognition of pregnancy and parturition (100). It has been established that omega-3 can significantly reduce dysmenorrhea and PMS symptoms presumably through a competitive action of prostaglandins of the 3 series (102). The effects were accompanied by alteration of net output of urinary metabolites, but the exact mechanisms behind were not determined. It was reported that series 1 PGs are anti inflammatory, but long significant alteration of PUFA composition toward DGLA is difficult because of intrinsic conversion into AA the precursor of pro-inflammatory series 2 PGs. It must be stressed that even though many reports militate in favour of increased omega 3 consumption in the diet, recovery of full body function following complete deprivation of FFA is optimal with omega 6 FA such as AA (103).

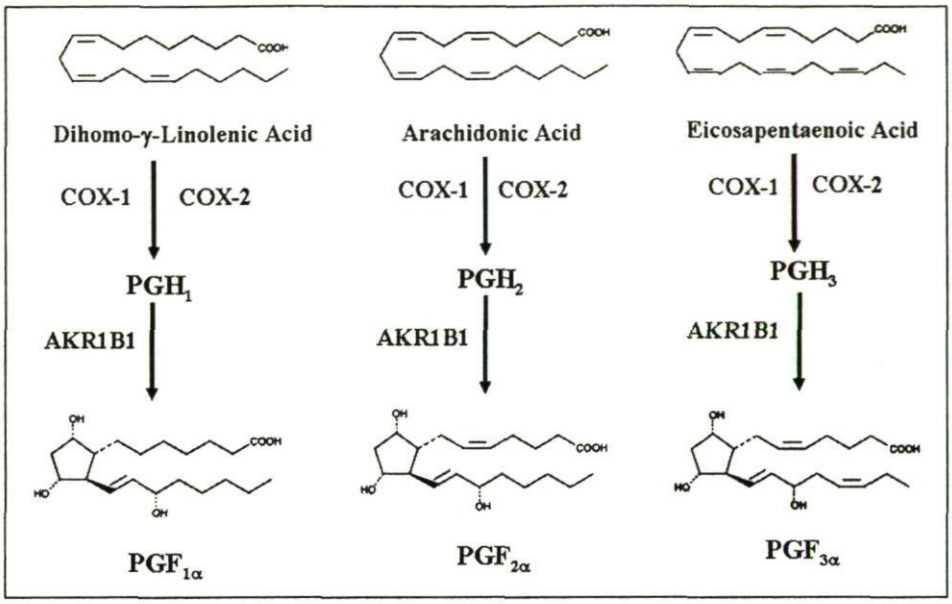

Fig. 6. Effect of dietary fatty acids on PG production. PGs can be generated from a variety of C-20 polyunsaturated fatty acids (PUFA) present in cell membranes in the form of phospholipids. These PUFA are poorly converted in mammals and must therefore be obtained from dietary intake. AA is the most abundant PUFA in the Western diet and is at the origin of the pro-inflammatory series 2 PGs. DGLA leads to the production of anti-inflammatory series 1 PGs whereas EPA, the omega 3 of fish origin leads to series 3 PGs. While there is no clear identification of the mechanisms responsible for the health benefits of omega 3 FFA, alteration in PG biosynthesis and signal transduction is a likely hypothesis.

Prostaglandin, ROS and antioxidants

There is a close association between the pathways generating reactive oxygen species (ROS) and PG biosynthesis. Reactive oxygen species (ROS) are generated by COXs during the process of PGs biosynthesis and can contribute directly to the regulation of reproduction (104) and initiation of menstruation (105). Interestingly, terminal PG synthases, especially aldose reductases can metabolize ROS. We have observed that AKR1B1 is increased by H2O2 whereas known antioxidants like curcumin vitamin E, N-acetyl cysteine and luteolin decreased *in vitro* PGs production in human endometrial cells treated with IL-1β.

PROSTAGLANDINS AND HUMAN PATHOLOGIES

Menstrual disorders

Menstrual disorders can affect women at any point in their childbearing years but are most prevalent during adolescence or the years just before menopause

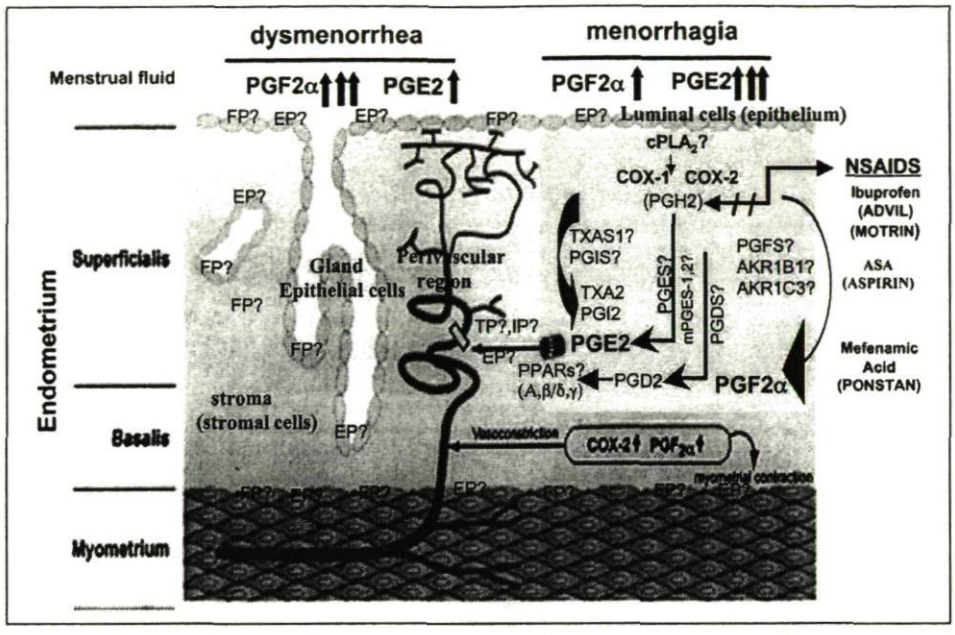

Fig. 7. Integrated views of PG biosynthetic and signalling pathways associated with menstrual disorders. The terminal synthases associated with the production of all PGs have been identified in the endometrium. However, PGE2 and PGF2α are produced in greater quantity and are the only members of the group with the chemical stability and penetration ability to generate the responses characteristic of menstrual disorders. Dysmenorrhea and menorrhagia are both associated with increased production of PGs, the former is preferentially associated with PGF2α inducing ischemia and pain and the latter with PGE2 exhibiting antithrombotic activity and hyperalgesia. The associations between specific biosynthetic enzymes, receptors and their precise sites of expression remain to be determined.

when sex hormones are shifting rapidly. The most common and debilitating menstrual disorders are dysmenorrhea or painful menstruation and menorrhagia or heavy menstrual bleeding (43). Locally released prostaglandins are considered as the primary mediators involved in the aberrant conditions and inhibition of their biosynthesis with non specific COX inhibitors (NSAIDs) is the primary therapeutic approach (*Fig 7*). Endometrial PGF2α is highest before the onset of menses. Vasoconstriction induced by PGF2α causes ischemia, accumulation of toxic catabolites, tissue necrosis, and desquamation. Myometrial contractility and abdominal discomfort (cramping) associated with menses are also caused by PGF2α. Increased concentrations of prostaglandins have been found in the endometrium and menstrual fluid of women who experience dysmenorrhea (106). The pain associated with uterine ischemia induced by PGF2α may be exacerbated by the hyper hyperalgesic effect of PGE2 on nerve terminals (107). The mechanisms behind dysfunctional uterine bleeding are not fully determined but PGs are again identified as important contributing factors. Increased PGE2 relative

to PGF2α levels in endometrium and menstrual fluid have been associated with menorrhagia (108), but altered PGI2 and TXA2 in the spiral arteries may also contribute significantly to this condition. In the case of menorrhagia, both NSAIDs and prothrombotic factors are used as therapeutic treatments. A recent review addresses the involvement of COX enzymes and prostaglandins in reproductive tract physiology and pathology (19). That review re-establishes the importance of COX expression in association with PGF2α and PGE2 in reproductive tract carcinoma, menorrhagia, dysmenorrhea and endometriosis through autocrine/paracrine mechanisms. Aberrations in uterine PG release or receptor expression were also demonstrated in association with premature labour (21).

Complications of metabolic disorders

Metabolic disorders result from complex interactions between genetic and environmental factors disturbing the normal immune and endocrine function. In turn, homeostasis is perturbed resulting in obesity, type 2 diabetes (T2D), increased cardiovascular morbidity and infertility. The contribution of prostaglandins (PGs) to the aetiology of metabolic disorders is poorly documented, but recent literature and our findings suggest that PGs may

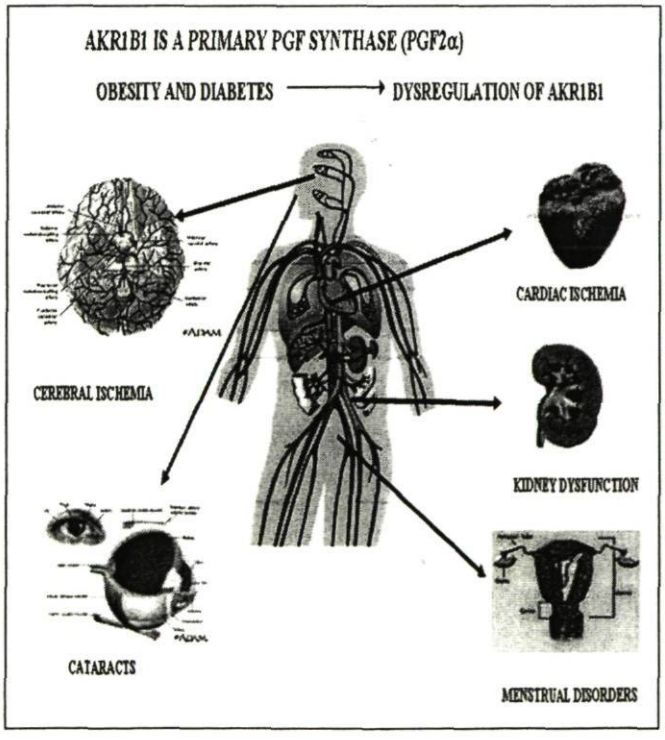

Fig. 8. PGFS activity and the aetiology of human pathologies. The generation of sorbitol from high levels of glucose observed in diabetes has been proposed as the primary cause of complications in many organs and systems. However, glucose at physiological concentrations is a poor substrate for AKR1B1. The demonstration of PGFS activity of AKR1B1 and increased expression in uterus, brain, heart and kidney in association with different pathologies, provide a strikingly coherent explanation of the effects observed in these organ and systems and warrant in depth investigation.

contribute to the development of associated complications. AKR1B1 and the polyol pathway responsible for conversion of glucose into sorbitol (*Fig. 5*) have been associated with several pathological conditions such as iron overload (109), alcoholic liver disease (110), heart failure (111), myocardial ischemia (112), vascular inflammation (113) and restenosis (114). Diabetes increases AKR1B1 expression and is associated with the impairment of NO-mediated vascular relaxation and decreased NO bioavailability, which may be a causative factor in other complications (115). However, recent studies have shown that AKR1B1 does not process glucose at physiological concentrations but is an excellent catalyst for the reduction of lipid peroxidation-derived aldehydes and their glutathione conjugates (116-121).

Interestingly, both AKR1B1 (122) and PGF2α (123) are increased in association with type 2 diabetes. This finding together with observation that this enzyme is expressed in adipose tissue, heart, skeletal muscle, eye and kidney (open an entire new field of investigation to study the potential contribution of PGF2α, its action relative to PGE2 and substrate interaction with steroids, glucose and their regulators in metabolic disorders such as obesity and diabetes. The newly described PGFS activity of AKR1B1 is also highly relevant to the documented association of this enzyme with cardiac (124-126) and cerebral ischemia (113, 127, 128) (*Fig. 8*).

Inhibitors of the aldose reductase activity of AKR1B1 were developed to correct aberrant responses associated with diabetes, but serious adverse side effects always occurred leading to their early withdrawal.

Table 1. Inhibitory effects and selectivity of some NSAIDs on Cox-1 and Cox-2 activity.

	Cox-1	Cox-2	Ratio Cox-2/Cox-1	Ref.
Drug	IC_{50} (μM)			
Aspirin	1.2	15.8	13.1	[130]
Flurbiprofen	0.1	0.4	4	[131]
Ibuprofen	3.3	37	11.4	[131]
Indomethacin	0.1	0.9	9	[131]
Mefenamic acid	25	2.9	0.12	[132]
Naproxen	1.1	36	32.7	[131]
Celecoxib (Celebrex*)	1.2	0.83	0.7	[132]
Nimesulide	12.5	0.4	0.03	[130]
NS-398	28.9	0.04	0.001	[130]
Rofecoxib (Vioxx*)	15	0.018	0.0012	[133]
Valdecoxib (Bextra*)	150	0.005	0.00003	[134]
Ketorolac	0.0014	0.14	100	[130]
SC-560	0.009	6.3	700	[135]

CONCLUSION

We have presented an integrated view of PGE2 and PGF2α biosynthesis, transport and signalling systems in the human and bovine endometrium. The net production of uterine PGs is governed by the anabolic enzymes COX-1, COX-2, PGES, PGFS and the catabolic enzyme PGDH (129) which are well conserved among species. Of particular interest is the identification of AKR1B1 as a functional PGFS. This activity appears as the missing link to understand the origin of diabetes complication affecting multiple tissues and systems and a promising pharmacological target to treat them (*Fig. 8*).

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Appendix 4

DIFFERENTIAL EXPRESSION AND REGULATION OF PROSTAGLANDIN TRANSPORTER (PGT) AND MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 4 (MRP4) IN THE BOVINE ENDOMETRIUM

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Differential Expression and Regulation of Prostaglandin Transporter (PGT) and Multidrug Resistance-associated Protein 4 (MRP4) in the Bovine Endometrium

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ABSTRACT

Prostaglandins (PGs) are involved in many female reproductive processes and their action is regulated at the biosynthesis, catabolism and signal transduction levels. Transmembrane transport of PGs is emerging as an additional site of regulation and PGT/*SLCO2A1*, a Solute Carrier Organic Anion family member was the first to be characterized. More recently MRP4/*ABCC4*, an ATP Binding Cassette (ABC) family member, was also shown to exert PG transport in transfected cells. We have proposed a role for PGT in the transport of $PGF_{2\alpha}$ in the utero-ovarian plexus during the luteolytic window in the bovine. In the present paper, we report for the first time the expression of ABC family members in the bovine endometrium during estrous cycle. We also show the modulation of PGT/*SLCO2A1* and MRP4/*ABCC4* genes expression in the bovine endometrial epithelial cell line (bEEL) in response to oxytocin (OT) or interferon-τ (IFNτ). In bovine endometrial samples, maximal expression of PGT /*SLCO2A1* and OATP-D /*SLCO3A1* transcripts was observed during the late luteal phase, whereas MDR1/ABCB1, MRP1/ABCC1 and MRP4/*ABCC4* expression was highest during early to mid luteal phase. Oxytocin (OT) induced upregulation of COX2 expression and $PGF_{2\alpha}$ production and this was correlated with an upregulation of MRP4/*ABCC4* but not PGT/*SLCO2A1* mRNA in bEEL cells. By contrast IFNτ increased PGT/*SLCO2A1* but not MRP4/*ABCC4* mRNA expression. Inhibition of PG production by NS-398 following OT treatment did not affect induction of ABCC4/*ABCC4* or COX2 expression in bEEL cells. Differential endometrial expression of the members of SLCO and ABC families during the bovine estrous cycle and contrasting regulation of PGT/*SLCO2A1* and MRP4/*ABCC4* by IFNτ and OT in bEEL cells suggest distinct temporal regulation of PG transit by these transporters.

INTRODUCTION

Prostaglandins (PGs) are key regulators of female reproductive function in mammals where they have been associated with ovulation, luteolysis, implantation, cervical ripening, parturition and postpartum involution of the uterus (Poyser 1995; Lim, Paria et al. 1997; Challis, Sloboda et al. 2002). Across species, the primary PGs involved in these processes are PGE_2 and $PGF_{2\alpha}$ (Poyser 1995). These PGs exert distinct and often opposite actions on many tissues and systems and are tightly regulated at the biosynthetic, catabolic, signal transduction and more recently at the selective transport levels (Fortier, Krishnaswamy et al. 2008). In ruminants, endometrial epithelial cells are the primary source of $PGF_{2\alpha}$ whereas stromal cells release more PGE_2 (Krishnaswamy, Danyod et al. 2009). In the reproductive system, $PGF_{2\alpha}$ is known to be a luteolytic and vasoconstrictive agent (McCracken, Custer et al. 1999) whereas PGE_2 is a luteoprotective and vasorelaxant mediator (Pratt, Butcher et al. 1979; Arosh, Banu et al. 2004). During the bovine estrous cycle, in absence of a viable embryo, $PGF_{2\alpha}$ is released by endometrial epithelial cells in response to oxytocin (OT) around day 16 to induce luteolysis. $PGF_{2\alpha}$ from the endometrium is transferred from the uterine to the ovarian compartment through a specialized structure called the utero-ovarian plexus (UOP) (Ginther 1981). We have shown that PGT/*SLCO2A1* which is responsible for this transfer is upregulated both in the endometrium and the UOP during the luteolytic window in the bovine (Banu, Arosh et al. 2003). We also showed an upregulation of COX2, a rate limiting biosynthetic enzyme for PGs during the same period (Arosh, Parent et al. 2002). By contrast, the trophoblastic interferon tau (IFNτ) acts as the embryonic signal repressing $PGF_{2\alpha}$ production by the endometrial epithelial cells, thus allowing maintenance of progesterone (P_4) production by the corpus luteum(Bazer, Burghardt et al. 2008). Using the bovine endometrial epithelial cell line bEEL, we have characterized a functional model to study the interaction between IFNτ and OT in the regulation of $PGF_{2\alpha}$ production (Krishnaswamy, Danyod et al. 2009).

PGs exist as organic anions and diffuse poorly through the plasma membrane in spite of their lipid nature. In the recent past, specialized carriers such as prostaglandin transporter (PGT/*SLCO2A1*) (Kanai, Lu et al. 1995; Schuster 1998) and Multidrug Resistance associated Protein 4 (MRP4/*ABCC4*) (Reid, Wielinga et al. 2003) have been shown to mediate the transport of PGs across the cell membranes. PGT/*SLCO2A1*, also called OATP2A1, belongs to the Solute Carrier Organic Anion Transporting Polypeptide family (OATP/*SLCO*) and is highly expressed

in several tissues including the uterus and ovary (Banu, Arosh et al. 2003; Banu, Arosh et al. 2005; Banu, Lee et al. 2008). It was initially considered that action of PGT was limited primarily to PG catabolism or inactivation (Schuster 1998); however, we have shown that it could also be associated with transport across adjacent tissues and physiological action (Banu, Arosh et al. 2003; Banu, Arosh et al. 2005). It has recently been shown that functional transporters are expressed during luteolysis in the ewe (Banu, Lee et al. 2008) as was shown for the cow (Banu, Arosh et al. 2003). PGT/*SLCO2A1* is believed to be involved in both uptake and release of PGs from the cells. Recently, MRP4/*ABCC4*, which belongs to class C of the ATP-Binding Cassette (ABCC) super family has also been shown to mediate the efflux of newly synthesized PGs from the cells (Reid, Wielinga et al. 2003; Russel, Koenderink et al. 2008). To our knowledge, no data is available on the expression pattern of these transporters in the female reproductive system.

Given the demonstrated importance of PGs and the potential regulatory function of their transport for effective luteolysis and recognition of pregnancy, we first evaluated the mRNA expression of different members of the ABC and SLCO families in the bovine endometrium and established their profile across the estrous cycle. Since IFNτ inhibits OT induced $PGF_{2\alpha}$ release from the endometrial epithelial cells in ruminants, we also investigated their effects on MRP4/*ABCC4* and PGT/*SLCO2A1* expression in the bEEL cells (Krishnaswamy, Danyod et al. 2009)

MATERIALS AND METHODS

Materials

The reagents were purchased from the following suppliers: Superscript III reverse transcriptase, DNA ladder, dithiothreitol, 5x first strand buffer, TRIzol and RPMI 1640 (without phenol) from Invitrogen Life Technologies Inc.(Burlington, ON, Canada). Random primer-pd(N)6, deoxy-NTPs, RNA Guard, rTaq DNA polymerase, PCR 10x buffer, from GE healthcare Canada (Baie d'Urfé, Qc, Canada). Plasmid pDrive (TA cloning kit), DNA purification kits and QuantiTect SYBR Green PCR Kit for quantitative real time PCR were from Qiagen (Mississauga, Ont, Canada) using LightCycler ® System (Roche Diagnostics, Laval, QC, Canada). Tissue culture plates from Sarstedt (St Leonard, QC, Canada); fetal bovine serum and antibiotics from Wisent Inc. (Montréal, QC, Canada). All oligonucleotide primers were chemically synthesized using

ABT 394 synthase (Perkin-Elmer, Foster City, CA). The other chemicals used were of molecular biology grade available from Laboratoire Mat or Fisher Biotech (Québec, QC, Canada). Oxytocin was from (Sigma-Aldrich, Oakville,ONT, Canada) and recombinant ovine IFNτ was a gift from Drs. F. W. Bazer and T. E. Spencer (Animal Biotechnology Laboratory, Texas A&M University, College Station, TX). NS-398 was from Cayman Chemicals, Ann Arbor, MI.

Preparation of endometrial tissues

Bovine uteri were collected at the local abattoir immediately after exsanguinations, placed on ice and brought to the laboratory within 1–1.5 h. The endometrial samples were classified based on the morphology of the ovaries as described previously (Arosh, Parent et al. 2002). The tissues were separated into 7 groups corresponding to 3 days period covering the entire estrous cycle and three representative tissues were used for each period. Endometrial strips were cut into small pieces, snap-frozen in liquid nitrogen, and stored at -80 C until used for analysis.

Cell culture

The bovine endometrial epithelial cell line (bEEL) was cultured as reported previously (Krishnaswamy, Danyod et al. 2009). Briefly, a frozen aliquot was grown in RPMI [Gibco-BRL (Invitrogen), Mississauga, Ontario, Canada] supplemented with 10% FBS and 1% streptopenicillin; confluent culture was trypsinized and seeded at $4X10^4$ cells per millilitre. Confluent cells were exposed overnight to 2% dextran-coated charcoal-treated FBS (DCC-FBS) and 1% penicillin-streptomycin before treatment with OT or IFNτ.

RT-PCR

Total cellular RNA was isolated from cells or endometrial tissues using TRIzol reagent according to the manufacturer's protocol. cDNA for each RNA sample was synthesized in 20-µL reactions using the SuperScript III First Strand synthesis system for reverse transcription- PCR (Invitrogen) following the manufacturer's protocol. First, RT-PCR was performed from the cDNAs generated from bEEL cells and endometrial tissues using specific primers for different members of SLCO or ABC gene families (Table1). RT-PCR reaction was done as follows: 3 µg of total RNA from the endometrial tissues was reverse transcribed and amplified with recombinant *Taq* polymerase (GE health Care, Canada). Amplification reaction was performed as follows: hot start at 95°C for 2 minutes and thermocycling is done as follows: 95°C

denaturation for 30 seconds, annealing at 58°C for 30 seconds and extension time at 72°C for 1minute during 35 cycles followed by 10 minutes at 72°C. Amplicons were resolved on 1.5% agarose gel; gel purified and cloned in pDrive vector (Qiagen). The identity of the cloned gene was validated by sequencing.

Quantitative PCR (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) reactions were performed using a Roche Light Cycler (Roche) and QuantiTect™ Syber® Green PCR kit (Qiagen). Samples from three independent experiments or different endometrial tissues were run in duplicate. Primers were designed in such a way that the amplified region spanned over intron–exon boundaries to avoid coamplification of genomic DNA (Table 1). Total RNA (3.0 μg) was reverse-transcribed by SuperScript™ III RT (Invitrogen) in a 20-μl reaction mixture according to the manufacturer's instructions. A RT-minus control (no enzyme) was included as a negative control. Two μl cDNA were used as the template in a final PCR reaction volume of 20 μl; a two μl aliquot of diluted cDNA 18s RNA (1 in10) was used to normalize the level of each sample analyzed. Thermocycling was initiated by a 15-min incubation at 95°C, followed by 40 cycles (MRP1/*ABCC1*, MRP4/*ABCC4*, OATPD/*SLCO3A1*), 42 cycles (MDR1/*ABCB1*), 45 cycles (COX2) and 50 cycles (PGT/*SLCO2A1*) at 95°C for 15 s; 55°C (MRP1/*ABCC1*, MRP4/*ABCC4*, MDR1/*ABCB1* and COX2), 60°C (OATPD/*SLCO3A1* and PGT/*SLCO2A1*) for 30 s; and 72°C for 20 s, and a single fluorescence reading was taken at the end of each cycle at 77°C (MRP4 and COX2), 78°C (MDR1/*ABCB1*), 80°C (MRP1/*ABCC1*), 82°C (OATPD/*SLCO3A1* and PGT/*SLCO2A1*). Each run was monitored with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. Comparative threshold cycle (C_t) values were determined by software using a fluorescence threshold automatically and further analysis was done. The amplified products were fractionated by agarose gel electrophoresis which showed single bands of predicted sizes for each sample and no product for the negative controls (water instead of cDNA). 18S RNA gene served as an internal control to normalize the expression of each gene.

Treatment of bEEL cells in culture

bEEL cells were treated with OT ($5x10^{-7}M$) or IFNτ ($20ngml^{-1}$) for 24 h to study the expression of MRP4 and PGT transcripts in relation with $PGF_{2\alpha}$ and PGE_2 accumulation in the culture medium in the presence or not of NS-398 (10nM), a selective inhibitor of COX2.

Enzyme Immunoassays (EIAs) of PGE_2 and $PGF_{2\alpha}$

Prostaglandins were assayed by competitive EIA using acetylcholinesterase –linked PG tracers (Cayman, Ann Arbor, MI, USA) as described previously (Asselin, Goff et al. 1996).

Statistical analysis

Data were analyzed by one way ANOVA with Fischer's PLSD as post-hoc test using Statview program. Data are presented as the Mean ± SEM (n=3).

Results

Identification of OATP and MRP gene family in the bovine endometrium

We first determined the endometrial expression profile of the members of the ABC and OATP families by RT-PCR. Within the ABC family members MRP1/*ABCC1*, MRP4/*ABCC4* and MDR1/*ABCB1* mRNAs were present (Fig.1) whereas MRP2/*ABCC2* was absent (result not shown). Bovine kidney served as a positive control for MRP2 to verify the quality of the primers. Of the OATP family members, PGT/*SLCO2A1*, OATPD/*SLCO3A1*, OATP2B1/*SLCO2B1* and OATP4C1/*SLCO4C1* transcripts were expressed in the endometrium (Fig.1). We detected the expression of OATP2B1/*SLCO2B1* in the tissue but not in the epithelial cell lines, suggesting that this transporter might be expressed only in a limited subtype of cells.

Expression profile of OATPs and MRPs across the estrous cycle

For the study of PG transporters gene expression in the bovine endometrium, we chose candidate genes presumed to transport PGs in other species (Mikkaichi, Suzuki et al. 2004; Dallas, Miller et al. 2006). By qRT-PCR analysis, we have found that PGT/*SLCO2A1* and OATPD/*SLCO1A3* of SLCO family are maximally expressed during the late luteal phase (Fig.2 A and B), whereas MDR1/*ABCB1*, MRP1/*ABCC1* and MRP4/*ABCC4* are predominantly expressed during early to mid luteal phase (Fig. 3 A, B and C) of the estrous

cycle. The PGT/*SLCO2A1* expression profile observed confirms our previously published results (Banu, Arosh et al. 2003). It is interesting to note that the pattern of expression is quite similar within the members of the same family but different between the families. Maximal expression occurs during early cycle for members of the ABC family and late cycle for OATPs.

Effects of OT and IFNτ on MRP4 and PGT expression in bEEL cells

Since the mRNA expression profiles were comparable within each of OATP and ABC family members, we chose PGT/*SLCO2A1* and MRP4/*ABCC4* to study the effect of OT or IFNτ in bEEL cells. Treatment of bEEL cells with IFNτ 20 $ngml^{-1}$ or OT $5X10^{-7}$ M for 24 hours showed contrasting responses for each treatment and transporter. Figure 4 illustrates that PGT/*SLCO2A1* mRNA was upregulated by IFNτ but not by OT, in contrast, MRP4/*ABCC4* mRNA was upregulated by OT but not by IFNτ (Fig. 4A and B). As expected for the concentrations used, OT strongly stimulated PG production while IFNτ did not. (Fig. 4 C and D).

Effects of COX2 inhibition on MRP4 and PGT expression

The specific COX2 inhibitor, NS-398 (10nM), practically abolished PG production (Fig. 5 D and E). As reported earlier, COX2 mRNA increased following OT $5X10^{-7}$ M treatment (Krishnaswamy, Danyod et al. 2009), but the same response was observed in the presence of NS-398, confirming that NS-398 inhibits COX2 activity but not transcription (Fig. 5A). Because OT stimulation of MRP4/*ABCC4* mRNA was not affected by NS-398, neither COX2 activity nor OT induced PG production is necessary for the regulation of MRP4/*ABCC4* expression (Fig.5B). Finally, we could also observe that NS-398 did not influence PGT/*SLCO2A1* mRNA expression (Fig. 5C).

Discussion

Mounting evidence suggests that PG transport is an additional check point in the regulation of PG action (Schuster 1998; Banu, Arosh et al. 2005). PGT, a member of the OATP family, and MRP4, an ABC protein are the best described membrane proteins shown to transport PGs (Kanai, Lu et al. 1995; Reid, Wielinga et al. 2003). Whether these transporters are the only ones involved in the transport of PGs is not clear because MRP4/*ABCC4* knockout mice exhibit no obvious reproductive abnormalities (Lin, Zhu et al. 2008). Since there are several members of OATP and MRP families that are expressed simultaneously, compensatory mechanisms may overcome specific deletions (Mikkaichi, Suzuki et al. 2004; Dallas, Miller et al. 2006). Since we were the first to propose a role for PGT/*SLCO2A1* in the bovine endometrium and reproductive function, we took a further initiative in investigating the expression of other OATP and ABC family members likely to transport PGs. In addition to PGT/*SLCO2A1* and MRP4/*ABCC4*, we detected OATP-D/*SLCO3A1*, MDR1/*ABCB1* and MRP1/*ABCC1* in the bovine endometrium (Fig1). MRP2/*ABCC2* was detected in the kidney but not in the endometrium. The differential expression profile of the two family members during the estrous cycle suggests that they might be under the regulation of steroid hormones, and could have complementary roles in the transport of their substrates. Progesterone (P_4) might be responsible for increased expression of some members of the ABC family during early to mid luteal phase because MDR1 another ABC member was shown to be upregulated by P_4 and repressed by estrogen (E_2) in mice endometrium (Schiengold 2006). By reverse analogy, OATPs might be either repressed by P_4 or stimulated by E_2, but this has to be investigated further.

We selected PGT/*SLCO2A1* and MRP4/*ABCC4* from OATP and ABC families respectively for further experiments *in vitro* as their role in PG transport is better documented (Schuster 1998; Reid, Wielinga et al. 2003). Since we have previously characterized PGT/*SLCO2A1* (Banu, Arosh et al. 2003), we used it as a positive control and focused mainly on MRP4/*ABCC4*, the newly identified PG transporter. We have used our newly characterized bEEL cells to investigate the modulation of these transporters in response to OT or IFNτ. IFNτ at 20ngml^{-1} did not generate any significant increase in PG production in bEEL cells (Fig. 4 C&D), in spite of a significant increase in COX2 levels (Krishnaswamy, Danyod et al. 2009). PGT/*SLCO2A1* expression was not affected by OT but significantly upregulated by

IFNτ (Fig. 4A), suggesting potential contribution of this transporter during recognition of pregnancy.

To investigate if MRP4/*ABCC4* upregulation in response to OT involved PG production, bEEL cells were treated with NS398 (10nM), a specific inhibitor of COX2 activity. NS398 completely inhibited OT induced PG production, but this inhibition had no effect on MRP4/*ABCC4* or COX2 expression (Fig.5 A and B). NS398 alone or in combination with OT did not affect PGT/*SLCO2A1* expression. Our results indicate that MRP4/*ABCC4* mRNA modulation by OT is independent of PG production. Co-regulation of COX2 and MRP4/*ABCC4* by OT do not require the production of PGs and may rely on the presence of a common transcription factor on their respective promoters. Our results on the expression and regulation of OATP and ABC family members during the bovine estrous cycle support the concept that transporters are important modulators of PG action and endometrial function. The distinct pattern of expression of the two family members and the selective response to OT and IFNτ adds some complexity but provides more precision to the regulation of endometrial function by PGs.

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Table and Figures

Table

Gene Symbol	Name	Sequence ID	RT-PCR Primers	qRT-PCR Primers
ABCC1	MRP1	NM_174223	5'GGAAATCGTCTCTACTCTCAGCA3'f(2048) 5'GGTAGACGTCTGAGTCGCAGTAT3'r(2368) (320bp)	5'GATCGTGTACTCCTCCAAGGAC3'f(822) 5'CTTCTGGGGACACTTGACAATC3'r(921)
ABCC2	MRP2	XM_599177	5'GCAGTCTGCGATAATGTTTCTCT3'f(1415) 5'TTATCAGAATTGCAATCATGTCG3'r(1691) (276bp)	
ABCC4	MRP4	XM_593336	5'CAGAGGCTTGGTTCTTGTTTCT3'f(2804) 5'TAATGAGTGCTGTCAGGTGCTT3'r(3196) (392bp)	5'ATATAGCCTAGATGGGCCTCTG3'f(3147) 5'GAACTTTTTCCAGCTCCTGTTC3'r(3248)
ABCB1	MDR1	XM_590317	5'AGTTCATTTGCTCCTGACTATGC3'f(3064) 5'CATTCAGCTGCTTTATTTCCTTG3'r(3384) (320bp)	5'ACTATGCCAAGGCCAAAGTG3'f(3080) 5'TAAATGCCACGTTTCCTTCC3'r(3202)
SLCO2A1	PGT	AY134618	5'TGTACATCTCCATCCTGTTTGC3'f(617) 5'GGGGAACCTTTTAATGAAATCC3'r(948) (331bp)	5'GTGGAGACGATGGGATTGAATA3'(1376) 5'AAGGAGATGAGGAAGATGGTTG3'(1571)
SLCO3A1	OATPD	BC123736	5'GACCCACCAGTACAAGTACGAAG3'(392) 5'AGGATTCCTATGTAGAGCGAGGA3'(658) (266bp)	5'CACAAGTAACCTGGACATCACC-3'(746) 5'GGGTCTCTCGTATTCTCTTTCG-3'(941)
SLCO2B1	OATP2B1	NM_174843	5'CGACTTTAACCCTGTCTGTGACT3'f(1748) 5'CACAGCCAGAGTCTTGTCTTCTT3'r(2051) (303bp)	
SLCO4C1	OATP4C1	XM_615216	5'TAACTGCCTGCGATCGTATTATT3'(1525)	

			5'CTAGAGATCGCTGCCTTTGATTA3'(1853) (328bp)	
PTGS2	COX-2	AF031698		5'CCAGAGCTCTTCCTCCTGTG3'(513) 5'GGCAAAGAATGCAAACATCA3'(673)
ACTB	□-ACTIN	AY141970	5'GACGACATGGAGAAGATCTGGCA3'(277) 5'GAGGATCTTCATGAGGTAGTCTGT3'(618) (341bp)	
	18SRNAr	DQ222453*		5'GTAACCCGTTGAACCCCATT3'(1579) 5'CCATCCAATCGGTAGTAGCG3'(1731)

*Sequence ID identical to human 18S RNAr

Legends:

Figure 1:

RT-PCR analysis of expression of OATP and ABC family members in the bovine endometrium. mRNA was extracted from a representative endometrial sample taken on day 11 of the estrous cycle and processed as described in material and methods. Amplified products were analyzed on 1.4% agarose gel and migrated at the predicted Mw using the1 kb DNA ladder as a marker in the first lane. The next three lanes represent MRP1, MRP4 and MDR1 belonging to ABC family followed by SLCO family members: PGT, OATPD, OATP2B1 and OATP4C1. The last lane represents β-actin.

Figure 2:

Real-time PCR (LightCycler) analysis of PGT/*SLCO2A1* and OATPD/*SLCO3A1* expression in the bovine endometrium during the estrous cycle. Total RNA was extracted from bovine endometrial tissues collected at different periods of the estrous cycle and was used for qRT-PCR analysis. Levels of expression (arbitrary units) were quantified using SYBR Green I. 18S

RNA as an internal standard. Amplification quality was validated by analysis of melting curves expressed as the first derivative of fluorescence over time –d(F1)/dT and agarose gel electrophoresis (not shown). The two methods showed a single peak or band at the expected temperature and size. Results are expressed as the mean ± SEM of PGT/*SLCO2A1* (A) or OATPD/*SLCO3A1* (B) relative to 18S mRNA expression levels. Columns with different superscripts are significantly different ($P < 0.05$).

Figure 3:

Real-time PCR (LightCycler) analysis of MDR1/*ABCB1*, MRP1/*ABCC1* and MRP4/*ABCC4* expression in the bovine endometrium during the estrous cycle. Samples were prepared and analyzed as described in figure 2. Results are expressed as the mean ± sem of MDR1/*ABCB1* (A) MRP1/*ABCC1* (B) or MRP4/*ABCC4* (C) relative to 18S mRNA expression levels. Columns with different superscripts are significantly different ($P < 0.05$).

Figure 4:

Effects of IFNτ and OT on PGT/*SLCO2A1* and MRP4/*ABCC4* expression in bEEL cells. Immortalized epithelial cells bEEL were grown to confluency and treated or not for 24h with IFNτ (20 ng/ml), or OT ($5x10^{-7}$M). The culture medium was recovered for analysis of $PGF_{2\alpha}$ (C) and PGE_2 (D) by EIA and the cell pellet used for mRNA extraction. MRP4/*ABCC4* (A) and PGT/*SLCO2A1* (B) mRNA levels were analyzed by qRT-PCR as described above. Results represent the mean ± SEM of 3 different experiments. Bars with different superscripts differ significantly ($P<0.05$).

Figure 5:

Effect of $PGF_{2\alpha}$ and PGE_2 production on MRP4/*ABCC4* and PGT/*SLCO2A1* expression and its regulation by OT in bEEL cells. bEEL cells were grown to confluency and treated or not for 24h with OT ($5x10^{-7}$M) in presence and absence of NS398 (10nM) to inhibit COX2 activity. The culture medium was recovered for analysis of $PGF_{2\alpha}$ (D) and PGE_2 (E) by EIA and the cell pellet used for mRNA extraction. COX2 (A) MRP4/*ABCC4* (B) and PGT/*SLCO2A1* (C) mRNA levels were analyzed by qRT-PCR as described above. Results

represent the mean ± SEM of 3 different experiments. Bars with different superscripts differ significantly ($P<0.05$).

Fig.1

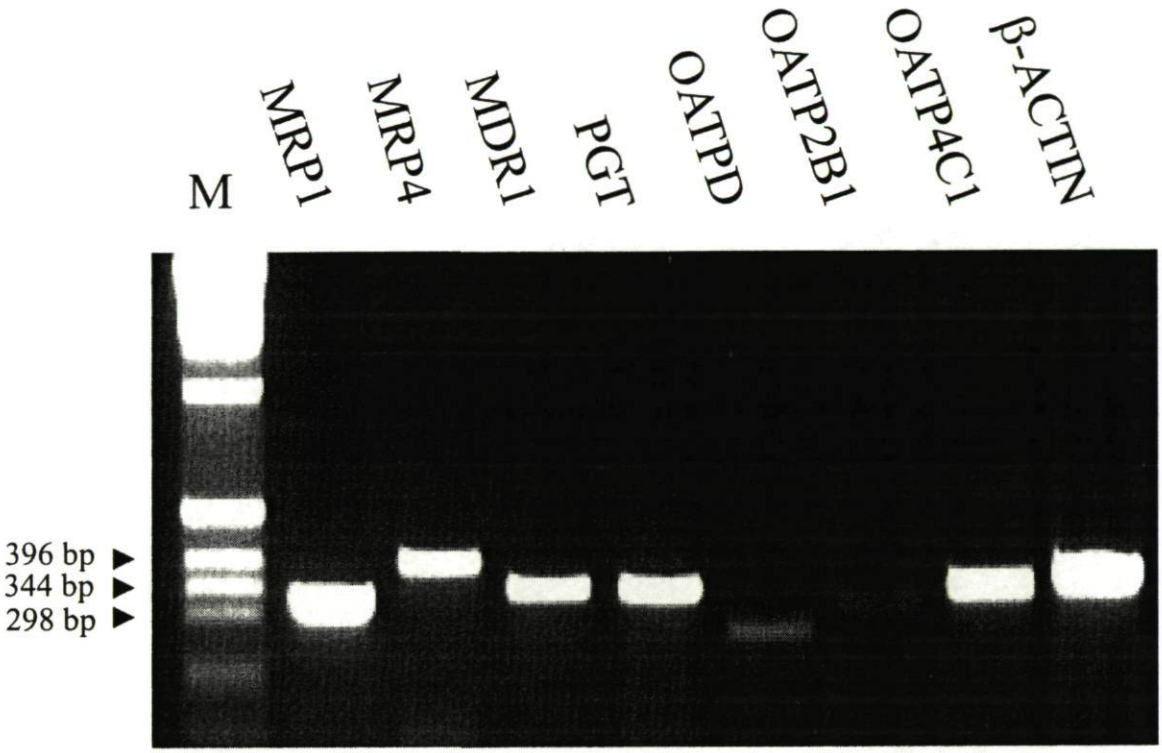
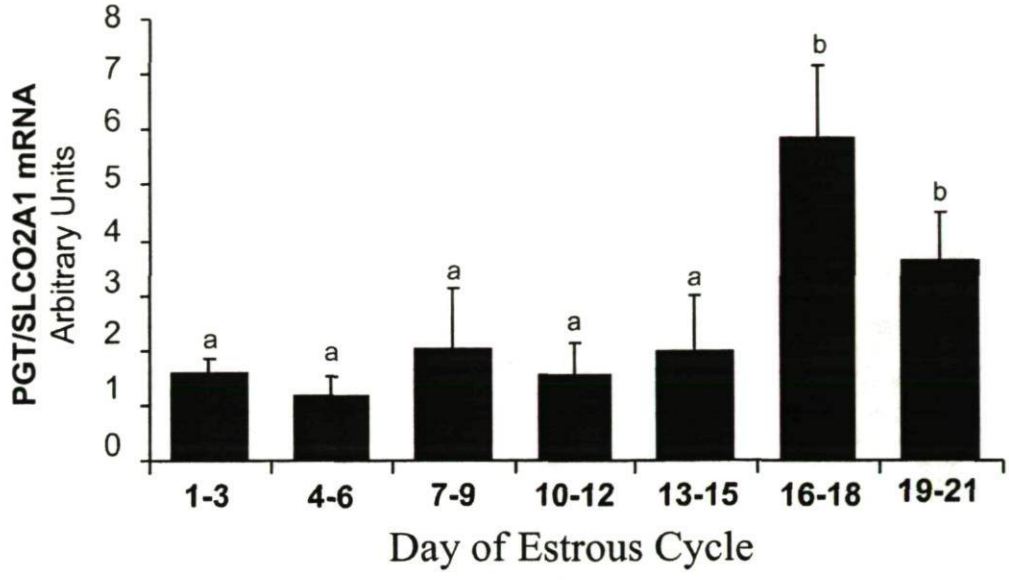


Figure 2

A


PGT/SLCO2A1 mRNA
Arbitrary Units
8
7
6
5
4
3
2
1
0
a
a
a
a
a
b
b
1-3
4-6
7-9
10-12
13-15
16-18
19-21
Day of Estrous Cycle

B

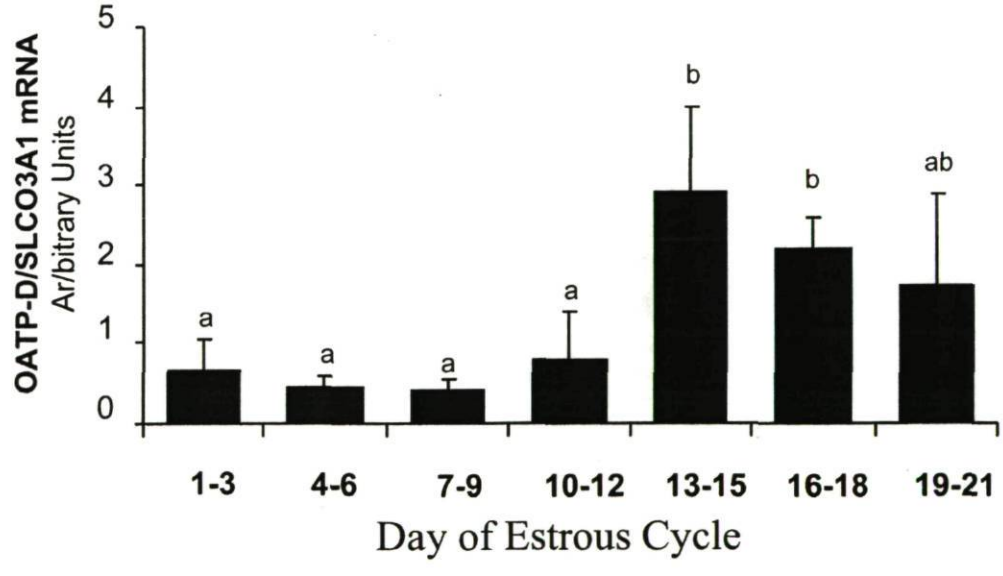
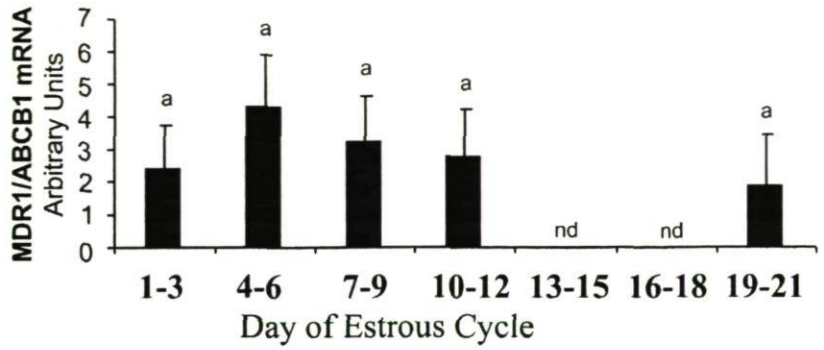
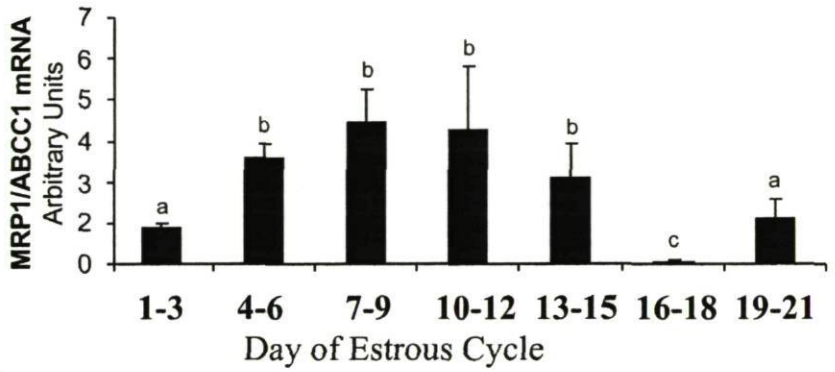

OATP-D/SLCO3A1 mRNA
Ar/bitrary Units
5
4
3
2
1
0
a
a
a
a
b
b
ab
1-3
4-6
7-9
10-12
13-15
16-18
19-21
Day of Estrous Cycle

Figure 3

A


MDR1/ABCB1 mRNA
Arbitrary Units
0
1
2
3
4
5
6
7
a
a
a
a
nd
nd
a
1-3
4-6
7-9
10-12
13-15
16-18
19-21
Day of Estrous Cycle

B


MRP1/ABCC1 mRNA
Arbitrary Units
0
1
2
3
4
5
6
7
a
b
b
b
b
c
a
1-3
4-6
7-9
10-12
13-15
16-18
19-21
Day of Estrous Cycle

C

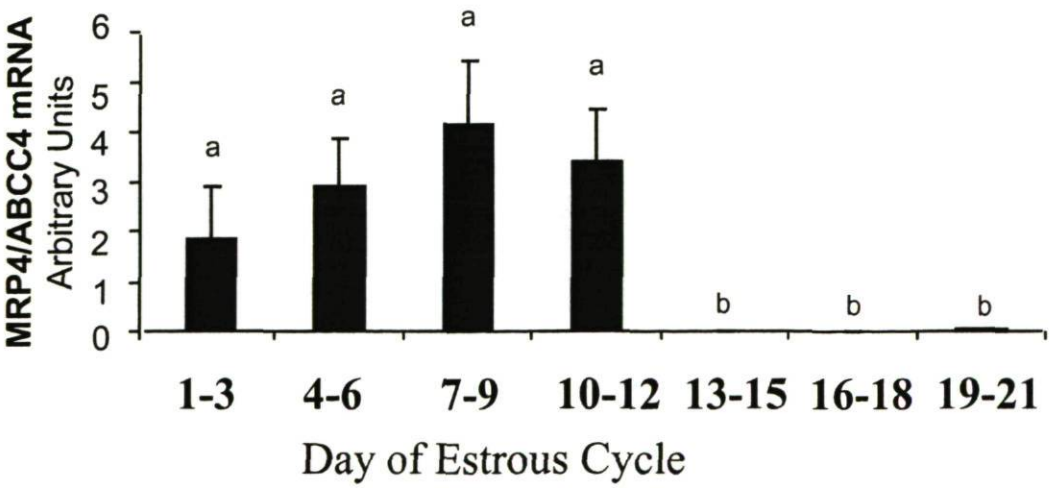
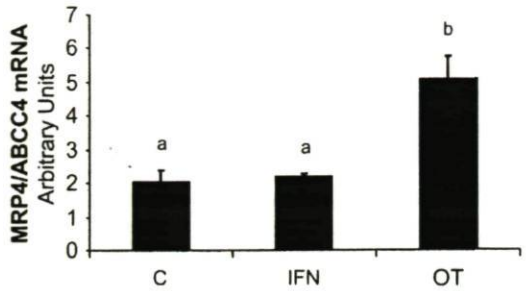

MRP4/ABCC4 mRNA
Arbitrary Units
0
1
2
3
4
5
6
a
a
a
a
b
b
b
1-3
4-6
7-9
10-12
13-15
16-18
19-21
Day of Estrous Cycle

Figure 4

A



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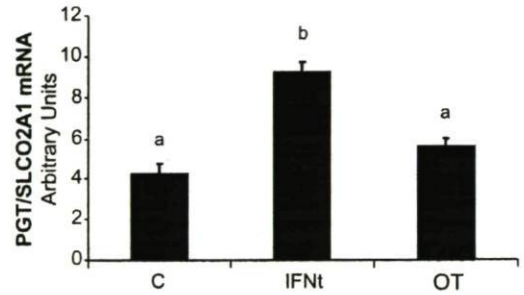

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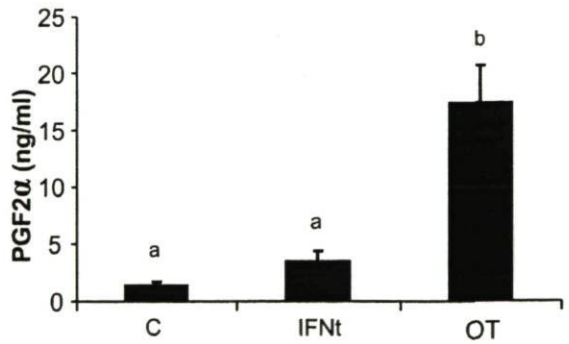

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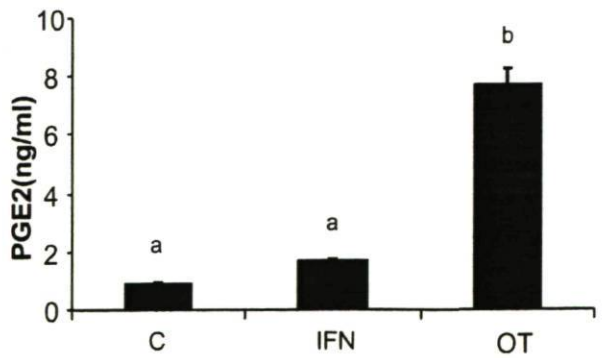
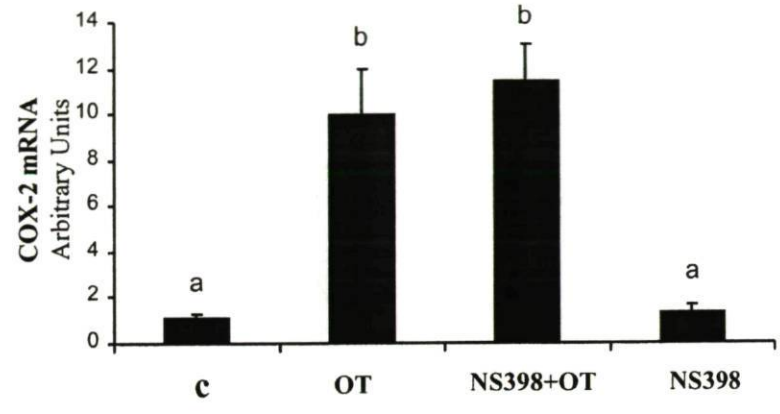
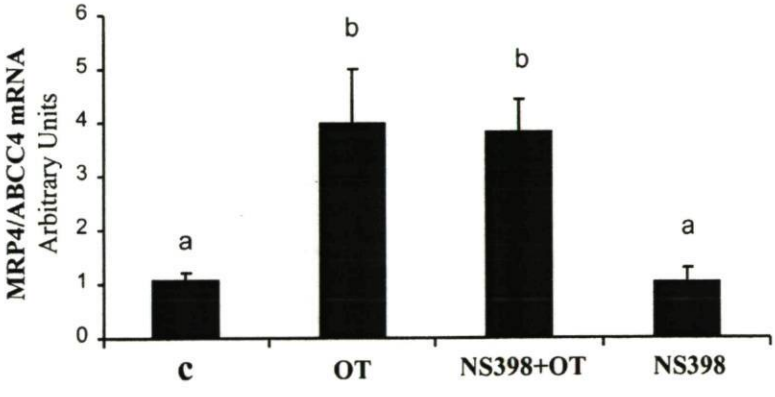


Figure 5

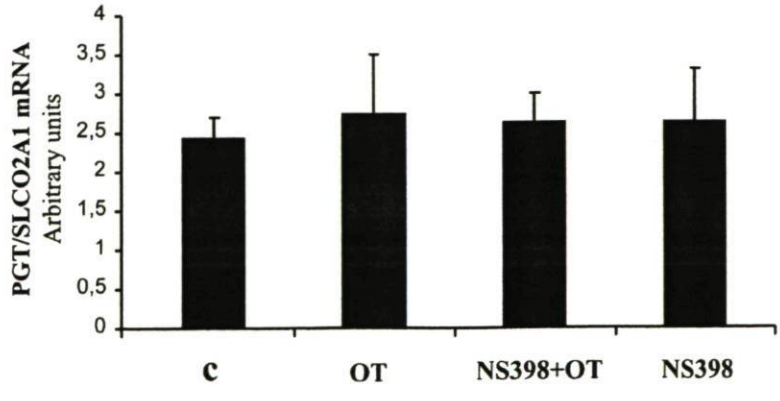
A


COX-2 mRNA
Arbitrary Units
14
12
10
8
6
4
2
0
a
b
b
a
c
OT
NS398+OT
NS398

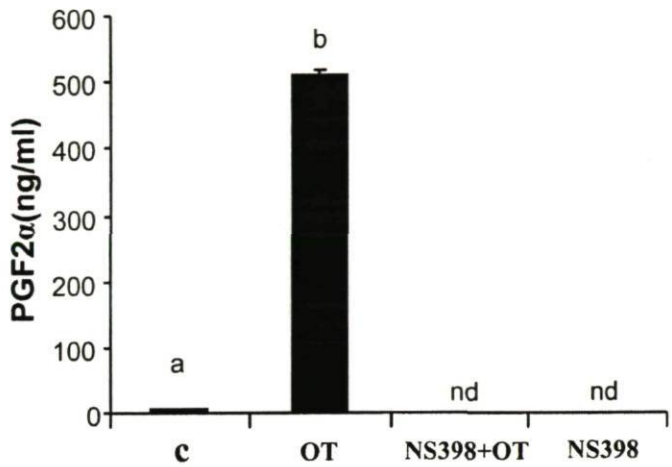
B


MRP4/ABCC4 mRNA
Arbitrary Units
6
5
4
3
2
1
0
a
b
b
a
c
OT
NS398+OT
NS398

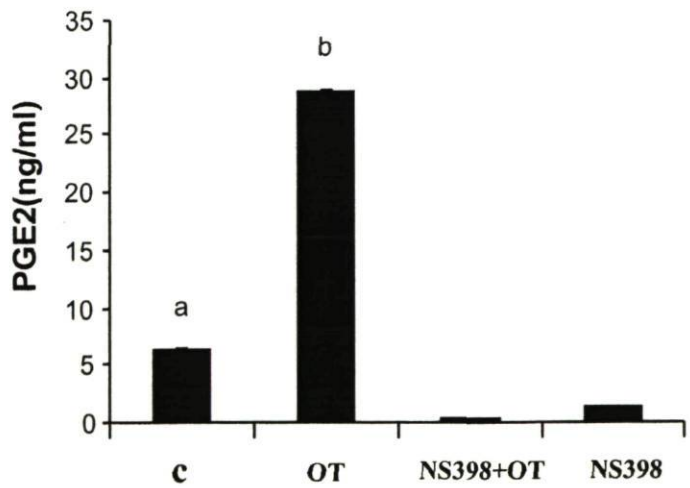
C


PGT/SLCO2A1 mRNA
Arbitrary units
4
3,5
3
2,5
2
1,5
1
0,5
0
c
OT
NS398+OT
NS398

E


600
500
400
300
200
100
0
PGF2α(ng/ml)
a
b
nd
nd
c
OT
NS398+OT
NS398

F


35
30
25
20
15
10
5
0
PGE2(ng/ml)
a
b
c
OT
NS398+OT
NS398