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A NOVEL INTEGRATED VIEW OF THE ROLE OF PROSTAGLANDINS FOR RECOGNITION AND ESTABLISHMENT OF PREGNANCY IN RUMINANTS

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SUMMARY

Prostaglandins are important regulators of fertility in most mammals. Prostaglandins $F_2\alpha$ ($PGF_2\alpha$) and E_2 (PGE_2) appear as the most active PGs in reproduction and often mediate opposite reactions at the time of establishment and termination of pregnancy. These prostaglandins and possibly also prostacyclin (PGI_2) are responsible for the regulation of luteolysis, implantation, and parturition. In cows, endometrial $PGF_2\alpha$ is the luteolytic hormone whereas PGE_2 may favour maternal recognition of pregnancy. During the bovine oestrous cycle, days 16-17 are considered as the "critical period" for either maternal recognition of pregnancy (MRP), in the presence of a viable embryo or luteolysis and return to a new oestrous cycle. In ruminants, trophoblastic interferon tau (IFNt) is known as the pregnancy recognition signal. We have found over the years that the mechanism of MRP is more complex than initially anticipated and involves regulation of PG action at the level of biosynthesis, catabolism, transport and receptors. For instance, in the bovine endometrium where the production of $PGF_2\alpha$ is abundant and tightly modulated, we have found that the PGFsynthase responsible for its production is not the enzyme previously identified in lung or liver but rather an aldose reductase with a new function, AKR1B5. This enzyme and others like PGH synthase 1 and 2 and PGE synthases are also tightly modulated to control the relative concentration of $PGF_2\alpha$ and PGE_2. Moreover, inspite of their lipid structure, PGs diffuse poorly through plasma membranes because they are charged negatively. We have identified a novel prostaglandin transporter bPGT that is expressed in the genital tract in a spatio-temporal manner in order to allow the transfer of PGs produced at critical periods of the oestrous cycle or during pregnancy. Finally, the action of individual PGs is regulated at the level of their receptors. A good understanding of PG action in the regulation of reproductive function requires that we consider all these factors. We intend to present here an integrated view of PG action on the function of the female genital tract at the critical period of recognition of pregnancy.

Keywords: Interferon tau; Luteolysis; Parturition; Pregnancy; Prostaglandin.

INTRODUCTION

A general overview of prostaglandins and reproduction

Prostaglandins (PGs) are 20-carbon unsaturated hydroxyl fatty acids mediating several physio-pathological events in mammalian species (1). In female reproduction, PGs play an important role in luteolysis, ovulation, fertilisation, implantation, pregnancy and parturition. Gene disruption studies of COX-1 and COX-2 enzymes, and FP and EP receptors have clearly indicated that PGE_2 and PGF_2 play important role during the reproductive process (Table 1) (2-6).

Table 1: Major phenotypes of mice deficient in prostanoid receptors and cyclooxygenases 1and 2 (knockout studies)

Disrupted Gene	Phenotypes
FP	Impaired parturition
EP1	Decreased aberrant foci formation to azoxymethane
EP2	Impaired ovulation, fertilisation, implantation, salt sensitive hypertension, impaired vasodepressor response, loss of bronchodilation, impaired osteoclastogenesis
EP3	Impaired febrile response, impaired duodenal secretion and mucosal integrity, enhanced vasodepressor response
EP4	Patent ductus arteriosis, impaired vasodepressor response, decreased inflammation, bone resorption
DP	Decreased allergic response
IP	Thrombotic tendency, decreased inflammatory swelling
TP	Bleeding tendency and resistance to thromboembolism
COX-1	Impaired parturition, still birth
COX-2	Impaired ovulation, fertilisation, implantation, decidualisation [Adapted from Narumiya and FitzGerald, 2001(5) Kobayashi and Narumiya, 2002 (6)]

Luteolysis and luteostasis

The corpus luteum (CL) is a transient ovarian endocrine gland formed from the ovulated follicle and responsible for progesterone (P_4) production during early or entire duration of pregnancy depending on species. Mechanisms involved in the control of the life span and function of CL have extensively been reviewed (Fig. 1) (7-9). In the cyclic cow, the CL goes through three phases, development, maintenance and regression (luteolysis). The process of luteolysis has been subdivided into functional luteolysis and structural luteolysis. In ruminants, endometrial production of prostaglandins (PGs) plays central role in the regulation of the oestrous cycle and establishment of pregnancy. PGF_2 and PGE_2 are the primary PGs produced in the uterine endometrium but their secretory pattern is different (10, 11). During the bovine oestrous cycle, days, 15-17 are the critical period for either luteolysis or pregnancy recognition (12). The bovine endometrium produces PGE_2 throughout the oestrous cycle, but its production is comparatively higher at mid and late luteal phases of the oestrous cycle (11). During luteolysis, endometrial PGF_2 is secreted in a series of pulses (4-5) within 24 h. The luteolytic process is governed by multiple events (Fig 2, 3). 1) P_4 autoregulates its own receptor (PR) in endometrium; 2) Estradiol from ovarian follicle acts through ER and prime the endometrium for the action of oxytocin (OT); 3) Decrease in

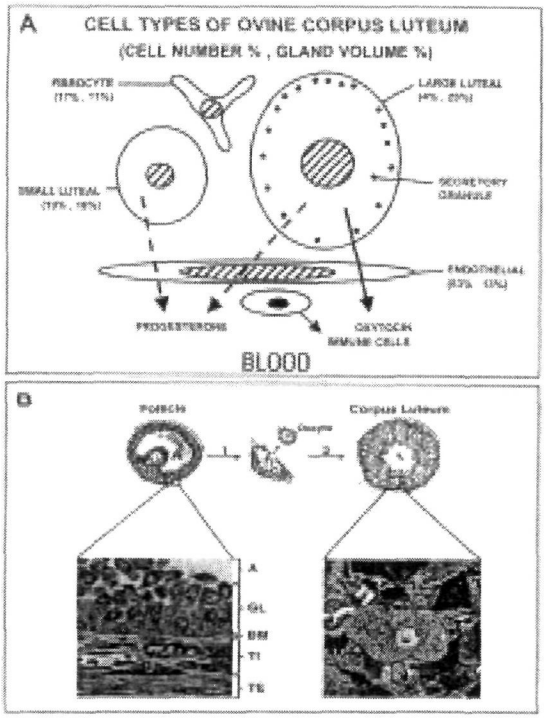

Figure 1 (A) Major cell types in ovine corpus luteum (CL). Relative number of cells (%), and relative cell volume (%) of each cell type in CL. **(B)** Development of a corpus luteum from a follicle. A-antrum follicle, GL-granulosal layer, BM- basement membrane, TI-theca interna, TE-theca externa, C-capillaries, SLC-small luteal cells, LLC-large luteal cells. Adapted from McCracken *et al.* (7) and Niswender *et al.* (8).

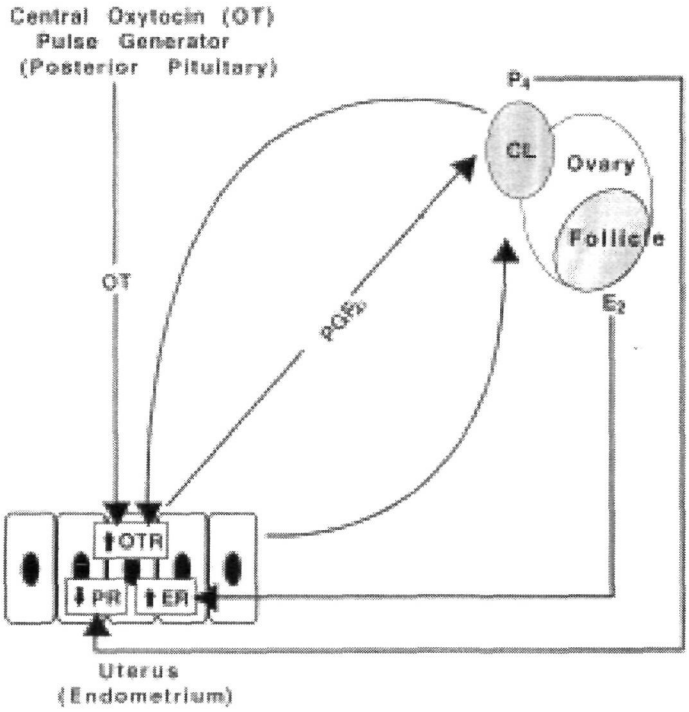

Figure 2 An existing hypothetical model of luteolysis in ruminants. At the time of luteolysis endometrial progesterone receptors (PR) are down-regulated and estradiol receptors (ER) are up-regulated. The increase in ER in turn increases oxytocin receptor (OTR). The timing of $PGF_{2\alpha}$ secretion is determined by the onset of OTR expression in endometrial epithelial cells. Oxytocin (OT) generated by the central oxytocin pulse generator (C.O.P) binds with OTR and initiates $PGF_{2\alpha}$ pulses and luteolytic process. A finite store of oxytocin in the CL may act to supplement OT signal and hence amplify $PGF_{2\alpha}$ pulses from uterus. Collectively, uterus is considered as the transducer that converts posterior oxytocin signals into pulses of $PGF_{2\alpha}$. Based on data from McCracken *et al.* (7), Niswender *et al.* (8) and Demmers *et al.* (69).

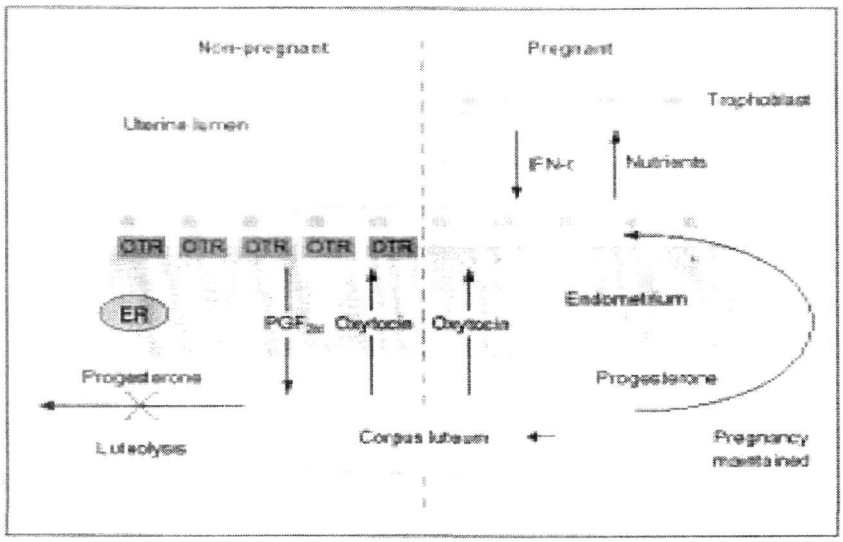

Figure 3 Hypothetical model explaining events occurring during transition from ovarian cyclicity to establishment of pregnancy in ruminants. In non-pregnant cows, luteolysis occurs involving complex events (see Fig 2). In pregnancy, the luteolytic mechanism is blocked by interferon tau (IFNt) secreted by the conceptus and rescuing the corpus luteum and maintaining the progesterone and pregnancy.

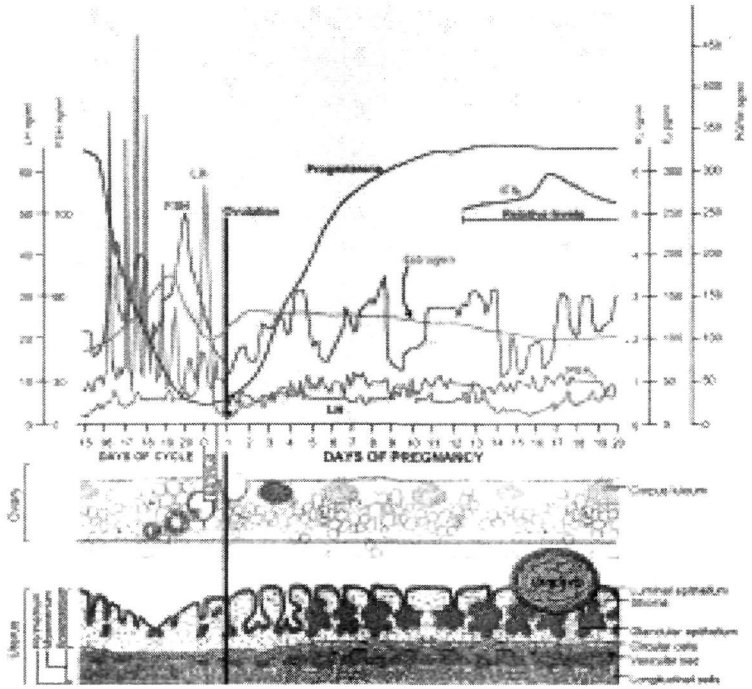

Figure 4 Integrated view of hormonal profiles, and associated ovarian and uterine changes during the process of recognition and establishment of pregnancy. Interferon tau (IFNt) secretion occurs during a precise time window. By blocking PGF_2 pulses (luteolysis) the conceptus ensures continued exposure of endometrium to high circulating concentration of progesterone which in turn maintains the secretory activity of the endometrial glands which provide the nutrients required for blastocyst growth, and other associated events. Data from Pineda *et al.* (20), Roberts *et al.* (21), Hafez *et al.* (22), Banks *et al.* (23), Demmers *et al.* (69), Bazer *et al.* (13) and Kindahl *et al.* (101).

PR and increase in ER, favour the expression of OTR in endometrium; 4) OT secreted by neurohypophysis and corpus luteum activates the endometrial OTR, and 5) Eventually leads to pulsatile secretion of luteolytic PGF_2 involving a positive feed back loop between the endometrium and the CL/ovary. All these events result in luteal regression and ceasing of P_4 production, leading to a new oestrous cycle (7-9, 13).

Recognition and establishment of pregnancy

During pregnancy, recognition of the presence of available embryo/conceptus prevents the pulsatile secretion of PGF_2. Bovine embryos enter into the uterus by day 4-5 (14). In ruminants, trophoblastic Interferon tau (IFNt) acts as the pregnancy recognition signal released by embryos to induce establishment of pregnancy. The changes in hormonal profile during recognition of pregnancy are shown in Fig 4. Events associated with PG production during transition from ovarian cyclicity to establishment of pregnancy in ruminants are schematically represented in Fig 3. Secretion of IFNt by the bovine blastocyst is highest between days 15 and 17, but observed for a period up to day 28. The exact mechanism by which IFNt inhibits the pulsatile secretory pattern of luteolytic PGF_2 is not clearly understood in bovine. In sheep, IFNt reduces the expression of uterine ER and OTR via paracrine mechanisms, thus preventing oxytocin-induced pulsatile secretion of PGF_2 thereby inhibiting luteolysis.

Inadequate reaction of the endometrium to IFNt or insufficient secretion of IFNt by the conceptus constitute major reasons for early embryonic losses and pregnancy failures. *In vitro* studies provided contrasting evidence on the action of

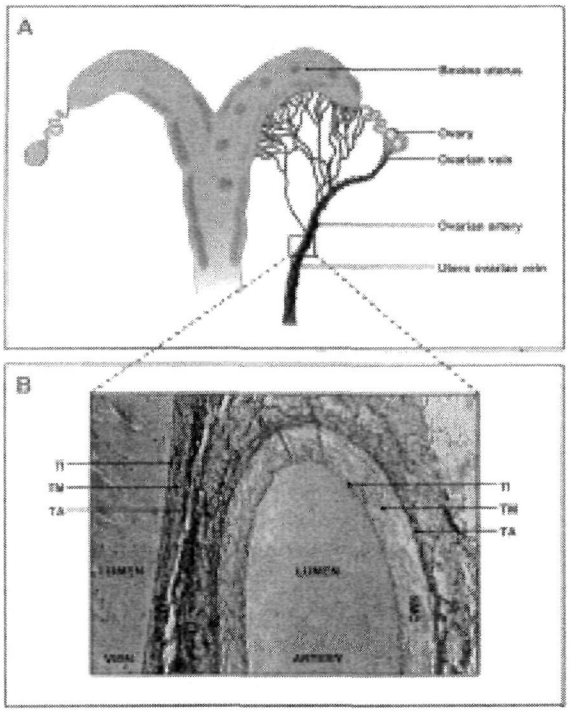

Figure 5 Prostaglandin transfer from the uterus to the ovarian compartment through utero-ovarian plexus (UOP) in ruminants. (A) Bovine uterus with ovary and arterial- venous system. (B) Cross section of UOP. TI-Tunica Intima, TM- Tunica Media, TA-Tunica Adventitia, SMC-Vascular smooth muscle cells. Adapted from Ginther *et al.*(25, 26).

IFNt in endometrium. IFNt was reported to decrease COX-2 expression and PGF_2 production (15) or to increase COX-2 expression and PGE_2 production (16). Other studies have documented that IFNt increases COX-2 expression and PGI_2 production in myometrium (17). Very recent evidence suggests that pregnancy upregulates COX-2 expression in the bovine and ovine endometrium *in vivo* (18, 19). High levels of PGE_2 have been found in the uterine vein during early pregnancy in the sheep (14). The exact role of PGE_2 in bovine endometrium is not fully elucidated. Therefore, it is important to explore how IFNt interacts with endometrial epithelial and stromal cells and controls the endometrial production of PGE_2 and PGF_2 during the process of recognition of pregnancy.

Intrauterine infusion of IFNt abrogates the development of the luteolytic mechanisms and extends the inter-oestrus interval and life span of the CL in sheep and cattle (13). But IFNt is not detectable in uterine venous or lymphatic drainage. It has been proposed in the 1970s that conceptus or uterine secretory products, most probably PGs, were released during early pregnancy to exert a luteoprotective effect in ruminants (20-23). PGs secreted from different uterine compartments are transported towards the vascular system. The endometrial PGF_2 and/or PGE_2 are transferred from the uterine to the ovarian compartment/ corpus luteum through the utero-ovarian plexus (UOP), a unique structure where the ovarian artery is more convoluted and coiled around the uterine vein at this specialised site of PG transfer (Fig 5A and B) (24-26). Surgical manipulation of UOP have shown that it allowed the transfer of secretory product(s) from the gravid uterus to rescue the CL at the time of establishment of pregnancy in cattle (27, 28). Several lines of evidence indicate that intrauterine administration of PGE_2 protects the CL from spontaneous as well as induced luteolysis (29-32). Recent studies suggest that endometrial PGs increase the auto amplification of luteal PGs production in ruminants(33-36). Treatment with PGE_2 stimulates luteal P_4 secretion both *in vivo* and *in vitro* in ruminants, a positive feedback loop between luteal PGE_2 and P_4 has been demonstrated (37-39). Further, treatment of indomethacin altered the luteal PGs and P_4 production and regression of CL in ewes (40). Very recently it has been proposed that endometrial PGF_2 initiates the functional luteolysis and luteal PGs contribute in structural luteolysis (33, 34). Accumulating evidence suggests a basic role for intraluteal PGF_2 and PGE_2 in luteal regression and maintenance in ruminants (7-9, 34). However, the underlying mechanisms are not known. Expression and regulation of FP have been extensively studied in luteal steroidogenic cells, but information pertaining to EP is largely unknown (41-43). COX-1 and-2 mRNAs are expressed in luteal steroidogenic cells (35, 36). PGF_2 treatment increases COX-2 expression in luteal cells (35). Clearly, the current dogma describing the return to a new oestrous cycle or establishment of pregnancy exclusively through the regulation of $PGF_2\alpha$ is overly simplistic and can not be supported if we take into account new data from our laboratory and other groups.

Prostaglandin biosynthesis and metabolism

Prostaglandins and other eicosanoids are produced from arachidonic acid (AA), an essential fatty acid stored in membrane phospholipids and liberated by $cPLA_2$. Cyclooxygenases 1 and 2 (COX-1 and COX-2) also known as prostaglandin endoperoxide H synthases 1 and 2 (PGHS-1 and PGHS-2) convert AA into PGH_2 and are the rate limiting enzymes in PGs biosynthesis. PGH_2 is then converted into different primary PGs including PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TXA_2 by cell-specific isomerases and synthases such as PGES, PGFS, PGDS, PGIS,

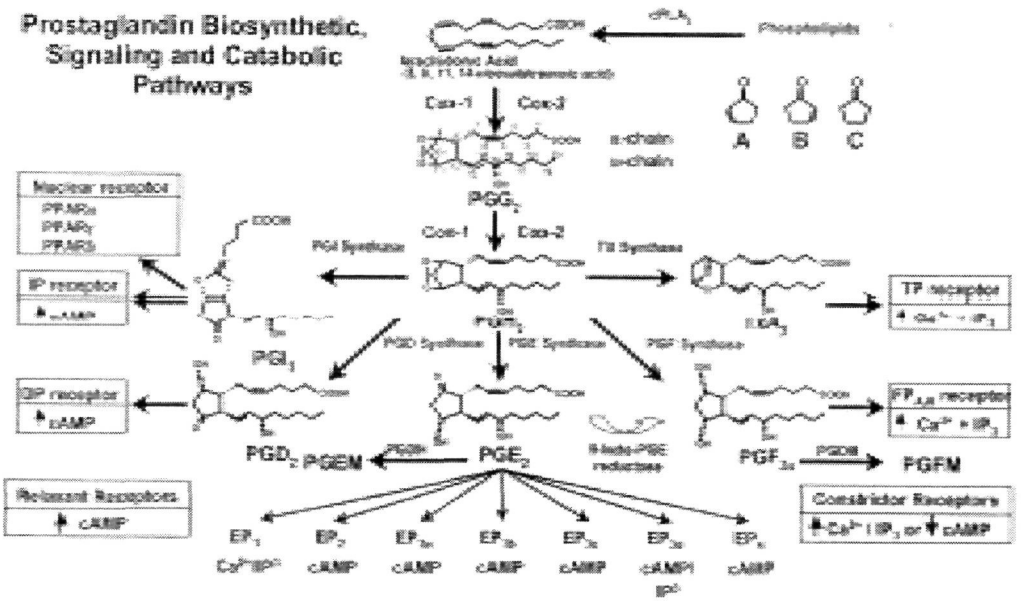

Figure 6 Prostaglandin biosynthetic, catabolic and signalling pathways. Based on data from Smith *et al.* (1, 44), Thoren *et al.* (100), Narumiya *et al.* (4), Coleman *et al.*(55) and Tai *et al.* (52).

TXAS, respectively. COX-1 is a constitutive enzyme important for housekeeping functions whereas COX-2 is an inducible enzyme involved in various physiological and pathological processes. The expression and regulation of COX-1 and COX-2 are tissue and species- specific (1, 44). Current evidence suggests that there are three forms of PGES, cytosolic PGES (cPGES) and membrane-bound PGES (mPGES)-1 and- 2. mPGES-1 is highly inducible by contrast mPGES-2 and cPGES are constitutively expressed in various cells and tissues. cPGES, mPGES-1 and mPGES-2 are respectively coupled with COX-1, COX-2, and both COXs, for the production of PGE_2. Several forms of PGFS were identified (50). Recently, we clearly described the characteristics of various PGFS isoforms in relation with PGF_2 production. We found that aldoketoreductase 1B5 (AKR1B5) is the most likely PGFS involved in the production of PGF_2 in bovine endometrium at the time of luteolysis (45). In some tissues, co-expression of COX-2 and PGES and/or PGFS has been demonstrated. The PG biosynthetic pathways are depicted in Fig 6 (46-51). PGs are primarily metabolised by the initial oxidation of the 15(S)-hydroxyl group catalysed by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). This enzyme is ubiquitously expressed in mammalian tissues. Two types of 15-PGDHs have been identified (52,53). The presence of PGDH has been reported in sheep CL (54).

Prostaglandin signalling

PGs exert their biological effects mainly through G-protein coupled rhodopsin type receptors designated EP, FP, DP, IP and TP respectively for PGE_2, PGF_2, PGD_2, PGI_2 and TXA_2. In addition, there are several splice variants of the EP3 (A-D), FP (A, B), and TP (A, B) receptors, which differ only in their C-terminal tails. Among the eight types and subtypes, the IP, DP, EP2, and EP4 receptors are coupled to adenylate cyclase and generate cAMP activating the PKA

signalling pathway, and have been termed "relaxant" receptors. A second group, TP, FP, and EP1 receptors are coupled to phospholipase C generating two second messengers, inositol triphosphate (IP_3) involved in the liberation of intracellular calcium (Ca^{++}) and diacyl glycerol (DAG), an activator of protein kinase C (PKC), and constitute a "contractile" receptor group. Finally, EP3 has a wide range of action from inhibition of cAMP production to increases in Ca^{++} and IP_3 and is referred to as the "inhibitory" receptor (4-6, 55). Peroxisome Proliferators-Activated Receptors (PPAR) have been proposed as nuclear receptors for PGD_2 and PGI_2 (56). Recently, EP2 and EP4 have been identified in the nuclear envelope suggesting the presence of functional nuclear receptors for PGE_2 (57). However, limited information is available on the putative actions of nuclear receptors. PGs signalling pathways are shown in Fig. 6 (57).

Prostaglandin transport

PGs predominate as charged anions and diffuse poorly through plasma membranes inspite of their lipid nature. The transfer of PGs through plasma membranes is poorly understood with proposed mechanisms starting from simple diffusion, passive transport, active transport, counter current to carrier-mediated transport. 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 (58). Recently, a novel prostaglandin transporter (PGT) was identified in rat, mouse and human. 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 in cell membranes of those capable of producing more PGs. Interestingly, PGT and cell surface PG receptors have comparable affinities for their substrates (59,60).

Overall, the available information converges to indicate that PGE_2 is an important mediator of diverse functions during the oestrous cycle and at the time of establishment, and maintenance of pregnancy in cattle. The available evidence suggests that selective production, transport and signalling of endometrial and luteal PGE_2 and $PGF_{2\alpha}$ are key factors governing the uterine and corpus luteum functions. To our knowledge, there is no integrated information available on these systems in bovine endometrium, myometrium and corpus luteum during the oestrous cycle and pregnancy in cattle.

Objectives

General

To determine the conditions necessary to effect selective action of PGE_2 or PGF_2

Specific

1) To study the expression of COX-1, COX-2, in cyclic and pregnant uterus. 2) To identify specific PGE and PGF synthases expressed in the endometrium. 3) To study the expression of PG receptors EP2, EP3, EP4 and FP in cyclic and pregnant uterus. 4) To study the mechanism of cellular transport of prostaglandins in bovine uterus. 5) To study the effect of IFNt on PG biosynthetic, transporting and signalling cascades during maternal recognition of pregnancy.

MATERIALS AND METHODS

The numerous reagents necessary for this study were purchased from the following suppliers : Superscript II RT, DNA and RNA ladders, dithiothreitol, T4 kinase, 5X forward reaction and first strand buffers and TRIzol (Invitrogen Life Technologies Inc, Burlington, ON); Random primer-pd(N)6,

dNTPs, RNA guard, rTaq DNA polymerase, PCR 10X buffer and Ready-To-Go DNA labelling kit (Amersham Pharmacia Biotech Montreal, PQ); prestained protein markers (New England Biolabs Inc, Mississauga, ON); Bright Star-plus nylon membrane and UltraHyb (Ambion Inc, Austin TX); Trans-Blot nitro-cellulose membrane (Bio-RAD Laboratories, Herculus,CA); [^{32}P] ATP and [^{32}P] dCTP (Perkin-Elmer life sciences, Markham, ON); Renaissance (Life Science Products Inc, Boston, NY); BioMax film (Eastman Kodak Corp, New York, NY); plasmid and mRNA purification kits (QIAGEN Inc, Mississauga, ON); Mayer's hematoxylin (Sigma- Aldrich Canada Ltd, Oakville, ON); LightCycler FasterStart DNA Master SYBR Green I mix and $MgCl_2$ (Roche Diagnostics, Laval, QC, Canada); Vectastain Elite ABC kit (Vector Laboratories Inc, Burlingame, CA). All oligonucleotide primers were chemically synthesised using ABT 394 synthase (Perkin-Elmer, Foster city, CA). The other chemicals used were molecular biological grade available from Laboratoire Mat or Fisher Biotech (Quebec, QC). Goat anti-rabbit biotinilated immunoglobulin (DAKO diagnostics of Canada Inc, Mississauga, ON); goat anti-rabbit or mouse IgG conjugated with horse radish peroxidase (Jackson Immunoresearch Laboratories, PA): monoclonal anti mouse ß actin antibody and anti human rabbit EP2 polyclonal antibody (Cayman Chemicals, Ann Arbor, MI) were used in this study. Antibodies against bovine PGES (61) PGFS (45), PGDH (53) PGT (62) were produced in our laboratories as described previously. Anti sheep COX-1 and COX-2 antibodies were donated by Dr. Stacia Kargman, Merk-Frost, Montreal, Canada. Anti bovine PGES (61) was generous gift from Dr. Jean Sirois, CRRA, University of Montreal, Canada. Recombinant ovine IFNt was kindly donated by Dr. F.W. Bazer and Dr. T.E. Spencer, Animal Biotechnology Laboratory, Texas A & M University.

Extraction of mRNA and protein from bovine uteri

Bovine uteri at different days of the oestrous cycle were collected at a local abattoir. Days of the oestrous cycle were determined by utero-ovarian morphology (63). Uteri were classified into 7 groups as days 1-3 (n=4), 4-6 (n=3), 7-9 (n=3), 10-12 (n=3), 13-15 (n=6), 16-18 (n=7), and 19-21 (n=5). Uterine horns were separated into endometrium and myometrium compartments.

Cross sections of tissues were prepared and processed for immunohistochemistry as described below. Tissues were cut into small pieces, snap frozen in liquid nitrogen and stored at -80°C until used. Total RNA was isolated using TRIzol according to the manufacturer's protocol. Total proteins were extracted and quantified (63). Expression of COX-1, COX-2 and PGT mRNA was studied using Northern blot. Expression of COX-1, COX-2, PGES (mPGES-1 102), PGFS (AKR1B5), PGDH, PGT and EP2 proteins were studied by Western blot as described previously (45, 53, 63, 62). EP2, EP3 and FP mRNAs were studied using real time quantitative RT-PCR (Light Cycler). Cellular localisation of PGFS, PGES and EP2 proteins were performed by immunohistochemistry.

Influence of IFNt *in vivo* on expression of prostaglandin systems in uterine tissues

Animal management and treatment protocols were the same as described previously (64, 65) Briefly, beef heifers (19±1 days cycle length) were used. Oestrus was synchronised with double PGF_2 a regimen at 11 days interval. One regular oestrous cycle was observed. Again oestrus was synchronised with single injection of PGF_2 on day 12 of the oestrous cycle. The induced oestrus was considered as day 0. On day 14, the animals were divided into control (n=3) and treatment (n=3) groups. Recombinant ovine IFNt (0.25 mg/dose = biological activity of $5x10^7$ antiviral unit / day) and 0.1% BSA in saline were infused intrauterine in treatment and control groups, respectively. A total of 4 doses at 12 h intervals were given. On day 16, all animals were slaughtered and reproductive tracts were collected. Uterine horns were identified as ipsiorcontralateral to the CL. The genital tracts were separated to isolate the endometrium, myometrium (only from ipsi-lateral horn), CL and UOP were separated (62, 63).

Quantitative RT-PCR (LightCycler)

LightCycler reaction using SYBR Green I (Roche Applied Science) and quantification was performed as we described (66). In brief, total RNA (1μg) was reverse transcribed using random primer and Superscript II RT. Sets of specific primers were deduced from the known sequences of bovine EP2, EP3, EP4 and FP (Table 1). LightCycler reactions were performed in a total volume of 20 μl in micro capillary tubes according to the manufacturer's instructions. Recombinant plasmids containing specific inserts of EP2, EP3, and FP, and the purified PCR product for GAPDH were used as templates. The plasmid DNAs or PCR products were quantified and serially diluted from 100 pg to 0.01pg /2 ml. Each reaction mixture contained 2 μl of cDNAs, 2 μl FasterStart DNA Master SYBR Green I mix, 2 μl of sense and antisense primers each (0.5μM), 1.6 ml of 25μM MgCl2 and 10.4 ml of PCR grade H_2O. The LightCycler programs for each gene were as follows: denaturation (95°C /10 min); PCR amplification and quantification (95 °C /10 sec, 60 °C /5 sec, 72°C / 20 sec) with single fluorescence measurement at specific temperature (acquisition) for 5 sec repeated for 30-50 cycles depending on the gene studied; a melting program (70-95 °C at rate of 0.1°C / sec with continuous fluorescence measurement), and finally a cooling step to 40 °C. At all steps the transition temperature was 20 °C /sec.

Northern blot analysis

Northern blotting and hybridisation were performed as we described (63). Briefly, total RNA (~20 μg) was loaded in each lane and electrophoresed on 1.2 % formaldehyde agarose gel. RNA was transferred overnight onto a nylon membrane in 10X SSC. The cDNA probes for COX-1, COX-2 and PGT were labelled with [a ^{32}P] dCTP (3000 Ci/ mmol) using Ready-To -Go DNA labelling kit. Prehybridisation for 2-3 h and hybridisation for overnight were carried out at 45°C using UltraHyb. The blots were stripped off by boiling in 1% SDS for 30 min and rehybridised with g-^{32}P(84) labelled oligoprobe specific to 18S ribosomal RNA. The blots were exposed to BioMax film and densitometry of autoradiograms was performed using an Alpha Imager (Alpha Innotec Corporation, Montreal, QC). Bovine COX-1 and COX-2 (112) and PGT cDNAs (62) were obtained and used as probes as described previously.

Western blot analysis

Western blot analysis was performed as we described (63). Briefly, total proteins (~20 μg) were loaded in each lane and electrophoresed on 10% SDS-PAGE followed by electrotransfer onto nitro-cellulose membrane. The following primary antibodies (raised in rabbit) were used for the respective protein: Anti-sheep COX-1 and COX-2 (1:3000), anti-bovine PGES (1:2000), PGFS (1:3000), PGDH (1:2000), and PGT (1:1000), and anti-human EP2 (1:500): Goat anti-rabbit IgG conjugated with horse radish peroxidase was used as the secondary antibody (1:20000). Chemiluminescent substrate was applied according to the manufacturer's instructions. The blots were exposed to BioMax films and densitometry was done using an Alpha Imager. ß actin (1:5000) was measured as an internal standard.

Immunohistochemistry

Cross sections were made in the middle portion of the uterine horns. Tissues were fixed in 4% paraformaldehyde for 4 h at 4°C and processed using standard procedures. Paraffin sections (3 μm) were made. Immunohistolocalisation was performed using Vectastain Elite ABC kit according

to the manufacturer's protocols, and as described (63, 67). Endogenous peroxidase activity was removed by fixing sections in 0.3% hydrogen peroxide in methanol. Tissue sections were blocked in 10% goat serum for 1 h at room temperature. The primary antibodies were the same as described above. The following concentrations were used: PGFS (1:1000), PGES (1:500) and EP2 (1:500). Incubation with the primary antibodies was done overnight at 4°C. The sections were further incubated with the second antibody (goat anti-rabbit IgG biotinilated, 1:200) for 30 min at room temperature. For the negative control, pre immune or control rabbit serum was used at the respective dilution used for primary antibodies. Between each step, tissues were washed in PBS. Finally, tissues were stained with Mayer's hematoxylin. Photos were captured using Spot program (Carsen group Incorp, Markham, ON), and quantification was done using Image–Pro-Plus (Media cybernetics, MD, USA). Pre-immune serum was used for the antibodies produced in our laboratory (PGFS) and control serum was used for commercial and donated antibodies (EP2, PGES) (control serum is the one collected without immunisation from the same species in which the antibody was raised).

STATISTICAL ANALYSIS

All numerical data are presented as the mean ± SEM. Data were analysed using two way ANOVA followed by Fischer's Protected LSD and Duncan New Multiple Range comparison and Scheffe's tests (SUPER ANOVA, ABACUS Concepts, Inc, Berkeley, CA). Differences were considered as statistically significant at 95% confidence level ($p < 0.05$).

RESULTS

Expression of PG biosynthetic enzymes in the uterus

The expression of COX-1, COX-2, PGES and PGFS, was studied during the oestrous cycle and following treatment *in vivo* with recombinant IFNt. COX-1 is not expressed at a significant level nor modulated in the endometrium or myometrium (Fig. 7 and 8). COX-2, PGES and PGFS proteins are expressed throughout the oestrous cycle in the endometrium, reaching maximal levels ($p< 0.05$) between days 13 to 21 (Fig. 7). Treatment with recombinant IFNt increases ($p< 0.05$) COX-2 protein expression in endometrium (~1.4 fold) but not in myometrium(Fig. 7 and Fig. 10). IFNt decreased PGFS protein expression in both endometrium and myometrium (Fig. 10). The level of expression of COX-2 is higher ($p<0.05$) in endometrium than in myometrium. Similar findings were obtained at the mRNA level, results not shown. ($p< 0.05$) while it has no effect on FP expression (Fig. 11).

DISCUSSION

We have presented an integrated view of PGE_2 and PGF_2 biosynthesis, transport and signalling systems in the uterus during the oestrous cycle, and establishment of pregnancy in cattle. The net production of uterine PGs is governed by the anabolic enzymes COX-1, COX-2, PGES, PGFS and the catabolic enzyme PGDH (1). COX-1 is expressed at low/undetectable levels in endometrium and by the anabolic enzymes COX-1, COX-2, PGEs and catabolic enzyme PGDH (1). COX-1 is expressed at low /undetectable levels in endometrium and myometrium. By contrast in endometrium COX-2 is highly expressed and modulated during the oestrous cycle. In this tissue, COX-2 and PGES are co-expressed on days 13-18 while COX-2 and PGFS are co-expressed on days 13-21 of the oestrous cycle (63). PGDH is highly expressed on days 13-18 of the

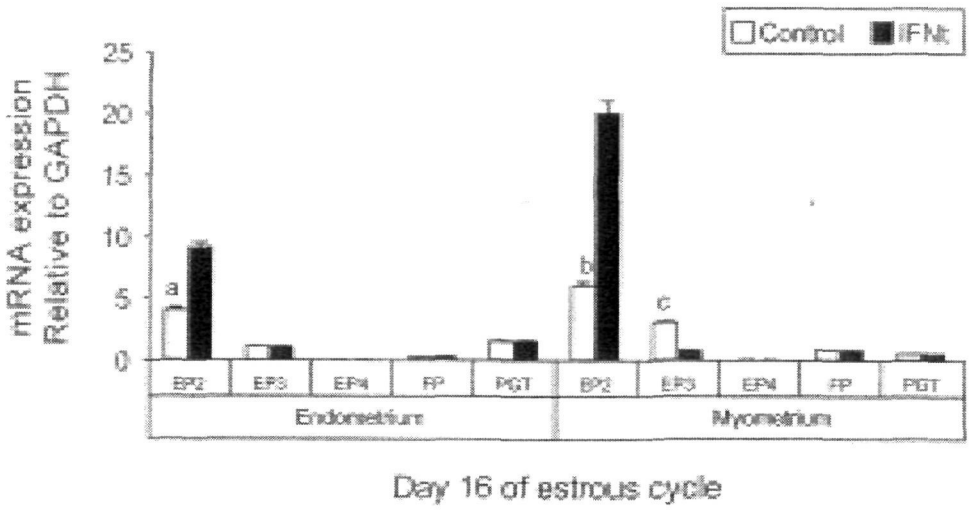

Figure 7 Densitometric analysis of expression of COX-1, COX-2, PGES and PGFS protein in bovine endometrial tissue collected at different days of the oestrous cycle. Quantification was done by densitometry of autoradiograms using an Alpha Imager. Each group (days of oestrous cycle) consisted of 3-8 samples. Values are presented as the mean ± SEM of Relative Integrated Density Values (IDV). Different letters (a, b,) indicate significant differences ($P < 0.05$), as determined by ANOVA followed by post-hoc multiple comparison tests. Within each protein enzyme, values with different letters are significantly different from others with or without superscripts. More details are given in *Materials and Methods*.

Expression of EP, FP and PGT in the uterus

In endometrium, EP2 is expressed throughout the cycle with maximal levels ($p< 0.05$) between days 10 and 18 at the mRNA and protein (not shown) levels (Fig. 8) . EP3, EP4 and FP are little or not expressed and not modulated during the cycle(Fig. 8). The PG transporter PGT is also expressed throughout the cycle, increased expression is observed on day 10-12 and maximal levels ($p< 0.05$) are found between days 16-21 at the mRNA (Fig 9) and at the protein (not shown) levels. IFNt increases ($p<0.05$) EP2 mRNA and protein (not shown) expression (~1.2 fold), but not EP3, FP and PGT mRNAs (Fig. 10).

In myometrium, EP2 mRNA and protein (not shown) expression is increased ($p< 0.05$) between days 10 and 18 and EP3 mRNA between days 13 and 21 (Fig. 11). EP4 is not expressed whereas FP and PGT mRNAs are expressed at a constant low level (Fig.9). IFNt increases EP2 mRNA (~ 4 fold) and protein (~ 2.7 fold, not shown) by contrast it decreases EP3 (2.8 fold) establishment of pregnancy in cattle. The net production of uterine PGs is governed cycle suggesting the presence of PG catabolism (53). Taken together, the data indicate that the COX-2 - PGES pathway responsible for endometrial production of PGE_2 is preferentially expressed during the implantation window whereas the COX-2 - PGFS pathway associated with endometrial production of PGF_2 is expressed during the luteolytic window. Cellular transport and signalling of PGE_2 and PGF_2 are complex events (4, 55, 59, 60). Selective localisation of PGT at the basal region of the luminal epithelial cells suggests that $PGF_{2\alpha}$ and PGE_2 produced in the epithelial cells are transported

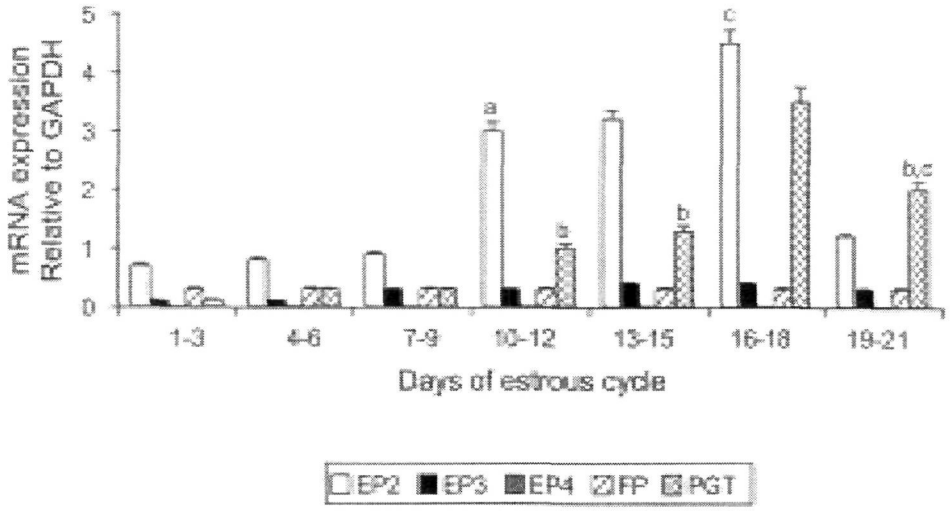

Figure 8 Influence of IFNt on the expression of COX-1, COX-2, PGES, PGFS and PGDH protein in endometrium and myometrium at the time of recognition of pregnancy. Uterine protein were extracted from control and IFNt animals on day 16 of the cycle. Levels of expression were determined by Western analysis of endometrial and myometrial samples. Quantification was done by densitometry of autoradiograms using an Alpha Imager and expressed relative to β-actin. Each group consisted of 3 samples. Values are presented as the mean ± SEM of Relative Integrated Density Values (IDV). Different letters (a, b,) indicate significant differences *($p < 0.05$)*, as determined by ANOVA followed by post-hoc multiple comparison tests. Within each protein enzyme, values with different letters are significantly different from others with or without superscripts. More details are given in *Materials and Methods*.

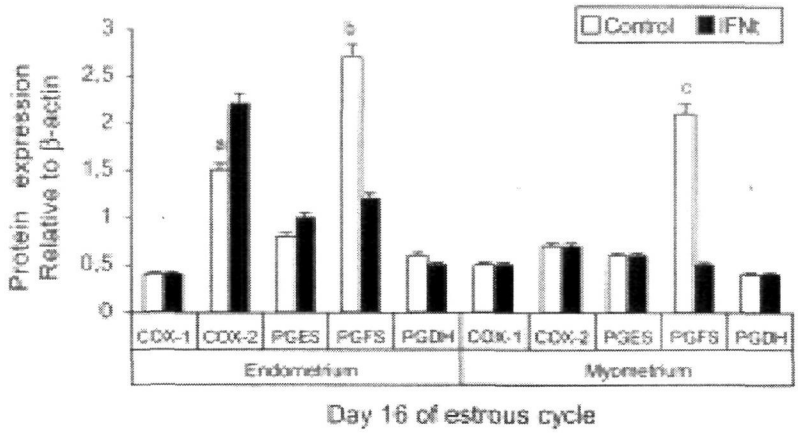

Figure 9 Densitometric analysis of expression of EP2, EP3, EP4, FP and PGT mRNA in bovine endometrial tissue collected at different days of the oestrous cycle. Quantification was done by real time RT-PCR (LightCycler). Each group (days of oestrous cycle) consisted of 3-6 samples. Values are presented as the mean ± SEM of ratios relative to GAPDH mRNA. Different letters (a, b, c) indicate significant differences ($p < 0.005$), as determined by ANOVA followed by post-hoc multiple comparison tests. For EP2 and PGT, values with different letters are significantly different from others with or without superscripts. More details are given in *Materials and Methods*.

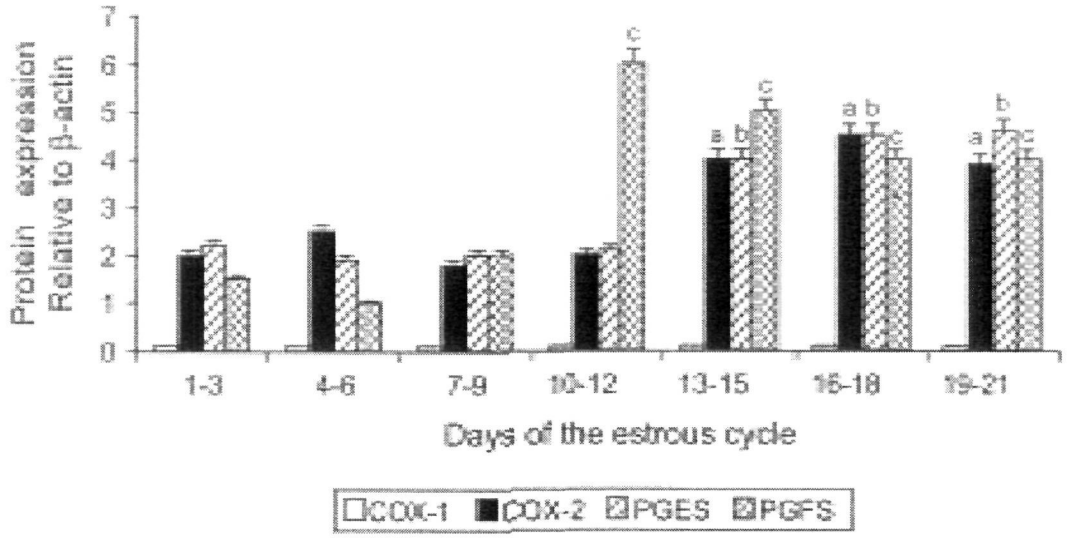

Figure 10 Influence of IFNt on the expression of EP2,EP3, EP4, FP and PGT mRNA in endometrium and myometrium at the time of recognition of pregnancy. Uterine mRNAs were extracted from control and IFNt animals on day 16 of the cycle. Levels of expression were determined by real time RT-PCR (LightCycler). Each group consisted of 3 samples. Values are presented as the mean + SEM of ratios relative to GAPDH mRNA. of endometrial and myometrial samples. Different letters (a, b, c) indicate significant differences ($P < 0.05$), as determined by ANOVA followed by post-hoc multiple comparison tests. For EP2 and EP3, values with different letters are significantly different from others with or without superscripts. More details are given in Materials and Methods.

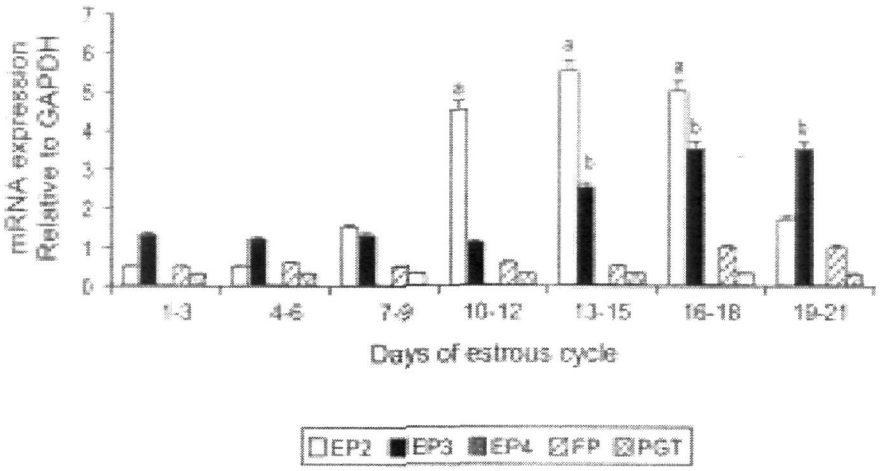

Figure 11 Densitometric analysis of expression of EP2,EP3, EP4, FP and PGT mRNA in bovine myometrial tissue collected at different days of the oestrous cycle. Quantification was done by real time RT-PCR (LightCycler). Each group (days of oestrous cycle) consisted of 3-6 samples. Values are presented as the mean $\pm$ SEM of ratios relative to GAPDH mRNA. Different letters (a, b) indicate significant differences *($p < 0.05$)*, as determined by ANOVA followed by post-hoc multiple comparison tests. For EP2 and EP3, values with different letters are significantly different from others with or without superscripts. More details are given in *Materials and Methods*.

towards the uterine compartment but not to the uterine lumen. The diffused expression of PGT in endometrial stroma and myometrial smooth muscle cells supports its role in transport of endometrial PGs towards the vascular system and ovarian compartment, and also suggests its role in autocrine and paracrine actions of PGs in endometrium and myometrium. The high expression of PGT in tunica intima and media of uterine vein compared with artery suggests that PGT contributes to the drainage of PGs from uterine compartments. Endometrial $PGF_{2\alpha}$ and/or PGE_2 are transferred from the uterine to the ovarian compartment through the UOP to bring forth their endocrine action, luteolysis or luteostasis in ruminants (67). It has been proposed since the 1970s that simple diffusion or counter current exchange (7, 25, 68) could be the mechanism involved in the transport of PGs across the UOP vascular wall. However, it was later found that PGs diffuse poorly through plasma membranes (59, 60) therefore, a carrier-mediated transport mechanism is inevitable for the transport of PGs across the biological membranes. Spatio-temporal expression of PGT in UOP adjacent to the CL between days 16-18 of the oestrous cycle coincides with the high production of endometrial PGs and presence of high levels of PGs in the uterine venous effluent. Selective localisation of PGT in UOP supports a role for PGT in transfer of PGs between sites of production and sites of action. In the present case, PGT is involved in cellular transport of PGs within the uterus, and from uterine to ovarian compartments. EP2, EP3 and FP receptors generate different second messenger systems cAMP versus Ca^{2+} and IP_3, and activate distinct signalling pathways. EP2 is maximally expressed between days 10 and 18 of the oestrous cycle in endometrium and myometrium. EP2 is localised in epithelial, stromal and glandular epithelial cells of endometrium and smooth muscle cells of the myometrium. FP and EP3 are expressed constantly at very low levels in endometrium whereas their expression levels are increased in myometrium at later stages of the oestrous cycle. Therefore, EP2 appears to be associated with a receptive uterus whereas EP3/FP are associated with termination of the oestrous cycle.

The process of MRP in ruminants is likely multi-dimensional involving a series of signals in sequence. There are two prevailing theories: 1) IFNt decreases luteolytic endometrial PGF_2 pulses (antiluteolytic signal) and 2) In addition to antiluteolytic effect, IFNt stimulates endometrial PGE_2 production which acts as a temporary luteotrophic signal at the time of establishment of pregnancy. During the bovine oestrous cycle, days 15-17 are the critical period. Endometrial PGF_2 is secreted in a pulsatile manner (4-5 pulses within 24 h). The luteolytic mechanisms involve a sequence of events: 1) P_4 autoregulates its own receptor PR in endometrium; 2) Estradiol from follicle acts through ER and prime the endometrium for the action of OT; 3) Decrease in PR and increase in ER, favour the expression of OTR in endometrium; 4) OT secreted by CL and neurohypophysis activates the endometrial OTR and its associated signalling and 5) eventually leads to pulsatile secretion of luteolytic PGF_2 involving positive feed back loop between endometrium and CL/ovary. During MRP, the presence of a viable embryo/conceptus prevents the pulsatile secretory pattern of PGF_2. The exact mechanism by which IFNt inhibits the pulsatile secretory pattern of the luteolytic PGF_2 is not clearly understood in bovine. In sheep, IFNt decreases the expression of uterine ER and OTR via paracrine mechanisms, thus preventing oxytocin-induced pulsatile secretion of PGF_2 and thus luteolysis. Recent studies have documented that IFNt decreased ER in bovine endometrium as well.

In the present study, IFNt decreases PGFS in luminal epithelium but it has no influence on PGES in any cell types. IFNt also increases COX-2 expression, and in a recent study we have shown that the up-regulation is more evident in luminal epithelium (64). Thus, the results indicate that IFNt increases the PGES: PGFS ratio which should result in increased endometrial PGE_2

relative to PGF_2 production. IFNt increases EP2 in luminal epithelium and stromal cells but not in glandular epithelium. It has no effect on FP and EP3. In myometrium, IFNt selectively increases EP2 in smooth muscle cell types and decreases EP3 signalling while it has no effect on FP. The effect of IFNt on EP2 is similar to what has been observed during early pregnancy. IFNt brings forth its effect mainly through type-I receptor. IFNt exerts its antiluteolytic effect in the endometrium in a paracrine manner to inhibit the pulsatile secretion of PGF_{2a} from endometrial epithelial cells (13, 14, 69-72). IFNt does not modulate PGT expression. Further, it is inferred that the effect of IFNt on endometrium is direct and on myometrium it might be indirect through PGE_2 and EP2 or by an unidentified mechanism. It is interesting to observe that binding of IFNt to its receptor in ovine endometrium does not stimulate increase in cAMP (13). IFNt does not reach other components of uterus and CL but activates several signal transduction across the uterine tissues and induces several uterine specific proteins (13). It is established that PGE_2 activates the EP2-cAMP-PKA signalling pathway that increases VEGF and ßFGF expression, and mitogenesis, angiogenesis, vasodilatation, endometrial receptivity, decidualisation, myometrial quiescence, and immunomodulation at the foeto-maternal interface during establishment of pregnancy in a variety of species (73- 84). The EP2 receptor is considered as a relaxant receptor in the myometrium of different species (77, 78, 85, 86). Butaprost, an agonist of EP2 increase cAMP production and reduces myometrial contraction (87), and abolishes oxytocin-induced myometrial activity (85). It has been proposed for several years that PGE_2 of embryonic origin could play a role in the establishment of pregnancy in ruminants (14). The presence of EP2 receptors on trophoblastic cells suggests that PGE_2 may play a role in embryonic development as well. Recently, COX-2 expression was found in trophoblastic cells of the ovine embryo between days 10 and 17 of pregnancy (88). A role for PGE_2 in embryogenesis (89) and in foetal development (90) has been proposed and its immunomodulatory role (91) at the time of establishment of pregnancy is well documented in ruminants. Taken together, our findings and available evidence indicate that PGE_2 could act through cAMP to effect the cross-talk between the different cell types in uterus and embryo at the time of establishment of pregnancy.

Rescue of CL and maintenance of P_4 secretion are the final events of the pregnancy recognition process. Early studies have documented that intrauterine infusion of IFNt extends the life span of the CL in sheep and cattle but IFNt is not detectable in uterine venous or lymphatic drainage (13). Study involving surgical separation of UOP suggests that secretory product(s) from the gravid uterus are transported through the UOP to rescue the CL at the time of establishment of pregnancy in cattle (27, 28). High levels of PGE_2 have been found in the uterine vein during early pregnancy in the sheep (14). PGE_2 increases luteal P_4, and a positive loop between luteal PGE_2 and P_4 have been documented (39, 99). Also, PGE_2 is involved in the autoregulation of its own production in different cell types (73-76, 92). The present results with our previous data and those of others strongly suggest that IFNt increases endometrial PGE_2 which is competitively transported to CL and influences the luteal PGE_2 biosynthesis and EP2 signalling thus favouring CL rescue. The PGFS (AKR1B5) we have evaluated in this study also possesses 20α-HSD activity (45), metabolising P_4 into the inactive 20α-OHP (93, 94). IFNt altered the expression AKR1B5 in endometrium and myometrium. The present findings suggest that IFNt regulates intrauterine and luteal P_4 metabolism and enhances P_4 action. It is well known that P_4 is the primary regulator involved in endometrial receptivity, myometrial quiescence and glandular proliferation and in turn provides conducive intrauterine environment for embryo/conceptus survival (12-14, 95, 96).

Early embryonic mortality remains as the major single cause of infertility in bovine. Up to 40% of early embryonic losses occur between days 15 and 17 of the oestrous cycle (12). Treatments targeting PG biosynthetic enzymes, transporter and receptors could represent novel therapeutic strategies to improve the conception rate in cattle. The information obtained from this study may potentially be applicable to other mammalian species as well.

Uterine receptivity and quiescence are both time and hormone dependent (13). Not much information is available on the regulation of PG systems in uterus. Ovarian steroids, oxytocin and cytokines have been documented as potential regulators of COX-2 and EP2, and other PG receptors during the oestrous cycle, establishment of pregnancy and at parturition (77-83, 86, 97, 98). Regulatory mechanisms of PGES, PGFS and PGT in CL are completely unknown. The precise hormonal regulation of PGE_2 and PGF_2 biosynthetic, transporting and signalling systems is tissue specific and likely to be different among endometrium, myometrium and CL. Future studies are required in this arena to unravel the molecular mechanisms involved in the regulation of uterine and luteal production and action of PGs.

Collectively, PG biosynthetic, transporting and signalling cascade in endometrium and myometrium indicate that IFNt selectively increases PGE_2 and decreases PGF_2 production while it inversely regulates EP2 and EP3 signalling during MRP in cattle. The present findings suggest an exquisite role for PGE_2 in endometrial receptivity, myometrial quiescence and luteal maintenance at the time of establishment of pregnancy in cattle. Moreover, establishment of pregnancy is not only due to inhibition of endometrial PGF_2 but also increased PGE_2 production in cattle.

CONCLUDING REMARKS

1. PGE_2 and PGF_2 biosynthetic, transporting and signalling systems are tightly regulated in endometrium, myometrium and CL during the oestrous cycle and pregnancy.
2. IFNt regulates PGE_2 and PGF_2 biosynthesis and signalling in a tissue specific and spatiotemporal manner in endometrium and myometrium.
3. Establishment of pregnancy in cattle not only relies on inhibition of endometrial PGF_2 but also on increased endometrial PGE_2 production and action.
4. Effect of IFNt on luteal function is probably indirect through altered endometrial PGE_2 and PGF_2 ratio.
5. In addition to PG biosynthetic enzymes and receptors essential for PGs production and action, the regulation of PGs trafficking across the cell membranes and compartments through PGT must be considered as an important contributing factor.
6. PGE_2 plays a pivotal role in recognition, establishment and maintenance of pregnancy in cattle.

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