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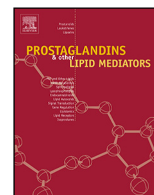

Lysophosphatidic acid modulates prostaglandin signalling in bovine steroidogenic luteal cells

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ABSTRACT

We examined whether lysophosphatidic acid affects prostaglandin biosynthesis, transport, and signalling in bovine steroidogenic luteal cells. The aim of the present study was to determine the influence of LPA on PGE2 and PGF2α synthesis and on the expression of enzymes involved in PG biosynthesis (PTGS2, mPGES-1, cPGES, mPGES-2, PGFS and 9-KPR), prostaglandin transporter (PGT), and prostaglandin receptors (EP1, EP2, EP3, EP4 and FP) in bovine steroidogenic luteal cells. We found that LPA inhibited $PGF_{2\alpha}$ synthesis in steroidogenic luteal cells. Moreover, LPA increased mPGES1 and cPGES and decreased PGFS expression in cultured bovine steroidogenic luteal cells. Additionally, LPA stimulated EP2 and EP4 receptor and PGT expression. This study suggests that LPA activity in the bovine CL directs the physiological intraluteal balance between the two main prostanoids towards luteotropic PGE_2.

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1. Introduction

The corpus luteum (CL) is an endocrine organ established from the remaining cells of the ovulated follicle. The mature CL consists of a heterogeneous cell population. There are at least two steroidogenic cell types: large luteal cells of granulosa cell origin and small luteal cells of theca cell origin [1,2]. Although progesterone (P4) is the major luteal hormone, the CL also produces other hormones, including prostaglandins (PGs) [3]. Phospholipase activity induces PG biosynthesis, which releases arachidonic acid (AA) from the phospholipid membrane. Thereafter, AA is converted into the PG precursor PG endoperoxide H_2 (PGH_2) by PG endoperoxide synthases (better known as cyclooxygenase) [4]. Prostaglandin E synthases (PGESs) and PGF synthase (PGFS) metabolize prostaglandin H_2 into PGE_2 and $PGF_{2\alpha}$, respectively. Additionally, an alternate pathway of $PGF_{2\alpha}$ synthesis is the conversion of PGE_2 into $PGF_{2\alpha}$ through PG 9-ketoreductase (9-KPR) activity [5]. Current evidence suggests that three forms of PGES exist: microsomal PGES1 and 2 (mPGES1 and mPGES2) and cytosolic PGES (cPGES) [6–9].

Newly synthesized PGs are transported through the plasma membrane by prostaglandin transporter (PGT) [10,11]. This molecule is a broadly expressed, 12-membrane-spanning domain integral membrane protein [12]. A previous study found that luteal PGT expression was lower in the early growing than in late growing, mature, and regressing phases of the oestrous cycle. Therefore, PGT is almost exclusively expressed in large luteal cells (LLCs) [13]. The same study suggested that PGT facilitated both the efflux and influx of available luteal PGE_2 and/or $PGF_{2\alpha}$ in a competitive manner to affect their autocrine and paracrine actions, as well as their catabolism during the different stages of the CL life span.

Prostaglandin E_2 and $PGF_{2\alpha}$ primarily exert their effects through G protein-coupled receptors designated EP and FP, respectively [14,15]. PGE receptor subtypes (EP1, EP2, EP3 (A–D) and EP4) and an FP receptor have been identified. EP2 and EP4 are coupled to adenylate cyclase and generate cAMP, which activates the protein kinase A signalling pathway. EP1 and FP receptors are coupled to phospholipase C to generate two second messengers, inositol triphosphate involved in the liberation of intracellular calcium (Ca^{2+}) and diacyl glycerol, an activator of protein kinase C. There are four of EP3 receptor isoforms, A–D. These EP3 receptors exhibit a wide range of action, from the inhibition of cAMP production to the increase in Ca^{2+} and inositol phosphate 3 [14,16]. Thus, FP and EP are expressed at high levels in the corpus luteum, particularly in the large luteal cells [13,17].

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Lysophosphatidic acid (LPA) is a naturally occurring, small, bioactive phospholipid. This ligand plays several roles in the female reproductive tract [18] by activating its G protein-coupled receptors LPAR1-6 [19]. Previous studies found that LPA stimulated *PTGS2* mRNA expression in the porcine endometrium [20] and increased PGE_2 synthesis in the ovine trophectoderm [21] and bovine endometrium [22]. Moreover, infusion of heifers with LPA prevented spontaneous luteolysis, prolonged the functional lifespan of the CL and stimulated luteotropic PGE_2 synthesis in vivo [23]. We have found that LPA is locally produced and released from the bovine CL [24] and that LPA reversed the inhibitory effect of NO donor on the $PGE_2/PGF_{2\alpha}$ ratio in cultured bovine steroidogenic luteal cells (bSLCs) [25]. The above studies identified a linkage between LPA signalling and PG biosynthesis in bovine CL. However, it is unknown whether LPA can differentially affect PG biosynthesis, transport, and signalling in bSLCs.

Taken together, the evidence suggests that $PGF_{2\alpha}$ and PGE_2 expression in the bovine CL depends not only on PTGS2 but also on PG synthases, transporter, and specific EP and FP receptors. According to previous results that described LPA as an additional luteosupportive factor in bSLCs [24] and an important modulator of oestrous cycle length in cows [25], we hypothesize that LPA can modulate PG biosynthesis, transport, and signalling in bSLCs. Therefore, the main objective of this study was to evaluate the effect of LPA on PG synthesis via the expression of enzymes responsible for their biosynthesis (PTGS2, mPGES-1, cPGES, mPGES-2, PGFS and 9-KPR), transport (PGT), and receptors (EP1, EP2, EP3, EP4 and FP) in bSLCs.

2. Materials and methods

2.1. Animals

All animal procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (Agreement no. 79/2008/N). For all experiments, healthy, normally cycling Holstein/Polish Black and White (75/25%; respectively) cows ($n = 40$) were used for ovary collection. The animals were culled because of their low milk production. Oestrus was synchronized by two injections of a $PGF_{2\alpha}$ analogue (dinoprost, Dinolytic; Upjohn & Pharmacia N.V.S.A., Belgium) as described previously [26]. Oestrus was detected by visual signs (i.e., vaginal mucus and standing behavior) and confirmed by a veterinarian using ultrasonography (USG) with a Draminski Animal Profi Scanner (Draminski Electronics in Agriculture, Olsztyn, Poland) and by per rectum examination. Only cows with behavioral signs of oestrus and the presence of a CL in the ovary confirmed by USG were chosen for the study ($n = 33$).

2.2. Collection of bovine CL

One day before slaughter, the animals were transported to the local abattoir (Zaklady Miesne "Warmia", Biskupiec, Poland). Bovine ovary CL tissue (Mid-CL; $n = 33$) were collected immediately after slaughter and kept on ice until further processing in the laboratory. Genital tracts on Days 8–10 of the oestrous cycle were selected for cell culture. For each experimental repetition, luteal cells were isolated from a pool of three CLs. The physiological stage of the reproductive cycle was confirmed by observation of ovarian morphology and the uterus according to criteria described by Miyamoto et al. [27].

2.3. Isolation and incubation of bSLCs

Enzymatic isolation of CL tissue and culture of steroidogenic cells was performed as described previously [28]. Cell viability was greater than 85%, as assessed by trypan blue staining. The cell suspension contained approximately 20–25% large steroidogenic luteal cells, 70–75% small steroidogenic luteal cells and less than 5% endothelial cells and fibroblasts, with no erythrocytes [25]. The final steroidogenic cell pellet was suspended in culture medium, consisting of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/Ham's F-12, 1:1 [v/v]; #D8900; Sigma) containing 10% calf serum (#16170-078; Gibco BRL) and 20 μg/ml gentamicin (#15750-060; Gibco BRL). Dispersed luteal cells were seeded at 5.0×10^4 viable cells/ml in 6-well plates (#3335; COSTAR®, Corning® CellBIND® Surface) and cultured at 37.5 °C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 h in culture, the medium was replaced with fresh DMEM/Ham's F-12 supplemented with 1% BSA (#A9056; Sigma), 5 ng/mL sodium selenite (#S1382; Sigma), 0.5 mM ascorbic acid (#A1417; Sigma), 5 μg/mL transferrin and 20 μg/mL gentamicin (#G-1397; Sigma).

After culture for 24 h, steroidogenic cells were exposed to an LPA agonist (1 oleoyl-sn-glycero-3-lysophosphatidic acid sodium salt, LPA; 10^{-6} M; #Alx 300-139-M005; Alexis) and LH (USDA-bLH-B-6; 100 ng/ml) for 6 h (mRNA evaluation) or 12 h (protein measurement). The LPA agonist dose was defined previously [24]. After incubation, the conditioned media were collected and frozen at −20 °C until use. For $PGF_{2\alpha}$ and PGE_2 measurements, the commercial $PGF_{2\alpha}$ high sensitivity EIA kit and the PGE_2 high sensitivity EIA kit (#ADI-931-001, #ADI-931-069, respectively; ENZO Life Sciences Inc., Farmingdale, NY, USA) were used and run according to the manufacturer's instructions. Gene expression for enzymes involved in PG synthesis (PG endoperoxide synthase*2–PTGS2, PGE2 synthases—cPGES,mPGES1,mPGES2* and*PGF_{2a} synthase—PGFS*), PG receptors (*EP1,EP2,EP3,EP4,FP*), prostaglandin transporter (*PGT*) and prostaglandin E2 9-ketoreductase (*9-KPR*) was quantitatively measured by real-time PCR.

2.4. Total RNA extraction, reverse transcription (RT) and real-time PCR

Total RNA was extracted from steroidogenic cells using TRIzol@ Reagent (#15596; Invitrogen) according to the manufacturer's instructions, and samples were stored at −80 °C. Subsequently, RNA content and quality were evaluated by spectrophotometric measurement and agarose gel electrophoresis. One microgram of total RNA from each sample was reverse transcribed using a Maxima@ First Standard cDNA Synthesis Kit (#K1642; Thermo Scientific). The RT reaction was performed in a total reaction volume of 20 μl following the manufacturer's instructions. Products were stored at −20 °C until real-time PCR amplification. mRNA expression for all genes examined was assessed by Real Time PCR using specific primers for: *PTGS2,mPGES1,mPGES2,cPGES,PGFS,EP1,EP2,EP3,EP4,FP,PGT*and*9-KPR*. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as the housekeeping gene. The primers were chosen using an online software package (http://frodo.wi.mit.edu/primer3/input.htm). The primers for all target genes are listed in Table 1.

Real-time PCR was performed with an ABI Prism 7900 sequence detection system using Maxima@ SYBR Green/ROX qPCR Master Mix (#K0222; Thermo Scientific). The PCR reactions were performed in 384-well plates. The total reaction volume was 10 μl and contained 1 μl diluted RT product (500 pg/μl), 10 pmol/μl each of forward and reverse primers and 5 μl SYBR Green PCR master mix. For relative quantification of mRNA expression levels (target gene versus housekeeping gene), miner software was used http://www.miner.ewindup.info/version2 Real time PCR was performed as follows: initial denaturation (10 min at 95 °C), followed by 40 cycles of denaturation (15 s at 95 °C) and annealing (1 min at 60 °C). Following each PCR reaction, melting curves were obtained by stepwise increases in the temperature from 60 °C to 95 °C to ensure single

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