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**—Original Article—**

# Lysophosphatidic Acid Action in the Bovine Corpus Luteum —An *In Vitro* Study

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**Abstract.** We examined whether the CL is a site for lysophosphatidic acid (LPA) synthesis and/or a target for LPA action in the bovine reproductive tract. LPA concentrations in the CL tissue increased towards the end of the cycle and were stable during early pregnancy. No changes in the expression of LPA receptors (LPARs) occurred during the estrous cycle. The expressions of LPAR2 and LPAR4 on days 17–19 of pregnancy were higher than those on the respective days of the estrous cycle and higher than those on days 8–10 of pregnancy. LPA stimulated P4 synthesis via 3βHSD stimulation but did not modulate the interferon–tau (IFNτ) influence on P4 synthesis in steroidogenic cells. Moreover, we found LPA-dependent stimulation of IFNτ action on 2,5'-oligoadenylate synthase (OAS1) and ubiquitin-like IFN-stimulated gene 15-kDa protein (ISG15) expression. The present study demonstrated that the CL might be a site of LPA synthesis and target of LPA action in the bovine reproductive tract. We postulate that during the estrous cycle and early pregnancy, LPA exerts autocrine and paracrine effects on the CL mainly via *LPAR2* and *LPAR4*. The stimulatory effect of LPA on P4 synthesis via 3βHSD stimulation and LPA-dependent stimulation of IFNτ action on OAS1 and ISG15 expression suggest that LPA is an additional auxiliary luteosupportive factor in steroidogenic cells.

**Key words:** Cow, Lysophosphatidic acid, Progesterone, Steroidogenic cells

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The corpus luteum (CL) is an endocrine gland that is temporarily formed in the ovary and undergoes regression at the end of the estrous cycle [1]. After ovulation, it forms from the Graafian follicle, grows and vascularizes rapidly. The bovine CL consists of a variety of cell types including large and small luteal cells, endothelial cells, fibroblasts and immune cells [2, 3]. In nonpregnant cows, the CL undergoes luteolysis and becomes nonfunctional around days 17–18 after ovulation [4, 5]. The main function of the CL both during the cycle and pregnancy is synthesis of progesterone (P4), which plays major roles in the regulation of the length of the estrous cycle and in the implantation of the blastocyst after fertilization [6]. During maternal recognition of pregnancy, the conceptus synthesizes and secretes interferon tau (IFNτ), which protects the CL and extends the estrous cycle [7]. In addition to its antiluteolytic effects, IFNτ increases expression of several IFN-stimulated genes (ISG), such as 2',5'-oligoadenylate synthetase (OAS1) and ubiquitin-like IFN-stimulated gene 15-kDa protein (ISG15) in the uterus [8], mammary gland and CL [9] in cattle. However, during maternal recognition of pregnancy, P4 is the main factor responsible for its successful establishment. Luteinizing hormone (LH) is the most important regulator of P4 synthesis [10, 11]. The growth and development of the early CL is supported by many factors, including LH, PGs ($PGE_2$ and $PGI_2$), oxytocin, noradrenaline and growth factors [12–15]. The CL can also autoregulate the synthesis of P4 [16].

Lysophosphatidic acid (LPA) has been shown to affect the reproductive processes in rats [17], pigs [18, 19], ewes [20] and cows [21–23]. LPA is a simple phospholipid that exerts many physiological and pathological actions on many cell types, including cell proliferation and differentiation [24], cytoskeletal rearrangement [25], cell-to-cell interactions [26] and tumorigenesis [27]. In mammals, LPA exerts its action via high affinity G-protein-coupled receptor (GPCR) types LPAR1/EDG2, LPAR2/EDG4, LPAR3/EDG7 and LPAR4/P2Y9 [28–30]. We have previously reported that LPA is locally produced and released from the bovine endometrium [21] and that LPA administered into the abdominal aorta stimulated P4 and $PGE_2$ secretion during the luteal phase of the estrous cycle *in vivo* [21]. Additionally, blocking the effect of endogenous LPA decreased the pregnancy rate. Moreover, during early pregnancy, LPA infused *intravaginally* stimulated P4 secretion [31]. However, it is unknown whether this stimulation *in vivo* is the result of a direct influence on the steroidogenesis of luteal cells or modulation of pregnancy recognition signal (IFNτ) action. Additionally, limited information is available on transcription and protein expression of LPA receptors (LPARs) in the bovine CL during the estrous cycle and early pregnancy.

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The aim of the present study was to determine whether CL is a site for LPA synthesis and/or a target for LPA action in the bovine reproductive tract. For this purpose we investigated (1) the concentrations of LPA in the bovine CL during the estrous cycle and early pregnancy, (2) the expression patterns of LPAR1, LPAR2, LPAR3 and LPAR4 mRNA and proteins in the bovine CL during the estrous cycle and early pregnancy, (3) the influence of LPA on P4 secretion by luteal cells in the bovine CL and (4) the influence of LPA on IFNτ action in the luteal cells in the bovine CL.

## Materials and Methods

### *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, normally cycling Holstein/Polish Black and White (75/25%; respectively) cows (n=90) from two dairy herds were used (2007–2009). The animals were culled because of their low milk production. Estrus was synchronized in the cows by two injections of an analogue of $PGF_{2\alpha}$ (dinoprost, Dinolytic; Upjohn & Pharmacia, Puurs, Belgium), as described previously [32]. Estrus was detected by the indicative signs (i.e., vaginal mucus, 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 estrus and presence of a CL in the ovary confirmed by USG were chosen for the study (n=75). Estrus was taken as day 0 of the estrous cycle. From these 75 cows, 18 were artificially inseminated at the detected estrus using semen from one bull. At sequential times after estrus detection and/or artificial insemination, cows were slaughtered, and the reproductive tracts were examined. The stages of the estrous cycle were confirmed by macroscopic observation of the ovaries and uterus [33], and pregnancy was confirmed by flushing the uterus for collection of an apparently normal conceptus, as described previously [21]. The cows were then divided into two groups: pregnant (n=12) and cyclic (n= 51).

### *Experimental procedure*

Experiment 1: LPA concentration and LPAR expression in the bovine CL during the estrous cycle and early pregnancy

Ovaries were obtained at a local slaughterhouse (Zaklady Miesne "Warmia," Biskupiec, Poland) within 20 min of exsanguination and were transported on ice to the laboratory within 40 min. The tissues for this experiment were collected on the following days of the estrous cycle and pregnancy: days 2–4 (n=6), 8–10 (n=7) and 17–19 (n=6) of the estrous cycle and days 8–10 (n=5) and 17–19 (n=7) of pregnancy.

The collected CLs were used to study concentrations of LPA and expression of LPAR mRNAs and proteins. Tissue samples were snap-frozen in liquid nitrogen and stored at −80 C until further use for mRNA and protein extraction. Cross sections of CL samples were also fixed for immunohistochemical analyses.

Experiment 2: The influence of LPA on IFNτ actions on steroidogenesis and on the expressions of interferon-stimulated genes in luteal cells of the bovine CL

Ovaries were collected after slaughter at days 8–10 of the estrous cycle, as described in experiment 1 (n=8; for each repetition, luteal cells were isolated from a pool of four CLs). The respective days of the estrous cycle were chosen because they represent a critical period in CL lifespan for P4 secretion [6]. The second reason for this selection is documented by Kimura *et al.* [34]; a dramatic increase in IFNτ production occurs during culture of *in vivo* derived embryos between days 9 and 14 after fertilization. Moreover, Kelemen *et al.* [35] reported that the presence of the fertilized ovum induces a Th2 lymphocyte shift on days 8–9 of early pregnancy, which enables pregnancy to proceed to term. Enzymatic dissociation of the luteal tissue and culture of steroidogenic cells of the CL were performed as previously described [36, 37]. Cell viability was greater than 85% as assessed by trypan blue staining. The cell suspension contained about 20–25% large steroidogenic CL cells, 70–75% small steroidogenic CL cells and less than 5% endothelial cells and fibroblasts, without erythrocytes [37]. The final pellet of steroidogenic cells was suspended in culture medium, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/Ham's F-12, 1:1 [v/v]; Sigma #D8900, Taufkirchen, Germany) containing 10% calf serum (Gibco BRL #16170-078, Grand Island, NY, USA) and 20 µg/ml gentamicin (Gibco BRL #15750-060). Dispersed luteal cells were seeded at $5.0 \times 10^4$ viable cells/ml in 6-well plates (Costar®, Corning® CellBIND® Surface, #3335, Corning B.V. Life Sciences, Amsterdam, The Netherlands) and cultured at 37.5 C in a humidified atmosphere of 5% $CO_2$ and 95% air. After a 24 h culture, the medium was replaced with fresh DMEM/Ham's F-12, supplemented with 0.1% BSA (Sigma #A9056), 5 ng/ml sodium selenite (Sigma; #S1382), 0.5 mM ascorbic acid (Sigma; #A1417), 5 µg/ml transferrin and 20 µg/ml gentamicin (Sigma; #G-1397). The cells were then exposed to an LPA agonist (1-oleoyl-sn-glycero-3-phosphate sodium salt, Alexis #Alx 300-139-M005; LPA; $10^{-6}$ M), recombinant ovine IFNτ (donated by Dr FW Bazer of Texas A&M University, College Station, TX, USA; $10^6$ AVU/ml) and IFNτ with LPA for 6 (mRNA evaluation) and 12 h (protein evaluation and measurement of P4 concentrations). The appropriate dose of LPA agonist was defined in preliminary experiments. LH (USDA-bLH-B-6; 100 ng/ml) was used as a positive control for P4 synthesis in steroidogenic cells [10]. After incubation, the conditioned media were collected and frozen at –20 C until measurement of P4 by an enzyme immunoassay (EIA) as previously described [38]. Gene expression of the enzymes involved in steroidogenesis (*cholesterol esterase, StAR protein, P450scc* and *3βHSD*) and interferon-stimulated genes (*interferon stimulated gene 15, ISG15* and *2,5'-oligoadenylate synthase, OAS1*) was quantitatively measured by real-time PCR. The cells for real-time PCR were disrupted with TRIzol reagent (Invitrogen #15596, Grand Island, NY, USA) and frozen at –80 C until they were processed for RNA isolation. The protein level for the enzymes involved in steroidogenesis in luteal cells was measured by Western blot analysis. The proteins were extracted using radioimmunoprecipitation assay buffer (RIPA buffer; 150 mM NaCl, 50 nM Tris base, pH 7.2, 0.1% SDS, 1% Triton ×100, 0.5% sodium deoxycholate and 5 mM EDTA) in the presence of a protease inhibitor cocktail (Roche; #11697498001, Indianapolis, Indiana, USA).

### *Lipid extraction from CL tissue and in vitro acylation of LPA*

1-Oleoyl-LPA contained in 100 mg of tissue was extracted with

**Table 1.** Primers used for real-time PCR

| Gene | Primer sequences | GenBank [acc. no.] | PCR product size [bp] |
|---|---|---|---|
| Cholesterol esterase | 5'GCCAGAGTGGAGTGGGTTTG'3<br>5'CGGGGGTCAGTGATCTTCAG'3 | NM_001013583.1 | 139 |
| StAR protein | 5'GGTGGTGGCACGTTTTCAAT'3<br>5'CCTTGTCCGCATTCTCTTGG'3 | Y17259.1 | 79 |
| P450scc | 5'CAGCATATCGGTGACGTGGA'3<br>5'GGCCACCAGAACCATGAAAA'3 | K02130.1 | 139 |
| 3βHSD | 5'TCCCGGATGAGCCTTCCTAT'3<br>5'ACTAGGTGGCGGTTGAAGCA'3 | NM_174343.2 | 116 |
| LPAR1 | 5'ACGGAATCGGGATACCATGA'3<br>5'CCAGTCCAGGAGTCCAGCAG'3 | NC_007306.3 | 86 |
| LPAR2 | 5'TTCTATGTGAGGCGGCGAGT'3<br>5'AGACCATCCAGGAGCAGCAC'3 | NC_007305.3 | 161 |
| LPAR3 | 5'TCCAACCTCATGGCCTTCTT'3<br>5'GACCCACTCGTATGCGGAGA'3 | NM_001192741.2 | 101 |
| LPAR4 | 5'CCACAGTACCTCCAGAAAGTTCA'3<br>5'TTGGAATTGGAAGTCAATGAATC'3 | NM_001098105.1 | 192 |
| ISG15 | 5'GGTATCCGAGCTGAAGCAGTT-3'<br>5'ACCTCCCTGCTGTCAAGGT-3' | NM_174366 | 86 |
| OAS1 | 5'-TAGGCCTGGAACATCAGGTC-3'<br>5'-TTTGGTCTGGCTGGATTACC-3' | NM_001040606 | 104 |
| GAPDH (*Bos taurus*) | 5'CACCCTCAAGATTGTCAGCA3'<br>5'GGTCATAAGTCCCTCCACGA3' | BC102589 | 103 |

one volume of 1-butanol. After agitation and centrifugation (5 min at 3000 *g*), the upper butanol phase was collected and evaporated under nitrogen at 50 C. After evaporation, lipids were incubated in the presence of semipurified recombinant rat lysophosphatidic acid acyl transferase (LPAAT) and [$^{14}$C]oleoyl-CoA as described by Saulnier-Blache *et al.* [39]. The products of the reaction were separated by two-dimensional thin-layer chromatography (2D-TLC) and autoradiographed. To convert radioactivity to picomoles, lipids visualized under iodine vapors were scraped and counted with 3 ml of scintillation cocktail, according to the procedures described by Saulnier-Blache *et al.* [39].

### *Total RNA extraction, reverse transcription (RT) and real-time PCR*

Total RNA was extracted from steroidogenic cells using TRIzol according to the manufacturer's instructions. RNA samples were stored at –80 C. Before use, RNA content and quality was evaluated by spectrophotometric measurement and agarose gel electrophoresis. One microgram of each sample of total RNA was reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen #205311, Tokyo, Japan). The RT reaction was performed in a total reaction volume of 20 µl according to the manufacturer's instructions, and products were stored at –20 C until real-time PCR amplification.

The expression of mRNA for all examined genes was conducted by real time PCR using specific primers for *LPAR1, LPAR2, LPAR3, LPAR4, cholesterol esterase, StAR protein, P450scc, 3βHSD, ISG15* and *OAS1* and *GAPDH* expression was used as an internal control. The primers were chosen using an online software package (http://frodo.wi.mit.edu/primer3/input.htm). The primers of all target genes are given in Table 1.

Real-time PCR was performed with an ABI Prism 7300 sequence detection system using Power SYBR Green PCR Master Mix (Applied Biosystems #4367659, Grand Island, NY, USA). The PCR reactions were performed on 96-well plates. Each PCR reaction well (20 µl) contained 2 µl of diluted RT product, 10 pmol/µl forward and reverse primers each and 10 µl SYBR Green PCR Master Mix. For the relative quantification of mRNA expression levels (target gene versus housekeeping gene), the Miner software was used http://www.miner.ewindup.info/. In the reaction, the primer length (20 bp) and GC-content of each primer (50–60%) were selected. Real-time PCR was performed under the following conditions: 95 C for 10 min, followed by 40 cycles at 94 C for 15 sec and at 60 C for 60 sec. Each PCR reaction was followed by obtaining melting curves by a stepwise increase in the temperature from 60 C to 95 C to ensure single product amplification. In order to exclude the possibility of genomic DNA contamination in the RNA samples, the reactions were also run either on blank-only buffer samples or in the absence of reverse transcriptase. The specificity of the PCR products for all examined genes was confirmed by sequencing.

### *Western blot analysis*

For immunoblotting, protein fractions were obtained from the tissue samples and total protein from the cells. Briefly, luteal tissues were homogenized on ice in RIPA buffer containing 150 mM NaCl, 50 nM Tris base, pH 7.2, 0.1% SDS, 1% Triton x-100, 0.5% sodium deoxycholate and 5 mM EDTA in the presence of the protease inhibitor cocktail (Roche #11697498001). Lysates were then sonicated and centrifuged at 10000 *g* for 15 min at 4 C. The protein samples were stored at −70 C for further analysis. The protein concentration was determined according to Bradford [40]. Equal amounts (50 µg) of membrane fraction were dissolved in SDS gel-loading buffer (50 mM Tris-HCl, pH 6.8; 4% SDS, 20% glycerol

and 2% β-mercaptoethanol), heated to 95 C for 5 min and separated by 12% SDS-PAGE. Separated proteins were electroblotted using a semidry transfer method onto polyvinylidene difluoride membranes (Immobilon-P Transfer Membrane; Millipore #IPVH00010, Billerica, MA, USA) in transfer buffer (0.3 mM Tris buffer, pH 10.4, 10% methanol; 25 mM Tris buffer, pH 10.4, 10% methanol; 25 mM Tris buffer, pH 9.4, 10% methanol, 40 mM glycine). After blocking in 5% nonfat dry milk in TBS-T buffer (Tris-buffered saline with 0.1% Tween-20) for 1.5 h at 25.6 C, the membranes were incubated overnight rabbit polyclonal anti-LPAR2, LPAR3, StAR protein, 3βHSD, ISG15 and OAS1 antibodies (concentration for all antibodies 1:100; Santa Cruz Biotechnology #sc-25490, #sc-25492, #sc-25806, #sc-28206, #sc-50366, #sc-98424, respectively, Santa Cruz, CA, USA), goat polyclonal anti-LPAR1, LPAR4, cholesterol esterase and P450scc=antibodies (concentration for all antibodies 1:100; Santa Cruz Biotechnology #sc-22207, #sc-46021, #sc-34878, #sc-18043, respectively) and monoclonal anti-GAPDH antibody produced in the mouse (concentration 0.05 μg/ml; Sigma #G8795) at 4 C. Subsequently, the enzymes were detected by incubating the membranes with an anti-rabbit IgG-alkaline phosphatase antibody produced in the goat (concentration 1:20000; for LPAR3, LPAR4, StAR protein, 3βHSD, ISG15 and OAS1, Sigma #A3687), donkey anti-goat IgG-alkaline phosphatase antibody (concentration 1:6000; for LPAR1, LPAR4, cholesterol esterase and P450scc, Santa Cruz Biotechnology #sc-2037) or anti-mouse IgG-alkaline phosphatase antibody produced in the goat (concentration for all antibodies 1:20000; for GAPDH, Sigma #A3562) for 1.5 h at 25.6 C. After washing again in TBS-T buffer, the immune complexes were visualized using an alkaline phosphatase visualization procedure. The specific bands were quantified using the Kodak 1D software (Eastman Kodak, Rochester, NY, USA). GAPDH was used as an internal control for protein loading.

*Immunohistochemistry*

Ten-micrometer-thick sections from all examined phases of the estrous cycle and early pregnancy were mounted on glass slides pre-coated with silane (Dako #S3003) and dried for 0.5 h at 60 C. Sections were deparaffinized, rehydrated and washed in tap water for 10 min, followed by immersion in methanol with 3% (v/v) $H_2O_2$ for 30 min. After 3 washes with PBS, sections were immersed in antigen retrieval buffer (0.1 M citrate acid, pH 6.0) at 95 C for 10 min. Sections were cooled for 30 min at room temperature, washed in PBS 3 times and then incubated with 10% (v/v) normal rabbit or goat serum (Invitrogen #85-9043, Grand Island, NY, USA and Leica Microsystems #RE7102-CE, Wetzlar, Germany) containing 1% (w/v) BSA diluted in PBS to block nonspecific protein binding. The same antibodies as for Western blot were used at the same dilutions in PBS containing 1% BSA (w/v). The sections were incubated overnight at 4 C with the same Western blotting analysis primary antibody. Negative control sections were incubated with PBS or normal rabbit or goat irrelevant IgG (concentration 1:100; Santa Cruz Biotechnology #sc-2027 and #sc-2028, respectively). Subsequently, the sections were washed in PBS 3 times, incubated with biotinylated anti-rabbit IgG antibody (Vector Laboratories #PK-4001, Burlingame, CA, USA) or with biotinylated anti-goat IgG antibody (Vector Laboratories #PK-4005) 1:300 in PBS for 60 min, washed 3 times in PBS and then incubated for 30 min at room temperature with VECTASTAIN avidin-biotin-peroxidase complex rabbit or goat (Vector Laboratories #PK-4001 and #PK-4005, respectively), according to the manufacturer's instructions. The sections were washed 3 times and visualized with 0.05% (w/v) 3,3'-diaminobenzidine (Dojindo #343-00901, Kumamoto, Japan) in PBS containing 0.01% (v/v) $H_2O_2$. They were then washed with distilled water, counterstained with hematoxylin, dehydrated, mounted with Entellan (Merck #HX612166, Darmstadt, Germany) and then examined under a light microscope.

*Statistical analysis*

Statistical analyses were conducted using the GraphPad Prism v. 5.0 software (GraphPad Software, San Diego, CA, USA). All numerical data are expressed as means ± SEM. and differences were considered to be statistically different at $P<0.05$. In Experiment 1, differences in concentrations of LPA (pmol/100 mg tissue) and expression of LPAR mRNAs and proteins in the CL tissue between cyclic and pregnant cows were assessed by one-way ANOVA followed by Bonferroni's multiple comparison test. In Experiment 2, the differences between control and experimental groups were assayed using one-way ANOVA followed by Bonferroni's multiple comparison test.

## Results

*LPA concentration in the luteal tissue*

Figure 1 shows that the concentrations of LPA in luteal tissue increased ($P<0.05$) between days 2–4 and 17–19 of the estrous cycle, but not between days 8–10 and 17–19 of pregnancy. However, the concentrations of LPA were higher ($P<0.05$) during the estrous cycle than on the corresponding days of pregnancy ($P<0.05$).

*LPAR1, LPAR2, LPAR3 and LPAR4 mRNA expression in luteal tissue*

Figure 2 shows the expression patterns of *LPAR1* (A), *LPAR2* (B), *LPAR3* (C) and *LPAR4* (D) mRNA on days 2–4, 8–10 and 17–19 of the estrous cycle and on days 8–10 and 17–19 of pregnancy**.** With specific primers, enabling amplification of *LPAR1, LPAR2, LPAR3* and *LPAR4*, one strong band migrating at 86, 161, 101 and 192 base pairs (bp; respectively) was observed for each primer in the CL tissue at all examined stages (data not shown).

*LPAR1* transcript abundance normalized to *GAPDH* mRNA expression did not differ during the estrous cycle ($P>0.05$) but was significantly higher on days 8–10 of pregnancy than on days 17–19 of pregnancy ($P<0.05$). Additionally, *LPAR1* transcript abundance on days 8–10 of pregnancy was higher than on all examined days of the estrous cycle ($P<0.05$).

Normalized *LPAR2* and *LPAR4* transcript abundances to *GAPDH* mRNA expression did not significantly change during the estrous cycle ($P>0.05$) but were higher on days 17–19 than on days 8–10 of pregnancy ($P<0.05$). Additionally, *LPAR2* and *LPAR4* transcript abundance on days 17–19 of pregnancy was higher than on all examined days of the estrous cycle ($P<0.05$).

Normalized *LPAR3* transcript abundance to *GAPDH* mRNA expression did not significantly change during either the estrous cycle or pregnancy ($P>0.05$).

*LPAR1, LPAR2, LPAR3 and LPAR4 protein expression in luteal tissue*

Figure 3 shows the expression patterns of LPAR1 (A), LPAR2 (B), LPAR3 (C) and LPAR4 (D) proteins on days 2–4, 8–10 and 17–19 of the estrous cycle and on days 8–10 and 17–19 of pregnancy.

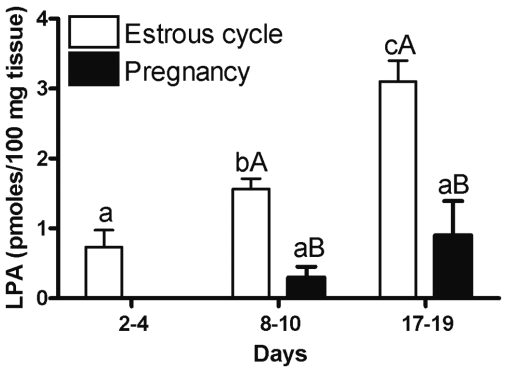


**Fig. 1.** Concentrations of lysophosphatidic acid (pmol/100 mg tissue) in CLs from cyclic cows (white bars) and pregnant cows (black bars). Small superscript letters: a and b indicate statistical differences in the respective LPA concentration within groups of cyclic or pregnant animals, respectively (P<0.05), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. Capital superscript letters: A and B indicate statistical differences in the respective LPA concentration between groups of animals on the same days of the estrous cycle and pregnancy, respectively (P<0.05), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

The relative level of LPAR1 protein tended to increase towards the end of the estrous cycle and early pregnancy, but the changes were not significant.

The relative levels of LPAR2 and LPAR4 proteins did not differ during the estrous cycle (P>0.05), but were higher (P<0.05) on days 17–19 than on days 8–10 of pregnancy. Additionally, the relative levels of LPAR2 and LPAR4 protein on days 17-19 of pregnancy were higher (P<0.05) than on all days of the estrous cycle examined.

The relative level of LPAR3 protein did not differ either during the estrous cycle or pregnancy (P>0.05).

*LPAR1, LPAR2, LPAR3* and *LPAR4* mRNA expressions and protein levels did not significantly change during the estrous cycle (P>0.05).

*Immunohistochemical localization of LPAR1, LPAR2, LPAR3 and LPAR4 in luteal tissue*

The membranes of both large and small luteal cells were positively immunostained for all examined LPARs (LPAR1-4) irrespective of the examined phases of the estrous cycle or early pregnancy. Figure 4 shows the luteal cells in the bovine CL immunostained during the mid-luteal stage. A moderate signal was observed for LPAR1 and LPAR2 (Fig. 4C, 4F). Pale immunostaining was found for LPAR3 in steroidogenic cells (Fig. 4I). The most intense immunostaining was seen for LPAR4 (Fig. 4L) in large and small CL cells. No positive staining was observed in the sections devoid of primary antibodies against LPARs or in sections with irrelevant antibodies (negative control; Fig. 4A, 4B, 4D, 4E, 4G, 4H, 4J, 4K).

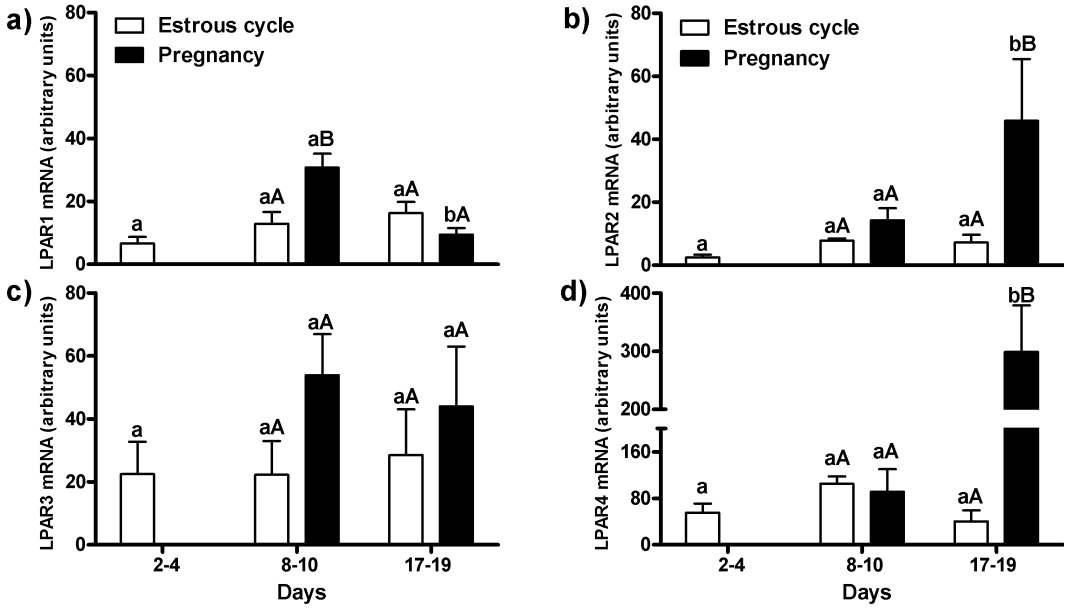


**Fig. 2.** Total receptor for LPA type 1 (LPAR1; A), receptor for LPA type 2 (LPAR2; B), receptor for LPA type 3 (LPAR3; C) and receptor for LPA type 4 (LPAR4; D) mRNA expression normalized to GAPDH gene expression in CLs of cyclic (white bars) and pregnant (black bars) cows. The abundance of mRNAs was determined using real-time PCR. Small superscript letters: a and b indicate statistical differences in mRNA quantitative expression within groups of cyclic or pregnant animals, respectively (P<0.05), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. Capital superscript letters: A and B indicate statistical differences in mRNA quantitative expression between groups of animals on the same days of the estrous cycle and pregnancy, respectively (P<0.05), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

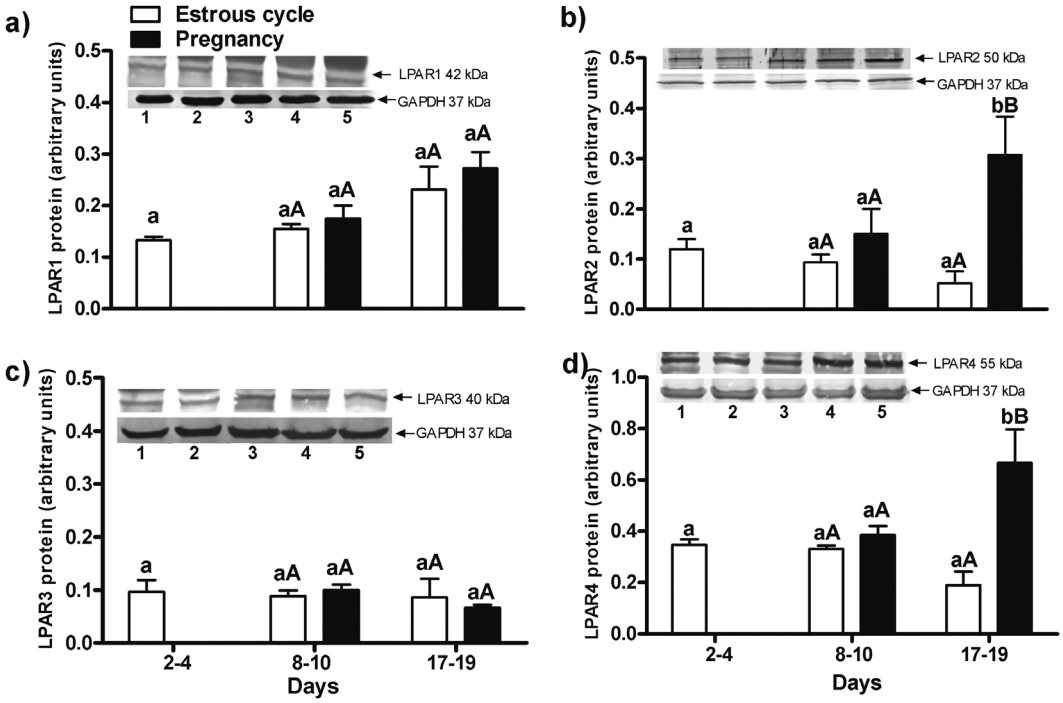


**Fig. 3.** Receptor for LPA type 1 (LPAR1; A), receptor for LPA type 2 (LPAR2; B), receptor for LPA type 3 (LPAR3; C) and receptor for LPA type 4 (LPAR4; D) proteins in CLs of cyclic (white bars) and pregnant (black bars) cows. Representative Western blot samples are shown in the upper panel. Small superscript letters: a and b indicate statistical differences in the respective protein level within groups of cyclic or pregnant animals, respectively ($P<0.05$), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. Capital superscript letters: A and B indicate statistical differences in protein level between groups of animals on the same days of the estrous cycle and pregnancy, respectively ($P<0.05$), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

Figure 5 shows the luteal cells in the bovine CL immunostained on days 17–19 of early pregnancy. A positive signal was observed for LPAR1, LPAR2, LPAR3 and LPAR4 (Fig. 5C, 5F, 5I, 5L) with no visible difference in the intensity in large and small CL cells. No positive staining was observed in the sections devoid of primary antibodies against LPARs or in sections with irrelevant antibodies (negative control; Fig. 5A, 5B, 5D, 5E, 5G, 5H, 5J, 5K).

*Effect of LPA, IFNτ and LPA with IFNτ on P4 production by steroidogenic cells*

Figure 6 shows the effect of LH, LPA, IFNτ and LPA with IFNτ on P4 production by cultured bovine steroidogenic cells. LH, LPA and LPA with IFNτ stimulated ($P<0.05$) production of P4 by cultured bovine steroidogenic cells obtained from cows in the mid-luteal stage of the estrous cycle. LPA stimulated P4 production by 60%. However, we did not find any modulation of IFNτ action by LPA on P4 production by steroidogenic cells ($P>0.05$).

*Effect of LPA, IFNτ and LPA with IFNτ on the expression of enzymes involved in steroidogenesis*

Figure 7 shows the effect of LPA, IFNτ and LPA with IFNτ on mRNA (A, B, C, D) and protein (E, F, G, H) expression of cholesterol esterase, StAR, P450scc and 3βHSD in cultured bovine steroidogenic cells. LPA and LPA with IFNτ stimulated 3βHSD gene and protein expressions in steroidogenic cells (D, H, respectively). LPA stimulated the expression of 3βHSD by 25%. We did not find any modulation of IFNτ action by LPA on the expression of an enzymes involved in steroidogenesis in steroidogenic cells ($P>0.05$).

*Effect of LPA, IFNτ and LPA with IFNτ on the expression of interferon-stimulated genes in the luteal cells of the bovine CL*

Figure 8 shows the effect of LPA, IFNτ and LPA with IFNτ on mRNA (A, B) and protein (C, D) expression of *interferon stimulated gene 15 (ISG15)* and *2,5'-oligoadenylate synthase (OAS1)* in cultured bovine steroidogenic cells. LPA did not influence ISG15 or OAS1 mRNA and protein expressions ($P>0.05$). IFNτ and LPA with IFNτ stimulated ISG15 and OAS1 mRNA and protein expressions ($P<0.05$). LPA augmented IFNτ–dependent stimulation of ISG15 and OAS1 mRNA and protein expressions in steroidogenic cells ($P<0.05$).

## Discussion

In the present study, we found that LPA is present in picomole concentrations in the bovine CL throughout the estrous cycle and early pregnancy. The lowest concentration of LPA at the beginning of the estrous cycle is comparable to that occurring during early pregnancy. The concentration of LPA increased from days 2–4 to days 17–19 of the estrous cycle. The detected presence of LPA in the

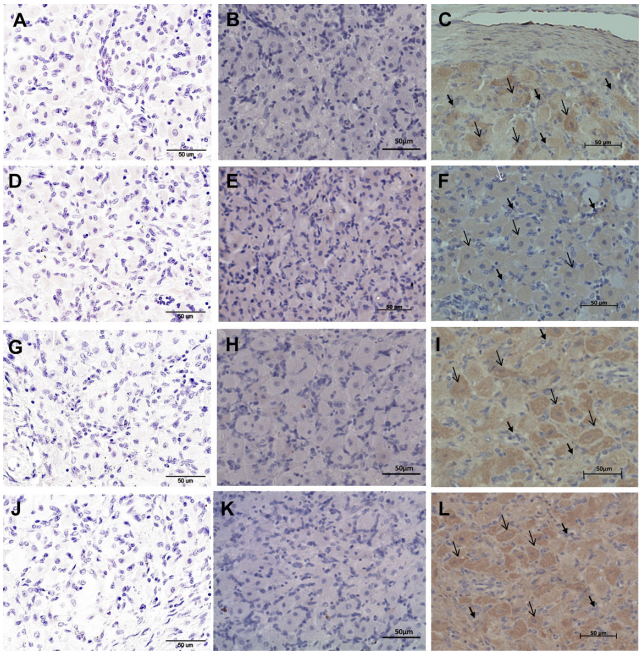


**Fig. 4.** Representative micrographs of receptor for LPA type 1 (LPAR1; C), receptor for LPA type 2 (LPAR2; F), receptor for LPA type 3 (LPAR3; I) and receptor for LPA type 4 (LPAR4; L) in CLs from the mid-luteal stage of estrous cycle. Control immunohistochemistry was performed by omitting the primary antibodies (images A, D, G and J) or using irrelevant IgG (images B, E, H, and K). Bars = 50 µm. Large, thin arrows represent large luteal cells, and small, thick arrows represent small luteal cells.

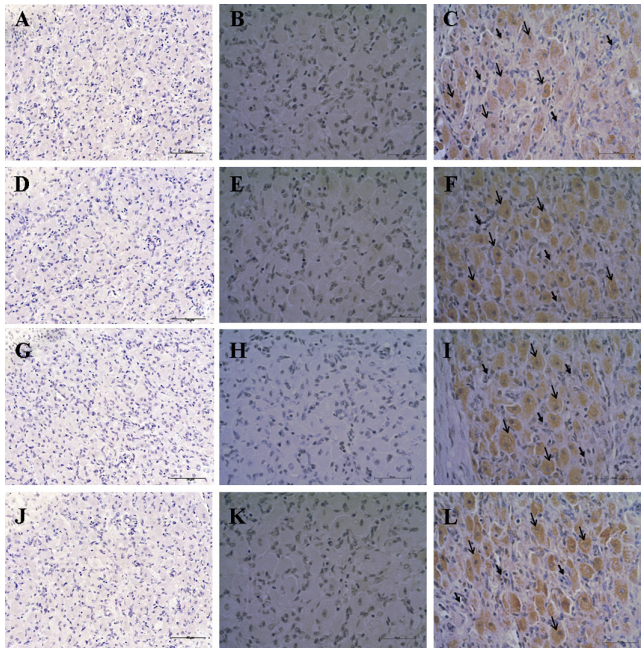


**Fig. 5.** Representative micrographs of receptor for LPA type 1 (LPAR1; C), receptor for LPA type 2 (LPAR2; F), receptor for LPA type 3 (LPAR3; I) and receptor for LPA type 4 (LPAR4; L) in CLs from days 17–19 of early pregnancy. Control immunohistochemistry was performed by omitting the primary antibodies (images A, D, G and J) or using irrelevant IgG (images B, E, H and K). Bars = 50 µm. Large, thin arrows represent large luteal cells, and small, thick arrows represent small luteal cells.

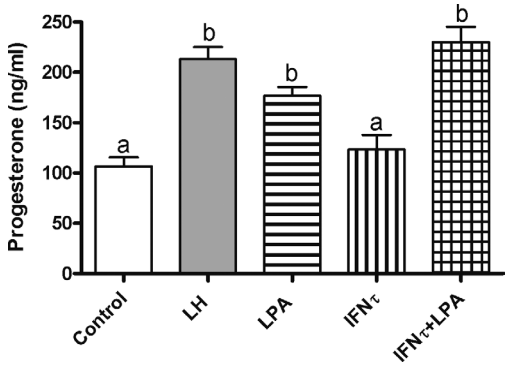


**Fig. 6.** Production of P4 by luteal cells isolated from bovine CLs from the mid-luteal stage of the estrous cycle in response to LPA, LH, IFNτ and IFNτ with LPA. LPA ($10^{-6}$ M), LH (100 ng/ml), IFNτ ($10^6$ AVU/ml) and IFNτ with LPA were added 12 h before the end of the culture. Different letters indicate significant differences ($P<0.05$) as determined by one-way ANOVA followed by Bonfferoni's multiple comparison test.

CL tissue indicates that this gland, in addition to the uterus [21], is a site of LPA synthesis during the estrous cycle. We previously showed that LPA concentrations were much higher in the blood plasma and bovine endometrial tissue [21] than the concentrations found in CL tissue in this study. Therefore, the concentrations of LPA originating from the blood plasma, uterus and CL under physiological conditions influence the functions of the luteal cells. In view of the cumulative effect of LPA originating from the endometrium, blood and CL, we decided to use micromole concentrations of LPA for *in vitro* experiments. On the other hand, the expressions of LPA receptors (especially LPAR2 and LPAR4) were 2–4 times higher during early pregnancy than during the respective days of the estrous cycle. The higher expression of LPA receptors during early pregnancy could explain their major role during early pregnancy in cattle, which is also consistent with our previous results [31]. Taking into consideration the probable role of LPA during pregnancy recognition (at the time of the highest production of IFNτ by the conceptus trophectoderm), one of the aims of the present study was to examine the potential influence of LPA on the modulation of IFNτ action in the luteal cells of the bovine CL.

The pleiotropic roles of LPA in reproductive physiology are demonstrated not only by the increased concentration of LPA in body tissues and fluids [41, 42] but also by the regulated expression of its receptors [43–46]. LPA exerts its biological effects via four G-protein-coupled membrane receptors (LPAR1/EDG2, LPAR2/EDG4, LPAR3/EDG7 and GPR23/p2y9/LPAR4) [28–30, 46, 47]. We found all types of LPARs in the CL tissue at both the mRNA and protein level. However, of the four LPARs examined, LPAR2 and LPAR4 were expressed the most strongly. We also found higher LPAR1 mRNA expression on days 8–10 of pregnancy than on days

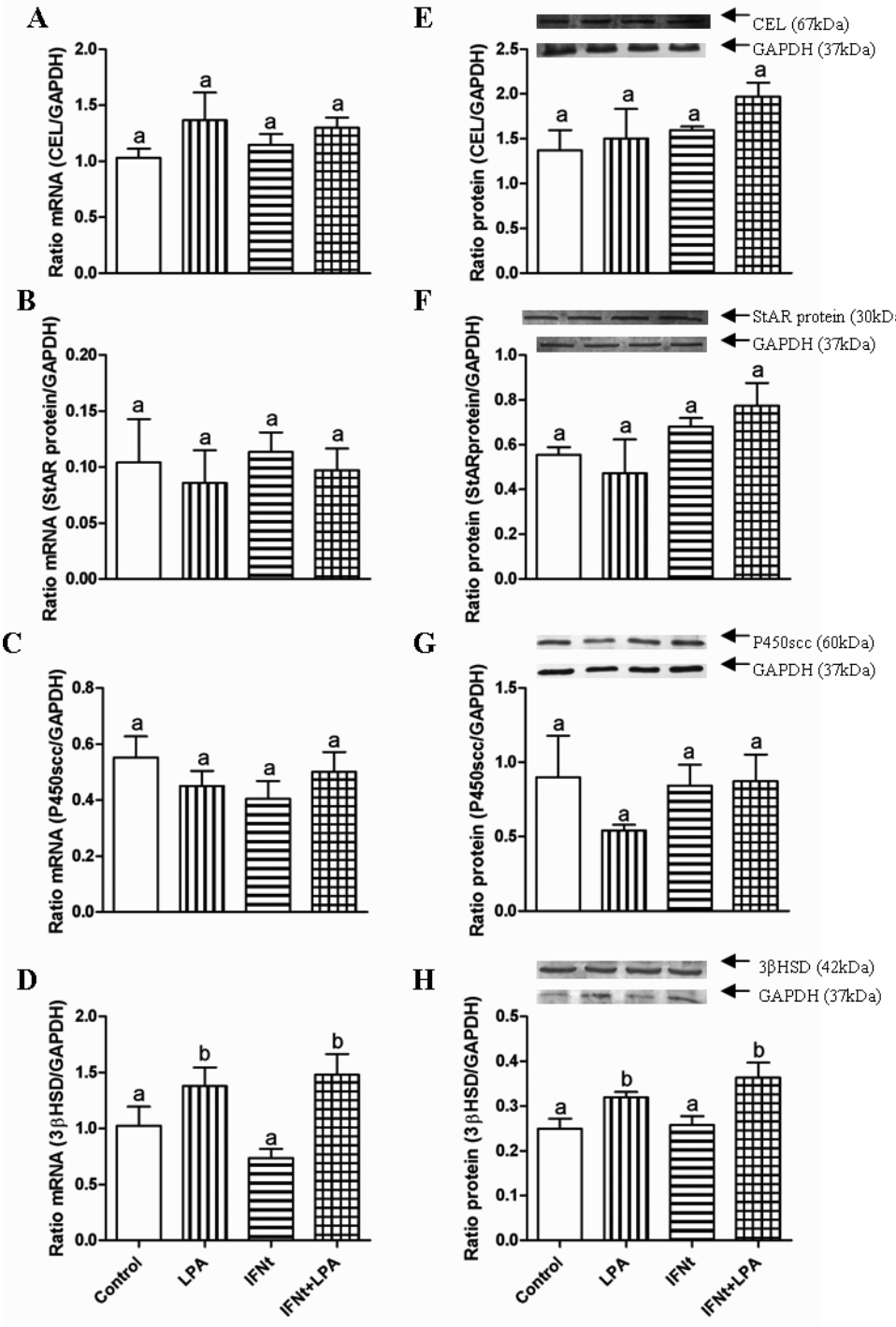


**Fig. 7.** Expression of mRNAs (A, B, C, D) and proteins (E, F, G, H) for cholesterol esterase (CEL), steroid acute regulatory (StAR) protein, cytochrome P450 side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase/5Δ-4Δ isomerase (3βHSD), respectively, in luteal cells isolated from CLs from the mid-luteal stage of the estrous cycle. LPA ($10^{-6}$ M), IFNτ ($10^{6}$ AVU/ml) and IFNτ with LPA were added 6 or 12 h before the end of the culture. All values are expressed as the mean ± SEM of CEL, StAR, P450scc and 3βHSD expression. Different letters indicate significant differences ($P<0.05$) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

17–19 of pregnancy and on other examined days of the estrous cycle. However, this result was not confirmed by the protein level for LPAR1. Therefore, we think that, because of posttranslational changes of this protein, the actual level of functional protein for LPAR1 in the cell did not differ during the estrous cycle or early pregnancy. The high mRNA and protein expressions of LPAR4 compared with the other receptors during the estrous cycle and early pregnancy, as well as the dynamic changes of LPAR2 and LPAR4 during early pregnancy, probably accounts for the contribution of LPA to different events during the estrous cycle and pregnancy, namely the contribution to P4 secretion or modulation of IFNτ action during early pregnancy. The expression of all LPARs in the bovine CL during the estrous cycle and early pregnancy does not completely agree with the results of Budnik and Brunswig-Spickenheier [48], who showed that LPA exerts its actions on bovine luteal cells only via LPAR2. We hypothesize that during the estrous cycle, LPA originating from the CL might exert

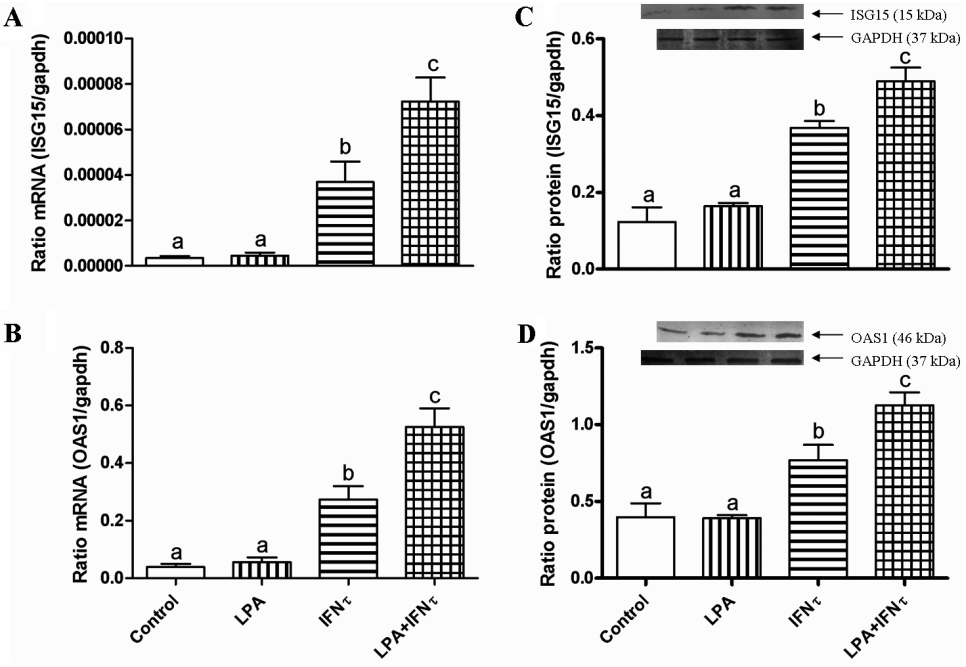


**Fig. 8.** Expression of mRNAs (A, B) and proteins (C, D) for 2,5'-oligoadenylate synthase (OAS1) and ubiquitin-like IFN-stimulated gene 15-kDa protein (ISG15), respectively, in luteal cells isolated from CLs from the mid-luteal stage of the estrous cycle. LPA ($10^{-6}$ M), IFNτ ($10^{6}$ AVU/ml) and IFNτ with LPA were added 6 or 12 h before the end of the culture. All values are expressed as the mean ± SEM of OAS1 and ISG15. Different letters indicate significant differences ($P<0.05$) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

potential autocrine and paracrine as well as endocrine roles at the time of CL development and maintenance (during early pregnancy), probably acting via all LPARs. During early pregnancy, LPAR2 and LPAR4 appear to be most important in mediating the actions of LPA. Therefore, we hypothesize that during early pregnancy LPA originating from the CL as well as from the uterus and blood plasma [21] probably exert a luteotropic and/or luteoprotective effect on the bovine CL via LPAR2 and LPAR4. This is in accordance with data previously reported showing that the infusion of LPA during early pregnancy in the cow increased the concentrations of P4 and $PGE_2$ in the periferic blood [31].

We previously showed that LPA administered into the *aorta abdominalis* [21] or intravaginally [31] affected P4 secretion in conscious cows during the luteal phase of the estrous cycle. Moreover, the infusion of heifers with LPA prevented spontaneous luteolysis and prolonged the functional lifespan of the CL [31]. In the present study, we investigated whether LPA has a direct effect on P4 secretion from bovine luteal cells *in vitro* and whether it modulates IFNτ action in the luteal cells. We found that LPA stimulated P4 secretion from steroidogenic CL cells of the mid-luteal phase. These results are important because the mid-luteal stage represents a critical period in the CL lifespan for secretion of P4 [6]. We hypothesize that at the examined time of estrous cycle, if the female becomes pregnant, continued secretion of P4 from the CL can also be supported by LPA. In the present study, we did not find any modulation of the pregnancy recognition signal (IFNτ) action on P4 secretion in the luteal cells of the bovine CL. On the other hand, Budnik and Brunswig-Spickenheier [48] reported that LPA inhibits LH-induced secretion of P4 by bovine CL cells *in vitro*. In contrast to our cells, the cells used in the study of Budnik and Brunswig-Spickenheier [48] were isolated differently and consisted of the four major kinds of luteal cells, steroidogenic, endothelial, fibroblasts and immune cells. Therefore, the inhibitory effect of LPA on LH-induced secretion of P4 documented by Budnik and Brunswig-Spickenheier [48], might have been mediated by various interactions between these cells. Moreover, the above authors cultured the cells for 7 days until they were confluent, while we cultured the cells until just after they became attached to the plates. Thus, their cells after 7–10 days of culture may not have the same physiological properties as cells at defined days of the estrous cycle and pregnancy. Finally, we stimulated luteal cells with LPA for a longer period of time and studied the direct effect of LPA and LPA with IFNτ on P4 production rather than the LPA modulation of LH-induced P4 production in bovine luteal cells.

The mechanism of action of luteosupportive substances such as P4 [49], $PGE_2$ [50, 51] and LH [10] is related to the increase in mRNA and protein expression of enzymes involved in steroidogenesis [52]. Thus, the present study evaluated effects of LPA on mRNA and protein expression for cholesterol esterase, known as the rate-limiting enzyme in the steroidogenic pathway [53], as well as steroid acute regulatory (StAR) protein, cytochrome P450 side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase/5Δ-4Δ isomerase (3βHSD), which are recognized as luteal markers [53], in the luteal CL cells. StAR protein transports cholesterol from the outer to the inner mitochondrial membrane [54, 55], cytochrome P450scc converts

cholesterol to pregnenolone, and 3βHSD converts pregnenolone into P4 in the smooth endoplasmic reticulum [56, 57]. We found that LPA only stimulated mRNA and protein expression of 3βHSD in the luteal cells and had no effect on mRNA and protein expressions for cholesterol esterase, StAR protein and cytochrome P450scc. We did not find any effect of LPA on the modulation of IFNτ action on the expression of the enzymes involved in steroidogenesis. The above findings partially agree with the results of Budnik and Brunswig-Spickenheier [47]. They did not observe any effect of LPA on the StAR protein expression in mitochondrial fractions derived from CLs in the mid-luteal phase of the estrous cycle. The observed stimulatory effect of LPA on the terminal enzyme responsible for P4 synthesis and P4 itself indicates that LPA can play a luteosupportive role during the estrous cycle in cows. In the present study, we proved that LPA augmented IFNτ−dependent stimulation of ISG15 and OAS1 mRNA and protein expressions in the steroidogenic cells. These two genes are expressed in the bovine CL of both cyclic and pregnant cows regardless of pregnancy status but are upregulated only during early pregnancy [8, 9].

The dynamic changes in the LPA concentration and LPARs expressions in the CL tissue during the estrous cycle and early pregnancy as well as the stimulatory effect of LPA on P4 synthesis and IFNτ action in the steroidogenic cells of the bovine CL indicate its potential autocrine and paracrine roles in the bovine CL. Further studies should evaluate the potential role of LPA on the luteolytic mechanism. We previously reported that LPA concentrations in bovine endometrial tissue did not significantly change due to day of the estrous cycle but were highest on days 17–19 of pregnancy [21]. The LPA concentration in the endometrial tissue [21] was twice the average concentration observed during the estrous cycle in the CL tissue in the present study. Together, the previous and present data indicate that both the uterus and CL are sites of LPA synthesis in the bovine reproductive tract. During the estrous cycle, LPA from the CL exerts mainly autocrine and paracrine effects on CL development and synthesis of P4, whereas during early pregnancy, LPA originating mainly from the uterus [21] affects the CL indirectly (e.g., via uterine $PGE_2$, as documented by Woclawek-Potocka *et al*. [21, 22], or increased IFNτ-dependent stimulation of interferon-stimulated gene expression) or directly if LPA reaches the ovary by a countercurrent mechanism as documented for $PGF_{2\alpha}$ [reviewed in 57].

In conclusion, our results indicate that the CL, in addition to the uterus, is also a site of LPA synthesis and a target for LPA action in the bovine reproductive tract. We postulate that during the estrous cycle, LPA originating from the CL exerts mainly an autocrine effect on CL development, whereas during early pregnancy, LPA originating mainly from the uterus [21] exerts a paracrine role on the bovine CL via *LPAR2* and *LPAR4*. Finally, our findings that LPA had a stimulatory effect on P4 synthesis via 3βHSD stimulation and that LPA increased IFNτ-dependent stimulation of interferon-stimulated gene expression strongly suggest that LPA is an additional auxiliary luteotropic factor in the steroidogenic cells of the bovine CL.

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