Effects of lysophosphatidic acid on tumor necrosis factor α and interferon γ action in the bovine corpus luteum

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A R T I C L E   I N F O
Article history:
Received 31 January 2013
Received in revised form 5 July 2013
Accepted 5 July 2013
Available online 13 July 2013

Keywords:
Apoptosis
Cow
Cytokines
Lysophosphatidic acid
Luteal cells

A B S T R A C T
We examined the effects of LPA on TNFα and IFNγ – induced decrease of P4 synthesis and on the cytokine – induced apoptosis of the cultured luteal cells. In the steroidogenic luteal cells LPA reversed the inhibitory effect of TNFα and IFNγ on P4 synthesis and also inhibited the stimulatory effects of TNFα and IFNγ on the expression of Bax, TNFR1, Fas and Fast, as well as caspase 3 activity. These results suggest that TNFα and IFNγ cannot induce apoptosis in the presence of LPA, which orientates the steroidogenic luteal cells towards the survival state. In conclusion our results indicate that LPA supports P4 synthesis and action in the bovine CL.

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1. Introduction

Lysophosphatidic acid (LPA) which is a simple, transmembrane lysophospholipid, has been shown to affect female reproductive function in several mammalian species (Tokumura et al., 1999; Seo et al., 2008; Liszewska et al., 2008) including cows (Woclawek-Potocka et al., 2009, 2010). We have previously reported that LPA is locally produced and released from the bovine endometrium (Woclawek-Potocka et al., 2009, 2010) and the corpus luteum (CL) (Kowalczyk-Zieba et al., 2012). We found much higher LPA concentrations in the blood plasma and bovine endometrium (Woclawek-Potocka et al., 2009) than the concentrations found in the CL (Kowalczyk-Zieba et al., 2012). The concentrations of LPA in endometrium were stable during estrous cycle but in the CL were the lowest at the beginning of the estrous cycle and then significantly increased from days 2–4 to days 8–10 and 17–19 of the estrous cycle (Woclawek-Potocka et al., 2009; Kowalczyk-Zieba et al., 2012). In the bovine endometrium, during estrous cycle, lysophosphatidic acid receptor type 1 (LPAR1) expression increased from early to late luteal stage and reached the highest level at late luteal stage (Woclawek-Potocka et al., 2009), whereas in the CL, the expressions of all LPA receptors (LPARs) were stable during the estrous cycle (Kowalczyk-Zieba et al., 2012). Moreover, LPA administered into the abdominal aorta stimulated progesterone (P4) and prostaglandin (PG) E2 secretion during the luteal phase of the estrous cycle and prolonged the CL lifespan in vivo (Woclawek-Potocka et al., 2009, 2010). However, it is unknown whether these luteotropic effects were the result of a direct influence on the CL functions or indirect on luteoprotective mechanisms including inhibition of the CL regression induced by local modulators in the cow.

The bovine CL is an ephemeral ovarian structure with its hormonal regulation well characterized (Pate et al., 2010). The mammalian CL is a heterogeneous tissue which consists of at least three cell types: steroidogenic luteal cells, endothelial cells and immune cells (Farin et al., 1986). The main function of the CL is to secrete P4, which is required for the establishment and maintenance of pregnancy (Niswender and Nett, 1988). However, if the female does not become pregnant, regression of the CL is crucial for regaining the cyclicity as it enables the development of a new ovulatory follicle. During estrous cycle, in the cow, luteolysing, endometrial PGF2α initiates the functional regression of the CL (McCracken et al., 1999). The first outcome of this functional regression of the CL is the inhibition of P4 production. Functional regression of the CL is always followed by the structural regression, characterized by the decrease of the size of the CL and by the loss of the integrity between luteal cells coexisting with progressing apoptosis of the cells (Juengel et al., 1993).

It has been documented before that tumor necrosis factor (TNF) α and interferon (IFN) γ, produced locally by luteal immune cells...
play an important role in functional luteolysis and apoptosis of steroidogenic and endothelial cells in the bovine CL (Friedman et al., 2000; Sakamoto et al., 2000; Hojo et al., 2010). Furthermore, since LPA is locally produced and released from the bovine CL (Kowalczyk-Zieba et al., 2012) and stimulates P4 and PGE2 secretion during the luteal phase of the estrous cycle in vivo (Woclawek-Potocka et al., 2009, 2010), we suspected that this lysophospholipid could support luteotropic and luteoprotective mechanisms acting directly on the luteal steroidogenesis or/and infiltrating TNFα and IFNγ – induced functional and structural regression of the CL.

It has been well documented that apoptosis occurs through two main pathways. In one pathway, high levels of caspase (Casp) 8 at the death-inducing signaling complex directly initiate cleavage of other downstream effector caspases, such as Casp3, thereby initiating the execution phase of apoptosis (Scaffidi et al., 1998). In the second-mitochondrial pathway, active Casp8 initiates the sequence of events that stimulate the binding of proapoptotic members of the Bcl2 family (Bax) to mitochondria and inhibits association of antiapoptotic members of the Bcl2 family (Bcl-2). This leads to the leak- 
age of cytochrome c from the mitochondria into the cytosol, which in turn promotes formation of the apoptosome and triggers activation of effector Casp3 (Scaffidi et al., 1998). Apoptosis on the receptor level can be initiated via several cytokine receptors including TNF super family receptors (TNFRs). Besides TNFR1 and TNFR2, the Fas antigen (Fas) also belongs to the TNF super family receptors which together with Fas ligand (FasL) transmit basic signals controlling intercellular apoptosis pathway (Nagata, 1997).

The aim of the present study was to determine whether LPA mediates the TNFα and IFNγ luteolytic action in the bovine CL. For this purpose we investigated: (1) the effects of LPA on TNFα and IFNγ – induced decrease of P4 synthesis in the cultured steroidogenic luteal cells, (2) the effects of LPA on TNFα and IFNγ – induced apoptosis of the cultured steroidogenic luteal cells.

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 the experiments, normally cycling Holstein/Polish Black and White (75/25%; respectively) cows (n = 30) from two dairy herds were used (Years 2007–2013). The animals were culled because of their low milk production. Estrus was synchronized in the cows by two injections of an analogue of PGF2α (dinoprost, 5 mg i.m with an 11-day interval; Dinolytic; Upjohn – Pharmacia N.V.S.A., Belgium). Estrus was detected by the indicative signs (i.e. vaginal mucus, standing behavior), and confirmed by a veterinarian using ultrasonography (USG) and by per rectum examination. Cows were monitored every day with USG and only cows with behavioral signs of estrus and confirmed by USG presence of the CL on the ovary were chosen for the study (n = 27). Estrus was taken as Day 0 of the estrous cycle. On day 8–12 of the estrous cycle the cows were slaughtered and the reproductive tracts examined. Stages of the estrous cycle were confirmed by macroscopic observation of the ovaries and uterus (Miyamoto et al., 2000).

2.2. Experimental procedure

2.2.1. Experiment 1: The influence of LPA on TNFα and IFNγ – induced decrease of P4 synthesis in the cultured steroidogenic luteal cells

Ovaries were collected after slaughter at Days 8–12 of the estrous cycle (n = 4; for each repetition, luteal cells were isolated from the pool of four CLs). Enzymatic dissociation of the luteal tissue and culture of steroidogenic cells of the CL were performed as previously described (Kowalczyk-Zieba et al., 2012). Cell viability was greater than 85% as assessed by trypan blue staining. The cell suspension contained about 20–25% of large steroidogenic luteal cells, 70–75% of small steroidogenic luteal cells and less than 5% of endothelial cells and fibroblasts, without erythrocytes as determined on the automated cell counter – Countess® purchased from Invitrogen (Kowalczyk-Zieba et al., 2012). The final pellet of steroidogenic cells was suspended in culture medium, Dulbecco modified Eagle medium and Ham’s F-12 medium (DMEM/Ham’s F-12, 1:1 [v/v]; Sigma; #D8900) containing 10% calf serum (Gibco BRL; #16170-078) and 20 μg/ml gentamicin (Gibco BRL; #15750-060). Dispersed luteal cells were seeded at 5.0 × 10^6 viable cells/ml in 6-well plates (COSTAR®, Corning® CellBIND® Surface; #3335) and cultured at 37.5 °C in a humidified atmosphere of 5% CO2 and 95% air. After a 24 h culture, cells were washed three times with PBS and 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 a LPA agonist (1 oleoyl-sn-glycero-3-lysophosphatidic acid sodium salt, Alexis; #ALS 300-139-M005; LPA; 435 ng/ml), TNFα + IFNγ (50 ng/ml each; Dainippon, Sumitomo Pharma Co., Ltd., Osaka, Japan) and LPA + TNFα + IFNγ for 6 (mRNA evaluation) and 12 h (protein evaluation and measurement of P4 concentration). The appropriate dose of LPA agonist was defined before (Kowalczyk-Zieba et al., 2012). After incubation, the conditioned media were collected and frozen at –20 °C until measurement of P4 by an enzyme immunoassay (EIA) as previously described (Woclawek-Potocka et al., 2005). Gene expression of the enzymes involved in steroidogenesis (Star protein, P450scc and 17α-HSD) was quantitatively measured by real-time PCR. The cells for real-time PCR were disrupted with TRIZOL reagent (Invitrogen; #15596) 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 Blotting analysis. The proteins were extracted using the Radio Immuno Precipitation Assay buffer (RIPA buffer) in the presence of the protease inhibitor cocktail (ROCHE; #11697498001).

2.2.2. Experiment 2: The influence of LPA on TNFα and IFNγ – induced apoptosis of the cultured steroidogenic luteal cells

Steroidogenic luteal cells were isolated and cultured in the same way as in Experiment 1. After the culture, the cells were exposed to LPA (435 ng/ml), TNFα + IFNγ (50 ng/ml each) and LPA + TNFα + IFNγ for 6 (mRNA evaluation) and 12 h (protein evaluation). Gene expression and protein level of the factors involved in apoptosis (proapoptotic Bax, antiapoptotic Bcl-2, TNFα, TNFR1, TNFR2, Fas, Fasl, Casp8 and Casp3) were quantitatively measured by Real-time PCR and Western Blotting, respectively. Additionally in the steroidogenic luteal cells after 12 h incubation with stimulators, cleaved caspase 3 activity was examined by the colorimetric Caspase 3 Assay Kit according to the manufacturer instructions (Casp-3-C). The cells for Real-time PCR were disrupted with TRIZOL reagent (Invitrogen; #15596) and frozen at –80 °C until they were processed for RNA isolation. The protein level for the factors involved in apoptosis in luteal cells was measured by Western Blotting analysis. The proteins were extracted using the Radio Immuno Precipitation Assay buffer (RIPA buffer) in the presence of the protease inhibitor cocktail (ROCHE; #11697498001).

2.3. 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). The RT reaction was performed in a total reaction volume of 20 µl, following manufacturer’s instructions and products stored at −20 °C until real-time PC amplification.

The expression of mRNA for all examined genes was conducted by Real time PCR using specific primers for StAR protein, P450scc, 3αHSD, proapoptotic Bax, antian apoptotic Bcl-2, TNFα, TNFR1, TNFR2, Fas, Fasl, Casp8 and Casp3. 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. The efficiency of PCR reaction for all the primers was between 90% and 110%.

Real-time PCR was performed with an ABI Prism 7900 sequence detection system using Power SYBR Green PCR master mix (Applied Biosystems; #4367659). The PCR reactions were performed in 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), miner software was used (http://www.miner.e windup.info/version2). 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 s and at 60 °C for 60 s. Each PCR reaction was followed by obtaining melting curves by 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.

2.4. Western blotting 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 mM Tris base pH 7.2, 0.1% SDS, 1% Triton X100, 0.5% Sodium Deoxycholate and 5 mM EDTA, in the presence of protease inhibitor cocktail (ROCHE; #11697498001). Lysates were then sonicated and centrifuged at 10,000 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 (1976). 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 on a 12% SDS–PAGE.Separated proteins were electrobotted using semi dry transfer method onto polyvinylidene difluoride (Immobilon-P Transfer Membrane; Millipore # IPVH00010) in transfer buffer (containing three different buffers: Anode buffer 1–0.3 M Tris buffer, pH 10.4, 10% methanol; Anode buffer 2–25 mM Tris buffer, pH 10.4, 10% methanol; Kathode buffer – 25 mM Tris buffer, pH 9.4, 10% methanol, 40 mM glycine). After blocking in 5% non-fat dry milk in TBS-T buffer (Tris-buffered saline, containing 0.1% Tween-20) for 1.5 h at 25.6 °C, the membranes were incubated overnight with: rabbit polyclonal anti-StAR protein, 3αHSD, proapoptotic Bax, antian apoptotic Bcl-2, Fas, Fasl, antibody (concentration for all antibodies 1:100; Santa Cruz Biotechnology; #sc-25806; #sc-28206; #sc-526; #sc-783; #sc-7886; #sc-834 respectively), rabbit polyclonal anti-Casp8 and Casp3 (concentration for both antibodies: 1:1000; Abcam; #ab-4052; #ab 90437), goat polyclonal anti-P450scc, TNFα, TNFR1 and TNFR2 antibody (concentration for all antibodies 1:100; Santa Cruz Biotechnology; #sc-18043; #sc-1348; #sc-1067; #sc-1074; respectively) and mouse polyclonal anti-GAPDH antibody (concentration 0.05 µg/ml; Sigma; #G8795) at 4 °C. The

| Gene       | Primer sequences                     | GenBank [acc. No.] | PCR product size [bp] |
|------------|--------------------------------------|--------------------|-----------------------|
| StAR protein | 5′GGTGCTGCCAGCTTTTCAAT3′, 5′CTTGCCTCCAGATCCTCCTG′ | Y17259.1           | 79                    |
| P450scc    | 5′CAGCATACTGAGCTATGCTGA′, 5′GCCAGCAAGACCATGAAA3′ | K02130.1           | 139                   |
| 3αHSD      | 5′TCCGGAGTACGCTCTCTAT3′, 5′ACTAGTGGCCTGTTAGCAG′ | NM_174343.2        | 116                   |
| Bax        | 5′GTCGCTGTGATGTCGGAG′, 5′CCGTCGGATGTCAGC′ | U92434.1           | 203                   |
| Bcl2       | 5′GGCTCCAGAGCGTGGTGT′, 5′GCTTTCCCCACACAGGAG′ | NM_173966.2        | 110                   |
| TNF        | 5′GCCAGTTGACCTCTCCCTC′, 5′TGGCTTCTTACCCAGGCT′ | U90937.1           | 110                   |
| TNFR1      | 5′CCAGTGCTGCTGCTATC′, 5′CCAGGAGCTGCTCTT′ | NM_00104090.2      | 113                   |
| TNFR2      | 5′CCAGGAGCTGCTGCTCTT′, 5′CCAGGAGCTGCTCTT′ | NM_174662.2        | 148                   |
| Fas         | 5′AACTGCTGGGTTGGCTCATG′ | NM_00108859.1      | 85                    |
| Fasl       | 5′CCAACAGCAGCAGGCACTTTA′ | NM_00108859.1      | 173                   |
| Casp8      | 5′CTGAGAGAGAGAGCTGG′, 5′CCGCTTCTGACGTTGAGG′ | Y102590.1          | 163                   |
| Casp3      | 5′CTGAGAGAGAGAGCTGG′, 5′CCGCTTCTGACGTTGAGG′ | NM_001077840.1     | 105                   |
| GAPDH (Bos taurus) | 5′ACCTCCAAGATTGTCACGA3′, 5′GGTCTAAATGTCCTACGCAG3′ | BC102589           | 103                   |
2.5. Statistical analysis

Statistical analyses were conducted using the software GraphPad PRISM v. 5.0 (GraphPad Software, Inc., San Diego, CA). All numerical data are expressed as mean ± S.E.M. and differences were considered to be statistically different at $P < 0.05$. Differences in P4 concentration and Casp3 activity were analyzed using one-way ANOVA tests followed by the Bonferroni Comparison Post Test, comparing all pairs of columns. Differences in gene and protein expressions were analyzed using one-way ANOVA followed by Bonferroni’s multiple comparison test, comparing all pairs of columns.

3. Results

3.1. Effect of LPA on TNF$\alpha$ and IFN$\gamma$ – induced decrease of P4 synthesis in the cultured steroidogenic luteal cells

LPA partially reversed the inhibitory effect of TNF$\alpha$ and IFN$\gamma$ on P4 synthesis in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 1; $P < 0.05$).

Fig. 1. Production of P4 by luteal cells isolated from bovine CL from the mid-luteal stage of estrous cycle in response to LPA, TNF$\alpha$ + IFN$\gamma$ and TNF$\alpha$ + IFN$\gamma$ with LPA. LPA (435 ng/ml), TNF$\alpha$ + IFN$\gamma$ (both 50 ng/ml) and TNF$\alpha$ + IFN$\gamma$ with LPA were added 12 h before the end of the culture. All values are expressed as the mean of four replicates ± SEM of P4 concentration in the culture medium. Different letters indicate significant differences ($P < 0.05$), as determined by one-way ANOVA followed by Bonferroni’s multiple comparison test.

Fig. 2. Expression of mRNAs (a–c) and proteins (d–f) for Steroid Acute Regulatory (StAR) protein, cytochrome P450 side-chain cleavage (P450scc) and 3$\beta$-hydroxysteroid dehydrogenase/5$\alpha$–4$\alpha$ isomerase (3$\beta$HSD), respectively in luteal cells isolated from CL from the mid-luteal stage of estrous cycle. Representative Western Blotting samples are shown in the upper panel (d–f). Cells were incubated with: LPA (435 ng/ml), TNF$\alpha$ + IFN$\gamma$ (both 50 ng/ml) and TNF$\alpha$ + IFN$\gamma$ with LPA for 6 (for mRNA expression measurement) or 12 (for protein level measurement) hours. All values are expressed as the mean of four replicates ± SEM of StAR, P450scc expression and 3$\beta$HSD. Different letters indicate significant differences ($P < 0.05$), as determined by one-way ANOVA followed by Bonferroni’s multiple comparison test.
None of the examined substances influence the expression of mRNA and protein of STAR protein or P450scc in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 2; P > 0.05). LPA stimulated the expression of mRNA and protein of 3βHSD in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 2c and d; P < 0.05). We did not find any LPA-induced modulation of the influence of TNFα and IFNγ on the expression of the enzymes involved in steroidogenesis in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 2; P > 0.05).

3.2. Effect of LPA on TNFα and IFNγ – induced apoptosis of the cultured steroidogenic luteal cells

LPA inhibited the stimulatory effect of TNFα and IFNγ on the expression of mRNA and protein of Bax in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 3a and c; P < 0.05). None of the examined substances influenced the expression of mRNA and protein of Bcl2 in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 3b and d; P > 0.05).

The administration of LPA alone did not influence the expression of mRNA and protein of TNFα, TNFR1 or TNFR2 in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 4; P > 0.05). TNFα together with IFNγ elevated the protein level of TNFα and TNFR1 in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 4d and e; P < 0.05). LPA inhibited only the stimulatory effect of TNFα and IFNγ on the protein level of TNFR1 in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 4e; P < 0.05).

Although, we did not find any effect of LPA on the expression of mRNA and protein of Fas (Fig. 5a and c; P > 0.05), LPA inhibited the expression of mRNA and protein of Fasl in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 5b and d; P < 0.05). We also found that in the presence of LPA, TNFα and IFNγ did not stimulate mRNA and protein expression of Fas and Fasl in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 5; P < 0.05).

TNFα and IFNγ stimulated the expression of mRNA and protein for Casp8 and Casp3 in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 6; P < 0.05). We did not find any influence of LPA on the stimulatory effect of TNFα and IFNγ on the expression of mRNA and protein of Casp8 and Casp3 in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 6; P > 0.05).

TNFα and IFNγ increased Casp3 activity induced by cytokines in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 7; P < 0.05). LPA decreased Casp3 activity induced by cytokines in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle (Fig. 7; P < 0.05).

4. Discussion

The present study showed that LPA reversed the inhibitory effect of TNFα and IFNγ on P4 synthesis as well as suppressed the cytokine-induced luteal cell apoptosis. Lyosphosphatidic acid synthesized in the blood plasma and area of reproductive system systemically and locally affects function of many organs. We have documented before that the crucial roles of LPA in the function of reproductive organs are demonstrated not only by the high concentration of LPA in body tissues and fluids but also by the regulated expression of its receptors in the target tissues. In this aspect, we have recently found that LPA concentrations in the blood plasma of uterine vein and bovine endometrial tissue (Woclawek-Potocka et al., 2009) were much higher than the concentrations found in luteal tissue (Kowalczyk-Zieba et al., 2012). However, the concentrations of LPA in endometrial tissue were high but stable during the estrous cycle (Woclawek-Potocka et al., 2009). On the other hand, in the bovine CL, the concentrations of LPA were increasing towards the end of the estrous cycle (Kowalczyk-Zieba et al., 2012). In the bovine endometrium, during estrous cycle, LPAR1 expression increased from early to late luteal cycle (Kowalczyk-Zieba et al., 2012) and TNFα together with IFNγ (both 50 ng/ml) and TNFα + IFNγ with LPA for 6 (for mRNA expression measurement) or 12 (for protein level measurement) hours. All values are expressed as the mean of four replicates ± SEM of Bax, Bcl2 and Bax/Bcl2 ratio. Different letters indicate significant differences (P < 0.05), as determined by one-way ANOVA followed by Bonferroni’s multiple comparison test.

Fig. 3. Expression of mRNAs (a and b) and proteins (c and d) for Bax, Bcl2 and Bax/Bcl2 ratio, respectively in luteal cells isolated from CL from the mid-luteal stage of estrous cycle. Representative Western Blotting samples are shown in the upper panel (c and d). Cells were incubated with: LPA (435 ng/ml), TNFα + IFNγ (both 50 ng/ml) and TNFα + IFNγ with LPA for 6 (for mRNA expression measurement) or 12 (for protein level measurement) hours. All values are expressed as the mean of four replicates ± SEM of Bax, Bcl2 and Bax/Bcl2 ratio. Different letters indicate significant differences (P < 0.05), as determined by one-way ANOVA followed by Bonferroni’s multiple comparison test.
stage and reached the highest level at late luteal stage (Woclawek-Potocka et al., 2009), whereas in the CL, the expressions of all LPA receptors (LPARs) were stable during the estrous cycle (Kowalczyk-Zieba et al., 2012). Moreover, the expression of LPARs were significantly higher during early pregnancy than during the respective days of the estrous cycle both in the CL and uterus (Woclawek-Potocka et al., 2009; Kowalczyk-Zieba et al., 2012). Therefore, we suspected that LPA originating from the activated platelets in blood plasma, uterus and CL, under physiological conditions influenced the functions of the luteal cells, especially during early pregnancy and the late luteal stage – when the luteolyzising cytokines act the most. In the first part of the present study we have documented that LPA reversed the inhibitory effect of TNFα and IFNγ on P4 synthesis. These data proclaim modulatory influence of LPA on the regulated by cytokines P4 synthesis in the bovine steroidogenic luteal cells.

It has been documented before that TNFα together with IFNγ inhibited P4 production in the cultured bovine steroidogenic luteal cells (Friedman et al., 2000; Petroff et al., 2001) as well as induced apoptosis of those cells in cow (Taniguchi et al., 2002). However, the direct influence of those cytokines on the expression of the enzymes involved in steroidogenesis has not been studied so far. In the organism, under physiological conditions, not only activated macrophages and lymphocytes produce TNFα and IFNγ but also fibroblasts and endothelial cells (Penny et al., 1999; Abbas et al., 2000). Whereas there are specific binding sites for TNFα throughout the estrous cycle (Sakumoto et al., 2000), the total amount of this cytokine and also IFNγ rise significantly just after initiation of luteolysis, as the reason of a great amount of lymphocytes infiltrating the CL at this time (Penny et al., 1999). In the in vivo study, Skarzynski et al. (2003) documented that TNFα in low concentrations caused luteolysis – decreased P4 level, which could be augmented by various factors, including IFNγ. In the conducted study, we demonstrated the modulatory effect of LPA on previously documented TNFα and IFNγ – induced P4 decrease in the cultured bovine steroidogenic cells (Korzekwa et al., 2006). The obtained data is consistent with our previous data that LPA administered into the aorta abdominalis (Woclawek-Potocka et al., 2009) or intravaginally (Woclawek-Potocka et al., 2010) increased P4 secretion in the cows during the luteal phase of the estrous cycle. Moreover, in heifers, LPA – dependent prevention of the spontaneous luteolysis and prolongation of the functional lifespan of the CL in vivo (Woclawek-Potocka et al., 2010), may also be explained by the data obtained in this study. We also suggest that the luteoprotective effects of LPA obtained in vivo may have resulted from both – direct influence of LPA on P4 secretion or indirect on

![Fig. 4. Expression of mRNAs (a–c) and proteins (d–f) for TNFα, TNFR1 and TNFR2, respectively in luteal cells isolated from CL from the mid-luteal stage of estrous cycle. Representative Western Blotting samples are shown in the upper panel (d–f). Cells were incubated with: LPA (435 ng/ml), TNFα + IFNγ (both 50 ng/ml) and TNFα + IFNγ with LPA for 6 (for mRNA expression measurement) or 12 (for protein level measurement) hours. All values are expressed as the mean of four replicates ± SEM of TNFα, TNFR1 and TNFR2. Different letters indicate significant differences (P < 0.05), as determined by one-way ANOVA followed by Bonferroni’s multiple comparison test.](image-url)
Fig. 5. Expression of mRNAs (a and b) and proteins (c and d) for Fas and FasL, respectively, in luteal cells isolated from CL from the mid-luteal stage of estrous cycle. Representative Western Blotting samples are shown in the upper panel (c and d). Cells were incubated with: LPA (435 ng/ml), TNFα + IFNγ (both 50 ng/ml), and TNFα + IFNγ with LPA for 6 (for mRNA expression measurement) or 12 (for protein level measurement) hours. All values are expressed as the mean of four replicates ± SEM of Fas and FasL. Different letters indicate significant differences (P < 0.05), as determined by one-way ANOVA followed by Bonferroni’s multiple comparison test.

Fig. 6. Expression of mRNAs (a and b) and proteins (c and d) for Casp8 and Casp3, respectively, in luteal cells isolated from CL from the mid-luteal stage of estrous cycle. Representative Western Blotting samples are shown in the upper panel (c and d). Cells were incubated with: LPA (435 ng/ml), TNFα + IFNγ (both 50 ng/ml), and TNFα + IFNγ with LPA for 6 (for mRNA expression measurement) or 12 (for protein level measurement) hours. All values are expressed as the mean of four replicates ± SEM of Casp8 and Casp3. Different letters indicate significant differences (P < 0.05), as determined by one-way ANOVA followed by Bonferroni’s multiple comparison test.
reversing the luteolysing actions of TNFα and IFNγ. These results are important because the mid-luteal stage represents a critical period in the CL lifespan for secretion of P4 (Niswender et al., 2000). We hypothesize that at the examined time of estrous cycle, if the female becomes pregnant, continued secretion of P4 from the CL can be directly supported by LPA or indirectly by reversing luteolysing action of TNFα and IFNγ.

In our study, we did not find any influence of LPA on the expression of mRNA and protein of STAR protein or P450scx in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle. However, LPA stimulated the expression of mRNA and protein of 3βHSD in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle. The above data, is consistent with the previous data obtained by Kowalczyk-Zieba et al. (2012). Moreover, documented in this study the lack of the direct influence of the examined cytokines on STAR protein, P450 scx and 3βHSD, is consistent with the previously published data (Petroff et al., 2001; Pate (1995). Pate (1995) documented that TNFα and IFNγ administered together inhibited LH – stimulated P4 synthesis in the cow. Similar data were obtained in rats and pigs concerning TNFα inhibitory effect on LH action in the steroidogenic luteal cells (Adashi et al., 1990; Pitzel et al., 1993). Moreover, the study of Neuviens et al. (2004) documented the inhibitory action of the selected inflammatory cytokines on LH-R expression and interpreted this influence as the direct marker of luteolysis.

In the second part of the present study we documented that LPA suppressed TNFα and IFNγ, – induced luteal cell apoptosis which is known to occur during structural luteolysis. Structural luteolysis is a recurrent in each estrous cycle process that eliminates the CL from the ovary. It has been established before that structural luteal regression in cattle occurs via apoptosis (Meidan et al., 1999; Pate and Landis Keyes, 2001). Apoptosis is controlled by mitochondrial regulatory proteins, Bcl-2 and Bax that belong to the Bcl-2 protein family. We showed in our study that LPA inhibited the stimulatory effect of TNFα and IFNγ on the expression of mRNA and protein of Bax in the cultured steroidogenic luteal cells. Neither LPA nor TNFα together with IFNγ influenced the expression of mRNA and protein of Bcl2 in the cultured steroidogenic luteal cells. LPA-dependent inhibition of the stimulatory effect of TNFα and IFNγ on the expression of Bax in the cultured steroidogenic luteal cells orients these cells towards the survival state. In addition, apoptosis on the receptor level can be also initiated via several cytokine receptors including TNF super family receptors (TNFRs). It was documented before that in various cell types, TNFα has two immunologically distinct receptors, type I (TNFR1; 55 kDa) and type II (TNFR2; 75 kDa) (Tartaglia and Goeddel, 1992) that induce different intracellular signaling pathways (Ihnatko and Kubes, 2007). It has been demonstrated that TNFα stimulated PG production and induced apoptotic cell death of cultured bovine luteal cells mainly acting via TNFR1 (Sakumoto et al., 2000; Taniguchi et al., 2002). On the other hand, TNF-α had the capacity to both shorten and prolong the CL lifespan, which depended on its concentration in the cow (Skarzynski et al., 2003, 2009). TNFR2 is the type of receptor for TNFα which is associated mainly with the prosurvival action of this cytokine in the organism (Fotin-Mleczek et al., 2002). It has previously been shown that both types of TNFα-receptors are expressed in the bovine CL (Sakumoto et al., 2000, 2011). In our study, the administration of LPA alone did not influence the expression of mRNA and protein of TNFα, TNFR1 or TNFR2 in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle. We also documented that TNFα together with IFNγ elevated the protein level of TNFα and TNFR1 in the cultured steroidogenic luteal cells. However, LPA inhibited only the stimulatory effect of TNFα and IFNγ on the protein level of TNFR1 in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle. This data prove that LPA can partially inhibit TNFα and IFNγ induced apoptosis in the cultured steroidogenic luteal cells via abrogation of the cytokine – dependent stimulatory influence on proapoptotic TNFR1 expression in these cells. Moreover, the Fas antigen (Fas) also belongs to the TNF super family receptors which together with Fas ligand (FasL) transmit basic signals controlling intercellular apoptosis pathway (Nagata, 1997). Although Fas mRNA has been shown in the bovine CL throughout the estrous cycle, Fas L alone did not induce the death of the cells (Taniguchi et al., 2002). In the studies of Taniguchi et al. (2002), the authors demonstrated higher sensitivity of the bovine luteal cells on the FasL that induced cell death in the presence of TNFα and IFNγ, which in turn was correlated with the increase of mRNA expression for Fas, induced by the examined cytokines. In our study we found that in the presence of LPA, TNFα and IFNγ did not stimulate mRNA and protein expression of Fas and FasL in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle. These data prove that LPA modulates TNFα and IFNγ induced apoptosis in the cultured steroidogenic luteal cells via its influence on Fas–FasL system. It has been also documented in the literature that, the inhibition of intra-luteal P4 action by various specific antagonists amplified Fas L-mediated apoptosis via the increase of Fas and initiation of Casp8 and Casp3 expressions as well as Casp3 activity in cultured bovine luteal cells (Okuda and Sakamoto, 2003). High levels of Casp8 directly initiate cleavage of an effector caspase Casp3, thereby initiating the execution phase of apoptosis (Scaffidi et al., 1998). During apoptosis executed through the mitochondrial pathway, active Casp8 stimulates the binding of proapoptotic Bax to mitochondria and inhibits association of antiapoptotic Bcl-2. This leads to the leakage of cytochrome c from the mitochondria into the cytosol, which in turn promotes formation of the apoptosome and triggers activation of the effector Casp3 (Scaffidi et al., 1998). The possible influence of LPA on the modulation of TNFα and IFNγ – induced cell death connected with activation of both Casp8 and Casp3 was also examined in our study. Lyposphatidic acid did not have any effect on the stimulatory effect of TNFα and IFNγ on the expression of mRNA and protein of Casp8 and Casp3 in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle. On the other hand, we demonstrated that LPA decreased cleaved Casp3 activity induced by cytokines in the cultured steroidogenic luteal cells at Days 8–12 of the estrous cycle. However, citing the work of Juengel et al. (1993) and Rueda et al. (1995), the onset of apoptosis in the bovine CL is not observed until P4 production has declined. In this aspect we can surmise that in the presence of LPA, P4 secretion is supported and also TNFα and IFNγ cannot induce apoptosis in the bovine CL. Lyposphatidic acid reversed TNFα and IFNγ – induced
apoptosis via inhibition of the stimulatory effect of the cytokines on the expression of mRNA and protein of Bax, Fas, FasL, system, TNFRI and Casp3 activity in the cultured steroidogenic luteal cells, which orientates these cells towards the survival state.

The results obtained in this study indicate that TNFα and IFNγ cannot induce apoptosis in the presence of LPA, which orientates the steroidogenic luteal cells towards the survival state. In conclusion our results indicate that LPA supports P4 synthesis and action in the bovine CL.

Acknowledgement

This research was supported by Grants-in-Aid for Scientific Research from the Polish Ministry of Sciences and Higher Education (MNISW – DPN/DWM/MZ/5751/08/09).

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