Lysophosphatidic Acid Stimulates Prostaglandin E_2 Production in Cultured Stromal Endometrial Cells Through LPA1 Receptor

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Lysophosphatidic acid (LPA) has been shown to be a potent modulator of prostaglandin (PG) secretion during the luteal phase of the estrous cycle in the bovine endometrium *in vivo*. The aims of the present study were to determine the cell types of the bovine endometrium (epithelial or stromal cells) responsible for the secretion of PGs in response to LPA, the cellular, receptor, intracellular, and enzymatic mechanisms of LPA action. Cultured bovine epithelial and stromal cells were exposed to LPA (10^{-5}–10^{-9} M), tumor necrosis factor α (TNFα; 10 ng/mL) or oxytocin (OT; 10^{-7} M) for 24 h. LPA treatment resulted in a dose-dependent increase of PGE_2 production in stromal cells, but not in epithelial cells. LPA did not influence $PGF_{2\alpha}$ production in stromal or epithelial cells. To examine which type of LPA G-protein–coupled receptor (LP-GPCR; LPA1, LPA2, or LPA3) is responsible for LPA action, stromal cells were preincubated with three selected blockers of LPA receptors: NAEPA, DGPP, and Ki16425 for 0.5 h, and then stimulated with LPA. Only Ki16425 inhibited the stimulatory effect of LPA on PGE_2 production and cell proliferation in the stromal cells. LPA-induced intracellular calcium ion mobilization was also inhibited only by Ki16425. Finally, we examined whether LPA-induced PGE_2 synthesis in stromal cells is via the influence on mRNA expression for the enzymes responsible for PGE_2 synthesis—*PGE_2 synthase* (*PGES*) and *PG-endoperoxide synthase 2* (*PTGS2*). We demonstrated that the stimulatory effect of LPA on PGE_2 production in stromal cells is via the stimulation of *PTGS2* and *PGES* mRNA expression in the cells. The overall results indicate that LPA stimulates PGE_2 production, cell viability, and intracellular calcium ion mobilization in cultured stromal endometrial cells via Ki16425-sensitive LPA1 receptors. Moreover, LPA exerts a stimulatory effect on PGE_2 production in stromal cells via the induction of *PTGS2* and *PGES* mRNA expression. Exp Biol Med 234:986–993, 2009

Key words: lysophosphatidic acid; prostaglandins; uterus; cow

Introduction

Prostaglandins (PGs) are produced from arachidonic acid (AA) liberated from phospholipid stores through the action of phospholipases. In the bovine uterus, *PG*-endoperoxide synthase 2 (PTGS2) converts AA to PGG_2 and then the peroxidative reduction of this intermediate to PGH_2 occurs (1). PGH_2 is an unstable endoperoxide intermediate that is converted by cell-specific synthases and isomerases (PGES or AKR1B5-PGFS) to distinct PGs (PGE_2 and $PGF_{2\alpha}$) (2). Prostaglandin $F_{2\alpha}$ and PGE_2 are secreted in the bovine endometrium throughout all phases of the estrous cycle, but the secretory pattern of these AA metabolites is different (3, 4). Endometrial $PGF_{2\alpha}$ is secreted in a pulsatile manner during the late luteal phase and luteolysis and is considered the luteolytic mediator (5, 6). It has been shown that endometrial production of PGE_2 is higher during the mid and late luteal phases of the estrous cycle as well as early pregnancy in the cow (4, 7). Moreover, PGE_2 has been proposed to have multiple roles as a luteoprotective signal and as an important mediator in endometrial receptivity and immune function at the fetal-maternal interface at the time of establishment of pregnancy (7–9). There are a few reports that the lipid messenger—lysophosphatidic acid (LPA)—can modulate the secretion of PGs in the endometrium in mice, pigs, and sheep (10–12). Moreover, we have recently found that LPA preferentially stimulates PGE_2 output from bovine endometrium during the luteal phase of the estrous cycle *in vivo* (13). We also

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found that LPA is locally produced and released from the bovine endometrium, both at the estrous cycle and early pregnancy, and that there is the specific receptor type 1 (LPA1) present in the bovine endometrium throughout the estrous cycle and early pregnancy (13). The gene expression of *LPA1* receptor was positively correlated with the expression of *PGES* during the estrous cycle and early pregnancy (13). These findings suggest that LPA of a uterine origin may, through the modulation of PG synthesis, play autocrine and/or paracine roles in the bovine uterus and serve as an important factor in the maintenance of early pregnancy, as it has been recently shown in mice, pigs, and sheep (10–12). However, there are no reports on the cell types of the bovine endometrium (epithelial or stromal cells) responsible for the secretion of PGs in response to LPA or the enzymatic mechanism of this LPA action.

The receptor and intracellular mechanisms of LPA action on PG output from the bovine endometrium are not understood. In mammals, LPA exerts its action via four high-affinity LPA G protein–coupled receptor (LP-GPCR) types: LPA1/EDG2, LPA2/EDG4, LPA3/EDG7, and the recently identified GPR23/p2y9/LPA4 (14–16). However, the precise roles of each receptor in various LPA functions and various kinds of cells are not known. Moreover, no single receptor-selective blocker for any of the LPA receptors has been reported so far. It has been reported that Ki16425 is an isoxazole derivative that has greater potency for LPA1 than LPA2 and LPA3 (17), N-acyl ethanolamide phosphate (NAEPA) selectively inhibited LPA-stimulated GTP binding in stably transfected LPA1- and LPA3-expressing RH7777 cells (18), whereas diacylglycerol pyrophosphate (DGPP) 8:0 preferentially inhibited the LPA3-induced actions (19). We have recently demonstrated that in the bovine endometrium, unlike in mice, pigs, and sheep (10–12, 20), there is only *LPA1* receptor expression (13). Therefore, in this study we wanted to check whether LPA action in the endometrial cells might be mediated via LPA1 LP-GPCR.

In the present study, we attempted to determine which cell types of the bovine endometrium are responsible for the secretion of PGs in response to LPA and which LP-GPCR is responsible for LPA action in the cells. We also attempted to determine whether LPA influences the expression of the enzymes responsible for PGE_2 synthesis (*PTGS2* and *PGES*) in the endometrial stromal cells.

Materials and Methods

Animals. All animal procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (Agreement No. 34/2005/N).

For all experiments, normally cycling Holstein/Polish Black and White (75/25%, respectively) cows (4–6 lactations; $n = 7$) were chosen. The animals were eliminated by the owner from two dairy cow herds (Years 2004–2006) because of their low milk production. Estrus was synchronized using double injections of an analogue of $PGF_{2\alpha}$ (dinoprost, Dinolytic; Upjohn-Pharmacia N.V.S.A., Belgium), as described recently by Skarzynski et al. (21) for the estrus synchronization of multiparous cows. The onset of the estrus was determined by observing the signs of estrus (i.e., vaginal mucus, standing behavior), and was confirmed by a veterinarian via ultrasonography (USG) examination using DRAMINSKI ANIMAL profi Scanner (Draminski Electronics in Agriculture, Olsztyn, Poland; www.draminski.com) and *per rectum* examination. Only the cows with behavioral signs of estrus were chosen for the study after positive USG and *per rectum* examination ($n = 5$). The day of estrus was designated Day 0 of the cycle.

Collection of Endometrial Tissues. Bovine uteri were obtained at a local slaughterhouse (Zaklady Miesne "Warmia" in Biskupiec, Poland) within 20 min of exsanguinations and were transported on ice to the laboratory within 40 min. The animals were slaughtered on Days 2–5 of the estrous cycle ($n = 9$). Estimation of the stages of the estrous cycle was additionally confirmed by macroscopic observation of the ovaries and uterus (4).

Isolation and Incubation of Endometrial Cells. The epithelial and stromal cells from the bovine endometrium were enzymatically separated (0.05% collagenase; Sigma Aldrich, St. Louis, MO, USA; #C0130) using procedures described previously (22). Cell viability, measured by trypan blue staining, was higher than 85%. The obtained two pellets consisted of enriched stromal cells and enriched epithelial cells (22). The homogeneity of the cells and contamination of the stromal and epithelial cell cultures (with epithelial cells and stromal cells, respectively) were evaluated using immunofluorescent staining for specific markers of epithelial (cytokeratin) and stromal cells (vimentin) (22).

The final pellets of both the stromal and epithelial cells were suspended in a culture medium, Dulbecco-modified Eagle medium and Ham's F-12 medium (DMEM/Ham's F-12, 1:1 [v/v]; Sigma-Aldrich, Inc., St. Louis, MO; #D8900) containing 10% calf serum (Gibco BRL, Grand Island, NY; #16170–078) and 20 μg/mL gentamicin (Gibco BRL; #15750–060). The cells of each type were separately seeded at a density of 1×10^5 viable cells/mL in different types of culture plates: 48-well plates (for PG secretion measurement; Costar, Cambridge, MA, USA; #150687), 96-well plates (for proliferation measurement in the cells; Sarstedt; #83.1835.300), 24-well plates (for gene expression; Becton Dickinson, Franklin Lakes, NJ, USA; #353847), or 4-well plates (for intracellular calcium ion measurement; Nunc, Roskilde, Denmark; #176740) and cultured at 38.5°C in a humidified atmosphere of 5% CO_2, 95% air. To purify the stromal preparation, the medium was changed 6 h after plating, at which time selective attachment of stromal cells had occurred. Alternatively, since the epithelial cells attached 24–48 h after plating, the medium in the epithelial cell culture was replaced 48 h after plating. The medium was changed every 2 days until confluency was reached.

When the cells were confluent (6–7 days after the start of the culture), the medium was then 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 various stimulators for the following experiments.

Experiment 1. *Determination of the Effective Dose of LPA on PG Secretion in the Endometrial Stromal and Epithelial Cells.* The bovine epithelial and stromal cells were cultured in 48-well plates until they were confluent. Afterwards, the cells were exposed to various concentrations of LPA (1-Oleoyl-sn-Glycero-3-Phosphate, Sodium; Sigma, #L7260; 10^{-5}–10^{-9} M). Tumor necrosis factor α (TNFα Dainippon Pharmaceutical Co. Ltd., Osaka, Japan; 10 ng/mL) and oxytocin (OT; Sigma, #O4375; 10^{-7} M) were used as positive control for stromal and epithelial cells, respectively (23). After 24 h incubation, the conditioned media were collected in tubes with 10 μL EDTA, 1% aspirin (Sigma; #A2093) solution (pH 7.3), and frozen at –20°C until measurement of PGE_2 and $PGF_{2\alpha}$ by enzyme immunoassay (EIA), as described previously (24). The anti $PGF_{2\alpha}$ and PGE_2 serum was purchased from Sigma (#P5539, #P5164; respectively). The PGF2α standard curve ranged from 0.016 ng/mL to 4 ng/mL, and the ED50 of the assay was 0.25 ng/mL. The intra- and interassay coefficients of variation were on average 7.1% and 11.3%, respectively. The PGE_2 standard curve ranged from 0.07 ng/mL to 20 ng/mL and the ID50 of the assay was 1.25 ng/mL. The intra- and inter-assay coefficients of variation were on average 6.9% and 9.7%, respectively. DNA content was measured spectrophotometrically and was used to standardize $PGF_{2\alpha}$ and PGE_2 concentrations (Murakami et al., 22). The results of $PGF_{2\alpha}$ and PGE_2 concentrations are expressed as ng/μg DNA.

In the second and third experiments, we examined LPA action only in stromal cells, since in Experiment 1, only stromal cells were the target for LPA-modulated PGE_2 synthesis in the bovine endometrium.

Experiment 2. *Determination of the LP-GPCR Responsible for LPA Action in the Endometrial Stromal Cells.* For PGE_2 secretion measurement, the bovine stromal cells were cultured in 48-well plates until they were confluent. Afterward, the cells were preincubated with three selected blockers of LPA receptors: N-acyl ethanolamide phosphate (NAEPA; Sigma, #N0912; 10^{-5} M), diacylglycerol pyrophosphate (DGPP; Sigma, #D5442; 10^{-5} M), and Ki16425 ($C_{23}H_{23}ClN_2O_5S$; 3-(4-[4-([1-(2-Chlorophenyl)ethoxy]carbonylamino)-3-methyl-5-isoxazolyl]benzylthio)propanoic acid; Sigma, #K0639; 10^{-5} M), for 0.5 h, and then stimulated with LPA for 24 h. After the incubation, the conditioned media were collected in tubes with 10 μL EDTA, 1% aspirin (Sigma; #A2093) solution (pH 7.3), and frozen at –20°C until measurement of PGE_2 by EIA.

For proliferation measurement in the cells, the bovine stromal cells were cultured in 96-well plates until they reached 50% of confluence. Afterward, the cells were preincubated with: NAEPA, DGPP, and Ki16425 (all 10^{-5} M) for 0.5 h, and then stimulated with LPA for 24 h. After the incubation, proliferation index of the cells was measured by MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method according to the instructions of the manufacturer (TOX-1 Kit including MTT; TOX-1; Sigma, #7H258). Insulin-like growth factor-1 (IGF-1; 100 ng/mL, Sigma, #12024) was used as a positive control for proliferation index measurement in the cells. Shortly, the culture medium was replaced with 100 μL of DMEM/Ham's F-12, and 10 μL of MTT was added to each well. The cells were then incubated for 6 h at 37°C. Next, MTT Solubilization Solution (100 μL; Tox-1, Sigma) was added to each well, and the absorbance (A) was read at 450 nm using a microplate reader (Labsystem). Cell proliferation was determined by dividing the mean A of the treated wells (A_{test}) by the mean A of the nontreated wells ($A_{control}$). The mean A of wells without the cells was subtracted from the mean A of all experimental wells. The cell proliferation was calculated as follows:

$$\text{number of alive cells}(\%) = 100 \times (A_{test})/(A_{control})$$

and presented as the percent of the control value.

For intracellular calcium ion measurement in the cells, the bovine stromal cells were cultured in 4-well plates until they reached 50% of confluence. The intracellular calcium ion mobilization was measured by the method described by Woclawek-Potocka et al. (25). In the method, cell-permeable form of the fluorescent calcium indicator Fura-2 (Fura-2 AM; Dojindo, Kumamoto, Japan, #384-0583) was used. Shortly after loading the cells with dye, the cells were incubated at 38°C for 40 min and then washed 4 times in calcium-free HBSS. Cells were then incubated in calcium-free HBSS supplemented with 0.1% BSA for 30 min at 37°C to allow hydrolysis of cytoplasmic Fura-2. For the time of the hydrolysis, three selected LP-GPCR blockers (NAEPA, DGPP, and Ki16425; all 10^{-5} M) were administered to the cells. The cells were then washed 3 times in calcium-free HBSS. Changes in the intracellular concentrations of intracellular calcium ion were monitored using an inverted microscope equipped with a fluorescent lamp and a Fura-2 filter. The intensity of fluorescence and the area occupied by the fluorescing cells were measured every 1 second, from 4 seconds before through 28 seconds after treatment with LPA (10^{-6} M), NAEPA, DGPP, and Ki16425 (all 10^{-5} M) or LPA (10^{-6} M) after 30 min of pretreatment with NAEPA (10^{-5} M), DGPP (10^{-5} M), and Ki16425 (all 10^{-5} M). Phorbol myristate acetate (PMA; 10^{-7} M) was used as a positive control of intracellular calcium ion exflux for both types of cells (27th second of the experiment). Digital interpretation for the computer-supported intracellular calcium ion visualization method was obtained using the density green and density mean of

Table 1. Primers Used for Real-Time PCR

Gene	Primer sequences	GenBank (acc. no.)	Position	PCR products (bp)
PGES (bos taurus)	5′-AGGACGCTCAGAGACATGGA-3′ 5′-TTCGGTCCGAGGAAAGAGTA-3′	NM174443	154–295	142
PTGS2 (bos taurus)	5′-TGTGAAAGGGAGGAAAGAGC-3′ 5′-GGCAAAGAARGCAAACATCA-3′	AF004944	138–252	115
GAPDH (bos taurus)	5′-CACCCTCAAGATTGTCAGCA-3′ 5′-GGTCATAAGTCCCTCCACGA-3′	BC102589	492–594	103

the examined cells in each separate photograph. Changes in intracellular calcium ion concentrations following treatments were shown on a graph as arbitrary units of intensity as analyzed by the computer software (Micro Image 4.0; Olympus Optical Co., Hamburg, Germany).

Experiment 3. *Determination of the Effect of LPA on PGE_2 Secretion and Expression of mRNA Transcripts for PTGS2 and PGES in Stromal Cells.* Cultured bovine stromal cells were exposed to LPA (10^{-6} M), Ki16425 (10^{-5} M), and the combination of 0.5-h preincubation with Ki16425 and then LPA, for either 4 h (for the expression of mRNA transcripts for *PTGS2* and *PGES* in the cells) or 24 h (for PGE_2 measurement in the culture medium). TNFα (10 ng/mL) was used as positive control for stromal cells. After 24-h incubation, the conditioned media were collected in tubes with 10 μL EDTA, 1% aspirin (Sigma; #A2093) solution (pH 7.3), and frozen at –20°C until measurement of PGE_2 by EIA. Gene expression for the enzymes responsible for PGE_2 synthesis (*PTGS2* and *PGES*) was quantitatively measured by real-time PCR in the cells. 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.

Total RNA Extraction, Reverse Transcription (RT), and Real-time PCR. Total RNA was extracted from stromal cells using TRIZOL according to manufacturer's instructions. RNA samples were stored at –80°C. Before use, RNA was verified by spectrophotometric measurement and agarose gel electrophoresis. Two micrograms of each sample of total RNA was reverse transcribed using a ThermoScript™ RT-PCR System (Invitrogen, Alab, Warsaw, Poland; #11146–016). The RT reaction was performed in total reaction volume of 20 μL, following manufacturer's instructions. RT 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 *PTGS2* and *PGES*, as described previously (13). Briefly, *GAPDH* expression was used as an internal control. The primers were chosen using an online software package (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). 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, Applera, Warsaw, Poland; #4367659). The PCR reactions were performed in 96-well plates. Each PCR reaction well (25 μL) contained 2.5 μL of diluted RT product, 200 pM forward and reverse primers each, and 12.5 μL SYBR Green PCR master mix. As standard curves, serial dilutions of appropriate cDNA were used for gene quantification. For quantification of the mRNA expression levels, the primer length (20 bp) and GC-contents 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 of 94°C for 15 sec, 56°C for 28 sec, and 72°C for 15 sec. 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 absence of the reverse transcriptase enzyme. The specificity of the PCR products for all examined genes was confirmed by gel electrophoresis and by sequencing. The results of *PTGS2* and *PGES* mRNA expression in the cells are expressed as arbitrary units normalized on the basis of *GAPDH* mRNA content.

Statistical Analysis. All analyses were done using one-way ANOVA tests followed by Bonferroni's Multiple Comparison Test (GraphPad PRISM; $P < 0.05$ was considered significant).

Patterns of intracellular calcium ion mobilization in Experiment 2 were estimated by tests for repeated measures. All tests were performed by computer using Prism 4 software (GraphPad PRISM; GraphPad Software, Inc., San Diego, CA).

Results

Effects of LPA on PGE2 Production in the Bovine Endometrial Cells. Figure 1 shows PGE_2 production in stromal (a) and epithelial (b) cells stimulated for 24 h with LPA (10^{-5}–10^{-9} M), TNFα (10 ng/mL; only stromal cells), and OT (10^{-7} M; only epithelial cells). TNFα and OT stimulated the production of PGE_2 in stromal and epithelial cells, respectively ($P < 0.01$), which accounts for appropriate responsiveness of the cells. LPA in doses 10^{-6} and 10^{-5} M stimulated PGE_2 secretion in stromal cells compared with controls ($P < 0.05$). However, the strongest effect on PGE_2 secretion in stromal cells was noted after LPA treatment in dose 10^{-6} M ($P < 0.01$). Therefore, this dose was chosen for the further experiments. LPA did not

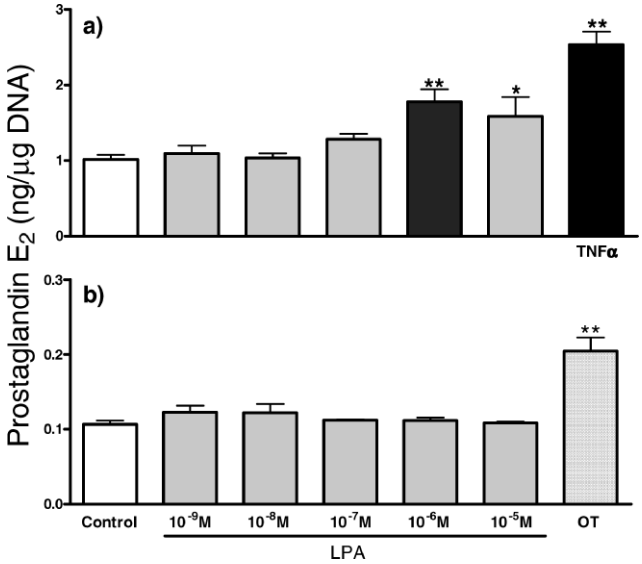

Figure 1. PGE_2 production in stromal (a) and epithelial (b) cells stimulated for 24 h with LPA (10^{-5}–10^{-9} M), TNFα (10 ng/mL; only stromal cells), and OT (10^{-7} M; only epithelial cells). Asterisks: *($P < 0.05$), **($P < 0.01$) indicate statistical differences between control and treated groups, as determined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

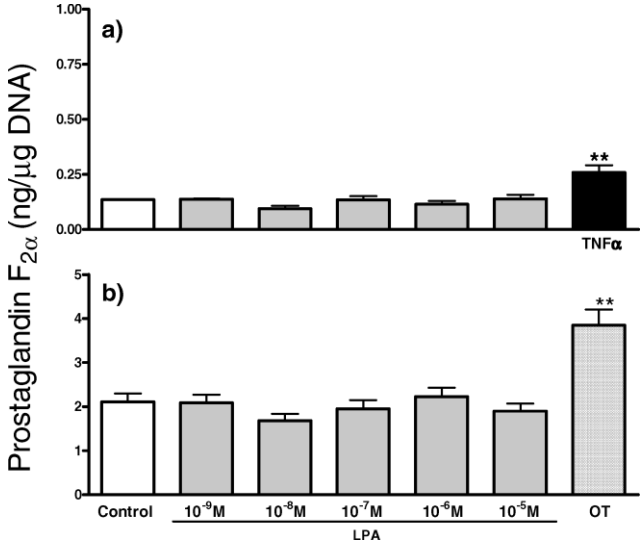

Figure 2. $PGF_{2\alpha}$ production in stromal (a) and epithelial (b) cells stimulated for 24 h with LPA (10^{-5}–10^{-9} M), TNFα (10 ng/mL; only stromal cells), and OT (10^{-7} M; only epithelial cells). Asterisks: *($P < 0.05$), **($P < 0.01$) indicate statistical differences between control and treated groups, as determined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

influence PGE_2 secretion in the epithelial cells compared with controls ($P > 0.05$).

Effects of LPA on $PGF_{2\alpha}$ Production in the Bovine Endometrial Cells. Figure 2 shows $PGF_{2\alpha}$ production in stromal (a) and epithelial (b) cells stimulated for 24 h with LPA (10^{-5}–10^{-9} M), TNFα (10 ng/mL; only stromal cells), and OT (10^{-7} M; only epithelial cells). TNFα and OT stimulated the production of $PGF_{2\alpha}$ in stromal and epithelial cells, respectively ($P < 0.01$), which accounts for appropriate responsiveness of the cells. LPA did not influence $PGF_{2\alpha}$ secretion in stromal and epithelial cells ($P > 0.05$).

On the basis of the results obtained in this experiment, for the further 2 experiments we used only stromal cells and only LPA effect on PGE_2 secretion.

Effects of Ki16425, NAEPA, and DGPP on LPA-Stimulated PGE_2 Production and Cell Viability in Stromal Cells. Figure 3 shows the effects of Ki16425, NAEPA, and DGPP on LPA-stimulated PGE_2 production (a) and cell viability (b) in stromal cells. NAEPA and DGPP did not affect the stimulatory effect of LPA on PGE_2 production in stromal cells ($P > 0.05$). The stimulatory effect of LPA on PGE_2 production by stromal cells was completely inhibited by Ki16425 only ($P < 0.05$). LPA and NAEPA significantly increased the number of the stromal cells to approximately 189% compared to control ($P < 0.05$). The stimulatory effect of LPA on the stromal cell number was completely inhibited by Ki16425 ($P < 0.05$). IGF-1 stimulated stromal cell number compared to control (190% of the control; $P < 0.01$), which accounts for appropriate responsiveness of the cells.

Effects of Ki16425, NAEPA, and DGPP on LPA-Stimulated Mobilization of Intracellular Calcium Ion in Stromal Cells. Figure 4 shows the intracellular calcium ion mobilization in stromal cells. Concentration of intracellular calcium ion in endometrial stromal cells was rapidly increased within 32 sec after LPA (10^{-6} M) treatment ($P < 0.05$). However, intracellular calcium ion mobilization was not observed in the cells pretreated for 30 min with Ki16425, as measured by area under the curve ($P > 0.05$). NAEPA and DGPP did not affect the stimulatory effect of LPA on intracellular calcium ion mobilization in stromal cells (data not shown; $P > 0.05$). None of the three selected antagonists of LPA receptors induced intracellular calcium ion mobilization in stromal cells (data not shown; $P > 0.05$). Phorbol 12-myristate 13-acetate (PMA; 10^{-7} M) induced the intracellular calcium ion mobilization in stromal cells ($P < 0.05$), which accounts for the appropriate responsiveness of the cells.

Effects of LPA on Expression of mRNA Transcripts for PTGS2 and PGES in Stromal Cells. Figure 5 shows PGE2 production (a), total quantification of *PTGS2* gene expression normalized to *GAPDH* mRNA transcripts expression (b), and total quantification of *PGES* gene expression normalized to *GAPDH* mRNA transcripts expression (c) in stromal cells stimulated for either 4 h (for the expression of mRNA transcripts for *PTGS2* and *PGES* in the cells) or 24 h (for PGE_2 measurement in the culture medium) with LPA (10^{-6} M), Ki16425 (10^{-5} M), and the combination of 0.5-h preincubation with Ki16425 and then LPA. TNFα stimulated the production of PGE_2 and *PTGS2* and *PGES* gene expression in stromal cells ($P < 0.05$),

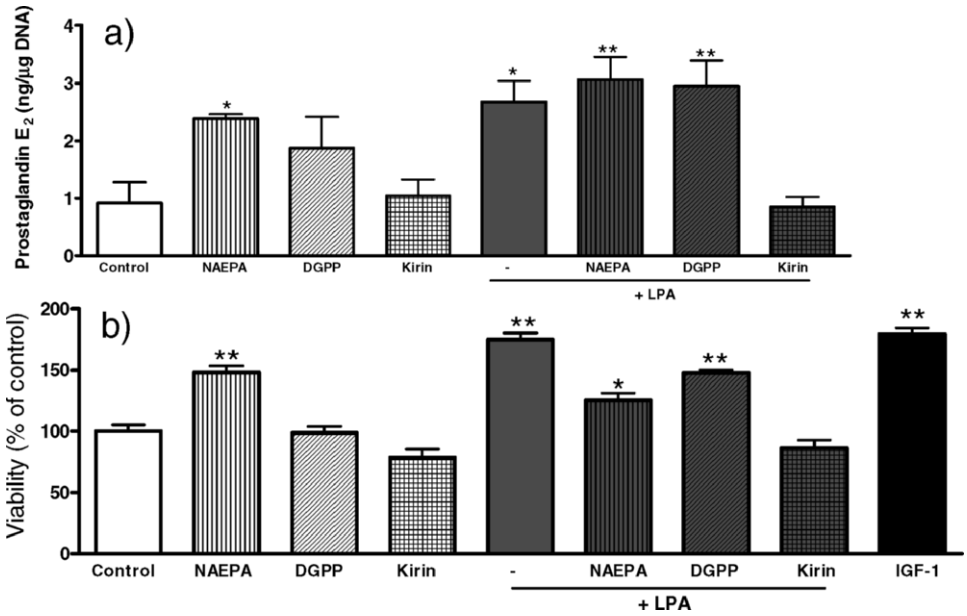

Figure 3. PGE_2 production (a) and cell viability (b) in stromal cells preincubated with NAEPA (10^{-5} M), DGPP (10^{-5} M), and Ki16425 (10^{-5} M), for 0.5 h, and then stimulated with LPA for 24 h. Asterisks: *($P < 0.05$), **($P < 0.01$) indicate statistical differences between control and treated groups, as determined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

which accounts for appropriate responsiveness of the cells. Ki16425 inhibited the stimulatory effect of LPA on PGE_2 production and *PTGS2* and *PGES* gene expression in stromal cells ($P < 0.05$).

Discussion

The present study demonstrated that LPA, which is locally synthesized by the bovine endometrium (13), had an influence on the secretory function of the bovine endometrial cells *in vitro*. LPA treatment resulted in a dose-dependent increase of PGE_2 production in stromal cells but not in epithelial cells. LPA did not influence $PGF_{2\alpha}$ production in stromal or epithelial cells. The chosen LPA dose had the strongest effect on PGE_2 synthesis in stromal cells and was not toxic to the cells. This study reports that only stromal cells of the bovine endometrium are the target for LPA action of PG production *in vitro*. Moreover, LPA modulated only PGE_2 synthesis and secretion in the bovine endometrial cells. The LPA-induced PGE_2 synthesis and secretion in the stromal cells is consistent only to a certain extent with our previous data obtained *in vivo* (13). In this study, we have shown that LPA stimulated both PGE_2 and $PGF_{2\alpha}$ during the luteal phase of the estrous cycle. However, the LPA-induced increase of $PGF_{2\alpha}$ synthesis was much less than the LPA-induced increase of PGE_2 synthesis (1.75 versus 11.5 times). The stimulation of $PGF_{2\alpha}$ synthesis *in vivo* might be explained by LPA-dependent activation of $PGF_{2\alpha}$ synthesis in the CL, myometrium, or endothelial cells of the blood vessels or by the stimulation of 9-keto-PGE_2 reductase (9kPGR) activity (26–30). However, confirmation of these hypotheses requires further investigation.

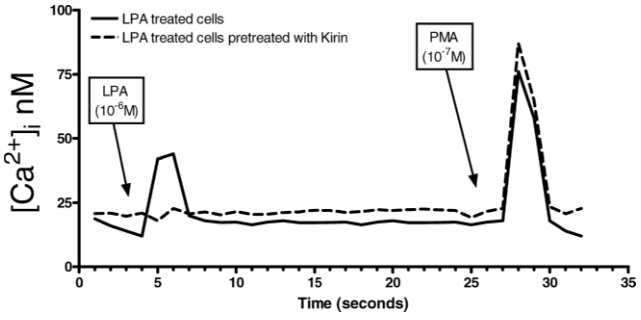

Figure 4. Changes in intracellular Ca^{2+} concentrations in endometrial stromal cells exposed to LPA (10^{-6} M; straight line) or LPA after pretreatment for 30 min with Ki16425 (10^{-5} M; dotted line) within 32 sec. Data are from a set of cells from one representative uterus. Similar profiles were obtained in 2 other experiments. Arrows indicate the time of treatment addition. Changes of calcium concentrations were shown as changes in the fluorescence of Ca^{2+}-FURA 2-AM complex. PMA (10^{-7} M) was used as a positive control.

In the present study we examined which LP-GPCR is responsible for LPA action in the cells. Lysophosphatidic acid in various cell types in different tissues exerts its biological effects via four G-protein coupled membrane receptors (LPA1/EDG2, LPA2/EDG4, LPA3/EDG7, and GPR23/p2y9/LPA4) (14, 15, 31, 32). However, we have shown before that there is only *LPA1* mRNA expression in the bovine endometrial tissue (13). Therefore, the data obtained in Experiment 2 confirm our assumption that only Ki16425 (most potent LPA1 blocker) inhibited the stimulatory effect of LPA on PGE_2 production, cell proliferation index, and intracellular calcium ion mobilization in stromal cells. We also showed that neither NAEPA nor DGPP affected the stimulatory effect of LPA on PGE_2 production,

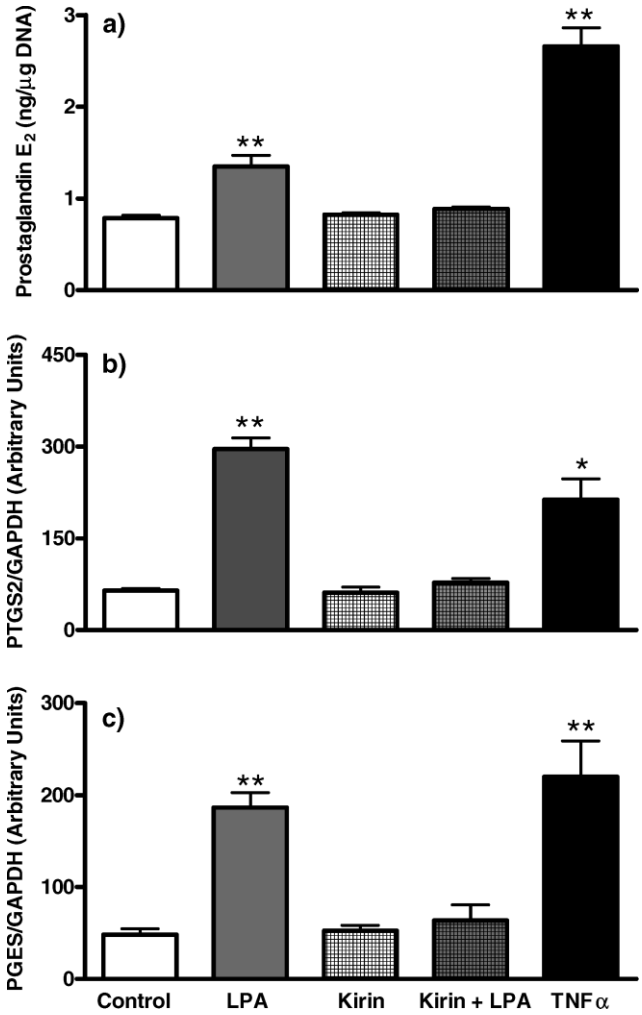

Figure 5. PGE_2 production (a), total quantification of *PTGS2* gene expression normalized to *GAPDH* mRNA expression (b), and total quantification of *PGES* gene expression normalized to *GAPDH* mRNA expression (c) in stromal cells stimulated for either 4 h (for the expression of mRNA transcripts for *PTGS2* and *PGES* in the cells) or 24 h (for PGE_2 measurement in the culture medium) with LPA (10^{-6} M), Ki16425 (10^{-5} M), and the combination of 0.5-h preincubation with Ki16425 and then LPA. Asterisks: *($P < 0.05$), **($P < 0.01$) indicate statistical differences between control and treated groups, as determined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

cell proliferation, and intracellular calcium ion mobilization in stromal cells. All of the selected chemicals have restricted selectivity to the respective LP-GPCRs; however, Ki16425 has the highest potency to LPA1 GPCR, as it was shown before by Ohta et al. (17). On the other hand, NAEPA and DGPP preferentially inhibited the LPA3- and LPA2-induced actions (18, 19). The LPA-induced stimulation of PGE_2 production, cell proliferation, and intracellular calcium ion mobilization in stromal cells *in vitro* agrees with our previous studies *in vivo* (13). We have demonstrated before that the infusion of Ki16425 caused the inhibition of endogenous LPA action via LPA1 receptor, leading to the decrease of P4 and PGE_2 concentrations. However, in contrast to our results, it has been shown that in the uteri of mice, pigs, and sheep, LPA action on PG synthesis was mediated mainly by LPA3 receptor (10–12). Summarizing this part of the study, LPA stimulates PGE_2 production, cell viability, and intracellular calcium ion mobilization in the cultured stromal endometrial cells via Ki16425-sensitive LPA1 receptors.

The data obtained in Experiment 3 confirmed the working hypothesis that LPA influences the expression of the enzymes responsible for PGE_2 synthesis (*PTGS2* and *PGES*) in the endometrial stromal cells. The results obtained in our study come together with the data of Ye et al. (10) and Seo et al. (11). Ye et al. (10) demonstrated that in mice, the targeted deletion of LPAR3 receptor resulted in the implantation defects accompanied by a reduction in *PTGS2* expression and levels of PGE_2 and PGI_2. Seo et al. (11) showed that LPA produced in the uterine endometrium may play a critical role in uterine endometrial function and conceptus development through LPA3-mediated *PTGS2* expression during implantation and establishment of pregnancy in pigs. Moreover, LPA-dependent PGE_2 stimulation via the increase of *PTGS2* and *PGES* mRNA expression in stromal cells is consistent with the study on other cell types (33–36). In these studies, LPA had been shown to increase PGE_2 synthesis in human monocytic cells (36) and rat mesanglial cells (33), and to up-regulate *PTGS2* expression in other cell types such as human ovarian cancer cells (35) and rat mesanglial cells (34).

The overall results indicate that LPA stimulates PGE_2 production, cell viability, and intracellular calcium ion mobilization in the cultured stromal endometrial cells via Ki16425-sensitive LPA1 receptors. Moreover, LPA exerts a stimulatory effect on PGE_2 production in stromal cells via the induction of *PTGS2* and *PGES* mRNA expression.

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