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Analysis of Lysophosphatidic Acid (LPA) Receptor and LPA-Induced Endometrial Prostaglandin-Endoperoxide Synthase 2 Expression in the Porcine Uterus

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Lysophosphatidic acid (LPA), a simple phospholipid-derived mediator with diverse biological actions, acts through the specific G protein-coupled receptors endothelial differentiation gene (EDG) 2, EDG4, EDG7, and GPR23. Recent studies indicate a critical role for LPA receptor signaling in embryo implantation. To understand how LPA acts in the uterus during pregnancy in pigs, we evaluated: 1) spatial and temporal expression of LPA receptors in the uterine endometrium during the estrous cycle and pregnancy and in early-stage concepti, 2) LPA levels in uterine luminal fluids from d 12 of the estrous cycle and pregnancy, 3) effects of steroid hormones on *EDG7* mRNA levels, and 4) effects of LPA on prostaglandin-endoperoxide synthase 2 (*PTGS2*) mRNA levels in the uterine endometrium using explant cultures. Of the four receptors, EDG7 was dominant, and its expression was regulated by pregnancy stage and status. *EDG7* expression was highest on d 12 pregnancy, and localized to the luminal and glandular epithelium, and *EDG7* mRNA levels were elevated by estrogen in the endometrium. *EDG7* expression was also detected in concepti of d 12 and 15. LPA with various fatty acyl groups was present in the uterine lumen on d 12 of both the estrous cycle and pregnancy. LPA increased *PTGS2* mRNA abundance in the uterine endometrium. These results indicate that LPA produced in the uterine endometrium may play a critical role in uterine endometrial function and conceptus development through EDG7-mediated *PTGS2* expression during implantation and establishment of pregnancy in pigs. (*Endocrinology* 149: 6166–6175, 2008)

IMPLANTATION for the establishment of pregnancy in pigs is a very complex process that requires well-coordinated interaction between the conceptus and maternal uterus. The process is regulated by the steroid hormones progesterone (P_4) and estrogen, as well as many other factors such as adhesion molecules, growth factors, and cytokines (1, 2). Beyond steroid hormones and prostaglandins (PGs), the involvement of lipid regulators in the processes of implantation and maternal recognition of pregnancy is poorly understood. Lysophosphatidic acid (LPA) has recently been identified as a potentially important factor in embryo implantation in mice (3).

LPA is a lysophospholipid (LP), *i.e.* a simple phospholipid composed of a glycerol or sphingoid backbone with a fatty acid of varied length and saturation (4). LPs include LPA, lysophosphatidylcholine, sphingosine 1-phosphate, and sphingosylphosphorylcholine. LPA, along with sphingosine 1-phosphate, is the best-characterized LP with identified receptors. There are four specific receptors for LPA: the G protein-coupled receptors designated as endothelial differentiation gene (EDG) 2, EDG4, EDG7, and GPR23 (also known as LPA_1, LPA_2, LPA_3, and LPA_4, respectively) (4). Through these receptors, LPA elicits many growth factor-like biological effects, such as cell proliferation, survival, migration, differentiation, and aggregation in various cell types (5). Expression of LPA receptors has been shown in many human and mouse tissues (4), but mechanisms regulating their expression are not well understood.

LPA is detectable in various body fluids, including serum (6), saliva (7), seminal plasma (8), and follicular fluids (9). In addition, elevated levels of LPA are detected in plasma and ascites from patients with ovarian cancer (10, 11). There has been no report on the presence of LPA in the uterus during the estrous cycle and pregnancy. LPA production and release into circulation involve hydrolysis of phospholipids by two enzymatic actions: phospholipase $A_{1/2}$ ($PLA_{1/2}$) plus lysophospholipase D (lysoPLD), or phospholipase D plus $PLA_{1/2}$ (6). Increased activity of lysoPLD correlates with the progress of pregnancy in humans (12). In pigs, PLA_2 activity is detectable in the uterine endometrium during the estrous cycle and pregnancy (13), but expression and activity of lysoPLD and phospholipase D have not been determined in the porcine uterine endometrium.

Targeted deletion of the *EDG7* gene in mice causes delayed implantation, aberrant embryo spacing, and reduced litter size due to down-regulation of prostaglandin-endoperoxide synthase 2 (*PTGS2*) (also called PG G/H synthase 2 and cyclooxygenase 2) expression and the ensuing lower level of PGE_2 and PGI_2 (3), which are essential for embryo implan-

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Abbreviations: DEPC, Diethylpyrocarbonate; DIG, digoxigenin; EDG, endothelial differentiation gene; E_2, estradiol-17β; ESR, estrogen receptor α; GE, glandular epithelium; ICI, ICI182,780; LE, luminal epithelium; LP, lysophospholipid; LPA, lysophosphatidic acid; lysoPLD, lysophospholipase D; PBST, PBS with 0.1% Tween 20; PG, prostaglandin; PGR, progesterone receptor; $PLA_{1/2}$, phospholipase $A_{1/2}$; P_4, progesterone; *PTGS2*, prostaglandin-endoperoxide synthase 2; SSC, standard saline solution; TBST, Tris-buffered saline with 0.1% Tween 20.

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tation in mice (14, 15). In pigs, PGs, especially $PGF_{2\alpha}$ and PGE_2, are produced in the uterine endometrium and play critical roles in regulating luteolysis and embryo implantation (16, 17). Indeed, *PTGS1* and *PTGS2* are expressed in the uterine endometrium at higher levels at diestrus and during early pregnancy in pigs (13, 18, 19). Elongated porcine concepti during early pregnancy also express *PTGS2* (20). Many factors, including growth factors, cytokines, pathogens, nitric oxide, and UV irradiation, can induce *PTGS2* expression by activating various transcription factors, such as nuclear factor-κB, CCAAT/enhancer binding protein, cAMP response element-binding protein, and activating transcription factor (21, 22). Some studies have shown that LPA increases *PTGS2* mRNA levels in ovarian cancer cells (23) and renal mesangial cells (24), but there is no information available on the role of LPA in *PTGS2* expression in the porcine uterus.

During the implantation period, the conceptus in pigs undergoes a dramatic morphological change and secretes various biological molecules, including estrogens, cytokines, and growth factors (25). Estrogen produced by conceptus plays a critical role in maternal recognition of pregnancy by redirecting $PGF_{2\alpha}$ secretion from the uterine vasculature to the uterine lumen where it is sequestered to prevent luteolysis (26). In addition, estrogen of conceptus origin and extended exposure of P_4 influence expression of many uterine genes, such as *FGF7*, *SPP1*, and *STAT1* (27–29). Our working hypothesis is that expression of LPA receptors is affected by estrogen of conceptus origin and ovarian P_4, and that LPA induces *PTGS2* expression in the porcine uterine endometrium as well as in concepti during the implantation period. Thus, to understand better the role of LPA and its receptor system in the uterine endometrium during implantation and pregnancy in pigs, we evaluated: 1) expression of LPA receptors in the endometrium and concepti, 2) the presence of LPA in the uterine lumen on d 12 of the estrous cycle and pregnancy, 3) the effect of steroid hormones on *EDG7* mRNA levels in the uterine endometrium, and 4) the effect of LPA on *PTGS2* mRNA levels in the uterine endometrium in pigs.

Materials and Methods

Animals and tissue collection

All experimental procedures involving animals were conducted in accordance with the National Research Council publication *Guide for the Care and Use of Laboratory Animals* (National Academy of Science, 1996). Sexually mature crossbred female pigs were assigned randomly to either cyclical or pregnant status. There were 24 gilts hysterectomized on d 12 and 15 of the estrous cycle, and d 12, 15, 30, 60, 90, or 114 pregnancy (n = 3 gilts/d/status). Pregnancy was confirmed by the presence of apparently normal concepti in uterine flushings or fetuses. Uterine flushings from d 12 and 15 of the estrous cycle and pregnancy were obtained by introducing and recovering 50 ml PBS (pH 7.4) at hysterectomy (25 ml/each horn). The uterine flushings were clarified by centrifugation (3000 × *g* for 10 min at 4C), aliquoted, and frozen at −80 C until analyzed. Endometrium dissected from the myometrium was collected from the middle portion of the uterine horn. Endometrial tissues were snap frozen in liquid nitrogen and stored at −80 C for RNA and protein extraction. For *in situ* hybridization and immunohistochemistry, cross-sections of endometrium and concepti were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 h and then embedded in paraffin.

Total RNA extraction and porcine LPA receptor cloning

Total RNA was extracted from endometrial tissues using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's recommendations. The quantity of RNA was assessed spectrophotometrically, and the integrity of the RNA was examined by gel electrophoresis using 1% agarose gels.

Two micrograms of total RNA were treated with Deoxyribonuclease I (Invitrogen) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to obtain cDNA. The cDNA templates were then diluted 1:5 with sterile water and amplified by PCR using *Taq* polymerase (Takara Bio Inc., Shiga, Japan), and specific primers based on mRNA sequences of mouse *EDG2* (GenBank accession no. NM_010336), human *EDG4* (GenBank accession no. BC025695), mouse *EDG7* (GenBank accession no. NM022983), or human *GPR23* (GenBank accession no. BC074722). The specific primers and PCR cycling conditions are listed in Table 1. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The identity of each amplified PCR product was verified by sequence analysis after cloning into the pCRII vector (Invitrogen).

Northern blot analysis

Total RNA (20 μg) was loaded into each lane and electrophoresed on 1% 3[*N*-morholino]propanesulfonic acid-formaldehyde agarose gels. RNA was transferred overnight onto a nylon membrane in 20× sodium chloride-sodium citrate (SSC). The RNA probes for LPA receptors were labeled with digoxigenin (DIG)-uridine 5′-triphosphate using the DIG RNA Labeling kit (Roche Applied Science, Indianapolis, IN). After transfer, the RNA was fixed to the blot by UV-cross linking (120 mJ). Prehybridization (30 min) and hybridization (7–8 h) were performed at 68 C using DIG Easy Hyb (Roche Applied Science). The blot was washed in low-stringency buffer (2× SSC and 0.1% sodium dodecyl sulfate) twice for 5 min each at room temperature and in high-stringency buffer (0.1× SSC and 0.1% sodium dodecyl sulfate) twice for 15 min each at 68 C. After stringency washing, signal was detected by an alkaline phosphatase reaction (Roche Applied Science) and exposure to x-ray film (Eastman Kodak Co., Rochester, NY).

Real-time quantitative RT-PCR

To analyze levels of *EDG2*, *EDG4*, *EDG7*, and *GPR23* mRNAs in the uterine endometrium, real-time RT-PCR was performed using the Applied Biosystems GeneAmp 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green method. cDNAs

TABLE 1. Summary of PCR primer sequences, annealing temperatures, and expected product sizes used for cloning partial cDNAs for LPA receptors

Primer	Sequence of forward (F) and reverse (R) primers (5′ → 3′)	Annealing temperature (C)	Product size (bp)	No. of cycles	GenBank accession no.
EDG2	F: GGT GGC AAT CTA CGT CAA CC R: AGT CCT CTG GCG AAC ATAGC	56	496	35	EF183523
EDG4	F: TTC CTC ATG TTC CAC ACT GG R: CCA TGA GCA GGA AGA CAA GC	54	355	35	EF183524
EDG7	F: TTT TCT GGA CTG TGT CCA ACC R: GGT TCA TGA CGG AGT TGA GC	56	302	35	EF183525
GPR23	F: TTT GAA GGC TTC TCC AAA CG R: TGA ATA GCT GGA AGG GAA GG	52	484	35	EF183526

TABLE 2. PCR primer sequences used for real-time RT-PCR of endometrial LPA receptor mRNA expression

Gene	Sequence of forward (F) and reverse (R) primers (5′ → 3′)	Annealing temperature (C)	Product size (bp)	No. of cycles	GenBank accession no.
EDG2	F: TGGGTATCGCGCCCATAACA R: TGGCCATTGCAATCGAGAGG	60	129	40	EF183523
EDG4	F: CGTGGTAGGCGGCTATGCAG R: CCGGCTCTCACTTCAGGGCT	60	141	40	EF183524
EDG7	F: TGCAGTTCAGGCCGTCCAGT R: GCCGGAGGACACCCATGAAG	60	111	40	EF183525
GPR23	F: TGCAAGGCACAAGGTGATTGG R: TGCATATGGCGGTCTTTGTGG	60	143	40	EF183526
ACTB	F: GTGGACATCAGGAAGGACCTCTA R: ATGATCTTGATCTTCATGGTGCT	60	137	40	U07786

were synthesized from 3 μg total RNA isolated from different uterine endometrial tissues, and newly synthesized cDNAs (total volume of 21 μl) were used for PCR. To maximize efficiency, primers were designed to amplify cDNA of less than 200 bp. The sequences and product sizes of primer pairs for each gene are listed in Table 2. The DyNAmo HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland) was used for amplification of LPA receptors. Final reaction volume is 20 μl, including 1 μl cDNA, 10 μl 2× premix, 2 μl of each primer, 0.4 μl 5-carboxy-x-rhodamine, and 4.6 μl diethylpyrocarbonate (DEPC)-treated ddH_2O. PCR cycle parameters were 50 C for 2 min and 95 C for 15 min, followed by 40 cycles of 95 C for 30 sec, 60 C for 30 sec, and 72 C for 30 sec. Data were analyzed using Applied Biosystems software. The results were reported as the relative expression to the level on d 12 of the estrous cycle after normalization of the transcript amount to the endogenous β-actin control by the $2^{-\Delta\Delta CT}$ method (30).

Explant culture

Endometrium was dissected from the myometrium and placed into warm phenol red-free DMEM/F-12 culture medium (Sigma-Aldrich Corp., St. Louis, MO) containing penicillin G (100 IU/ml) and streptomycin (0.1 mg/ml), as described previously (27) with some modifications. The endometrium was minced with scalpel blades into small pieces (2–3 mm^3), and aliquots of 500 mg were placed into T25 flasks with serum-free modified DMEM/F-12 containing 10 μg/ml insulin (Sigma-Aldrich; catalog no. I5500), 10 μg/ml transferrin (Sigma-Aldrich; catalog no. T1428), and 10 ng/ml hydrocortisone (Sigma-Aldrich; catalog no. H0396). Endometrial explants were cultured immediately after mincing in the presence of ethanol (control), estradiol-17β (E_2) (50 ng/ml; Sigma-Aldrich; catalog no. E8875), P_4 (3 ng/ml; Sigma-Aldrich; catalog no. P0130), E_2 plus P_4, E_2 plus P_4 plus ICI182,780 (ICI) (an estrogen receptor antagonist; 50 ng/ml; Tocris, Ballwin, MO), or E_2 plus P_4 plus RU486 [a P_4 receptor (PGR) antagonist; 30 ng/ml; Sigma-Aldrich; catalog no. M8046], for 24 h with rocking in an atmosphere of 5% carbon dioxide in air at 37 C. To determine the effects of LPA on *PTGS2* expression, explant tissues were treated with 0, 1, 10, or 100 μM LPA (Sigma-Aldrich; catalog no. L7260) or 10 μM LPA plus 10 μM Ki16425 (Sigma-Aldrich; catalog no. K0639), an EDG2/7 antagonist, for 24 h with rocking in an atmosphere of 5% carbon dioxide in air at 37 C. Explant tissues were then harvested, and total RNA was extracted for Northern blot analysis of *EDG7* or *PTGS2* mRNA levels. Experiments for *EDG7* expression were conducted using the endometria from three separate gilts on d 12 of the estrous cycle, and experiments for *PTGS2* expression were conducted using the endometria from three separate gilts on d 12 pregnancy. These experiments were conducted using endometrium from three individual gilts. Treatments were performed in triplicate using tissues obtained from each gilt.

Nonradioactive in situ hybridization

The nonradioactive *in situ* hybridization procedure was performed as described previously (31), with some modifications. Sections (5 μm) were rehydrated through successive baths of xylene, 100% ethanol, 95% ethanol, DEPC-treated water, and DEPC-treated PBS. Tissue sections were permeabilized with DEPC-treated PBS containing 0.3% Triton X-100. After washing in DEPC-treated PBS, tissue sections were digested with 5 μg/ml Proteinase K (Sigma-Aldrich) in Tris-EDTA [100 mM Tris-HCl, and 50 mM EDTA (pH 7.5)] at 37 C. After postfixation in 4% paraformaldehyde, sections were incubated for 2 × 15 min in PBS containing 0.1% active DEPC, and equilibrated for 15 min in 5× SSC. The sections were prehybridized for 2 h at 68 C in hybridization mix (50% formamide, 5× SSC, and 500 μg/ml herring sperm DNA; 200 μl on each section). Sense and antisense *EDG7* riboprobes labeled with DIG-uridine 5′-triphosphate were denatured for 5 min at 80 C and added to the hybridization mix. The hybridization reaction was performed at 68 C overnight. Prehybridization and hybridization were performed in a box saturated with a 5× SSC-50% formamide solution to avoid evaporation, and no coverslips were used. After hybridization, sections were washed for 30 min in 2× SSC at room temperature, 1 h in 2× SSC at 65 C, and 1 h in 0.1× SSC at 65 C. Probes bound to the section were immunologically detected using sheep anti-DIG Fab fragments covalently coupled to alkaline phosphatase and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) as a chromogenic substrate, according to the manufacturer's protocol (Roche Applied Science).

Protein isolation and immunoblot analysis

Endometrial tissues were homogenized in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.2 mM Na_3VO_3, 0.2 M phenylmethylsulfonylfluoride, and 0.5 μg/ml NaF) at a ratio of 100 mg tissue/1 ml buffer, and the cellular debris was removed by centrifugation (16,500 × *g* for 5 min). The concentration of proteins in lysates and uterine luminal flushings was determined using a Bradford protein assay (Bio-Rad Laboratories, Inc., Richmond, CA) with BSA as the standard. Proteins (20 μg) were loaded in each lane and electrophoresed on 12% SDS-PAGE gels, followed by electrotransfer onto nitrocellulose membranes. Nonspecific binding was blocked with 5% (wt/vol) fat-free milk in Tris-buffered saline with 0.1% Tween 20 (TBST buffer) for 1 h at room temperature. The blot was incubated overnight at 4 C with 0.1 μg/ml rabbit polyclonal anti-EDG7 antibody (GeneTex, San Antonio, TX; catalog no. GTX16456) diluted in 2% milk/TBST. The blot was washed in TBST at room temperature three times for 10 min each, incubated with peroxidase-conjugated goat antirabbit secondary antibody (1:20,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at room temperature, and rinsed again for 30 min at room temperature with TBST. Immunoreactive proteins were detected by chemiluminescence (SuperSignal West Pico; Pierce Chemical Co., Rockford, IL) according to the manufacturer's recommendations using Kodak Biomax XAR Film. Blots were reblotted with rabbit polyclonal antiactin antibody (1:5000; Sigma-Aldrich) to assess consistent loading. The OD of EDG7 and actin bands in the immunoblots was quantified by scanning densitometry using an Epson1680 (Epson, Long Beach, CA) and GelPro Analyzer (Media Cybernetics, Silver Spring, MD). Values are presented as the ratio of each EDG7-integrated OD to the corresponding actin-integrated OD.

Immunohistochemical analysis

Immunohistochemistry was used to determine which type of cells in the porcine endometrium expresses EDG7 proteins. Sections (5 μm) were deparaffinized and rehydrated in an alcohol gradient. For antigen retrieval, tissue sections were boiled in citrate buffer (pH 6.0) for 10 min. Tissue sections were washed with PBS with 0.1% Tween 20 (PBST) three times, and a peroxidase block was performed with 0.5% H_2O_2 in meth-

anol for 30 min. Tissue sections were then blocked with 10% normal goat serum for 30 min at room temperature. Three micrograms per milliliter of rabbit anti-EDG7 (GeneTex; catalog no. GTX70731) were added and incubated overnight at 4 C in a humidified chamber. For each tissue tested, purified normal rabbit IgG was substituted for primary antibody as a negative control. Tissue sections were washed with PBST three times. Biotinylated goat antirabbit secondary antibody (Vector Laboratories, Burlingame, CA) was added and incubated for 1 h at room temperature. After washes with PBST, a streptavidin peroxidase conjugate (Zymed Laboratories, Inc., San Francisco, CA) was added to the tissue sections, which were then incubated for 10 min at room temperature. The sections were washed with PBST, and 3-amino-9-ethylcarbozole in N,N-dimethylformamide color development substrate (Zymed Laboratories) was added to the tissue sections, which were then incubated for 10 min at room temperature. The tissue sections were then washed in water, counterstained with Mayer's hematoxylin, and coverslipped.

Analysis of LPA concentrations

LPA concentrations in the uterine lumen on d 12 of the estrous cycle and pregnancy were analyzed from uterine luminal flushings using an electrospray ionization tandem mass spectrometry method (Seoul Clinical Laboratories, Seoul, Korea), as described previously (32). Briefly, 40 μl 6 M hydrochloric acid and 800 μl methanol/chloroform (2:1) containing LPA 14:0 as internal standard were added to each 200 μl uterine flushing sample. The uterine flushing sample was vortexed for 1 min, incubated for 20 min at −10 C, and centrifuged at 25,000 × *g* for 10 min at 4 C. The lower phase was transferred to a new microcentrifuge tube and incubated for 30 min at −50 C to remove lipid residues. The lower phase (100 μl) was transferred to a 96-well microplate (Costar, Cambridge, MA), evaporated under a gentle nitrogen stream at 40 C, and redissolved in 100 μl methanol. Ten microliters of the solution were directly injected into the turbo electrospray ion source of an electrospray ionization tandem mass spectrometry (API 4000; Applied Biosystems).

Statistical analyses

Data from real-time RT-PCR or immunoblot analyses for LPA receptors and *PTGS2* expression were subjected to least squares ANOVA using the General Linear Models procedures of SAS Institute Inc. (Cary, NC). As sources of variation, the model included day, pregnancy status (cyclical or pregnant), and their interactions to evaluate the steady-state level of LPA receptor mRNAs and EDG7 protein, and treatment and animal to evaluate the effect of steroid hormones on *EDG7* mRNA levels and the effect of different LPA doses on *PTGS2* mRNA levels. Preplanned contrasts (control *vs.* E_2; control *vs.* P_4; E_2 *vs.* E_2 plus P_4; E_2 plus P_4 *vs.* E_2 plus P_4 plus ICI; and E_2 plus P_4 *vs.* E_2 plus P_4 plus RU486 for *EDG7* expression, and 0 *vs.* 1; 0 *vs.* 10; and 0 *vs.* 100 for *PTGS2* expression) were used to test for effects of treatments in the explant cultures. Data are presented as least squares means with SE. Data from LPA concentration measurements and data from Ki16425 treatments were subjected to the *t* test procedure of SAS, and are presented as means with SE.

Results

Levels of LPA receptor mRNAs in the uterine endometrium during the estrous cycle and pregnancy in pigs

To determine whether LPA receptors were expressed in the porcine uterine endometrium, we cloned partial cDNAs for EDG2, EDG4, EDG7, and GPR23 by RT-PCR (Table 1). Northern blot hybridization analysis using these partial cDNAs detected *EDG7* mRNA with a single 2.4-kb transcript, which is similar in size to that in the mouse (33), in total endometrial RNA from cyclical and pregnant pigs, but *EDG2*, *EDG4*, and *GPR23* mRNAs were not detected by Northern blot analysis (data not shown).

Real-time RT-PCR analysis showed that mRNA levels for *EDG7*, but not *EDG2* and *EDG4*, were regulated by pregnancy status and stage (Fig. 1). During pregnancy, steady-state levels of *EDG7* mRNA were highest on d 12 but decreased thereafter ($P < 0.01$). Levels of *EDG7* mRNA on d 12 pregnancy were significantly higher than those on d 12 of the estrous cycle (day × status; $P < 0.01$). *GPR23* mRNA levels by real-time RT-PCR were below the detection limit in most endometrial tissues.

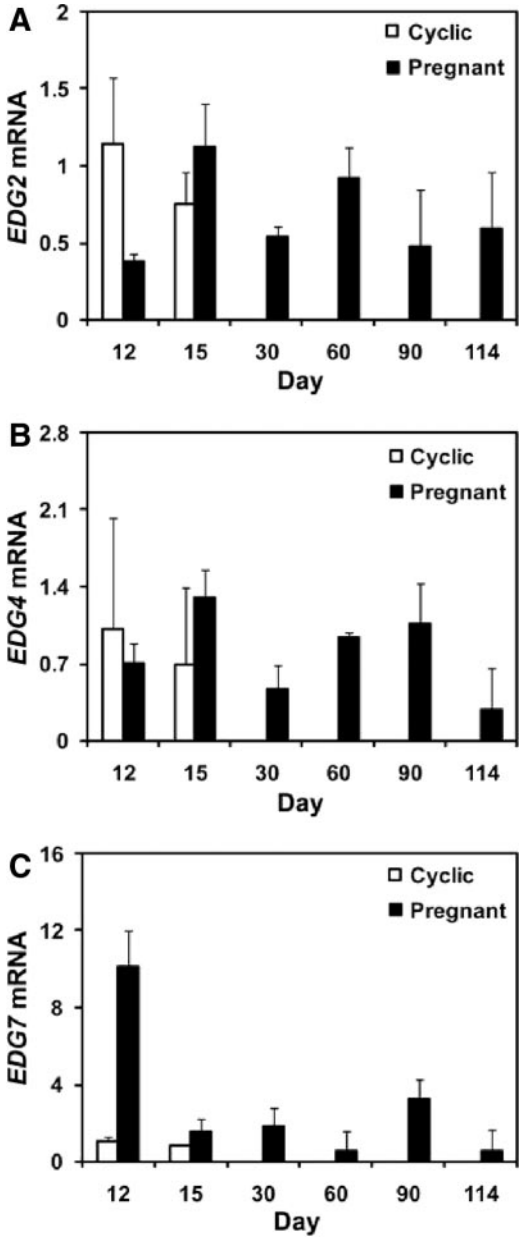

FIG. 1. Real-time RT-PCR analysis of mRNA levels for EDG2 (A), EDG4 (B), and EDG7 (C) in the uterine endometrium during the estrous cycle and pregnancy in pigs. Endometrial tissue samples from cyclical and pregnant gilts (n = 3 per each day) were tested. Abundance of mRNA was presented as the relative expression to the level on d 12 of the estrous cycle after normalization of the transcript amount to the endogenous β-actin control. *EDG7* mRNA levels were highest on d 12 in pregnant gilts ($P < 0.01$), and higher on d 12 pregnancy than on d 12 of the estrous cycle (day × status; $P < 0.01$), whereas *EDG2* and *EDG4* mRNA levels were not affected by the pregnancy status and stage.

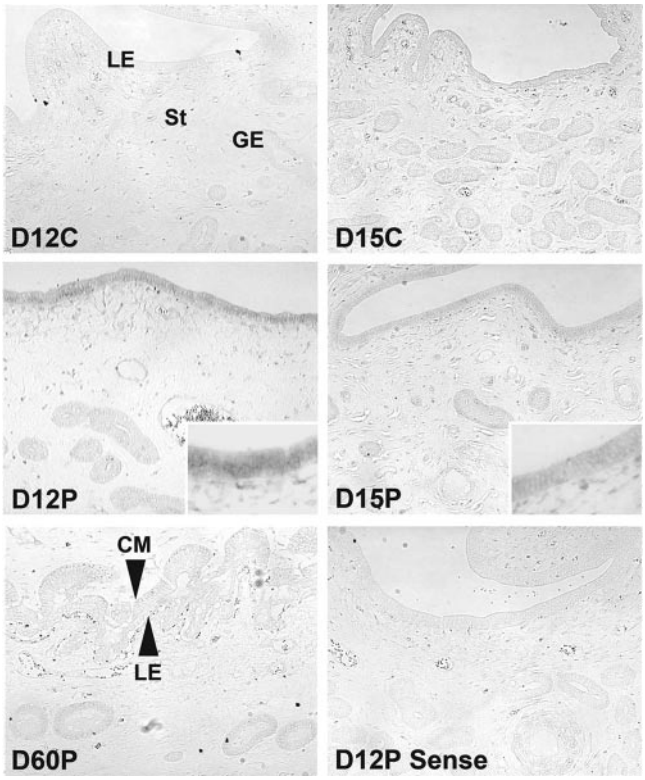

FIG. 2. *In situ* hybridization analysis of *EDG7* mRNA in the uterine endometrium during the estrous cycle (C) and pregnancy (P) in pigs. *EDG7* mRNA was localized to the LE and GE during the estrous cycle and early pregnancy, and especially by the LE on d 12 pregnancy. A representative uterine section from d 12 pregnancy is shown hybridized with a DIG-labeled sense *EDG7* cRNA probe (Sense) as a negative control. A higher magnification image of *EDG7* mRNA localization in LE cells is shown in *inset* on d 12 and 15 pregnancy. Original magnification, ×200. *Inset*, ×400. CM, Chorionic membrane; D, d; St, stroma.

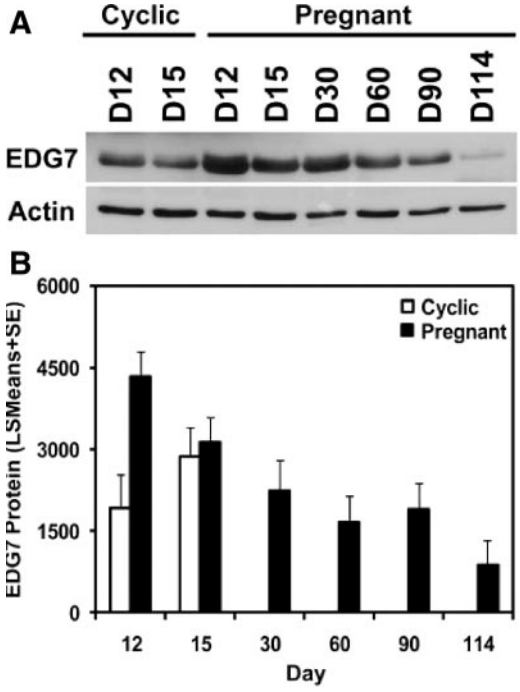

FIG. 3. Immunoblot analysis of EDG7 proteins in the uterine endometrium during the estrous cycle and pregnancy in pigs. A, Endometrial tissues from cyclical and pregnant gilts [n = 3 per each day (D)] were tested. An EDG7 protein of approximately 42 kDa was detected. Actin was used as a loading control. B, The ratio of EDG7 protein density to actin density obtained by scanning densitometry. EDG7 protein levels were highest on d 12 in pregnant gilts ($P < 0.05$), and higher on d 12 pregnancy than on d 12 of the estrous cycle (day × status; $P < 0.05$).

Localization of EDG7 mRNA in the uterine endometrium during the estrous cycle and pregnancy in pigs

Nonradioactive *in situ* hybridization was used to determine which types of cells produce *EDG7* mRNA in the uterine endometrium (Fig. 2). The *EDG7* mRNA signal was barely detected in the endometrium on d 12 of the estrous cycle but was weakly detected in the luminal epithelium (LE) and glandular epithelium (GE) on d 15 of the estrous cycle. On d 12 pregnancy, *EDG7* mRNA was strongly detected in the LE and moderately so in the GE of the endometrium but was not detected in the stroma. After d 15 pregnancy, *EDG7* mRNA was only rarely detected in the endometrium.

Expression and localization of EDG7 protein in the porcine uterine endometrium during the estrous cycle and pregnancy

Having determined that *EDG7* mRNA was localized in the uterine endometrium in a stage and cell type-specific manner, we then examined protein expression. Immunoblot analysis detected a single band of EDG7 protein with a 42-kDa form across all stages of the estrous cycle and pregnancy (Fig. 3). Steady-state levels of EDG7 protein were the highest on d 12 during pregnancy and decreased thereafter ($P < 0.05$), and levels of EDG7 protein on d 12 pregnancy were higher than those on d 12 of the estrous cycle (day × status; $P < 0.05$).

Localization of EDG7 protein in the porcine endometrium during the estrous cycle and pregnancy was determined by immunohistochemistry (Fig. 4). On d 12 and 15 of the estrous cycle, EDG7 was weakly detected in the GE, whereas on d 12 and 15 pregnancy, EDG7 was weakly detected in the LE and strongly in the GE. Interestingly, EDG7 protein in the LE on d 15 pregnancy was localized primarily to the basal side of the cells. During mid- to late-stage pregnancy, EDG7 protein was rarely detected in the uterine endometrium, but it was detected in endoderm cells of allantoic membranes starting at d 60 pregnancy.

Expression of EDG7 mRNA and protein in the conceptus during early pregnancy

To determine whether concepti also expressed EDG7, we performed *in situ* hybridization and immunohistochemistry using concepti from d 12 and 15 pregnancy (Fig. 5). EDG7 mRNA and protein were expressed in concepti from d 12 and 15 pregnancy.

LPA concentrations in the uterine lumen on d 12 of the estrous cycle and pregnancy

Having determined that EDG7, a receptor for LPA, was expressed in the maternal-fetal interface, we then asked if LPA was present in the uterine lumen and if LPA levels

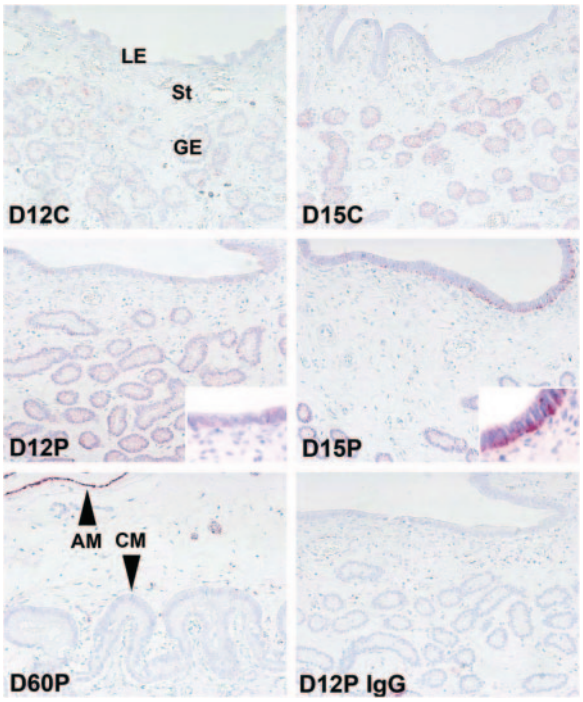

FIG. 4. Immunohistochemical analysis of EDG7 protein in the uterine endometrium during the estrous cycle (C) and pregnancy (P) in pigs. Immunoreactive EDG7 protein was detected on luminal epithelial cells (LE) and glandular epithelial cells (GE), and later on the allantoic membrane (AM). A higher magnification image of EDG7 protein localization in LE cells is shown in *inset* on d 12 and 15 pregnancy. Original magnification, ×200. *Inset*, ×400. CM, Chorionic membrane; D, d; St, stroma.

differed in the uterine lumen between d 12 of the estrous cycle and pregnancy. Because the LPA molecule is composed of various different fatty acid side chains, we measured the concentrations of LPA with different fatty acyl groups, including palmitoyl (LPA 16:0), stearoyl (LPA 18:0), oleoyl (LPA 18:1), linoleoyl (LPA 18:2), and arachidonyl (LPA 20:4) groups in uterine luminal flushings from d 12 of the estrous cycle and pregnancy (Table 3). Concentrations of LPA 16:0, LPA 18:0, and LPA 18:2 in the uterine lumen on d 12 pregnancy were significantly higher than those on d 12 of the estrous cycle ($P < 0.01$ for LPA 16:0 and LPA 18:2, and $P = 0.06$ for LPA 18:0).

Effect of steroid hormones on EDG7 mRNA levels in the uterine endometrium

Next, we asked if the abundance of *EDG7* mRNA in the endometrium was affected by the steroid hormones E_2 and P_4. Uterine endometrial explant tissues from gilts on d 12 of the estrous cycle were treated with control, E_2, P_4, E_2 plus P_4, E_2 plus P_4 plus ICI, or E_2 plus P_4 plus RU486. As shown in Fig. 6, *EDG7* mRNA levels were increased by E_2 (control *vs.* E_2; $P < 0.01$), but P_4 did not have any effect on *EDG7* levels, even when combined with E_2 (control *vs.* P_4, E_2 *vs.* E_2 plus P_4; $P > 0.05$). E_2-induced *EDG7* mRNA levels were inhibited by the addition of ICI, an estrogen receptor antagonist (E_2 plus P_4 *vs.* E_2 plus P_4 plus ICI; $P < 0.01$) but were not affected by the presence of RU486, a PGR antagonist (E_2 plus P_4 *vs.* E_2 plus P_4 plus RU486; $P > 0.05$).

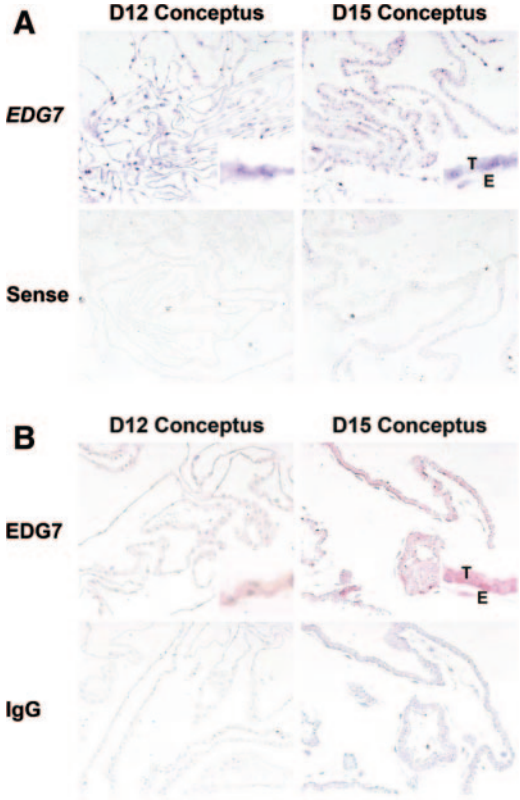

FIG. 5. Expression of EDG7 mRNA and protein on concepti from d 12 and 15 pregnancy by *in situ* hybridization (A) and immunohistochemistry (B). EDG7 mRNA and protein were detected on conceptus trophectoderm (T). A higher magnification image of *EDG7* expression in concepti is shown in *inset* on d 12 and 15 pregnancy. Original magnification, ×200. *Inset*, ×400. D, d; E, endoderm.

Effect of LPA on PTGS2 mRNA levels in the uterine endometrium

Because deletion of the *EDG7* gene down-regulates *PTGS2* expression in mice (5), we examined whether LPA plays a role in *PTGS2* expression in the porcine uterine endometrium. Uterine endometrial explant tissues from gilts on d 12 pregnancy were treated with 0, 1, 10, or 100 μM LPA. As shown in Fig. 7, *PTGS2* mRNA levels in endometrial tissues increased significantly after treatment with 10 or 100 μM LPA ($P < 0.05$). The LPA-induced increase of *PTGS2* expression was reduced by cotreatment with Ki16425, an EDG2/7 antagonist ($P < 0.05$) (Fig. 8).

Discussion

We observed that: 1) EDG7 was expressed in the uterine endometrium in a cell type- and stage-specific manner during the estrous cycle and pregnancy; 2) EDG7 expression was detectable in conceptus trophectoderm on d 12 and 15 pregnancy; 3) several LPA species were present in the uterine lumen on d 12 of the estrous cycle and pregnancy, and levels of some LPA species differed depending on pregnancy status; 4) *EDG7* mRNA levels were increased by estrogen; and 5) LPA up-regulated *PTGS2* expression in the uterine endometrium. To our knowledge, this is the first report characterizing the LPA receptor system and its function in the uterus in pigs.

TABLE 3. Concentration (nM) of LPA in uterine flushings from d 12 of the estrous cycle and pregnancy in gilts

Status	LPA 16:0[a]	LPA 18:0[b]	LPA 18:1	LPA 18:2[a]	LPA 20:4
d 12 estrous cycle	124.65 ± 26.52	267.57 ± 4.90	75.14 ± 10.82	128.72 ± 25.90	117.29 ± 17.74
d 12 pregnancy	268.10 ± 27.72	480.00 ± 74.24	67.55 ± 9.74	325.02 ± 33.06	127.87 ± 36.20

[a] Day 12 of the estrous cycle *vs.* pregnancy ($P < 0.01$).
[b] Day 12 of the estrous cycle *vs.* pregnancy ($P = 0.06$).

There are four specific receptors for LPA (4). In our study, expression of all four receptors was detected by RT-PCR, but only *EDG7* expression was detectable by Northern blotting, and its expression was regulated by pregnancy status and pregnancy stage in the uterine endometrium in pigs. Steady-state levels of *EDG7* expression were highest on d 12 pregnancy, the period when implantation begins, and estrogens are produced by the conceptus trophectoderm. These results are similar to the findings that *EDG7* mRNA levels in the uterine endometrium are highest on embryonic d 3.5, the implantation period in mice (3). Targeted deletion of the *EDG2* and *EDG4* genes in mice does not affect reproductive function (34), but deletion of the *EDG7* gene causes problems in implantation and embryo spacing (3). These reports and our observations indicate that EDG7 is the predominant LPA receptor in the uterine endometrium and plays an important role in implantation, whereas EDG2, EDG4, and GPR23 are not critical for embryo implantation because of their low levels of expression and no regulation during pregnancy in the uterine endometrium.

Expression of EDG7 mRNA and protein was localized to the endometrial LE and GE. On d 12 pregnancy, *EDG7* mRNA was localized mainly in the LE and somewhat in the GE, whereas EDG7 protein was present mainly in the GE and weakly in the LE. On d 15 pregnancy, the signal intensity of *EDG7* mRNA was low in the LE and GE, but expression of EDG7 protein was apparent in the LE and GE, revealing a discrepancy in LE expression of EDG7 mRNA and protein on d 12 and 15 pregnancy. This difference may be because translation of EDG7 protein takes longer in the LE compared with the GE, or that GE expression of EDG7 begins before d 12 pregnancy. Alternatively, this may be due to a lack of sensitivity of the nonradioactive technique for *in situ* hybridization. Interestingly, EDG7 in the LE on d 15 pregnancy was localized to the basal side of the cells. This distribution indicates that LPA may act as an autocrine and paracrine mediator in epithelial cell-to-epithelial cell and epithelial cell-to stromal cell communications because LPA can be derived from epithelial cells, stromal cells, and blood vessels (4).

Allantoic fluid contains substantial amounts of water, electrolytes, carbohydrates, proteins, and other nutrients, and provides a reservoir of nutrients secreted by the uterine endometrial epithelium and fetus (35). Accumulation of allantoic fluid is affected by steroid hormones (estrogen and P_4) and protein hormone (prolactin) in pigs. EDG7 protein was detected in endoderm cells of allantoic membranes from d 60 to term, although *EDG7* mRNA was not detected. This discrepancy may be caused by a low level of mRNA levels or low sensitivity of the *in situ* hybridization technique using nonradioactive-labeled probes applied in this study. We did not measure the LPA levels in allantoic fluids, but it is possible that LPA, if present in allantoic fluids, may influence the function of the allantoic membrane via the EDG7 signaling pathway in fetal development during late-stage pregnancy.

It has been shown that human serum contains 2–20 μM LPA, and the major LPA species in human serum, in decreasing order, are LPA 18:1, LPA 16:0, LPA 18:2, LPA 20:4, and LPA18:0 (36). Several other body fluids such as saliva (7), seminal plasma (8), and follicular fluids, (9) also contain LPA. The results of this study establish that uterine luminal fluids contain LPA species, as expected from the discovery of the enzymatic system that generates LPA in the porcine uterus. We did not measure LPA levels in uterine endometrial tissue extracts because endometrial tissue extracts contain various materials that originate from blood that is known to contain LPA (37). The levels of LPA species in uterine luminal fluids seem to be comparable to serum because the levels shown in Table 3 are diluted with flushing solution. LPA 16:0, LPA 18:0, and LPA 18:2 were the major LPA species in uterine luminal fluid on d 12 pregnancy, whereas LPA 18:0 was the major LPA species on d 12 of the estrous cycle. Interestingly, uterine luminal fluids on d 12 pregnancy contained more LPA 16:0, LPA 18:0, and LPA 18:2 than those on d 12 of the estrous cycle. In insect Sf9 cells, different LPA species with varied length and position of the acyl chain have different potencies to activate cellular responses; LPA 18:1, LPA 18:2, LPA 18:3, and LPA 20:4 induce more Ca^{2+}

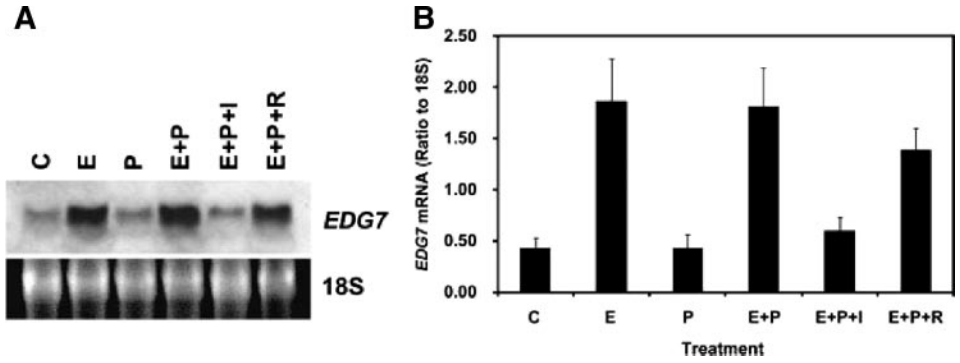

FIG. 6. Effect of estrogen and P_4 on *EDG7* mRNA levels in porcine uterine endometrial explant cultures by Northern blot (A) and densitometric (B) analyses. Endometrial explants from gilts on d 12 of the estrous cycle were cultured in DMEM/F-12 in the presence of control (C), E_2 (E) (50 ng/ml), P_4 (P) (3 ng/ml), E_2 plus P_4, E_2 plus P_4 plus ICI (I) (50 ng/ml, an estrogen receptor antagonist), or E_2 plus P_4 plus RU486 (R) (30 ng/ml, a PGR antagonist), at 37 C for 24 h. E_2 increased *EDG7* mRNA levels ($P < 0.01$) when E_2 alone or E_2 plus P_4 groups were compared with control or P_4 alone groups, respectively. All experiments were repeated with endometrium from each of the three gilts in triplicate for each treatment.

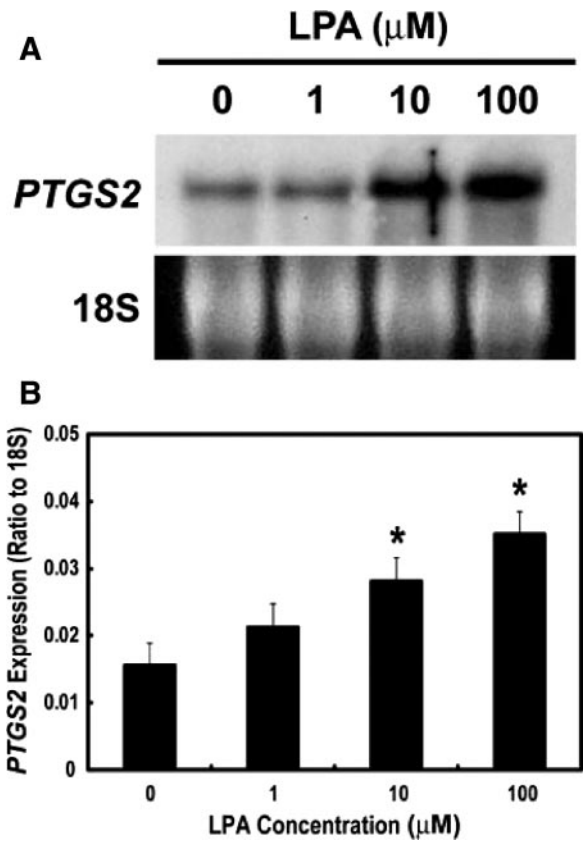

FIG. 7. Effect of LPA on *PTGS2* mRNA levels in the uterine endometrium by Northern blot (A) and densitometric (B) analyses. Endometrial explants from gilts on d 12 pregnancy were cultured in DMEM/F-12 in the presence of 0, 1, 10, or 100 μM LPA at 37 C for 24 h. Increasing doses of LPA elevated *PTGS2* mRNA levels significantly ($P < 0.05$). All experiments were repeated with endometrium from each of the three gilts in triplicate for each treatment, *, $P < 0.05$.

response than LPA 16:0 and LPA 18:0 through EDG7 (37). Accordingly, we speculate that increased LPA 16:0, LPA 18:0, and LPA 18:2, and/or the changed ratio of LPA species levels in the uterus may activate cellular responses for implantation in the uterine endometrium as well as in the conceptus trophectoderm. However, the detailed mechanisms and significance of changes in LPA composition depending on pregnancy status are not clear. To understand these, it may be necessary to determine the affinity of each LPA species for EDG7, the signaling pathways induced by each LPA species, and any enzymatic preference to generate specific LPA species in the uterus during pregnancy. The source or types of cells that produce LPA in the uterine lumen also need to be identified because this was not pursued in this study.

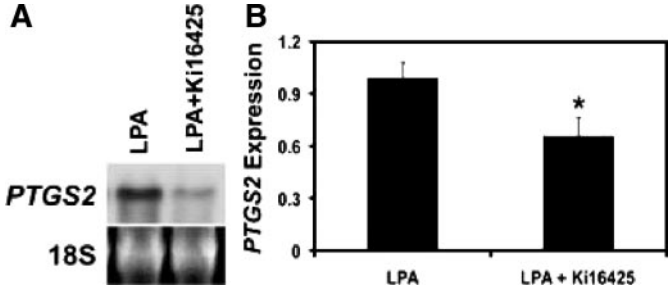

FIG. 8. Inhibition of LPA-induced *PTGS2* expression by an EDG2/7 antagonist. Endometrial explants from gilts on d 12 pregnancy were cultured in DMEM/F-12 in the presence of 10 μM LPA or 10 μM LPA with 10 μM Ki16425, an EDG2/7 antagonist, at 37 C for 24 h. Cotreatment of LPA with Ki16425 significantly reduced LPA-induced *PTGS2* expression ($P < 0.05$). All experiments were repeated with endometrium from each of the three gilts in triplicate for each treatment.

Before this work, not much was known regarding regulation of *EDG7* expression. Interestingly, the pattern of *EDG7* expression during the estrous cycle and pregnancy was very similar to that of *FGF7* in the porcine uterine endometrium, in that *FGF7* expression is highest on d 12 pregnancy and decreases thereafter (38). In pigs, conceptus trophectoderm secretes estrogen for maternal recognition of pregnancy around d 12 pregnancy (1). The estrogen of conceptus origin induces *FGF7* expression in the uterine endometrium (26). Thus, we hypothesized that *EDG7* expression might also be induced by estrogen of conceptus origin. Indeed, our experiments using endometrial explant cultures demonstrated that estrogen induced *EDG7* expression, but P_4 did not affect *EDG7* expression. Increased expression of *EDG7* in uterine luminal epithelial cells corresponded to the period temporally associated with the loss of the PGR and expression of estrogen receptor α (ESR1) in the uterine epithelia. PGR is not detectable in the LE and GE on d 12–18 pregnancy, but ESR1 is expressed in the LE and GE on d 12 pregnancy (39, 40). However, it remains unclear whether estrogen induction of *EDG7* expression is associated with down-regulation of PGR in the LE, and whether estrogen acts directly on the *EDG7* gene to induce *EDG7* expression or indirectly through other unknown mechanisms. Further research is required to elucidate the relationship between *EDG7* expression, steroid hormones, and their receptors in the porcine uterus. Recently, it was shown that *EDG7* expression is increased by P_4 and inhibited by estrogen in mice (41). However, the mice in that study were ovariectomized, which causes altered expression of steroid receptors in the uterus. The pattern of ESR1 and PGR expression in the uterine endometrium is generally altered by ovariectomy in various species (42–44).

Since the first report that LPA induces cell proliferation and differentiation similar to that induced by growth factors via G protein-coupled receptors (45), many studies have shown that LPA participates in diverse physiological functions such as angiogenesis, neuronal and cardiovascular development, immunomodulation, smooth muscle contraction and relaxation, and tumorigenesis (4). Not much is known about LPA function in the uterus, but a recent report suggested that LPA-LPA receptor signaling plays an important role in the embryo implantation process (3). Deletion of the *EDG7* gene in mice causes delayed implantation, aberrant embryo spacing, hypertrophic placentas, and embryonic death, along with reduction of *PTGS2* expression and the consequent reduced secretion of PGE_2 and PGI_2 (3). PTGS2 is an enzyme involved in the production of PGs (45). In the pig, PGs, especially $PGF_{2\alpha}$ and PGE_2, play critical roles in the processes of luteolysis, implantation, and maternal recognition of pregnancy (1, 16, 17), and are produced by the uterine endometrium during the estrous cycle and early pregnancy

and by concepti (46–48). Inhibition of PG synthesis by indomethacin results in pregnancy failure (49). PTGS2 expression is observed in uterine endometrial epithelial cells during the estrous cycle and pregnancy, and its expression increases at diestrus and during early pregnancy (18, 19). We hypothesized that LPA might induce PTGS2 expression, and observed LPA-induced *PTGS2* expression in the uterine endometrium from gilts on d 12 pregnancy. This result suggests that LPA via PTGS2 expression may modulate uterine production of $PGF_{2\alpha}$ and PGE_2, which are important regulators for implantation and maternal recognition of pregnancy in pigs. PTGS2 induction by LPA has also been shown in *in vitro* cell culture studies using ovarian cancer cells (23) and renal mesangial cells (24). Induction of *PTGS2* expression is mediated by several transcription factors, including nuclear factor-κB, CCAAT/enhancer binding protein, cAMP response element-binding protein, and activating transcription factor (21, 22). Because activation of EDG7 by LPA causes stimulation of cAMP, mitogen-associated protein kinases, and phospholipase C and Ca^{2+} signaling pathways (4), it is likely that one or more signaling pathways are activated to induce *PTGS2* expression in the uterine endometrium. Further detailed studies are needed to clarify the signaling molecules activated by EDG7 to induce *PTGS2* expression.

Conceptus trophectoderm during early pregnancy also expresses EDG7 mRNA and protein. Expression of EDG7 in the conceptus and the presence of its ligand, LPA, in uterine luminal fluids suggest that LPA may also play an important role in the development of the conceptus and implantation. Because elongated concepti express PTGS2 (20), and substantial amounts of PGs are produced by conceptus trophectoderm (48), it is possible that LPA activates PTGS2 expression to induce PG synthesis in concepti. Further research is required to elucidate the function of LPA in conceptus development.

In summary, we found that LPA, present in the porcine uterus, activated *PTGS2* expression via EDG7, which was expressed in the uterine endometrium during the estrous cycle and pregnancy in a cell type- and stage-specific manner and up-regulated by estrogen of conceptus origin. Although LPA's function in inducing *PTGS2* expression in the uterine endometrium in pigs has been demonstrated in this study, LPA may have additional biological activities that need to be investigated. Overall, our findings suggest that the LPA-EDG7 signaling system plays a critical role in uterine function for the implantation and establishment of pregnancy in pigs.

Acknowledgments

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