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Expression of Lysophosphatidic Acid Receptor 3 in the Uterine Endometrium of Pigs with Somatic Cell Nuclear Transfer Cloned Conceptuses

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ABSTRACT

Lysophosphatidic acid (LPA) is a small lipid molecule that plays an important role through LPA receptors (LPARs) in reproductive processes. Our previous study has shown maximal expression of *LPAR3* in the uterine endometrium on day (D) 12 of pregnancy in pigs, the period when conceptus secretes various molecules such as estrogen and interleukin-1β (IL1B) and initiates implantation. We determined that endometrial expression of *LPAR3* was increased by conceptus estrogen in the previous study, but the effect of IL1B on *LPAR3* expression has not been determined. Thus, in this study we examined whether *LPAR3* expression was also affected by IL1B. Endometrial explant cultures from D12 of the estrous cycle showed that levels of endometrial *LPAR3* expression did not changed in response to IL1B. We also investigated *LPAR3* expression in the uterine endometrium on D12 and D30 of pregnancy from gilts with conceptuses derived from somatic cell nuclear transfer (SCNT). The expression of *LPAR3* mRNA was lower in endometria from gilts with conceptuses resulting from SCNT compared with those from gilts with embryos resulting from natural mating on D12 of pregnancy, but it was not different between them on D30 of pregnancy. Our results indicate that estrogen of conceptus origin is responsible for induction of *LPAR3* expression during the peri-implantation period and appropriate LPA signaling is impaired in the uterine endometrium with SCNT-derived conceptuses during the implantation period in pigs.

(**Key words :** Pig, Uterus, Interleukin-1β, LPAR3, SCNT)

INTRODUCTION

Embryo implantation is a process that conceptus trophectoderm adheres to uterine luminal epithelium, and requires appropriate interactions between the conceptus and maternal uterus (Bowen and Burghardt, 2000). During pre-implantation period, pig conceptus undergoes dramatic trophoblastic elongation and secretes various molecules, which induce changes in maternal uterus to become receptive to embryo implantation (Bazer et al., 1998; Burghardt et al., 1997). A variety of molecules including steroid hormones, cytokines, adhesion molecules, and growth factors are involved in this process. Among these molecules, steroid hormones and prostaglandins are essential lipid components in the process of implantation and maternal recognition of pregnancy, but involvement of other lipid molecules is poorly understood. Recent studies indicated that lysophosphatidic acid (LPA) affects reproductive processes in vertebrates (Ye and Chun, 2010).

LPA is a lysophospholipid composed of a glycerol backbone with a fatty acyl chain at a sn-1 or sn-2 position and a free phosphate group (Ishii et al., 2004). There are at least six specific receptors for LPA, designated as LPAR1-6. Depending on the type of LPAR activated, LPA specifies its biological function, such as cell proliferation, survival, migration, differentiation, and aggregation in various cell types (Gardell et al., 2006). Among LPA receptors, LPAR3 appears to be the most important receptor for embryo implantation in the uterus during early pregnancy in several species. In mice, LPAR3 is associated with the process of embryo implantation and embryo spacing (Ye et al., 2005). In pigs, endometrial expression of *LPAR3* is highest on day (D) 12 of pregnancy when the conceptus implantation begins (Seo et al., 2008). LPA is detected in the uterine lumen in pigs (Seo et al., 2008).

During the peri-implantation period, uterine endometrium is under the control of estrogen secreted by conceptus and progesterone produced by corpora lutea (CL). Estrogen secreted from conceptus blocks luteolytic action of $PGF_{2\alpha}$ for sustained progesterone secretion from CL. Also, estrogen affects expression of many endometrial genes (Ka et al., 2001; White et al., 2005; Joyce et al., 2007; Choi et al., 2010). In addition to estrogen, pig conceptus secretes

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cytokines such as interleukin-1β (IL1B), interferon-γ (IFNG), interferon-δ (IFND) during the peri-implantation period (Spencer and Bazer, 2004), and these cytokines regulate expression of uterine endometrial genes in pigs (Joyce et al., 2007; White et al., 2009). Our previous study showed that estrogen of conceptus origin increases *LPAR3* expression in the uterine endometrium during pregnancy in pigs (Seo et al., 2008). However, effect of cytokines on *LPAR3* expression has not been determined. Especially, IL1B is produced by the porcine conceptus at the same time of estrogen production, but studies on its effect on the uterine endometrium are very limited.

The efficiency of production of cloned animals by somatic cell nuclear transfer (SCNT) is still very low (Campbell et al., 2005). Various signaling molecules, including steroid hormones, growth factors, adhesion molecules, and cytokines are involved in interactions between the conceptus and the maternal uterus, but the low cloning efficiency is associated with inappropriate maternal-fetal interaction during pregnancy (Bazer et al., 1998; Burghardt et al., 1997; Bauersachs et al., 2009; Mansouri-Attia et al., 2009). Our previous study showed that the IL1B signaling between the conceptus and the maternal uterus is impaired in pigs pregnant with SCNT cloned embryos (Seo and Ka, unpublished data). Thus, we hypothesized that LPA signaling, similar to IL1B signaling, would be impaired in the maternal-conceptus interface in the uterus with SCNT-derived conceptus in pigs.

Therefore, objectives of this study were to determine 1) the regulation of *LPAR3* mRNA levels by a cytokine, IL1B, and 2) expression of *LPAR3* in the uterine endometria with SCNT-derived conceptuses.

MATERIALS AND METHODS

1. Animals and Tissue Preparation

All experimental procedures involving animals were conducted in accordance with the Guide for Care and Use of Research Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Yonsei University. Sexually mature crossbred gilts were assigned randomly to either cyclic or pregnant status. Gilts were hysterectomized on either D12 of the estrous cycle, D12 of pregnancy, or D30 of pregnancy (n = 3 gilts/day/status). Pregnancy was confirmed by the presence of apparently normal conceptuses with filamentous morphology in uterine flushings on D12 of pregnancy or conceptuses on D30 of pregnancy.

Endometrial tissue samples were obtained from gilts carrying embryos generated by SCNT on D12 and D30 of pregnancy as described previously (Ka et al., 2008 Kim et al., 2009). Uterine endometrial tissues were obtained from three gilts on D12 of pregnancy that carried SCNT embryos and three gilts with embryos resulting from natural mating. Endometrial tissues were obtained on D30 of pregnancy from a gilt with SCNT embryos and three gilts with embryos resulting from natural matings. The tissue was from three different areas of placentation of each conceptus from gilts with SCNT embryos. Uterine tissues from gilts carrying conceptuses resulting from natural mating were classified as Non-NT.

2. Explant Cultures

Endometrial tissue of D12 of the estrous cycle was dissected from the myometrium and placed into warm phenol red-free Dulbecco modified Eagle medium / F-12 culture medium (DMEM/F-12; Sigma, St. Louis, MO) containing penicillin G (100 IU/ml) and streptomycin (0.1 mg/ml) as described previously (Ka et al., 2001), 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 (catalog number I5500; Sigma), 10 ng/ml transferrin (catalog number T1428; Sigma), and 10 ng/ml hydrocortisone (catalog number H0396 Sigma). To determine the effects of IL1B on *LPAR3* expression, explant tissues were treated with 0, 1, 10, 100 ng/ml IL1B (catalog number I9401; Sigma) in the presence of both E_2 (50 ng/ml) and P_4 (3 ng/ml) at 37°C for 24 h. Explant tissues were then harvested and total RNA was extracted for real-time RT-PCR analysis of *LPAR3* mRNA levels. These experiments were conducted using endometria from three gilts on D12 of the estrous cycle in triplicates.

3. Total RNA Extraction

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

4. Quantitative Real-Time RT-PCR

To analyze levels of *LPAR3* mRNAs in the uterine endometrium, real-time RT-PCR was performed using the Applied Biosystems StepOnePlus System (Applied Biosystems, Foster City, CA) using the SYBR Green method. Complementary DNAs were synthesized from 4 μg of total RNA isolated from different uterine endometrial tissues, and newly synthesized cDNAs (total volume of 21 μl) were diluted 1:4 with sterile water and then used for PCR. Specific primers based on porcine *LPAR3* (GenBank accession number EF183525; forward, 5'- TGC AGT TCA GGC CGT CCA GT-3' reverse, 5'- GCC GGA GGA CAC CCA TGA AG-3') and porcine ribosomal protein L7 (*RPL7*) (GenBank accession number NM_001113217; forward, 5'-AAG CCA AGC ACT ATC ACA AGG AAT ACA-3' reverse, 5'-TGC AAC ACC TTT CTG ACC TTT GG-3') were designed to amplify cDNAs of 111 bp and 172 bp, respectively. The Power SYBR Green PCR Master Mix (Applied Biosystems) was used for PCR reactions. Final reaction volume was 20 μl including 2 μl of cDNA, 10 μl of 2X Master mix, 2 μl of each primer, and 4 μl of dH_2O. PCR conditions were 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. The results are reported as the expression relative to the level detected on D12 of pregnancy with Non-NT conceptuses or D30 of pregnancy with Non-NT conceptuses after normalization of the transcript amount to the endogenous *RPL7* control by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

5. Statistical Analysis

Data from real-time RT-PCR analysis for effect of IL1B dose on *LPAR3* mRNA levels were analyzed by least squares regression analysis of SAS (Cary, NC) and are presented as least squares means with standard errors (SE). Data for comparison of *LPAR3* levels in endometria with SCNT-derived conceptuses and conceptuses derived from natural matings on D12 of pregnancy were subjected to the Student's *t* test procedure of SAS, and are presented as means with standard errors.

RESULTS

1. Effects of IL1B on *LPAR3* Expression in Uterine Endometrium from D12 of the Estrous Cycle

Because *LPAR3* expression increased dramatically in the uterine endometrium on D12 of pregnancy (Seo et al., 2008), we hypothesized that IL1B of conceptus origin in addition to estrogen might also affect *LPAR3* expression. We measured *LPAR3* mRNA levels in endometrial explant tissues from gilts on D12 of the estrous cycle that were treated with 0, 1, 10, or 100 ng/ml of IL1B in the presence of both E_2 and P_4. As shown in Fig. 1, *LPAR3* mRNA levels were not affected by increasing doses of IL1B (dose, $P > 0.05$).

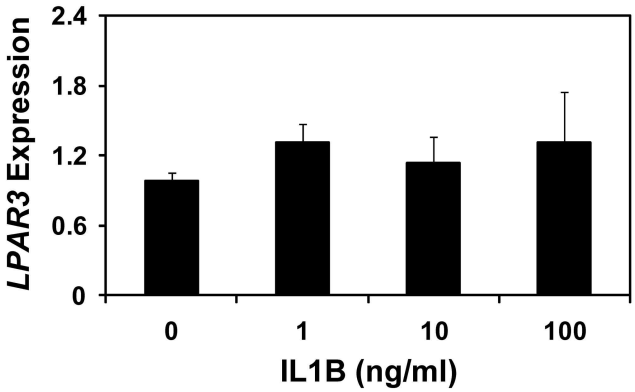

Fig. 1. Effects of IL1B on *LPAR3* mRNA levels in porcine endometrial explant cultures by real-time RT-PCR analyses.
Endometrial explants from gilts on day (D) 12 of the estrous cycle were cultured in DMEM/F-12 with 0, 1, 10, 100 ng/ml IL1B in the presence of both E_2 (50 ng/ml) and P4 (3 ng/ml), at 37°C for 24 h. All experiments were repeated in triplicate with endometria from each of three gilts. *LPAR3* mRNA levels were not affected by increasing doses of IL1B. Data are presented as least squares means with standard errors for relative units of expression.

2. Analysis of *LPAR3* in Uterine Endometria Carrying Conceptuses Derived from SCNT or Conceptuses from Natural Mating on D12 and 30 of Pregnancy

We hypothesized that low survival rate of cloned embryo by SCNT was due, at least in part, to inappropriate or insufficient maternal-fetal interactions during the peri-implantation period and that expression of *LPAR3* mRNA would be less abundant in uteri of gilts with SCNT-derived conceptuses. Thus, we compared levels of *LPAR3* mRNAs in gilts carrying SCNT-derived conceptuses with those carrying non-NT conceptuses on D12 and D30 of pregnancy. On D12 of pregnancy, *LPAR3* mRNA expression was significantly lower in endometria of gilts carrying SCNT-derived con-

ceptuses ($P < 0.05$; Fig. 2). *LPAR3* mRNA levels were not different in endometria of gilts with SCNT-derived conceptuses and non-SCNT embryos on D30 of pregnancy ($P > 0.05$ Fig. 3).

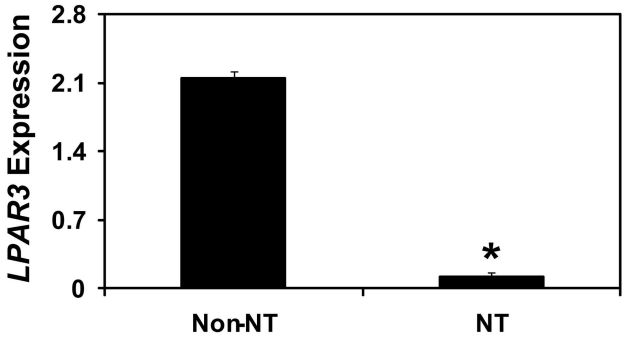

Fig. 2. Analysis of *LPAR3* mRNA levels in uterine endometria of pigs with embryos derived by SCNT (NT) as compared to embryos derived from natural mating (Non-NT) on day (D) 12 of pregnancy by real-time RT-PCR.
Uterine endometrial tissues were obtained from three gilts on D12 of pregnancy that carried SCNT embryos and three gilts with embryos resulting from natural mating. Abundance of mRNA is presented as expression relative to that for *LPAR3* mRNAs in uterine endometria from gilts with non-NT embryos after normalization of transcript amounts to *RPL7* mRNA. Expression of *LPAR3* was less in endometria of gilts carrying conceptuses derived from NT on D12 of pregnancy as noted by the asterisks ($P < 0.05$). Data are presented as means with standard error.

DISCUSSION

LPA has diverse physiological functions depending on cell type and type of activated LPAR (Ishii et al., 2004). Recently, it has been reported that LPA plays an important role in establishment and maintenance of pregnancy in many species including mice (Ye et al., 2005), pigs (Seo et al., 2008), cows (Woclawek-Potocka et al., 2009) and sheep (Liszewska et al., 2009). Deletion of *Lpar3* gene in mice causes significant problems in pregnancy such as delayed implantation, aberrant embryo spacing, hypertrophic placentas, embryonic death, reduced expression of prostaglandin-endoperoxide synthase 2 (*PTGS2*), and decreased level of secretion of prostaglandin (PG) E_2 and PGI_2. Our previous study has shown that LPA is present in the uterine lumen and *LPAR3* is expressed in the uterine endometrium and

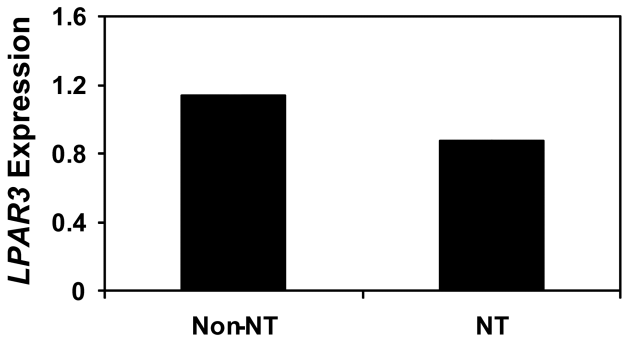

Fig. 3. Analysis of *LPAR3* mRNA levels in uterine endometria of pigs with embryos derived by SCNT (NT) as compared to embryos derived from natural mating (Non-NT) on day (D) 30 of pregnancy by real-time RT-PCR.
Endometrial tissues were obtained on D30 of pregnancy from a gilt with SCNT embryos and three gilts with embryos resulting from natural matings. Abundance of mRNA is presented as expression relative to that for *LPAR3* mRNAs in uterine endometria from gilts with non-NT embryos after normalization of transcript amounts to *RPL7* mRNA. *LPAR3* mRNA was not different in endometria of gilts with SCNT-derived conceptuses and conceptuses derived from natural mating on D30 of pregnancy. Data are presented as means.

conceptus in pigs (Seo et al., 2008). Expression of *LPAR3* is highest on D12 of pregnancy when conceptus implantation begins, and LPA increases *PTGS2* expression in the uterine endometrium on D12 of pregnancy in pigs. Thus, LPA in the uterine lumen may play an important role in uterine endometrial function and conceptus development through *LPAR3* during implantation and establishment of pregnancy in pigs.

During the implantation period in pigs, the conceptus experiences trophoblastic elongation from spherical to filamentous form and secretes various biological molecules including estrogens, IL1B, IFNG and IFND (Spencer and Bazer, 2004). Estrogen of conceptus origin protects CL by changing secretory direction of $PGF_{2\alpha}$ from the uterine vasculature (endocrine) to the uterine lumen (exocrine) (Jaeger et al., 2001). Also, estrogen of conceptus origin induces expression of many uterine endometrial genes including fibroblast growth factor 7 (*FGF7*) (Ka et al., 2001), secreted phosphoprotein 1 (SPP1) (White et al., 2005), signal transducer and activator of transcription 1 (*STAT1*) (Joyce et al., 2007), and transient receptor potential vanilloid type 6 (*TRPV6*)

(Choi et al., 2010). In addition, infusion of conceptus secretory molecules containing IFNG increased endometrial *STAT1* expression in pigs (Joyce et al., 2007).

In addition to estrogen, conceptuses secrete considerable levels of IL1B protein into uterine lumen during the peri-implantation period of pregnancy (Ross et al., 2003). IL1B also affects expression of uterine endometrial genes including *PTGS2* (White et al., 2009), prostaglandin E synthase (*PTGES*) (Franczak et al., 2010), and *SAL1* (Seo et al., 2011). Thus, we hypothesized that *LPAR3* expression might be induced by estrogen or cytokines of conceptus origin. Indeed, our previous study using explant cultures showed estrogen increases *LPAR3* expression in the uterine endometrium on D12 of pregnancy (Seo et al., 2008). However, effect of IL1B on *LPAR3* expression was not determined. Present study showed that IL1B did not affect *LPAR3* expression in the uterine endometrium. Therefore, estrogen is likely the major factor for *LPAR3* induction in the uterine endometrium during the peri-implantation period in pigs. Further study is needed to investigate whether IFNG and IFND affect *LPAR3* expression.

SCNT technique is widely used to produce cloned animals, but the survival rate of cloned embryos to term is very low (Campbell et al., 2005). There are evidences showing that the high rate of pregnancy failure is associated with abnormal placental development (Kim et al., 2005; Chae et al., 2006; Jouneau et al., 2006). Recent study has shown that expression of uterine endometrial genes, including nuclear receptor subfamily 2, group F, member 2 (*NR2F2*) and gap junction protein, alpha 1 (*GJA1*), important for implantation and placentation is altered in endometria carrying SCNT-derived embryos during the implantation period in cow, indicating that abnormal placental development in pregnancy of cloned embryo may be due to inappropriate embryo-maternal communication during implantation period (Mansouri-Attia et al., 2009). Thus, we hypothesized that endometrial responsiveness to conceptus signals such as estrogen and IL1B during the implantation period is altered in uteri of pigs carrying SCNT-derived conceptuses. In fact, our recent data showed that during the peri-implantation period IL1B and estrogen synergistically increase endometrial responsiveness to IL1B by regulating IL1 receptor type I (*IL1R1*) and IL1 receptor accessory protein (*IL1RAP*) expression, which leads to induction of endometrial expression of salivary lipocalin (*SAL1*). However, this IL1B-induced *SAL1* expression was not observed in uteri of pigs carrying SCNT-derived conceptuses due to deficiencies in the conceptus to secrete E_2 and IL1B, suggesting impairment of IL1B signaling in SCNT-derived pregnancy (Seo and Ka, unpublished data). In addition, present study showed that endometrial expression of *LPAR3*, an estrogen-responsive gene, decreased in pigs carrying SCNT-derived conceptuses on D12 of pregnancy. This result indicates that endometrial responsiveness to LPA might be also impaired due to deficiencies of E_2 secreted by SCNT-derived conceptuses. Collectively, lack of conceptus signal results in insufficient endometrial receptivity to conceptuses and this may be the major reason for pregnancy failure in SCNT cloned embryos in pigs. In the present study, levels of *LPAR3* expression were not different between endometria of gilts with SCNT-derived conceptuses and non-SCNT embryos on D30 of pregnancy. *LPAR3* expression is maintained at low levels after the implantation period in pigs (Seo et al., 2008). Thus, it seems that once pregnancy is established, *LPAR3* levels stays low on D30 of pregnancy, regardless of conceptus origin whether they are derived from SCNT or natural mating.

In summary, we determined in pigs that 1) IL1B did not influence *LPAR3* expression in the uterine endometrium during pregnancy, and 2) *LPAR3* expression decreased in uteri of gilts carrying SCNT-derived conceptuses on D12 of pregnancy. Our results suggest that estrogen of conceptus origin is responsible for induction of *LPAR3* expression during peri-implantation period and appropriate LPA signaling in uterine endometrium is critical for successful implantation of SCNT-derived conceptuses in pigs.

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