ORIGINAL PAPER

FSH-induced Activation of PI 3-kinase-PKB Pathway is Essential for LH Receptor Formation in Cumulus Cells during Meiotic Resumption of Porcine Oocytes

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Abstract. It has been reported that the PI 3-kinase-protein kinase B (PKB) pathway is activated by FSH stimulation and plays an important role in follicular growth and maturation. However, the critical role of the PI 3-kinase-PKB pathway in cumulus cell differentiation during *in vitro* maturation of porcine oocytes remains unclear. The present study showed that both PI 3-kinase and PKB were detected by Western blotting in cumulus cells of COCs that were cultured with or without FSH. However, positive signals against anti-phospho-PKB specific antibody, which recognized PKB phosphorylated at Ser473, were observed when COCs were cultured with FSH for 20 hr. The addition of the PI 3-kinase inhibitor LY294002 to FSH-containing medium suppressed the phosphorylation of PKB in cumulus cells. We already reported that expression of the LH receptor (LHR) gene is one of the cumulus cell responses to FSH stimuli. Four types of LHR mRNA were detected in FSH-stimulated cumulus cells by RT-PCR. The large form was identified as a part of the porcine full-length LHR cDNA, which was able to bind its ligand. The level of full-length type LHR mRNA was significantly decreased by the addition of LY294002 to FSH-containing medium. These results suggest that FSH up-regulates LHR formation through a PI 3-kinase-PKB dependent pathway in cumulus cells of COCs during *in vitro* maturation of porcine oocytes.

Key words: PI 3-kinase, Akt/PKB, LH receptor, Cumulus cells, Pig oocytes

Introduction

It is well documented that FSH stimulates adenylcyclase with a resultant increase in cAMP synthesis [1,2]; however, signal transduction of the cAMP-protein kinase A (PKA) pathway alone does not represent the expression pattern of the many genes activated by FSH [3]. Actually, FSH stimulation has been shown to be mediated by several pathways, for example the MAP kinase pathway [4] and the intracellular Ca^{2+} pathway [5] in addition to the cAMP-PKA pathway. Several researchers have shown that FSH also increases Akt/protein kinase B (PKB) phosphorylation and activation in a way that is dependent on phosphatidylinositol 3-kinase (PI 3-kinase) in granulosa cells [6,7]. In cumulus cells, we have reported that PKB activity significantly decreases in a dose-dependent manner in the relation to the PI 3-kinase inhibitor, LY294002, when porcine cumulus-oocyte complexes (COCs) were cultured with FSH [8], suggesting that FSH stimulation also activates the PI 3-kinase-PKB pathway in cumulus cells as well as granulosa cells.

Recently, it has been reported in rat granulosa cells that the transcriptional activity of the LH receptor (LHR) gene was up-regulated by stimulation with FSH and that the induction was significantly suppressed by LY294002 [9]. In our previous study [10,11], we showed that the expression of the LHR gene in cumulus cells of porcine COCs was up-regulated by FSH and that the binding of LH to its receptor induced progesterone production. Furthermore, high-level progesterone production was significantly reduced by LY294002 during in vitro maturation of COCs [8]. Judging from these reports including our previous study, we estimated that the PI 3-kinase-PKB pathway in cumulus cells plays an important role in LHR formation.

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In this study, to investigate the role of the PI 3-kinase-PKB pathway in the formation of LHR in cumulus cells, COCs were cultured with both FSH and PI 3-kinase inhibitor, LY294002 for 20 hr. The expression of the LHR gene was analyzed by RT-PCR technique. The protein level of PI 3-kinase and the phosphorylated status of PKB were detected by Western blotting.

Materials and methods

1) In vitro cultivation of porcine COCs

Isolation of porcine COCs was described previously [12]. Briefly, porcine ovaries were collected from 5- to 7-month-old prepubertal gilts at a local slaughterhouse. Oocytes were collected from the surfaces of intact healthy antral follicles measuring from 3 to 5 mm in diameter. Oocytes having evenly granulated cytoplasm with at least 4 layers of unexpanded cumulus cells were selected and were washed 3 times with maturation medium. The COCs were cultured (20/well) for 20 hr in 300 μl of the maturation medium supplemented with or without 20 ng/ml highly purified porcine FSH (NIDDK, Torrance, CA, USA) at 39℃ in a humidified incubator (95% air, 5% CO_2). The maturation medium was modified NCSU37 [13] supplemented with 10% (v/v) FCS (Gibco BRL, Grand Island, NY, USA), 7 mM Taurine (Sigma St Louis, MO, USA) and 4 mM hypoxanthine (Sigma). After cultivation, COCs were collected for RNA and protein isolation. In the case of treatment with PI 3-kinase inhibitor, LY294002 (Sigma), COCs were cultured with FSH and 5 μM LY294002 for 20 hr. LY294002 was dissolved in DMSO (Sigma) at 5 mM, and stored in -30℃. The final concentration was obtained by dilution (1:1000) with the maturation medium. Inhibitor-free medium supplemented only with 0.1% (v/v) DMSO to the maturation medium served as a control. The LY294002 concentration used herein was the same as that used in our previous study [14].

2) RNA isolation

After cumulus cells were separated from COCs, they were washed three times in PBS. Total RNA was extracted from cumulus cells using SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the instruction manual, and dissolved in 20 μM nuclease-free water. The final RNA concentrations were determined by absorbance using a spectrophotometer.

3) RT-PCR for LHR mRNA

Oligonucleotide primers used for amplification of the LHR were designed from known cDNA sequences of four porcine LHR isoforms (GenBank accession number: M29525) [15]. The upstream primer (5'-CCAATCTCCTAGATGCCACATTGAC-3') is identical to nucleotides 861-885 of the porcine cDNA, and the downstream primer (5'-GCTCAGCAACAGAAAGAAATCCC-3') represents the reverse complement of nucleotides 1959-1981. These primer pair predicts 185-, 411-, 855- and 1121-base pair (bp) DNA fragments.

β-actin was used as a control for reaction efficiency and variations in concentrations of mRNA in the original RT reaction. The β-actin primers were based on the mouse sequences (GenBank accession number: NM009609; Tokunaga et al., 1986). The upstream primer (5'-CTACAATGAGCTGCGTGTG-3') is identical to nucleotides 192-211 of the mouse cDNA, and the downstream primer (5'-TAGCTCTTCTCCAGGGAGGA-3') represents the reverse complement of nucleotides 622-641. The primer pair predicts a 450 bp DNA fragment.

RT-PCR was performed according to a coupled one-step procedure using the Access RT-PCR System (Promega) with some modifications [10]. Briefly, 10 ng of total RNA was reverse tanscribed at 48℃ for 45 min, denatured at 94℃ for 2 min, and amplified for 26 or 32 cycles of denaturation at 94℃ for 30s, primer annealing at 58℃ (LH receptor) or 56℃ (β-actin) for 1 min, and extension at 68℃ for 2 min, with a final extension step of 7 min at 68℃. The amplified products were analyzed by electrophoresis on 2% agarose gels. The intensity of the objective bands was quantified by densitometric scanning using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA).

4) Western blotting analysis

Cumulus cells were lysed in Laemmli sample buffer and protein extracts stored at -80℃ until use. After denaturing by boiling for 5 min, 10 μl of each protein sample was separated by SDS-PAGE on either 7.5 or 12.5% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA), then transferred onto PVDF membrane (Amersham Biosciences, Uppsala Sweden). The membrane was blocked with 5% (w/v) nonfat dry milk (Amersham Biosciences) in 0.1% (v/v) Tween 20 (Sigma)/ PBS (T-PBS). Primary antibodies

were added in 5% (w/v) BSA (Sigma) in PBS-T, and incubated overnight at 4℃ . Rabbit anti-phospho-PKB polyclonal antibody (Cell Signaling Technology, Inc., Beverly, MA) which recognized specifically PKB phosphorylated at Ser473 was used at 1:2000 dilution. A rabbit polyclonal antibody against PKB (Cell Signaling Technology) was used at a dilution of 1:2000. A mouse monoclonal anti PI 3-kinase antibody (Transduction Laboratories, Lexington, KY, USA) which recognized the regulatory subunit of PI 3-kinase (p85 α) was used at 1:500 dilution. After four washes in T-PBS, the membranes were incubated for 1.5 hr with a 1:2000 dilution of either goat anti-rabbit IgG horseradish-peroxidase-labeled antibody (Cell Signaling Technology) or goat anti-mouse IgG horseradish-peroxidase-labeled antibody (Cell Signaling Technology) in 5% (w/v) nonfat dry milk in PBS-T at room temperature. After five washes of 10 min each with T-PBS, peroxidase activity was visualized using the ECL Plus Western blotting detection system (Amersham Biosciences), according to the manufacturer's instructions.

5) Statistical analysis

Statistical analyses of all data from three or four replicates for comparison were carried out by one-way ANOVA followed by Duncan's multiple-range test (Statview; Abacus Concepts, Inc., Berkeley, CA).

Results and discussion

Recently, Gonzalez-Robayna et al. [6] have shown that in granulosa cells FSH increases PKB phosphorylation and activation in a way that is cAMP-dependent and PI 3-kinase-dependent. In rat Sertoli cells, the PI 3-kinase-PKB pathway has been shown to be related to FSH-regulation of cell functions [16]. As mentioned before, we have already demonstrated that the inhibition of PI 3-kinase activity by LY294002 in cumulus cells interferes with gonadotropin-stimulated progesterone production, and with the close of gap junctional communications in cumulus cells during meiotic resumption of porcine oocytes [8,14]. However, there is little information about the regulation of the PI 3-kinase-PKB pathway and the roles of this pathway in FSH-regulation of cumulus cell function during meiotic resumption of oocytes. The present study showed that both PI 3-kinase and PKB were detected in cumulus cells by Western blotting analysis when COCs were cultured for 20 hr without FSH stimulation (Fig. 1 A,C). The bands were also observed in cumulus cells of COCs that were cultured with FSH (Fig. 1 A,C). However, the phosphorylated status of

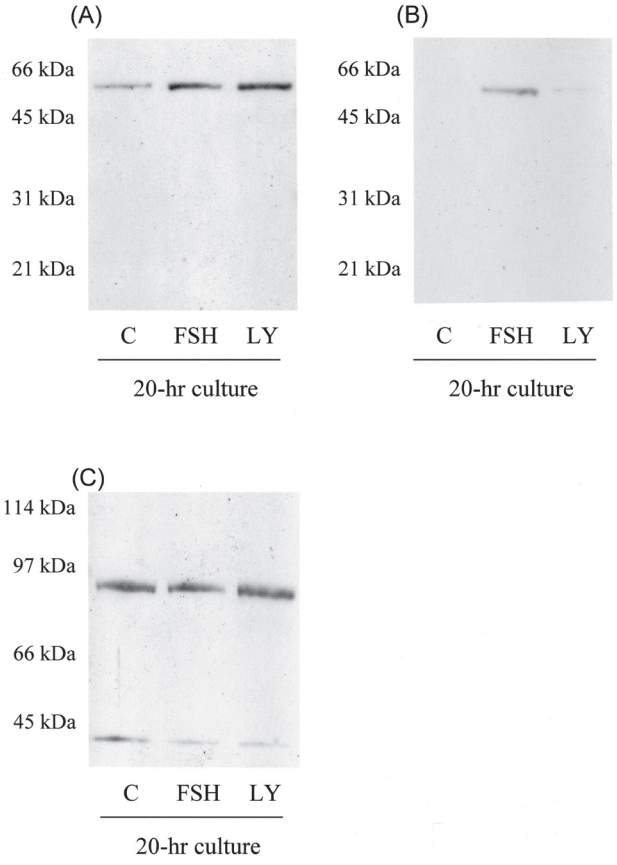

Fig. 1 The effects of LY294002 on the phosphorylation of PKB in cumulus cells of COCs cultured for 20 hr

A. Western blotting analysis of total PKB in cumulus cells of COCs. After COCs were cultured for 20 hr, COCs were collected for protein. Total PKB was detected by a rabbit anti-PKB polyclonal antibody which recognized both phosphorylated PKB and non-phosphorylated PKB. The positions of molecular weight markers (in kDa) are indicated on the left. C (control); COCs were cultured without FSH, FSH; COCs were cultured with 20 ng/ml FSH, LY; COCs were cultured with FSH and 5 μM LY294002.

B. Western blotting analysis of phosphorylated PKB in cumulus cells of COCs. Phosphorylated PKB at Ser473 was detected by anti-phospho-PKB polyclonal antibody. The positions of molecular weight markers (in kDa) are indicated on the left. C (control); COCs were cultured without FSH, FSH; COCs were cultured with 20 ng/ml FSH, LY; COCs were cultured with FSH and 5 μM LY294002.

C. Western blotting analysis of PI 3-kinase in cumulus cells of COCs. Regulatory subunit of PI 3-kinase (p85 α) was detected by mouse anti-PI 3-kinase monoclonal antibody. The positions of molecular weight markers (in kDa) are indicated on the left. C (control); COCs were cultured without FSH, FSH; COCs were cultured with 20 ng/ml FSH, LY; COCs were cultured with FSH and 5 μM LY294002.

PKB in cumulus cells was up-regulated by culture with FSH for 20 hr (Fig. 1 B), suggesting that the stimulation with FSH increased the activity of PI 3-kinase-dependent pathway in cumulus cells as well as granulosa cells.

In early antral follicles, FSH has been shown to be a major survival factor through the generation of cAMP [17]. Wang et al. [18] reported that FSH was involved in an apoptosis suppressor for granulosa cells through activation of the PI 3-kinase-PKB pathway. In our previous study [8], few TUNEL-positive signals were detected in COCs cultured with FSH and 5 μM of PI 3-kinase inhibitor, LY294002, whereas a high concentration of this drug (25 μM) increased apoptosis of cumulus cells. However, the inhibitory effects of the addition of LY294002 to FSH-containing medium on progesterone production by COCs and GVBD in oocytes reached a maximum at 5 μM [8]. In the present study, when COCs were cultured with FSH and 5 μM LY294002, the intensity of the phosphorylated status of PKB was markedly reduced and the level was comparable with that in cumulus cells of COCs cultured without FSH (Fig. 1 B). Judging from these results, FSH-induced PKB phosphorylation via the PI 3-kinase dependent pathway plays an important role in the responses of cumulus cells to FSH stimuli.

Zeleznik et al. [7] revealed that the overexpression of dominant-negative PKB resulted in a decrease of the differentiation-associated mRNA, LHR and aromatase mRNA in FSH-stimulated granulosa cells. We have reported by RT-PCR that at least three splice variants of LHR (LHR2: 855; LHR3: 411; LHR4: 185 bp) were detected in cumulus cells, in addition to the large form (LHR1: 1121 bp) [10,11]. The large form (LHR1) was identified as a part of the porcine full-length LHR cDNA (DDBJ/EMBL/GeneBank accession number; M29525), using the Basic Local Alignment Search Tool (BLAST, DDBJ) [11]. Loosfelt et al. [15] reported that when the cDNA containing the full-length open reading frame of LHR was transfected into COS-7 cells, hCG bound to the receptors. In the present study, the full length type of LHR mRNA was expressed in cumulus cells of COCs that were cultured for 20 hr with FSH (Fig. 2). The level was significantly higher than that in cumulus cells of COCs cultured without FSH (Fig. 2). The increase of the full length type LHR mRNA level in cumulus cells was significantly diminished by the addition of LY294002 to FSH-containing medium (Fig. 2). Thus, the PI 3-kinase-PKB pathway up-

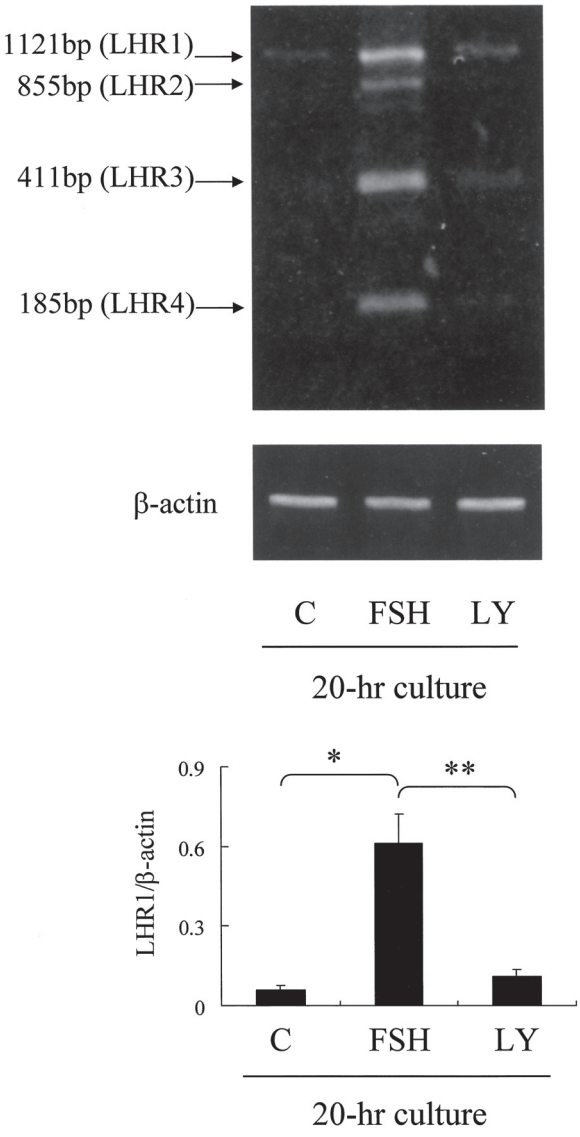

Fig. 2. Effects of LY294002 on the expression of LHR gene in cumulus cells of COCs cultured for 20 hr
After COCs were cultured for 20 hr, COCs were collected for RNA, and LHR mRNA was detected by RT-PCR. The intensity of the amplified bands was quantified by densitometric scanning. The respective values of LHR were normalized according to those of β -actin to evaluate arbitrary units of the relative abundance of the targets. *; The addition of FSH significantly increased the level of full length of LHR mRNA (LHR1) ($P<0.01$). **; The addition of LY294002 to FSH-containing medium significantly affected the level of full length of LHR mRNA (LHR1) ($P<0.01$). C (control); COCs were cultured without FSH, FSH; COCs were cultured with 20 ng/ml FSH, LY; COCs were cultured with FSH and 5 μM LY294002.

regulates the level of LHR mRNA in cumulus cells when COCs are cultured with FSH.

We also reported that the binding of LH to LHR on cumulus cells significantly increased the level of cAMP in cu-

mulus cells of COCs [11]. The elevation of cAMP in cumulus cells stimulated progesterone production by cumulus cells, then both decreased the proliferative activity of cumulus cells and induced differentiation, such as close of gap junctional communication and cumulus cell expansion [19,20]. Moreover, the high concentration of progesterone secreted by COCs improved the rate of early embryonic development to the blastocyst stage after in vitro fertilization [21]. Therefore, the FSH-induced LHR formation via the PI 3-kinase-PKB dependent pathway in cumulus cells, is very important in the production of in vitro matured porcine oocytes with a high developmental competence.

In summary, the present study shows that the phosphorylation of PKB in cumulus cells is induced by stimulation with FSH. When the phosphorylation of PKB was suppressed by a PI 3-kinase inhibitor, LY294002, FSH-induced LHR mRNA expression was significantly decreased in cumulus cells of COCs. These results suggest that FSH up-regulates LHR formation through the PI 3-kinase-PKB dependent pathway in cumulus cells of COCs during in vitro maturation of porcine oocytes.

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