Epidermal growth factor receptor activation by protein kinase C is necessary for FSH-induced meiotic resumption in porcine cumulus–oocyte complexes

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Abstract

It is proved that epidermal growth factor (EGF)-like factors mediate gonadotropin-induced rodent oocyte maturation via EGF receptor (EGFR). However, the detail kinetics and signal pathway between FSH and EGF/EGFR is not clear in large animals. In the present study, we investigated the roles of EGFR and protein kinase C (PKC) in FSH-induced porcine oocyte meiotic resumption. Porcine cumulus–oocyte complexes were cultured in NCSU37 medium containing 10% porcine follicular fluid and germinal vesicle breakdown (meiotic resumption) was detected after different treatments. The results showed that EGF-like factor amphiregulin (AR) and EGFR mRNA were expressed in porcine cumulus cells, but not oocytes. FSH significantly induced AR mRNA expression with maximum at 4 h and activated EGFR phosphorylation at 8 h. AR (1–100 ng/ml) dose-dependently induced meiosis resumption of porcine oocyte. The specific EGFR inhibitor, AG1478, but not AG43 (the inactive analog of AG1478), completely blocked FSH, EGF, and

AR-induced oocyte meiotic resumption; the inhibitory effect of AG1478 on FSH action gradually decreased when the inhibitor was added at 6 h or later and disappeared when it was added at 11 h; EGF reversed the inhibitory effect on FSH when AG1478 was added within 6 h. FSH triggered porcine oocyte meiotic resumption (at 20 h) later than that of EGF and AR (at 18 h). All these results supported that endogenously produced EGFR activator(s), possibly AR (maximum at 4 h) and EGFR activation (began at 6 h and finished within 11 h), in cumulus cells is necessary for FSH-induced porcine oocyte meiotic resumption (began at 18 h). Furthermore, PKC activator PMA mimicked but PKC inhibitor chelerythrine chloride inhibited FSH action, and AG1478 also suppressed PMA-induced porcine oocyte meiotic resumption. These data together suggested that EGFR activation, by PKC signal pathway, participates in FSH-induced porcine oocyte meiotic resumption.

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Introduction

In most mammalian, oocytes are arrested at the diplotene stage (germinal vesicle (GV) stage) of the first meiotic prophase until a surge of luteinizing hormone (LH) from the pituitary stimulates the immature oocyte to resume meiosis (GV breakdown, GVBD) and ovulate (Mehlmann 2005). The actions of gonadotropins, follicle-stimulating hormone (FSH), and LH on mammalian oocyte meiotic resumption are believed to be mediated in large part through increasing the production of cyclic AMP (cAMP; Lindner *et al.* 1974, Downs *et al.* 1988, Eppig 1989) and subsequent activation of mitogen-activated protein kinase (MAPK; Shimada & Terada 2002a, Su *et al.* 2002, Liang *et al.* 2005), but the downstream pathway of cAMP is unclear. Recently, it is reported that LH stimulates the mRNA expression of epidermal growth factor (EGF)-like factors, including amphiregulin (AR), epiregulin (ER), and betacellulin (BTC) in mouse preovulatory follicles.

These EGF-like growth factors trigger mouse follicle meiotic resumption and cumulus expansion via paracrine mechanism, and AG1478, the EGF receptor (EGFR) inhibitor, successfully blocks LH-induced meiotic resumption (Park *et al.* 2004, Conti *et al.* 2006). The similar results are obtained using rat preovulatory follicles cultured *in vitro* (Ashkenazi *et al.* 2005, Tsafiri *et al.* 2005). Furthermore, EGF-like factors mediate the stimulatory actions of gonadotropin-releasing hormone on rat oocyte meiosis and ovulation (Motola *et al.* 2006). These results suggest that EGF-like factors, acting on EGFR signal pathway, are involved in LH-induced rodent follicle-enclosed oocytes meiotic resumption and ovulation.

It is reported that mouse and pig cumulus–oocyte complexes (COCs) also express EGF-like growth factors, AR, and ER by autocrine mechanism (Shimada *et al.* 2006, Yamashita *et al.* 2007). By the well-characterized COCs *in vitro* culture model, FSH has been widely used for promoting COCs maturation *in vitro* (Meinecke &

Meinecke-Tillmann 1979, Singh *et al.* 1993, Ding & Foxcroft 1994, Shimada & Terada 2002b, Schoevers *et al.* 2003, Zhang *et al.* 2005, Silvestre *et al.* 2007), since LH cannot directly induce COCs maturation (Byskov *et al.* 1997, Fu *et al.* 2007) for lack of its receptor in cumulus cells (Peng *et al.* 1991, Shimada *et al.* 2003, Fu *et al.* 2007). Furthermore, FSH induces AR gene (Shimada *et al.* 2006) and protein expression in mouse cumulus cells, which can induce mouse COCs maturation, and AG1478 blocks FSH- and AR-induced oocyte maturation (Downs & Chen 2008). These results suggest that the expression of EGF-like factors in cumulus cells as well as granulosa cells also play an important role in FSH-induced mouse cumulus-enclosed oocyte maturation.

EGF, by the same receptor EGFR as that of EGF-like factors (Harris *et al.* 2003), has also been shown to mimic FSH-induced porcine oocyte meiotic maturation and development competence of oocytes (Singh *et al.* 1993, Ding & Foxcroft 1994, Singh *et al.* 1997, Abeydeera *et al.* 1998, 2000, Prochazka *et al.* 2000). Although many studies have provided evidence to demonstrate that EGF-like factors production and EGFR phosphorylation are involved in the gonadotropins-induced rodent oocyte maturation, the detail kinetics and signal pathway between FSH and EGF/EGFR is not very clear especially in large animals.

Protein kinase C (PKC) signal pathway is involved in FSH-induced oocyte maturation (Su *et al.* 1999, Downs *et al.* 2001, Lu *et al.* 2001, Fan *et al.* 2004, Jin *et al.* 2006). Further study indicates that FSH activates EGFR by PKC during mouse COCs maturation (Downs & Chen 2008). On the other hand, PKC may be involved in EGF-induced maturation of porcine oocytes *in vitro* (Coskun & Lin 1995). Whether the signal pathway between rodent and porcine oocyte maturation is same or not remains to be investigated.

In this study, we have extensively examined in detail the kinetics of FSH-induced EGF and/or EGF-like factors mRNA expression, EGFR phosphorylation, and meiotic resumption. We also examined the possible relationship between PKC and EGFR activation during FSH-induced porcine oocyte meiotic resumption.

Materials and Methods

Chemicals

Unless specified, all chemicals and reagents were purchased from Sigma. FSH from porcine pituitary and EGF from mouse submaxillary glands were prepared as stock solutions in distilled PBS containing 0.1% BSA. PKC activator phorbol 12-myristate 13-acetate-4-O-methyl ester (PMA) and PKC inhibitor chelerythrine chloride were dissolved to form 10^{-3} M and 1 mM stock solutions respectively. The final concentrations of FSH, EGF, PMA, and chelerythrine chloride for culture were 0.05 units/ml, 10 ng/ml, 10^{-7} M, and 5 μ M respectively. These concentrations were shown to be efficient in previous studies (Singh *et al.* 1993, Abeydeera

et al. 2000, Lu *et al.* 2001, Jin *et al.* 2006). AG1478 (Biosource, Camarillo, CA, USA) and AG43 (Alexis biochemicals, San Diego, CA, USA) were dissolved in anhydrous dimethylsulfoxide (DMSO) to form 10 mM stock solutions. All stock solutions were kept at -20°C . Prior to use, they were diluted with culture medium, and the final concentration of DMSO was $<0.1\%$, which had no significant effect on oocyte maturation (data not shown).

Porcine COCs collection and culture

Porcine ovaries were collected from prepubertal gilts at a local abattoir and delivered within 3 h after slaughter in a bottle with sterile saline maintained at 37°C . The COCs were collected from healthy follicles 3–6 mm in diameter and placed in Tyrode's lactate-HEPES medium with 0.1% polyvinyl alcohol (Tatemoto *et al.* 2001). Porcine follicular fluid (pFF) was harvested at the same time and centrifuged at 1000 g at 4°C for 15 min. The supernatant fluid was collected, supplemented with 100 IU/ml penicillin G potassium and 50 μ g/ml streptomycin sulfate, sterilized by a 0.45–0.22 μ M filter, and stored at -20°C . Only COCs with uniform ooplasm and compact cumulus were chosen for further culture. The COCs were cultured in modified North Carolina State University 37 (NCSU37) medium (Funahashi *et al.* 1994) supplemented with 1.0 mM glutamine, 0.23 mM sodium pyruvate, 0.6 mM cysteine, 100 IU/ml penicillin G potassium, 50 μ g/ml streptomycin sulfate, and 10% pFF. Groups of 50 COCs were transferred into individual well of a 24-well culture dishes (Nunc; Nunc, Roskilde, Denmark) with 0.5 ml maturation medium. The maturation medium was equilibrated for more than 4 h before culture. The cultures were carried out in an atmosphere with 5% CO_2 of air at 38.5°C for 24 h.

Evaluation of cumulus expansion and oocyte meiosis resumption

Cumulus expansion was assessed after 24 h culture using a subjective scoring method according to Downs (1989) and Vanderhyden (1993). Briefly, no response was scored as 0, minimum observable response was scored as 1, expansion of outer COCs layers was scored as 2, expansion of all COCs layers except the corona radiata was scored as 3, and expansion of all layers was scored as 4. A cumulus expansion index (CEI) was calculated as previously described (Fagbohun & Downs 1990).

Oocytes were denuded mechanically by repeated pipetting to remove cumulus cells at the end of culture. The denuded oocytes (DOs) were mounted on glass slides after rinsing with saline, fixed in acetic alcohol (acetic acid:alcohol, 1:3, v/v) for more than 24 h, stained with 1% acetic orcein for 5–10 min, and examined by phase contrast microscopy (Leica, Wetzlar, Germany) to evaluate the stage of meiosis. Oocytes were assessed for maturation scoring for GV_s (meiotic arrest), GVBD (meiotic resumption), and the first polar body (PB1, the first meiotic maturation). As very few oocytes had PB1

emission (<10%) after 24 h culture *in vitro*, we considered GVBD percentage only in this research. Oocytes that had degenerated were not included.

RNA extraction and semi-quantitative RT-PCR

A total of 200 COCs, 400 oocytes, and cumulus cells from 400 COCs were pooled for RNA extraction respectively. Total RNA was prepared from cultured cells using TRIzol reagent (Invitrogen Ltd) according to the manufacturer's suggested procedure. The total RNA concentration was determined from spectrophotometric analysis at A260/280.

Verification of changed expression was done by RT-PCR for EGF, AR, heparin-binding EGF (HB-EGF), and EGF receptor (EGFR). The unique PCR primers specific for the genes designed using Oligo 6.0 software and sequence data from the National Center for Biotechnology Information database (Table 1). First-strand cDNA was created by RT (Promega Reverse Transcription System) from 1 µg total RNA. The RT proceeded for 1 h at 42 °C. DNA was amplified by an initial incubation at 94 °C for 5 min followed by 24–33 cycles at 94 °C denaturation for 30 s, annealing for 30 s (annealing temperature for specific primer see in Table 1), 72 °C extension for 30 s, and a final extension at 72 °C for 5 min. The number of cycles was determined following pre-testing a range of cycles in which the product showed linear expression. β-Actin served as the internal standard. The PCR products were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. Relative intensities were quantified using Gel-pro Analyzer 4.0 (Media Cybernetics, Bethesda, MD, USA).

Western blot analysis

To detect phosphorylated EGFR, 50 porcine COCs for each treatment were lysated using 1× SDS supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate for 20 min on ice, and stored at −20 °C. Before

electrophoresis, samples were heated up to 100 °C for 5 min, cooled on ice immediately, and then centrifuged at 12 000 g for 5 min. Protein samples were run on an SDS-polyacrylamide electrophoresis gel and transferred onto nitrocellulose membranes (Amersham Biosciences) by electrophoresis (Bio-Rad, Hercules). Stained proteins markers (weight range 6.5–175 kDa, New England Biolabs Inc., Ipswich, MA, USA) were run simultaneously as a standard. The membrane was blocked in TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.6)) containing 5% nonfat dry milk or 5% BSA (for anti-phosphotyrosine) for 1 h at room temperature and incubated with primary antibody diluted in TBST containing 5% nonfat dry milk (5% BSA for phosphotyrosine) overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine (HRP-PY99, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted to 1:500, goat anti-phospho-EGFR (phospho-Tyr1173, Santa Cruz; Gall *et al.* 2005) was diluted to 1:300, and mouse monoclonal anti-β-tubulin (Sigma) was diluted to 1:5000. The blots were washed thrice with TBST and incubated with HRP-conjugated anti-goat or anti-mouse secondary antibody (diluted to 1:5000) except for blot incubated with HRP-PY99. After the membranes were washed in TBST, ECL Plus reagent (Pierce Biotechnology, Rockford, IL, USA) was used to detect the peroxidase activity and the signal was visualized by autoradiography. Immunoreaction signals were analyzed using gel-pro Analyzer 4.0. Each experiment was repeated at least thrice.

Experimental design

Experiment 1 To test the possibility that EGFR signaling mediates FSH-induced porcine oocyte meiotic resumption, we first examined the effect of FSH on EGF family members' gene expression and EGFR phosphorylation during oocyte meiotic resumption. The COCs were cultured with or without FSH for different times (0, 1, 2, 4, 6, 8, 16, and 24 h). After culturing, a total of 200 COCs were pooled for RNA extraction and followed by RT-PCR. EGF, AR, HB-EGF, and EGFR mRNA expressions were examined. β-Actin served as the internal

Table 1 Primers used for determination of pig epidermal growth factor (EGF), amphiregulin (AR) heparin-binding (HB)-EGF, and EGF receptor (EGFR) mRNA by RT-PCR

Gene	GeneBank accession no.	Primers	PCR cycles	Annealing temp. (°C)	Product length (bp)
EGF	NM_214020	F: 5'-TCTGAACCCGGACGGATTG-3' R: 5'-GACATCGCTCGCCAACGTAG-3'	33	53	202
Amphiregulin	NM_214376	F: 5'-CACCTGGAAGCAGTAACCT-3' R: 5'-ATGACTTGGCAGTGACCC-3'	32	52	321
HB-EGF	NM_214299	F: 5'-AAGAAGAAAGGCAAAGGGTTAGGGA-3' R: 5'-AACATGAGAAGCCCCACAATGAC-3'	31	53	269
EGFR	NM_214007	F: 5'-CCTTGGGAAGCTTGGAGATCACCTAC-3' R: 5'-TGTGCTTAGAAAGTCGCTGTTGAC-3'	32	52	344
β-actin	SSU07786	F: 5'-GGGAGATCGTGCCTGGGACATC-3' R: 5'-CCGCCAGACAGCACCGTGTT-3'	24	53	289

F, forward; R, reverse.

standard. To further reveal which component (cumulus cells or oocyte) contributes to the secretion of these factors, these genes were also examined in oocytes and cumulus cells from 400 COCs. Next, COCs were incubated with or without FSH for indicated periods (0, 4, 6, and 8 h), and then the EGFR phosphorylation was analyzed by western blotting. The COCs cultured with EGF for indicated times (0, 30, 60, and 120 min) served as the positive control.

Experiment 2 Based on the results of Experiment 1, the functional effect of AR on porcine oocyte meiotic resumption was determined. Porcine COCs were cultured with 0, 1, 5, 10, 25, 50, and 100 ng/ml AR and the oocyte was scored for GVBD after 24 h.

Experiment 3 The kinetics of AR-, EGF-, and FSH-induced porcine oocyte meiotic resumption were examined. Porcine COCs were cultured in NCSU37 medium supplemented with AR, EGF, or FSH respectively, and oocytes were scored for GVBD after 14, 16, 18, 20, 22, and 24 h of culture.

Experiment 4 The effects of EGFR-specific inhibitor, AG1478, on FSH-induced cumulus expansion and meiotic resumption were detected. EGF and AR were used as the general stimulators of EGFR. The COCs were cultured with FSH, EGF, AR, and/or different concentrations of AG1478 (0–10 μ M) for 24 h. Cumulus expansion and nuclear status of oocytes were determined at the end of the culture period. The time course of EGFR function in FSH-induced meiotic resumption was also examined.

Experiment 5 The relationship between EGFR and PKC during FSH-induced porcine oocyte meiotic resumption was examined. PKC activator, PMA with the concentration of 10^{-7} M, and the PKC inhibitor, chelerythrine chloride with the concentration of 5 μ M, were used. The effect of PKC on EGF family members' genes expression was also detected.

Statistical analysis

The percentage of GVBD was calculated as number of oocytes that underwent GVBD divided by total number of surviving oocytes. GVBD includes all stages such as GVBD, metaphase I, anaphase I, telophase I, and metaphase II. Experiments were performed at least thrice and the values are given as means $\pm$ S.E.M. Meiosis resumption frequencies (% GVBD) were subjected to arcsine transformation and analyzed by ANOVA followed by Duncan's multiple range tests. $P < 0.05$ was considered statistically significant.

Results

FSH-stimulated AR gene expression and EGFR kinase phosphorylation

By RT-PCR, we observed that COCs originally expressed EGF, AR, HB-EGF, and EGFR mRNA (Fig. 1A–E). Further

research showed that the EGF and HB-EGF mRNA were strongly expressed both in cumulus cells and oocytes, EGFR mRNA strongly expressed only in cumulus cells, and AR had little if any expression in both cumulus cells and oocytes (Fig. 1F). With FSH treatment, the AR mRNA expression was significantly increased after 1 h (6-fold), peaked at 4 h (17-fold), maintained at 6 h and then gradually decreased (Fig. 1A–E). This increase may be contributed to cumulus cells response, since FSH had no effect on AR mRNA expression in oocytes (Fig. 1F). FSH had no significant effect on the other genes expression.

EGFR is a 170 kDa tyrosine protein kinase and its C-terminus contains three conserved tyrosines (tyr-1068, tyr-1148, and tyr-1173) that are phosphorylated upon ligand activation (Downward *et al.* 1984). By western blot, we detected the tyrosine-phosphorylated band around 175 kDa, and FSH had the tendency to induce tyrosine phosphorylation after 6 h of culture and significantly increased this phosphorylation level at 8 h (Fig. 2A and B); this band was confirmed by an antibody that specifically recognizes the EGFR phosphorylated at one of the conserved tyrosine phosphorylation site Tyr-1173 (phospho-Tyr1173; Gall *et al.* 2005; Fig. 2C). EGF rapidly induced EGFR phosphorylation within 30 min and declined after 2 h (Fig. 2D).

AR dose-dependently induced porcine oocyte meiotic resumption

AR dose-dependently stimulated porcine oocyte GVBD; 10 ng/ml AR began to significantly induce porcine oocyte meiotic resumption (55.3 vs 37.1% GVBD, $P < 0.05$) and 100 ng/ml AR further augmented this stimulatory effect (74.6% GVBD, $P < 0.001$; Fig. 3). The concentration at 100 ng/ml of AR was preferred thereafter.

The kinetics of FSH-, EGF-, and AR-induced porcine oocyte meiotic resumption

As shown in Fig. 4, almost all oocytes in groups were arrested at GV stage during the first 14 h of culture. Oocytes in control group spontaneously resumed meiosis from 16 to 24 h with a slow speed. FSH treatment kept oocyte arrested at GV stage from 16 h (5.2% GVBD) to 18 h (5.9% GVBD) and then had a rapidly stimulatory effect from 20 h (32.0% GVBD) to 24 h (74.5% GVBD); EGF treatment began to stimulate GVBD at 18 h (26.7% GVBD) and kept up to 24 h (63.8% GVBD). AR had the similar kinetics as that of EGF. During the period between 18 and 22 h, the rate in the FSH group was faster and then exceeded that in the EGF and AR group.

The effects of EGFR-specific inhibitor, AG1478, on FSH-induced cumulus expansion and meiotic resumption

On cumulus expansion and meiotic resumption, 1 μ M AG1478 had no effect, but 10 μ M AG1478 inhibited spontaneous cumulus expansion (CEI, 0.08 vs 0.47,

Figure 1 FSH-induced EGF family members mRNA expression of porcine COCs. Porcine cumulus–oocyte complexes (COCs) were cultured in NCSU37 medium containing 10% porcine follicular fluid (pFF) with or without 0.05 units/ml FSH for the indicated period (0, 1, 2, 4, 6, 8, 16, and 24 h). A total of 200 COCs, 400 oocytes, and cumulus cells from 400 COCs were pooled for RNA extraction respectively. The genes expressions were analyzed by semi-quantitative RT-PCR using specific primers as shown in Table 1. Relative intensities were quantified using Gel-pro analyzer 4.0 software. For reference, the 0 h COC value was set as 1. The ratio between the intensity of the bands was reported as the mean $\pm$ S.E.M. $P < 0.05$ was considered statistically significant. Groups with a common letter are not significantly different. (A) Representative gel showing gene expression of the epidermal growth factor (EGF), amphiregulin (AR), heparin-binding EGF (HB-EGF), and EGF receptor (EGFR) examined in COCs and β -actin as standard. (B–E) Summary of the relative density of EGF, AR, HB-EGF, and EGFR genes respectively. (F) Representative gel showing expression of the four genes examined in cumulus cells and oocytes respectively and β -actin as standard. C, control; F, FSH.

Figure 2 FSH-induced EGFR kinase phosphorylation. For indicated periods, 50 porcine cumulus–oocyte complexes (COCs) were cultured in NCSU37 containing 10% porcine follicular fluid (pFF) with or without 0.05 units/ml FSH. In each treatment, 50 COCs were lysated, separated by 5–7.5% SDS-PAGE, and analyzed by western blot. Immunoblots were performed by (A and B) anti-phosphotyrosine (PY99), (C and D) EGFR-phospho-specific Tyr1173 antibody, and β -tubulin served as internal standard. Relative intensities were quantified using Gel-pro analyzer 4.0 software. For reference, the 0 h COCs value was set as 1. The ratio between the intensity of the bands was reported as the mean $\pm$ S.E.M. of the three experiments performed. $P < 0.05$ was considered statistically significant. Groups with a common letter are not significantly different. C, control; F, FSH.

$P < 0.001$) and meiotic resumption (GVBD, 11.3% vs 28.0%, $P < 0.01$). AG1478 with a concentration of 1 μ M was preferred thereafter. As shown in Table 2 and Fig. 5A, AG1478 significantly inhibited FSH-induced porcine cumulus expansion (CEI, 1.75 for FSH + AG1478 and 2.28 for FSH, $P < 0.01$) and meiotic resumption (GVBD, 27.1% vs

Figure 3 Effect of AR on porcine oocyte meiotic resumption. Porcine cumulus–oocyte complexes (COCs) were cultured in modified NCSU37 medium containing 10% porcine follicular fluid (pFF) supplemented with increasing concentration of amphiregulin (AR) for 24 h. $P < 0.05$ was considered statistically significant. Groups with a common letter are not significantly different.

73.1%, $P < 0.001$). As the positive control, AG1478 completely blocked EGF-induced cumulus expansion (CEI, 0.33 for EGF + AG1478 and 1.74 for EGF, $P < 0.001$) and meiotic resumption (GVBD, 24.0 vs 66.7%, $P < 0.001$). AG1478 had the similar effect on AR-induced cumulus expansion and oocyte meiotic resumption as that of EGF. AG43, the inactive analog of AG1478, had no effect on FSH-, EGF-, and AR-induced cumulus expansion and oocyte meiotic resumption.

As shown in Fig. 5B, FSH was added at the beginning of the culture period, AG1478 completely suppressed FSH action when it was added before 4 h of the culture period, and the inhibitory effect was gradually weakened when it was added after 6 h and disappeared after being added at 11 h (GVBD; 29.8, 48.4, 63.6, and 80.9% for FSH 0 h + AG1478, FSH

Figure 4 The kinetics of FSH, EGF, and AR-induced porcine oocyte meiotic resumption. Porcine cumulus–oocyte complexes COCs were cultured in NCSU37 containing 10% porcine follicular fluid (pFF) supplemented with 0.05 units/ml FSH, 10 ng/ml epidermal growth factor (EGF), or 100 ng/ml amphiregulin (AR) for indicated time. Bars indicated the percentage of oocyte at GVBD.

Table 2 Effect of AG1478 on follicle-stimulating hormone- (FSH), epidermal growth factor- (EGF), and amphiregulin (AR)-induced porcine cumulus expansion

Treatment	Total no.	CEI
Control	149	0.4687 ± 0.0215 ^a
AG1478	149	0.3245 ± 0.0785 ^a
AG43	150	0.4308 ± 0.0214 ^a
EGF	149	1.7420 ± 0.0654 ^b
EGF+AG1478	149	0.3287 ± 0.2133 ^a
EGF+AG43	150	1.5333 ± 0.0940 ^b
AR	150	1.7938 ± 0.0213 ^b
AR+AG1478	150	0.4496 ± 0.0878 ^a
AR+AG43	150	1.5970 ± 0.1642 ^b
FSH	150	2.2795 ± 0.0686 ^c
FSH+AG1478	151	1.7518 ± 0.1065 ^b
FSH+AG43	149	2.2600 ± 0.0702 ^c

CEI, cumulus expansion index. This represents the mean value for the complexes, with 0 equal to no expansion and 4 equal to maximal expansion. $P < 0.05$ was considered statistically significant. Treatments with a common superscript letter are not significantly different. The significance between letters a and b, b and c, a and c are $P < 0.01$, $P < 0.01$ and $P < 0.001$ respectively. AG1478, 1 μ M; AG43, 1 μ M.

6 h+ AG1478, FSH 8 h+ AG1478, and FSH 11 h+ AG1478 respectively). The above results indicated that FSH needs a period of time to induce EGFR activator(s) production and then to activate EGFR. So, we added exotic EGF before the endogenous ligand secretion (at the beginning of the culture together with FSH) to activate EGFR in advance, and then added AG1478 at 2, 4, 6, and 8 h. The results showed that EGF reversed the inhibition of AG1478 on FSH action when AG1478 was added within 6 h (Fig. 5C). These findings suggested that FSH-stimulated porcine oocyte GVBD involved inducing EGFR ligands secretion and EGFR activation.

The relationship between PKC and EGFR signaling during FSH-induced porcine oocyte meiotic resumption

As shown in Fig. 6, PKC activator PMA-promoted oocyte GVBD (71.2% GVBD versus 38.4% GVBD), and EGFR kinase inhibitor AG1478 significantly inhibited this effect (50.7% GVBD). Chelerythrine chloride, an inhibitor of PKC, completely blocked FSH-induced oocyte meiotic resumption (45.7% GVBD versus 74.5% GVBD, $P < 0.001$), but had no effect on EGF- or AR-induced oocyte meiotic resumption. Chelerythrine chloride and PMA was ineffective on EGF, AR, HB-EGF, and EGFR mRNA expression with or without FSH treatment respectively (Fig. 7), suggesting that PKC participates in FSH-induced EGFR activation not by stimulating the expression of EGF, AR, and HB-EGF.

Discussion

It is reported that FSH influences folliculogenesis by up-regulating EGF receptors in the porcine granulosa cells (Fujinaga *et al.* 1992). Meanwhile, FSH-dependent activation

Figure 5 Effect of AG1478 on FSH-induced oocyte meiotic resumption. (A) Porcine cumulus-oocyte complexes (COCs) were cultured in NCSU37 containing 10% porcine follicular fluid (pFF) supplemented with 0.05 units/ml FSH, 10 ng/ml epidermal growth factor (EGF), 1 μ M AG1478 and/or AG1478 inactive analog AG43 for 24 h. (B) COCs were cultured in NCSU37 containing 10% pFF and 0.05 units/ml FSH. At different time of culture (0, 2, 4, 6, 8, 9, 10 and 11 h), 1 μ M AG1478 was added. (C) COCs were cultured in NCSU37 containing 10% pFF, 0.05 units/ml FSH, and 10 ng/ml EGF, and 1 μ M AG1478 was added at different time of culture (2, 4, 6, and 8 h). Bars indicated the percentage of oocyte at GVBD after total culture for 24 h. $P < 0.05$ was considered statistically significant. Groups with a common letter are not significantly different. C, control; E, EGF; F, FSH.

Figure 6 The relationship between PKC and EGFR signaling during FSH-induced porcine oocyte meiotic resumption. Porcine cumulus-oocyte complexes (COCs) were pre-cultured with 1 μ M AG1478 or 5 μ M protein kinase C (PKC) inhibitor chelerythrine chloride for 30 min, and 10^{-7} M PKC activator phorbol 12-myristate 13-acetate-4-O-methyl ester (PMA), 10 ng/ml epidermal growth factor (EGF), 100 ng/ml amphiregulin (AR), or 0.05 units/ml follicle-stimulating hormone (FSH) was added to correspondent groups and the cultures were continued for 24 h before GVBD scoring. Bars indicated the percentage of oocyte at GVBD after total culture for 24 h. $P < 0.05$ was considered statistically significant. Groups with a common letter are not significantly different. CC, chelerythrine chloride.

of EGFRs is beneficial for cumulus expansion of porcine COCs isolated from the small follicles (Prochazka *et al.* 2003). Most recently, it is reported that tumor necrosis factor α -converting enzyme, a member of a disintegrin and metalloprotease that is critical for activation of EGFR signaling under a variety of circumstances (Sahin *et al.* 2004), participates in the gonadotropin-induced porcine cumulus expansion and oocyte maturation (Yamashita *et al.* 2007). All these studies indicate that EGF family members and EGFR may play an important role in porcine oocyte maturation.

In this study, we demonstrated here with detailed kinetics that FSH-induced porcine oocyte meiotic resumption involves endogenous EGFR activator(s) (possibly AR) production and EGFR activation. The specific EGFR inhibitor, AG1478 but not AG43 (the inactive analog of AG1478), blocked FSH-, EGF-, and AR-induced cumulus expansion and oocyte meiotic resumption. The inhibitory effect of AG1478 on FSH action gradually decreased when the inhibitor was added after 6 h and disappeared when the inhibitor was added at 11 h; EGF reversed the inhibitory effect on FSH when AG1478 was added within 6 h. All the results indicate that the time course is reasonable, FSH endogenously induced the AR mRNA expression with maximum at 4 h, possibly by which it began to activate EGFR at 6 h and is finished within 11 h. Western blotting also confirmed that FSH began to induce EGFR phosphorylation at 6 h, but with significant difference at 8 h. The meiotic resumption was triggered at 20 h, which was later

Figure 7 Effect of PMA and chelerythrine chloride on EGF family genes expression. Porcine cumulus-oocyte complexes (COCs) were pre-cultured with or without protein kinase C (PKC) inhibitor chelerythrine chloride (5 μ M) for 30 min and then further cultured with FSH and PKC activator phorbol 12-myristate 13-acetate-4-O-methyl ester (PMA; 10^{-7} M) for 4 h. A total of 200 COCs were pooled for RNA extraction. (A) Representative gel showing gene expression of the epidermal growth factor (EGF), amphiregulin (AR), heparin-binding EGF (HB-EGF), and EGF receptor (EGFR) examined in COCs and β -actin as standard. (B) Summary of the relative density of EGF, AR, HB-EGF, and EGFR genes respectively. The genes expressions were analyzed by semi-quantitative RT-PCR using specific primers as shown in Table 1. Relative intensities were quantified using Gel-pro analyzer 4.0 software. For reference, the 0 h COC value was set as 1. The ratio between the intensity of the bands is reported as the mean $\pm$ S.E.M. $P < 0.05$ was considered statistically significant. Groups with a common letter are not significantly different. C, control; F, FSH; CC, chelerythrine chloride.

than that of EGF and AR (at 18 h). Further, PKC activator PMA mimicked but PKC inhibitor chelerythrine chloride inhibited FSH action, and AG1478 also suppressed PMA-induced porcine oocyte meiotic resumption, suggesting that PKC participates in FSH-induced EGFR activation. Our results in porcine oocyte meiotic resumption were consistent with recent reports that gonadotropins induce rodent oocyte maturation by stimulating EGF-like factors expression and EGFR signaling (Park *et al.* 2004, Ashkenazi *et al.* 2005, Shimada *et al.* 2006, Downs & Chen 2008).

AG1478 completely blocked FSH and EGF action when all were synchronously added, while the inhibitory effect of AG1478 on FSH action gradually decreased when the inhibitor was added after 6 h and EGF reversed the inhibitory effect on FSH when AG1478 was added at 2–8 h, which suggested that the action of AG1478 to inhibit EGFR kinase may be quicker than that of EGF to activate EGFR when they were simultaneously added so that EGF reversed the inhibitory effect when the AG1478 was added delayingly and also suggested that FSH-stimulated EGFR activation needs a period of time possibly through inducing EGFR ligand(s) synthesis. The reason for the delay of EGFR activation to meiotic resumption is not entirely clear. It is possible that a specific downstream component of EGFR mediated this response. Recent studies reveal that EGF-induced porcine oocyte maturation involves phosphatidylinositol 3-kinase (PI3-kinase) and the rapid phosphorylation of MAPK (Keely *et al.* 1998, Gall *et al.* 2005, Li *et al.* 2007, Liang *et al.* 2007), which indicates FSH-induced oocyte maturation through EGFR, PI3-kinase, and MAPK. The endogenous EGF and/or EGF-like factors stimulated by FSH may be more efficient in stimulating maturation than those provided exogenously, since during the period between 18 and 22 h the rate in the FSH group was faster and then exceeded that in the EGF and AR groups.

It is reported that EGF is present in porcine follicular cells and fluid (Hus *et al.* 1987, Singh *et al.* 1995). EGF-like factors AR and HB-EGF mRNA have been detected in porcine corpora lutea and COCs (Kennedy *et al.* 1993, Yamashita *et al.* 2007). In our study, EGF, HB-EGF but few, if any, AR gene expressions were also detected in porcine COCs, and 10 μ M AG1478 inhibited spontaneous meiotic resumption and cumulus expansion. These results suggested that EGF and/or EGF-like factors were locally synthesized in porcine COCs and involved in oocyte maturation. The amount of initial EGF and/or EGF-like factors in COCs may be too small to strongly induce meiotic resumption. However, the local EGF in COCs may be very important for FSH-induced oocyte resumption since anti-EGF antiserum alone can completely block FSH action (Downs & Chen 2008).

Porcine cumulus cells isolated from small and large follicles contain similar amounts of EGFR protein, and no signal of it was detected in oocytes (Prochazka *et al.* 2003). We also showed that porcine cumulus cells but not oocyte expressed EGFR mRNA. In our and other studies, EGF and AR dose-dependently resumed porcine COCs meiosis, but had no effect on DO (Coskun & Lin 1993, Singh *et al.* 1993). All these findings suggest that EGF and/or EGF-like factors act on cumulus cell to mediate FSH-induced oocyte maturation (Downs *et al.* 1988, Park *et al.* 2004, Yamashita *et al.* 2007, Downs & Chen 2008).

PKC signaling mediates FSH-induced oocyte maturation (Su *et al.* 1999, Downs *et al.* 2001, Lu *et al.* 2001, Fan *et al.* 2004, Jin *et al.* 2006). In our study, AG1478 successfully blocked FSH- and PMA-induced porcine oocyte meiotic resumption, suggesting that FSH may activate EGFR by PKC signaling

(Downs & Chen 2008). On the other hand, PMA had no effect on the EGF, AR, HB-EGF and EGFR gene expression, consistent with previous report that PMA alone is ineffective on AR, ER, and BTC mRNA expression in mouse granulosa cells (Shimada *et al.* 2006). Furthermore, PKC inhibitor chelerythrine chloride successfully inhibited FSH-induced porcine GVBD, but it did not block FSH-induced AR gene expression. These results indicate that PKC, possibly by transcription-independent mechanism, activates EGFR. There is still the possibility that additional EGF-like factors serve a redundant function in regulating meiotic resumption. It is well known that EGF and EGF-like factors are expressed as transmembrane precursors, which are cleaved at one or more sites in the extracellular domain to release a soluble EGF domain for which the metalloprotease activity is responsible (Dong *et al.* 1999, Sahin *et al.* 2004). The PKC activator PMA is a commonly used activator of ectodomain shedding (Massague & Pandiella 1993, Hooper *et al.* 1997, Le Gall *et al.* 2003, Sahin *et al.* 2004), by which PKC may participate in FSH-induced oocyte maturation. Thus, FSH-induced oocyte meiotic resumption may be mediated by shed EGF and/or EGF-like factors via PKC, in addition to the dependence on *de novo* synthesis of EGF-like growth factors. The PMA alone can stimulate oocyte maturation, indicating that a pool of EGF and/or EGF-like factors precursors may be already present on COCs. If so, it is easy to explain that anti-AR antiserum have no effect on FSH-induced mouse oocyte maturation (Downs & Chen 2008). The PKC inhibitor chelerythrine chloride could not block exotic EGF- and AR-induced porcine oocyte maturation, since they are already soluble mature peptides. That PKC inhibitor inhibits EGF-induced porcine oocyte maturation is previously reported (Coskun & Lin 1995). Possibility for this contradiction may be the different culture media.

In conclusion, we have shown that FSH endogenously stimulated EGFR activator(s) (possibly AR) expression with maximum at 4 h, which activates EGFR pathway at 6 h, and EGFR activation finished within 11 h; AR functionally mimicked FSH action on porcine oocyte meiotic resumption, supporting that EGFR activation is necessary for FSH-induced porcine oocyte meiotic resumption. The PKC activator PMA mimicked but the PKC inhibitor chelerythrine chloride inhibited FSH-induced porcine oocyte meiotic resumption, and AG1478 successfully blocked FSH and PMA, suggesting that PKC is necessary for FSH-induced EGFR activation. Together, this study revealed that gonadotropin-induced oocyte maturation of both mouse and pig share the same mechanism, i.e. EGF-like factor(s) induced by gonadotropins and EGFR activation play a physiological role in gonadotropin-induced oocyte maturation.

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