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ORIGINAL PAPER

Mitogen-activated protein kinase regulates FSH-induced expression of tissue-type plasminogen activator through an activator protein 1 response element

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Abstract We have analyzed a possible role of mitogen-activated protein kinase (MAPK) and activator protein-1 (AP-1) in the regulation of FSH-induced tissue type plasminogen activator (tPA) production in granulosa cells (GCs) prepared from DES-treated immature rats; Treatment of the cells in the presence of FSH with MAPK inhibitors, such as UO126 or SB203580, significantly decreased the FSH-induced tPA production, suggesting that multiple signaling pathways may be involved in FSH-regulated tPA expression. We further examined possible signaling action involved in FSH-activated ERK1/2 and p38 MAPK on tPA production, and observed that FSH receptor occupancy led to both ERK1/2 and p38 MAPK phosphorylation. Such action might be through a protein kinase A-dependent pathway because the observed activation was destroyed by the addition of its specific inhibitor H89 to the culture. The inhibition of ERK1/2 and p38 MAPK activation by their specific inhibitors remarkably reduced FSH-induced tPA mRNA and its protein production. We further examined whether AP-1 located in the tPA promoter is involved in FSH-regulated tPA production, and demonstrated that FSH significantly stimulated AP-1 expression, whereas inclusion of H89, UO126, or SB20358 in the culture significantly decreased FSH-induced AP-1 expression. In summary, FSH-induced ERK1/2 and p38 MAPK activation is capable of regulating tPA production in cultured primary GCs, and that the transcript factor AP-1 may be important in the regulation of FSH-induced tPA expression.

Keywords FSH · Granulosa cells · MAPKs · tPA · AP-1

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Introduction

Plasminogen activator (PA) is a rate-limiting protease for converting plasminogen to plasmin. PA system is involved in extracellular matrix remodeling, such as ovulation, embryogenesis, embryo implantation, fibrinolysis, angiogenesis, inflammation, and tumor metastasis [1–3]. Two types of PA, tissue-type PA (tPA) and urokinase-type PA (uPA), and a PA inhibitor, PAI-1, have been reported in rat [4] and monkey [5] ovaries. Our previous studies have demonstrated that GCs and oocyte of rat and rhesus monkey produced tPA, while theca cells produced PAI-1, the coordinated expression of tPA and PAI-1 regulated by gonadotrophins in the ovary-induced ovulation [6, 7]. Evidence has also shown that rat GCs in vitro express tPA in response to FSH, LH, GnRH, VIP, and growth factors via different signaling pathways [4, 8–12]. GnRH is likely to stimulate tPA mRNA level through protein kinase C pathway [13], while growth factors EGF and TGF-alpha induced tPA mRNA and activity in GCs through a pathway independent of protein kinase A and C [14]. FSH induces a maximal level of tPA mRNA just after 3–6 h stimulation [15] and the hormone alone is capable of inducing ovulation in hypophysectomized rat [16]. In a classical model, FSH first binds to its cognate G-protein-coupled receptor and activates membrane-associated adenylyl cyclase,

resulting in the elevation of intracellular Cyclic AMP level. cAMP subsequently activates its dependent protein kinase A (PKA), resulting in the phosphorylation of cellular proteins and expression of specific genes involved in ovulation [17, 18]. However, the cellular signaling events occurring downstream of PKA in GCs remain unknown. It is uncertain whether activation of cAMP-PKA signaling pathway alone is sufficient to account for the stimulation of tPA expression in GCs.

Rat tPA promoter at position −178 to −185 contains an element CRE (TGACGTCA) which can be activated by phosphorylation of cAMP-responsive element binding protein (CREB) [19]. In contrast, mouse tPA promoter has no CRE, but TPA-responsive element [20]. It is unclear how FSH induces tPA expression in different cells. Mitogen-activated protein kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis [21]. Recently, it has been demonstrated that in addition to stimulating cAMP-PKA pathway, FSH is also capable of promoting activation of MAP kinases such as extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38 MAPK [22, 23]. Members of MAPK superfamily could regulate acute cellular responses and transcriptional events via phosphorylation for the target enzymes and transcription factors [24, 25]. This study was designed to investigate the role of MAPK signaling cascade in FSH-regulated tPA expression and to examine the possible involvement of transcript factor AP-1 in the signal pathways in vitro.

Results

FSH induced ERK1/2 and p38 MAPK activation in a time-dependent manner in cultured granulosa cells

GCs were collected from ovaries of DES-treated immature rats and incubated with FSH for 72 h, and the medium tPA activity was measured. Activation of ERK1/2 and p38 MAPK in the cells was assessed by immunoblotting with a phospho-specific anti-active ERK antibody that detects dually phosphorylated ERK1/2 and with a specific corresponding antibody for p38 MAPK, respectively. As shown in Figs. 1a and 2a, the treatment of the GCs with FSH (100 ng/ml) resulted in a transient activation of ERK1/2 and p38 MAPK. Phosphorylated ERK1/2 and p38 MAPK both were detected within 5 min, and peaked at 20 min, and then gradually decreased to a low level at 60 min. Re-probing the membranes using an antibody recognizing both the phosphorylated and nonphosphorylated forms of ERK1/2 and p38 MAPK indicated that the total protein levels of the ERK1/2 and p38 MAPK were not changed by

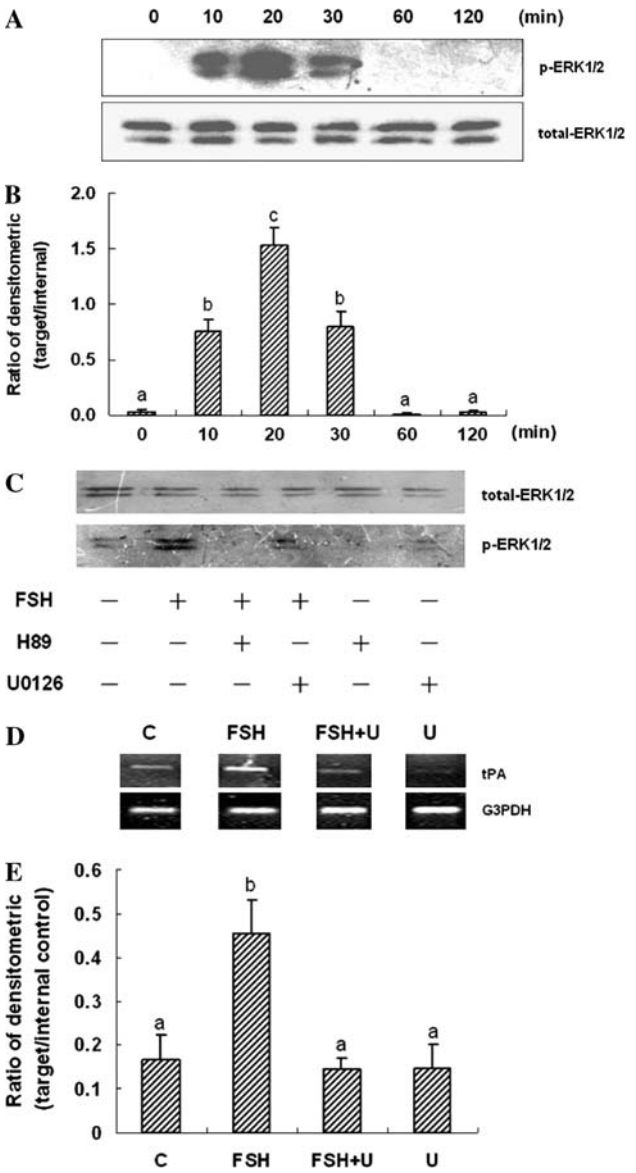

Fig. 1 Phosphorylation of ERK1/2 in response to FSH in cultured granulosa cells. GCs were harvested from ovaries of DES-treated immature rats and cultured overnight at 37°C in 5% serum McCoy's 5a media. The GCs (2×10^6 viable cells) were incubated with FSH (100 ng/ml) for various times. **a** Western blot analysis of phospho-MAPK, MAPK; **b** Western blotting quantitative analysis; **c** GCs were cultured with PKA inhibitor H89 (10 μM) or ERK1/2 inhibitor U0126 (10 μM) for 20 min, followed by treatment with 100 ng/ml FSH for 20 min. Data are represented as percentage ratio of the amount of phosphorylated protein over the total amount of protein in each sample and expressed as means ± SEM ($n = 3$). **d**, **e** Inactivation of ERK1/2 activity inhibits FSH-induced tPA mRNA expression in cultured granulosa cells. Different letters represent values significantly different at $P < 0.05$

the FSH treatment. The 20-min incubation period was then chosen for subsequent experiments. To confirm specificity of FSH-induced ERK1/2 activation, the cells were pre-treated with 10 μM MPKA inhibitor H89 or 10 μM ERK1/2 inhibitor U0126 for 20 min, followed by treatment

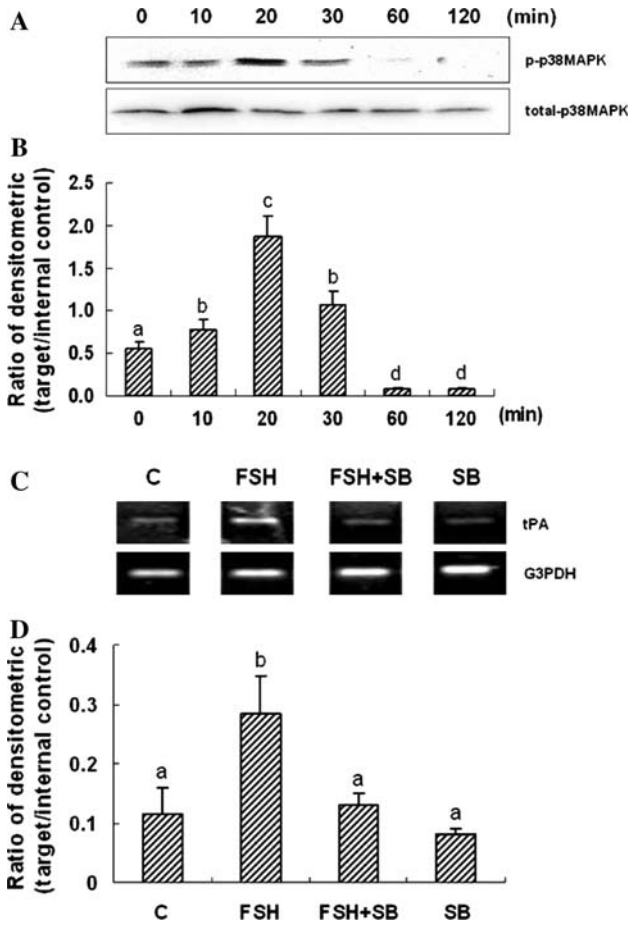

Fig. 2 Phosphorylation of p38 MAPK in response to FSH in cultured granulosa cells. GCs were cultured as described in Fig. 1. The cell proteins were collected for western blot of p38 MAPK activation. **a** Western blot of phospho-MAPK and MAPK. **b** Western blotting quantitative analysis. **c**, **d** Inactivation of p38 MAPK activity inhibits FSH-induced tPA mRNA expression in cultured granulosa cells. Different letters represent values significantly different at $P < 0.05$

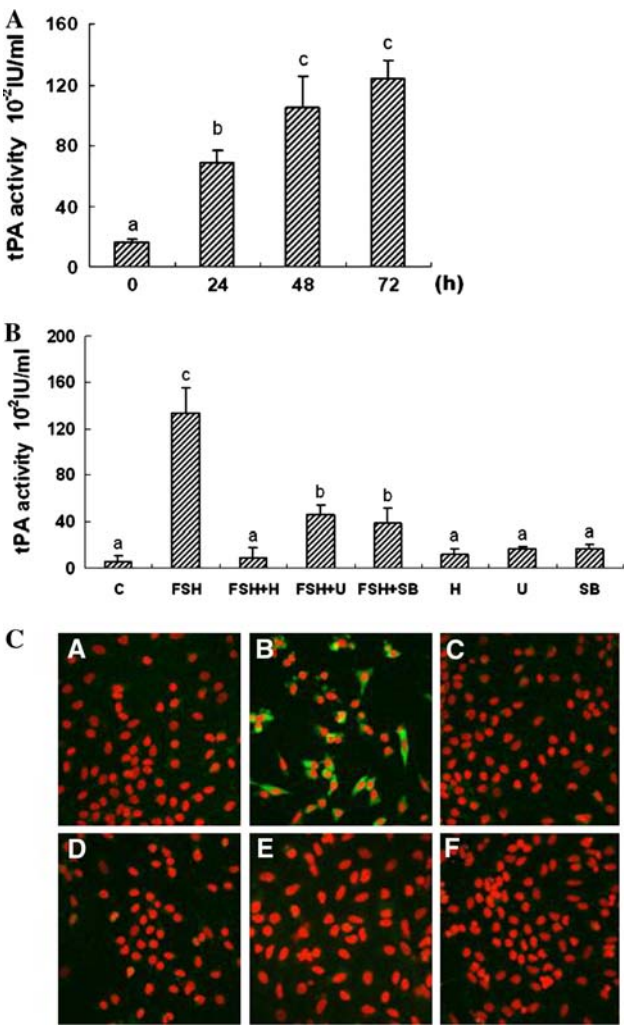

Fig. 3 tPA activity and subcellular expression in cultured granulosa cells. GCs were cultured as Fig. 1. **a** The GCs (2 x 10^6 viable cells) were treated with 100 ng/ml FSH for the indicated time points. Media were collected at the end of incubation. Activity of tPA in the media was determined by colorimetry (tPA activity kit, Sun co. Shanghai China); **b** GCs were cultured with H89 (10 μM) or U0126 (10 μM) or SB203580 (20 μM) for 20 min, followed by treatment with 100 ng/ml FSH for 24 h. Activity of tPA in the media was determined by tPA activity kit; **c** Inactivation of ERK1/2 and p38 MAPK activity inhibits FSH-induced tPA subcellular expression in cultured granulosa cells. **a** Cells without any treatment. **b** Cells treated with FSH only. **c** Cells treated with FSH and U0126. **d** Cells treated with FSH and SB203580. **e** Cells treated with U0126 only. **f** Cells treated with SB203580 only. Different letters represent values that were significantly different at $P < 0.05$

with 100 ng/ml FSH for 30 min. ERK1/2 was no longer phosphorylated in response to FSH in the GCs pretreated with H89 or U0126 (Fig. 1c). H89 or U0126 alone was not able to change the phosphorylation status of ERK1/2. Collectively, these results indicated that FSH-induced phosphorylation of ERK1/2 kinase and p38 MAPK is in a time-dependent manner, and ERK1/2 activation by FSH may be involved in PKA signaling.

FSH-induced tPA production is time dependent

GCs obtained from follicles of the DES-treated immature rats were cultured with FSH for a period of 0 to 72 h. The tPA activity in the conditioned media was analyzed. As shown in Fig. 3a, FSH-induced tPA production was in a time-dependent manner. The FSH-induced magnitude of tPA activity was significant at 24 h, reaching the relatively highest level at 72 h ($P < 0.01$).

Inactivation of ERK1/2 and p38 MAPK activity inhibited FSH-induced tPA production

GCs were incubated with FSH in the presence or absence of ERK1/2 specific inhibitor U0126 (10 μM) or p38 MAPK-specific inhibitor SB203580 (20 μM) for 48 h. As shown in Fig. 3b, the FSH-induced tPA activity was

suppressed significantly by U0126 and SB203580. The tPA mRNA level in the GCs treated with FSH in the presence of U0126 or SB203580 was also analyzed by RT-PCR. As shown in Figs. 1d and 2c, inhibition of ERK1/2 and p38 MAPK activation caused a marked decrease in FSH-enhanced tPA mRNA expression ($P < 0.05$). Immunocytochemical data using the specific tPA antibody revealed that the protein was localized in mitochondria of GCs treated with FSH, and the staining was obviously reduced by the addition of U0126 or SB203580 (Fig. 3c).

FSH increased c-Jun mRNA level

FSH could increase c-Jun mRNA production measured by RT-PCR as shown in Fig. 4a. FSH induced a marked increase in c-Jun mRNA level that was maximal by 2 h after the hormone treatment, and declined at 4 h thereafter.

FSH increased binding of AP-1 response element to tPA promoter

To determine whether FSH increases the binding of AP-1 response element to tPA promoter area, nuclear extracts from FSH-treated GCs were used for gel-mobility shift experiments. As shown in Fig. 5a, AP-1 DNA binding activity reached peak value at 2 h, and decreased at 4 h after the hormone treatment. HeLa nuclear extract was prepared and used as a positive control. The unlabeled AP-1 consensus oligo as oligonucleotides was used as a specific competitor. As shown in Fig. 5b, inactivation of PKA, ERK1/2, or p38 MAPK activity could inhibit the FSH-induced AP-1 DNA binding activity.

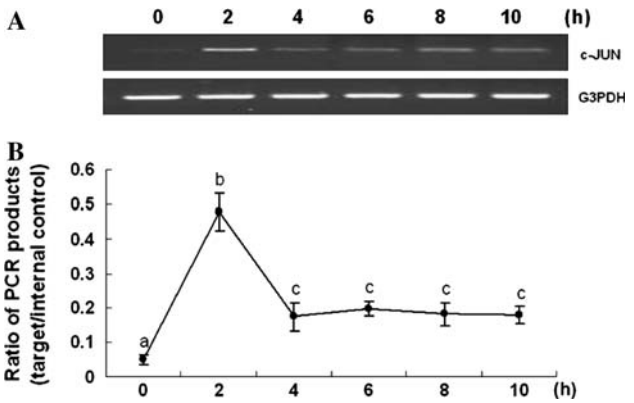

Fig. 4 FSH time dependently induces c-JUN mRNA expression in cultured granulosa cells. GCs were cultured as Fig. 1. The GCs (3×10^6 viable cells) were treated with 100 ng/ml FSH for the indicated times. The total RNA in the cells was extracted and the levels of c-JUN mRNA were evaluated by semi-quantitative RT-PCR. G3PDH was used as the internal control. Different letters represent values that were significantly different at $P < 0.05$

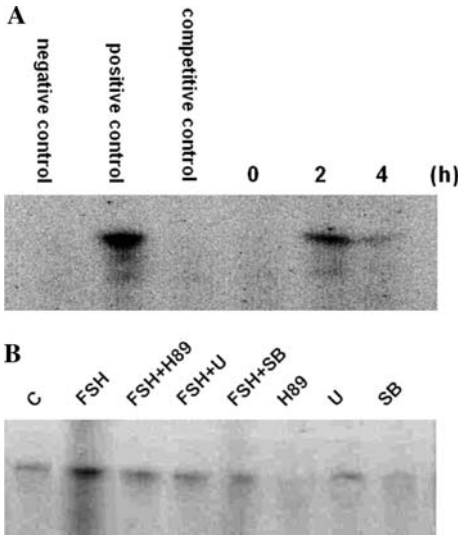

Fig. 5 FSH induces Ap1 DNA binding activity in cultured granulosa cells. GCs were cultured as Fig. 1. **a** The GCs (2×10^6 viable cells) were treated with 100 ng/ml FSH for the indicated times. The nuclear proteins were prepared for analyzing AP1 binding activity by Gel Shift Assay kit (Promega co. USA). The binding activity to AP1 reached the peak value at 2 h after FSH treated. **b** GCs were cultured with H89 (10 μM) or U0126 (10 μM) or SB203580 (20 μM) for 20 min, followed by treatment with 100 ng/ml FSH for 2 h; the AP1 binding activity was analyzed by the Gel Shift Assay kit

Discussion

We have demonstrated in the present study that FSH-induced ERK1/2 and p38 MAPK activation is capable of regulating tPA production in cultured primary GCs, and the transcript factor AP-1 may be important in the regulation of the FSH-induced tPA expression. It is well known that the peak levels of LH and FSH from pituitary at mid-cycle are key factors for controlling ovulation in mammals [26–29]. PA system has been demonstrated to be involved in ECM degradation [30, 31]. FSH and LH are the principal regulators in mammalian ovaries and play a critical role in the regulation of tPA expression in GCs [32, 33]. LH is the well-known hormone inducing ovulation in mammals. FSH was also reported to be capable of inducing ovulation in hypophysectomized rat [16]; the molecular signaling mechanism, however, is unclear. Gonzales-Robayna et al. have reported that FSH may play a role in A-kinase-signaling pathway involved in the regulation of aromatase and sgk expression during transition of granulosa cells to luteal cells [17]. Richards has also suggested that FSH controlling granulosa cell gene expression is via cAMP-PKA signaling pathway [18]. Our present data further demonstrated that FSH-induced ERK1/2 and p38 MAPK signaling cascade could regulate tPA expression in rat GCs.

Rat tPA promoter contains an element CRE, that can be activated by phosphorylation of CRE binding protein (CREB); therefore, it is suggested that FSH may regulate

tPA expression via CRE activation through cAMP-PKA pathway. However, it has been reported that tPA genes from primate and mouse have no CRE element, but a TPA-response element (TRE), which is regulated by PKC pathway. Interestingly, we have demonstrated that GnRH, in addition to FSH, could induce tPA expression in rat granulosa cells [8, 33]. In addition to CRE, the rat tPA promoter also contains a few nuclear factor binding sites, such as nuclear factor 1 (NF 1), SP1 binding sites, and GC box binding factor. Evidence has shown that these factors seem to play a role in cAMP activation of tPA gene constitutive expression [20, 34, 35]. These data suggest that FSH and GnRH may induce tPA gene activation by different intracellular mechanisms that do not converge at the gene level. Therefore, tPA can be regulated through several signal pathways in different species. Galway et al. [14] reported that epidermal growth factor stimulated tPA activity and mRNA level in cultured rat GCs, and this regulation was independent of PKA or PKC. A report has also shown that FSH regulated tPA activity in B103 cell line via nuclear factor NF1 [34]. Recently, a few targets have been identified downstream MAPK including 90-kDa ribosomal S6 protein kinase, cytoplasmic phospholipase A2, MAPK-activated protein kinase-2, and transcriptional factors such as c-myc and Elk-1 [36–39]. Our current results indicate that transcript factor AP-1 was involved in phosphorylation of MAPK-regulated tPA expression.

In summary, our study demonstrated for the first time that inhibition of ERK1/2 and p38 MAPK activation by their specific inhibitors U0126 and SB203580 significantly suppressed FSH-induced tPA mRNA and protein levels. It is therefore suggested that ERK1/2 and p38 MAPK signaling may play an important role in the regulation of FSH-induced tPA expression in GCs. Inactivation of ERK1/2 or p38 MAPK activity significantly decreased FSH-induced DNA binding activity of AP-1, which is an important transcriptional factor in tPA promoter area. It is therefore suggested that signaling from MAPK to AP-1 may be involved in the regulation of FSH-induced tPA expression.

Materials and methods

Materials and reagents

Culture materials were purchased from Corning Inc. (Corning, NY, USA). FSH (NIADDK-hFSH-I-3) was obtained from the National Hormone and Pituitary Distribution Program (National Institutes of Health, Maryland, USA), and National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases. McCoy's 5a medium (M4892), PKA inhibitor H89 (B1427), ERK1/2-specific inhibitor U0126, specific p38 MAPK inhibitor SB203580, diethylstilbestrol (DES), 4-androstene-3,17-dione (androstenedione), soybean trypsin inhibitor, aprotinin, and leupeptin were all purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Trizol Reagent (15596-018) and SuperScript III Rnase H-Reverse Transcriptase (18080-044) from Life Technologies, Inc (Gaithersburg, MD, USA). Antibodies for p38 MAPK, phospho p38 MAPK,ERK1/2, and phospho ERK1/2 were from New England Biolabs, Inc. (Beverly, MA, USA). Monoclone antibody against tPA was obtained from Biopool Company (Umea, Sweden).

Animals

Immature female Sprage–Dawley (SD) rats (23 days old) were obtained from the Experiment Animal Center, Chinese Academy of Sciences (Beijing, China) and maintained under 16 h light, 8 h dark schedule with food and water ad libitum. The rats were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Academic Committees of Institute of Zoology, Chinese Academy of Sciences.

Primary cell culture

Female SD rats (23 days old) were injected with 0.5 mg DES/day for 3 consecutive days to stimulate follicular development and to increase GC numbers. Then GCs were harvested by puncturing individual ovarian follicles with 25 gauge needles and collected by centrifugation (500 g for 5 min). The cells were washed three times with fresh serum-free McCoy's 5a medium; an aliquot of cells was mixed with trypan blue and stained for determining cell number and viability. The cells were then cultured overnight for adhesion in serum-free McCoy's 5a medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37°C in an atmosphere of 5% CO_2 and 95% air, and further incubated in fresh medium in the presence or absence of various reagents.

Confocal immunohistochemistry

GCs were seeded onto 24 × 24-mm^2 coverslips placed in six-well plates at a density of 2 × 10^5 cells/coverslip. The cells were cultured overnight in serum-free McCoy's 5a medium for adhesion, and then treated with or without 10 μM U0126 or 20 μM SB203580 for 20 min, followed by incubation in the presence or absence of 100 ng/ml FSH for 24 h. By the end of incubation, the media were removed and the cells were fixed with 4% paraformaldehyde for 30 min, and followed by incubation for 1 h with

2% goat serum in phosphate-buffered saline (pH 7.4) containing 0.01% Triton-100 X (PBS-triton buffer). Then the cells were incubated at 4°C overnight with monoclone tPA antibody (1:100). The cells were then washed with phosphate-Triton buffer and further incubated at room temperature for 1.5 h with second antibody (1:100) conjugated fluorescein. The antibody binding was visualized using a Leica confocal microscope (Leica Microsystems, Bensheim, Germany). For negative controls, the cells were incubated with nonimmune rabbit serum, followed by the second antibodies.

RNA extraction and RT-PCR assays

The GCs (3×10^6 viable cells) were cultured in a six-well plate in 2 ml McCoy's 5a in the presence or absence of 100 ng/ml FSH, or 20 μM U0126, 20 μM SB203580. After 6 h culture, total cell RNA in each group was isolated with Trizol Reagent according to the instruction of the manufacturer. Each RNA sample was pooled, respectively, from three replicate well preparations, quantified by measuring the absorbance at 260 nm, and stored at −80°C until assay. Total RNA (2 μg) was reversely transcribed to first strand cDNA with 200 U SuperScript III RNase H-Reverse Transcriptase in the presence of 0.5 mM deoxy-NTPs and 25 μg/ml oligo (deoxythymidine) in a total volume of 20 μl for 60 min at 50°C. After reverse transcription step, the reaction mixture was split into aliquots to which specific primer pairs for tPA were added. PCR was performed in a total volume of 50 μl with 1 μl reverse-transcribed product, 2.5 U Taq polymerase (TaKaRa Biotechnology Co., Ltd, Dalian, China), 100 μM deoxy-NTP, 0.4 μM primers in 1* PCR buffer. Amplification was carried out under the following conditions: denaturation at 94°C for 20 s, annealing at 58°C for 20 s, and extension at 72°C for 30 s. There were 28 cycles. Aliquots of the PCR products were electrophoresed on 1.5% agarose gels, and visualized after ethidium bromide staining, photographed, and scanned. The relative integrated density of each band was digitized by multiplying the absorbance of the surface area. The levels of tPA products were calculated with that of G3PDH, which was used as an internal control.

Western blotting analysis

The GCs (2×10^6 viable cells) were cultured for the indicated times in a six-well plate with McCoy's 5a alone (control) or supplemented with 100 ng/ml FSH in the presence or absence of 10 μM H89, 10 μM U0126, or 20 μM SB203580, respectively. The protein sample in each culture was prepared. Protein extract (20 μg) from each sample was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and then probed with an antibody that recognizes the non-phosphorylated and phosphorylated (total) isoforms of ERK1/2 and p38 MAPK and with an antibody that only recognizes phosphorylated (active) isoform. The antibody binding was detected by enhanced chemiluminescence (ECL).

EMSA

GCs were cultured with 100 ng/ml FSH in the presence or absence of 10 μM U0126 or 20 μM SB203580 for 2 h, and the cell nuclear protein was isolated for EMSA. About 2 μg of nuclear protein was end-labeled with 40 k cpm of ^{32}P-labeled oligonucleotide coding for the region of interest. Oligonucleotide sequence for AP1 was purchased from Beyotime Co., China.

Statistical analysis

All experiments were repeated three times with GC preparations obtained from three different separate experiments. Values were presented as means ± SEM ($n = 3$). Data were analyzed by ANOVA. Differences between groups were analyzed by Tukey's multiple-comparison test (SPSS Standard Version 15.0. SPSS Inc. Chicago, IL, USA). Different letters represent values significantly different at $P < 0.05$. For immunofluorescence, one representative picture was shown from three similar independent experiments.

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