

Activation of the p38 MAPK pathway by follicle-stimulating hormone regulates steroidogenesis in granulosa cells differentially

Fu-Qing Yu^{1,2}, Chun-Sheng Han¹, Wei Yang¹, Xuan Jin¹, Zhao-Yuan Hu¹ and Yi-Xun Liu¹

¹State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China

²Graduate School of the Chinese Academy of Sciences, 19 Yu-quan Road, Beijing 10009, China

(Requests for offprints should be addressed to Y-X Liu; Email: liuyx@panda.ioz.ac.cn)

Abstract

In the present study, we started out to test whether the follicle-stimulating hormone (FSH)-activated p38 MAPK signaling cascade was involved in the regulation of steroidogenesis in granulosa cells (GCs). GCs were prepared from the ovaries of DES-treated immature rats and cultured in serum-free medium. Treatment of GCs with FSH (50 ng/ml) induced the phosphorylation of p38 MAPK rapidly with the phosphorylation being observed within 5 min and reaching the highest level at 30 min. Such activation was protein kinase A-dependent as indicated by the results using specific inhibitors. FSH stimulated the production of progesterone and estradiol as well as the expression of the steroidogenic acute regulatory protein (StAR) in a time-dependent manner, with a maximum level being observed in the production of progesterone and StAR at 48 h. Moreover, the potent p38 MAPK inhibitor SB203580 (20 μ M) augmented FSH-induced progesterone and StAR production, while reduced FSH-induced estradiol production at the same time ($P < 0.01$). RT-PCR data showed that inclusion of

SB203580 in the media enhanced FSH-stimulated StAR mRNA production, while decreased the FSH-stimulated P450arom mRNA expression ($P < 0.05$). Immunocytochemical studies showed that FSH treatment together with the inhibition of p38 MAPK activity resulted in a higher expression of StAR in mitochondria than FSH treatment alone. FSH also significantly up-regulated the protein level of LRH-1, a member of the orphan receptor family that activates the expression of P450arom in ovaries and testes. p38 MAPK inactivation down-regulated the basal and FSH-induced LRH-1 expression significantly. The intracellular level of DAX-1, another orphan receptor that inhibits StAR expression, also decreased upon p38 MAPK being inactivated. For the first time, the present study suggests that FSH-activated p38 MAPK signal pathway regulates progesterone and estrogen production in GCs differentially, and that the transcription factors LRH-1 and DAX-1 might play important roles in the process.

Journal of Endocrinology (2005) **186**, 85–96

Introduction

Gonadotropins exert their functions on ovarian follicles through the activation of hormone sensitive adenylate cyclase that causes an elevation of intracellular cyclic AMP (cAMP). This results in an increase in ovarian estradiol and progesterone production. *In vivo* studies have shown that during the follicular phase of the menstrual/estrous cycle, follicle-stimulating hormone (FSH) promotes granulosa cell (GC) differentiation and then the cells become competent to produce copious amounts of estradiol but no progesterone (Smith *et al.* 1975). To the contrary, GCs from early antral follicles cultured *in vitro* in the presence of FSH secrete both estradiol and progesterone (Liu *et al.* 1989). Further studies indicate that the pathways utilized by FSH in estrogen synthesis are not completely the same as those in FSH-induced progesterone production

(Zeleznik *et al.* 2003). In the classical model, FSH binds to its cognate G-protein-coupled receptor and then activates the membrane-associated adenylyl cyclase leading to an increase in cAMP level. cAMP subsequently activates cAMP-dependent protein kinase A (PKA), resulting in the phosphorylation of cellular proteins and the expression of specific genes involved in the regulation of progesterone and estradiol production (Richards 1994, Gonazalez-Robayna *et al.* 1999). However, the cellular signaling events occurring downstream of PKA in GCs remains unknown. It is uncertain whether activation of the cAMP-PKA signaling pathway alone is sufficient to account for the divergent steroidogenic properties of GCs. Recently, it has been demonstrated that in addition to stimulating the cAMP-PKA pathway, FSH also promotes the activation of MAP kinases such as ERK1/2 and p38 MAPK (Tajima *et al.* 2003, Maizels *et al.* 1998,

Gonzalez-Robayna *et al.* 2000). Moore *et al.* (2001) have identified ERK1/2 as the first intracellular signaling molecules that differentially regulate FSH-induced progesterone and estradiol synthesis in GCs. Whether p38 MAPK would manifest a similar action is still an open question.

Steroidogenic acute regulatory protein (StAR) is a rate-limiting protein in steroid biosynthesis. StAR participates in the transport of cholesterol from mitochondrial outer membrane to inner membrane, where cytochrome P450 side-chain cleavage enzyme (P450_{scc}) resides (Christenson & Strauss 2000, Stocco 2000). StAR expression is regulated by several transcription factors such as DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita, critical region on the X chromosome gene-1), SF-1 (steroidogenic factor-1), and AP-1 (activator protein-1) (Christenson & Strauss 2000, Stocco 2000, Wooton-Kee & Clark 2000, Manna *et al.* 2004). DAX-1 is a transcriptional repressor of steroidogenic proteins and inhibits steroidogenesis at multiple levels in the Y-1 mouse adrenocortical tumor cell line (Lalli *et al.* 1998), while SF-1 promotes the transcription of the StAR gene (Morohashi & Omura 1996, Gyles *et al.* 2001). DAX-1 co-localizes with SF-1 in various steroidogenic tissues and blocks SF-1 activity through their direct interaction (Ikeda *et al.* 1996, Zazopoulos *et al.* 1997, Ito *et al.* 1997). Recent studies have shown that the AP-1 family member c-Fos and c-Jun are involved in the attenuation of mouse StAR expression induced by epidermal growth factor (EGF) in mLTC-1 Leydig tumor cells (Manna *et al.* 2002). c-Fos has also been demonstrated to inhibit StAR gene transcription in Y-1 cells (Shea-Eaton *et al.* 2002).

Liver receptor homologue-1 (LRH-1) is another member of the orphan nuclear hormone receptor family, and is very similar to SF-1 in its structure (Galarneau *et al.* 1996). It is also known as cholesterol 7 α -hydroxylase (CYP7A)-promoter binding factor, α -fetoprotein transcription factor, human B1-binding factor and NR5A2 (Galarneau *et al.* 1996, Li *et al.* 1998, Nitta *et al.* 1999, Nuclear Receptors Nomenclature Committee 1999). Originally, LRH-1 was cloned from mouse liver as a liver-enriched transcription factor. It regulates the expression of genes involved in cholesterol metabolism and bile acid synthesis, including CYP7A (Goodwin *et al.* 2000, Lu *et al.* 2000), sterol 12 α -hydroxylase (CYP8B1) (Castillo-Olivares & Cil 2000) and the cholesteryl ester transfer protein (Luo *et al.* 2001). Recently, it has been reported that LRH-1 expression is abundant in testes and ovaries and is highly restricted to the estrogen-biosynthesizing cells, including GCs (Hinshelwood *et al.* 2003), Leydig cells, as well as pachytene spermatocytes and round spermatids (Pezzi *et al.* 2004). Moreover, LRH-1 has been found to be a potential regulator of P450_{arom} (P450_{arom}) gene transcription in ovaries, testes and human pre-adipocytes (Hinshelwood *et al.* 2003, Pezzi *et al.* 2004, Clyne *et al.* 2002), suggesting that it may also play a role in steroidogenesis in addition to bile acid production.

FSH induces the phosphorylation/activation of p38 MAPK in GCs of immature rats. Activation of the p38 signaling pathway results in the activation of Elk-1 ternary complex factor that binds to the c-fos promoter (Whitmarsh *et al.* 1997), and the activation of myocyte enhancer family (MEF-2)-dependent c-jun gene expression (Han *et al.* 1997), suggesting a potential role of p38 MAPK in the regulation of the expression of these immediate early genes. In addition, p38 kinase signaling pathway participates in the transactivation of LRH-1 promoter in GCs (Falender *et al.* 2003).

Based on the descriptions above, we hypothesize that an FSH-activated p38 MAPK signaling cascade is involved in the regulation of GC steroidogenesis, and start out in the present study to test this hypothesis by using *in vitro* cultured rat GCs. For the first time, we reported that p38 MAPK signaling pathway mediated FSH's differential regulation on the steroidogenesis in GCs. Inhibition of p38 MAPK activity significantly augmented FSH-induced StAR expression and progesterone production, and at the same time attenuated FSH-induced P450_{arom} expression and subsequent estradiol production. The orphan nuclear receptors LRH-1 and DAX-1 may be involved in the signal transduction.

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), National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases. McCoy's 5a medium (M4892), PKA inhibitor H89 (B1427), anti- α -tubulin antibody (T6074), 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). p38 MAPK inhibitor SB203580 (B475449) from Calbiochem (San Diego, CA, 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 9212 and phospho (thre180/try182) p38 MAPK were from New England Biolabs, Inc. (Beverly, MA, USA) Anti-DAX-1 antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) The antibody against StAR was generously provided by Dr D M Stocco (Texas Tech University, USA) and the antibody against LRH-1 by Dr Luc Belanger (Universite Laval, Canada).

Animals

Immature female Sprague-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. All the protocols had the approval of the Institutional Committee on Animal Care and Use.

Primary cell culture

Female SD rats (23 days old) were injected with 0.5 mg DES/day for 3 consecutive days to increase follicular GC numbers. Then the GCs were harvested by puncturing the 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, and an aliquot of the cells was mixed with trypan blue stain for determining the cell number and viability. The cells were 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% CO₂ and 95% air, and then further incubated in fresh medium with the presence or absence of the various reagents for the indicated times.

Steroid radioimmunoassay (RIA)

The GCs (5×10^5 viable cells) were cultured in a 24-well plate with 500 µl medium in the presence or absence of FSH (50 ng/ml) with or without SB203580 (20 µM). For the assessment of progesterone and estradiol production, 100 nM androstenedione, a substrate for P450arom, was added to the medium. By the end of culture, the conditioned media were collected and stored at -20 °C until assay. The levels of progesterone and estradiol in the media were measured by the standard RIA procedures as reported previously (Liu *et al.* 1989).

Confocal immunohistochemistry

GCs were seeded onto 24 × 24-mm 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 further incubated at 37 °C in the medium with or without 50 ng/ml FSH, and/or 20 µM SB203580 for the indicated times. By the end of incubation, the media were removed and the cells were fixated with 4% paraformaldehyde for 30 min, and followed by incubation for 1 h with 2% goat serum in the phosphate-buffered saline (pH 7.4) containing 0.01% Triton-100X (PBS-triton buffer). Then the cells were incubated at 4 °C overnight with the antibody to StAR (1:200), LRH-1 (1:200) or DAX-1 (1:150). The cells were then washed with phosphate-Triton buffer and further incubated at room temperature for 1.5 h with the second antibody (1:100) conjugated fluorescein. The anti-

body binding was visualized using a Leica confocal microscope (Leica Microsystems, Bensheim, Germany). For the negative controls, the cells were incubated with non-immune 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 with 2 ml of McCoy's 5a in the presence or absence of 50 ng/ml FSH and/or 20 µM SB203580. After 24 h culture, the total cell RNA in each culture was isolated with Trizol Reagent according to the instructions of the manufacturer. Each RNA sample was pooled from three replicate wells, quantified by measuring absorbance at 260 nm and stored at -80 °C until assay. Total RNA (2 µg) was reverse 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 the reverse transcription step, the reaction mixture was split into three aliquots to which specific primer pairs for StAR (Ariyoshi *et al.* 1998), P450arom (Hickey *et al.* 1990), L19 (Chan *et al.* 1987) 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 55 °C for 20 s, and extension at 72 °C for 30 s. The cycles were 22 for StAR, 26 for P450arom and 22 for L19. The sizes of PCR products were 120 bp for StAR cDNA, 220 bp for P450arom and 195 bp for L19. 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 StAR and P450arom PCR products were compared with that of L19, 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 50 ng/ml FSH and/or 20 µM SB203580. The protein sample in each culture was prepared as described by Seger *et al.* (2001). Protein extract (20 µg) from each sample was separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes, then probed with an antibody that recognizes the nonphosphorylated and phosphorylated (total) isoforms of p38 MAPK and with an antibody that only recognizes the phosphorylated (active) isoform. LRH-1 and StAR immunoreactivities

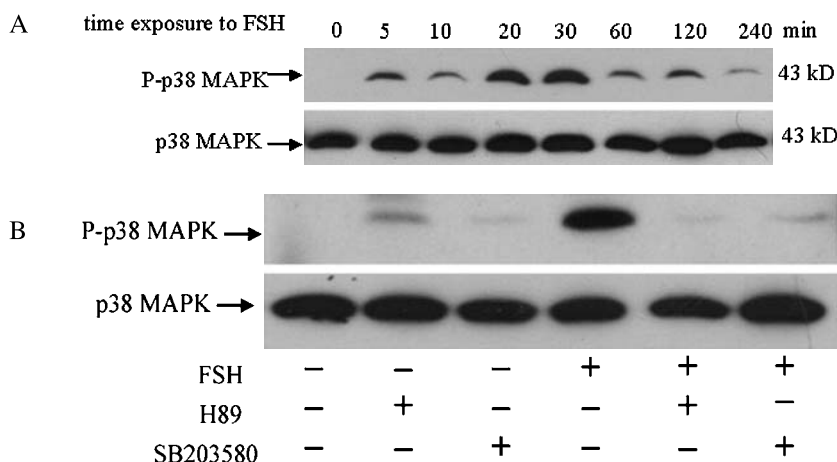


Figure 1 FSH induced p38 MAPK activation in a time- and PKA-dependent manner in cultured primary granulosa cells (GCs). GCs were harvested from the ovaries of DES-primed immature rats and cultured overnight at 37 °C in serum-free McCoy's 5a media. (A) The GCs (2×10^6 viable cells) were treated with 50 ng/ml FSH for the different time spans indicated. (B) GCs were cultured with PKA inhibitor H89 (10 μ M) or p38 MAPK inhibitor SB203580 (20 μ M) for 20 min, followed by the treatment of 50 ng/ml FSH for 30 min. Subsequently, the cell lysates were prepared and subjected to immunoblotting analysis using antibodies recognizing either phosphorylated p38 MAPK or total p38 MAPK. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL).

were also detected by their respective specific antibodies. The antibody binding was detected by enhanced chemiluminescence (ECL).

Statistical analysis

All the experiments were repeated three times with GC preparations obtained from separate groups. The values were presented as the mean \pm S.D. ($n=3$). Differences between the groups were analyzed by statistical significance using analysis of variance (SPSS Standard Version 10.0.1. SPSS Inc. Chicago, IL, USA). P value < 0.05 was accepted as statistical significance. For the immunofluorescence data, one representative picture was shown from three similar independent experiments.

Results

FSH induced p38 MAPK activation in GCs in a time- and PKA-dependent manner

The p38 MAPK gains its kinase activity upon being phosphorylated. To study p38 MAPK activation induced by FSH in GCs, immature rats were treated with diethylstilbestrol (DES), and the ovaries were collected. GCs were subsequently isolated and cultured *in vitro* in the presence or absence of FSH (50 ng/ml) for different time spans. The amount of phosphorylated p38 MAPK was measured by Western blotting using its specific antibody.

As shown in Fig. 1A, FSH stimulated the activation of p38 kinase in the primary GCs rapidly, and the phosphorylated p38 MAPK was detected within 5 min, peaked at 30 min, and then gradually decreased to a low level at 4 h. Re-probing the membranes using an antibody recognizing both the phosphorylated and nonphosphorylated forms of p38 MAPK indicated that the total protein level of p38 MAPK was not changed by FSH treatment. The 30-min incubation period was then chosen for subsequent experiments. To confirm the specificity of FSH-induced p38 MAPK activation, the cells were pretreated with 10 μ M PKA inhibitor H89 or 20 μ M p38 MAPK inhibitor SB203580 for 20 min, followed by treatment with 50 ng/ml FSH for 30 min. p38 MAPK was no longer phosphorylated in response to FSH in the GCs pretreated with H89 or SB203580 (Fig. 1B). H89 or SB203580 alone was not able to change the phosphorylation status of p38 MAPK. Collectively, these results indicated that FSH induced the phosphorylation of p38 kinase in a time- and PKA-dependent manner.

FSH stimulated the production of progesterone, estradiol and StAR protein

Steroidogenesis of the cultured GCs in response to FSH was studied. We first performed dose-response experiments of progesterone, estradiol, and StAR production in response to FSH with 24 h incubation and concentration range of 0, 10, 20, 50, 100 and 200 ng/ml. The results

(data not shown) showed that when the concentration of FSH increased to 50 ng/ml, the levels of progesterone, estradiol and StAR protein reached the maximal levels. Fifty nanograms per milliliter of FSH was accordingly chosen for the subsequent experiments. As shown in Fig. 2, progesterone (Fig. 2A) and StAR (Fig. 2C) production were stimulated by FSH in a time-dependent manner (0–72 h). The magnitude of progesterone production was significant only by 24 h ($P < 0.01$), reaching the peak level at 48 h, and starting to decline at 72 h. StAR protein was detected at 6 h, the magnitude became significant at 12 h ($P < 0.01$), increased gradually up to 48 h and then dropped slightly by 72 h. The time course of StAR expression was similar to that of progesterone synthesis. At the same time, estradiol production was also induced by FSH in a time-dependent manner. However, no decrease in its production was observed by 72 h (Fig. 2B).

Inhibition of p38 MAPK activity changed FSH-induced progesterone and estradiol production differentially

To investigate whether p38 MAPK signaling pathway plays a role in FSH-induced progesterone and estradiol production, the GCs were incubated with FSH in the presence or absence of 20 μ M p38 MAPK specific inhibitor SB203580 for 48 h. This concentration was chosen according to several reports that utilized this inhibitor (Gonzalez-Robayna *et al.* 2000, Shiota *et al.* 2003), COCs (cumulus-oocyte complexes; Villa-Diaz & Miyano 2004) and α T3 cells (Roberson *et al.* 1999). As shown in Fig. 3, FSH-induced progesterone production was further increased by SB203580; in contrast, FSH-stimulated estradiol production was suppressed significantly. mRNA levels of the two key regulators of steroidogenesis, StAR and P450arom, were analyzed by RT-PCR in the GCs treated with FSH in the presence or absence of SB203580. As shown in Fig. 4, inhibition of p38 MAPK activity by SB203580 caused a marked increase in FSH-promoted StAR mRNA expression, whereas the same treatment reduced P450arom mRNA production significantly.

Inactivation of p38 MAPK enhanced FSH-induced StAR protein expression

The effect of MAPK inactivation on FSH-induced StAR protein expression and localization was studied by Western blotting and immunocytochemistry techniques. As shown in Fig. 5, FSH stimulated StAR protein expression markedly and inhibition of p38 kinase by SB203580 augmented the expression further. Immunocytochemical data using StAR specific antibody revealed that StAR protein was localized in the mitochondria of GCs treated with FSH (Fig. 6A), and the staining was enhanced by the addition of SB203580 (Fig. 6B).

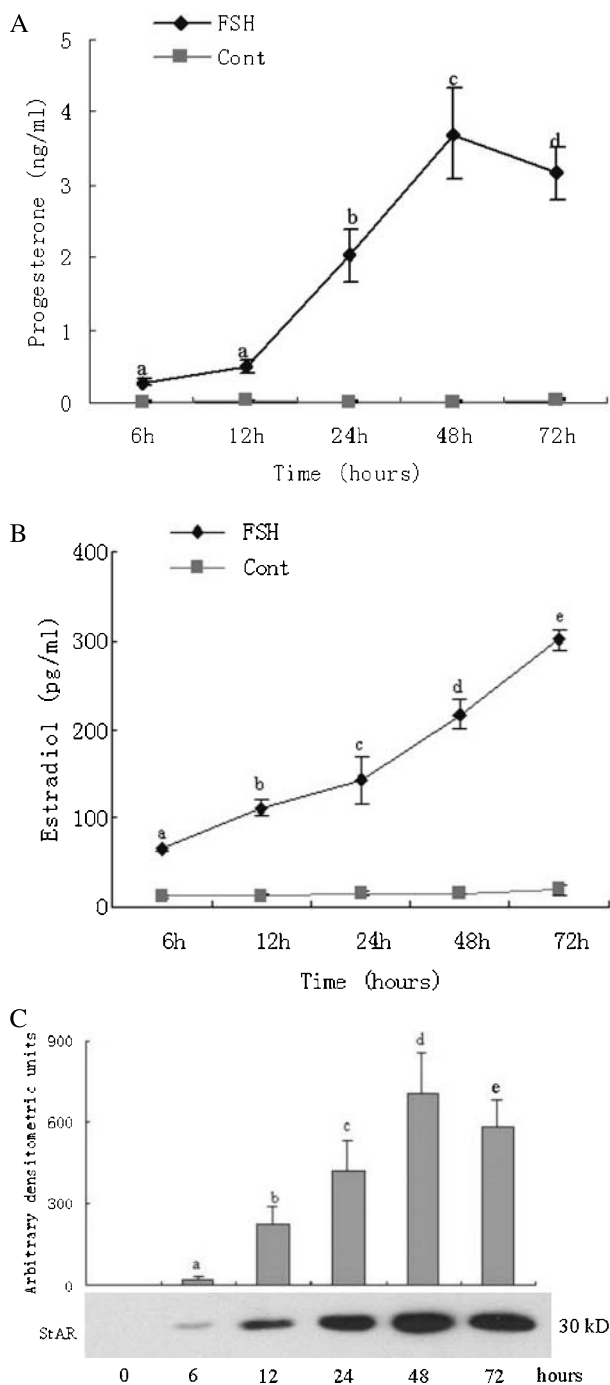


Figure 2 FSH stimulated the production of progesterone (A), estradiol (B), and StAR protein (C) in a time-dependent manner. The GCs were cultured as described in Fig. 1. 5×10^5 viable cells were incubated with FSH (50 ng/ml) for RIA analysis of progesterone and estradiol. 2×10^6 viable cells were incubated with FSH (50 ng/ml) for Western blotting analysis of StAR protein. These experiments were repeated three times. Data were expressed as means \pm s.d. ($n=3$). Different letters represent values that were significantly different at $P < 0.05$.

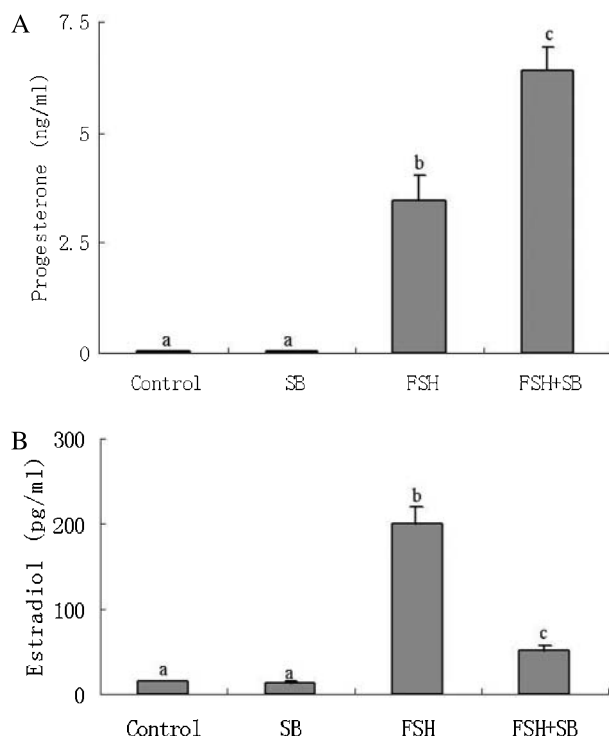


Figure 3 Inhibition of p38 MAPK activity changed FSH-induced steroid synthesis differentially. Four groups of GCs (5×10^5 viable cells per group) were cultured overnight in serum-free media. Two groups were then treated with p38 MAPK inhibitor SB203580 (20 μ M) for 20 min, and the other two without SB203580 treatment. One SB203580 treated group and one without SB203580 treatment were further treated with FSH (50 ng/ml) for 48 h. The levels of progesterone (A) and estradiol (B) in the media were measured by RIA. These experiments were repeated three times. Data are expressed as means \pm s.d. ($n=3$). Bars with different letters indicate significantly different means at $P<0.01$.

FSH-induced LRH-1 and DAX-1 protein expression and their changes by p38 MAPK inactivation

It has been demonstrated that LRH-1 is a critical transcriptional activator for P450arom expression, which can activate P450arom expression by binding to its promoter II (Luo *et al.* 2001, Hinshelwood *et al.* 2003). DAX-1 is a transcriptional repressor of steroidogenic enzymes and blocks steroid production at multiple levels in steroidogenic cells (Lalli *et al.* 1998). Therefore, the intracellular levels of LRH-1 and DAX-1 in response to FSH were analyzed in the GCs with or without p38 MAPK inactivated by SB203580. As shown in Fig. 7, FSH significantly induced LRH-1 protein expression, and the p38 kinase inhibitor SB203580 suppressed both the basal and the FSH-induced LRH-1 expression. The localization of LRH-1 was examined by confocal immunohistochemistry (Fig. 8). LRH-1 was mainly localized in the nucleus and occasionally appeared in the cytoplasm. Treatment of the cells with FSH augmented the nuclear staining, whereas

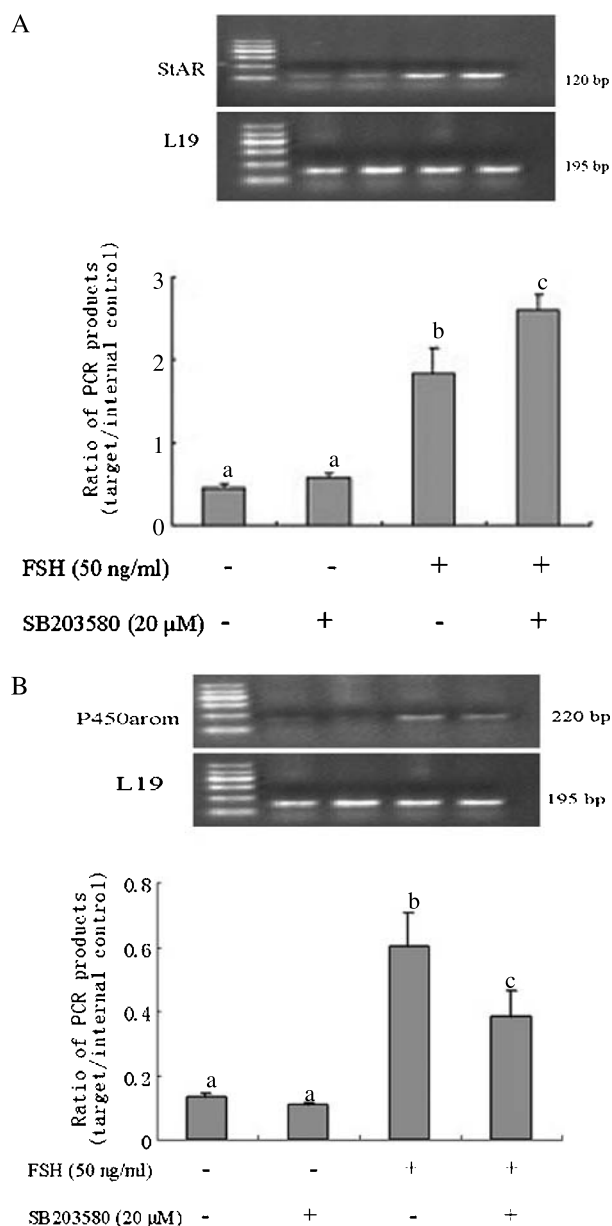


Figure 4 Inactivation of p38 MAPK altered FSH-induced transcription of StAR and P450arom genes differentially. 3×10^6 GCs were cultured and treated with p38 MAPK inhibitor SB203580 and FSH as described in Fig. 3 with time length of FSH treatment being 24 h. The total RNA in the cells was extracted and the levels of StAR (A) and P450arom (B) mRNA were evaluated by semi-quantitative RT-PCR. L19 was used as the internal control. The experiments were repeated three times. Data were shown as means \pm s.d. ($n=3$). Bars with different letters indicate significantly different means at $P<0.05$.

SB203580 reduced the staining. The intracellular localization of DAX-1 and its change in response to FSH and/or SB203580 treatment were similar to those of LRH-1 (Fig. 9).

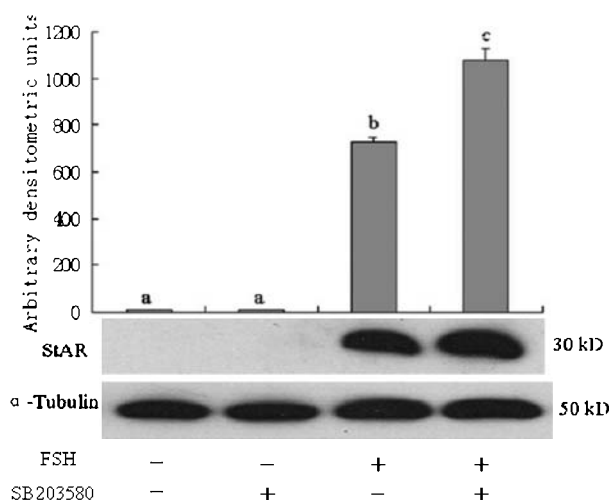


Figure 5 p38 MAPK inactivation enhanced FSH-induced StAR protein expression. 2×10^6 GCs were cultured and treated with p38 MAPK inhibitor SB203580 and FSH as described in Fig. 3. The cells' lysates (20 μ g total protein) from each treatment group were subjected to SDS-PAGE and Western blotting using anti-StAR or anti- α -tubulin antibodies. Densitometric values of StAR expression were normalized by α -tubulin expression. The experiments were repeated three times. Data are expressed as means \pm s.d. ($n=3$). Bars with different letters indicate significantly different means at $P<0.01$.

Discussion

FSH is a principal regulator in mammalian ovaries and plays a critical role in the process of steroidogenesis in GCs. The molecular mechanism of this event, however, is not completely understood. Although cAMP-PKA plays an important role in FSH-stimulated steroidogenesis in GCs (Richards 1994, Gonzalez-Robayna *et al.* 1999), it is only a part of the mechanism of FSH's action and can't

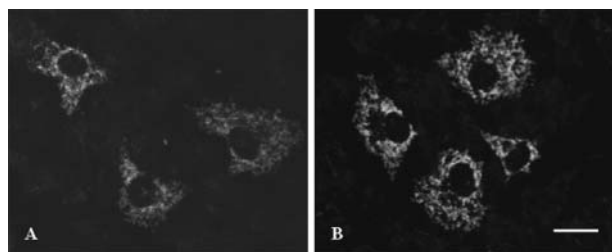


Figure 6 Expression and subcellular localization of StAR in cultured GCs treated with FSH in the absence or presence of SB203580 pretreatment. 2×10^5 viable GCs were seeded onto 24×24 -mm coverslips and cultured overnight. The cells were pretreated with 20 μ M SB203580 for 20 min and then incubated with 50 ng/ml FSH for 48 h. (A) cells treated with FSH only. (B) cells treated with FSH and SB203580. After the experiment, the sub-confluent GCs were incubated with the anti-StAR antibodies, and probed with goat anti-rabbit IgG conjugated to fluorescein. The localization of StAR in mitochondria was manifested by dotted green fluorescence. Magnification is 1000 fold. Bar represents 10 μ m.

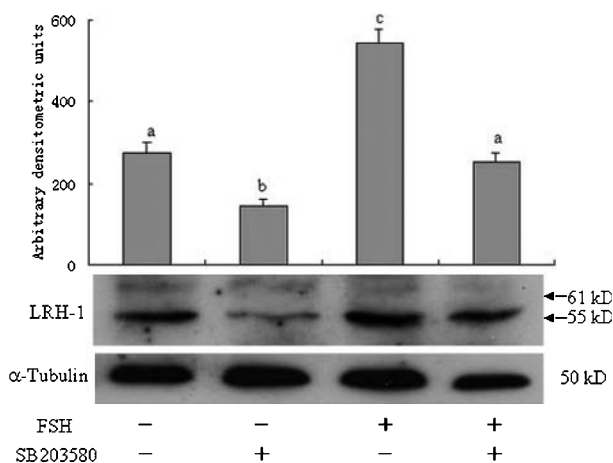


Figure 7 Inhibition of p38 MAPK activity blocked FSH-induced LRH-1 protein expression. 2×10^6 GCs were cultured and treated with SB203580 and FSH as described in Fig. 3 with FSH treatment time of 9 h. The cell lysates were prepared and subjected to Western blotting with anti-LRH-1 antibody. Densitometric values of LRH-1 expression were normalized by α -tubulin expression. These experiments were repeated three times. Data were expressed as means \pm s.d. ($n=3$). Bars with different letters indicate significantly different means at $P<0.01$.

completely explain the different regulation of steroidogenesis elicited by FSH. So it can be speculated that there may also be other signaling molecules participating in the modulation of steroidogenesis. Recently, it has been demonstrated that gonadotropins can activate multiple MAPK pathways including ERK1/2 and p38 MAPK cascades (Das *et al.* 1996, Maizels *et al.* 1998, Gonzalez-Robayna *et al.* 2000). Further studies have shown that FSH-activated ERK1/2 pathway was involved in the regulation of steroidogenesis in GCs (Seger *et al.* 2001, Dewi *et al.* 2002, Tajima *et al.* 2003). The present study demonstrated that FSH-activated p38 MAPK signaling cascade also regulated steroidogenesis in GCs differentially.

The p38 MAPK is a mammalian homolog of the yeast protein HOG-1, originally identified as an intracellular mediator of osmotic stress (Han *et al.* 1994, Raingeaud *et al.* 1995) and activated by factors such as osmotic manipulation, cytokines and hormones (Raingeaud *et al.* 1995, Roberson *et al.* 1999). Our present data showed that the addition of FSH to rat GCs activated p38 kinase rapidly in a time-dependent manner, reaching the peak level at 30 min. By comparison, FSH activated ERK1/2 kinases more instantly, with a peak activity occurring at 20 min after treatment (Fu-Qing *et al.* 2005). The activation of ERK1/2 and p38 kinase by FSH was consistent with earlier work by others (Das *et al.* 1996, Maizels *et al.* 1998, Gonzalez-Robayna *et al.* 2000). Using PKA specific inhibitor H89, we also demonstrated that PKA activity was required for the FSH-stimulated p38 kinase phosphorylation. The observation was similar to that of

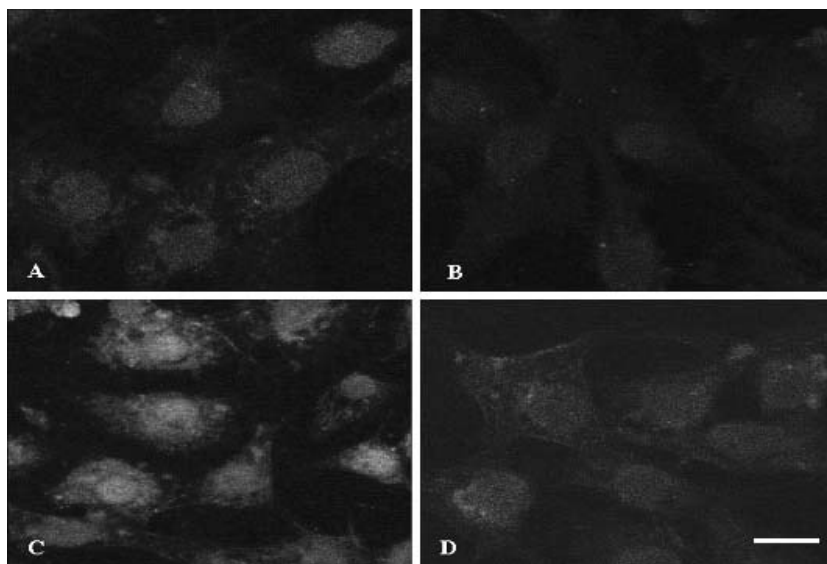


Figure 8 Expression and subcellular localization of LRH-1 in cells treated with SB203580 and FSH. The GCs were treated with SB203580 and FSH and then stained with anti-LRH-1 antibody as described in Fig. 6 with FSH treatment time of 9 h. (A) cells without any treatment. (B) cells treated with SB203580 only. (C) cells treated with FSH only. (D) cells treated with FSH and SB203580. Magnification $\times 1000$.

Maizels *et al.* (1998), in which they found that FSH and forskolin respectively promoted p38 MAPK phosphorylation in primary GCs in a PKA-dependent manner. The detailed mechanism of PKA-dependent p38 activation is currently unknown. In contrast, p38 kinase

phosphorylation in preovulatory GCs treated by hCG was not inhibited by PKA inhibitor H89 (Salvador *et al.* 2002). Therefore, it appears that the PKA-dependence of p38 MAPK activation may be related to the stages of cell differentiation and/or the stimuli employed.

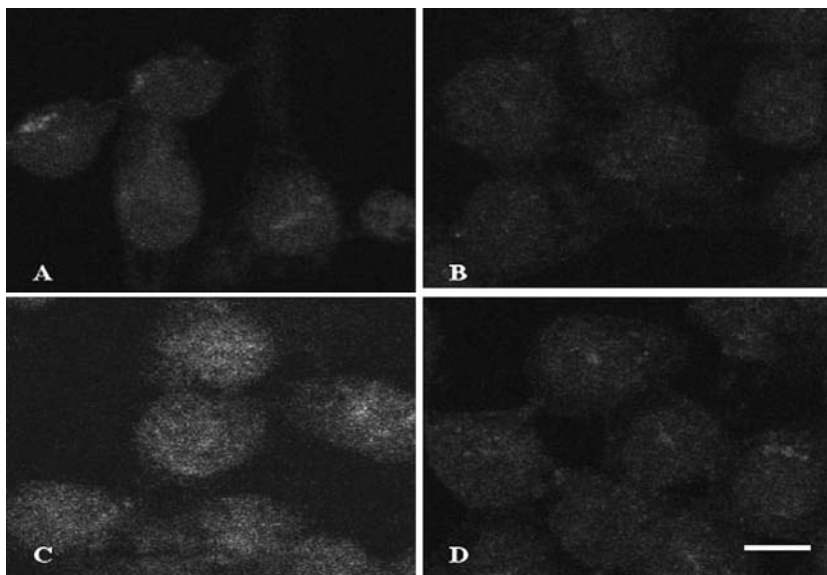


Figure 9 Expression and subcellular localization of DAX-1 in cells treated with SB203580 and FSH. The GCs were treated with SB203580 and FSH and then stained with anti-DAX-1 antibody as described in Fig. 6 with FSH treatment time of 9 h. (A) cells without any treatment. (B) cells treated with SB203580 only. (C) cells treated with FSH only. (D) cells treated with FSH and SB203580. Magnification is $\times 1000$.

A few studies have shown that SB 203580 is a highly specific inhibitor for p38 MAPK but not for p38 MAPK-kinases such as MKK3 or MKK6, and that it prevents the phosphorylation of p38 MAPK in many experimental systems such as GCs (Maizels *et al.* 1998, Gonzalez-Robayna *et al.* 2000, Shiota *et al.* 2003), COCs (Villa-Diaz & Miyano 2004) and α T3 cells (Roberson *et al.* 1999). Although the exact mechanism by which SB 203580 blocks phosphorylation of p38 MAPK is not known, the inhibitor may bind to the enzyme to mask a critical phosphorylation site (thereby inhibiting its activation) or alter the confirmation of the kinase preventing its phosphorylation and activation (Ge *et al.* 2002). So far five isoforms of p38 MAPKs have been identified, and they are divided into two groups, the p38 α / β / β 2 and the p38 γ / δ , based on their ability to respond to different stimuli (Kumar *et al.* 1997). Studies have shown that SB 203580 is a highly specific inhibitor for p38 MAPK and that it only prevents the phosphorylation of p38 α / β / β 2 (Cuenda *et al.* 1995, Gum *et al.* 1998). Therefore, p38 α / β / β 2 probably account for the results obtained in this study, although we cannot rule out the possible role of p38 γ / δ during steroidogenesis induced by FSH.

p38 MAPK pathway modulates cytoskeletal reorganization and cell survival in a variety of cell types including GCs. The function is through the activation of mitogen-activated protein kinase activated protein kinases-2 and -3 (MAPKAPK-2/-3) and subsequent phosphorylation of the downstream small heat shock protein (sHSP) HSP-25/27 (Guay *et al.* 1997, Maizel *et al.* 1998). For the first time, we provided the data to show that p38 kinase was involved in the regulation of steroidogenesis through modifying the expression of steroidogenic enzymes in the primary culture of GCs. We demonstrated that FSH induced StAR protein expression in GCs in a time-dependent manner, and the time course was similar to that of FSH-stimulated progesterone production. Inhibition of p38 MAPK activity augmented FSH-induced StAR mRNA and protein levels, and FSH-stimulated progesterone production while it inhibited the FSH-promoted P450arom mRNA and estradiol production. It implied that activated p38 MAPK differentially regulated the FSH-stimulated steroidogenesis in GCs, reminiscent of Moore *et al.*'s observation on the differential regulatory effects of ERK1/2 on FSH-induced steroid synthesis (Moore *et al.* 2001).

Estradiol synthesis is mainly dependent on the level of P450arom activity. Even in the absence of StAR, the aromatizable androgen precursors such as androstenedione are able to enter into the microsome where they are converted to estradiol by P450arom. Recent studies have shown that LRH-1 is a potential regulator of P450arom that binds to its promoter II in rodent GCs (Hinshelwood *et al.* 2003), Leydig cells (Pezzi *et al.* 2004) and human preadipocytes (Clyne *et al.* 2002). Promoter II is the principal promoter of P450arom (Hickey *et al.* 1990, Lanzino *et al.* 2001). Moreover, the co-localization of

LRH-1 and P450arom to multiple testis cells types (Pezzi *et al.* 2004) and GCs (Hinshelwood *et al.* 2003, Liu *et al.* 2003) suggests that LRH-1 may exert an important effect on estrogen production. Our data showed that LRH-1 protein was mainly located in the nucleus of GCs, and that inactivation of p38 kinase with SB203580 significantly repressed both the basal and the FSH-induced LRH-1 expression, which correlated well with the action of SB203580 on FSH-induced P450arom mRNA expression and estrogen synthesis. By transfection analyses, Falender *et al.* (2003) reported that p38 MAP kinase signaling pathways impacted the transactivation of the LRH-1 reporter gene in GCs. Collectively, those observations suggest that FSH-activated p38 kinase may promote P450arom and estrogen production through inducing LRH-1 expression.

The findings by Saxena *et al.* (2004) are very interesting and valuable in elucidating the regulation mechanism of the divergent steroidogenic properties of GCs by FSH. To the best of our knowledge, it is the first paper reporting a direct role for LRH-1 in the induction of progesterone but not estrogen biosynthetic pathway during GC differentiation. At the same time, we also notice that our findings regarding the induction of LRH-1 on estrogen production are contrary to those by Saxena *et al.* We noticed that Saxena *et al.* (2004) had used undifferentiated GCs as experiment models to study the role of LRH-1 on estrogen biosynthesis. The cells themselves can express LRH-1 in the presence of FSH. It is very possible that endogenous LRH-1 expression induced by FSH may be enough to promote estrogen production in the cells. Exogenous LRH-1 by Ad-LRH-1 may have no further effect on P450arom expression and estrogen level. Thus, only if the effect of endogenous LRH-1 is excluded, a correct conclusion can be made on whether LRH-1 can stimulate estrogen synthesis during FSH-induced GC differentiation.

It has been demonstrated that DAX-1 blocks steroidogenesis by inhibiting the transcription of the StAR gene (Ikeda *et al.* 1996, Ito *et al.* 1997, Zazopoulos *et al.* 1997, Lalli *et al.* 1998, Christenson & Strauss 2000). In the present study, we also investigated whether DAX-1 might play a role in mediating the steroidogenic signal elicited by FSH and transduced by p38 MAPK. The immunocytochemistry data showed that the orphan receptor was mainly located in the nucleus although a weak fluorescent signal was also observed in the cytoplasm. Treatment of the cells with FSH for 9 h clearly increased the nuclear staining. The inactivation of p38 MAPK by inhibitor SB203580 reduced such augmentation. These findings were similar to those about the action of ERK1/2 on DAX-1 and progesterone production in which ERK1/2 inactivation down-regulated DAX-1 level and up-regulated progesterone synthesis in GCs in response to FSH/LH (Seger *et al.* 2001, Tajima *et al.* 2003). In addition, a recent study with cycling human ovaries also showed that DAX-1 protein

expression in GCs increased as follicles developed from primordial to dominant stages (Sato *et al.* 2003), suggesting that gonadotropins might play an important role in the induction of *DAX-1* gene expression during follicular development. To the contrary, there have been reports indicating that the expression of the orphan receptor was down-regulated by tropic hormones including FSH (Tamai *et al.* 1996, Yazawa *et al.* 2003) and Angiotensin II (Osman *et al.* 2002). To thoroughly explain the discrepancy, further investigation with the same experimental protocols is needed. Moreover, the precise mechanism underlying the regulation of *DAX-1* gene by tropic hormones still remains an open question.

It has also been reported that LRH-1 might participate in progesterone synthesis as an activator of StAR, P450 scc and 3 β -HSD genes in human tissue (Sirianni *et al.* 2002, Peng *et al.* 2003, Kim *et al.* 2004). Accordingly, down-regulation of LRH-1 should cause a decrease in StAR expression and progesterone production. However, we observed in the present study that inhibition of p38 kinase resulted in an attenuation of FSH-induced LRH-1 expression and an enhancement of StAR and progesterone levels. Such a discrepancy could be explained by the different cell types used and the stimuli employed. In addition, possible crosstalks between other MAPK cascades and transcription factors including *DAX-1* and LRH-1 and their different proportional activities may contribute to the discrepancy. For example, the decrease in the expression of *DAX-1*, a known repressor of StAR gene, in response to MAPK inactivation could be responsible for the elevation of StAR expression.

In summary, we demonstrated for the first time that p38 MAPK played different roles in transducing FSH's progesterone and estradiol steroidogenic signals in GCs. Inhibition of p38 MAPK activity augmented FSH-induced StAR and progesterone production, while significantly reduced FSH-induced P450arom mRNA expression and estradiol production. Inactivation of p38 MAPK decreased the expression of LRH-1 and *DAX-1*, which are known to be the transcription activator and repressor of P450arom and StAR genes respectively. Therefore, it seems that steroidogenic signal initiated by FSH diverges from p38 MAPK to LRH-1 and *DAX-1* regulated expression of P450arom and StAR that are key enzymes in progesterone and estradiol production.

Acknowledgements

We acknowledge Dr Dougals M Stocco for his generous supply of anti-StAR antibody, Dr Luc Belanger for anti-LRH-1 antibody and Dr J P Raynard for NIADDK hormones. This work was supported by funds from NSFC (No: 90208025, 30370200, 30170451, 0170542), CAS 'Chuangxi' (KSCX-2-SW-201) and '973' project (G1999055901). The authors declare that there is no

conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 12 March 2005

Accepted 8 April 2005