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Signal pathway of GnRH-III inhibiting FSH-induced steroidogenesis in granulosa cells

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1. ABSTRACT

Gonadotrophin-releasing hormone type 1 and type 2 have been demonstrated to inhibit follicle-stimulating hormone (FSH)-induced granulosa cell (GC) steroidogenesis. A third type of GnRH (GnRH-III) was also purified from salmon, its action on the FSH-regulated GC function, however is not clear. In the present study we demonstrated that the FSH-induced estrogen and progesterone production in cultured DES-treated GCs was significantly inhibited by GnRH-III. Furthermore, the FSH-stimulated steroidogenic acute regulatory protein and the enzymes for steroidigenesis, such as HSD3B2,aromatase and cytochrome P450 side-chain cleavage were also significantly suppressed by this peptide. The inhibitory action of GnRH-III on the FSH-induced steroidogenenisis was demonstrated via Akt and p38 mitogen-activated protein kinase signaling pathways through suppressing its own receptor expression. Further studies indicated that FSH could stimulate NR5A2 and upstream stimulatory factor (USF) activation, and their induction was significantly suppressed by the GnRH-III. Therefore, it is suggested that GnRH-III inhibiting FSH-induced steroidogenenisis in GCs might be by suppressing FSH-induced its own receptor expression via NR5A2 and USF transcriptional factors.

2. INTRODUCTION

GnRH is a decapeptide secreted by hypothalamus and is a key regulator of reproductive system (1). Primate brain was originally thought to contain only one form of GnRH, known as mammalian GnRH (GnRH-I). Lately, a second form of GnRH (GnRH-II) has been demonstrated with a characteristic of chicken GnRH-II (cGnRH-II), and expressed in human and other primates (2). A third isoform of GnRH, GnRH-III, purified from salmon (sGnRH) was also demonstrated, and expressed in human pituitary stalk (3). Immunohistochemical studies showed that GnRH-III-containing neurons were observed in the hypothalamus and midbrain of human and rat (3), suggesting that GnRH-III might have functions distinct from those of GnRH-I and GnRH-II.

In addition to its well-documented role in gonadotropin biosynthesis and secretion(1), GnRH (GnRH-I and –II) has been implicated as an autocrine/paracrine regulator in several extrapituitary tissues, including gonad (4). There are several reports about multitude effects of the GnRH attributed to its receptor (GnRHR)-mediated signaling in extrapituitary tissues(5) and induced transcription of several genes involved in the follicular

maturational process and ovulation(6). Both GnRH-I and GnRH-II inhibited gonadotropin-induced progesterone production in hGLCs, and GnRH-II appears to be more potent in this regard (7). However, the role of the third form of GnRH in mammalian gonad has not been reported.

FSH is essential to regulate granulosa cell steroidogenesis (8, 9).The first action of FSH activating its signaling pathway is binding to its receptor, and triggers intracellular signaling pathways (10). Evidence has shown that GnRH-I possesses anti-gonadotropic effect in the ovary by downregulating FSHR expression (11) via inhibiting cAMP production (12, 13), leading to suppression of steroidogenic enzymes(14, 15). Similar to the effects of GnRH-I, GnRH-II also exerts its anti-gonadotropic effects by reducing ovarian FSHR level (16).However, whether GnRH-III also exerting an anti-gonadotropic effect in the ovary remains to be elucidated. The purpose of this study is designed to examine if GnRH-III via the same signal pathways could possess an anti-FSH action in the cultured granulosa cells.

3. MATERIALS AND METHODS

3.1. Materials

Ovine FSH (oFSH-20), GnRH-III(salmon GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2), McCoy's 5a medium, diethylstilbestrols (DES) and 4-androstene-3, 17-dione (androstenedione) were obtained from Sigma (St. Louis, MO). Trizol was purchased from Invitrogen Corp. (Carlsbad, CA); Random primer was purchased from TAKARA Biotechnology. Brilliant SYBR Green QPCR Master Mix was purchased from Tiangen Biotech CO., LTD.

3.2. Antibodies

Anti-Phospho-Akt (Ser^{473}) (no. 9271), anti-Akt (no. 9272), anti-Phospho-p38 MAPK (Thr^{180}/Tyr^{182})(no. 9211), anti-p38 MAPK (no. 9212) were purchased from CST (Cell Signaling Technologies, Beverly, MA); The polyclonal antibodies against StAR (ab3343) were from Abcam; The polyclonal antibodies against HSD3B2(sc30821), USF1(sc229) and USF2(sc862) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); The monoclonal antibody against beta-actin was from Sigma (St. Louis, MO).

3.3. Animals

Immature female Sprage–Dawley (SD) rats (23 days old) were obtained from the Experiment Animal Center (Beijing, China) and maintained under 16 hrs light, 8 hrs 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.

3.4. Granulosa cell preparation and culture

Twenty-three-day-old female Sprague-Dowley rats were injected with 1 mg of DES/day (dissolved in oil) for 3 consecutive days to increase follicular granulosa cells numbers, Animals were then slaughtered. Ovaries were removed and granulosa cells were harvested by puncturing the individual ovarian follicles with 25 gauge needles, Granulosa cells were extracted, and cell suspensions were centrifuged at 1000 rpm for 5 min. Pellets were resuspended in culture medium (McCoy's 5a medium; Sigma). The cells were 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^o 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.

3.5. RNA extraction and real-time PCR

The granulosa cells (3×10^6M vial cells) were cultured in 35mm well with different treatment. The culture medium was then removed and total cellular RNA was extracted using TRIzol (Invitrogen Co.) and quantified by measuring absorbance at 260 nm. The first-strand cDNA was synthesized (2μg total RNA) by using random primers and Superscript III reverse transcriptase (Invitrogen, San Diego, CA). Then the cDNA was used as the template, and real-time PCR was carried out using Sybr Green (Sybr Green PCR Master Mix; Tiangen, Co.). Reactions were run for 40 cycles (95 ^{o} for 1 min and 40 cycles at 95 ^{o} for 15 s, 60 ^{o} for 1min). Specific PCR settings were used in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Melting curve analyses were performed after real-time PCR reactions to monitor PCR product purity. The threshold cycle (CT) numbers were determined for the amplified cDNA for each investigated mRNA and for the housekeeping gene, 18S rRNA in each sample during real-time PCR. The relative quantification of gene expression across treatments was evaluated using the ΔΔCT method. Real-time PCR quantification of gene expression level in each sample was the mean of triplicate real-time PCR experiments. Values are presented as the mean±S.E.M of triplicate independent experiments. The primers specific to the candidate genes were designed using primer 3 software. The primers for FSHR(NM_199237) were forward 5'-tggaagaattgcctaatgacg-3' and reverse 5'-gggagctttttcaagcggta-3'; NR5A2 (NM_021742, forward 5'-gtcgcattcaacaacctcct-3' ; reverse 5'-tccaccagctggaagttctc-3'); CYP19 (NM_017085.1, forward 5'-cagagtatccggaggtggaa-3'; reverse 5'-catgaccaagtccacgacag-3'); CYP11A (NM_017286, forward 5'-ctgggacttaaggcagaagc-3'; reverse 5'-tccattgcagagtcatggag-3'); 18S rRNA (forward 5'- cgcggttctattttgttggt-3'; reverse 5'- agtcggcatcgtttatggtc-3')(17)

3.6. Western blotting

The granulosa cells (3×10^6M vial cells) were cultured in 35mm well with different treatment. The culture medium was then removed and ganulosa cells were lysed in cold lysis buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, supplemented with 100μg/ml phenylmethylsulfonyl fluoride and 1μg/ml aprotinin) for 30min. The supernatants after centrifugation (10,000×g, 10 min) were collected, and the total protein concentrations were determined by colorimetry, using BSA as a standard. Twenty five

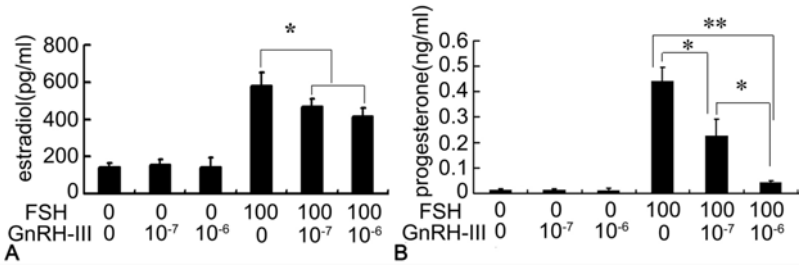

Figure 1. GnRH-III inhibited FSH-induced estrogen and progesterone production in cultured primary granulosa cells. Granulosa cells obtained from DES-primed immature rat ovaries were cultured in the serum-free McCoy's 5a containing androstendione (10^{-7}M) alone (control) or supplemented with 100 ng/ml FSH and/or GnRH-III□$10^{□7}$M and $10^{□6}$M□for 48 hrs. The contents of estradiol (Fig 1A) and progesterone (Fig1B) in the media were measured by standard RIA procedures. Data are presented as mean ± S.E.M. (n =3). Bars with * among the groups indicated significant different. *, significant different at P<0.05; **, P<0.01.

micrograms total protein of each sample per lane were separated by 12% SDS PAGE and transferred to the nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). After blocking in 5% nonfat milk in 0.09% NaCl, 0.05% Tween 20, 100 mm Tris-HCl (pH 7.5) for 1 hr at room temperature, the membranes were incubated with the primary antibodies: p38 MAPK(1:1000), p-P38 MAPK(1:1000), Akt(1:1000), p-Akt(1:1000), STAR (1:1000) , HSD3B2 (1:200), USF1(1:200), USF2(1:200) and beta-actin (1:2000) respectively, in the blocking solution overnight at 4°. The membranes were washed three times and then incubated with the corresponding peroxidase-conjugated secondary antibodies (1:2000) for 1 hr at room temperature. Reactive bands were visualized by Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL). Band intensities were determined by Quantity One software (Bio-Rad).

3.7. Estradiol and progesterone RIA

Granulosa cells (1×10^{6}M vial cells) were cultured overnight in a twenty four-well plate with 0.5ml medium. After 16hrs incubation, the cells were cultured in the presence or absence of FSH with or without GnRH-III in serum-free medium contained androstendione (10^{-6}M) for 48 hrs. The media were then collected for analysis of progesterone (P4) and estradiol (E2) concentrations, which were measured by the standard RIA procedures.

3.8. Data analysis and statistics

All the experiments were repeated at least three times with granulosa cells preparations obtained from separate groups. The values were presented as the mean ± S.E.M. Statistical significance was determined using SPSS 14.0 software for multiple group comparisons. Significance was accepted at $p < 0.05$ or $p < 0.01$.

4. RESULTS

4.1. GnRH-III inhibits FSH-induced estrogen and progesterone production

To investigate the action of GnRH-III on FSH-induced steroidogenesis, granulosa cells prepared from the ovaries of DES-treated immature rat were cultured in a serum-free McCoy'5a medium for 16 hrs, the cells were further incubated for 48 hrs in the serum-free medium containing androstendione (10^{-7}M) in the presence or absence of FSH(100ng/ml) and GnRH-III(10^{-6}M or 10^{-7}M). As shown in Figure 1, the basal level of estrogen and progesterone in the untreated control group was low, treatment of the cells with GnRH-III alone for 48 hrs showed that both hormone levels were no obvious changes as compared with that of the control group. The levels of estrogen and progesterone were dramatically increased in the FSH treated group. Addition of GnRH-III ($10^{□7}$M or $10^{□6}$M) significantly reduced the FSH-induced progesterone (50% and 90%) and estradiol (20% and 30%) production respectively. Please added the figures

4.2. GnRH-III inhibits FSH-induced key enzymes of steroidogenesis

In order to determine the inhibitory site of GnRH-III suppressing FSH action, we examined its possible effect on the four key enzymes StAR, HSD3B2, CYP11A and CYP19 because their important role in steroidogenesis (18, 19). As shown in Figure 2A and 2B, FSH induced a marked increase in StAR, HSD3B2 expression, while GnRH-III alone did not significantly affect HSD3B2 production, but slightly stimulated StAR expression. However, addition of GnRH-III to the cell culture significantly decreased the FSH-induced StAR and HSD3B2 expression. As shown in Figure 2C and 2D, FSH induced a dramatically increase in *CYP11*A and *CYP19* expression. GnRH-III alone did not obviously affect the *CYP19* mRNA level, but slightly stimulated *CYP11A* mRNA expression. FSH concomitant treatment of the cells with GnRH-III, a significant decrease in FSH-induced *CYP11A* and *CYP19* mRNA expression was observed.

4.3. GnRH-III action on FSH-induced phosphorylation of Akt and p38 MAPK

We examined possible signaling pathways of GnRH-III inhibiting FSH-induced estrogen and progesterone production. As shown in Figure 3A, GnRH-III alone had no obvious effect on Akt phosphorylation in the granulosa cells, while FSH significantly stimulated Akt activation. Concomitant treatment of FSH with GnRH-III significantly decreased the FSH-induced Akt phosphorylation. We also examined the possible effect of

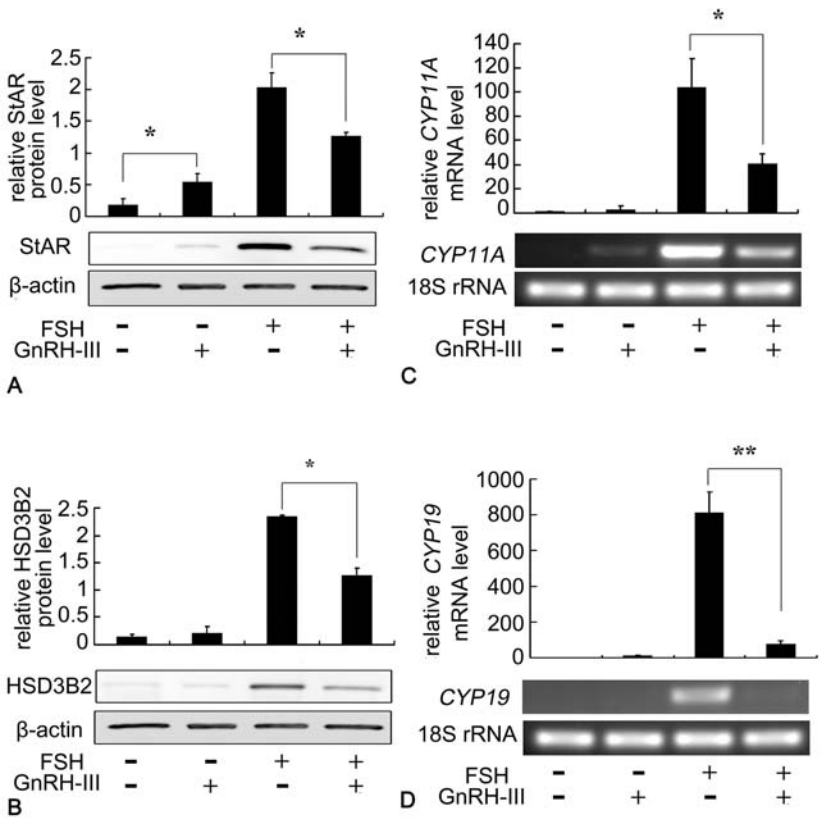

Figure 2. GnRH-III inhibited expression of FSH-induced StAR, HSD3B2, CYP11A and CYP19. Granulosa cells were cultured in the serum-free McCoy's 5a medium containing androstendione (10-7M) alone (control) or supplemented with 100 ng/ml FSH and/or GnRH-III(10^{-6}M) for 48 hrs. The cell lysates were prepared and subjected to immunoblotting analysis using StAR (1:1000) and HSD3B2 (1:200) antibodies, immunoreactive bands were visualized (Fig 2A and 2B); Total RNA in the cell lysate was extracted by Trizol and reversely transcribed, the relative mRNA levels of CYP11A and CYP19 were measured by real-time qPCR and expressed as a fold change in expression relative to control values (no treatment) and normalized to 18S rRNA, and then agarose gel electrophoresis (Fig 2C and 2D). Bars with * among the groups indicate significantly different. Data are presented as mean± SEM (n=3). *, significant different at $P<0.05$; **, $P<0.01$.

the peptide on FSH-induced p38 MAPK activation, figure 3B showed that both FSH and GnRH-III were capable of increasing p38 MAPK phosphorylation. It is interesting to note that concomitant treatment of FSH with GnRH-III significantly decreased the FSH induced p38 MAPK activation.

4.4. GnRH-III inhibits FSH-induced FSH receptor expression

As shown in Figure 4, FSH receptor expression increased when the granulosa cells were treated with FSH (100ng/ml), while GnRH-III did not stimulate FSHR expression. Concomitant treatment of the cells with GnRH-III, the FSH induced FSHR expression was significantly inhibited.

4.5. GnRH-III inhibits FSH-induced NR5A2 expression

Reports have shown that NR5A2 is a transcription factor binding to the promoter of CYP11A (21) and CYP19 (22) and FSHR (23)**.** Evidence indicated NR5A2 enhanced the reporter activity driven by 5'-flanking DNA from StAR, HSD3B2 (20), and was stimulated by FSH (23). We further examined whether GnRH-III inhibited FSH-induced steroidogensis via inhibiting the hormone-induced NR5A2 expression. As shown in Figure 5, FSH greatly induced NR5A2 expression in the cultured granulosa cells, concomitant treatment with GnRH-III significantly decreased the FSH-induced NR5A2 production.

4.6. GnRH-III inhibits FSH-induced expression of upstream stimulatory factor1 and 2

E-box is the most important element of FSHR. USF1 and USF2 format either heterodimers or homodimers can bind to the E-box regulating FSHR transcription. We examined whether GnRH-III exerts a possible effect on expression of USF1 and USF2. As shown in Figure 6A and B, FSH stimulated USF1 and USF2 expression in granulosa cells. When the cells were treated with GnRH-III in combination with FSH, the FSH-induced USF1 and USF2 expression was markedly decreased, suggesting that GnRH-III is capable of inhibiting the FSH-induced FSHR expression via suppressing USF1 and USF2 production.

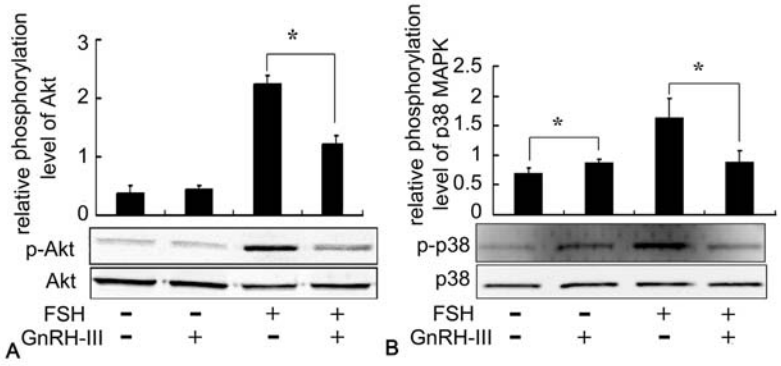

Figure 3. GnRH-III inhibited FSH-stimulated phosphorylation of Akt and p38 MAPK. After the granulosa cells were cultured in McCoy's 5a medium alone(control) or supplemented with FSH(100 ng/ml) and/or GnRH-III 10^{-6}M for 30 min, the cell lysates were prepared and subjected to immunoblotting analysis using antibodies recognizing either phosphorylation of Akt or total Akt (Figure 3A), and phosphorylation of p38 MAPK or total p38 MAPK (Fig 3 B); Each bar represents means ± S.E.M. (n = 3). * Significant different at P<0.05.

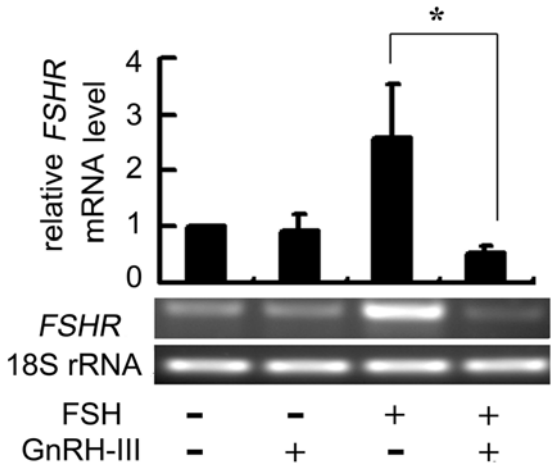

Figure 4. GnRH-III inhibited FSH-induced FSHR expression. Granulosa cells were cultured with FSH (100ng/ml) or GnRH-III(1μM) alone or in combination. After 24hr culture, total RNA in the cell lysates were extracted by Trizol and reversely transcribed; relative levels of FSHR mRNA were measured by real-time PCR and expressed as a fold change relative to the control values (no treatment) and normalized to 18S rRNA, and then agarose gel electrophoresis (Fig4). Each bar represents means ± S.E.M. (n = 3). *significant different at P<0.05; **P<0.01.

5. DISCUSSION

Vertebrate GnRHs were clustered into four major clades: GnRH-I (mGnRH, hypothalamic/preoptic forms), GnRH-II (cGnRH, the midbrain form), GnRH-III (sGnRH, a fish-specific terminal nerve form), and GnRH-IV (the lamprey forms)(24). It was generally believed that only GnRH-I and GnRH-II exist in mammals. However, recently GnRH-III was also observed in the brain of human, bovine and rat (3, 25). Both GnRH-I and GnRH-II inhibit gonadotropin-induced steroid production in rat granulosa cells (11, 26-30), but whether GnRH-III having the same effect as GnRH-I and GnRH-II in mammal granulosa cells is uncertain. In order to investigate the mechanism of GnRH-III inhibiting FSH action, we systematically examined signaling pathways involved in GnRH-III suppressing action on FSH-induced steroidogenesis in cultured rat granulosa cells. We proposed that GnRH-III might also interact with the same receptor of GnRH-I and GnRH-II, a transmembrane G-protein coupled receptor, to activate the complex intracellular signaling pathway. We observed GnRH-III also significantly inhibited FSH-induced estrogen and progesterone production in less extent in rat granulosa cells. Chen *et al* reported that Akt signaling played an important role in steroidogenesis (31), and FSH and some other factors could stimulate Akt phosphorylation (32). We demonstrated that GnRH-III alone is not capable of stimulating Akt phosphorylation in the cells, but concomitant treatment with FSH could inhibit the hormone-induced phosphorylation. It is therefore suggested that GnRH-III inhibiting FSH–stimulated estrogen and progesterone production may be via Akt signaling.

Increasing evidence has demonstrated that p38 MAPK participates in a signaling cascade controlling

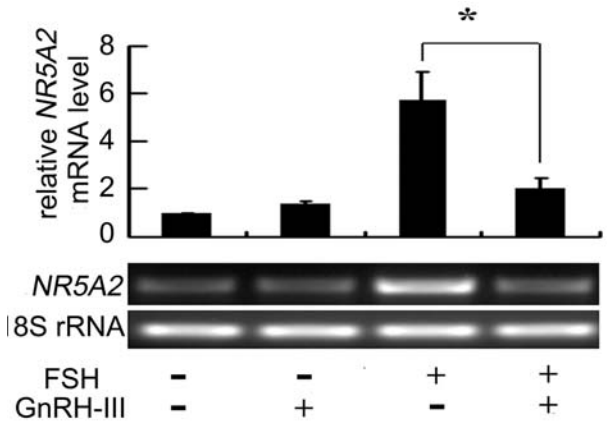

Figure 5. GnRH-III inhibited expression of FSH-induced NR5A2. Granulosa cells were cultured in the serum-free McCoy's 5a medium containing androstendione (10^{-7}M) alone (control) or supplemented with FSH(100 ng/ml) and/or GnRH-III□10^{-6}M□ for 48 hrs, the total RNA in the cell lysate was extracted by Trizol and reversely transcribed, the relative mRNA levels of NR5A2 were analyzed by real-time qPCR and expressed as a fold change in expression relative to the control values (no treatment) and normalized to 18S rRNA. Bars with * among the groups indicate significant different. Data are presented as mean± SEM (n=3). * Significant different at P<0.05.

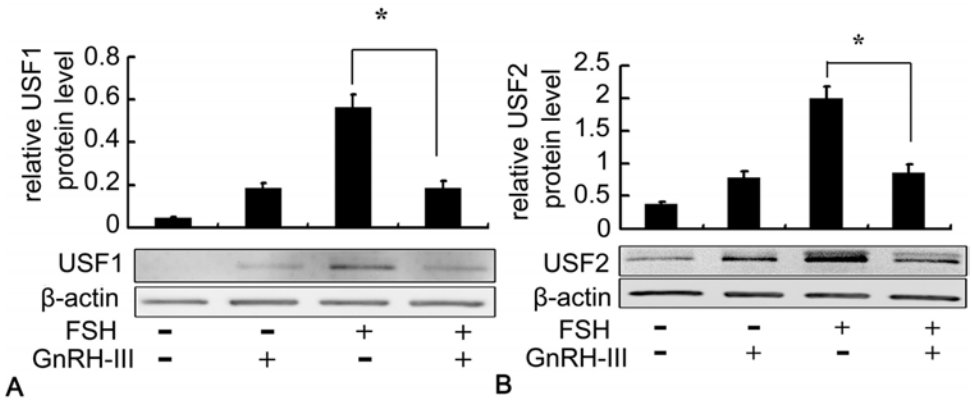

Figure 6. GnRH-III suppressed expression of upstream stimulatory factor 1(USF1) and 2 (USF2). Granulosa cells were cultured in McCoy's 5a medium alone (control) or supplemented with 100 ng/ml FSH and/or GnRH-III□10^{-6}M□for 48 hrs. The cell lysates were prepared and subjected to immunoblotting analysis using USF1 and USF2 antibodies. Each bar represents means ± S.E.M. (n = 3). *significant different at P<0.05.

cellular response to cytokines and stress (33). Phosphorylation of p38 MAPK is important in steroidogenesis (34). We observed that FSH or GnRH-III alone was capable of stimulating p38 MAPK phosphorylation, however, the extent of GnRH-III-induced phosphorylation was less than that of FSH. However, GnRH-III concomitant treatment of the cells with FSH could inhibit the FSH-stimulated p38 MAPK phosphorylation. How GnRH-III via p38 MAPK signaling inhibiting FSH–induced steroidogensis is not clear.

GnRH-I and GnRH-II possess anti-gonadotropic effect in rat ovary by suppressing FSHR expression via inhibiting gonadotropin-stimulated cAMP production (16). It is well known that FSH stimulates its own receptor expression (11, 35). In order to investigate whether GnRH-III exerting the anti-gonadotropic effect is also through this mechanism, we designed experiments to detect the FSHR content in the presence of FSH. Our data showed that addition of GnRH-III to the granulosa cell culture significantly inhibited the FSH-induced FSHR production. Therefore we concluded that GnRH-III, a fish special GnRH, is also capable of inhibiting FSH-induced steroidogensis by suppressing FSHR expression in rat granulosa cells.

Transcriptional studies of FSHR have identified several DNA elements and proteins important for its promoter function. There are several nuclear transcriptional factors, such as USF1 and USF2 involved in regulation of FSHR (36). Evidence showed that ovarian FSHR mRNA levels in Usf1-/-and Usf2-/- mice were reduced to 60% and 40% of wild type, respectively (37). USF1 and USF2 form both homodimers and heterodimers between themselves (38, 39) and USF dimers regulate FSHR transcription by binding the E-box of its promoter (40). Hermann *et al* reported that USF heterodimers were more prevalent and active to direct FSHR transcription in the ovary than

homodimers (37). Report also indicated that NR5A2 is a transcription factor binding to the promoter FSHR. In the present studies, we observed that FSH alone could remarkably induce NR5A2, USF1 and USF2 expression; However, concomitant treatment of the cells with FSH and GnRH-III greatly decreased the FSH-induced NR5A2, USF1 and USF2 production. It is the first time we demonstrated that GnRH-III could suppress FSH-induced NR5A2, USF1 and USF2 expression leading to decrease in FSHR mRNA level.

In conclusion, GnRH-III, similar to GnRH-I and GnRH-II, is also capable of antagonizing FSH action on steroidogensis in rat granulosa cells. GnRH-III inhibiting FSH-induced steroidogenenisis in granulosa cells might be by suppressing FSH-induced FSHR expression via NR5A2 and USF transcriptional factors.

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