Modulatory Action of Nitric Oxide on the Expression of Transcription Factor Genes, c-fos and c-jun, in Developing Porcine Granulosa Cells *In Vitro*

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Abstract. The present study was performed to clarify the synthesis of nitric oxide (NO) and its effect on the expression of transcription factor genes (c-fos, c-jun and ATF-4) during the differentiation of granulosa cells. Granulosa cells prepared from porcine ovarian follicles (1–4 mm diameter) were matured with FSH for 48 h. From 40 h to 48 h of ovine FSH stimulation, nitrite and nitrate increased by 2 folds, and this was accompanied by an increase of cyclic GMP. The cells were exposed to either NO scavenger (carboxy-PTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide) or NO donor (NOC18: 2,2'-(hydroxynitrosohydrazino)bis-ethanamine) before or after NO generation, and further stimulated with ovine LH until 48 h. Removal of endogenous NO (NO scavenger) induced a serious impairment in the LH-induced synthesis of progesterone, whereas NO donor had no significant effect on the synthesis. In the semi-quantitative reverse transcriptase-PCR of the transcription factor mRNAs, removal of endogenous NO resulted in a reduction of c-fos expression, an increase of c-jun and no changes in ATF-4 expression. In contrast, NO donor had no significant effects on the expression of these transcription factor genes. Consequently, the transient generation of NO may have critical roles in the LH-induced expression of transcription factor genes and thereafter transformation of the granulosa cell to the luteal cell.

Key words: Nitric oxide, Transcription factor genes, Granulosa cell differentiation.

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Nitric oxide (NO) is a short-lived messenger molecule that mediates a variety of cellular functions. NO is generated from molecular O₂ and L-arginine by nitric oxide synthase (NOS) [1–4]. Constitutive isoforms such as endothelial (eNOS) and neural NO (nNOS) synthases [5, 6], and an inducible isoform (iNOS) [7] have been identified.

The expression of mRNAs and proteins for eNOS and iNOS in the rat ovary has been shown during follicular development, ovulation and pseudo-pregnancy [8, 9]. In rat and human ovarian cells, NO has been shown to be involved in folliculogenesis, ovulation and steroidogenesis [10–15]. In our previous study using rat granulosa cells [16], NO was proposed as functioning as a modulator in cell differentiation, because removal of NO during cell differentiation suppressed

expression of the EGF receptor. In contrast, NO has been reported to inhibit steroidogenesis in rat, human and porcine ovarian cells [17–20].

FSH induces the transformation from immature to mature granulosa cells, then the mature cells are further transformed into luteinized cells by LH. The process of cell differentiation is generally accepted to be mediated by the cyclic AMP signaling pathway [21]. Protein kinase C is also an essential element in the expression of genes during the differentiation of granulosa cells [22]. The differentiation process is also accompanied by the expression of several proto-oncogenes in many cell types. The transient or constitutive expression of the transcription factors such as proto-oncogenes (c-fos, c-jun, c-myc), and the cyclic AMP response element binding protein (CREB or ATF-4) family is promoted by protein kinase C and/or protein kinase A [23, 24]. The major actions of such transcription factors are related to the transcription of several late gene responses that are regulators of cell differentiation. Consequently, we raised the interesting possibility of NO participation in the expression of the transcription factors. In this study using porcine granulosa cells, we investigated NO modulation in the expression of c-fos, c-jun and ATF-4 by a semi-quantitative reverse transcriptase (RT)-PCR.

Materials and Methods

Materials

Ovine FSH (NIDDK-oFSH-20) and LH (NIDDK-oLH-26) were supplied by Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA, USA). Ham's F-10 and DMEM were obtained from GIBCO Laboratories (Grand Island, NY, USA). 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide (carboxy-PTIO) 2,2'-(hydroxynitrosohydrazino)bis-ethanamine (NOC18) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) were obtained from Dojindo (Kumamoto, Japan). Sodium pentacyanonitrosylferrate-(III)dihydrate (SNP) was obtained from Nacalai Tesque (Kyoto, Japan); ISOGEN from Wako (Osaka, Japan), T-primed first-strand kit from Amersham Pharmacia Biotech (Tokyo, Japan), and AmpliTaq Gold polymerase from Perkin-Elmer (Norwalk, CT, USA). All the other chemicals used were of reagent grade and obtained from commercial sources.

Cell culture

The porcine ovaries were obtained at a local slaughterhouse. Granulosa cells were prepared from small-sized (1–4 mm diameter) follicles by aspirating with a 20-gauge needle and then filtering through a fine stainless mesh. They were treated with 50 μ g/ml DNase I at 37 C for 5 min, and washed with Ham's F10 and DMEM (1:1) supplemented with 10 mM HEPES, 50 μ g/ml gentamycin, 20 U/ml nystatin [25]. Cell viability was determined to be >95% by trypan blue exclusion. The cells were seeded at 2×10^5 /well in 48-well plates or at 10^6 /dish in 35 mm dishes coated with fibronectin (Becton-Dickinson, Oxnard, CA, USA) and cultured for 24 h with 110 nM hydrocortisone, 1 μ g/ml insulin, 5 μ g/ml transferrin and 0.1% (w/v) bovine serum albumin at 39 C in humidified 95% air 5% CO₂. FSH was then added to the cultures (culture 0 day), and the cells were matured by culture for an additional 48 h before washing and exposure to LH. Carboxy-PTIO, SNAP, and SNP were dissolved in culture medium and added to cultures at 30 h and 46 h after the start of culture. NOC18 was dissolved in 0.1 M NaOH which was adjusted to pH 7.2–7.5 with 50 mM Tris-HCl (pH 7.0), and added to cultures at the same times.

Measurements of nitrite and nitrate

Medium samples were collected at indicated times. After the removal of proteins from the cultured media with 50% methanol, soluble fractions were analyzed with an automated NO detector HPLC system (ENO-20, EICOM, Kyoto, Japan) [26]. Nitrite and nitrate were separated by a reverse phase separation column packed with polystyrene polymer, and nitrate was reduced to nitrite with copper-plated cadmium filings. Nitrite was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The mobile phase was 10% methanol containing 0.15 M NaCl/NH₄Cl and 0.5 g/l EDTA-4Na, and the flow rate was 0.33 ml/min. The absorbance of the product dye was measured at 540 nm by a flow-through spectrophotometer. Medium cultured without cells was used as a blank for measurements of nitrite and nitrate.

RNA isolation and cDNA preparation

After removing the media, ISOGEN was added

to each well to dissolve the cells (10^6). Each resulting solution was extracted with chloroform and centrifuged at $12000 \times g$ for 15 min. The aqueous phase was collected and precipitated with isopropylalcohol at room temperature, and the precipitate was washed with 75% (v/v) ethanol. After drying, the recovered RNA was dissolved in 20 μ l diethylpyrocarbamate-treated water. The reverse transcription was performed using a T-primed first-strand kit as follows: 10 μ l RNA samples mixed with the reagent were incubated at 37 C for 1 h, after which diethylpyrocarbamate-treated water was added to each tube (final volume, 50 μ l), and the mixture heated at 70 C for 5 min.

Analysis of granulosa cells for mRNA of c-fos, c-jun and ATF-4 using reverse transcriptase-PCR (RT-PCR)

RT-PCR was performed on total RNA using a Program Temp Control system PC-800 (ASTEC, Fukuoka, Japan). The oligonucleotides specific for c-fos [27], c-jun [27], ATF-4 [28] and β -actin [29] were synthesized by Hokkaido System Science (Sapporo, Japan). The sequences of these oligonucleotides are described in Table 1. A mouse β -actin cDNA fragment was selected as a control. Amplification of each cDNA (1 μ l of RT template) was performed in 10 μ l of $1 \times$ PCR buffer, 0.2 mM dNTPs, 0.25 U AmpliTaq Gold polymerase, and 0.2 μ M each of the synthetic primers. After an initial denaturation step (95 C for 9 min), the amplification step for porcine c-fos, c-jun and ATF-4 consisted of 35 cycles under a thermal profile of 95 C for 30 sec (denaturation), 54 C for 30 sec (annealing), and 72 C for 2 min (synthesis). The products were resolved on a 2% (w/v) agarose/

TBE gel containing ethidium bromide, and quantified as a reference gene of β -actin using a densitometry program (NIH image Ver. 1.58).

Assays of cyclic nucleotides and progesterone

Medium samples were collected at indicated time, heated and succinylated. Cyclic nucleotides were determined by specific radioimmunoassays [30] and calculated with the use of a competitive assay computer program. There was 0.07% cyclic GMP cross-reactivity with the cyclic AMP antiserum and 0.15% cyclic AMP cross-reactivity with the cyclic GMP antiserum. The lower limits of detection for both assays were 0.09 pmol/ml, and the intra-assay and inter-assay variabilities were <10%. In some experiments, medium samples collected were diluted with assay buffer and determined for progesterone using an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA). The detection limit was 10 pg/ml, and the intra-assay and inter-assay variabilities were <10%.

Statistical analysis

The data are expressed as means $\pm$ SE; differences between them were analyzed using Student's t-test following analysis of variance.

Results

Cyclic GMP formation and NO synthesis during development

LH receptor mRNA was expressed 24 h after FSH stimulation of porcine granulosa cells, as revealed by RT-PCR (data not shown). Thereafter,

Table 1. Oligonucleotide primers used for PCR analysis

mRNA	Sequences	Nucleotide number	Expected PCR fragment (bp)
c-fos	Sense: 5'-CCGAAGGGAAAGGAATAAGATGG-3' Antisense: 5'-TCGGTGTCTGTAGAGAAGACC-3'	551-573 782-762	232
c-jun	Sense: 5'-CGGAAAAGGAAGCTGGAGAGGATC-3' Antisense: 5'-ACGTTGACGACGCAATCGTAC-3'	998-1021 1176-1156	179
ATF-4	Sense: 5'-GCAGAGCAGGAGGCCCTCACTG-3' Antisense: 5'-CATTTGTGTGTCGATTTCGGGATCGG-3'	1126-1147 1341-1316	216
β -actin	Sense: 5'-TT(CT)TACAA(TC)GAGCTGCGTGTGG-3' Antisense: 5'-AA(CG)GTCTC(GA)ACATGATCTGGGTC-3'	348-369 460-437	113

an increase of cyclic GMP level was observed 48 h after FSH stimulation (Fig. 1). Nitrite and nitrate were measured using an automated NO detector-HPLC system. The concentrations of nitrite and nitrate were relatively constant until 42 h, after which they significantly increased, by 2 folds at 48 h ($P < 0.05$). These results indicate that NO synthesis is transiently promoted 42–48 h after FSH stimulation and NO in turn stimulates guanylate cyclase.

Effect of NO scavenger or NO donor on the cellular functions

In order to assess the role of endogenous NO in the functions of granulosa cells, the cells were treated with either carboxy-PTIO, a potent antagonist of NO action [31], or three types of NO donors (NOC18, SNAP and SNP). NOC18 (50 μ M) caused a marked increase in cyclic GMP synthesis during 5 h incubation with the cells in the presence of 0.1 mM 3-isobutyl-1-methylxanthine, as compared to other NO donors (Fig. 2). NOC18 automatically produced NO in a short time and the cells were permeable to NO in this condition.

The effects of carboxy-PTIO or NOC18 were investigated on cyclic AMP formation and progesterone synthesis induced by LH. Carboxy-PTIO (200 μ M) was added to cultures before NO generation (30 h), and the cells were further cultured until 48 h. Then the cells were washed twice with culture medium, and stimulated with LH for 60 min. As shown in Table 2, cyclic AMP formation decreased by 50% compared with the control. When carboxy-PTIO was added after NO generation (46 h), however, it had little effect on LH-induced cyclic AMP formation. In contrast to carboxy-PTIO, NOC18 (50 μ M) caused no significant effect on LH-induced cyclic AMP formation. In addition, progesterone was measured after 48 h stimulation with LH. When carboxy-PTIO was added before NO generation, progesterone was decreased by 60% (Table 1). However, progesterone synthesis was not significantly influenced in the other groups. These data suggest that the removal of endogenous NO results in serious impairment to the functions of developing granulosa cells.

Effect of NO scavenger on the levels of c-fos, c-jun and ATF-4 mRNAs

After maturation of the granulosa cells induced

Fig. 1. Changes of cyclic GMP and NO metabolites (nitrite and nitrate) levels during differentiation of porcine granulosa cells. Granulosa cells were cultured for the indicated times with FSH (0.3 ng/ml), and then media were removed for cyclic GMP determination by a specific radioimmunoassay (column). For measurement of NO metabolites (circle), cells and media were collected together. After removal of proteins with 50% methanol, soluble fractions were analyzed with an automated NO detector HPLC system as described in Materials and Methods. Data are the means $\pm$ S.E. of three independent experiments.

Fig. 2. Effect of NO donors on cyclic GMP accumulation by porcine granulosa cells. Granulosa cells were cultured for the indicated times with 50 μ M NO donors (NOC18, SNAP, SNP) in the presence of FSH (0.3 ng/ml) and 0.1 mM 3-isobutyl-1-methylxanthine, and then media were removed for cyclic GMP determination by a specific radioimmunoassay. Data are the means from closely agreeing duplicate experiments.

Table 2. Cyclic AMP formation and progesterone synthesis in porcine granulosa cells stimulated with LH

Addition	Time of addition	cAMP pmol/ml	Progesterone ng/ml
Non	—	17.5 ± 2.1 (100)	40.5 ± 1.2 (100)
carboxy-PTIO	30 h	8.4 ± 1.4 (48)*	24.9 ± 0.8 (61)**
carboxy-PTIO	46 h	12.7 ± 0.8 (73)	43.3 ± 1.1 (107)
NOC18	30 h	23.4 ± 0.8 (134)	40.2 ± 0.8 (99)
NOC18	46 h	12.4 ± 0.2 (71)	42.7 ± 0.5 (105)

* $P < 0.05$ vs control (non treatment), ** $P < 0.01$ vs control.

The porcine granulosa cells (2×10^5 /well) were cultured with FSH (0.3 ng/ml); 200 μ M 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide (carboxy-PTIO) or 50 μ M 2,2'-(hydroxynitrosohydrazino)bis-ethanamine (NOC18) was then added to cultures at 30 h or 46 h of culture, and cells were cultured until 48 h. Thereafter, the cells were washed and challenged for 60 min with LH (100 ng/ml) in the presence of 0.1 mM 3-isobutyl-1-methylxanthin (IBMX), then the media were removed for cAMP assay. In the progesterone assay, the cells were challenged for 48 h with LH (100 ng/ml) without IBMX, after which the media were removed for progesterone assay. The data are means $\pm$ S.E. of four experiments. Values in parentheses represent percentages of the respective control value (non treatment).

Fig. 3. Time dependent changes in the levels of transcription factors c-fos, c-jun and ATF-4 mRNAs in mature granulosa cells in response to LH. The porcine granulosa cells (10^6 /dish) were cultured with FSH (0.3 ng/ml) for 48 h, then the cells were stimulated with LH (100 ng/ml) for 30 or 60 min. Total RNA samples were prepared and analyzed by RT-PCR, as described in Materials and Methods.

by FSH, LH-induced expressions of c-fos, c-jun and ATF-4 mRNAs were tested. A maximal expression of c-fos was observed 30 min after exposure to LH, whereas c-jun and ATF-4 were consistently expressed (Fig. 3). Fig. 4 shows the effect of carboxy-PTIO pretreatment on the levels of c-fos, c-jun and ATF-4 mRNAs. When carboxy-PTIO was added before NO generation, LH-induced expression of c-fos mRNA was decreased to about 50% of the control. In contrast, the c-jun mRNA expression was increased by about 250%, and no significant changes were observed in ATF-4 mRNA expression. When carboxy-PTIO was added after NO generation, however, no significant changes were observed in LH-induced expression of c-fos, c-jun and ATF-4 mRNAs.

Effect of NO donor on the levels of c-fos, c-jun and ATF-4 mRNAs

In order to compare with the effect of endogenous NO, we next tested the effect of NOC18 pretreatment on LH-induced expression of c-fos, c-jun and ATF-4 mRNAs. NOC18 was also added to cultures before or after NO generation, which were stimulated with LH. In contrast to carboxy-PTIO, no changes were observed in the expression of these transcription factors' mRNAs (Fig. 5).

Discussion

In this study, we investigated NO synthesis and

Fig. 4. LH-induced expression of transcription factor genes *c-fos*, *c-jun* and *ATF-4* in porcine granulosa cells pretreated with NO scavenger. The porcine granulosa cells (10^6 /dish) were cultured with FSH (0.3 ng/ml); 200 μ M 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxy-3-oxide (carboxy-PTIO) was then added to cultures at 30 h or 46 h of culture, and cells were cultured until 48 h. The cells were washed and stimulated by LH (100 ng/ml) for 30 min, then total RNA samples were prepared and analyzed by RT-PCR, as described in Materials and Methods. The results presented are obtained from two independent RT-PCR experiments performed for two different mRNA preparations.

Fig. 5. LH-induced expression of transcription factor genes *c-fos*, *c-jun* and *ATF-4* in porcine granulosa cells pretreated with NO donor. The porcine granulosa cells (10^6 /dish) were cultured with FSH (0.3 ng/ml); 50 μ M 2,2'-(hydroxynitrosohydrazino)bis-ethanamine (NOC18) was then added to cultures at 30 h or 46 h of culture, and cells were cultured until 48 h. The cells were washed and stimulated by LH (100 ng/ml) for 30 min, then total RNA samples were prepared and analyzed by RT-PCR, as described in Materials and Methods. The results presented are obtained from two independent RT-PCR experiments performed for two different mRNA preparations.

its possible role during maturation of granulosa cells prepared from small follicles of porcine ovaries, and found that NO was synthesized at the critical period of cell maturation after FSH stimulation. NO synthesis may have an important role in the differentiation program of granulosa cells, i.e., transformation of the granulosa cell into the luteal cell. eNOS protein is localized in the theca and granulosa cells of gonadotropin-treated immature rats and the developing corpus luteum [9]. Cyclic GMP increase is seen in the *in vitro* differentiation of rat granulosa cells [16]. In the present study, significant increases of cyclic GMP and NO metabolites (nitrite + nitrate) were also observed near the end of the maturation, suggesting activation of cytosolic guanylate cyclase by the transient synthesis of NO.

We think that the transient generation of NO has an important role in the function of developing

granulosa cells. We previously reported that NO might take part in the regulation of EGF receptor expression in developing rat granulosa cells stimulated by FSH; addition of an NO donor, SNP, stimulated expression of the EGF receptor, whereas carboxy-PTIO (blocking NO action) had an inhibitory effect on the expression [16]. In the present study, NOC18 was used as an NO donor, because it produced NO in a short time, resulting in a marked increase in cyclic GMP synthesis. We theorized that 50 μ M NOC18 would produce 100 μ M NO, 20 times the NO produced by the cultured cells. When granulosa cells were exposed to 50 μ M NOC18 during the critical period of NO release (40–48 h), the progesterone secretion did not decrease after LH stimulation of the cells. In contrast, there are many reports that a high concentration (more than 1 mM) of NO donors negatively regulates steroidogenesis after exposure

in the rodent testes [32], human granulosa-luteal cells [17], cultured rat Leydig cells [33], rat luteinized ovarian cells [18], and porcine granulosa cells [19, 20]. Estradiol secretion from FSH-stimulated porcine granulosa cells is suppressed during 2 h-incubation with NOC18 [19]. Another NO donor, S-nitroso-N-acetylpenicillamine, was reported as having inhibited progesterone secretion from porcine granulosa cells [20]. In these reports, NO donors and gonadotropin were added simultaneously to cell cultures, whereas in our system LH was added after removal of NO donor and the cells were stimulated for 24 h by LH. NO inhibits the functions of cytochrome P450 enzymes, including the P450 aromatase, by directly binding to the enzymes [17, 18, 34]. Since NO is a short-lived molecule, the progesterone synthesis might not have been affected by NO remaining in the medium after removal of NO donor.

However, in porcine granulosa cells exposed to carboxy-PTIO at the critical period of NO release, the progesterone secretion from LH-stimulated granulosa cells decreased to 61% of control, and cyclic AMP production decreased to about 50% of control. We paid attention to the expression of immediate early genes such as c-fos, c-jun, and ATF-4 after LH stimulation. After granulosa cells were exposed to carboxy-PTIO or NOC18 during the critical period of NO release, the cells were stimulated by LH for 30 min, and then the expression of three transcription factor genes was estimated by a semi-quantitative RT-PCR. A significant decrease of c-fos was observed after treatment with carboxy-PTIO at the critical period. In contrast, c-jun expression significantly increased, and ATF-4 expression was not influenced. When carboxy-PTIO or NOC18 was added after the critical period, however, expression of these transcription factor genes was not significantly altered. Expression of c-fos and junB was reported in PC12 cells 30 min after activation of cytosolic

guanylate cyclase by SNP [35]. In porcine granulosa cells, however, LH-stimulated expression of c-fos was not enhanced after a 2 h incubation with NOC18. Enhancement of c-jun expression was observed after an 18 h culture with carboxy-PTIO. This contradiction is not clear, but is probably due to partially different types of cells.

At present, it is not clear why the expression of c-fos and c-jun was altered after exposure to NO antagonist. Expression of c-fos is regulated through a well-characterized mechanism of phosphorylation of the dimeric transcription factor, CREB or ATF-4 [36], nuclear actions of protein kinase C [23, 24] and Ca^{2+} /calmodulin-dependent protein kinase [37]. Protein kinase A and protein kinase C are essential elements in expression of the genes which are induced by LH in cultured granulosa cells [22]. Changes of c-fos and c-jun expression produced by blocking NO action might result from alterations in signaling pathways of these protein kinases, and analyses of signaling pathways remain to be investigated. However, the change of the levels of AP-1 factors probably affects expression of several genes related to cell functions.

In conclusion, NO was released from cultured porcine granulosa cells during the critical period of maturation. NO might have an important role in the progress of granulosa cell differentiation through modulation of LH-induced expression of c-fos and c-jun.

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