Expression of endothelial nitric oxide synthase gene in cultured porcine granulosa cells after FSH stimulation

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ABSTRACT
The present study was designed to investigate nitric oxide (NO) synthesis and the expression of endothelial NO synthase (eNOS) gene in cultured porcine granulosa cells. Granulosa cells prepared from small follicles (1–4 mm diameter) were cultured in plastic dishes coated with fibronectin in chemically defined medium, and matured after 48 h of stimulation with FSH. The concentrations of nitrite and nitrate remained relatively constant until 42 h of stimulation, after which they increased significantly up to twofold at 48 h. NO synthesis was accompanied by an increase in cGMP. Gene expression for eNOS was studied by RT-PCR, and a PCR product of the expected size amplified. eNOS mRNA was expressed in the presence of FSH, but not in the absence of FSH. Although eNOS mRNA was not expressed in the initial period, it was expressed after 12 h of stimulation with FSH, and remained at a relatively constant level until 48 h. Expression of eNOS mRNA preceded expression of LH receptor mRNA, which showed a maximal level at 24 h of stimulation. These observations suggest that eNOS expression is not related to a rapid synthesis of NO in developing granulosa cells, and that the activation of NO synthesis is rigidly regulated in the initial period of development.

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INTRODUCTION
Nitric oxide (NO) is a short-lived messenger molecule that mediates a variety of cellular functions. It is generated by three isoforms of NO synthase (NOS) that are classified into constitutive isoforms such as endothelial (eNOS) and neural NO (nNOS) synthases (Bredt et al. 1991, Lamas et al. 1992) and an inducible isoform (iNOS) (Lowenstein et al. 1992). NO is also synthesized by rat and human ovaries and has been shown to be involved in folliculogenesis, ovulation, apoptotic cell death of follicular cells and steroidogenesis (Ellman et al. 1993, Ben-Shlomo et al. 1994, Chun et al. 1995, Shukovski & Tsafri 1995, Hattori et al. 1996, Bonello et al. 1996, Yamauchi et al. 1997). A recent study using rat granulosa cells showed that NO might function as a modulator in cell differentiation, because exposure of the cells to an NO donor caused an increase in the epidermal growth factor receptor contents during cell differentiation (Hattori et al. 1996). In contrast, it has been reported that NO may inhibit steroidogenesis in rat, human and porcine ovarian cells (Adams et al. 1992, Van Voorhis et al. 1994, Olson et al. 1996, Punta et al. 1996).

iNOS is transcriptionally regulated, whereas nNOS and eNOS are considered to be constitutively expressed. However, evidence accumulated in recent years indicates that these two isoforms are also subject to expressional regulation (Weiner et al. 1994, Van Voorhis et al. 1995, Bryant et al. 1995, Arnet et al. 1996, Jablonka-Shariff & Olson 1997, Vega et al. 1998, El Dwairi et al. 1998, Srivastava et al. 1999, Hangai et al. 1999, Venkov et al. 1999). Changes in their expression may have physiological and pathophysiological consequences. Nitrite, an NO metabolite, is accumulated in luteinized ovarian
cells of the rat (Olson et al. 1996), suggesting an important function of NO during the periovulation period. In our previous study (Nishida et al. 2000), we found that NO was synthesized by cultured porcine granulosa cells. Northern blot analysis has shown that iNOS and eNOS are expressed in the rat ovary (Van Voorhis et al. 1995). Expression of iNOS is stimulated by interleukin-1β (IL-1β) in cultured rat granulosa cells, but follicle-stimulating hormone (FSH) can not induce iNOS mRNA (Tabraue et al. 1997). Therefore, there is no report concerning expression of the eNOS gene in cultured granulosa cells. To extend understanding of the regulation of eNOS in granulosa cells, it is necessary to investigate the expression of eNOS gene using a cell culture system. In this study, we therefore used RT-PCR analysis to identify eNOS mRNA in cultured porcine granulosa cells, and investigated the expression of eNOS mRNA and NO synthesis during cell maturation.

MATERIALS AND METHODS

Chemicals

Ovine FSH (NIDDK-oFSH-20) was 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); ISOGEN was from Nippon Gene Co. (Toyama, Japan); the T-primed first-strand kit was from Amersham Pharmacia Biotech (Tokyo, Japan); AmpliTaqGold polymerase was from Perkin-Elmer (Norwalk, CT, USA); the restriction enzyme SmaI was from New England Biolabs (Beverly, MA, USA). All other chemicals used were of reagent grade and obtained from commercial sources.

Cell culture

Porcine ovaries were obtained at a local slaughterhouse. Granulosa cells were prepared from medium-sized (1–4 mm diameter) follicles by aspiration with a 20 gauge needle and filtering through a fine stainless mesh. They were treated with 50 µg DNase I/ml at 37 °C for 5 min and washed with Ham’s F-10 and DMEM (1:1) supplemented with 10 mM Hapes, 50 µg gentamycin/ml, and 20 IU nystatin/ml (Mondschein et al. 1990). Cell viability was determined to be >95% by trypan blue exclusion. Cells were seeded at 2 × 10^6/dish in a 35 mm dish coated with human fibronectin ( Falcon; Becton-Dickinson, Oxnard, CA, USA) with 100 nM androstenedione, 110 nM hydrocortisone, 1 µg insulin/ml, 5 µg transferrin/ml and 0·1% (w/v) bovine serum albumin at 39 °C in a humidified atmosphere of 95% air and 5% CO_2_. FSH was then added to the cultures (culture 0 day), and the cells were cultured for the times indicated.

Nitrite and nitrate assays

Nitrite and nitrate were separated on a reverse-phase separation column packed with polystyrene polymer (NO-PASK, 4.5 × 50 mm) with an automated NO detector–HPLC system (ENO-20, EICOM, Kyoto, Japan) as described previously (Hattori et al. 2000a). Nitrate was reduced to nitrite with copper-plated cadmium filings (NO-RED). 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/NH_4Cl and 0·5 g EDTA–4Na/l, applied at a flow rate of 0·33 ml/min. The absorbance of the dye product was measured at 540 nm by a flow-through spectrophotometer (NOD-10).

cGMP assay

cGMP was assayed by a specific radioimmunoassay (Hattori et al. 1996) and calculated with the use of a competitive assay computer program. There was 0·15% cross-reactivity between cAMP and the cGMP antiserum. The lower limits of detection for both assays were 0·09 pmol/ml, and the intra-assay and interassay variabilities were <10%.

RNA isolation

The total RNAs were extracted from granulosa cells using ISOGEN, according to the instruction manual, and dissolved in 20 µl diethylpyrocarbonate-treated water.

RT-PCR

Single-stranded cDNA was synthesized from these RNA preparations in a reverse transcription (RT) reaction in a final volume of 10 µl, using a T-primed first-strand kit, according to the instruction manual. The RT reaction was performed at 45 °C for 1 h and followed by heating for 5 min at 70 °C and then diethylpyrocarbonate-treated water was added (final volume 50 µl). After an initial denaturation step (95 °C, 10 min), the PCR reaction was performed in 10 µl of 1 × PCR buffer, 1 or 3 µl cDNA, 0·2 mM dNTPs, 0·25 U AmpliTaqGold polymerase, and 0·2 µM each of the synthetic primers. The primer sets were designed from known cDNA sequences of the porcine eNOS (GenBank/Accession NO. U59924) and four porcine luteinizing hormone
(LH) receptor isoforms (Loosfelt et al. 1989) (Table 1). LH receptor amplified products were 411 bp and 185 bp (Hattori et al. 2000b). The amplification step consisted of 40 cycles (eNOS) and 34 cycles (LH receptor) using denaturation (95 °C, 30 s), annealing (54 °C, 30 s) and extension reactions (72 °C, 2 min) before a final extension step of 10 min at 72 °C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for reaction efficiency and variations in concentrations of mRNA in the original RT reaction. The amplified products were analysed by electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed under UV light. The intensities of the bands were quantified as a reference gene of GAPDH using a densitometry program (NIH Image Version 1.58).

The eNOS PCR product was checked by the restriction enzyme, SmaI, and the resulting products were analysed by electrophoresis on 2% agarose gels.

Statistical analysis
The data are expressed as means ± s.e.; differences between them were analysed using Student’s t-test after analysis of variance.

RESULTS

cGMP formation and NO synthesis during development
An increase in cGMP was observed after 48 h of stimulation with FSH (Fig. 1). Nitrite and nitrate were assayed using an automated NO detector–HPLC system. The concentrations of nitrite and nitrate remained relatively constant until 42 h of stimulation, after which they increased significantly up to twofold at 48 h (P<0.05). Production of cGMP stimulated by an NO donor was observed at the initial periods of the cell development (Nishida et al. 2000). These results indicate that NO synthesis is transiently promoted after 42–48 h of FSH stimulation, and that NO in turn stimulates guanylate cyclase.

Detection of eNOS mRNA by RT-PCR analysis
To determine whether eNOS mRNA was present in cultured granulosa cells, we performed RT-PCR on total RNA isolated from the cells. As shown in Table 1.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sequences</th>
<th>Nucleotide number</th>
<th>Expected PCR fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>Sense: 5’AGCGGCTGCATGACATTTGAG3’</td>
<td>3092–3111</td>
<td>456</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’AAAGCTCTGGGTGGTGATGCG3’</td>
<td>3547–3526</td>
<td></td>
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<tr>
<td>LH-R</td>
<td>Sense: 5’CCAAATCTCCTAGTGCCCACATTTGAC3’</td>
<td>861–885</td>
<td>1121</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’GCTCAGCAACAGAAAGAATCCC3’</td>
<td>1981–1959</td>
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<td></td>
<td></td>
<td>411</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>185</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: 5’ACCACAGTCCATGCCATC3’</td>
<td>861–880</td>
<td>452</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’TCCACCACCTGTGGCTGTA3’</td>
<td>1312–1293</td>
<td></td>
</tr>
</tbody>
</table>

LH-R, LH receptor.

TABLE 1. Oligonucleotide primers used for PCR analysis

(LH) receptor isoforms (Loosfelt et al. 1989) (Table 1). LH receptor amplified products were 411 bp and 185 bp (Hattori et al. 2000b). The amplification step consisted of 40 cycles (eNOS) and 34 cycles (LH receptor) using denaturation (95 °C, 30 s), annealing (54 °C, 30 s) and extension reactions (72 °C, 2 min) before a final extension step of 10 min at 72 °C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for reaction efficiency and variations in concentrations of mRNA in the original RT reaction. The amplified products were analysed by electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed under UV light. The intensities of the bands were quantified as a reference gene of GAPDH using a densitometry program (NIH Image Version 1·58).

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FIGURE 1. Changes in the concentrations of cGMP and NO metabolite (nitrite and nitrate) during maturation of porcine granulosa cells. Granulosa cells were cultured for the indicated times with FSH (10 ng/ml), and then media were removed for cGMP assay by a specific radioimmunoassay (columns). Nitrite and nitrate (○) were assayed using an automated NO detector–HPLC system as described in Materials and Methods. Data are the means ± s.e. of three independent experiments. NOx, *P<0.05 compared with 0 h; cGMP, #P<0.05 compared with 30 h.
stimulated with FSH, whereas it was not detected in the cells stimulated without FSH.

**Time-dependent changes in the levels of granulosa cell eNOS mRNA**

The kinetics of eNOS mRNA expression was examined in granulosa cells stimulated with FSH by using RT-PCR analysis, and compared with changes in LH receptor mRNA expression. After stimulation with FSH for the times indicated, cDNA was prepared from total RNA isolated. Figure 4 shows a gel electrophoresis of RT-PCR products for eNOS and LH receptor. The transcript of eNOS was detected after 12 h of FSH stimulation, and remained constant until 48 h (Fig. 4a and c). In contrast, LH receptor mRNA reached a maximal level at 24 h of stimulation, and decreased progressively to 48 h (Fig. 4b and c).

**DISCUSSION**

It has been demonstrated using immunocytochemical staining and Northern blot analysis that eNOS and iNOS are expressed in the rat ovary (Van Voorhis et al. 1995, Jablonka-Shariff & Olson 1997). In the present study using RT-PCR analysis, expression of eNOS mRNA was also detected in cultured porcine granulosa cells. Van Voorhis et al. (1995) reported that eNOS mRNA levels increased after stimulation with gonadotrophin and peaked in ovaries containing ovulatory follicles, before declining in the luteal phase. Jablonka-Shariff & Olson (1997) reported that the levels of immunostaining...
Expression of granulosa cell eNOS

of eNOS increased in the granulosa cells and theca cell layer during follicle growth of rat ovaries that was induced by pregnant mare’s serum gonadotrophin. In contrast, a slight increase in iNOS was observed in the theca cell layer and stroma during the hormonal stimulation. These reports indicate that expression of granulosa cell eNOS is dependent upon gonadotrophin stimulation. However, no reports have described expression of eNOS mRNA during in vitro development of granulosa cells. In the present study, eNOS mRNA was expressed after 12 h of FSH stimulation, then the mRNA levels remained relatively constant until 48 h of stimulation. A maximal expression of LH receptor mRNA was detected after 24 h of stimulation. A similar finding for LH receptor mRNA expression was also reported using Northern blots and receptor binding studies (Goxe et al. 1992). As NO may be involved in ovulation (Shukovski & Tsafriri 1995, Bonello et al. 1996, Yamauchi et al. 1997) and differentiation of the granulosa cells (Hattori et al. 1996, Nishida et al. 2000), it is reasonable to propose that expression of eNOS mRNA preceded maximal expression of LH receptor mRNA in cultured granulosa cells. eNOS has been considered to be a constitutively expressed protein, but there are many reports of regulation of the expression of its gene (Weiner et al. 1994, Bryant et al. 1995, Van Voorhis et al. 1995, Arnet et al. 1996, Jablonka-Shariff & Olson 1997, El Dwairi et al. 1998, Vega et al. 1998, Hangai et al. 1999, Srivastava et al. 1999, Venkov et al. 1999). In post-ischaemic rat retina, eNOS mRNA increased to a peak at 12 h, and decreased progressively beyond 24 h (Hangai et al. 1999). Expression of eNOS mRNA increases within 3 h in bovine aortic endothelial cells exposed to ethanol (Venkov et al. 1999). Mechanical forces seem to induce expression of eNOS gene in these cells, and the expression is relatively quick. However, it is also known that expression of eNOS is regulated by hormonal stimulation (Weiner et al. 1994, Bryant et al. 1995, Van Voorhis et al. 1995, Jablonka-Shariff & Olson 1997). In human corpus luteum, expression of eNOS is detected in high levels in mid and early corpus luteum, and decreased in late corpus luteum (Vega et al. 1998). In the development of rat skeletal muscle, the level of eNOS mRNA increases during the late gestational and early postnatal periods (El Dwairi et al. 1998).

Expression of eNOS was continuously observed after 12 h of FSH stimulation, but NO synthesis did not occur until approximately 42 h. A maximal synthesis of NO was observed after approximately 48 h of FSH stimulation, and the synthesis was accompanied by a transient increase in cGMP. These findings indicate that NOS expression is not necessarily related to a rapid synthesis of NO and that the activation of NO synthesis may be rigidly
regulated in the granulosa cells. It seems that NO is not required at least for the initial stage of granulosa cell development. Although it is not known what stimulus activates eNOS in granulosa cells, eNOS requires Ca\(^{2+}\) and calmodulin for its activity. Several signalling pathways may be involved in the synthesis of NO, depending on the cell type. The Ca\(^{2+}\)/calmodulin system and tyrosine kinase-dependent pathway were reported to be involved in the activation of eNOS (Tsukahara et al. 1994a,b). Gonadotrophin may regulate the expression of eNOS and iNOS during ovarian follicular development and luteinization (Van Voorhis et al. 1995, Jablonka-Shariff & Olson 1997), but there has been no report of the regulation of NOS activation.

It has been reported that iNOS mRNA is not detectable in rat ovaries containing ovulatory follicles after injection of gonadotrophin (Van Voorhis et al. 1995); however, iNOS mRNA was not determined in the present study. In cultured rat granulosa cells, IL-\(\beta\) induces activation of iNOS, but FSH can not induce iNOS mRNA (Tabraue et al. 1997). In addition, it has been reported that iNOS is expressed in the theca cell layer and stroma surrounding immature follicles in rat ovaries (Jablonka-Shariff & Olson 1997). As iNOS inducers such as IL-\(\beta\) were not contained in our culture system, NO released from porcine granulosa cells might result from activation of eNOS. The mRNA levels for these isoenzymes are differentially regulated, and eNOS, but not iNOS, seems to be necessary for the process of maturation of granulosa cells. The characteristics of eNOS – that is, it is a constitutively expressed gene producing small amounts of synthesized NO – may be significant for the function of granulosa cells.

In conclusion, the present study using RT-PCR demonstrated that eNOS mRNA was expressed before that of LH receptor mRNA in FSH-stimulated granulosa cells, but that NO synthesis occurred in the terminal stages of cell maturation.

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