EXPERIMENTAL STUDY

Effects of nitric oxide on steroidogenesis in porcine granulosa cells during different stages of follicular development

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Abstract

Background: We have previously demonstrated that nitric oxide (NO) inhibits steroidogenesis via a cGMP-independent process, by inhibiting P450 aromatase activity in porcine granulosa cells (PGCs) derived from medium-sized (3–5 mm) ovarian follicles (M-PGC).

Objective: To determine whether the NO/NOS system exerts any significant effects on steroidogenesis in PGCs derived from small follicles (<3 mm) (S-PGC) in comparison with those derived from medium follicles.

Design and methods: PGCs, namely S-PGC and M-PGC, were incubated with the NO donor, NOC18, and a competitive blocker of NOS, N\textsuperscript{G}-monomethyl-L-arginine (LNMMA), either alone or in the presence of FSH (200 ng/ml) or hCG (5 IU/ml).

Results: NOC18 significantly \( P < 0.01–0.001 \) suppressed basal (unstimulated) and gonadotropin-stimulated estradiol (E2) release from both S-PGC and M-PGC in a 2-h culture. NOC18 significantly \( P < 0.01–0.001 \) decreased basal and gonadotropin-stimulated progesterone release from S-PGC, but not from M-PGC. In addition, NOC18 significantly \( P < 0.05–0.001 \) inhibited aromatase activity in S-PGC. LNMMA had a significantly \( P < 0.01–0.001 \) stimulatory effect on the basal release of E2 and progesterone from M-PGC; however, it had no significant effect on basal steroidogenesis in S-PGC in a 24-h culture. In the presence of gonadotropin, LNMMA significantly \( P < 0.01–0.001 \) stimulated the release of E2 and progesterone from both S- and M-PGC, and this stimulatory effect was weaker in S-PGC than in M-PGC. These results demonstrate that NO inhibits E2 secretion by directly inhibiting the aromatase activity in S-PGC, as in M-PGC. It has been shown that the NO system suppresses the differentiation of S-PGC; however, the extent of suppression decreased with the progression of follicular growth. In addition, the activity of NOS in S-PGC was weaker than that in M-PGC.

Conclusion: We strongly suggest that the NO/NOS system in PGC regulates steroidogenesis differently during different phase of follicular development.

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Introduction

Nitric oxide (NO), a highly reactive free radical gas, has recently been implicated in a variety of physiological and pathological roles, including reproductive functions. NO is synthesized by conversion of L-arginine to L-citrulline by a family of enzymes known as NO synthase (NOS), which includes inducible NOS (iNOS), constitutive endothelial NOS (eNOS) and neural NOS (nNOS) (1). Many physiological processes are regulated by NO and its absence or inadequate production or release may lead to several pathological conditions. Recently, the NO/NOS system has been found to be involved in modulating the hypothalamic–pituitary axis (2) and endometrial (3) and ovarian functions (4, 5). The possible roles of the NO/NOS system in the control of ovarian function are based on the modulatory effects of NO/NOS-related agents on granulosa cell (GC) function (5).

In our previous studies, we have demonstrated that the NO/NOS system is localized in porcine granulosa cells (PGC) derived from medium size follicles (M-PGC), and that it inhibits steroidogenesis by inhibiting the P450 aromatase activity in basal (unstimulated) and gonadotropin-stimulated M-PGC (6). In a subsequent series of experiments, the influence of the NO/NOS system on ovarian functions throughout the process of GC maturation was investigated. We have therefore, in the present study, extended our work to the earlier phases of GC maturation that occur before luteinization and compared the effects of NO/NOS on the steroidogenic functions of immature PGC derived from small
follicles (S-PGC) with those of PGC from medium-sized follicles (M-PGC).

Materials and methods

Drugs and reagents

The chemicals used were obtained from the following sources. Dulbecco’s modified Eagle’s medium (DMEM), porcine follicle stimulate hormone (FSH) and human albumin fraction V were purchased from Sigma Chemical Co. (St Louis, MO, USA); Dulbecco’s phosphate-buffered saline (D-PBS), fetal calf serum (FCS) and antibiotic–antimycotic solution containing 10,000 IU/ml penicillin G, 10 mg/ml streptomycin, and 25 μg/ml amphotericin B were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA); Nω-monomethyl-L-arginine (LNMMA) and Nω-monomethyl-D-arginine (DNMMA) were from Calbiochem (La Jolla, CA, USA); NOC 18 (l-hydroxy-2-3,3-bis(2-methyl-D-arginine (DNMMA) were from Calbiochem Co. (La Jolla, CA, USA); NOC 18 (l-hydroxy-2-3,3-bis(2-aminoethyl)-l-triazene) was from Dojindo Laboratories (Kumamoto, Japan); Δ^4-androstenedione and charcoal-activated powder from were Wako Pure Chemical Industries (Osaka, Japan); plastic multiple tissue culture plates were from Falcon (Becton Dickinson Co., Lincoln Park, NJ, USA); hCG was supplied by Mochida Pharmaceutical Co. (Tokyo, Japan).

Cell culture

The study was approved by the ethics committee of Tokyo Medical and Dental University Hospital, Japan. Ovaries were obtained from 4- to 6-month old Yorkshire pigs from a local slaughterhouse. PGC were immediately isolated from small (<3 mm)- and medium (3–5 mm)-sized follicles by needle aspiration as described previously (7, 8). The cells were rinsed twice with serum-free DMEM, and then suspended in DMEM containing 10% FCS and 1% antibiotic–antimycotic solution. The number of granulosa cells was counted with a haemocytometer and the viable cells were assessed by the dye exclusion method using 0.4% Trypan Blue. Cells were seeded into 24-well culture plates at a density of 5 x 10^5 viable cells/well and cultured cells were washed twice with D-PBS and the protein content of the cells in each well was measured (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, CA, USA).

Measurements of estradiol and progesterone

Estradiol and progesterone concentrations in the media were measured by chemiluminescence enzyme immunoassay (Johnson & Johnson Clinical Diagnostics Ltd, Amersham, Bucks, UK). The intra- and interassay coefficients of variation for both the assay kits did not exceed 10% (7, 8).

P450 aromatase assay

Cytochrome P450 aromatase activity was measured using a modified tritiated water production assay (9). To initiate the assay, [1β-3H]-androstenedione (899.1 GBq/mmol) (New England Nuclear, Boston, MA, USA) was added to the culture media to yield a concentration of 20.5 nmol/l. NOC18 (0.01–1.0 mmol/l) was added to this media and cultures were incubated for 2 h with or without FSH (200 ng/ml) or hCG (5 IU/ml). Thereafter, 0.9 ml of the culture media (from a total of 1 ml) was removed and mixed with 0.45 ml 20% trichloroacetic acid and 0.45 ml 5% charcoal suspension. The mixtures were vortexed intermittently over a 30-min period, centrifuged for 30 min at 1600 g and the supernatants were removed and filtered. Radioactivity was then measured using a liquid scintillation counter (Tri-cab 1900, Packard, CT, USA). The data are presented as disintegrations per min of 3H2O produced per 2-h culture/μg protein (percentage of control).

Statistics

Statistical analysis was performed by post hoc testing (analysis of variance) to determine the statistical significance of the differences, with P < 0.05 being accepted as denoting statistical significance. All experiments were repeated at least three times to ensure reproducibility of the results, and data are expressed as the mean ± s.d. of groups consisting of six wells. The results of one representative experiment are reported in each figure. Data were analyzed using StatViewR (Abacus Concepts Inc., Berkeley, CA, USA) on a Macintosh personal computer (Apple Computer Inc, Cupertino, CA, USA).

Results

NOC18 significantly (P < 0.001) suppressed the basal release of E2 from S-PGC by 27.1 ± 5.1% at a concentration of 1 mmol/l. NOC18 in a concentration of 1 mmol/l also significantly (P < 0.001) suppressed the E2 release from FSH-stimulated S-PGC (by 26.7 ± 7.5%).
and hCG-stimulated S-PGC (by 18.1 ± 6.7%) (Fig. 1a). In M-PGC, NOC18 in a concentration of 1 mmol/l significantly (P < 0.01–0.001) suppressed the basal E2 release (by 27.1 ± 4.8%), FSH-stimulated E2 release (by 49.5 ± 5.1%), and hCG-stimulated E2 release (by 19.0 ± 2.7%) in 2-h cultures (Fig. 1b). NOC18 in a concentration of 1 mmol/l also significantly (P < 0.001) decreased basal progesterone release (by 53.3 ± 3.7%), FSH-stimulated progesterone release (by 54.8 ± 5.2%), and hCG-stimulated progesterone release (by 51.8 ± 4.6%) from S-PGC (Fig. 2a), but no significant changes in the basal and gonadotropin-stimulated progesterone release from M-PGC were noted (Fig. 2b). In addition, NOC18 significantly (P < 0.05–0.001) inhibited the basal and gonadotropin-stimulated aromatase activities in S-PGC. These results were almost the same as those for M-PGC (Fig. 3).

LNMMA had no significant effect on basal E2 release from S-PGC (Fig. 4a), but had a significant (P < 0.01) effect on E2 release from M-PGC in a 24-h culture (Fig. 4b). In the presence of gonadotropin, however, LNMMA significantly (P < 0.01–0.001) stimulated the release of E2 from both S- and M-PGC (Fig. 4). In addition, although LNMMA had no significant effect on basal release of progesterone from S-PGC (Fig. 5a), it significantly (P < 0.001) enhanced the basal progesterone release from M-PGC in a 24-h culture (Fig. 5b). In the presence of gonadotropin, however, LNMMA significantly (P < 0.01) stimulated the release of progesterone from both S- and M-PGC (Fig. 5). Especially in the case of stimulation with FSH, the progesterone concentration was significantly increased (by 29.1 ± 6.8%) in the presence of 1 mmol/l LNMMA in S-PGC (Fig. 5a) and (by 189.1 ± 5.4%) in the presence of 1 mmol/l LNMMA in M-PGC (Fig. 5b). The

**Figure 1** Concentration-related effect of NOC 18 (an NO donor) on basal [(a)], FSH-stimulated [(b)], and hCG-stimulated [(c)] accumulation of estradiol in cultured porcine granulosa cells [(a)] derived from small follicles (S-PGC) and [(b)] derived from medium-sized follicles (M-PGC) during a 2-h incubation. Each point represents the mean of six wells; bars show S.D. *P < 0.01; **P < 0.001 compared with control.

**Figure 2** Concentration-related effect of NOC 18 on basal [(a)], FSH-stimulated [(b)], and hCG-stimulated [(c)] accumulation of progesterone [(a)] in cultured S-PGC and [(b)] in M-PGC during 2-h incubation. Each point represents the mean of six wells; bars show S.D. *P < 0.01; **P < 0.001 compared with control.
stimulatory effect of LNMMA on the release of progesterone in the presence of FSH was clearly weaker in S-PGC than in M-PGC. DNMMMA, the inactive stereoisomeric analog of LNMMA, had no effect on the release of E2 and progesterone from either basal or gonadotropin-stimulated PGC under the same experimental conditions (data not shown).

Discussion
We have previously demonstrated that the NO/NOS system is localized in cultured M-PGC and that it suppresses steroidogenesis via a cGMP-independent process, by inhibiting P450 aromatase activity in M-PGC (6). In this study, we attempted to clarify the possible roles of NO/NOS in proliferation and maturation of PGC from immature to moderately mature GC. At first, we examined steroidogenesis in PGC derived from different maturational stages of the follicles. The results of the present study reveal that the NO donor, NOC18, significantly suppressed the basal (unstimulated) and gonadotropin-stimulated E2 release by inhibiting the aromatase activity in PGC derived from immature follicles also. These results imply that NO has inhibitory effects on the release of E2 from S-PGC and M-PGC via suppression of the P450 aromatase activity.

![Figure 3](image3.png)

**Figure 3** Concentration-related effect of NOC18 on (a) basal (without gonadotropin), (b) FSH-stimulated and (c) hCG-stimulated accumulation of aromatase activity in cultured S-PGC and in M-PGC during 2-h incubation. Data expressed as the percentage of control (100%), as represented by a culture in the absence of NOC18. Each column represents the mean of six wells; bars show S.D. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control cells. DPM, disintegrations per minute

![Figure 4](image4.png)

**Figure 4** Concentration-related effect of LNMMA on basal (□), FSH-stimulated (●), and hCG-stimulated (○) accumulation of estradiol (a) in cultured S-PGC and (b) in M-PGC during 24-h incubation. Each point represents the mean of six wells; bars show S.D. *P < 0.01; **P < 0.001 compared with control.
We also investigated the effects of NO/NOS on the regulation of luteinization in cultured PGC. NOC18 inhibited progesterone release from S-PGC; however, it had no significant effect on progesterone release from M-PGC. Therefore, the NO/NOS system was also involved in the suppression of differentiation of PGC derived from immature follicles; however, the extent of this suppression decreased with the progression of follicular growth. Thus we strongly suggest that the NO/NOS system in the PGC regulates steroidogenesis differently during different phases of follicular development.

In our previous work, we demonstrated that LNMMA, a competitive blocker of NOS, significantly stimulated the basal release of E2 and progesterone from M-PGC (6). In contrast, it had no significant effect on basal steroidogenesis in S-PGC in the present study. As it is well established that gonadotropin plays a central role during the maturation of ovarian follicles (10, 11) and that it induces proliferative and differentiative functions in cultured immature granulosa cells (12), we examined the effects of NO/NOS on the gonadotropin-induced steroidogenic functions in immature PGC. LNMMA significantly stimulated the release of E2 and progesterone from both S-PGC and M-PGC in the presence of gonadotropin, and the stimulatory effect in S-PGC was weaker than that in M-PGC. These findings suggest that the NOS activity in PGC, inhibited by LNMMA, increases during the process of follicular growth associated with the functions of gonadotropin, and to a larger extent in M-PGC than in S-PGC. These findings suggest that the NOS activity in PGC, inhibited by LNMA, increased in the process of follicular growth associated with the functions of gonadotropin.

Two distinct types of NOS have been identified: an inducible Ca<sup>2+</sup>-independent enzyme (iNOS) (13) and a constitutive Ca<sup>2+</sup>-calmodulin-dependent enzyme (cNOS), which consists of eNOS (14) and nNOS (15). Immunocytochemical studies have shown the expression of eNOS in human granulosa-luteal cells (5). It is suggested that the preovulatory increase in NO<sub>3</sub>/NO<sub>2</sub> production (16) induced by eNOS may have an autocrine/paracrine effect on the granulosa or neighboring theca cells (5). As our previous study showed that eNOS and iNOS were localized in M-PGC (6), the cellular source of follicular NO is believed to be GC. Van Voorhis et al. (17) have revealed that the increased E2 concentrations seen after gonadotropin stimulation may lead to the induction of eNOS in the rat ovary. Our studies also suggest that the inhibitory effects of NO on PGC steroidogenesis may be mediated by eNOS because the inhibitory effect of the NO donor on E2 secretion was greater than that on gonadotropin stimulation. In contrast, NO production by iNOS has been reported to be important for the rupture of the rat follicle during ovulation (18) and in the developmental state of rat follicles, acting in concert with gonadotropin (19).

The physiological functions of NO/NOS in follicular development have not been clarified. Antebay et al. (20) reported an association between follicular size and follicular NO<sub>3</sub>/NO<sub>2</sub> concentration in women undergoing in vitro fertilization. They demonstrated that the follicular NO<sub>3</sub>/NO<sub>2</sub> concentrations were correlated with the follicular size in follicles measuring 15–30 mm. Increased follicular NO concentrations may stimulate fluid accumulation and thereby, follicular growth, by causing vasodilatation. The follicular NO concentrations in these cases may represent local synthesis, and local vasodilatory actions. Furthermore, a significant negative correlation was found between human follicular fluid NO<sub>3</sub>/NO<sub>2</sub> concentration and ovarian vasodilatation, and a potential role for NO in preovulatory follicular expansion has been proposed.
(20). In addition, we have already demonstrated that nitrite is detected in the culture medium of PGC and in porcine follicular fluid (6), therefore we speculate that NO production by GC may act in a paracrine fashion to cause local vasodilatation and mediate, in part, the increase in blood flow to the developing follicle and corpus luteum (21).

In conclusion, we have shown that NO inhibits E2 secretion by directly inhibiting aromatase activity in S-PGC, as in M-PGC. It has also been shown that NO significantly inhibits progesterone secretion in S-PGC, different from its effect in M-PGC, indicating that NO/NOS may suppress the early luteinization of GC in the immature follicle, but that the extent of this suppression decreases with the progression of follicular maturation. NO/NOS may also act as a luteinization inhibitor by attenuating key biosynthetic steps leading to progesterone production mainly in S-PGC. Conversely, the activity of NOS in S-PGC is less than that in M-PGC, and it is likely that the NO/NOS system in PGC regulates steroidogenesis differently during different phases of follicular development. The NOS activity is closely related to the regulation of steroidogenesis, and the level of activity is different during the different stages of GC maturation. These data suggest that the NO/NOS system may have an important role in the local regulation of ovarian hormonal function, follicle growth and GC differentiation via direct effects on GC (15). These findings support the hypothesis that NO is an autocrine/paracrine regulator of the functions of GC.

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References