Inhibitory effects of nitric oxide on estrogen production and cAMP levels in rat granulosa cell cultures

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Abstract

Previous studies demonstrated inhibitory effects of nitric oxide (NO) and cGMP on ovarian steroidogenesis. This study examined the effects of NO on estrogen levels and cAMP accumulation from immature cultured rat granulosa cells. Granulosa cells were incubated with media alone (control), FSH or FSH plus increasing concentrations of the NO generator, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA/NO). While FSH increased estrogen levels 15-fold compared with controls, DETA/NO inhibited FSH-stimulated aromatase activity in a dose-dependent manner. Time-course studies revealed that the inhibitory effects of DETA/NO on aromatase activity persisted throughout the 72 h culture period. Treatment with DETA/NO also inhibited the stimulatory effects of forskolin on estrogen

production, indicating that NO can influence steroidogenesis by actions downstream of the FSH receptor. Incubation of cells with FSH plus DETA/NO increased cGMP accumulation over 100-fold, compared with cells treated with media or FSH alone. In this regard, a cGMP analog mimicked the inhibitory effects of NO on FSH- and forskolin-stimulated estrogen production, indicating a potential mechanism of NO action. NO also decreased FSH-stimulated (cAMP) accumulation from cultured cells, indicating an antagonistic effect of NO on the second messenger mediating FSH actions. These findings demonstrate that NO inhibits estrogen production from rat granulosa cells, potentially reflecting actions on the second messengers cGMP and cAMP.

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Introduction

Estradiol (E2) plays essential roles in the establishment and maintenance of the female reproductive system. Ovarian E2 production is dependent upon the induction of the enzyme cytochrome P450 aromatase, which catalyzes the conversion of androgens to estrogens. The induction of aromatase mRNA and protein in ovarian granulosa cells is dependent upon exposure to follicle-stimulating hormone (FSH), which acts through its G protein-coupled receptor to activate adenyl cyclase and stimulate production of the second messenger cAMP (Erickson & Hsueh 1978, Hsueh *et al.* 1984, Hickey *et al.* 1988). In addition to the stimulatory effects of FSH, the induction of aromatase activity in granulosa cells is modulated by a number of other hormones, growth factors and peptides (Hsueh *et al.* 1984, Adashi & Resnick 1986, Mendelson *et al.* 1986, Krasnow *et al.* 1990, LaPolt & Hong 1995).

Nitric oxide (NO) is a diffusible gas which has been implicated as a signaling molecule in a variety of cellular functions, including vasodilation, neurotransmission and macrophage toxicity (Moncada *et al.* 1991). Previous studies suggest that NO may play important roles in the ovary, influencing processes such as ovulation, follicular

development, oocyte maturation, apoptosis and steroidogenesis (Shukovski & Tsafiriri 1994, Chun *et al.* 1995, Snyder *et al.* 1996, Masuda *et al.* 1997, Yamauchi *et al.* 1997, Matsumi *et al.* 1998b, Jablonka-Shariff *et al.* 1999a). Two forms of NO synthase (NOS), the enzyme catalyzing NO production, are expressed in the ovary in a regulated manner (Van Voohris *et al.* 1995, Jablonka-Shariff & Olson 1997, Matsumi *et al.* 1998b). Although the literature is not in complete agreement, it appears that the main form of NOS expressed in granulosa cells of small follicles is inducible NOS (iNOS), the expression of which apparently decreases during follicular growth (Van Voorhis *et al.* 1995). In addition, iNOS is expressed primarily in follicles that are functionally immature, rather than developed follicles (Matsumi *et al.* 1998b). In contrast, endothelial NOS (eNOS) is expressed in ovarian thecal and stromal cells, in mural granulosa cells of maturing follicles, and in corpora lutea (Jablonka-Shariff & Olson 1997). The regulated expression of NOS isoforms in the ovary is consistent with a physiological role of NO in ovarian functions. In addition, factors known to modulate granulosa cell function, such as interleukin-1, may exert their influences at least in part through induction of NO (Ben-Shlomo *et al.* 1994).

The actions of NO are mediated by activation of soluble guanylyl cyclase, resulting in increased cGMP production (Ignarro 1990). However, NO also influences enzyme activity by interactions between NO and heme or sulfhydryl moieties on proteins (Stamler 1994). In this regard, previous studies indicate that NO directly inhibits the activity of enzymes such as cytochrome P450 side chain cleavage and aromatase (Snyder *et al.* 1996, Hanke *et al.* 1998), as well as two isoforms of adenylyl cyclase (Duhe *et al.* 1994, McVey *et al.* 1999). While NO has been reported to inhibit estrogen production in both human granulosa-luteal cells and porcine granulosa cells in a cGMP-independent manner (Snyder *et al.* 1996, Masuda *et al.* 1997), our previous findings demonstrate an inhibitory effect of cGMP on FSH-stimulated E2 production in rat granulosa cells (LaPolt & Hong 1995), suggesting an additional mechanism of NO action. Furthermore, some actions of cGMP and NO are known to reflect modulation of adenylyl cyclase and phosphodiesterase activity, resulting in decreased cAMP production and accumulation (Macfarland *et al.* 1991, McVey *et al.* 1999). Therefore, it is conceivable that NO and cGMP may exert significant influences on the levels of cAMP, the second messenger mediating FSH actions. The present studies examined further the effects of NO and cGMP on steroidogenesis and second messenger signaling in cultured rat granulosa cells.

Materials and Methods

Hormones and reagents

Ovine FSH (oFSH-19-SIAFP; 94 × NIH-oFSH-S1) was obtained from the National Hormone and Pituitary Program, National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Bethesda, MD, USA. Forskolin (FSK) and dibutyryl cGMP ((Bu)₂cGMP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The NO generator (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate (DETA/NO) was obtained from Alexis Corporation (San Diego, CA, USA). McCoy's 5A medium (modified, serum-free), trypan blue, streptomycin sulfate, penicillin and L-glutamine were obtained from Life Technologies (Rockville, MD, USA). The levels of cGMP and cAMP were measured using commercial RIA kits (Amersham Corp., Arlington Heights, IL, USA). All other chemicals were reagent grade obtained from Fisher Scientific (Pittsburgh, PA, USA).

Granulosa cell culture

Granulosa cells were obtained from immature, estrogen-treated Sprague-Dawley female rats (Harlan Sprague Dawley, Indianapolis, IN, USA) as previously described

(LaPolt & Hong 1995). Cells were cultured in 12 × 75 mm polypropylene culture tubes (Falcon Plastics, Oxnard, CA, USA) at a concentration of 125 000 viable cells/0.5 ml McCoy's 5A medium supplemented with antibiotics and 10⁻⁷ M androstenedione, with or without added hormones and reagents. Cell viability was assessed using trypan blue exclusion staining. Incubations were performed at 37 °C in a humidified 5% CO₂-95% air atmosphere for up to 72 h. After incubation, medium was collected and stored at -70 °C until assayed for E2 content, as an indication of aromatase activity. For cAMP and cGMP RIAs, cells and media were heated for 10 min at 90 °C to inactivate phosphodiesterases, and then frozen in a dry ice/methanol bath and stored at -70 °C until assay.

Estrogen RIA and data analysis

The estrogen concentrations in conditioned granulosa cell medium were determined by RIA, as previously reported (LaPolt & Hong 1995). Each cell culture experiment was analyzed within the same assay. Experiments were performed in triplicate, and each experiment was repeated at least three times. Differences between treatment groups were determined by one-way or two-way ANOVA, and a confidence level of *P* < 0.05 was considered statistically significant.

Results

Effects of DETA/NO on FSH-induced estrogen production by cultured rat granulosa cells

Treatment of granulosa cells with FSH (1 ng/ml) markedly increased estrogen levels compared with cells treated with culture medium alone (control; Fig. 1). To examine the effects of NO on granulosa cell function, we utilized the NO generator DETA/NO, which has a long half-life of NO release (Shimaoka *et al.* 1995). Treatment of granulosa cells with FSH plus increasing doses of DETA/NO (30–250 µM) resulted in decreasing levels of estrogen production (Fig. 1), indicating that NO inhibits FSH-induced aromatase activity in a dose-dependent manner.

Time-course of FSH and DETA/NO actions on E2 production in rat granulosa cells

To determine the time-course during which NO inhibits FSH-induced estrogen production, cells were cultured with media alone (control), FSH (1 ng/ml) or FSH plus DETA/NO (120 µM) (Fig. 2). Estrogen levels in the conditioned media were measured at 0, 12, 24, 36, 48 or 72 h of culture. Untreated cells (control) produced low amounts of estrogen throughout 72 h of culture. In contrast, cells treated with FSH alone showed elevated levels

Figure 1 Dose-dependent effects of NO on FSH-stimulated estrogen production from rat granulosa cells. Cells were treated for 72 h with media alone (C; control), FSH (1 ng/ml) or FSH plus increasing concentrations of the NO generator, DETA/NO (30–250 μ M). After culture, media were collected to determine estrogen concentrations by E2 RIA.

Figure 3 Dose-dependent effects of NO on FSK-stimulated estrogen production from rat granulosa cells. Cells were treated for 72 h with media alone (C; control), FSK (10^{-4} M) or FSK plus increasing concentrations (30–250 μ M) of the NO generator, DETA/NO.

Figure 2 Time-course for inhibition of estrogen production by NO. Cells were treated with media alone (control), FSH (1 ng/ml) or FSH plus DETA/NO (120 μ M) for 12, 24, 36, 48, 60 or 72 h, followed by assay of media to determine estrogen concentrations.

of estrogen production beginning at 36 h of incubation, with the greatest accumulation occurring at 72 h. Cotreatment of cells with FSH plus DETA/NO completely prevented any increase in estrogen production throughout the entire culture period. These results indicate that while FSH stimulates estrogen production in a time-dependent manner, NO exerts a continuous suppression of FSH-induced aromatase activity.

Effects of DETA/NO on FSK-induced estrogen production by cultured rat granulosa cells

The inhibitory effects of NO on FSH-induced aromatase activity may reflect influences of NO on the FSH receptor (receptor expression, ligand binding and/or signal transduction) and/or on post-receptor events required for cAMP production and signaling. To determine whether NO might act at a post-FSH receptor site we directly stimulated cAMP production with the adenylyl cyclase activator FSK, bypassing the FSH receptor and associated guanine nucleotide-binding protein. As anticipated, treatment of cells with FSK alone (10^{-4} M) markedly elevated estrogen levels in comparison with untreated control cells (Fig. 3). In contrast, cotreatment of cells with FSK and increasing doses of DETA/NO (30–250 μ M) progressively inhibited estrogen production, indicating that NO

Figure 4 Influence of FSH and DETA/NO on cGMP accumulation from cultures of rat granulosa cells. To determine the relationship between the induction of aromatase activity and cGMP levels, cells were treated for 72 h with media alone (C; control), FSH (1 ng/ml) or FSH plus DETA/NO (120 μ M).

can inhibit estrogen production from rat granulosa cells at one or more post-FSH receptor sites.

Effects of NO on cGMP accumulation of cultured rat granulosa cells

Since NO mediates many of its effects through cGMP-dependent signaling pathways by activating soluble guanylyl cyclase, we next established whether NO could stimulate cGMP levels in rat granulosa cell cultures. Cells treated with media alone or with FSH (1.0 ng/ml) exhibited low levels of cGMP accumulation in cells and media after 72 h of culture. In contrast, cultures cotreated with FSH plus DETA/NO (120 μ M) displayed an over 100-fold increase in cGMP accumulation, compared with control cells (Fig. 4). These data demonstrate that DETA/NO effectively stimulates cGMP accumulation in cultured rat granulosa cells, and is consistent with the stimulatory effects of NO on guanylyl cyclase activation. Furthermore, these findings suggest a potential role of cGMP as a second messenger mediating the actions of NO on ovarian steroidogenesis.

Influence of a cGMP analog on FSH- and FSK-induced aromatase activity

Previous studies have reported that exogenous cGMP does not have a significant effect on steroidogenesis in human granulosa-luteal cells (Van Voorhis *et al.* 1994) and in

Figure 5 Effects of (Bu)₂cGMP on FSH-stimulated estrogen production from rat granulosa cells. Cells were treated for 72 h with media alone (control), FSH (1 ng/ml) or FSH plus (Bu)₂cGMP (2 mM), followed by measurement of estrogen concentrations by RIA.

porcine granulosa cells (Masuda *et al.* 1997). However, we have previously found that the cGMP analog (Bu)₂cGMP inhibits aromatase activity in rat granulosa cells (LaPolta & Hong 1995). To further confirm the effects of cGMP on FSH-induced aromatase activity, granulosa cells were cultured with FSH and (Bu)₂cGMP. As shown above, treatment of cells with FSH alone (1.0 ng/ml) markedly stimulated estrogen production, compared with controls. In contrast, estrogen production from cells cotreated with FSH plus (Bu)₂cGMP (2 mM) was significantly reduced, compared with that in cells incubated with FSH alone (Fig. 5). Similarly, while FSK (10⁻⁴ M) markedly increased estrogen concentrations compared with control cells, cotreatment with FSK plus (Bu)₂cGMP (2 mM) inhibited estrogen levels compared with cells cultured with FSK alone (Fig. 6). Higher levels of (Bu)₂cGMP (4 mM) did not further inhibit FSH- or FSK-stimulated E2 levels (data not shown).

Effects of FSH and NO on cAMP accumulation in cultured rat granulosa cells

To address whether NO might inhibit estrogen production by decreasing FSH-induced cAMP levels, cells were incubated for 72 h with medium alone (control), FSH (1.0 ng/ml) or FSH plus a maximally inhibiting dose of DETA/NO (120 μ M), and accumulated levels of cAMP in the cells and media were determined by RIA. As

Figure 6 Influence of $(\text{Bu})_2\text{cGMP}$ on FSK-stimulated estrogen production from rat granulosa cells. Cells were treated for 72 h with media alone (control), FSK (10^{-4} M) or FSK plus $(\text{Bu})_2\text{cGMP}$ (2 mM).

expected, cAMP levels were very low in control cells. In contrast, treatment of cells with FSH markedly increased cAMP accumulation, as anticipated (Fig. 7). Cotreatment of cells with FSH and DETA/NO, however, significantly decreased cAMP levels compared with those seen from cells treated with FSH alone. These findings are consistent with the hypothesis that NO inhibits FSH-stimulated estrogen production in part by decreasing levels of the second messenger mediating FSH action.

Discussion

During follicular development, FSH acts through the cAMP-dependent protein kinase A pathway to induce aromatase activity in granulosa cells. However, the stimulatory effect of FSH on estrogen production is influenced by other hormones, growth factors and gonadal peptides, indicating the presence of diverse mechanisms regulating ovarian steroidogenesis. Results of the present study contribute further evidence that NO is a modulatory factor that effectively inhibits FSH- and cAMP-dependent estrogen production. Using cultures of rat granulosa cells, we have demonstrated that the NO generator, DETA/NO, inhibits FSH-induced aromatase activity in a dose-dependent manner at one or more post-FSH receptor sites. Furthermore, our data indicate that the effects of NO may be mediated, at least in part, through increased levels of the

Figure 7 Inhibition of FSH-stimulated cAMP levels by DETA/NO. Granulosa cells were treated for 72 h with media alone (C; control), FSH (1 ng/ml) or FSH plus DETA/NO (120 μM), followed by measurement of cAMP accumulation.

second messenger cGMP, and by attenuating FSH-induced cAMP levels. Overall, the data presented in this study add to the body of evidence indicating that NO and cGMP act as modulators of ovarian functions.

It is unlikely that the inhibitory effects of NO and cGMP on granulosa cell functions are due to deleterious effects of these factors on cell viability. Previous studies have indicated an inverse relationship between NO expression and apoptosis in granulosa cells (Matsumi *et al.* 1998a), and that treatment with NO donors prevents apoptosis in ovarian cells (Chun *et al.* 1995, Basini *et al.* 1998). Similarly, treatment of ovarian follicles with cGMP analogs prevents programmed cell death (Chun *et al.* 1995, McGee *et al.* 1997). In addition, NO does not appear to influence granulosa cell proliferation in culture (Basini *et al.* 1998). Thus, the observed effects on NO and cGMP on granulosa cell functions is believed to be independent of influences on cell number.

The site of action of NO and the detailed mechanisms by which NO inhibits ovarian steroidogenesis are not completely understood. We have demonstrated that NO inhibits FSK-induced aromatase activity in addition to FSH-induced steroidogenesis, indicating that NO can act distally to receptor activation. However, this result does

not rule out additional effects of NO on the FSH receptor or its associated G protein. Previous studies have demonstrated that NO donors can inhibit FSH-induced E2 production in human granulosa-luteal cells and porcine granulosa cells (Snyder *et al.* 1996, Masuda *et al.* 1997, Kagabu *et al.* 1999). Snyder *et al.* (1996) proposed that NO directly inhibits aromatase activity by binding to a sulfhydryl group near the active site of the enzyme.

In addition to these potential direct effects on steroidogenic enzyme activity, the inhibitory effects of NO may also result from other signaling mechanisms. Snyder *et al.* (1996) also observed a significant inhibitory effect of NO on aromatase mRNA levels, indicating actions not limited to those directly affecting enzyme activity. Our results indicate that DETA/NO markedly increases levels of the second messenger cGMP in FSH-treated granulosa cell cultures. While others have reported that cGMP analogs have no effect on aromatase activity in luteinized human (Van Voorhis *et al.* 1994) and 'moderately mature' porcine granulosa cells (Masuda *et al.* 1997), our previous (LaPolt & Hong 1995) and present findings clearly demonstrate that treatment of immature rat granulosa cells with a cGMP analog effectively inhibits FSH- and FSK-induced estrogen levels in a dose-dependent manner. This discrepancy may reflect differences in the maturational state of granulosa cells used, as we used functionally immature cells as compared with the more mature granulosa cells used by the other investigators. This may suggest that cGMP acts by blocking the early initial events leading to granulosa cell maturation. In addition, studies in which no effect of cGMP on E2 production was found used shorter incubation times (16–24 h) and serum-based cell culture (compared with our use of serum-free conditions for 72 h), as well as different species. It is clear from our observations that NO may inhibit the induction of estrogen production by a cGMP-dependent mechanism, in addition to having potential direct effects on aromatase activity.

The inhibitory effect of NO on FSH-induced aromatase activity is associated with an inhibition of FSH-stimulated cAMP levels from granulosa cell cultures. A dose of DETA/NO that maximally inhibited FSH-stimulated E2 production resulted in a 43% reduction in cAMP. This inhibitory effect of NO on cAMP accumulation is consistent with the recent observation that NO inhibits cAMP levels in cultured human granulosa-luteal cells (Kagabu *et al.* 1999). The mechanism through which NO inhibits cAMP levels in granulosa cells is not clear. Previous reports demonstrate direct inhibitory effects of NO on two isoforms of adenylyl cyclase (Duhe *et al.* 1994, McVey *et al.* 1999). Thus, NO may antagonize FSH-induced cAMP production by direct interactions with adenylyl cyclase. On the other hand, it is known that cGMP induces activity of a cGMP-stimulated phosphodiesterase (PDE2) that increases hydrolysis of cAMP (Rosman *et al.* 1997). For example, the inhibitory effect of atrial natriuretic peptide on adrenal aldosterone production and

cAMP levels is related to the induction of PDE2 (MacFarland *et al.* 1991). While it is not presently known whether PDE2 is expressed in the ovary, it is conceivable that the high levels of cGMP induced by treatment with DETA/NO inhibit cAMP levels by increasing PDE2 activity in granulosa cells. Clearly, further studies are required to elucidate the diverse molecular and biochemical mechanisms mediating the inhibitory effects of NO and cGMP on steroidogenesis of granulosa cells.

The physiological relevance of these *in vitro* studies is suggested by the pattern of NOS expression in the ovary, and by findings utilizing mice lacking NOS expression. One isoform of NOS (iNOS) is expressed primarily in follicles that are functionally immature (Matsumi *et al.* 1998b), and iNOS expression decreases during follicular growth (Van Voorhis *et al.* 1995). This may suggest that the acquisition of steroidogenesis in mature follicles is associated with decreased production of NO. On the other hand, while transgenic mice with a knockout of the iNOS gene display relatively normal patterns of cyclicity and steroidogenesis, knockout of the eNOS gene results in higher E2 levels on proestrus, consistent with an inhibitory effect of NO on aromatase activity (Jablonka-Shariff *et al.* 1999b). In this regard, eNOS expression increases in mural granulosa cells during follicular development (Jablonka-Shariff & Olson 1997), indicating an inhibitory tone on estrogen production by granulosa cells. Together, these studies indicate that NO and cGMP play significant roles in the modulation of ovarian functions.

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