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Nitric Oxide Inhibits Aromatase Activity: Mechanisms of Action

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NO synthase is present in human ovarian granulosa-luteal cells and NO inhibits estradiol secretion by granulosa cells in culture. These findings suggest that NO is an autocrine regulator of ovarian steroidogenesis. The purpose of this investigation was to explore the mechanisms through which NO exerts an inhibitory effect on cytochrome P450 aromatase activity. To examine the effect of NO on aromatase mRNA levels, human granulosa-luteal cells were cultured in the presence or absence of the NO donor SNAP for 16 h. Using a probe for human aromatase, Northern blots revealed a 26% decrease in aromatase mRNA in cells exposed to SNAP. Because this modest decrease in mRNA is unlikely to explain a rapid and profound reduction in estradiol secretion that we have observed, we looked for direct effects of NO on cytochrome P450 aromatase activity. Aromatase activity was assayed in placental microsomes and granulosa-luteal cells by measuring the release of ^3H_2O from [1β-^{3}H] androstenedione. NO (10^{-4}–10^{-3}M), added as a saturated saline solution, reduced aromatase activity by as much as 90% in a concentration-dependent, non-competitive manner. In contrast, carbon monoxide (CO), a gas known to bind to the heme iron in aromatase, had no effect on aromatase activity when added alone nor could CO reverse the NO-induced inhibition of aromatase. These data suggest that NO binding to the heme is insufficient to inhibit aromatase activity. NO has been reported to alter protein function by reacting with the sulfhydryl group of cysteines, forming a nitrosothiol group. Because a cysteine sulfhydryl group is thought to participate in the catalytic mechanism of all P450 enzymes, experiments were designed to test whether NO might inhibit aromatase via such a mechanism. Addition of increasing amounts of mercaptoethanol, a chemical with free sulfhydryl groups, blocked the NO-induced inhibition of aromatase in microsomes. *N*-Ethylmaleimide, a chemical which covalently modifies sulfhydryl groups, reduced aromatase activity in a concentration-dependent manner. We conclude that NO inhibits aromatase both by decreasing mRNA for the enzyme and by an acute, direct inhibition of enzyme activity. We hypothesize that the direct inhibition occurs as a result of the formation of a nitrosothiol on the cysteine residue adjacent to the heme in aromatase. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

Endogenously derived nitric oxide (NO) plays important regulatory roles in a number of physiological processes including regulation of vascular tone, platelet function, neurotransmission, and host-defense mechanisms [1]. We have demonstrated that nitric oxide synthase (NOS) is present in human granulosa-luteal cells and the product of NOS, nitric oxide, inhibits estradiol secretion by granulosa-luteal cells in culture [2]. These data are suggestive that NOS may be involved in the regulation of steroidogenesis. The inhibitory effect of NO on estradiol secretion seems to be independent of guanylate cyclase activation since estradiol secretion from granulosa-luteal cells cultured with 8 bromo-cGMP was not inhibited [2]. The purpose of this report is to further explore the mechanisms behind the inhibitory effect of NO on aromatase activity.

NO exerts its biological effects through a variety of mechanisms. Many of the actions of NO are attributable to the binding of NO to iron-containing

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enzymes [3]. For example, NO binds to the heme in guanylate cyclase causing enzyme activation [4]. NO also exerts some of its biological effects by binding to the sulfhydryl group of cysteine which, if in the active site of an enzyme, can alter the enzyme's function [5]. This is reported to be the mechanism by which NO activates calcium-dependent potassium channels in smooth muscle cells [6]. Finally, NO has been reported to exert some of its biological effects by altering gene transcription [7].

Given the known mechanisms through which NO acts, we hypothesized that NO could potentially inhibit aromatase activity in granulosa-luteal cells by: (1) direct binding to the cytochrome P450 heme iron in aromatase displacing oxygen and thereby blocking enzyme activity; (2) binding to the sulfhydryl group of a key active site cysteine, forming a nitrosothiol group which could inhibit aromatase activity; and/or (3) inhibition of transcription of aromatase mRNA. Experiments were designed to test these hypotheses.

METHODS

Preparation of aromatase

Aromatase was prepared from both human granulosa-luteal cells and human placenta. Human granulosa-luteal cells were obtained from the follicular aspirates of women undergoing *in vitro* fertilization procedures as previously described [8]. During the course of the study, tissue samples from eight different human placentae were obtained within 1 h of delivery. To obtain partially purified aromatase, tissues were homogenized individually in ice cold 0.1 M Tris–HCl buffer, pH 7.4, with a Polytron homogenizer. The homogenates were centrifuged at $8000 \times g$ for 10 min. The postmitochondical supernatant was recovered and centrifuged at $35{,}000 \times g$ for 1.5 h to obtain microsomal pellets. The pellets were resuspended in 10 ml of Tris–HCl buffer and the protein content of the homogenates assayed using Coomassie Brilliant Blue G-250 (Bio-Rad Inc., Hercules, CA) and human serum albumin as a standard. Homogenates were aliquoted, snap-frozen in a dry ice-acetone bath, then stored at −70°C until use.

Aromatase assay

Aromatase activity was assayed by a modification of a tritiated water production assay which relies on the stereospecific release of tritium from [1β-^{3}H] androstenedione (New England Nuclear, 15–30 Ci/mmol as described) [9]. In brief, 100 μg of protein were incubated with 250 μM NADPH (Sigma), 6.5 mM glucose-6-phosphate (Sigma), 1.25 U glucose-6-phosphatase dehydrogenase (Sigma), and 6.25 mM $MgCl_2$ and 6 nM [1β^3H] androstenedione plus unlabeled androstenedione to make a final concentration of 50 nM androstenedione. For comparison, selected parallel experiments were also performed with a saturating substrate concentration (10 μM androstenedione) to assure that proportional results were obtained when either saturating or unsaturating substrate concentrations were used. Test substances were added in volumes of 50–250 μl. The total volume of the assay was 500 μl. The assay mixture was incubated at 37°C in a shaking water bath under air. Assays were stopped after 2–15 min by the addition of cold chloroform. The aqueous phase was subsequently extracted with dextran-coated charcoal and an aliquot of that supernatant taken for scintillation counting to determine the production of ^3H_2O as the index of aromatase activity. Assay mixtures containing no microsomal protein were extracted and the activity in these blank assays was subtracted from all tubes. All data were corrected to obtain total activity by using the ratio of unlabeled/labeled substrate. Recovery of ^3H_2O averaged 32% as determined in separate assay tubes run in each experiment. All assay data points were performed in duplicate. Experimental variables were repeated a minimum of three times in separate experiments.

Preparation and culture of cells

Human granulosa-luteal cells were obtained from women undergoing oocyte retrieval for assisted reproductive techniques as previously described [8]. Briefly, ovarian follicular aspirates were combined and granulosa-luteal cells were separated from contaminating red blood cells by a 30 min, $3000 \times g$ centrifugation step over a 50% (vol/vol) Percoll/saline solution (Pharmacia LKB Biotechnology, Piscataway, NJ). Granulosa-luteal cells were cultured at a concentration of 2×10^7 cells per well in Medium 199 (M199; Gibco-BRL Life Technologies, Grand Island, NY) and 10% fetal calf serum for 48 h to allow adherence. This culture medium was replaced with M199 containing 0.2% bovine serum albumin and 10^{-7} M androstenedione. Experimental dishes had 10^{-4} M SNAP or human chorionic gonadotropin added at 100 ng/ml. After 16 h, the culture medium was removed and estradiol was measured by radioimmunoassay. RNA was extracted from the granulosa-luteal cells attached to the plate.

Northern analysis

Total RNA was isolated using a single-step guanidinium thiocyanate-phenol-chloroform extraction described by Chomczynski and Sacchi [10]. RNA was quantitated by optical density and 10 μg samples of total RNA were subjected to electrophoresis through a 1.2% agarose-formaldehyde gel containing ethidium bromide. The resolved RNA was transferred on to a Nytran membrane by capillary transfer and fixed by u.v. irradiation of the membrane for 3 min and air drying overnight. A human aromatase cDNA probe was kindly provided by Dr Evan R. Simpson, Dallas, TX. This cDNA probe was labeled with ^{32}P using a random

primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The Nytran membrane was prehybridized for 6 h at 42°C with 50% formamide followed by overnight hybridization at 42°C with the aromatase cDNA probe (specific activity = 8×10^8 CPM/μg probe). After hybridization, membranes were washed twice at room temperature in 2 SCC, followed by two more washes in 1 SSC and 1% SDS at 65°C for 30 min, followed by one wash in 0.1 SSC at room temperature. The membrane was then exposed to X-ray film. Relative quantities of mRNA were compared by densitometry using a scanning densitometer and ImageMaster™ software from PDI, Inc. Equal loading of total RNA was ensured by comparing the intensity of the 28s and 18s ribosomal RNA bands detected on the membrane by ethidium bromide staining.

Chemicals

NO- and CO-saturated solutions were prepared by bubbling NO or CO anaerobically through oxygen-depleted saline in a gas-tight flask. A saturated aqueous solution of NO has a molarity of approximately 2.0 mM and a saturated solution of CO has a molarity of approximately 1.0 mM [11]. Gas-saturated solutions (50–250 μl) were injected directly into the assay tubes using gas-tight microliter syringes. The concentrations of gases given in the text and figures are based on these reported data and are thus approximations, not measured values. *S*-Nitroso-l-acetylpenicillamine [SNAP] was a gift of H. Hodson, Wellcome Research Laboratories, Beckenham, U.K. *N*-Ethylmaleimide[NEM], and mercaptoethanol were purchased from Sigma Inc., St Louis, MO.

RESULTS

The cellular level of aromatase is thought to be regulated primarily by changes in gene transcription [12]. Because NO reduced estradiol production from cultured granulosa-luteal cells, we examined the effect of NO on aromatase gene expression. Human granulosa-luteal cells cultured for 16 h in the presence of 10^{-4} M SNAP, an NO donor, had an average decrease of 26% in aromatase mRNA by Northern analysis as compared to untreated cells (Fig. 1). We reported previously that 10^{-4} M SNAP reduced estradiol secretion by 70% during an overnight culture [2]. Over the same period of time, human chorionic gonadotropin increased mRNA for aromatase by 155%. In other experiments, measurable reductions in aromatase activity by cultured cells were observed within 1 h of adding SNAP (data not shown). The rapid onset of inhibition by NO and the relatively small decrease in aromatase mRNA apparent even after an overnight culture lead to the hypothesis that NO affects aromatase activity directly.

Pilot experiments were performed using microsomes from both human granulosa-luteal cells and human placenta. NO reduced aromatase activity identically, regardless of tissue source. Data reported herein were obtained from experiments using microsomes derived from the placenta due to the larger quantities and greater availability of placental tissue.

NO inhibited microsomal aromatase activity in a dose-dependent fashion between 0.2 mM and 1 mM concentrations. Aromatase activity was reduced by as much as 80%. In contrast, carbon monoxide (CO) at approximately the same concentrations as NO had no effect on aromatase activity (Fig. 2). In a second series of experiments, microsomes were pre-incubated with 0.5 mM CO for 30 s before adding substrate and 0.8 mM NO. Despite prior exposure of the microsomal fraction to CO, aromatase activity was reduced by 75%. This is the same extent of inhibition as observed in the absence of CO (Fig. 3).

NO inhibited aromatase activity at all concentrations of androstenedione tested (Fig. 4). A Lineweaver–Burke plot of the data (see insert) revealed non-competitive inhibition with the maximum velocity reduced from approximately 100 fmol/mg protein/4 min to 20 fmol/mg protein/4 min in the presence of 0.8 mM NO. The saturating concentration of androstenedione for partially purified aromatase was determined to be ~8 μM for 100 μg of granulosa cell or placental microsomal protein. Identical effects of NO on aromatase activity were observed at both unsaturated and saturating concentrations of androstenedione over short incubation periods (results not shown).

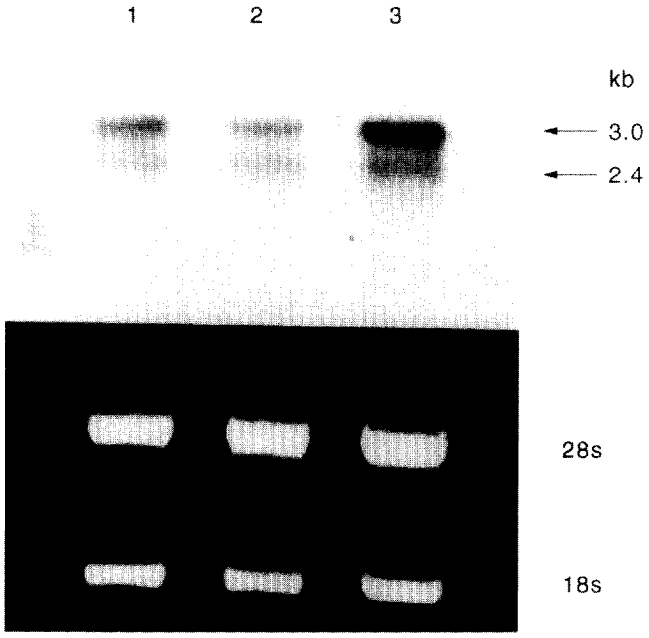

Fig. 1. Northern blot of aromatase mRNA from granulosa-luteal cells cultured *in vitro* in the absence (lane 1) or presence (lane 2) of 10^{-4} M SNAP for 16 h. Lane 3 is aromatase mRNA from cells cultured in the presence of 100 ng/ml human chorionic gonadotropin. Molecular weight markers are expressed as kilobases (kb) of single stranded DNA.

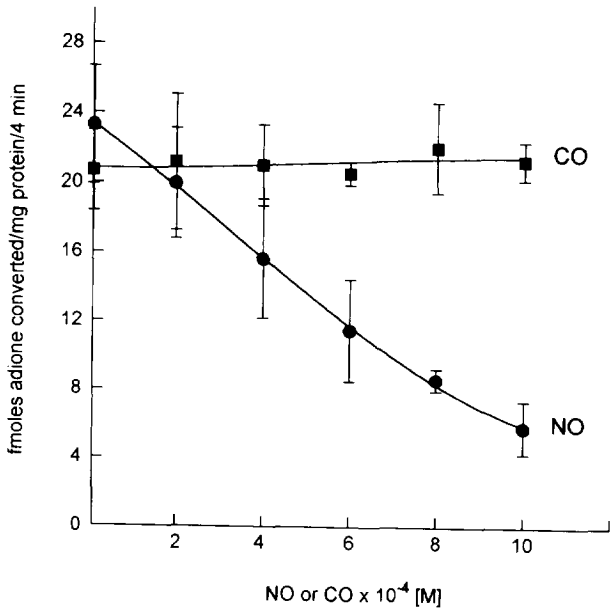

Fig. 2. The effect of NO and CO concentration on the activity of microsomal aromatase. NO- or CO-saturated saline was added to partially purified aromatase in microsomal preparations and the assay incubated for 4 min. Data represent the mean ± the standard error of the mean of six separate experiments, each point assayed in duplicate.

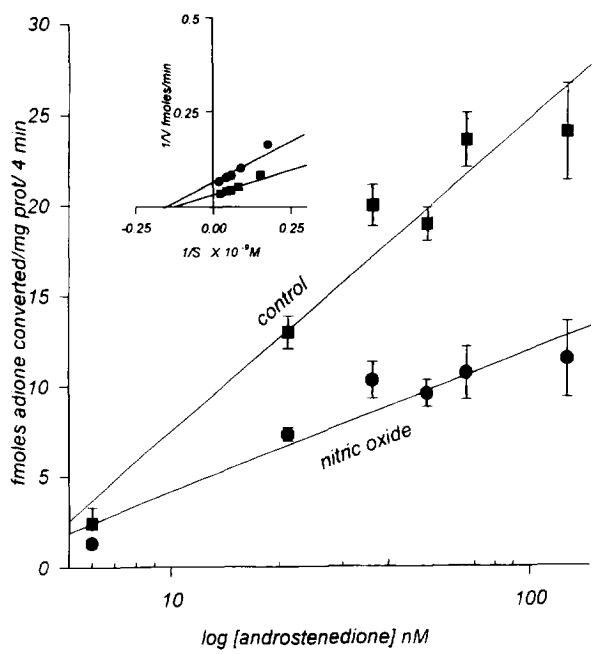

Fig. 4. The effect of 0.8 mM NO on aromatase enzyme velocity at varying substrate concentrations. The insert is a Lineweaver–Burke plot of the same data demonstrating non-competitive inhibition of aromatase by NO. Data represent the mean of three separate experiments, each point assayed in duplicate.

Cytochrome P450 enzymes have a conserved cysteine residue whose sulfhydryl group projects toward the heme. The sulfur is thought to interact with the iron during the catalytic process [19]. We hypothesized that NO may inhibit aromatase by binding to the conserved sulfhydryl group in the enzyme and thus blocking electron transfer. To test this hypothesis, mercaptoethanol, which has a free reactive sulfhydryl group, was included in the aromatase assay buffer. In sufficient concentration, mercaptoethanol would be expected to block the inhibitory effect of NO on aromatase. Mercaptoethanol was added in increasing concentrations (10^{-9}–10^{-4} M) to aromatase assays in the absence or presence of 0.8 mM NO. The presence of mercaptoethanol blocked the inhibitiory action of NO on aromatase in a concentration-dependent fashion. No concentration of mercaptoethanol tested had any effect on aromatase activity in the absence of NO (Fig. 5).

N-Ethylmaleimide [NEM], an agent that has been used widely to acetylate and render sulhydryl groups inactive [16], was added to aromatase assays at concentrations ranging from 10^{-6} M to 10^{-2} M (Fig. 6A). The addition of NEM reduced aromatase activity by greater than 60% at the highest concentration tested (10^{-2} M). The rate of aromatase inactivation by NEM is rapid. Approximately half of the overall inhibition due to NEM was expressed within 4

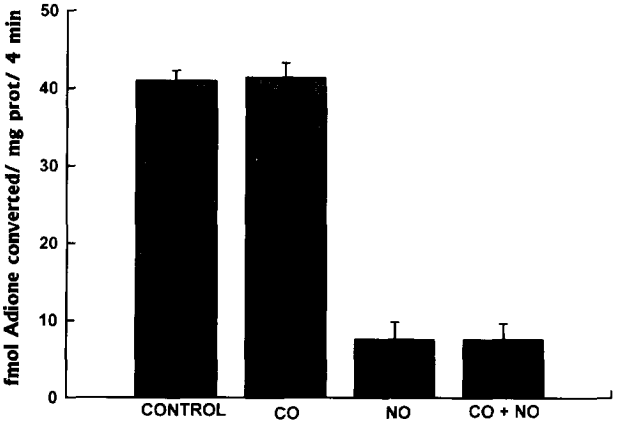

Fig. 3. The effect of preincubation of microsomes with 0.5 mM CO. Microsomes were preincubated with CO for 30 s prior to the addition of substrate and 0.8 mM NO. Data represent the mean ± the standard error of the mean from two separate experiments, each point assayed in duplicate.

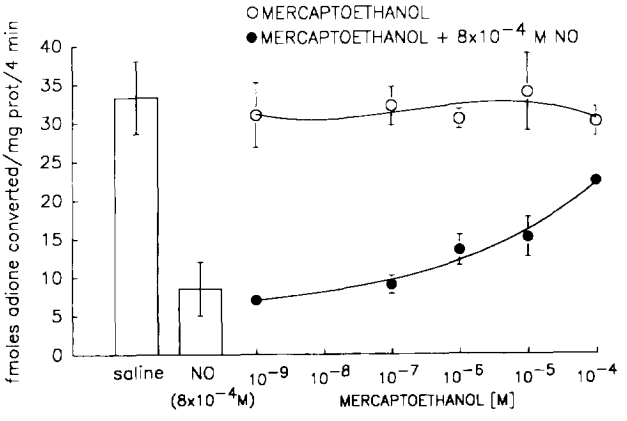

Fig. 5. The effect of increasing concentration of mercaptoethanol on the inhibition of aromatase by 0.8 mM NO. Data are the mean ± the standard error of the mean from three experiments each point assayed in duplicate.

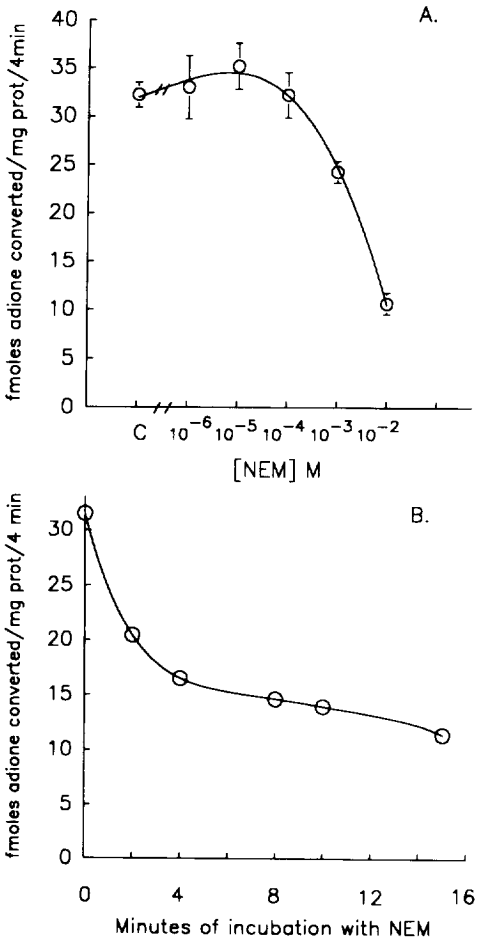

Fig. 6. (A) The effect of increasing concentration of a thiol acetylating agent, *N*-ethylmaleimide (NEM), on aromatase activity. (B) The rate of inactivation of aromatase by NEM. Data represent the mean of three separate experiments, each point assayed in duplicate.

min. The initial rapid decline in enzyme activity was followed by a slower rate of enzyme inactivation through 15 min of preincubation with NEM (Fig. 6B). Other acetylating agents, *p*-chloromercuriphenylsulfonic acid (10^{-6}–10^{-4}M) and iodoacetamide (10^{-4}–10^{-2} M) also reduced aromatase activity in a manner parallel to NEM (data not shown).

Glucose-6-phosphate dehydrogenase also contains a cysteine residue associated with the active site of the enzyme. Because this enzyme is used to regenerate NADPH in the aromatase assay used in this study, the possibility of NO affecting the regeneration of NADPH and thus causing a reduced activity measurement of aromatase was of concern. Separate aromatase assays were performed with a 10 times normal concentration of NADPH added to the assay buffer instead of an NADPH-regeneration system. As seen in previous assays, the addition of 0.8 mM NO reduced aromatase activity 53% (Fig. 7). This demonstrated that NO had a direct affect on aromatase in the absence of G6PDH, a potential NO target.

Data from Wink *et al.* [11] suggest that NO inhibition of hepatic cytochrome P450 activity is, in part, irreversible. NO (0.8 mM) was added to the aromatase assay mixture for periods ranging from 2 to 25 min (37°C under air) prior to initiation of the assay by addition of substrate. The rate of conversion of androstenedione to estrone and ^3H_2O, measured in the subsequent 2 min, was unaffected by the period of time NO was present (see Table 1). Inhibition of the aromatase activity by NO was greater than 60% at all preincubation times tested. No reversibility of the NO-induced aromatase inhibition was observed for 25 min after NO was added to the assay.

DISCUSSION

Estradiol is synthesized by cytochrome P450 aromatase which converts androgens to estrogens. Changes in the level of estrogen biosynthesis are closely related to changes in the transcription of aromatase [12]. Therefore, research regarding the regulation of estrogen biosynthesis has been focused mainly on the control of transcription of the aromatase gene [12, 14]. In contrast, there is little information regarding post-transcriptional regulation of aromatase activity by autocrine or paracrine factors. We have previously shown that NOS is localized in human granulosa-luteal cells and that NO inhibits aromatase activity [2]. The results of this paper suggest that NO may inhibit aromatase activity both by decreasing aromatase mRNA levels and by a direct inhibitory effect on the enzyme. Inhibition of aromatase transcription by NO is unlikely to account for all of the inhibition in estradiol secretion that we have observed in cell cultures. Although aromatase mRNA was decreased by 26% after 16 h of exposure to NO, the degree of inhibition was not nearly as great as the inhibition in estradiol

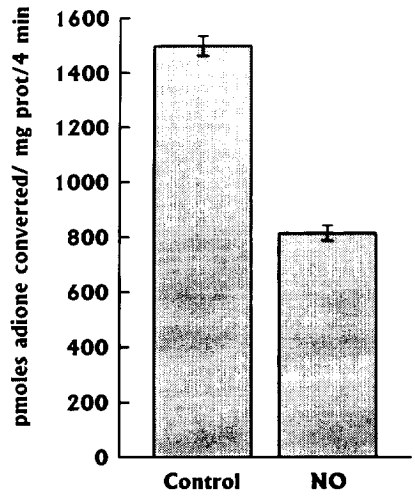

Fig. 7. The effect the addition of 0.8 mM NO to aromatase assays in the absence of the NADPH regenerating system (e.g. glucose-6-phosphate and glucose-6-phosphate dehydrogenase) but containing a 10-fold excess of NADPH (10 mM) and a saturating concentration of substrate (androstenedione, 10 μM).

Table 1. Rate of aromatase activity after preincubation with 0.8 mM NO

	Picomol adione converted/mg prot/2 min					
	2 min	5 min	10 min	15 min	20 min	25 min
Control	406 ± 23	495 ± 33	471 ± 70	315 ± 28	356 ± 9	354 ± 12
0.8 mM NO	107 ± 9	154 ± 9	90 ± 26	78 ± 2	85 ± 13	81 ± 5

Data are the mean and standard error of four replicate assays from one of two experiments in which placental microsomes were incubated with NO at 37°C for the times stated. A saturating concentration (10 μM) of androstenedione was added for 2 min after each time interval

secretion seen in these cultures. In addition, we have seen significant inhibition in aromatase activity in cultured granulosa-luteal cells after only 1 h of NO exposure [unpublished data]. This effect is likely to be due to direct inhibition of aromatase by NO rather than alterations in aromatase mRNA levels.

Several independent lines of evidence suggest that NO directly inhibits aromatase activity by nitrosylation of cysteines. NO inhibited aromatase in microsomes in a dose-dependent and non-competitive fashion. Carbon monoxide, which is known to bind the ferrous heme group of cytochrome P450 enzymes, had no inhibitory effect on aromatase activity. This result is consistent with previous reports [15, 16]. When microsomes were pretreated with CO to occupy the heme, NO reduced aromatase activity by 73%, the same extent as if CO was not present. Therefore, it is unlikely that the activity of aromatase is regulated completely by interaction of NO with the heme. NEM, a chemical that covalently acetylates sulfhydryl groups [13], also inhibited aromatase activity in a dose-dependent fashion. Since modification of sulfhydryl groups in aromatase blocks enzyme activity and NO is known to bind sulfhydryl groups to form nitrosothiols, we propose that this mechanism may be a second mechanism for NO's inhibitory effect. Indirect evidence for this hypothesis is provided by the experiments in which the magnitude of the NO-induced inhibition of aromatase was reduced by the addition of mercaptoethanol which contains sulfhydryl groups that can compete with the cysteines of aromatase for NO. Finally, there is relatively little evidence of reversibility in the microsomal assay for at least 25 min. NO has an extremely short half life (5.2 s) in the presence of oxygen [23]; thus an effective concentration in solution sufficient to maintain NO presence on the heme would be expected to be maintained only briefly. In contrast, because the nitrosylthiol is formed through a covalent linkage [24], it would be expected that any inhibition of aromatase activity due to the formation of a nitrosylthiol would be maintained much longer than if the inhibition were due only to NO binding to the heme. This latter inhibition, which is dependent on the NO concentration in the assay buffer, should be rapidly reversible as NO is either oxidized or diffuses from the assay buffer. These data, combined with the data from NEM and mercaptoethanol experiments, are strongly supportive of a hypothesis that the formation of a nitrosylthiol has a significant role in blocking P450 aromatase activity.

Molina *et al.* [25] have reported that glucose-6-phosphate dehydrogenase (G6PD, the enzyme that the enzyme used to regenerate the supply of NADPH in the aromatase assay) has a cysteine residue that affects the active site of this enzyme in much the same manner as we are proposing for aromatase. To test whether the inhibition of aromatase activity observed in the microsomal assay was due in part to an effect of NO on the G6PD we incubated NO with the microsomal protein for 30 s and then added substrate at saturating concentrations and 10 times the normal concentration of NADPH to initiate the assay. The effect of the addition of 0.8 mM NO was the reduction of aromatase activity by 53%, which was a smaller reduction than when 0.8 mM NO was added to the complete assay system. These experiments demonstrated that NO affects the microsomal cytochrome P450 aromatase directly. Because in every experiment where NO was added to the complete assay system, aromatase activity was reduced by > 60%, the difference in activity between these two protocols might be explained by the additional action of NO on G6PD to reduce the availability of NADPH (Fig. 7).

The hypothesis that nitrosylation of sulfhydryl groups in cysteine causes aromatase inhibition requires the presence of a conserved cysteine on the enzyme. There are several cysteines in aromatase including one crucial cysteine at amino acid 437 in the active site of the enzyme [17]. The importance of this cysteine has been demonstrated by the complete absence of aromatase activity in a patient with a missense mutation coding for a tyrosine rather than the conserved cysteine at this location [17]. *In vitro* mutational analysis has confirmed the importance of this amino acid for aromatase function [18]. All cytochrome P450 enzymes have a required cysteine residue that is positioned below and in close association with the heme iron [19]. There is evidence that the interaction of the heme iron and the cysteine sulfhydryl provides a "catalytic push" by transferring electrons to a peroxide during the oxidation of the substrate [20]. The active site configuration of aromatase is consistent with the presence of a reactive sulfur in the active site of the enzyme [20].

NO has been shown to inhibit hepatic cytochrome P450 enzymes both *in vitro* and *in vivo*. Wink *et al.* [11] demonstrated that exogenously applied NO inhibited

the *in vitro* activity of the hepatic cytochrome P450 enzyme CYP2B1. Stadler *et al.* [21] reported that endogenously produced NO-inhibited cytochrome P450 metabolism of hepatocytes in culture. Khatsenko *et al.* [22] has demonstrated that NO inhibits cytochrome P450 dependent metabolism *in vivo* in the livers of rats treated with immunostimulants. All groups speculated that the mechanism of NO's inhibitory action was via the binding of hemes in these enzymes since NO is known to bind to both ferric and ferrous forms of heme in P450 enzymes. In addition, Wink *et al.* [11] speculated that formation of nitrosothiols could be contributing to the inhibition that they observed and Stadler *et al.* [21] reported an NO-dependent inhibition of cytochrome P450 enzyme gene transcription.

We demonstrated that a steroidogenic P450 enzyme can be inhibited by NO *in vitro*. Our results suggest that NO inhibits aromatase both by a direct effect on the enzyme and indirectly by altering mRNA levels for the enzyme. This finding is consistent with a dual mechanism of action found for NO's inhibitory effect on hepatic cytochrome P450 enzymes [21] and for the "feedback" inhibition of NO on NOS activity [7]. Whether or not NO inhibits ovarian steroidogenesis *in vivo* is the subject of ongoing investigation.

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REFERENCES

1. Monacada S., Palmer R. M. J. and Higgs E. A.: Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmac. Rev.* **43** (1991) 109–142.
2. Van Voorhis B. J., Dunn M. S., Snyder G. D. and Weiner C. P.: Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology* **135** (1994) 1799–1806.
3. Ignarro L. J.: Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu. Rev. Pharmacol. Toxicol.* **30** (1990) 535–560.
4. Ignarro L. J., Adams J. B., Horwitz P. M. and Wood K. S.: Activation of soluble guanylate cyclase by NO-hemoproteins involves NO-heme exchange. Comparison of heme-containing and heme-deficient enzyme forms. *J. of Biol. Chem.* **261** (1986) 4997–5002.
5. Stamler J. S.: Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* **78** (1994) 931–936.
6. Bolotina V. M., Najibi S., Palacino J. J., Pagano P. J. and Cohen R. A.: 1994 Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Science* **368** (1994) 850–853.
7. Park S. K., Lin H. L. and Murphy S.: Nitric oxide limits transcriptional induction of nitric oxide synthase in CNS glial cells. *Biochem. Biophys. Res. Comm.* **201** (1994) 762–768.
8. Van Voorhis B. J., Dunn M. S., Falck J. R., Bhatt R. K., Van Rollins M. and Snyder G. D.: Metabolism of arachidonic acid to epoxyeicosatrienoic acids by human granulosa cells may mediate steroidogenesis. *J. Clin. Endorcinol. Metab.* **76** (1993) 1555–1559.
9. Lephart E. D. and Simpson E. R.: Assay of aromatase activity. *Meth. Enzymol.* **206** (1991) 477–483.
10. Chomczynski P. H. and Sacchi N.: Single-step methods of RNA isolation by guanidinium thiocynate-phenol-chloroform extraction. *Analyt. Biochem.* **162** (1987) 156–160.
11. Wink D. A., Osawa Y., Darbysbire J. F., Jones C. R., Eshenaur S. C. and Nims R. W.: Inhibition of cytochromes P450 by nitric oxide and a nitric oxide releasing agent. *Archs Biochem. Biophys.* **300** (1993) 115–123.
12. Simpson E. R., Mahendroo M. S., Means G. D., Kilgore M. W., Hinshelwood M. M., Grahm-Lorence S., Amarneh B., Ito Y., Fisher C. R., Michael M. D., Mendelson C. R. and Bulun S. E.: Aromatase Cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr. Rev.* **15** (1994) 342–355.
13. Smyth D. G., Blumenfeld O. O. and Konigsberg W.: Reactions of N-ethylmaleimide with peptides and amino acids. *Biochem. J.* **91** (1964) 589–595.
14. Mendelson C. R., Evans C. T. and Simpson E. R.: Regulation of aromatase in estrogen-producing cells. *J. Steroid Biochem.* **27** (1987) 753–757.
15. Canick J. A. and Ryan K. J.: Properties of the aromatase enzyme systems associated with the mitochondrial fractions of human placenta. *Steroids* **32** (1978) 499–509.
16. Meigs R. A. and Ryan K. J.: Enzymatic aromatization of steroids. *J. Biol. Chem.* **246** (1971) 83–87.
17. Conte F. A., Grumbach M. M., Ito Y., Fisher C. R. and Simpson E. R.: A syndrome of female pseudohermaphroditism, hypergonadotropic hypogonadism and multicyctic ovaries associated with missense mutations in the gene encoding aromatase. *J. Clin. Endocr. Metab.* **78** (1994) 1287–1292.
18. Ito Y., Fisher C. R., Conte F. A., Grumbach M. M. and Simpson E. R.: Molecular basis of aromatase deficiency in an adult female with sexual infantilism and polycystic ovaries. *Proc. Natl Acad. Sci. USA* **90** (1993) 11673–11677.
19. Black S. D. Coon M. J.: Comparative structures of P-450 cytochromes. In *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Edited by P. R. Oritz de Montellano) Plenum Press, New York and London, (1986) pp. 161–216.
20. Poulos T. L.: The crystal structure of cytochrome P-450$_{cam}$ In: *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Edited by P. R. Oritz de Montellano) Plenum Press, New York and London, (1986) pp. 161–216.
21. Stadler, J., Trockfeld, J., Schmalix, W. A., Brill, T., Siewert, J. R., Greim, H., and Doehmer, J.: Inhibition of cytochromes P4501A by nitric oxide, *Proc. Natl Acad. Sci. USA*, **91** (1994) 3559–3563.
22. Khatsenko, O. G., Gross, S. S., Rifkind, A. B. and Vane, J. R.: Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants, *Proc. Natl Acad. Sci. U.S.A.* **90** (1993) 11147–11151.
23. Kelm M. and Schrader J.: Control of coronary vascular tone by nitric oxide. *Circ. Res.* **66** (1990) 1561–1575.
24. Radi R., Beckman J., Bush K. and Freeman B. A.: Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266** (1991) 4244–4250.
25. Molina y Vedia L., McDonald B., Reep B., Brune B., Di Silvio M., Billiar T. R. and Lapetina E. G.: Nitric oxide-induced S-nitrosylation of gylceraldehyde-3-phosphate dehydrogenasee inhibits enzymatic activity and increases endogenous ADP-ribosylation. *J. Biol. Chem.* **267** (1992) 24929–24932.