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Nitric Oxide Inhibits Leydig Cell Steroidogenesis*

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ABSTRACT

Testicular macrophages as well as endothelial cells, which are intimately associated with Leydig cells, constitute a potential source of paracrine nitric oxide (NO) in the testis. In the present study, we investigated the effect of NO donors on MA-10 murine Leydig tumor cell line and rat Leydig cell steroidogenesis. We show that NO donors inhibit human CG-induced steroidogenesis in both type of cells. We also studied NO mechanism of action. Contrary to what is observed in many other systems, NO inhibitory effect on Leydig cell steroidogenesis is not mediated by cyclic GMP (cGMP) because NO fails to increase cGMP production, and cGMP analogs do not reproduce NO effect. NO does not modify the production of cAMP, the main second messenger that mediates gonadotropin action. When we studied NO effect over the steroidogenic pathway in MA-10 cells, we found that NO was inhibiting the conversion of cholesterol to pregnenolone. Taken together these results show an inhibitory effect of NO donors on Leydig cell steroidogenesis, and suggest that NO can be directly inhibiting cholesterol side-chain cleavage enzyme (cytochrome $P450_{scc}$) as it does with other heme proteins, including different cytochromes P450. (*Endocrinology* **137:** 5337–5343, 1996)

NITRIC OXIDE (NO), which is synthesized from L-arginine by the enzyme NO synthase (NOS), is a highly chemically reactive inorganic free radical gas. This small molecule has been known to be involved in the regulation of a wide range of biological functions such as vasodilatation and regulation of normal vascular tone, inhibition of platelet aggregation, neuronal transmission, and cytostasis (for reviews see Refs. 1 and 2). NO exerts many of its physiological actions by directly reacting with heme or nonheme iron and iron-sulfur complexes located either at the active or allosteric site of key cellular enzymes, activating or inhibiting them (3, 4). For example, the activation of heme-containing soluble guanylate cyclase mediates NO effects in many systems. Cytochromes P450 of Leydig cell steroidogenic pathway are also heme-enzymes, and we thought they might also be possible targets for NO.

NO also seems to play a role in the regulation of endocrine systems because it has been reported to modulate secretion of some hormones such as insulin (5), LHRH (6), GH (7), PRL (8), and LH (9). As far as steroidogenic systems are concerned, it has been shown that endogenously produced NO, as well as NO-releasing agents inhibit steroidogenesis in granulosa-luteal cells (10). NO can be produced by many different cell types, including vascular endothelial cells, macrophages, and mast cells (1, 2). These cell types are present in the interstitium of the testis, representing a potential source of NO release in this organ. Indeed, NO synthesis in the testis has been recently reported (11). Leydig cells are generally found forming clusters of cells around blood vessels where endothelial cells are present (12). Testicular macrophages, which are known to modulate Leydig cell biosynthesis of steroids (13), are physically associated with them (12, 14). This close relation between NO-producing cells and Leydig cells suggests a possible role for NO in the paracrine regulation of Leydig cell steroidogenesis. In addition, immunoreactivity for at least one isoform of NOS has been observed in human and MA-10 Leydig cells, indicating that NO could also be produced by these cells (15).

The present series of experiments examined whether NO could exert any effect on steroid biosynthesis in Leydig cells and studied its mechanism of action. The results showed a direct effect of exogenously added NO on Leydig cell steroidogenesis, suggesting a possible paracrine/autocrine role for this molecule on the regulation of testicular steroidogenesis.

Materials and Methods

Materials

Purified human CG (hCG) (CR-127, 14, 900 IU/mg) was a gift from the National Hormone and Pituitary Program, NIDDK, NIH (Bethesda, MD) and human LH (hLH) (batch 161178, 7600 IU/mg) was provided by Dr. P. Torjensen (Hormonlab Aker Hospital, Oslo, Norway). Diethylamine/nitric oxide complex sodium salt (DEA/NO), diethylenetriamine nitric oxide adduct (DETA/NO), and S-nitroso-N-acetylpenicilamine (SNAP) were purchased from Research Biochemicals International (Natick, MA). The NO releasing abilities for DEA/NO (16–18), DETA/NO (16, 17), and SNAP (10, 19, 20) have been documented. Metrizamide, atrial natriuretic peptide (atriopeptin II), ferrous hemoglobin, 22R-hydroxycholesterol, cAMP, cyclic GMP (cGMP), $(Bu)_2$cAMP, $(Bu)_2$cGMP, 8-bromo-cGMP, 2′-O-monosuccinyladenosine-3′,5′-cyclic monophosphate tyrosyl methyl ester (TME-cAMP), and 2′-O-monosuccinylguanosine-3′,5′-cyclic monophosphate tyrosyl methyl ester (TME-cGMP) were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase was from Worthington (Freehold, NJ). Cell culture supplies and plasticware were obtained from GIBCO-BRL (Gaithersburg, MD) and Corning (Corning, NY), respectively. $Na^{125}I$ was from Dupont-NEN (Boston, MA). TME-cAMP and TME-cGMP were radiolabeled with $Na^{125}I$ in our lab by the method of chloramine-T (sp. act.: 600 Ci/mmol), described by Birnbaumer (21). Specific antibodies for

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cAMP and cGMP were purchased from Chemicon (Temecula, CA). Other reagents used were of the best grade available and were obtained from commonly used suppliers.

Cellular culture of MA-10 Leydig cells

MA-10 cells, a clonal strain of cultured mouse Leydig tumor cells, which secrete progesterone rather than testosterone as a major steroid, were generously provided by Dr. Mario Ascoli (University of Iowa College of Medicine). The origin and handling of the MA-10 cells have been described (22, 23). Cells were plated in 24 × 16-mm well plates on day 0, at a density of 7.5×10^4 cells/well, and in a total volume of 1 ml growth medium (Waymouth MB752/1 modified to contain 1.1 g/liter $NaHCO_3$, 20 mM HEPES, 50 μg/ml gentamycin, and 15% horse serum, pH 7.4). The wells were maintained in a humidified atmosphere containing 5% CO_2 and were used on day 3. At this time, the cell density was about 6×10^5 cells/well. On this day, the cells were washed twice with 1 ml warm serum-free medium supplemented with 1 mg/ml BSA (assay medium). Incubations were performed in a total volume of 1 ml assay medium at 37 C with the corresponding additions as described in each figure or table. After a total of 4.5 h incubation, medium was removed and kept to determine progesterone, cAMP, and cGMP. Progesterone was measured by RIA in suitable aliquots as described (24, 25). cAMP and cGMP were determined as indicated below.

Preparation of rat Leydig cells and testosterone production in vitro

Isolated Leydig cells were prepared by collagenase dispersion of testis from 60-day-old male Sprague-Dawley rats (200–250 g) (Charles River descendants, Animal Care Lab, Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina) as previously described (26, 27). The animals had full access to water and to Purina formula chow. They were housed (3 rats/cage) under controlled room temperature (24 C) and lighting (lights on, 0700–1900 h). Animals were killed by decapitation according to protocols for animal use, approved by the institutional animal care and use committee (IBYME-CONICET) that follows the NIH guidelines. Interstitial cells were resuspended in 5 ml of a 1.7 mM Tris, 140 mM NH_4Cl solution, pH 7.2, as described (28, 29) and incubated for 10 min at 34 C. Incubation was terminated by dilution with medium 199–0.1% BSA and centrifuged. This procedure eliminates any interference caused by the presence of red blood cells and does not affect the cell response to gonadotropin stimulation (28). The cell pellet was resuspended in 10 ml medium 199–0.1% BSA and purified by fractionation on 16% metrizamide (26, 28). The purity of Leydig cells obtained was over 87% as assessed by histochemical staining for 3β-hydroxysteroid dehydrogenase activity (30). Cells were collected and resuspended in medium 199, containing 1.2 g/liter $NaHCO_3$, 20 mM HEPES, 0.1 mM 1-methyl-3-isobutylxanthine (MIX), and 0.5% BSA. The incubation was done in plastic tubes in a volume of 600 μl (3×10^5 cells/tube), at 34 C with shaking, and for a total of 3.5 h as described in Fig. 8. The samples were centrifuged at 1000 × *g* for 10 min, and the supernatants were stored at −20 C. Testosterone was determined by RIA from the unextracted medium (27, 28).

[^{35}S]Methionine incorporation to cellular and secreted proteins

MA-10 cells were preincubated with or without DEA/NO for 30 min as described in Table 1. [^{35}S]Methionine was then added (in the absence or presence of hCG). After a total of 4.5 h of incubation at 37 C, media were collected (for secreted proteins), and cellular extracts were obtained with 300 μl 0.2% SDS for 10 min (for cellular proteins). Aliquots of the incubation media and of the SDS extracts were precipitated with trichloracetic acid (TCA) as follows. Ten microliters of the samples were mixed with 1 ml PBS containing 1 mg/ml BSA at 0 C. One milliliter 40% TCA was then added, and samples were kept in ice to precipitate for 15–20 min. After that the samples were filtered through Whatman (Maidstone, UK) GF-A and were washed twice with 1 ml TCA 20% and once with 1 ml cold ethanol. The incorporated radioactivity was measured in a liquid scintillation counter.

TABLE 1. [^{35}S]Methionine incorporation into proteins in MA-10 cells treated with an NO donor

Treatment[a]	Incorporated [^{35}S]methionine (cpm)	
	Secreted proteins	Cellular proteins
Control	10594 ± 487	36745 ± 5467
DEA/NO	9804 ± 654	40087 ± 3220
hCG	9967 ± 355	42432 ± 3125
hCG + DEA/NO	10265 ± 412	39965 ± 3664

[a] Cells were preincubated with or without DEA/NO (1 mM) for 30 min. [^{35}S]Methionine was added in the presence or absence of hCG (10 ng/ml) and incubation was continued for 4 h. [^{35}S]Methionine incorporation (cpm) was measured in aliquots of SDS-cellular extracts (cellular proteins) and incubation media (secreted proteins). Results from a representative experiment are shown. Results represent mean ± SEM of three culture wells per condition.

Determination of intracellular and extracellular cAMP and cGMP in MA-10 Leydig cells

The methodology for the treatment of the cells and the determination of intracellular and extracellular cAMP and cGMP have been previously published (31–33).

After incubation at the indicated times (20 min or 4.5 h), the wells were placed on ice, and the medium was quickly aspirated and kept to determine extracellular cAMP and cGMP. One milliliter absolute ethanol was then added, and the cells were scraped and disrupted by ultrasonic oscillation. The extracts were heated for three periods of 1 min each in boiling water to destroy endogenous protein kinase. After centrifugation in an Eppendorf microfuge (Brinkmann Instruments, Westbury, NY) for 3 min, supernatants were evaporated by heating at 45C under N_2. The extracts were resuspended in 50 mM sodium acetate buffer (pH 6.0). Unknown samples and standards of cAMP or cGMP were acetylated and assayed by RIA using the protocol described by Steiner *et al.* (34) modified as follows (31–33). At the end of the incubation, the antigen-antibody complexes were precipitated by the addition 50 μl 2% BSA and 2 ml cold ethanol (95%) and centrifuged at 2000 rpm for 20 min, supernatants were aspirated, and pellets were counted in a Packard Auto-Gamma (Packard Instrument Co., Downers Grove, IL).

Statistical analysis

Statistical analysis between means was calculated by ANOVA followed by the Tukey-Kramer's multiple comparisons test. $P < 0.05$ was considered significant.

Most of the experiments shown in this study were done with the three NO donors: DEA/NO, DETA/NO, and SNAP, obtaining similar results with all of them. Except for Fig. 1, we show the effect of only one NO donor, DEA/NO, to make the figures easier to understand.

Results

Effects of NO-releasing agents on MA-10 Leydig cell progesterone production

To assess the possibility that NO may affect progesterone production, MA-10 Leydig tumor cells were preincubated with increasing concentrations of different NO-releasing agents (SNAP, DETA/NO, or DEA/NO: 0–1 mM, 30 min) before being stimulated with a maximal hCG dose (10 ng/ml, 4 h). It can be seen in Fig. 1 that all of the NO donors used inhibited in a dose-dependent manner the hCG-induced progesterone accumulation in the incubation media (for 1 mM doses: 64%, 57%, and 60% of inhibition, respectively). Addition of NO donors alone to MA-10 Leydig cells had no significant effect on basal progesterone formation.

The inhibitory effect of NO donors on MA-10 Leydig cells was unaccounted for by cytotoxicity, as the percentages of

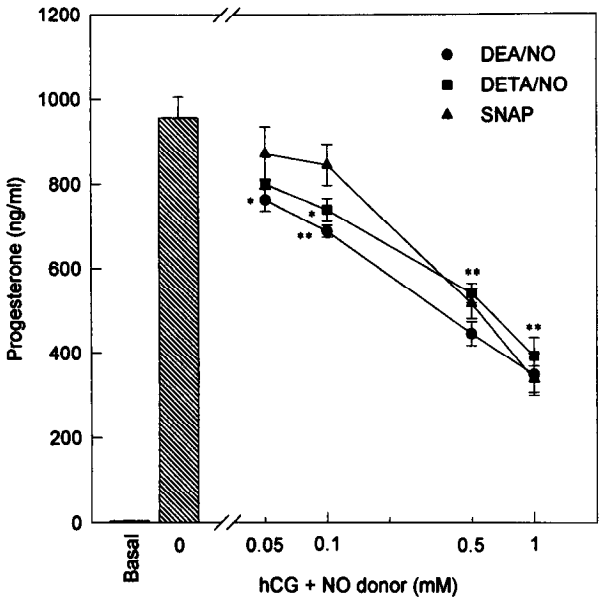

FIG. 1. Effect of different NO donors on hCG-stimulated progesterone accumulation in MA-10 Leydig cells. Cells were preincubated with increasing concentrations (0–1 mM) of DEA/NO, DETA/NO, or SNAP for 30 min. hCG (10 ng/ml) was added, and incubation was continued for an additional 4 h. Basal, Control values in absence of hCG. Each *bar* or *point* shows mean ± SEMof four independent experiments, each performed with two culture wells per condition. *, $P < 0.05$ *vs.* hCG alone. **, $P < 0.001$ *vs.* hCG alone.

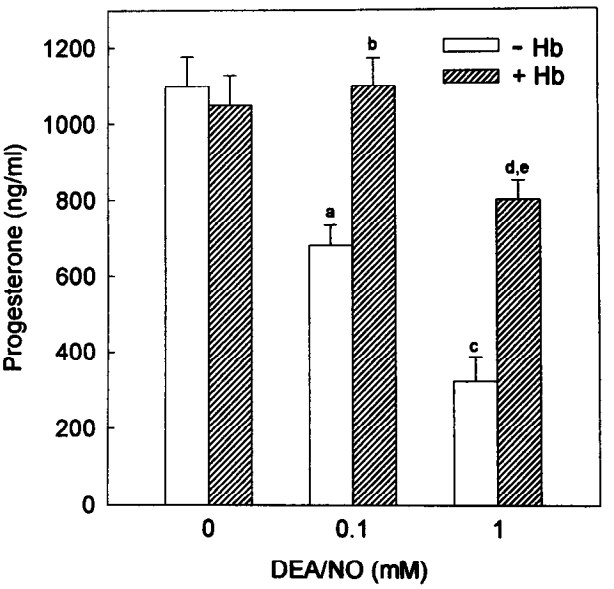

FIG. 2. Effect of NO donor DEA/NO alone and in combination with hemoglobin (Hb) on stimulated-progesterone secretion by MA-10 cells. Cells were preincubated for 30 min with DEA/NO (0–1 mM) with or without hemoglobin (Hb: 160 μM). hCG (10 ng/ml) was added, and incubation was continued for 4 h. Each *bar* shows mean ± SEM of four independent experiments, each performed with two culture wells per condition. a, $P < 0.01$ *vs.* control without DEA/NO. b, $P < 0.01$ *vs.* DEA/NO (0.1 mM); c, $P < 0.001$ *vs.* control without DEA/NO. d, $P < 0.001$ *vs.* DEA/NO (1 mM); e, $P < 0.05$ *vs.* control without DEA/NO.

viable cells per dish determined using trypan blue exclusion stain after a total of 4.5 h of incubation were similar between the NO donors-treated cells (93%) and the controls (94%). To discard a nonspecific inhibitory effect on cellular functions, we also evaluated the incorporation of [^{35}S]methionine into proteins as an index of protein synthesis. As shown in Table 1, there were no changes in the amount of labeled amino acid incorporated into cellular and secreted proteins in cells treated with a NO donor compared with controls.

To confirm that the inhibitory effect of NO donors on MA-10 Leydig cell steroidogenesis was mediated by NO, hemoglobin (160 μg/ml) was added together with DEA/NO. This protein, which can bind released NO, completely prevented DEA/NO effect for the 0.1 mM dose and partially for the 1 mM (Fig. 2). Incomplete reversal for the highest dose of DEA/NO was probably caused by the concentration of hemoglobin used, which may not have been enough to capture all released NO. Higher concentrations of hemoglobin were toxic to the cells. The addition of hemoglobin (160 μg/ml) also completely prevented DETA/NO (0.1 mM) effect and partially prevented SNAP (0.5 mM) effect (data not shown).

To further characterize NO effect on Leydig cell steroidogenesis, we examined the reversibility of this effect. Table 2 shows that the inhibitory effect of DEA/NO (1 mM) on activated steroid biosynthesis (for hCG-stimulated cells: 52% of inhibition at the end of DEA/NO treatment in day 1) was completely reverted 24 h after removal of the NO donor: MA-10 Leydig cells fully recovered their capacity to respond to a steroidogenic stimuli in day 2. $(Bu)_2$cAMP, instead of hCG, was used in day 2 to bypass a possible decrease in the steroidogenic response caused by down-regulation of hCG

TABLE 2. Recovery of MA-10 Leydig cell steroidogenesis after NO removal

Day 1 treatment[a]	Progesterone (ng/ml)	
	Day 1	Day 2 + $(Bu)_2$cAMP
Control	1.8 ± 0.4	654 ± 44
DEA/NO	2.1 ± 0.3	695 ± 51
hCG	830 ± 53	712 ± 33
hCG + DEA/NO	389 ± 41[b]	742 ± 27

[a] Cells were preincubated with DEA/NO (1 mM) for 30 min. hCG (10 ng/ml) was added and incubation was continued for 4 h. Media were collected for progesterone determination (day 1). Cells were washed and reincubated with NO-free fresh medium for an additional 24 h. At the end of incubation, cells were stimulated with $(Bu)_2$cAMP (1 mM) for 4 h (day 2). Results represent the mean ± SEM of three independent experiments, each performed with two culture wells per condition.
[b] $P < 0.001$ vs. hCG alone in Day 1.

receptors in those cells that had been treated with hCG in day 1. The reversibility of NO effect on these cells is important if we consider this molecule as a possible physiological messenger in the testis. These results also confirm that NO is not causing cellular damage to these cells.

NO site(s) of action

To study NO mechanism of action on MA-10 Leydig cells, we examined the effect of this molecule on cellular biochemical steps known to be modulated by NO or involved in the LH/hCG action. Because NO exerts many of its physiological effects in other systems by activating a soluble guanylate cyclase and consequently increasing cGMP levels, we studied the possibility that NO inhibitory effect on MA-10 Leydig

cells could also be mediated by the activation of this pathway. Atrial natriuretic peptide (ANP), which is known to stimulate a particulate guanylate cyclase in MA-10 Leydig cells (35), was used as a positive control for extracellular cGMP measurement in 4.5-h incubations. Although ANP produced a pronounced increase in extracellular cGMP levels, NO donors did not increase the production of this nucleotide, either in the presence or absence of hCG (Fig. 3). NO donors also failed to modify intracellular (control: 1.03 ± 0.14; 1 mM DEA/NO: 1.22 ± 0.25; 10^{-8} M ANP: 25.59 ± 6.29 pmol/ml, n = 2) and extracellular (control and 1 mM DEA/NO: nondetectable; 10^{-8} M ANP: 7.40 ± 0.91 pmol/ml, n = 2) cGMP levels in 20-min incubations. Moreover, the cGMP analogs, $(Bu)_2$cGMP or 8-Br-cGMP (0.1–5 mM), did not reproduce the NO donors' inhibitory effect. Although none of the analogs produced any change on hCG-stimulated progesterone production (Fig. 4B), only 8-Br-cGMP, a more potent analog than $(Bu)_2$cGMP, produced an increase in basal steroidogenesis (Fig. 4A). The high concentrations of 8-Br-cGMP (5 mM) needed to elicit a steroidogenic response may reflect a cross-stimulation of cAMP-dependent protein kinase, as has been already described (36).

We next examined whether NO could affect the production of cAMP, the main second messenger that mediates LH/hCG action on Leydig cells. NO donor DEA/NO did not produce any change in basal or hCG-stimulated extracellular cAMP accumulation in 4.5-h incubation (Fig. 5). It did not produce any change in intracellular (control: 5.9 ± 1.0; 1 mM DEA/NO: 6.2 ± 1.2; 10 ng/ml hCG: 118.0 ± 22.0 pmol/ml, n = 2) or extracellular (control: 0.24 ± 0.07; 1 mM DEA/NO: 0.32 ± 0.16; 10 ng/ml hCG: 38.2 ± 3.7 pmol/ml, n = 2) cAMP accumulation in 20-min incubations. In addition, NO donors still inhibited, in a dose-dependent manner, progesterone production stimulated by $(Bu)_2$cAMP (Fig. 6). These data suggest that the NO site(s) of action must be at least at one step beyond cAMP synthesis.

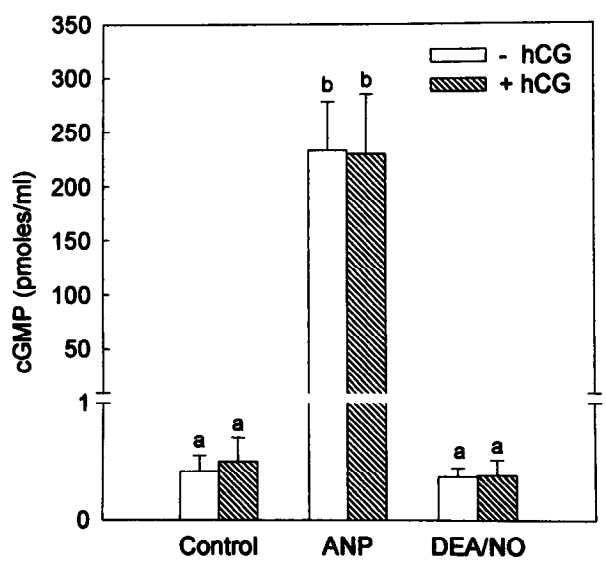

FIG. 3. Effect of NO and ANP on cGMP production in MA-10 cells. Cells were preincubated with or without ANP (10^{-8} M) or DEA/NO (1 mM) for 30 min in the presence of MIX (0.25 mM). incubation was continued for 4 h in the absence or presence of hCG (10 ng/ml). Each *bar* shows mean ± SEM of three independent experiments, each performed with two culture wells per condition. Different *letters* indicate significant differences ($P < 0.001$) between treatments.

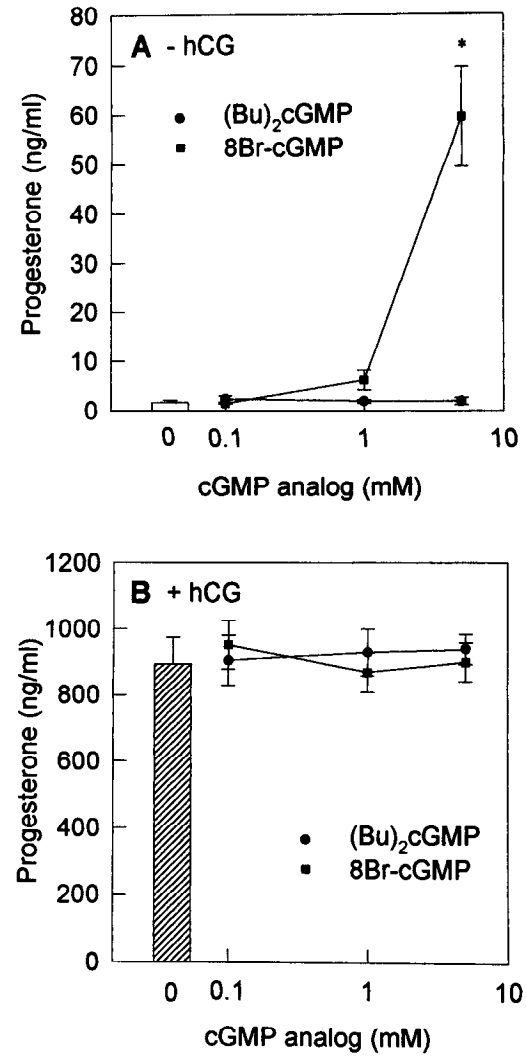

FIG. 4. Effect of cGMP analogs on progesterone accumulation in MA-10 cells in the presence or absence of hCG. Cells were incubated for 4.5 h with increasing concentrations of $(Bu)_2$cGMP or 8-Br cGMP (0–5 mM): A, Basal conditions; B, in the presence of hCG (10 ng/ml). Each *bar* or *point* shows mean ± SEM of three independent experiments, each performed with two culture wells per condition. *, $P < 0.001$ *vs.* control without cGMP.

To study the effect of NO on the steroidogenic pathway from cholesterol to progesterone, MA-10 Leydig cells were incubated with DEA/NO in the presence of 22R-hydroxycholesterol (60 μM), a cholesterol substrate derivative that readily passes through cell membranes, or pregnenolone (50 μM) (Fig. 7). It can be seen that NO inhibitory effect was completely prevented by the addition of pregnenolone, indicating that NO is not affecting 3β-hydroxysteroid dehydrogenase activity (Fig. 7B). In contrast, an inhibition of progesterone synthesis was still observed when 22R-hydroxycholesterol was used as a substrate for progesterone synthesis (Fig. 7C). These data suggest that the inhibitory effect of NO on stimulated steroidogenic pathway seems to occur at least at a step related to cholesterol side-chain cleavage ($P450_{scc}$) activity.

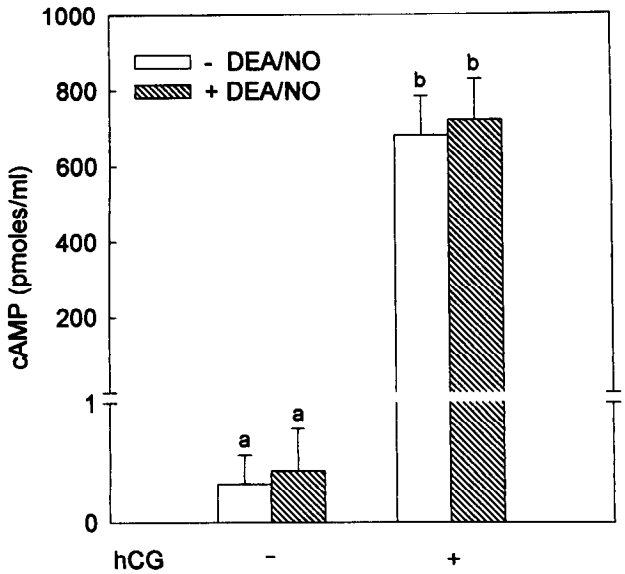

FIG. 5. Effect of NO donor DEA/NO on cAMP production in MA-10 cells. Cells were preincubated with or without DEA/NO (1 mM) for 30 min in the presence of MIX (0.25 mM). Incubation was continued for 4 h in the presence or absence of hCG (10 ng/ml). Each *bar* shows mean ± SEM of three independent experiments, each performed with two culture wells per condition. Different *letters* indicate significant differences ($P < 0.001$) between treatments.

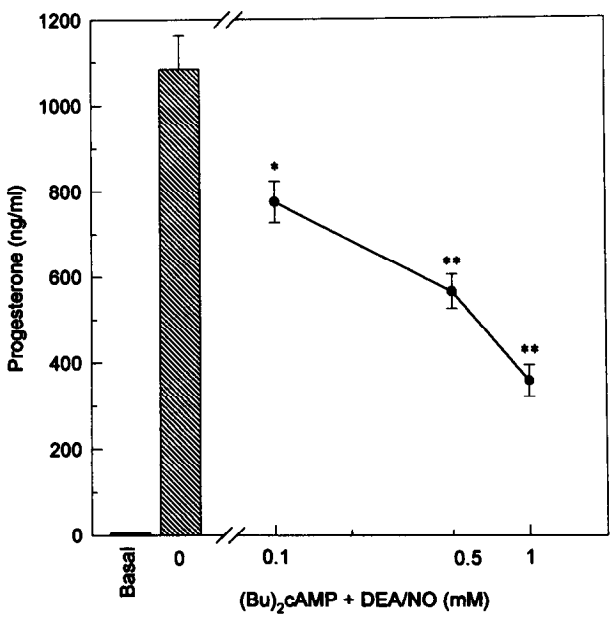

FIG. 6. Effect of NO donor DEA/NO on $(Bu)_2$cAMP-stimulated progesterone accumulation in MA-10 cells. Cells were preincubated for 30 min with increasing concentrations of DEA/NO (0–1 mM). $(Bu)_2$cAMP (1 mM) was added, and cells were incubated for another 4 h. Basal, Control values in absence of $(Bu)_2$cAMP. Each *bar* or *point* shows mean ± SEM of four independent experiments, each performed with two wells per condition. *, $P < 0.01$ *vs.* $(Bu)_2$cAMP alone. **, $P < 0.001$ *vs.* $(Bu)_2$cAMP alone.

Effect of NO-releasing agent on rat Leydig cell testosterone production

Although MA-10 cells are known to conserve most of the features characteristic of Leydig cells, and they represent a widely used model to study Leydig cell steroidogenesis, they

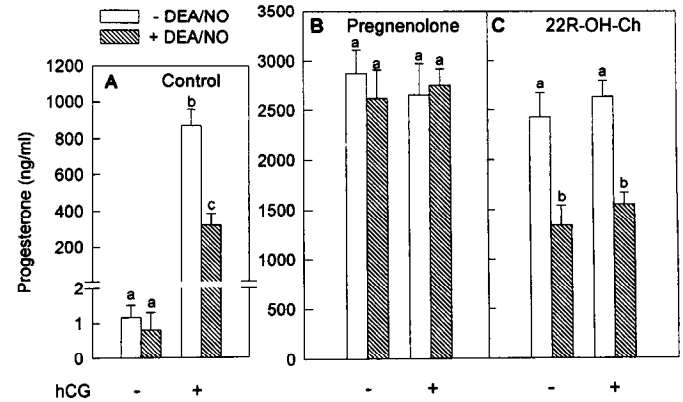

FIG. 7. Site of action of NO donor DEA/NO on steroid production. MA-10 cells were preincubated in the presence or absence of DEA/NO (1 mM) for 30 min. Incubation was continued for another 4 h with or without hCG (10 ng/ml) and without any further addition (A), in the presence of pregnenolone (50 μM) (B), or with 22R-hydroxycholesterol (22R-OH-Ch, 60 μM) (C). Each *bar* shows mean ± SEM of four independent experiments, each performed with two culture wells per condition. Different *letters* indicate significant differences ($P < 0.01$) between treatments within each pannel.

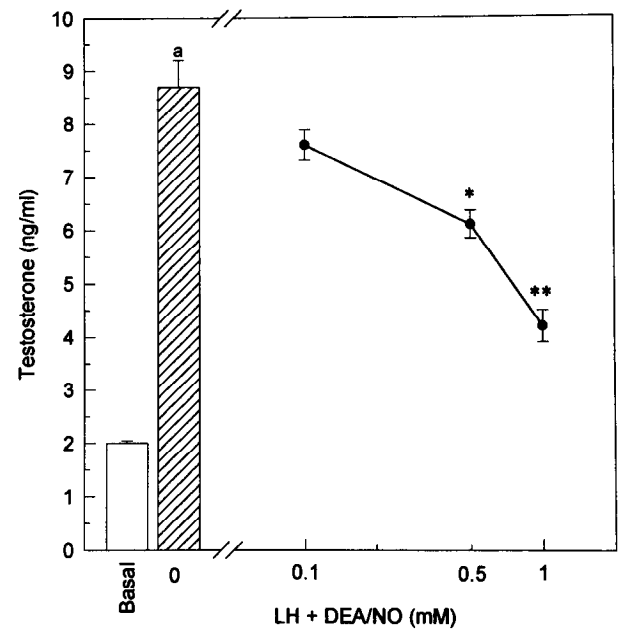

FIG. 8. Effect of NO on testosterone production in rat Leydig cells. Purified Leydig cells were preincubated for 30 min at 34 C with increasing concentrations of DEA/NO (0–1 mM). LH (4 ng/ml) was added, and incubation was continued for 3 h. Basal, Control values in the absence of LH. Each *bar* or *point* shows mean ± SEM of three independent experiments, each performed with triplicate incubations per condition. a, $P < 0.001$ *vs.* basal. *, $P < 0.01$ *vs.* LH alone. **, $P < 0.001$ *vs.* LH alone.

are still tumoral cells that have undergone a series of mutations. It was therefore necessary to validate the cellular model by showing that the NO inhibitory effect on MA-10 Leydig cell steroidogenesis was not exclusive for this tumoral cell line and can be extrapolated to normal Leydig cells. To do so we examined NO donor effect on testosterone biosynthesis in a suspension of purified rat Leydig cells. As shown in Fig. 8, DEA/NO (0–1 mM) also produced a dose-dependent inhibition on testosterone synthesis in rat Leydig

cells, confirming that NO was indeed exerting an inhibitory effect on Leydig cell steroidogenesis.

We also tested the effect of nitric oxide on an unpurified testis cell preparation. We found that NO donors inhibited testosterone production in these cells in a similar fashion to the one shown for the purified preparation (data not shown). These results indicate that even in the presence of other testicular cell types, NO is able to exert its inhibitory effect on Leydig cell function.

Discussion

In the present study, we demonstrate that NO inhibits hCG-induced steroidogenesis both in MA-10 and rat Leydig cells. The fact that we obtained the same inhibitory effect with three different NO donors, and that this effect was prevented by the addition of hemoglobin, confirmed that the inhibition was in fact caused by NO and not by a coproduct of NO donors' breakdown.

It is very interesting to note that, contrary to what is observed in many other systems, NO effect in these cells is not mediated by cGMP because it failed to increase its production, and nucleotide analogs did not reproduce its inhibitory effect. NO did not affect the production of cAMP, the main second messenger that mediates LH/hCG action in Leydig cells, and still inhibited $(Bu)_2$cAMP-stimulated steroidogenesis. These data clearly indicate that NO must be acting through a different mechanism that does not involve typical regulation of cGMP or cAMP synthesis. It was observed that the NO inhibitory effect was prevented when exogenously added pregnenolone was used as a substrate for progesterone synthesis, but was still seen in the presence of 22R-hydroxycholesterol. These results suggest that although 3β-hydroxysteroid dehydrogenase activity is not affected, the conversion of cholesterol to pregnenolone, which is catalyzed by the enzyme $P450_{scc}$, is at least one step that is inhibited by NO.

$P450_{scc}$ belongs to the family of cytochrome P450 enzymes, which have a heme moiety involved in its mechanism of action and can be inhibited by the binding of CO to its heme iron. NO, as it was mentioned, can also bind to the heme iron of enzymes and modulate their activity. Moreover, NO and CO can bind to some common heme proteins and exert similar effects on them (37–39). Indeed, it has been shown that cytochromes P450 are also inhibited by NO binding to its heme iron (40, 41). Because our results indicate that the conversion of cholesterol to pregnenolone is inhibited, it is tempting to think that NO could be inhibiting $P450_{scc}$ enzyme by directly binding to its heme iron as it does CO (42, 43), and in this way inhibit MA-10 Leydig cell progesterone formation. This hypothesis is reinforced and even generalized by results obtained in granulosa-luteal cells by Van Voorhis *et al.* (10). They reported that NO inhibited estradiol secretion without modifying cGMP levels but by inhibiting the activity of aromatase, which is also a cytochrome P450 enzyme. P450 17α-hydroxylase/17:20 lyase enzyme might also be inhibited by NO in normal Leydig cells. Nevertheless, we do not discard other possible sites of action of NO on Leydig cell steroidogenesis between cAMP and pregnenolone formation.

In a recent work, Davidoff *et al.* (15) also showed, as we did, that treatment of MA-10 or TM3 Leydig cells with an NO donor does not result in increased cGMP formation. However, they did not study the effect of NO on Leydig cell steroidogenesis. In this study we demonstrate a clear direct effect of NO on Leydig cell steroidogenesis and show an alternative mechanism for NO action in these cells, different from the classical cGMP pathway.

Our *in vitro* results are in accordance with reported *in vivo* effects of NO donors and NO synthase inhibitors on male rat testosterone levels (44, 45). In those studies male rats treated with an NO donor showed a significant decrease in both serum and testicular interstitial fluid testosterone levels, as well as an increase when treated with a NOS inhibitor. Our results suggest that a direct action of NO on Leydig cell steroidogenesis could account for the *in vivo* effects described. However, in *in vitro* testis slices studies, Adams *et al.* (45) reported no effect of NO donor isosorbide dinitrate (ISDN) on testicular steroidogenesis. The reason for the discrepancy between these and our results could probably be attributed to the kind of NO donor used in their *in vitro* studies, because we tested ISDN in parallel experiments with all other three NO donors, and we observed that ISDN produced no inhibitory effect either in MA-10 or in rat Leydig cells (data not shown).

Steroidogenesis in Leydig cells has long been recognized to be under the control of the hypothalamic-pituitary-testis axis. However, much data over the past several years clearly indicate that a subtle regulation of testicular function can be locally controlled. Many factors produced in the testis have been demonstrated to exert autocrine and paracrine effects on Leydig cell function (for review see Ref. 46). As mentioned above, NO has been shown to be synthesized in the testis (11). NO-producing cells, mainly endothelial cells and macrophages, are often observed in intimate association with Leydig cells. For example, in rodents, Leydig cells are found forming clusters around blood vessels where NO-producing vascular endothelial cells are present. On the other hand, macrophages, which are relatively numerous in the testis, are often found forming membrane digitations with Leydig cells (14) and were described to modulate testosterone biosynthesis (13). In addition, the observation that at least one isoform of NOS is present in Leydig cells (15), indicates that NO could also be produced by these cells. These studies together with our results showing a direct effect of exogenously added NO on Leydig cell steroidogenesis, suggest a possible paracrine/autocrine regulatory role of NO on testicular steroidogenesis. The concentrations of NO donors used in this study are comparable with those in which these compounds reproduce endogenously produced NO effects in other systems, suggesting that we could well be mimicking endogenously produced NO. Present studies are being performed to study paracrine or autocrine effects of endogenously produced testicular NO on Leydig cell function.

NO seems to play an important role in the regulation of male reproductive function and fertility. NO has been implicated as an important mediator of penile erection (47, 48). It has been reported to be synthesized all along the male reproductive tract and to affect spermatozoa motility (49, 50). It has also been shown to modulate both LHRH and LH release (6, 9). In addition, sex hormones such as testosterone were reported to induce enzyme NOS (51). In this study we

report a direct effect of NO on Leydig cell steroidogenesis, suggesting a possible new physiological site of action that may contribute to a fine net of regulation exerted by this molecule on male reproductive function.

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