Effect of nitric oxide on rat adrenal zona fasciculata steroidogenesis

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Abstract

The present study was designed to investigate the role of nitric oxide (NO) in the regulation of adrenocortical function. Different NO donors, such as sodium nitroprusside (SNP), S-nitroso-L-acetyl penicillamine, diethylamine/NO complex sodium salt and diethylenetriamine NO adduct, significantly decreased corticosterone production both in unstimulated and in corticotropin-stimulated zona fasciculata adrenal cells, in a dose-dependent manner. The effect of SNP was reversed by ferrous hemoglobin. A selective inhibitor of NO synthase, L-N^G-nitro-arginine significantly increased corticosterone secretion. The effect of SNP was not mediated by cGMP as permeable cGMP analogs did not reproduce its inhibitory effect. SNP significantly inhibited the steroidogenesis stimulated by 8Br-cAMP and 22(R)-OH-cholesterol, but was ineffective when corticosterone was produced in the presence of exogenously added pregnenolone. Moreover, the conversion of [^{3}H]cholesterol to [^{3}H]pregnenolone and the production of pregnenolone or progesterone (assessed by RIA) were significantly decreased by SNP. Taken together, these results suggest that NO may be a negative modulator of adrenal zona fasciculata steroidogenesis.

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Introduction

Nitric oxide (NO), synthesized from L-arginine by the enzyme NO synthase (NOS), is a small, gaseous and reactive molecule that has been found to be involved in the regulation of a wide range of biological functions as an intercellular and intracellular signal. Among others, its role as a neurotransmitter has been demonstrated in the central and peripheral nervous system, as well as a regulator of vasodilatation and normal vascular tone, and as a cytotoxic agent in immunological reactions (Moncada *et al.* 1991, Nathan 1992, Bredt & Snyder 1994). Some actions of NO result from binding to the iron atom in the heme group of guanylate cyclase, which stimulates the production of cGMP. Moreover, NO can also bind to nonheme iron in numerous enzymes and modify its activity (Ignarro 1989, Snyder & Bredt 1991) .

Increasing evidence suggests that NO participates in endocrine modulation, being implicated in the control of the hypothalamo–pituitary axis (Ceccatelli *et al.* 1993, Rettori *et al.* 1993, Duvilansky *et al.* 1995) and in the activity of pancreatic β-islets (Schmidt *et al.* 1992).

In particular, several reports suggest that NO may be involved in the regulation of steroid biosynthesis. It has been shown that NO inhibits steroidogenesis in granulosa–luteal cells (Van Voorhis *et al.* 1994, Dave *et al.* 1997) and human chorionic gonadotropin (hCG)-induced steroidogenesis in both MA-10 and rat Leydig cells (Del Punta *et al.* 1996). Moreover, arginine analogs such as N^G-monomethyl-L-arginine (L-NMMA) and N^ω-nitro-L-arginine-methyl ester (L-NAME) that inhibit NOS activity increase basal and hCG-stimulated testosterone production by interstitial cells. This effect could not be attributed to cAMP accumulation nor to any alteration on specific hCG binding (Welch *et al.* 1995).

As for the adrenal gland, NOS activity in cytosol and stimulation of a soluble guanylate cyclase activity induced by NO were demonstrated in rat whole adrenal, bovine cortex and medulla cytosol (Palacios *et al.* 1989). However, the role of NO in the regulation of adrenal steroidogenesis remains controversial. Adams *et al.* (1991) reported that *in vivo* treatment with L-NAME resulted in a dose-dependent increase in testosterone and corticosterone secretion without any effect at the anterior pituitary level, but an increase in plasma adrenocorticotropin (ACTH) concentrations induced by the administration of L-NAME *in vivo* was also described (Giordano *et al.* 1996). On the contrary, it has been reported that corticosterone secretion in isolated perfused adrenals is inhibited by L-NAME (Cameron & Hinson 1993). Concurrently, Nakayama *et al.* (1996) found that L-NMMA prevented the ACTH-stimulated production of aldosterone in isolated rat adrenal glands. Recently, however, a direct inhibitory effect of NO on angiotensin II and ACTH-induced aldosterone synthesis in rat and human adrenal glomerulosa cells was reported by Natarajan *et al.* (1997). The present study was

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intended to assess the effect of NO on basal and ACTH-stimulated corticosterone production in isolated adrenal cells. Its mechanism and possible site of action along the steroidogenic pathway were also examined.

Materials and Methods

Chemicals

ACTH (lyophilized), was obtained from Elea Laboratories, Buenos Aires, Argentina, 9-α-fluoro-11-β,17,21-trihydroxy-16-α-methylpregna-1,4-diene-3,20-dione (dexamethasone) was from Fluka, A. G., Buchs, Switzerland, 8-bromo cyclic AMP (8Br-cAMP), 8-bromo cyclic GMP (8Br-cGMP), dibutyryl cyclic GMP (db-cGMP), 22(R)-OH-cholesterol, ferrous hemoglobin, pregnenolone and sodium nitroprusside (SNP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). S-nitroso-N-acetyl-penicillamine (SNAP), diethylamine/NO complex sodium salt (DEA/NO), diethylenetriamine NO adduct (DETA/NO), and L-N^G-nitro-arginine (L-NNA) were purchased from Research Biochemicals International (Natick, MA, USA). [7-^{3}H]Cholesterol (10–30 Ci/mmol), [1,2,6,7-^{3}H]progesterone, [1,2,6,7-^{3}H] corticosterone and [7-^{3}H]pregnenolone were from New England Nuclear (Boston, MA, USA). Progesterone and corticosterone antisera were provided by Dr A Bélanger, Laval University, Quebec, Canada. Pregnenolone antiserum was a kind gift of Dr S Campo, Hospital de Niños, Buenos Aires, Argentina. All other reagents were commercial products of the highest grade available.

Animals

Adult Wistar rats were used throughout the experiments. Animals had free access to water and Purina formula chow. Dexamethasone was supplied in the drinking water (10 μg/ml) 16 h before killing. Animals were killed by decapitation according to protocols for animal use approved by the institutional animal care and use committee that follow NIH guidelines. Adrenals were rapidly excised and kept on ice.

Zona fasciculata cell preparation and treatment

Preparation of zona fasciculata cells followed published procedures (Neher & Milani 1978). Cells were suspended in Krebs–Ringer bicarbonate buffer (pH 7·4) under O_2/CO_2 (95:5%), containing 10 mM glucose, 0·5% (w/v) BSA and 0·1 mM 3-isobutyl-1-methylxanthine and aliquoted in 1 ml fractions containing 10^5 cells per tube.

SNP, SNAP, DEA/NO and DETA/NO were freshly prepared just before use. SNP was dissolved in acetate buffer 50 mM pH 5, SNAP was dissolved in dimethyl sulfoxide (DMSO). DEA/NO and DETA/NO were dissolved in incubation buffer. Equivalent amounts of acetate buffer, DMSO (0·1%) or incubation buffer were added to the controls as vehicle. According to the manufacturer, the half-lives of dissociation for DEA/NO and SNP are in the range of minutes, while for DETA/NO and SNAP they are 20 and 26 h respectively.

Preincubations in the presence of SNP, SNAP, DEA/NO or DETA/NO were carried out for 15 min at 37 °C, then ACTH or other stimuli were added to the cell suspensions. In another set of experiments cell suspensions were preincubated in the presence of 0·1 mM L-NNA for 30 min. After the appropriate additions, incubations proceeded for 60–90 min at 37 °C, with shaking (100 cycles/min). Incubations were stopped by cooling the tubes in ice/water and cells were pelleted by centrifugation at 500 ***g*** for 15 min. Supernatants were assayed for corticosterone by RIA after extraction with methylene chloride. Cell viability was assessed by the Trypan Blue dye exclusion test as determined by microscopy. Briefly, the cell pellets were resuspended in Krebs–Ringer bicarbonate buffer and aliquots of the suspensions were diluted 1:1 with the Trypan Blue solution (0·4%). Counting of the cells (approximately 100 cells per tube in each case) was performed within 5 min.

Side-chain cleavage cytochrome P450 activity

Incubation of adrenal quarters with SNP were performed in Krebs–Ringer bicarbonate buffer (pH 7·4) containing 10 mM glucose for 15 min, followed by a 20 min incubation in the presence of ACTH. Adrenals were homogenized in 0·32 M sucrose and the mitochondrial fraction was obtained by differential centrifugation. Mitochondrial pellets were suspended in 10 mM Tris, 0·25 M sucrose, 0·4 mM β-mercaptoethanol, 0·1 mM EDTA, 5 mM $MgCl_2$ pH 7·4 and 100 μl aliquots were used per tube (approximately 0·4 mg protein/ml). After the addition of 50 μM cyanoketone, in order to block pregnenolone metabolism (Farese *et al.* 1970), and [^{3}H]cholesterol (1–2 μCi/ml), mitochondrial fractions were preincubated for 25 min at room temperature (final volume 150 μl). The reaction was started by addition of 100 μl NADPH (1 mM). After 20 min at 37 °C, the tubes were placed in an ice/water bath. The [^{3}H]pregnenolone recovery of extraction with methylene chloride (70–80%) was determined by adding a tracer amount of [^{14}C]pregnenolone (5000 d.p.m.). The organic phase was evaporated and the dried residue reconstituted in methylene chloride, spotted on silica gel plates and developed using cyclohexane:ethyl acetate (3:2) as the mobile phase. The position of pregnenolone was revealed by placing the plates in an iodine-saturated chamber. Silica was scraped off each 1 cm section and subjected to liquid scintillation counting (Romero *et al.* 1996). Results were expressed as the pregnenolone fraction of total radioactivity, corrected by recovery, per mg of mitochondrial protein.

Assessment of pregnenolone and progesterone production

Adrenal quarters were preincubated as previously described, in the presence of 1 mM SNP for 15 min, and then incubated for 20 min after addition of ACTH. Mitochondrial fractions were obtained and resuspended as before. To determine pregnenolone production in control and SNP-treated mitochondrial fractions, 50 μM cyanoketone was added to the incubation mix, and tubes were incubated in the presence of NADPH (1 mM) for 30 min at 37 °C, in a 150 μl volume. The reaction was stopped by placing the tubes in an ice/water bath. Pregnenolone was extracted with 1·5 ml cyclohexane and suitable aliquots were used for RIA. To assess progesterone production, cyanoketone was substituted in the experimental protocol by the inhibitors of progesterone metabolism (Neher *et al.* 1982): metopyrone (0·27 mM) and 1-(β-guanidinoethyl)-3-(4-pyridyl)indole sulfate (Ba 40.028) (1·6 mM) as inhibitors of 11β-, 18- and 19-hydroxylation, and 21-hydroxylation respectively. At the end of the incubation progesterone was extracted with cyclohexane and its concentration estimated by RIA.

Statistical analysis

Data points represent mean ± S.E.M. Statistical analysis was performed by Student's *t*-test, one-way analysis of variance (ANOVA) followed by Dunnett's test or two-way analysis of variance (MANOVA) followed by Tukey's test where indicated.

Results

In order to evaluate the effect of *in vitro* treatment with SNP on isolated adrenal cells, corticosterone production, in basal conditions, was assessed in the presence of increasing concentrations of SNP (20 μM–1 mM). Corticosterone accumulation was significantly decreased by SNP with a threshold concentration of 100 μM (Fig. 1A). Hemoglobin (an NO trapping agent), which was ineffective *per se*, completely prevented the effect of 1 mM SNP (Fig. 1B).

SNP significantly inhibited steroidogenesis in ACTH-stimulated zona fasciculata cells, starting at 50 μM, in a dose-dependent manner (Fig. 2A) and corticosterone production induced by different concentrations of ACTH was significantly decreased by 1 mM SNP (Fig. 2B). SNAP, DEA/NO and DETA/NO also produced a significant inhibition of basal and submaximally ACTH-stimulated steroidogenesis as shown in Fig. 3.

The percentage of viable cells was assessed in each experiment and it was not significantly different for SNP, SNAP, DEA/NO or DETA/NO at the highest concentration used (84 ± 4%, 86 ± 5%, 83 ± 4% and 87 ± 3% respectively), in comparison with control cells (87 ± 5%). Similar results were obtained in the presence of ACTH (data not shown).

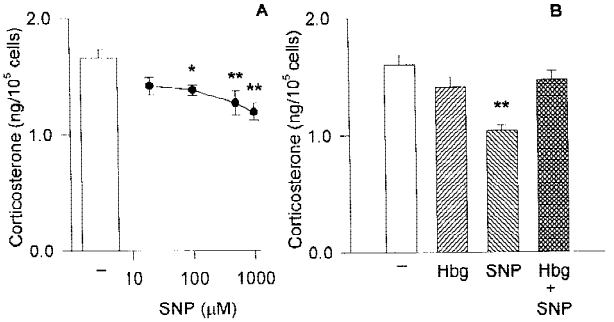

Figure 1 Effect of SNP on corticosterone secretion in rat adrenal zona fasciculata cells. Unstimulated cells were incubated for 60 min at 37 °C in the presence of increasing concentrations (20 μM–1 mM) of SNP (A) or 1 mM SNP with or without hemoglobin (Hbg, 50 μM) (B). Corticosterone production was assessed by RIA. Each bar or point represents the mean ± S.E.M. of three independent experiments each performed in triplicate. *P<0·05, **P<0·01 vs control by Dunnett's test.

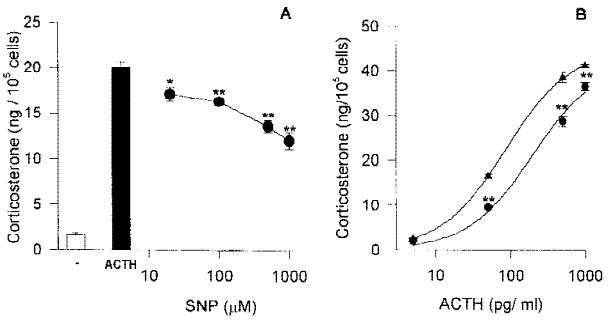

Figure 2 Effect of SNP on ACTH-stimulated corticosterone secretion in rat adrenal zona fasciculata cells. (A) Cells were preincubated with increasing concentrations of SNP (20 μM–1 mM) for 15 min and further incubated with ACTH (50 pg/ml) for an additional 60 min. Each bar or point represents the mean ± S.E.M. of three independent experiments performed in triplicate. *P<0·05, **P<0·01 vs ACTH, by Dunnett's test. (B) Cells were preincubated without (▲) or with 1 mM SNP (●) for 15 min and further incubated with increasing concentrations of ACTH (5-1000 pg/ml) for an additional 60 min. Each point represents mean ± S.E.M. of three independent experiments, performed in triplicate. **P<0·01 vs ACTH alone by two-way ANOVA and Tukey's test

The participation of endogenously produced NO in the modulation of adrenal cell steroidogenesis was studied using a specific inhibitor of NOS. L-NNA significantly stimulated corticosterone production in control and submaximally ACTH-stimulated adrenal cells (Fig. 4). In the presence of L-arginine (0·5 mM), which significantly inhibited both basal and ACTH-induced steroidogenesis, L-NNA stimulation was not observed (data not shown).

To study the role of cGMP as a second messenger of NO action, the effect of the permeable analogs of cGMP, 8Br- and db-cGMP on adrenal steroidogenesis was examined. No inhibitory effect was observed for 8Br-cGMP either on basal or on ACTH-stimulated

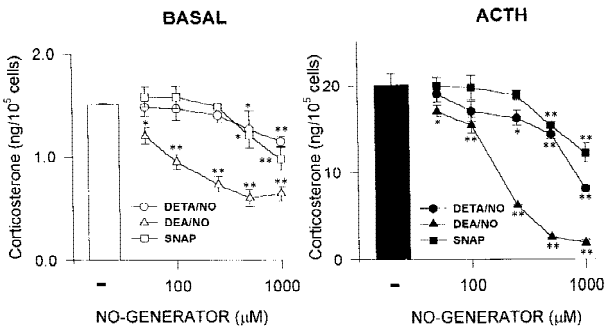

Figure 3 Effect of different NO donors on basal and ACTH-stimulated corticosterone production by rat adrenal zona fasciculata cells. Cells were preincubated for 15 min with increasing concentrations of SNAP, DEA/NO or DETA/NO, and the incubation proceeded for 60 min in the absence or the presence of ACTH (50 pg/ml). Each point represents the mean ± S.E.M. of three independent experiments, each performed in triplicate. *P<0·05; **P<0·01 without vs with NO donors by Dunnett's test.

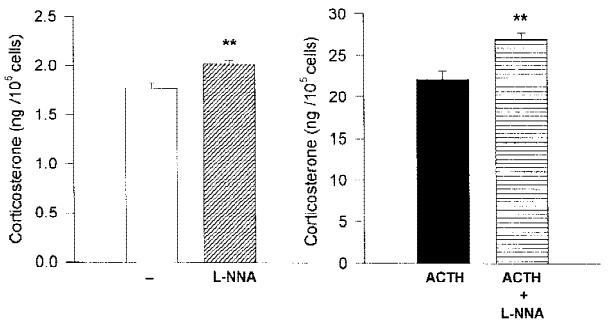

Figure 4 Effect of an inhibitor of NOS, L-NNA, on steroidogenesis in rat adrenal zona fasciculata cells. Cells were preincubated in the absence or in the presence of 0·1 mM L-NNA for 30 min. ACTH (50 pg/ml) was added where shown and incubation proceeded for 90 min. Corticosterone was assessed by RIA. Each bar represents the mean ± S.E.M. of three independent experiments, performed in triplicate. **P<0·01 vs basal or ACTH by Student's *t*-test.

Table 1 Effect of cGMP analogs on corticosterone accumulation in rat adrenal zona fasciculata cells. Cells were incubated for 60 min in the presence of 8Br-cGMP alone or in combination with ACTH. Corticosterone was assessed by RIA. Each value represents the mean ± S.E.M. of four independent experiments performed in triplicate. Statistical analysis was performed by ANOVA for each group (e.g. control, ACTH)

	Corticosterone (ng/10^5 cells)
Additions	
None	1·3 ± 0·2
8Br-cGMP (0·1 mM)	1·4 ± 0·1
8Br-cGMP (1 mM)	3·8 ± 0·4**
ACTH (50 pg/ml)	18·6 ± 0·6
ACTH (50 pg/ml)+8Br-cGMP (0·1 mM)	17·0 ± 0·8
ACTH (50 pg/ml)+8Br-cGMP (1 mM)	21·7 ± 2.4

**P<0·01 vs control by Dunnett's test.

Table 2 Effect of SNP on corticosterone synthesis under different stimuli. Adrenal zona fasciculata cells were preincubated in the absence or presence of SNP for 15 min, 22(R)-OH-cholesterol, 8Br-cAMP or pregnenolone was added and incubation proceeded for another 60 min. Corticosterone was assessed by RIA. Each value represents the mean ± S.E.M. of four independent experiments performed in triplicate

	Corticosterone (ng/10^5 cells)
Additions	
None	1·3 ± 0·1
SNP (1 mM)	0·9 ± 0·1**
22(R)-OH-cholesterol (5 μM)	93·4 ± 3·0
22(R)-OH-cholesterol (5 μM)+SNP (1 mM)	74·5 ± 3·7**
8Br-cAMP (5×10^{-6} M)	4·8 ± 0·4
8Br-cAMP (5×10^{-6} M)+SNP (1 mM)	3·1 ± 0·2**
8Br-cAMP (10^{-4} M)	46·5 ± 3·7
8Br-cAMP (10^{-4} M)+SNP (1 mM)	29·8 ± 2·7**
Pregnenolone (5 μM)	39·8 ± 3·5
Pregnenolone (5 μM)+SNP (1 mM)	38·3 ± 3·7

**P<0·01 vs same treatment without SNP, by Student's *t*-test.

corticosterone production (Table 1). 8Br-cGMP at 1 mM produced an increase in basal steroidogenesis. db-cGMP was also unable to inhibit steroidogenesis (data not shown).

To investigate the possible site of inhibition of NO, the effect of SNP on the biochemical steps involved in ACTH action was examined. As shown in Table 2, SNP inhibited adrenal steroidogenesis stimulated by 8Br-cAMP or 22(R)-OH-cholesterol (a cholesterol analog that freely diffuses between the mitochondrial membranes). On the contrary, corticosterone production in the presence of exogenously added pregnenolone was not affected by SNP.

The conversion of [^{3}H]cholesterol to [^{3}H]pregnenolone was assessed in mitochondrial fractions from adrenal glands treated with ACTH in the presence or absence of 1 mM SNP. The results shown in Fig. 5 indicate that while ACTH increased (1·8-fold) this conversion, SNP treatment resulted in a significant inhibition of [^{3}H]pregnenolone synthesis, both in unstimulated and stimulated glands. Similar results were obtained when mitochondrial fractions, instead of whole adrenals, were directly exposed to SNP (data not shown). To further confirm these results, we analyzed pregnenolone or progesterone production by mitochondria from control and stimulated glands exposed to SNP. Mitochondrial fractions were incubated for 25 min with NADPH and cyanoketone (for pregnenolone estimation) or metopyrone and Ba 40.028 (for progesterone determination). SNP significantly inhibited the production of both steroids as shown in Table 3.

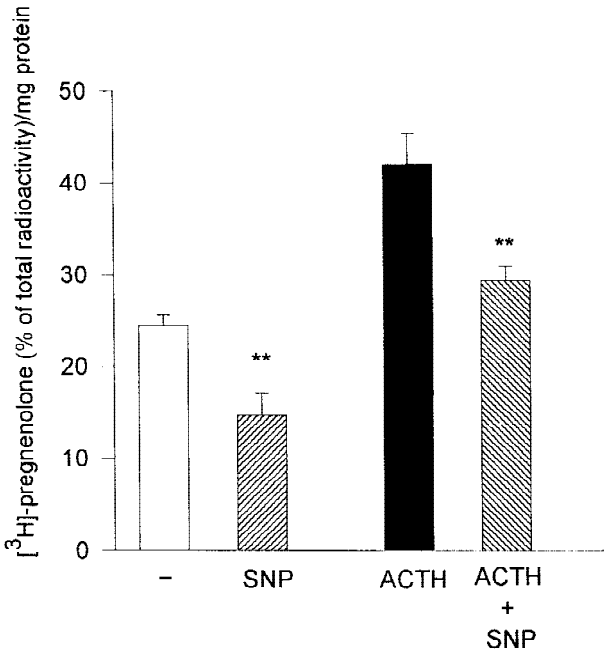

Figure 5 Effects of SNP on [^{3}H]pregnenolone synthesis. Adrenal glands were preincubated with or without 1 mM SNP for 15 min before addition of ACTH (1 ng/ml). Mitochondrial fractions, obtained as described elsewhere, were incubated in the presence of [^{3}H]cholesterol (1–2 μCi/ml), and cyanoketone (50 μM) for 25 min at room temperature. NADPH (1 mM) was added and incubation proceeded for 20 min at 37 °C. The incubation mix was extracted with methylene chloride and processed as described in Materials and Methods. Results are expressed as the pregnenolone fraction of total radioactivity, corrected by recovery, per mg of mitochondrial protein. Each bar represents the mean ± S.E.M. of three independent experiments, performed in triplicate. $^{**}P<0{\cdot}01$ without vs with SNP by Student's *t*-test.

Table 3 Effect of SNP on pregnenolone or progesterone production by mitochondrial fractions. Adrenal glands were preincubated without or with SNP for 15 min and further incubations, without or with ACTH, proceeded for an additional 30 min. Mitochondrial fractions were incubated in the presence of cyanoketone (50 μM) and NADPH (1 mM) for 30 min at 37 °C, in a 150 μl volume. To assess progesterone production, cyanoketone was substituted by metopyrone (0·27 mM) and Ba 40.028 (1·6 mM). Both steroids were assessed by RIA. Each point represents the mean ± S.E.M. of three independent experiments, performed in triplicate.

	Pregnenolone (ng/mg protein)	**Progesterone** (ng/mg protein)
Control	522·4 ± 29·2	400·9 ± 21·7
SNP (1 mM)	302·0 ± 10·4**	221·6 ± 17·2**
ACTH (1 ng/ml)	735·2 ± 49·7	633·3 ± 14·1
ACTH (1 ng/ml)+SNP (1 mM)	491·6 ± 43·7**	454·3 ± 29·0**

**$P<0{\cdot}01$ without vs with SNP by Student's *t*-test.

Discussion

NO has been postulated as an autocrine/paracrine regulator of steroidogenesis in several tissues (Van Voorhis *et al.* 1994, Welch *et al.* 1995, Del Punta *et al.* 1996, Nakayama *et al.* 1996, Dave *et al.* 1997). The present results indicate that SNP, widely used as a NO generating compound (Moncada *et al.* 1991), significantly decreases rat adrenal corticosterone synthesis.

In the present study the inhibitory effect of SNP on steroidogenesis is probably associated with the release of NO, as different NO donors, such as SNAP, DEA/NO and DETA/NO, also produced a significant inhibition of corticosterone biosynthesis. Moreover, the addition of ferrous hemoglobin, which binds free NO, as reported in several studies (Bredt & Snyder 1989, Ignarro 1989, Del Punta *et al.* 1996, Ientile *et al.* 1996) prevented SNP inhibition, ruling out a deleterious effect of the ferrocyanide ions generated by SNP breakdown.

The fact that no changes in cell viability were observed for any of the doses of SNP, nor for the other NO donors, together with the prevention by pregnenolone of SNP inhibitory effect, suggests that the decrease in corticosterone production is unlikely to be a result of an increase in cell death and is rather associated with NO effects on certain specific points along the steroidogenic pathway.

SNP and other NO donors inhibited basal and ACTH-stimulated steroid biosynthesis in a dose-dependent manner. The effect of both SNP and DEA/NO was already evident at 50–100 μM, while 250–500 μM was the threshold for DETA/NO and SNAP effects. Presumably, the higher concentrations required to attain a significant effect are related to the longer half-lives of these compounds.

This inhibitory effect is in agreement with the report by Adams *et al.* (1991) in which the *in vivo* stimulatory effect of L-NAME suggested that testicular and adrenal steroidogenesis are negatively regulated by endogenous NO. However, it has been shown that the administration of L-NAME induces the activation of the hypothalamic–pituitary–adrenal axis through an increase of ACTH plasma levels (Giordano *et al.* 1996). Since these experiments were performed *in vivo*, systemic effects induced by the inhibition of the basal NO tone, such as an increase in blood pressure (Cameron & Hinson 1993) cannot be ruled out.

As SNP and other NO donor effects were assessed in isolated cells, the results reported herein suggest, for the first time, a direct effect of NO on zona fasciculata steroidogenesis. Moreover, an inhibitor of NOS, L-NNA, significantly increased basal and ACTH-stimulated corticosterone production in zona fasciculata cells, suggesting a possible modulatory role for endogenously produced NO. Also in this sense, L-arginine not only significantly inhibited basal and ACTH-induced steroidogenesis, but prevented L-NNA stimulation of corticosterone production.

There is much evidence to suggest that NO might act via the activation of soluble guanylate cyclase (Ignarro 1989, Snyder & Bredt 1991). Although it has been demonstrated that NO also increases cGMP levels in adrenal cells (Palacios *et al.* 1989), exposure to the nucleotide permeable analogs 8Br- and db-cGMP did not reproduce the effect of SNP either on basal or on ACTH-stimulated adrenal steroidogenesis. This suggests, as has been reported for other steroidogenic cells (Van Voorhis *et al.* 1994, Del Punta *et al.* 1996), that the cGMP pathway is not involved in the NO-mediated regulation of steroidogenesis. The increase in corticosterone production elicited by the highest concentration of 8Br-cGMP could reflect its stimulation of the cAMP-dependent protein kinase, as has already been described (Lincoln & Cornwell 1993).

In order to determine the site of action of SNP along the steroidogenic pathway, corticosterone biosynthesis was examined under different stimuli. As shown in Table 2, SNP significantly inhibited the steroidogenesis stimulated by 8Br-cAMP and 22(R)-OH-cholesterol, but was ineffective in the presence of exogenous pregnenolone. These results suggest that the NO site(s) of action must be at least at a step beyond cAMP synthesis and cholesterol transport between the mitochondrial membranes, but not after pregnenolone synthesis. In fact, SNP also inhibited pregnenolone and progesterone synthesis from endogenous cholesterol. Moreover, the conversion of [^{3}H]cholesterol to [^{3}H]pregnenolone, as an index of cytochrome P450 scc activity, was significantly inhibited by SNP. As this assay involves preincubation of adrenal glands in the presence of SNP, and the loading of mitochondria with the radio-labeled compound, both a decrease in the availability of [^{3}H]cholesterol to the enzyme and the inhibition of [^{3}H]cholesterol to [^{3}H]pregnenolone conversion itself may account for the results reported herein. However, as 22(R)-OH-cholesterol stimulation of corticosterone synthesis was still impaired by SNP, the activity of the cytochrome P450 scc appears as a target for SNP action. The existence of an additional effect of SNP on cholesterol transport between the mitochondrial membranes is under current investigation.

The molecular events triggered by SNP that could explain these effects remain to be established. It has been shown that NO reacts directly with heme or nonheme iron and iron–sulfur complexes, located either at the active or allosteric site of key enzymes, producing activation or inhibition (Bredt & Snyder 1994). Indeed, this is the proposed mechanism for the activation of soluble guanylate cyclase (Moncada *et al.* 1991, Bredt & Snyder 1992, Schmidt 1992). Since it has been reported that NO inhibits hepatic cytochrome P450 enzymes both *in vitro* and *in vivo* (Wink *et al.* 1993) by binding to the heme moiety of the protein, it is tempting to speculate that the effect of NO on adrenal steroidogenesis could be explained by this mechanism. On the other hand, P450 enzymes have a conserved cysteine residue whose sulfhydryl group projects towards the heme. This sulfur is thought to interact with the iron during the catalytic process. Whether nitrosylation of that cysteine of rat adrenal cytochrome P450 scc occurs, as has been proposed for the mechanism of NO-induced inhibition of aromatase activity in granulosa–luteal cells (Snyder *et al.* 1996), deserves further examination.

As the metabolism of exogenously added pregnenolone was not impaired by SNP, the other P450 enzymes involved in the conversion of pregnenolone to corticosterone (e.g. the 21-hydroxylase from the endoplasmic reticulum, and the mitochondrial 11β-hydroxylase) seemed not to be significantly affected by SNP. The reasons for this apparent specificity are not clear.

The physiological relevance of the present results awaits further investigation. However, the identification of NOS activity in rat zona fasciculata cells (Palacios *et al.* 1989), together with the significant inhibitory effect of several NO donors, and the fact that an inhibitor of this enzyme increases the production of corticosterone support the hypothesis that NO may play a role as a paracrine or autocrine regulator of adrenal steroidogenesis. Moreover, NO donors also partially prevented ACTH-induced steroidogenesis. The role of ACTH as a pleiotropic regulator in the zona fasciculata of the adrenal gland is well known. The existence of signals able to modulate its function, even partially, may contribute to the avoidance of an 'all-or-none' kind of response, providing the gland with a higher capacity to respond to a wide range of physiological demands. In this context, we consider that NO should be included in this category of biological modulators.

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