Expression of Nitric Oxide Synthases in Rat Adrenal Zona Fasciculata Cells

CORA B. CYMERYNG, SEBASTIÁN P. LOTITO, CECILIA COLONNA, CARLA FINKIELSTEIN, YAEL POMERANIEC, NATALIA GRIÓN, LUCIANA GADDA, PAULA MALOBERTI, AND ERNESTO J. PODESTÁ

Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155 5° (C1121ABG), Buenos Aires, Argentina

Nitric oxide (NO) synthase (NOS) expression was analyzed in rat adrenal zona fasciculata. Both neuronal NOS and endothelial NOS mRNAs were detected by RT-PCR, immunohistochemistry, and immunoblot analysis. The biochemical characterization of adrenal zona fasciculata NOS enzymatic activity confirmed the presence of a constitutive isoform. In a cell line derived from mouse adrenal cortex, only endothelial NOS expression was detected by both RT-PCR and immunoblot analysis. Nitrate plus nitrite levels in Y1 cell incubation medium were increased in the presence of L-arginine and the calcium ionophore A23187, but not D-arginine, indicating enzymatic activity. Moreover, a low, but significant, conversion of L-arginine to L-citrulline, abolished by the NOS inhibitor, N^G-nitro-L-arginine, was detected in Y1 cells. The effect of L-arginine on pregnenolone production was examined. L-Arginine decreased both basal and ACTH-stimulated pregnenolone production in Y1 cells. The inhibitory effect of L-arginine could be attributed to endogenously generated NO, because it was blocked by N^G-nitro-L-arginine, and it was mimicked by the addition of a NO donor, diethylenetriamine-NO. An inhibitory effect of NO on pregnenolone production from 22R-hydroxycholesterol and on steroidogenic acute regulatory protein expression was also determined. Taken together, these results suggest that at least part of the adrenal NO could derive from steroidogenic cells and modulate their function. (*Endocrinology* 143: 1235–1242, 2002)

NITRIC OXIDE (NO) has been identified as a major biological signal, exerting both inter- and intracellular effects. It has been implicated in the modulation of platelet function and in the regulation of blood flow, macrophage cytotoxicity, and neurotransmission (1). NO is generated by the oxidation of L-arginine in a complex enzymatic reaction catalyzed by a family of NO synthases (NOS) that have been purified and cloned from various tissues and species (2–4). Two constitutively expressed types first characterized in neurons and vascular endothelium are calcium-, calmodulin-dependent NOS [neuronal NOS (nNOS) and endothelial NOS (eNOS)], whereas the inducible type of NOS (iNOS), binds calmodulin tightly at normal intracellular Ca^{2+} concentrations, and its activity is thus generally considered to be Ca^{2+} independent (5).

Increasing evidence suggests that NO is involved in the regulation of steroid biosynthesis. In this sense it has been shown that NO inhibits steroidogenesis in granulosa, luteal, and MA-10 cells as well as in rat Leydig cells under hCG stimulation (6–9). As for the adrenal gland, a direct inhibitory effect of NO on angiotensin II- and ACTH-induced aldosterone synthesis has been described in rat and human glomerulosa cells (10, 11), and we have recently shown that several NO donors significantly decreased both basal and ACTH-induced corticosterone production in rat zona fasciculata (ZF) cells (12).

The presence of NOS in steroidogenic tissues has been demonstrated in both ovarian stroma and follicular granulosa cells from human, rat, and rabbit (6, 13–15). Moreover, NOS was localized in the vascular endothelium of the rat testis (16) as well as in human Leydig cells (17), whereas the messenger for an iNOS was detected in both Leydig (18) and Sertoli (19, 20) cells.

We have demonstrated that L-arginine significantly increases both cGMP and nitrite levels, suggesting the presence of NOS activity in rat adrenal ZF cells (21). As the adrenal cortex shows a complex array of steroidogenic and nonsteroidogenic cells (*e.g.* endothelial cells, macrophages, fibroblasts, and neuronal cells), whether steroid production is under the control of NO generated within steroidogenic cells or otherwise produced by other cell types remains an open question.

To date, the expression of NOS in adrenal cells remains a subject of debate. Palacios *et al.* (22) demonstrated that NOS activity was Ca^{2+} dependent in rat whole adrenal gland and bovine cortex and medulla. nNOS mRNA and immunoreactivity were detected in the adrenal cortex of stressed rats (23, 24), whereas eNOS expression was reported in rat zona glomerulosa cells (10). However, other researchers demonstrated that only bovine adrenal endothelial cells, not adrenal glomerulosa cells, contain detectable levels of eNOS (25).

In the present work we analyzed the expression of different isoforms of NOS in both ZF adrenal gland and in a cell line (Y1) derived from a mouse adrenocortical tumor.

Materials and Methods

Chemicals

ACTH was obtained from Elea Laboratories (Buenos Aires, Argentina). Anti-NOS antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). AG 50W-X8 cation exchange resin and peroxidase-conjugated anti-IgG antibodies were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). ECL reagents came from Amersham Pharmacia Biotech (Little Chalfont, UK). N^G-Nitro-L-arginine (L-NNA) and diethylenetriamine NO adduct (DETA-NO) were purchased from Research Biochemicals International (Natick, MA). The Nitrate/Nitrite Colorimetric Assay Kit was obtained from Cayman Chemical Co. (Ann Arbor, MI). TRIzol reagent was from Life Technologies, Inc. (Gaithersburg, MD). All other reagents were commercial products of the highest grade available.

Animals

Adult Wistar rats were used throughout the experiments. The animals had full access to water and Purina chow (Ralston Purina Co., St. Louis, MO) and were killed by decapitation according to protocols for animal use approved by the institutional animal care and use committee following NIH guidelines. Adrenal glands were rapidly excised and kept on ice while being decapsulated and demedullated.

ZF tissue preparation

ZF adrenal tissue was homogenized in 0.2 ml/gland of 10 mM HEPES (pH 7.4), 320 mM sucrose, 0.1 mM EDTA, 0.1 mM dithiothreitol with the following protease inhibitors: 2 μg/ml pepstatin, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 2 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged at $800 \times g$ for 10 min. The supernatant was further centrifuged at $9,000 \times g$ for 20 min. The $9,000 \times g$ supernatant (postmitochondrial supernatant) was centrifuged at $105,000 \times g$ for 60 min to obtain a cytosol fraction (supernatant) and a microsomal fraction (pellet).

Preparation and culture of cells

Methods for the culture of Y1 mouse adrenal tumor cells (American Type Culture Collection, Manassas, VA) have been published previously (26). To detach the cells from the monolayer, 1 ml of a 0.05% trypsin and 0.53 mM EDTA solution was added to each 25-cm² flask. After 2 min of incubation at 37 C, 2 ml growth medium (Ham's F-10) containing FBS (2.5%) and horse serum (12.5%) was added to the dispersed cells. Cell suspensions were centrifuged at $800 \times g$ for 15 min, and the pellet was washed once with 10 ml PBS and resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 10 mM glucose, 0.5% (wt/vol) BSA under 95% O_2-5% CO_2, counted, and aliquoted in fractions containing 10^5 cells/tube. To determine nitrate plus nitrite production, cells were incubated for 210 min at 37 C in the presence of increasing concentrations of L-arginine (0.1–2.5 mM) or 2.5 mM D-arginine as indicated. In another set of experiments cells were incubated in Ham's F-10 medium including 2 mM Ca^{2+} and 1 mM L-arginine in the presence or absence of ACTH (10 mIU/incubation) and/or 10 μM A23187. Nitrate plus nitrite levels were determined with Griess reagent in aliquots of the incubation medium after nitrate reductase treatment. To assess pregnenolone production, cells were preincubated for 15 min at 37 C in the presence of cyanoketone (10 μM), an inhibitor of 3β-hydroxy-Δ^5-steroid dehydrogenase, to block further conversion of pregnenolone to progesterone, then incubated for 15 min with increasing concentrations of L-arginine (0.1–2.5 mM) and further incubated in the presence or absence of ACTH (0.2 mIU/incubation) for 2 h. When indicated, 1 mM L-NNA was added together with cyanoketone 15 min before 0.5 mM L-arginine addition. In another set of experiments DETA-NO (100–1000 μM) was added 15 min after cyanoketone, and cells were further incubated for 2 h in the presence or absence of ACTH (0.2 mIU/incubation). DETA-NO was prepared just before use. Incubations were stopped by cooling the tubes in an ice-water bath, and cells were pelleted by centrifugation at $500 \times g$ for 15 min. The supernatants were assayed for pregnenolone levels by RIA. Cell viability was assessed by the trypan blue dye exclusion test as determined by microscopy. No significant difference was observed for any of the treatments.

RT-PCR

Total RNA was isolated from rat adrenal zona fasciculata or Y1 cells using TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's instructions and stored in diethylpyrocarbonate-treated water at −20 C. Before the RT step, RNA was subjected to deoxyribonuclease I treatment (deoxyribonuclease I amplification grade, Life Technologies, Inc.) to eliminate any possible DNA contamination. RT was then performed on total RNA (5 μg). Briefly, cDNA synthesis was carried out using 200 U Moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI), 8 μM oligo(deoxythymidine)$_{20}$, 0.5 mM of each dNTP, and 25 U rRNAsin ribonuclease inhibitor in a total reaction volume of 25 μl. In selected tubes the reverse transcriptase was omitted as a control of amplification from contaminating cDNA or genomic DNA. The reaction was carried out at 42 C for 60 min and was terminated at 90 C for 5 min.

PCR reactions were carried out in a Perkin-Elmer Corp. (Foster City, CA) thermal cycler (GeneAmp PCR System 9600) and were performed using 4 μl cDNA for the amplification of NOS genes products. The cDNA was added to 46 μl of the following reaction mixture: 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 400 nM of each specific oligonucleotide primer, and 1.25 U AmpliTaq Gold DNA polymerase (PE Applied Biosystems). The sequence for the oligonucleotide primers were based on published sequences for murine nNOS and eNOS isoforms (GenBank accession no. D14552 and U53142, respectively) and rat iNOS isoform (GenBank accession no. M87039). Primer pairs for nNOS were 5'-TTTCTGTCCGTCTCTTCAAACGCAAAGTGG-3' (nucleotides +145 to +174) for the forward sense primer and 5'-GCGGGAGACTGTTCGTTCTCT GAATACGGG-3' (nucleotides +943 to +914) for the reverse antisense primer. Primer pairs for iNOS were 5'-CACGGAGAACAGCAGAGTTGG-3' (nucleotides +212 to +232) for the forward sense primer and 5'-GGAACACAGTAATGGCCGACC-3' (nucleotides +979 to +959) for the reverse antisense primer. For eNOS the primer pairs used were 5'-CTGTGTCCAACATGCTGCTAGAAATTG-3' (nucleotides +1008 to +1034) for the forward sense primer and 5'-TAAAGGTCTTCTTCCTGGTGATGCC-3' (nucleotides +1493 to +1469) for the reverse antisense primer. For amplifying NOS gene sequences, PCRs were carried out with a first step at 94 C for 10 min and then 35 cycles of denaturation at 94 C for 1 min, annealing at 55 C for 30 sec, and elongation at 72 C for 1 min, plus a final incubation at 72 C for 10 min. Reaction products (10 μl) were electrophoresed on a 1.5% agarose gel in 40 mM Tris-acetate and 2 mM EDTA, pH 8; stained with ethidium bromide; and photographed.

PCR bands of the expected size were cloned into pGEM-T easy (Promega Corp.) and sequenced using the T7 Sequenase Quick-Denature plasmid sequencing kit (Amersham Pharmacia Biotech, Arlington Heights, IL).

When total RNA from Y1 cells was used, the identity of the PCR product obtained with eNOS primers was confirmed by transferring the electrophoresed DNA to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech) and probing with the 485-bp internal fragment of the rat adrenal zona fasciculata eNOS isoform ^{32}P labeled by nick translation (GenBank accession no. AF110508).

Northern analysis

Total RNA from Y1 cells was prepared by homogenization in TRIzol reagent according to the manufacturer's instructions. Samples of RNA (24 μg) were resolved on 1.2% agarose/2.2 M formaldehyde gels and transferred onto Hybond-N$^+$ nylon membranes (Amersham Pharmacia Biotech, Buenos Aires, Argentina). A cDNA probe for steroidogenic acute regulatory protein (StAR) was prepared by RT-PCR from total RNA from Y1 cells. Primers were designed according to the published sequence of mouse StAR. The forward (5'-AAAGGATTAAGGCACCAAGCTGTGC-3') and reverse (5'-CTCTGATGACACCACTCTGCTCCGG-3') primers were used to amplify a 588-bp fragment. The PCR product was sequenced to confirm its identity. After prehybridization for 8 h at 42 C, blots were hybridized overnight with the [α-^{32}P]dCTP-radiolabeled cDNA probe at 42 C. The hybridization solution contained 6× SCC, 5× Denhardt's solution, 0,5% formamide, and 100 μg/ml denatured salmon sperm DNA. Blots subsequently were washed twice with 2× SSPE (150 mM NaCl, 10 mM PO_4NaH_2, 1 mM EDTA)/0.5% SDS at room temperature and twice with 1× SSPE/0.1% SDS at 65 C. StAR

hybridization signals were quantified using a Storm PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). After signal quantitation, the membranes were stripped and rehybridized to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Relative mRNA levels were calculated after correcting for RNA loading by normalizing the primary hybridization signal with the GAPDH signal.

Immunoblot analysis

Y1 cells were washed twice in PBS and lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 10 μg/ml leupeptin, 1 mM PMSF, and 2 μg/ml pepstatin A. Alternatively, proteins from Y1 cells were isolated from the phenol-ethanol supernate obtained after RNA and DNA extraction with TRIzol reagent. Samples from ZF (cytosolic or microsomal fraction) or Y1 cells were boiled for 5 min in SDS-PAGE loading buffer and electrophoresed on 7.5% or 12% poly-acrylamide gels. After electrophoresis, proteins were transferred to poly-vinylidene difluoride membranes for 1 h at 15 V in a Bio-Rad Laboratories, Inc. Trans-Blot SD system in transfer buffer [48 mM Tris-HCl (pH 9.2), 39 mM glycine, and 1.3 mM SDS]. Polyvinylidene difluoride membranes were blocked in buffer [50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.25% Tween 20] and 1% BSA for 60 min at room temperature and then incubated overnight in a 1:1000 dilution of the respective NOS or StAR antiserum at 4 C. Polyclonal antisera against a synthetic peptide consisting of amino acid sequence 88–98 of the mouse StAR protein was a gift from Dr. D. Stocco, Department of Cell Biology and Biochemistry, Texas Tech University (Lubbock, TX). Membranes were washed with 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.25% Tween 20 buffer and then incubated for 2 h with a 1:5000 dilution of a goat antirabbit IgG antibody-horseradish peroxidase conjugate. The filters were washed, and the bands were visualized by chemiluminescence (ECL Western Blotting Analysis System, Amersham Pharmacia Biotech) and autoradiography.

NOS activity

NOS activity was determined in postmitochondrial fractions from rat adrenal ZF and homogenates of Y1 cells by monitoring the conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline. Protein fractions were brought to 100 μl in a reaction mixture that contained final concentrations of 10 mM HEPES (pH 7.4), 0.75 mM β-NADPH, 1.25 mM $CaCl_2$, 20 μM L-arginine, 187 nM L-[2,3-^{3}H]arginine, 5 μM tetrahydrobioterin, 50 nM calmodulin, 0.5 μM flavin mononucleotide, 0.5 μM flavin adenine dinucleotide, 0.5 mM dithiothreitol. When indicated, cofactors were omitted or $CaCl_2$ was substituted by 1 mM EGTA. The reaction mixture was incubated for the indicated periods of time, and the reaction was terminated by the addition of 250 μl ice-cold stop buffer (10 mM EGTA, 10 mM EDTA, and 50 mM HEPES, pH 5). L-[^{3}H]Citrulline was separated by anion exchange chromatography and quantified by liquid scintillation spectroscopy. NOS enzyme activity is indicated as picomoles of [^{3}H]citrulline formed per mg protein/min.

Immunohistochemistry

Rat adrenals were dissected out and immersion-fixed in 4% paraformaldehyde in 0.01 M PBS for 2 h at room temperature and left overnight at 4 C. The tissue was stored in PBS containing 12% sucrose at 4 C, sections were cut at 16-μm thickness in a cryostat, thaw-mounted onto gelatinized glass slides, and processed for indirect immunohistochemistry. Briefly, sections were rinsed in PBS and incubated in blocking solution (PBS with 0.3% Triton X-100 and 1.5% goat serum) for 1 h at room temperature, followed by the incubation with the respective rabbit polyclonal antibodies against nNOS or eNOS (1:100 and 1:50, respectively; Santa Cruz Biotechnology, Inc.) or P450 side-chain cleavage enzyme (P450scc; 1:500; Chemicon International, Inc., Temecula, CA), in a humid chamber for 24 h at 4 C. After rinsing in PBS, the sections were incubated with goat antirabbit IgG conjugated to cy3 (Molecular Probes, Inc., Eugene, OR). In control experiments tissues were incubated with normal rabbit IgG diluted in blocking solution and then with the secondary antisera as described above. Preabsorption experiments were also performed for eNOS and nNOS antisera using the corresponding blocking peptide to the synthesized antibody. Immunostained sections were mounted in FluorSave reagent (Calbiochem, La Jolla, CA), and fluorescence was visualized at the appropriate wavelength for the sec-

ondary antibody using a Carl Zeiss 510 confocal laser scanning microscope.

Statistical analysis

Statistical analysis of results was performed by one-way ANOVA, followed by Tukey 's or Dunnett's *t* test.

Results

To identify the isoform(s) of NOS expressed in rat adrenal zona fasciculata, total RNA was isolated from decapsulated and demedullated adrenal glands, reversed transcribed to cDNA with oligo(deoxythymidine) primers, and amplified by PCR with specific primers for the different NOS isoforms. Amplicons with the expected size were obtained with primers for nNOS and eNOS, whereas iNOS mRNA was not detected (Fig. 1). Nonreverse transcribed mRNAs were used as controls for each amplification. The identity of the PCR products was confirmed by DNA sequencing and comparison with published sequences for nNOS and eNOS. The sequence of the rat cDNA for eNOS (AF110508) that was not available in the GenBank was 95% homologous to its murine counterpart (U53142).

The presence of mRNAs for both isoforms in rat adrenal ZF was confirmed by Northern blot analysis. Bands of 10.9 kb (nNOS) and 4.4 kb (eNOS) were identified using the corresponding PCR products as probes (data not shown). The expression of the respective proteins was assessed by Western blot analysis. By using specific antibodies raised against the three NOS isoforms, immunoreactive bands of 155 kDa corresponding to nNOS and of 135 kDa corresponding to eNOS were detected in the postmitochondrial fractions (Fig. 2). At least under this experimental conditions, iNOS protein was not detected (data not shown). To localize the isoforms of NOS within the adrenal gland we performed immunohistochemical staining of sections of adrenal tissue. As shown in Fig. 3 both NOS isoforms were stained within ZF and zona glomerulosa cells. A similar staining pattern was obtained with an antibody directed against cytochrome P450scc, a marker for steroidogenic cells.

The activity of adrenal ZF NOS was assessed through the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. This activity was calcium and calmodulin dependent, as both 1 mM EGTA
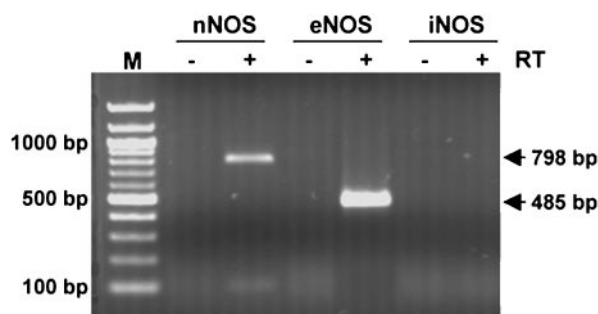

FIG. 1. Identification of transcripts for NOS isoforms in rat adrenal ZF. RNA from ZF was subjected to deoxyribonuclease I treatment, reverse transcribed with oligo(deoxythymidine); amplified by PCR with specific oligonucleotide primers for nNOS, iNOS, or eNOS as indicated; and analyzed by agarose gel electrophoresis and ethidium bromide staining. Negative controls were performed in the absence of RT (−RT). Size markers are shown on the *left*: 100-bp DNA ladder.

and 100 μM trifluoroperazine (a calmodulin inhibitor) caused significant inhibition, whereas the presence of 1 mM N^G-nitro-L-arginine methyl ester or 1 mM L-NNA completely blocked adrenal enzymatic activity (Fig. 4).

Only eNOS mRNA was detected in Y1 cells (Fig. 5A). The identity of the RT-PCR obtained product was confirmed by
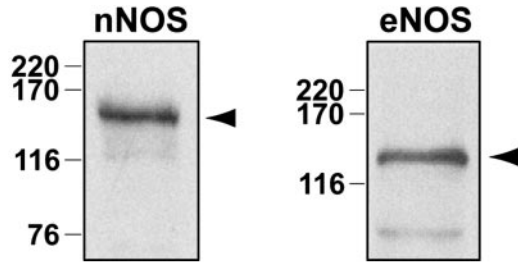

FIG. 2. Western blot analysis of NOS isoforms in rat adrenal ZF. Equal amounts of protein (80 μg) from ZF postmitochondrial fractions were analyzed by Western blotting using NOS-specific antibodies, as described in *Materials and Methods*. Numbers on the *left* indicate molecular mass in kilodaltons. *Arrows* show nNOS- or eNOS-specific immunoreactivity.

Southern blot analysis using the 485-bp cDNA from rat ZF eNOS as a probe (Fig. 5B). The expression of eNOS in Y1 cells was confirmed by immunoblot as a protein band of 135 kDa (Fig. 5C). Antibodies raised against nNOS and iNOS failed to detect any protein band (data not shown).

L-[^{3}H]Citrulline conversion from L-[^{3}H]arginine was also determined in Y1 cells. Although the activity in Y1 cells was significantly lower compared with the activity in the adrenal ZF postmitochondrial fraction, this parameter was significantly reduced in the presence of L-NNA (Fig. 6A). Furthermore, L-arginine, but not D-arginine, significantly increased Y1 cell production of nitrate plus nitrite, the soluble metabolites of NO (Fig. 6B). A significant increase in nitrate plus nitrite levels was observed when the cells were incubated in the presence of the calcium ionophore A23187 in both basal and stimulated conditions (Fig. 6C).

The effect of L-arginine on steroid production was examined in Y1 cells. L-Arginine significantly inhibited pregnenolone production in a dose-dependent manner in both control and ACTH-stimulated cells (Fig. 7, A and B). This effect was reversed by L-NNA and was reproduced by an NO
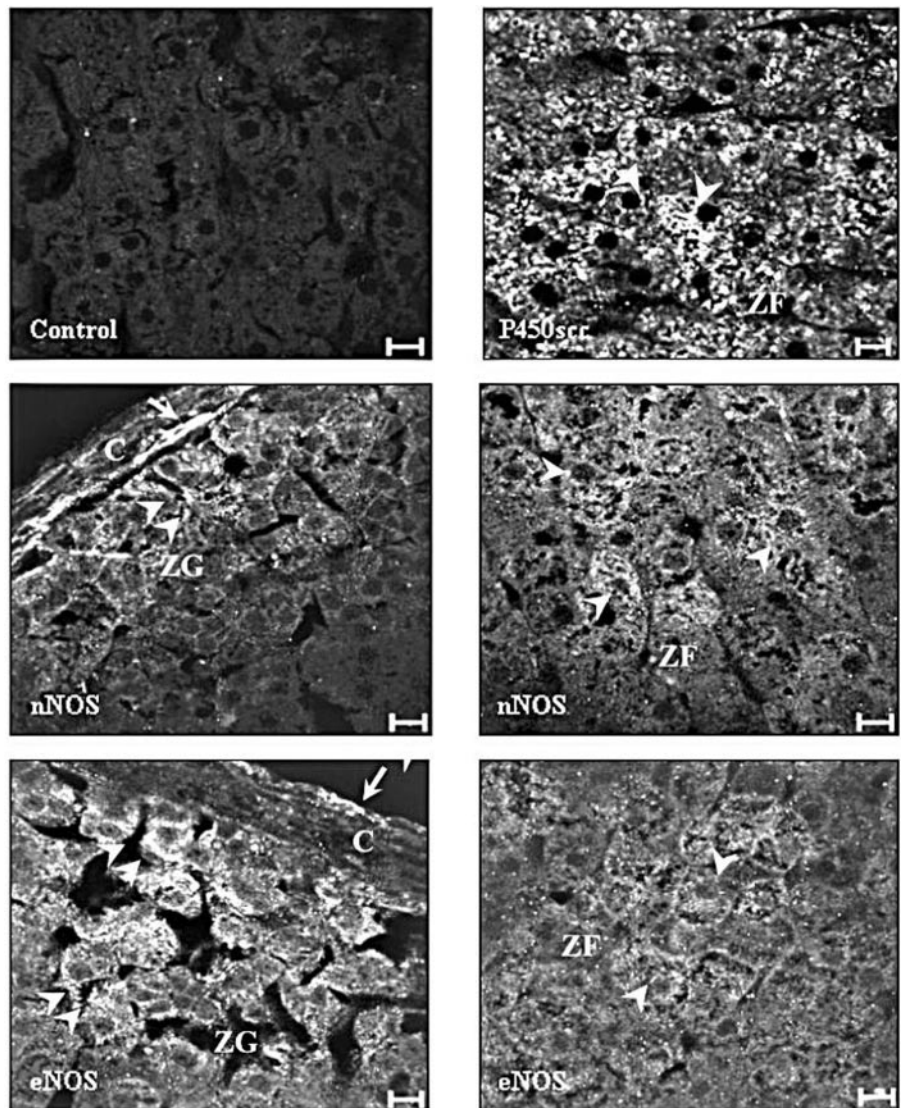

FIG. 3. Adrenal gland immunofluorescence micrographs showing control (first antibody omitted, *left upper panel*), P450scc (*right upper panel*) nNOS (*middle panels*), and eNOS (*lower panels*) staining. Both neuronal and endothelial NOS immunoreactivity is found in ZF cells (*right middle* and *lower panels*, *arrowhead*) and ZG cells (*left middle* and *lower panels*, *double arrowhead*). P450scc shows a similar staining pattern. NOS staining is also observed in the blood vessel walls and fibers on the capsule (*arrow*). ZG, Zona glomerulosa; C; capsule. Magnification, ×100. *Bar*, 10 μm.
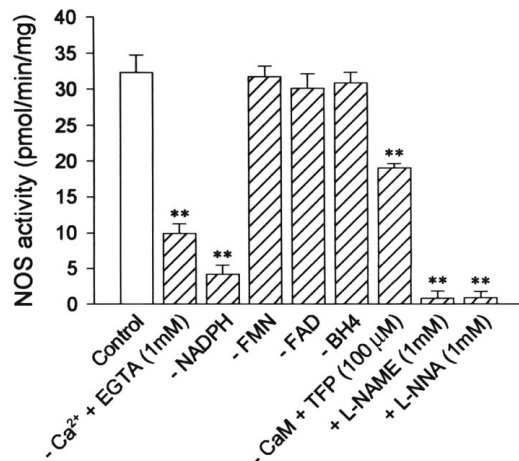
FIG. 4. Effects of cofactors and inhibitors on NOS activity in rat adrenal ZF. NOS activity was determined in the postmitochondrial fraction by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as described in *Materials and Methods*. NOS activity is expressed as the mean ± SEM (picomoles per min/mg) of four independent experiments. Signs denote the addition (+) or omission (−) of cofactors or inhibitors.
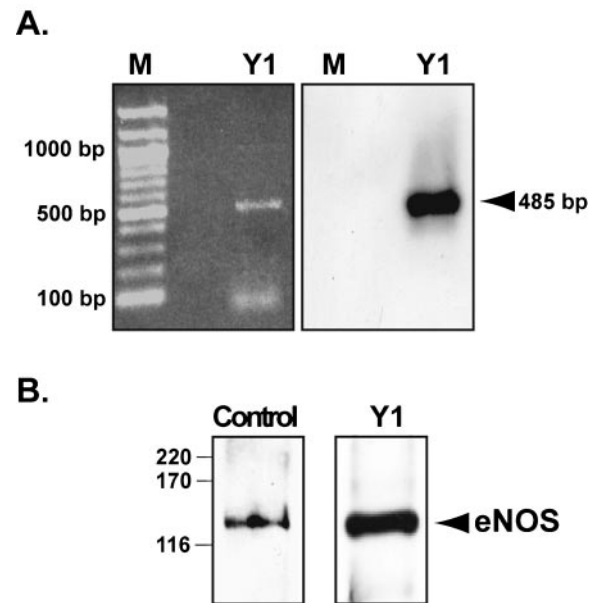

FIG. 5. Expression of eNOS in Y1 cells. A, RT-PCR of Y1 cells total RNA with specific oligonucleotide primers for the eNOS isoform. *Left panel*, Ethidium bromide staining. Size markers (100-bp ladder) are shown on the *left*. *Right panel*, Southern blot analysis of the RT-PCR product shown in the *left panel* using a probe from rat eNOS. B, Western blot analysis of eNOS from Y1 cells. Cell lysates were resolved on 7.5% SDS-PAGE and analyzed by immunoblot with a specific antiserum against eNOS. Control, Endothelial cells lysate; Y1, Y1 cells homogenate (80 μg). Numbers on the *left* indicate molecular mass in kilodaltons. The *arrow* shows eNOS-specific immunoreactivity.

donor, DETA-NO (Fig. 7, C and D). The effect of DETA-NO and the calcium ionophore A23187 on steroidogenesis was analyzed in Y1 cells incubated in the presence of the cholesterol permeable analog, 22*R*-hydroxycholesterol (Table 1). Results showed that pregnenolone production was significantly inhibited in the presence of A23187 and increasing concentrations of DETA-NO.

The effect of DETA-NO on ACTH induction of StAR expression in Y1 cells was studied. As illustrated in Fig. 8, two major mRNA species with sizes of 1.6 and 3.5 kb were detected in ACTH-stimulated Y1 cells. Stimulation of the cells in the presence of increasing concentrations of DETA-NO resulted in a dose-dependent decrease in both StAR mRNAs and a 30-kDa immunoreactive protein detected with an anti-StAR protein antibody (Fig. 8).

Discussion

The present results demonstrate that eNOS and nNOS are expressed in rat adrenal zona fasciculata, whereas only eNOS is expressed in Y1 cells. The biochemical characterization of the enzymatic activity supports the presence of functional constitutive isoforms of NOS in rat adrenal ZF. Although the inducible isoform was not detected in either rat adrenal ZF or Y1 cells, the possibility that iNOS could be expressed in these systems in response to appropriate stimuli cannot be ruled out.

As for NOS expression in endocrine glands, the eNOS isoform was detected in rat adrenal glomerulosa (10) and human ovarian follicular cells (6). In ovaries obtained from hypophysectomized and hormone-treated rats, both iNOS and eNOS isoforms were identified, whereas all three NOS isoforms were detected in the prepubertal ovary (7, 27, 28). On the other hand, nNOS protein and iNOS mRNA were detected in human and rat Leydig cells (17–20).

In a recent work we have shown that L-arginine induces several effects in the adrenal gland, presumably through the involvement of an endogenous NOS (21). The present results

demonstrate that NO could be produced by the NOS isoforms, nNOS and eNOS, in rat adrenal ZF. According to our immunohistochemical studies, steroidogenic cells within the adrenal cortex express both NOS isoforms, displaying a similar localization pattern as cytochrome P450scc. Kishimoto *et al.* (24) demonstrated the presence of nNOS mRNA and NOS immunoreactivity in the adrenal cortex of stressed rats. However, these researchers observed negligible levels of both parameters in the adrenal cortex of nonstressed rats (24). In contrast to those results, we detected nNOS mRNA and protein in basal conditions. Moreover, in our experimental setting NOS activity was 10 times higher than that reported.

Endothelial and neuronal cells have a close anatomical proximity to steroidogenic cells in the adrenal cortex. As these cells release NO, it was suggested that vascular or neuronal NO could be an effective means to regulate steroid production (11). In addition, NO could also be generated within steroid-producing cells. In this sense eNOS was detected in Y1 cells, a pure population of steroidogenic cells. The isoform detected in Y1 cells is an active enzyme, as a low, albeit significant, L-arginine to L-citrulline transformation, which was abolished by L-NNA, was observed. Moreover, an increase in nitrate plus nitrite levels was produced in the presence of exogenous L-arginine, but not D-arginine. A number of studies indicate that NO production under physiological conditions can be increased by extracellular arginine despite saturating intracellular arginine concentrations. One possible explanation for this paradox is that intracellular arginine is sequestered in one or more pools that are poorly

FIG. 6. L-Citrulline and nitrate plus nitrite levels in Y1 cells. A, Y1 cells homogenates were incubated for 60 min in the presence of 20 μM L-[^{3}H]arginine with or without the addition of 1 mM L-NNA as described in *Materials and Methods*. Values are the mean $\pm$ SEM of three independent experiments, each performed in triplicate. **, $P < 0.01$ (*vs.* control, by *t* test). B, Cells were incubated for 210 min at 37 C in the presence of increasing concentrations of L-arginine (0.1–2.5 mM) or 2.5 mM D-arginine as indicated. C, Cells were incubated for 210 min at 37 C in the presence of 1 mM L-arginine and 2 mM Ca^{2+} with or without the addition of ACTH (10 mIU/incubation) and/or 10 μM A23187. Aliquots of the incubation medium were assayed for nitrate plus nitrite levels. Values in B and C are the mean $\pm$ SEM of three independent experiments, each performed in triplicate. *, $P < 0.05$; **, $P < 0.01$ (*vs.* control, by Dunnett's *t* test).
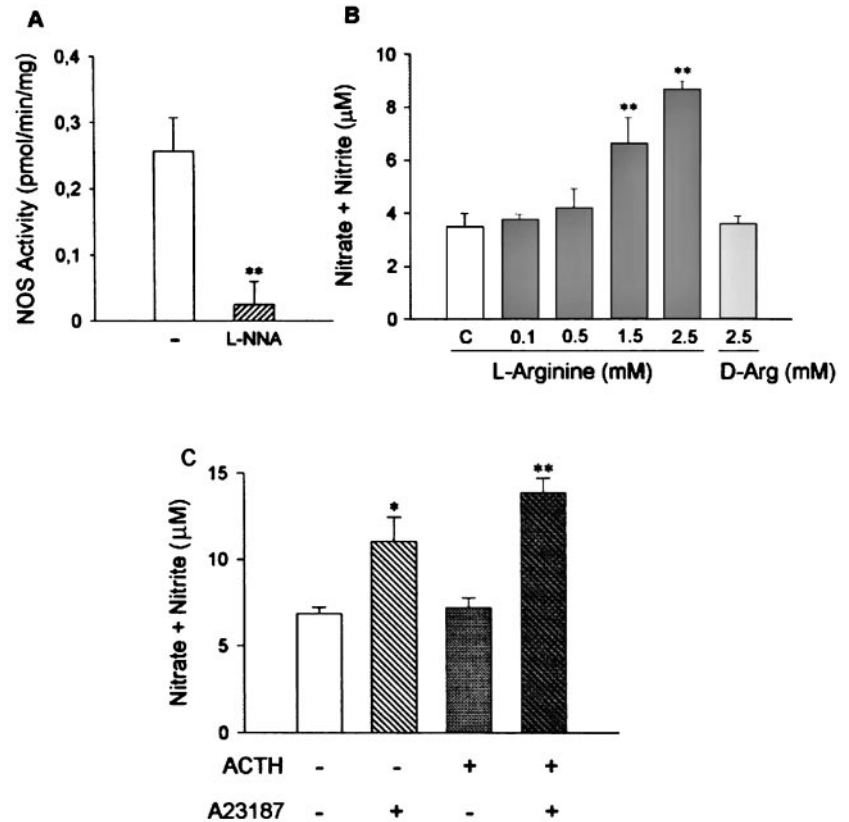
FIG. 7. Basal and ACTH-stimulated pregnenolone production by Y1 cells incubated in the presence of L-arginine or DETA-NO. Cells were preincubated for 15 min at 37 C in the presence of cyanoketone (10 μM), then incubated for 15 min with increasing concentrations of L-arginine (0.1–2.5 mM; A and B) or DETA-NO (100–1000 μM) and further incubated for 2 h in the absence (A and C) or presence (B and D) of ACTH (0.2 mIU/incubation). When indicated, L-NNA (1 mM) was added together with cyanoketone 15 min before L-arginine (0.5 mM) addition. Pregnenolone production was assessed in the incubation medium by RIA. Each value represents the mean $\pm$ SEM of three independent experiments, each performed in quadruplicate. *, $P < 0.05$; **, $P < 0.01$ (*vs.* control, by Dunnett's test).
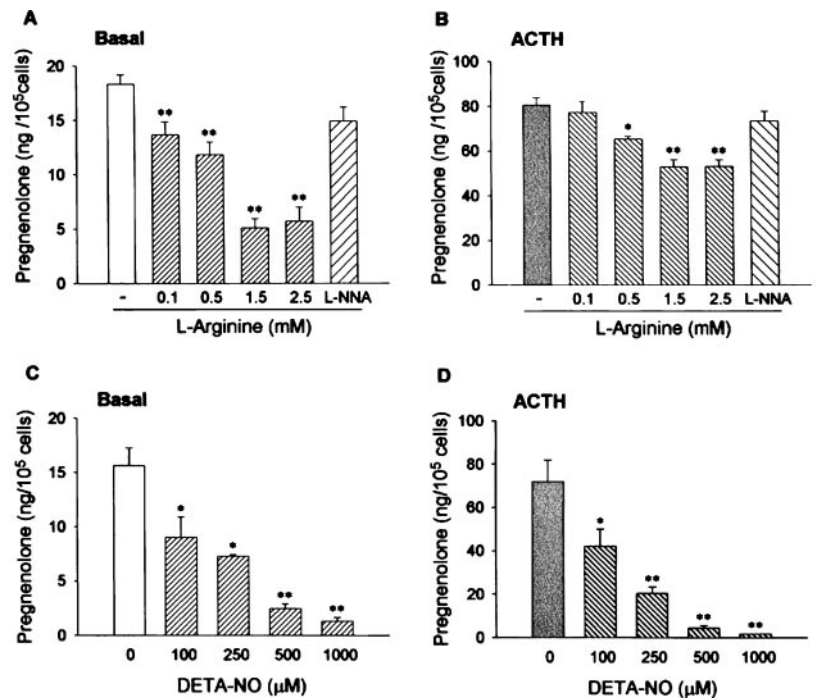
accessible, if at all, to NOS, whereas extracellular arginine transported into the cells is preferentially delivered to NOS (29, 30). A further increase in nitrite plus nitrate levels was observed when the cells were incubated in the presence of exogenous L-arginine and a calcium ionophore, suggesting that although intracellular calcium concentrations may be

sufficient to sustain NOS activity, it could be increased by a calcium influx from the extracellular compartment.

Depending on species, nutritional state, and diet, the L-arginine plasmatic concentration ranges from 70–180 μM (31, 32). L-Arginine significantly decreased steroid production in basal and ACTH-stimulated Y1 cells, with a threshold con-

TABLE 1. Effect of DETA-NO or the calcium ionophore A23187 on pregnenolone production from 22-R-OH-cholesterol in Y1 cells

Additions	Pregnenolone (ng/10^5 cells)
None	22.5 ± 7
22(R)-OH-cholesterol (5 μM)	526.9 ± 9
22(R)-OH-cholesterol (5 μM) + A23187 (10 μM)	473.7 ± 9^a
22(R)-OH-cholesterol (5 μM) + ACTH (10 mIU/ml)	572.4 ± 20
22(R)-OH-cholesterol (5 μM) + ACTH (10 mIU/ml) + A23187 (10 μM)	333.6 ± 11^b
22(R)-OH-cholesterol (5 μM) + DETA-NO (100 μM)	385.7 ± 3^c
22(R)-OH-cholesterol (5 μM) + DETA-NO (250 μM)	89.3 ± 9^c

Y1 cells were preincubated for 30 min in the presence of 5 μM cyanoketone and further incubated with the indicated additions for 150 min. Pregnenolone levels were assessed by RIA. Each value represents the mean ± SEM of three independent experiments, performed in quadruplicate.

[a] $P < 0.05$ *vs.* same treatment without A23187.
[b] $P < 0.001$ *vs.* same treatment without A23187.
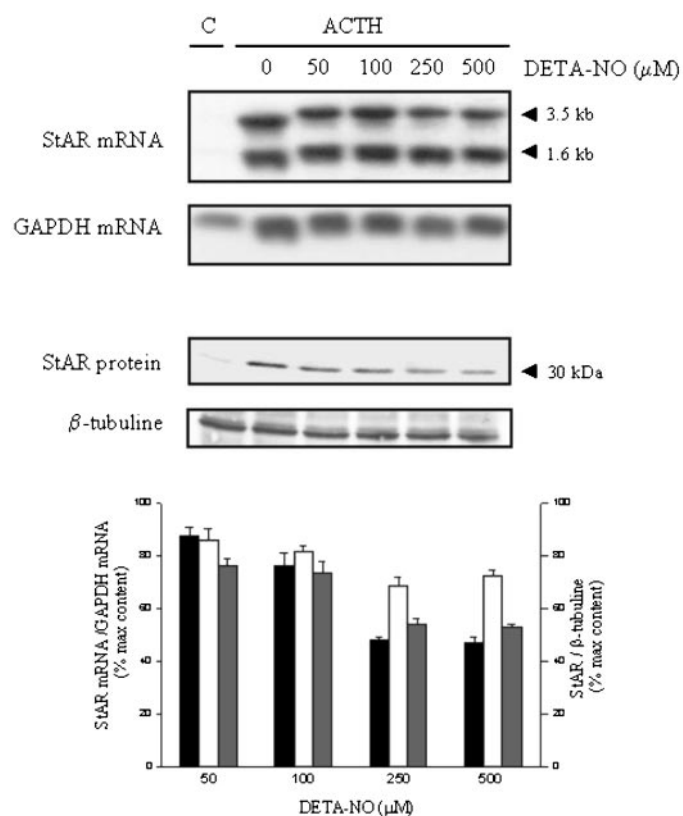[c] $P < 0.001$ *vs.* same treatment without DETA-NO, by Tukey's test.

FIG. 8. Effect of DETA-NO on StAR mRNAs and protein levels in Y1 cells under ACTH stimulation. Y1 cells were incubated for 120 min with ACTH (10 mU/ml) in the presence of increasing concentrations of DETA-NO (50, 100, 250, and 500 μM). Total RNAs and proteins were obtained as described in *Materials and Methods. Upper panels* show a representative Northern blot analysis of total RNAs probed with cDNA fragments of StAR and GAPDH. *Lower panels* show Western blot analysis of total cell proteins immunoblotted with StAR antibody. Protein loading was verified by immunoblotting the same membrane with β-tubuline. The histogram shows data representing the signal integration quantitated by densitometric scanning of StAR mRNAs signal normalized to GAPDH mRNA (■, 3.5 kb StAR mRNA; □, 1.6 kb StAR mRNA) and StAR protein normalized to β-tubuline protein (▨).

centration of 100 μM in nonstimulated conditions. The availability of L-arginine to eNOS depends on many factors, including the transport mechanism and the affinity of other enzymes that use L-arginine as a substrate. We have previously shown that the K_m of the L-arginine transport system

in ZF isolated cells is 150 μM (21). In addition, it seems likely that overcoming a certain threshold is necessary for the detection of the inhibitory effect of L-arginine-derived NO on Y1 cell steroidogenesis. In fact, the range of effective concentrations of L-arginine in Y1 cells is similar to that observed in isolated ZF cells. The effect of L-arginine on Y1 steroidogenesis was reproduced by an exogenous NO donor and blocked by L-NNA. Therefore, it seems likely that the inhibitory effect of L-arginine involves its local transformation to NO.

As the inhibitory effect of NO on steroidogenesis was observed in the presence of cyanoketone, an inhibitor of pregnenolone metabolism, one site of action for NO must be at least before pregnenolone synthesis. Moreover, as the effect of NO generated from both an NO donor and L-arginine was still evident in the presence of a permeable analog of cholesterol, the activity of the mitochondrial P450scc appears to be a target for NO action in Y1 cells. This is in agreement with the inhibitory effect of NO on cytochrome P450scc demonstrated in several steroidogenic systems (9, 11, 12, 21). However, as cholesterol availability to cytochrome P450scc is the rate-limiting step in steroid production the existence of an additional effect of NO on the transfer of cholesterol from the outer mitochondrial membrane to the inner membrane was also investigated. StAR plays a key role in the intramitochondrial movement of cholesterol (33); it is implicated in the rapid synthesis and secretion of steroids by adrenal cells under ACTH stimulation. The role of NO on StAR expression had not been addressed until very recently. In that sense, Kamidono *et al.* (34) demonstrated that in primary cultures of rat Leydig cells, StAR mRNA induction by LH was not affected by the NO generator, sodium nitroprusside. However, our results showed that in ACTH-stimulated Y1 cells an additional inhibitory site for NO was localized at the level of StAR expression (both mRNA and protein levels were affected), indicating that cholesterol transport between mitochondrial membranes could be another target for NO action in adrenal cells. The precise mechanism of NO inhibition on StAR expression and the functional significance of this inhibitory action of NO are currently under investigation.

To a minor extent the rat adrenal cell preparation is normally contaminated with endothelial cells, macrophages, or other cell types. In almost every putative contaminant of our preparation, the presence of NOS is highly probable. Thus, a pure population of steroidogenic cells, free from the con-

tamination of other cell types normally present in the adrenal gland, could be a useful tool to study the role of autocrine NO in the modulation of steroid biosynthesis. It is noteworthy that Y1 cells derive from an adrenocortical tumor, so care must be taken when extrapolating results to normal steroidogenic cells. In this sense although Y1 cells metabolize cholesterol to hydroxyprogesterone instead of corticosterone, they behave like normal steroidogenic cells in several respects, including the stimulation of steroid production by ACTH (28). Moreover, the inhibitory response to L-arginine observed in this cell line was similar to that obtained with ZF adrenal cells (21).

The adrenal gland is capable of responding to physiological demands with flexibility and subtlety in a manner that depends on the interactions of numerous cell types, each contributing its own signal to the system, and each responding in varied ways to the signals from the cells around it. As several NO-producing cells, mainly endothelial cells and macrophages, are often observed in intimate association with adrenal endocrine cells, the putative autocrine function of NO described herein may be one of the pathways through which NO participates in the regulation of adrenal physiology.

Acknowledgments

Address all correspondence and requests for reprints to: Dr. C. B. Cymeryng, Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155 5° (C1121ABG), Buenos Aires, Argentina. E-mail: cymeryng@fmed.uba.ar.

References

1. **Moncada S, Palmer RMJ, Higgs EA** 1991 Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109–142
2. **Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH** 1991 Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 351:714–718
3. **Lamas S, Marsden PA, Li GK, Tempst P, Michel T** 1992 Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc Natl Acad Sci USA 89:6348–6352
4. **Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan C** 1992 Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 256:225–228
5. **Cho HJ, Xie QW, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Nathan C** 1992 Calmodulin is a subunit of nitric oxide synthase from macrophages. J Exp Med 176:599–604
6. **Van Voorhis BJ, Dunn MS, Snyder GD, Weiner CP** 1994 Nitric oxide: an autocrine regulator of human granulosa-luteal cells steroidogenesis. Endocrinology 135:1799–1806
7. **Olson LM, Jones-Burton CM, Jablonka-Shariff A** 1996 Nitric oxide decreases estradiol synthesis of rat luteinized ovarian cells: possible role for nitric oxide in functional luteal regression. Endocrinology 137:3531–3539
8. **Dave S, Farrance DP, Whitehead SA** 1997 Evidence that nitric oxide inhibits steroidogenesis in cultured rat granulosa cells. Clin Sci 92:277–284
9. **Del Punta K, Charreau EH, Pignataro OP** 1996 Nitric oxide inhibits Leydig cell steroidogenesis. Endocrinology 137:5337–5343
10. **Natarajan R, Lanting L, Bai W, Bravo EL, Nadler J** 1997 The role of nitric oxide in the regulation of aldosterone synthesis by adrenal glomerulosa cells. J Steroid Biochem Mol Biol 61:47–53
11. **Hanke CJ, Drewett JG, Myers CR, Campbell WB** 1999 Nitric oxide inhibits aldosterone synthesis by a guanylyl cyclase-independent effect. Endocrinology 139:4053–4060
12. **Cymeryng CB, Dada LA, Podestá EJ** 1998 Effect of nitric oxide on rat adrenal zona fasciculata steroidogenesis. J Endocrinol 158:197–203
13. **Hesla JS, Preutthipan S, Maguire MP, Chang TS, Wallach EE, Dharmarajan AM** 1997 Nitric oxide modulates human chorionic gonadotropin-induced ovulation in the rabbit. Fertil Steril 67:548–552
14. **Chatterjee S, Gangula PR, Dong YL, Yallampalli C** 1996 Immunocytochemical localization of nitric oxide synthase-III in reproductive organs of female rats during oestrous cycle. Histochem J 28:715–723
15. **Jablonka-Shariff A, Olson LM** 1997 Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. Endocrinology 138:460–468
16. **Burnett AL, Ricker DD, Chamness SL, Maguire MP, Crone JK, Bredt DS, Snyder SH, Chang TS** 1995 Localization of nitric oxide synthase in the reproductive organs of the male rat. Biol Reprod 52:1–7
17. **Davidoff MS, Middendorff R, Mayer B, Holstein AF** 1995 Nitric oxide synthase (NOS-I) in Leydig cells of human testis. Arch Histol Cytol 58:17–30
18. **Tatsumi N, Fujisawa M, Kansaki M, Okuda Y, Okada H, Arakawa S, Kamodona S** 1997 Nitric oxide production by cultured rat Leydig cells. Endocrinology 135:1799–1806
19. **Stephan JP, Guillemois C, Jegou B, Bauche F** 1995 Nitric oxide production by Sertoli cells in response to cytokines and lipopolysacharide. Biochem Biophys Res Commun 213:218–224
20. **O'Bryan MK, Schlatt S, Gerdprasert O, Phillips DJ, de Kretser DM, Hedger MP** 2000 Inducible nitric oxide synthase in the rat testis: evidence for potential roles in both normal function and inflammation-mediated infertility. Biol Reprod 63:1285–1293
21. **Cymeryng C, Dada LA, Colonna C, Mendez CF, Podestá EJ** 1999 Effects of L-arginine in rat adrenal cells: involvement of nitric oxide synthase. Endocrinology 140:2962–2967
22. **Palacios M, Knowles RG, Palmer RMJ, Moncada S** 1989 Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. Biochem Biophys Res Commun 165:802–809
23. **Tsuchiya T, Kishimoto J, Nakayama Y** 1996 Marked increases in neuronal nitric oxide synthase (nNOS) mRNA and NADPH-diaphorase histostaining in adrenal cortex after immobilization stress in rats. Psychoneuroendocrinology 21:287–293
24. **Kishimoto J, Tsuchiya T, Emson P, Nakayama Y** 1996 Immobilization-induced stress activates neuronal nitric oxide synthase (nNOS) mRNA and protein in hypothalamic-pituitary-adrenal axis in rats. Brain Res 720:159–171
25. **Hanke CJ, Cambpell WB** 2000 Endothelial cell nitric oxide inhibits aldosterone synthesis in zona glomerulosa cells: modulation by oxygen. Am J Physiol Endocrinol Metab 279:E846–E854
26. **Schimmer BP** 1979 Adrenocortical Y1 cells. Methods Enzymol 52:570–574
27. **Van Voorhis BJ, Moore K, Strijbos PJLM, Nelson S, Baylis SA, Grzybicki D, Weiner CP** 1995 Expression and localization of inducible and endothelial nitric oxide synthase in the rat ovary. J Clin Invest 96:2719–2726
28. **Srivastava VK, Hiney JK, Rettori V, Dees WL** 1999 Effects of ethanol on intraovarian nitric oxide production in the prepubertal rat. J Endocrinol 161:69–75
29. **Kurz S, Harrison D** 1997 Insulin and the arginine paradox. J Clin Invest 99:369–370
30. **Mc Donald K, Zharikov S, Block E, Kilberg S** 1997 A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric oxide synthase may explain the "arginine paradox." J Biol Chem 272:31213–31216
31. **WU, G and Morris, S** 1998 Arginine metabolism: nitric oxide and beyond. Biochem J 336:1–17
32. **Kitiyakara C, Chabrashvili T, Jose P, Welch WJ and Wilcok CS** 2001 Effects of dietary salt intake on plasma arginine. Am J Physiol 280:R1069–R1075
33. **Stocco DM, Clark BJ** 1996 Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev 17:221–244
34. **Dobashi M, Fujisawa M, Yamazaki T, Okuda Y, Kanzaki M, Tatsumi N, Tsuji T, Okada H, Kamidono S** 2001 Inhibition of steroidogenesis in Leydig cells by exogenous nitric oxide occurs independently of steroidogenic acute regulatory protein (StAR) mRNA. Arch Androl 47:203–209