Characterization of L-arginine transport in adrenal cells: effect of ACTH

Esteban M. Repetto,1 Vanesa Pannunzio,1 Francisco Astort,1 Camila Martínez Calejman,1 Marcos Besio Moreno,2,3 Omar P. Pignataro,2,3 and Cora B. Cymeryng1
1Departamento de Bioquímica Humana, Facultad de Medicina; 2Instituto de Biología y Medicina Experimental, and 3Departamento de Química Biológica Facultad de Cs Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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Repetto, Esteban M., Vanesa Pannunzio, Francisco Astort, Camila Martínez Calejman, Marcos Besio Moreno, Omar P. Pignataro, and Cora B. Cymeryng. Characterization of L-arginine transport in adrenal cells: effect of ACTH. Am J Physiol Endocrinol Metab 291: E291–E297, 2006. First published February 14, 2006; doi:10.1152/ajpendo.00413.2005.—Nitric oxide synthesis depends on the availability of its precursor L-arginine, which could be regulated by the presence of a specific uptake system. In the present report, the characterization of the L-arginine transport system in mouse adrenal Y1 cells was performed. L-arginine transport was mediated by the cationic/neutral amino acid transport system y+L and the cationic amino acid transporter (CAT) y+ in Y1 cells. These Na+-independent transporters were identified by their selectivity for neutral amino acids in both the presence and absence of Na+ and by the effect of N-ethylmaleimide. Transport data correlated to expression of genes encoding for CAT-1, CAT-2, CD-98, and y+L. A similar expression profile was detected in rat adrenal zona fasciculata. In addition, cationic amino acid uptake in Y1 cells was upregulated by ACTH and/or cAMP with a concomitant increase in nitric oxide (NO) production.

Although ACTH has been largely recognized as the primary regulator of adrenal cortex development and function, locally produced factors may synergize or antagonize its biological effects. Several lines of evidence support the inclusion of NO among the autocrine/paracrine regulators of adrenal function, as was also demonstrated for other endocrine tissues (9, 11, 18, 26, 35). In previous reports (6, 7) we have demonstrated that NO donors inhibit steroid synthesis in adrenal cells. Moreover, we also reported that adrenal steroidogenesis is negatively modulated by an endogenous NO synthase (NOS) activity. In addition, it has been recently demonstrated that NO is involved in the modulation of adrenal blood flow (28, 41).

Because incubation of adrenal cells in the presence of L-arginine resulted in a significant inhibition of steroid production and a concomitant increase in nitrite plus nitrate and cGMP levels (6, 8), we hypothesized that the endogenous production of NO could depend on extracellular L-arginine levels, and therefore, the activity of the L-arginine transport system could modulate NO production in adrenal cells.

Four distinct transport mechanisms, systems y+, y+L, b0,+ and B0,+ cooperate for L-arginine transport in mammalian cells (12). These transporters have been classified in terms of their ion dependency and their specificity and relative affinity for their substrates. The y+ system is selective for cationic amino acids; the other three cationic amino acid carriers (y+L, b0,+ and B0,+ ) also transport neutral amino acids in an Na+-dependent, Na+-independent, and Na+- and Cl–-dependent manner, respectively. Both systems y+L and b0,+ are heterodimeric amino acid transporters comprised of a heavy-chain subunit (4F2hc and rBAT, respectively) and a light-chain subunit (y+LAT1, y+LAT2, and b0,+ respectively) (3, 12, 37a).

Despite the significant role of NO in the modulation of adrenal function, L-arginine transport systems in this tissue have not been previously examined. Therefore, we undertook the biochemical characterization of L-arginine transport systems in Y1 adrenal cells. Y1 cells, derived from murine adrenal cortex, are a useful model for investigating adrenal cells because they behave as normal steroidogenic cells in several aspects, including the stimulation of steroid production by ACTH in a cAMP-dependent pathway (32, 33) and the ACTH-dependent induction of both early and delayed genes (38). We also identified the transporters operating in Y1 cells and in rat adrenal zona fasciculata (ZF). Finally, we studied the effects of ACTH on L-arginine transport.

MATERIALS AND METHODS

ACTH was obtained from ELEA Laboratories (Buenos Aires, Argentina). Sodium nitrite, N-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, 8Br-cAMP, and L-arginine were purchased from Sigma Chemical (St. Louis, MO). Fetal calf serum, penicillin, and streptomycin were from Invitrogen (Life Technologies, Buenos Aires, Argentina), and L-[2,3,4,5-3H]arginine (60 Ci/mmol) was from NEN Life Sciences (Boston, MA). All other chemicals were of the highest quality available.

Cell culture and treatments. The cell lines used in these studies were 1) Y1, an ACTH- and cAMP-responsive subclone of the mouse adrenocortical tumor cell line isolated by Yasumura et al. (39) and 2) a cAMP-resistant, protein kinase-defective mutant derived from Y1 designated Kin-8 (27). Y1 and Kin-8 cells were generously provided by Dr. Bernard Schimmer (University of Toronto). Cells were grown as monolayers in plastic tissue culture dishes in growth medium (Ham’s F-10) containing heat-inactivated fetal bovine (2.5%) and horse (12.5%) serum, 200 U/ml penicillin G, and 270 μg/ml streptomycin sulfate. Cells were incubated in a humidified atmosphere of 5% CO2 in air at 37°C (32). Treatments were initiated by replacing the complete culture medium with fresh Ham’s F-10 without serum for the specified time periods, with or without the addition of the following agents: ACTH (10 mU/ml), 8Br-cAMP (500 μM), forskolin (1 μM), (N-[2-(p-bromocinnamyl)amino]ethyl)-5-isouquinoline-sulfonamide HCl) or H-89 (10 μM), wortmannin (100 nM), LY-294002 (50 μM).

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Address for reprint requests and other correspondence: C. B. Cymeryng, Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155 5° (1121ABG), Buenos Aires, Argentina (e-mail: cymeryng@fmed.uba.ar).

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Transcriptional/translational upregulation of transporters was determined by treating Y1 cells with either actinomycin D (1 μg/ml) or cycloheximide (1 μg/ml) before ACTH addition.

Cell viability was assessed by the trypsin blue dye exclusion test, as determined by microscopy. No significant difference was observed for any of the treatments.

**RNA Isolation and RT-PCR.** Total RNA was extracted from Y1 cells with TRIzol reagent (Invitrogen). RNA (2 μg) was pretreated with RNase-free DNase (deoxyribonuclease I, amplification grade; Invitrogen), heated at 70°C for 10 min, placed on ice for 1 min, and then incubated with a mixture containing 0.5 mM dNTPs mix, 25 ng/μl (8 μM) random primers, 1× first-strand buffer, 25 units of rRNase inhibitor, 200 units of MMLV reverse transcriptase (Promega, Madison, WI), and water to a final volume of 25 μl for 1 h at 42°C. The reaction was stopped by being heated at 90°C for 5 min. The reaction mixture was brought to 100 μl with diethylpyrocarbonate-treated water and stored at −70°C. In selected tubes the reverse transcriptase was omitted as a control of amplification from contaminating cDNA or genomic DNA.

PCR reactions were carried out in a Tpersonal Thermocycler (Biometra Biomedizinische Analytik, Göttingen, Germany) and were performed using 2 μl of cDNA for the amplification of transporter gene products. The cDNA was added to 18 μl of the following reaction mixture: 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP, 500 nM of each specific oligonucleotide primer, and 0.625 U Taq polymerase (Life Technologies). The sequence for the oligonucleotide primers that were based on published sequences is shown in Table 1. GAPDH was used in the semiquantitative RT-PCR protocol as a constitutively expressed housekeeping gene.

For amplifying the genes coding for L-arginine transporters, PCRs were carried out with a first step at 94°C for 3 min and then the corresponding number of cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and elongation at 72°C for 90 s plus a final incubation at 72°C for 10 min. Reaction products were electrophoresed on 1.5% agarose gels in 40 mM Tris acetate and 2 mM EDTA, pH 8, stained with ethidium bromide, photographed, and quantitated by a video documentation system (GelPro Imager; Image Processing Solutions, North Reading, MA). The identity of the obtained amplions was confirmed by sequencing using the T7 Sequenase Quick-Genature plasmid sequencing kit (Amersham Pharmacia Biotech, Arlington Heights, IL) after being cloned into pGEM-T easy (Promega).

### L-Arginine transport assay.

Unidirectional transport of L-[3H]arginine was measured in Y1 cells. The cells were plated at a density of 2 × 10^5 cells per well (1,000 cells/mm^2) in 12-well trays. In all the experiments, Y1 cells were incubated for the indicated periods of time with the appropriate additions in fresh medium. After the treatments, the cells were washed twice with 1 ml of buffer A (5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 5.6 mM D-glucose, and 20 mM HEPES, pH 7.4) containing either 140 mM NaCl or choline chloride, and the uptake was measured by adding 500 μl of buffer A containing L-[2, 3-3H]arginine (final concentration 1 μCi/ml) to each well. Transport was rapid, time dependent, and apparently linear for up to 2 min (data not shown). Accordingly, L-arginine influx was measured over 1 min at 37°C at the indicated external L-arginine concentrations. Incubations were terminated by rinsing the cells three times with 1 ml buffer A. Cells were detergent solubilized in 0.5% Nonidet P-40 and 0.1% SDS, and the radioactivity in the extracts was quantified by liquid scintillation counting. To correct for nonspecific uptake or binding to the cell surface, L-[2,3-3H]arginine was added and immediately removed from the cells, and the fraction of the radioactivity associated was determined. These values were subtracted from each data point. L-[3H]arginine uptake was expressed as picomoles of L-arginine per milligram protein per minute. Calculations of Km and Vmax of L-arginine uptake were carried out using GraphPad Prism version 4.03 software (GraphPad Software) to perform regression analyses and rectangular hyperbola transformations.

**Measurement of L-arginine efflux.** For efflux experiments the cells were preloaded for 20 min with 50 μM L-[3H]arginine in buffer A. Subsequently, the cells were washed three times with 400 μl of buffer A. To initiate efflux, the buffer was aspirated and replaced by 400 μl of fresh buffer or by buffer supplemented with the indicated additions. The buffer was replaced every 30 s and radioactivity was counted in the incubation medium by liquid scintillation. After the indicated times, the cells were lysed as described above, and the radioactivity retained by the cells was also determined. Greater than 80% of the released radioactivity was identified as authentic L-arginine by TLC.

**Measurement of nitrite accumulation.** Nitrite levels were determined in cell-free medium at the end of the culture periods by a spectrophotometric assay that was based on the Griess reaction, as described previously (6). The nitrite contents given in RESULTS were expressed as nanomoles of nitrite per milligram of protein.

**Statistical analysis.** All values are means ± SE of n experiments. Statistical analysis was performed by one-way ANOVA followed by Dunnett’s or Tukey’s t-test using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego CA).

**RESULTS**

Characterization of L-arginine uptake in Y1 cells. The biochemical characterization of L-arginine uptake in Y1 cells is shown in Fig. 1. L-Arginine was transported into the cells in an Na^+ independent manner, as sodium replacement by choline had no effect on this parameter. Figure 1 also shows the effect of selected amino acids on L-arginine uptake. The transport of L-[3H]arginine was significantly inhibited by L-lysine and L-ornithine and by the addition of unlabeled L-arginine, but not D-arginine. The neutral amino acids L-leucine and L-glutamine significantly inhibited L-arginine uptake, although neither L-

**Table 1. Primers used for RT-PCR analysis**

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<tr>
<th>GenBank Accession No.</th>
<th>Species</th>
<th>cDNA</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Product Size (position), bp</th>
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<td>NM_013111</td>
<td>Rat</td>
<td>SLC7A1</td>
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<tr>
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<td>5′-CATGCTGGTGCGTGGAGAAGG-3′</td>
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<td>GAPDH</td>
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<tr>
<td>XM 233305</td>
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<td>SLC6A4</td>
<td>5′-GCTGATCCAGTTGAGGATG-3′</td>
<td>5′-CTCAGCCTCCTCTTTGCC-3′</td>
<td>535 (156–690)</td>
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CAT, cationic amino acid transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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cystine nor L-valine had any effect. Addition of 5 mM L-leucine or L-glutamine almost completely blocked L-arginine uptake in the presence of Na+; although neither of them had a significant effect when Na+ was replaced by choline (data not shown). To analyze transstimulation effects, L-arginine uptake was measured after the cells were preloaded for 1 h with L-lysine or L-glutamine (2 mM). Figure 2 shows that loading Y1 cells with L-lysine or L-glutamine stimulated L-arginine influx ~20- and 3-fold, respectively. This effect was not prevented by addition of cycloheximide.

Characterization of L-arginine efflux in Y1 cells. To study the transport systems mediating the outward flux of L-arginine in Y1 cells, L-[3H]arginine efflux was determined in Na+ or choline HEPES buffer (Fig. 3). A significant and Na+-independent L-arginine efflux was observed in the absence of any added amino acid. Both L-lysine (1 mM) and L-glutamine (1 mM) induced a marked efflux of preloaded L-[3H]arginine (Fig. 3). In the absence of Na+, only L-lysine increased L-arginine efflux.

After the cells with N-ethylmaleimide (NEM) were pretreated, a selective inhibitor of system y, L-arginine efflux, was almost abolished, although L-lysine or L-glutamine induced efflux was also significantly inhibited by this treatment (Table 2).

Expression profile of L-arginine transporters in Y1 cells and in rat adrenal ZF. Analysis of the expression of genes involved in cationic amino acid transport in Y1 cells was performed by RT-PCR. Results shown in Fig. 4A indicate the expression of transcripts encoded by SLC3A2 (heavy chain, CD98/4F2hc) and SLC7A9 (light chain, y+LAT-2), whereas only a very low signal was detected for SLC7A7 (y+LAT-1) and SLC7A9 (heavy chain, rbat). Transporters SLC7A1 and SLC7A2 coding for system y+ cationic amino acid transporter (CAT)-1 and CAT-2, respectively, were also detected. Both splice variants of CAT-2 gene were detected in Y1 cells (data not shown). RT-PCR analysis performed in total RNA from rat adrenal ZF showed the expression of both y+ and y+-LAT transport systems (Fig. 4B).

Effect of ACTH on L-arginine transport and NO production in Y1 cells. Incubation of Y1 cells with ACTH resulted in a significant increase in L-arginine uptake. In addition, ACTH significantly increased nitrite levels in the incubation medium (Fig. 5, A and B). The effect of ACTH on L-arginine uptake and nitrite levels was reproduced by 8Br-cAMP and forskolin (Fig. 5, A and B). ACTH failed to increase L-arginine uptake in the presence of a PKA inhibitor (H-89) and in Kin-8 Y1 cells (a cPKA-deficient cell line derived from Y1; Fig. 6). Incubation in the presence of 3 μM chelerytrine (a PKC inhibitor) had no effect on ACTH-induced L-arginine influx (data not shown), whereas inhibition of phosphatidylinositol 3-kinase (PI3K) by wortmannin or LY-294002 did not inhibit the increase in L-arginine uptake by ACTH (data not shown). L-arginine efflux significantly decreased in the presence of ACTH (control: 71.37 ± 9.16 dpm.min⁻¹.μg⁻¹ protein; ACTH: 54.83 ± 16.7 dpm/min.μg protein; P < 0.05).

Cycloheximide treatment significantly inhibited L-arginine uptake both in control and ACTH-stimulated cells, although actinomycin D had no effect on this parameter (Fig. 7). ACTH treatment did not modify the messenger levels for the transporters studied or endothelial (e)NOS in Y1 cells (Figs. 4A and 8).

DISCUSSION

The foregoing results indicate the involvement of both y+ and y+L carriers in L-arginine transport in Y1 cells. We also demonstrate a stimulatory effect of ACTH on L-arginine transport and nitrite levels, suggesting that NOS activity in Y1 cells...
could be upregulated by ACTH, probably through an increase in its substrate availability.

As already mentioned, four transport systems have been described for l-arginine transport, namely y\(^+\), y\(^+\)-L, b\(^{+\,+}\) and B\(^{++}\). Because l-arginine transport in Y1 cells was Na\(^+\)-independent, the presence of Na\(^+\)-dependent transport systems, such as B\(^{++}\), was ruled out. In addition, a contribution of b\(^{+\,+}\) system was also discarded on the basis of the Na\(^+\) dependency of the inhibitory effect of neutral amino acids and the absence of the effect of l-cystine (a specific substrate for b\(^{+\,+}\) system).

The involvement of system y\(^+\)L in l-arginine uptake and efflux in Y1 cells is supported by several observations: 1) neutral amino acids, such as l-leucine or l-glutamine, that interact weakly with system y\(^+\) (12) strongly inhibited l-arginine uptake in the presence of Na\(^+\), whereas l-valine had no effect; 2) inhibition of l-arginine uptake by l-leucine or l-glutamine was lower in the absence of Na\(^+\) than in its presence; 3) l-arginine uptake was highly stimulated by cationic amino acids (L-lysine) and by neutral amino acids (L-glutamine) on the trans side of the membrane, an effect that did not depend on protein synthesis because it was not impaired by cycloheximide; 4) the expression of both components of the heterodimer, y\(^+\)-LAT-2 (SLC7A6) and CD-98 (SLC3A2, 4F2hc), was demonstrated in Y1 cells; and 5) the basic amino acids L-lysine or L-ornithine significantly reduced L-arginine uptake in an Na\(^+\)-independent way (a characteristic shared by system y\(^+\)).

The effect of NEM on the efflux of l-arginine in Y1 cells suggests the activity of y\(^+\) system, whereas RT-PCR studies demonstrated the expression of two members of the CAT family (SLC7A1/CAT-1 and SLC7A2/CAT-2). CAT proteins have previously been involved in the influx as well as in the efflux of cationic amino acids (4). Results obtained in trans-stimulation experiments indicate that both y\(^+\) and y\(^+\)-LAT systems are involved in l-arginine transport, suggesting that both systems cooperate for l-arginine transport in Y1 cells. Our results also support the involvement of the y\(^+\)-LAT-2 carrier, in addition to the previously demonstrated y\(^+\) system, in l-arginine transport in rat adrenal ZF (6). The coexistence of several CATs of high and low affinity in the same cell type has been previously demonstrated in human placental microvascular endothelial cells (13), INF-γ-stimulated human monocytes (29), human platelets (33a), and a rat thyroid cell line (37). The physiological relevance of each transport system in l-arginine transport in adrenal cells is presently difficult to determine. However, considering the physiological concentration of l-arginine in the extracellular medium (150–200 μM) and the reported K\(_m\) for both transport systems (12), it seems likely that both transporters cooperate for l-arginine influx in adrenal cells. Alternatively, it has been reported that system y\(^+\)L functions as an obligatory exchanger of cationic and neutral amino acids (2, 36). Therefore, l-arginine efflux in exchange for neutral amino acids and Na\(^+\) could be a mechanism through which steroidogenic cells provide other NO producing adrenal cells (endothelial and neural cells) with this amino acid. By this mechanism, l-arginine-derived NO synthesized by endothelial cells within the adrenal cortex could be involved in the regulation of blood flow by histamine (42) or angiotensin II (16) or in the effects of the vascular endothelial growth factor or endocrine gland derived-VEGF (34a). Moreover, it is tempting to speculate that NO produced by adrenal endothelial cells could be involved in the modulation of steroid secretion in the ZF because it was demonstrated in zona glomerulosa cells (19).

The key role of ACTH in adrenal growth and differentiation has been widely recognized. Several specific genes, particularly those related to steroid synthesis, and transcription factors are regulated by ACTH mainly via PKA, the most important signaling pathway involved in the response to this hormone. The present results demonstrate that ACTH increased l-arginine uptake in Y1 cells. This effect seems to be mediated by the cAMP-dependent PKA, since ACTH action was mimicked by 8Br-cAMP and forskolin and inhibited by H-89. In addition, ACTH had no effect on l-arginine uptake in the PKA-defective cell line Y1-Kin-8. Moreover, on the basis of inhibitor studies, the involvement of PKC or PI3K activities in the stimulatory effect of ACTH in l-arginine uptake was also discarded.

### Table 2. Effect of NEM on l-[\(^3\)H]arginine efflux in Y1 cells

| l-Arginine Efflux, dpm/μg protein \(^{-1}\) min\(^{-1}\) |
|-----------------|-----------------|
| Control         | 83.77 ± 1.66    |
| NEM, 0.5 mM     | 18.5 ± 3.05*    |
| L-Lys, 1 mM     | 167.16 ± 15.63  |
| L-Lys, 1 mM + NEM 0.5 mM | 118.68 ± 6.52† |
| L-Gln, 1 mM     | 183.33 ± 16.9   |
| L-Gln, 1 mM + NEM 0.5 mM | 101.93 ± 15.32‡ |

Values are expressed as means ± SE, n = 5. NEM, N-ethylmaleimide. Y1 cells were preloaded with 50 μM l-[\(^3\)H]arginine for 20 min, with 0.5 mM NEM added during the last 10 min of the incubation. Efflux was measured in the absence or presence of 1 mM l-lysine or l-glutamine in standard uptake solution containing Na\(^+\). *P < 0.05 vs. control without NEM; †P < 0.05 vs. l-Lys without NEM; ‡P < 0.001 vs. l-Gln without NEM.
is, to our knowledge, the first report on the modulation of CAT by ACTH via PKA.

Although ACTH had no significant influence on the messenger levels of the l-arginine transporters, stimulation of l-arginine uptake apparently involves de novo protein synthesis. This aspect is currently under investigation, although at present, an additional ACTH-dependent posttranslational mechanism cannot be discarded. However, there have been no reports on PKA-dependent phosphorylation of l-arginine transporters.

The expression and activity of eNOS in Y1 cells and the effect of increasing extracellular l-arginine concentrations on nitrite production have been previously demonstrated (8). In the present report we showed that in addition to increasing l-arginine uptake, ACTH also augmented nitrite levels. This effect could be the consequence of the coordinate regulation of l-arginine uptake and NOS activity as it was described in other cellular types. In endothelial cells, a direct correlation of l-arginine uptake and intracellular eNOS activity in response to stimulation by different agents has been demonstrated (20) and NOS activity has been associated with CAT-1 transporters (40). The activity of systems y⁺L and y⁺LAT has also been shown to modulate NO production in platelets (33a).

The increase in nitrite levels by ACTH could also reflect a direct effect of the hormone on eNOS expression levels or enzymatic activity. In this sense, upregulation of eNOS expression by db-cAMP has been demonstrated in cardiomyocytes (34) and in vascular endothelial cells (25), although PKA-dependent phosphorylation and activation of eNOS was also shown in platelets and adipocytes (23, 30). However, because the magnitude of the effect of ACTH on l-arginine uptake and nitrite production in Y1 cells was similar, we hypothesized that the observed increase in NO production is probably due to the stimulation of l-arginine uptake by ACTH. In agreement, eNOS messenger levels were not significantly affected by ACTH treatment.

Our results suggest that, although ACTH increases steroid production in adrenal cells, by increasing NO synthesis ACTH could exert a negative control of steroidogenesis. A dual effect was also observed in ZG, where ACTH acutely increased aldosterone production while decreasing the transcription of aldosterone synthase (1). Because the increase in NO, observed in Y1 cells, is probably involved in the long-term response to the hormone, it seems reasonable to suggest that, in addition to the inhibitory effects of NO on cytochrome P450scc activity, there could be an additional inhibitory effect of NO on the
transcription of proteins involved in the steroidogenic pathway, as it was previously demonstrated for steroidogenic acute regulatory protein (8).

Our current hypothesis is that NO belongs to a group of autocrine/paracrine modulators of steroidogenesis whose combinatorial effects are involved in the fine tuning of the cellular responses to hormones, therefore providing the gland with a mechanism to avoid an “all or none” kind of response. This group of modulators includes, among others, growth factors (15), serotonin (5), endorphins, and pituitary adenylate cyclase-activating polypeptide (14), galanin (22), and endothelins (10).
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