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Effects of Nitric Oxide on Aldosterone Synthesis and Nitric Oxide Synthase Activity in Glomerulosa Cells from Bovine Adrenal Gland

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This study investigated the effects of two NO-releasing agents, diethylenetriamine-NO (deta-NO) and sodium nitroprusside (SNP), on basal, ACTH-, and angiotensin II (AngII)-stimulated aldosterone production in glomerulosa cells from bovine adrenal gland. NO donors inhibited basal and ACTH- or AngII-stimulated aldosterone synthesis in a concentration-dependent manner. Deta-NO and SNP also provoked a concentration-dependent stimulation of cGMP production. However, cGMP was not responsible for the inhibition of aldosterone secretion, because a cGMP analog did not reproduce the inhibitory effect. Moreover, soluble guanylyl cyclase or protein kinase G inhibitors did not revert the inhibitory effect of NO on aldosterone production. NO donors did not modify ACTH-stimulated cAMP production or AngII-stimulated PLC activity stimulation, but inhibited 22[R] hydroxycholesterol- or pregnenolone-stimulated aldosteronogenesis. NO can be synthesized in bovine glomerulosa cells because nitrite production was determined and characterization of NOS activity was also performed. Nitrite accumulation was not modified in the presence of ACTH, AngII, or other factors used to induce iNOS. NOS activity that showed a Michaelis–Menten kinetic was NADPH- and calcium-dependent and was inhibited by two competitive inhibitors, L-NAME and L-NMMA. These results show that NO inhibits aldosterone production in glomerulosa cells acting on P450scc and other P450-dependent steroidogenic enzymes, and these cells display NOS activity suggesting that NO can be produced by constitutive NOS isozymes.

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Introduction

Nitric oxide (NO) is an important intracellular and intercellular messenger controlling many physiological processes *(1,2)*. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS), which has been found in a variety of cell types. Three isozymes of NOS have been identified: NOS I or neuronal (nNOS), NOS II or inducible (iNOS), and NOS III or endothelial (eNOS) *(3–5)*. nNOS and eNOS are calcium-dependent and constitutive. Both generate NO in small amounts. In contrast, iNOS is calcium-independent and inducible by cytokines. It can produce large amounts of NO, which can be cytotoxic or can inhibit pathogens.

Previous studies have shown that NO regulates steroidogenesis in different tissues. Nitric oxide inhibits Leydig cell steroid production, and this effect is not mediated by cyclic GMP *(6)*. Tatsumi et al. *(7)* have reported the presence of iNOS and NO production in cultured rat Leydig cells. Moreover, Van Voorhis et al. *(8)* have detected eNOS messenger RNA in human granulosa and luteal cells and showed that NO donors caused a concentration-dependent decrease in estradiol and progesterone secretion. Immunofluorescent and immunoblotting experiments have shown that rat luteinized ovaries express the endothelial and inducible isoforms of NOS *(9)*, and the authors suggest that NO participates in functional luteal regression by inhibiting estradiol secretion.

Several works have been published showing that NO regulates steroidogenesis in adrenal cortex. Cymeryng et al. *(10,11)* have described that NO donors or exogenously added L-arginine inhibits corticosterone secretion in rat adrenal zona fasciculata cells. This effect is not mediated by cGMP and could be generated by a direct inhibition of cytochrome

P450scc. Endogenous NO synthesized by nNOS and/or eNOS could be involved in the inhibition of corticosterone secretion *(12)*.

As regards mineralocorticoid synthesis, sodium nitroprusside (SNP), a NO donor, has been reported to inhibit aldosterone production in bovine adrenal gland but a cGMP analog was found to be an stimulator of the aldosterone response rather than an inhibitor *(13)*. In chicken adrenocortical cells *(14)*, the inhibition by 8-bromo cGMP of aldosterone secretion suggests that cyclic GMP mediates the SNP-provoked inhibition on steroidogenesis. Aldosterone synthesis inhibition by NO has also been described in human and rat adrenal glomerulosa cells, and the presence of eNOS in these cells has been reported by Natarajan et al. *(15)*. In calf glomerulosa cells NO inhibits aldosterone synthesis and increases cGMP levels, but, in contrast with the results described above, the mechanism of steroid inhibition appears to be independent of guanylyl cyclase *(16,17)*. However, Hanke and Campbell *(18)* have observed that only bovine adrenal endothelial cells but not adrenal glomerulosa cells, contain detectable levels of eNOS.

The aim of this work was to study the role of NO on basal, ACTH- and angiotensin II (AngII)-stimulated aldosterone production in primary culture of bovine glomerulosa cells, focusing on the effects of NO on ACTH and AngII transduction mechanisms. In addition, NO production was measured and characterization of NOS activity was also performed.

Results

Effects of NO-Releasing Agents on Steroidogenesis in Bovine Adrenal Glomerulosa Cells

Effect of deta-NO and SNP on Basal, ACTH-, and AngII-Stimulated Aldosterone Synthesis in Primary Culture of Bovine Adrenal Glomerulosa Cells

Basal aldosterone levels were 0.4 ± 0.1 ng/10^6 cells × 2 h. When cells were incubated with different concentrations of diethylenetriamine-NO (deta-NO) or SNP, basal aldosterone production significantly decreased in a concentration-dependent manner (Fig. 1A). Maximal inhibitions observed were 46.5 ± 10.2% and 27.4 ± 8.9% for deta-NO (1 m*M*) and SNP (1 m*M*), respectively.

Aldosterone levels increased from 0.4 ± 0.1 to 2.7 ± 0.3 ng/10^6 cells × 2 h when cells were stimulated with ACTH (10 n*M*). deta-NO and SNP evoked a concentration-dependent inhibition of ACTH-stimulated aldosterone production (Fig. 1B). Maximal inhibitions observed on ACTH-stimulated aldosterone synthesis were 77.0 ± 3.4% and 56.3 ± 6.6% for deta-NO (1 m*M*) and SNP (1 m*M*), respectively.

Both NO donors also caused a concentration-dependent inhibition of 100 n*M* AngII-induced aldosterone synthesis (maximal response for AngII: 2.0 ± 0.3 ng/10^6 cells). Maximal inhibitions observed were 78.1 ± 6.6% for deta-NO (1 m*M*) and 60.5 ± 9.0% for SNP (1 m*M*) (Fig 1C).

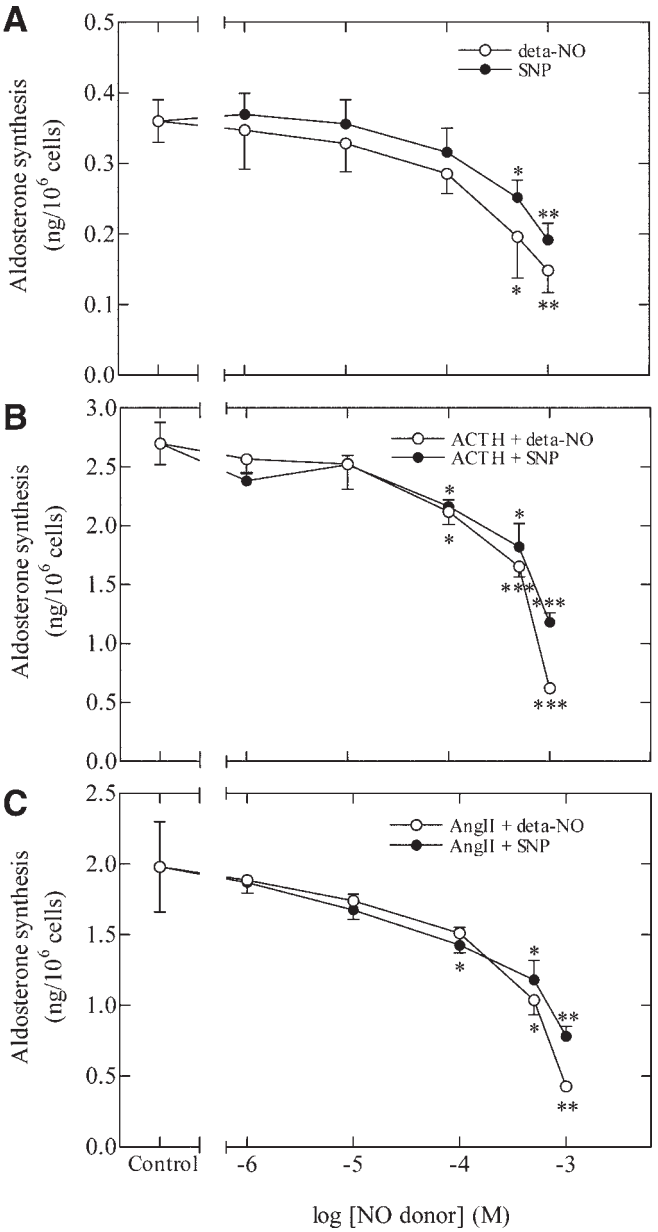

Fig. 1. Effects of NO donors on basal, ACTH-, and AngII-stimulated aldosterone secretion in zona glomerulosa cells from bovine adrenal gland. Glomerulosa cells were preincubated with increasing concentrations (0–1 m*M*) of deta-NO or SNP for 10 min. Vehicle (**A**), 10 n*M* ACTH (**B**), or 100 n*M* AngII (**C**) were then added and cells were incubated for 2 h at 37°C. Aldosterone production was measured as described in *Methods*. Results are expressed as mean ± SEM from five independent experiments, each performed in triplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs respective control.

Hemoglobin, a NO scavenger, was utilized to confirm that the inhibitory effect of deta-NO and SNP on glomerulosa cells' aldosterone secretion was mediated by releasing NO and not by a nonspecific effect of these donors. Hemoglobin (10–300 μ*M*) was added to the samples in order to

evaluate its effects on aldosterone secretion, and no effect was found. When hemoglobin (100 μ*M*) was added simultaneously with deta-NO or SNP, it completely prevented the inhibitory effects of both NO releasing agents (0.1, 0.5, and 1 m*M*) on basal or agonist-stimulated aldosterone synthesis.

On the other hand, deta-NO and SNP increased cGMP intracellular levels in a concentration-dependent manner with a maximal stimulation of 20- and 16-fold, respectively, over basal value (0.47 ± 0.04 pmol/10^6 cells). Neither AngII (100 n*M*) nor ACTH (10 n*M*) alone had effects on cGMP production and both hormones did not modify the cGMP production induced by NO donors (data not shown).

Because inhibition of aldosterone production in the presence of NO donors was accompanied by a sharp increase in cGMP production, we conducted experiments to examine the role of cGMP and soluble guanylyl cyclase on NO-dependent aldosterone inhibition. The effect of a soluble and not hydrolyzable cGMP analog, 8-Br-cGMP, on aldosterone synthesis was studied. 8-Br-cGMP (1–500 μ*M*) had no effect on basal, AngII, or ACTH-stimulated aldosterone production. Two different soluble guanylyl cyclase inhibitors, methylene blue (20 μ*M*) and ODQ (2 μ*M*), were used. Both inhibitors completely blocked the deta-NO- and SNP-induced increase in cGMP production at all concentrations of NO donors assayed, but were not capable of abolishing the inhibition on basal or ACTH- and AngII-stimulated aldosterone production. In addition, Rp-8-Br-PET-cGMP, a protein kinase G (PKG) inhibitor, was used to avoid cGMP-dependent phosphorylations. When Rp-8-Br-PET-cGMP (10 μ*M*) was preincubated for 30 min before the assay, it could not block deta-NO-dependent inhibition on aldosterone production.

Taken together, our results suggest that cGMP does not mediate the NO-dependent inhibition on aldosterone production.

Effects of NO-Releasing Agents on ACTH- and AngII-Stimulated Second Messengers

In order to evaluate the possible site(s) of action of NO in inhibiting aldosterone synthesis, the effects of deta-NO and SNP on ACTH and AngII transduction mechanisms were studied. We first examined whether NO could affect the production of cAMP, the main second messenger that mediates ACTH physiological action on bovine zona glomerulosa cells. Figure 2A shows that ACTH produced a 15-fold increase over basal value in the intracellular levels of cAMP (control: 5.5 ± 0.3 pmol/10^6 cells; ACTH 84.4 ± 8.3 pmol/10^6 cells), but when deta-NO or SNP were added to the cells, no significant differences were found.

The action of angiotensin II in glomerulosa cells is mediated by the activation of phospholipase C in the plasma membrane. The subsequent hydrolysis of phosphatidylinositol bisphosphate results in the release of the second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG). The following experiments were designed to examine the

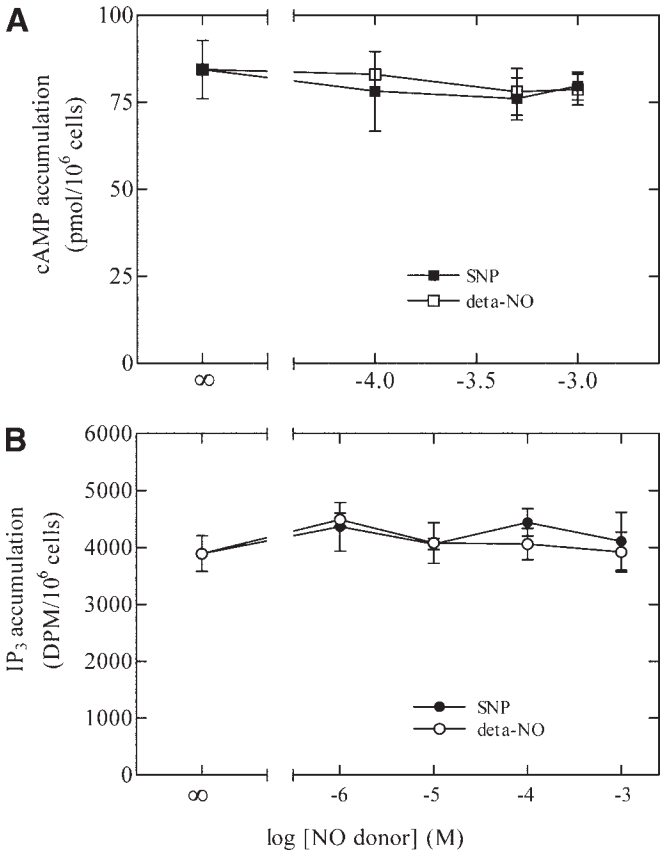

Fig. 2. Effects of NO donors on cAMP and IP_3 production in zona glomerulosa cells from bovine adrenal gland. Zona glomerulosa cells were preincubated with increasing concentrations (0–1 m*M*) of deta-NO or SNP, in the presence of 0.5 m*M* IBMX for 10 min. Vehicle, 10 n*M* ACTH, or 100 n*M* AngII were then added and cells were incubated for 20 min or 15 min, for cAMP (**A**) and IP_3 (**B**), respectively, at 37°C. Intracellular cAMP and IP_3 accumulation were determined as described in *Methods*. Results are expressed as mean ± SEM from three independent experiments, each performed in triplicate.

effects of SNP and deta-NO on AngII-stimulated PLC activity. AngII increased IP_3 levels up to twofold over basal value, but NO donors did not modify AngII-stimulated IP_3 levels (Fig. 2B). Similar results were obtained when total IPs was analyzed (data not shown). These results suggest that NO-dependent inhibition on aldosterone production is independent of cAMP production or PLC activation.

Effects of NO Donors on 22[R]Hydroxycholesterol- (22R-chol) and Pregnenolone-Stimulated Aldosterone Production

The following experiments were conducted to study the effects of NO on the steroidogenic enzymes involved in aldosterone synthesis. Glomerulosa cells were incubated with NO donors in the presence of a permeable cholesterol analog, 22R-chol (10 μ*M*) or pregnenolone (10 μ*M*). 22R-chol stimulated basal aldosterone synthesis (control: 0.4 ± 0.1; 22R-chol: 3.2 ± 0.3 ng Aldo/10^6 cells × 2 h). It can be

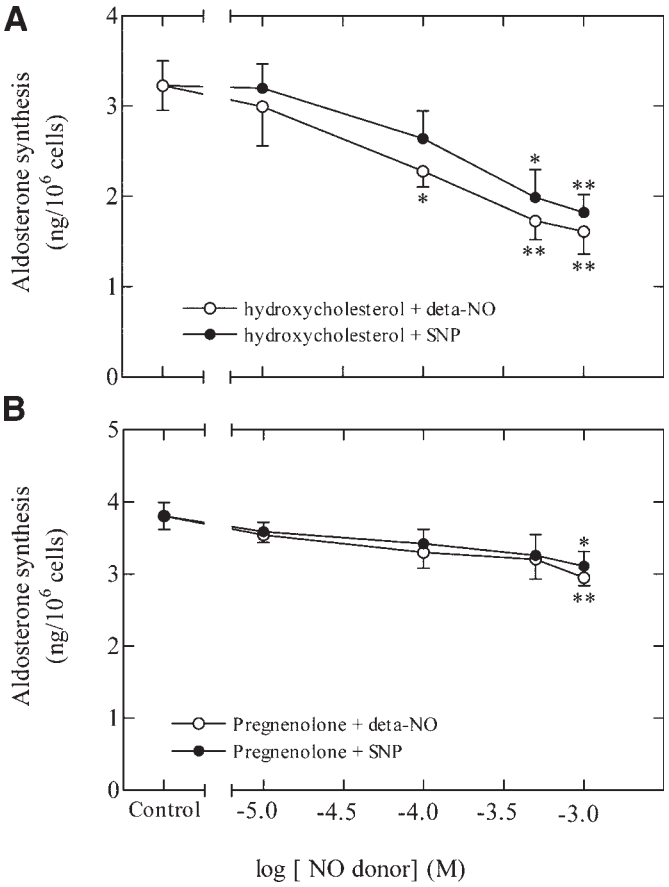

Fig. 3. Effects of NO donors on 22(R)-hydroxycholesterol- and pregnenolone-stimulated aldosterone secretion in zona glomerulosa cells from bovine adrenal gland. Zona glomerulosa cells were preincubated with increasing concentrations (0–1 m*M*) of deta-NO or SNP for 10 min; 10 μ*M* 22(R)-hydroxycholesterol (**A**) or 10 μ*M* pregnenolone (**B**) were then added and cells were incubated for 2 h at 37°C. Aldosterone production was measured as described in *Methods*. Results are expressed as mean ± SEM from five independent experiments, each performed in triplicate. **p* < 0.05; ***p* < 0.01 vs respective control.

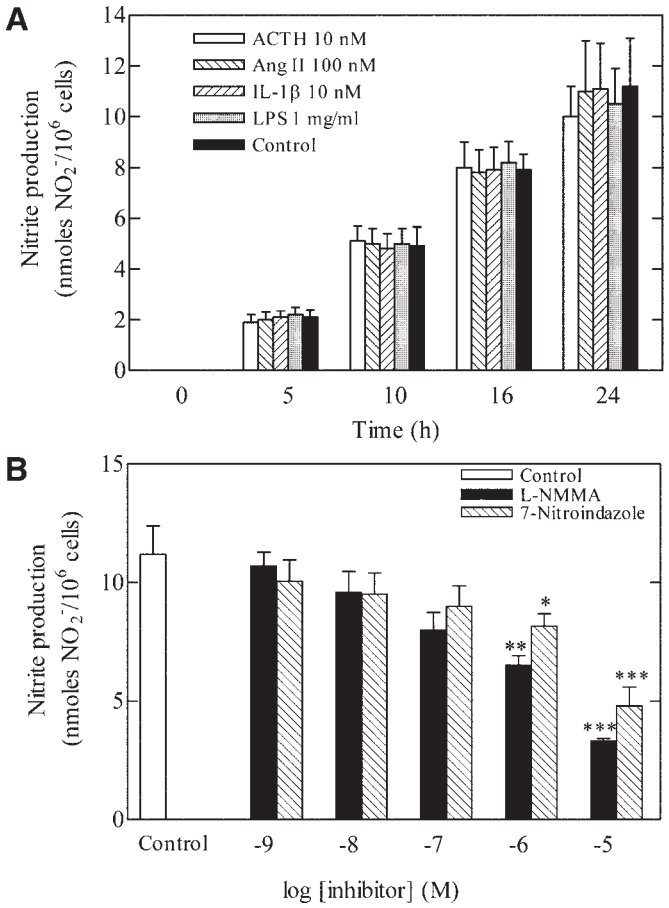

Fig. 4. Nitrite production by zona glomerulosa cells from bovine adrenal gland. Time course of nitrite production (**A**) by glomerulosa cells. Cells were incubated for the times indicated at 37°C, in the absence or presence of 10 n*M* ACTH, 100 n*M* AngII, 10 n*M* IL-1β, or 1 mg/mL LPS; (**B**) the effects of L-NMMA and 7-nitroindazole, two NOS inhibitors. Cells were incubated with increasing concentrations (0–10 μ*M*) of L-NMMA and 7-nitroindazole for 24 h at 37°C. Nitrite accumulation was measured by Griess' method. Results are expressed as mean ± SEM from three independent experiments, each performed in triplicate. **p* < 0.05; ***p* < 0.01; ****p* < 0.001 vs control.

seen that deta-NO and SNP evoked a concentration-dependent inhibition on aldosterone production stimulated by 22R-chol. Maximal inhibitions observed were 50.1 ± 15.5% and 43.5 ± 13.5%, respectively (Fig. 3A).

Pregnenolone stimulated basal aldosterone synthesis (control: 0.4 ± 0.1; Pregnenolone: 3.8 ± 0.2 ng Aldo/10^6 cells × 2 h). NO donors caused a small inhibition, only significant for highest concentrations of deta-NO or SNP (Fig. 3B). Maximal inhibitions observed were 18.3 ± 6.6% and 22.4 ± 3.9%, respectively.

The NO-mediated inhibitory effect on aldosterone production in the presence of both steroidogenic precursors suggests an inhibition of several steroidogenic enzymes.

NO Synthesis in Bovine Adrenal Glomerulosa Cells

Two different approaches were used: a) Incubation of primary culture of zona glomerulosa cells during the times indicated in the figures and measurement of nitrites accumulated in the incubation medium by Griess reaction; (b) characterization of NO synthase activity in zona glomerulosa cell extracts by conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline.

Nitrite Production in Primary Culture of Bovine Adrenal Glomerulosa Cells

Figure 4A shows the time course of nitrite production in glomerulosa cells cultured in control medium (black bars). Nitrite accumulation was linear between 5 and 24 h. Nitrites

were not detectable in incubation times shorter than 5 h. Neither 10 n*M* ACTH nor 100 n*M* AngII increased the nitrite production. Moreover, neither LPS nor IL-1β, extensively used to induce iNOS, increased the nitrite production.

Two competitive NOS inhibitors, L-NMMA and 7-nitroindazole *(19,20)*, inhibited nitrite accumulation (Fig. 4B). Maximal inhibitions observed (for 10 μ*M* inhibitor) were 70.3 ± 3.0% and 57.2 ± 12.3% for L-NMMA and 7-nitroindazole, respectively. Particularly, 7-nitroindazole is considered more specific for nNOS *(21,22)*. These data suggest that bovine glomerulosa cells can synthetize NO, but the iNOS does not appear to be involved.

NO Synthase (NOS) Activity in Bovine Glomerulosa Cell Extracts

To further confirm that glomerulosa cells produce NO, NO synthase activity was measured and partially characterized in incubations of zona glomerulosa cell extracts. The time course study (Fig. 5A) showed that [^{3}H]L-citrulline formation increased during the first 25 min, the time at which it reached its peak, and remained constant until 60 min.

When different concentrations of L-arginine were added to the reaction, NOS showed a Michaelis–Menten kinetic (Fig. 5B). After transformation of data into a Lineweaver–Burk plot (Fig. 5C), kinetic parameters of NOS were calculated (K_m = 24.2 μ*M* and V_{max} = 19.5 pmol/mg protein × minute). NOS activity was dependent on NADPH and was maximal at 0.1 m*M* of NADPH.

L-NAME and L-NMMA are two structural analogs of L-arginine, which can bind to NOS acting as competitive inhibitors. When different concentrations of L-NAME or L-NMMA (10 n*M* to 0.1 m*M*) were added to the reaction, both analogs were able to inhibit NOS activity in a concentration-dependent manner (Fig. 6). NOS activity was completely inhibited by 0.1 m*M* L-NAME and L-NMMA. Half-maximal effects were observed at about 0.1 μ*M* of L-NAME and 5 μ*M* of L-NMMA.

Figure 7 shows NOS activity in the presence of increasing concentrations of free Ca^{2+} (calculated as explained in *Methods*). Calcium ion concentration had a biphasic effect on NOS activity. An initial phase of concentration-dependent stimulation was observed between 0.01 and 0.5 μ*M*. NOS activity reached a plateau at 0.5 μ*M* and this was followed by a concentration-dependent enzyme activity decrease.

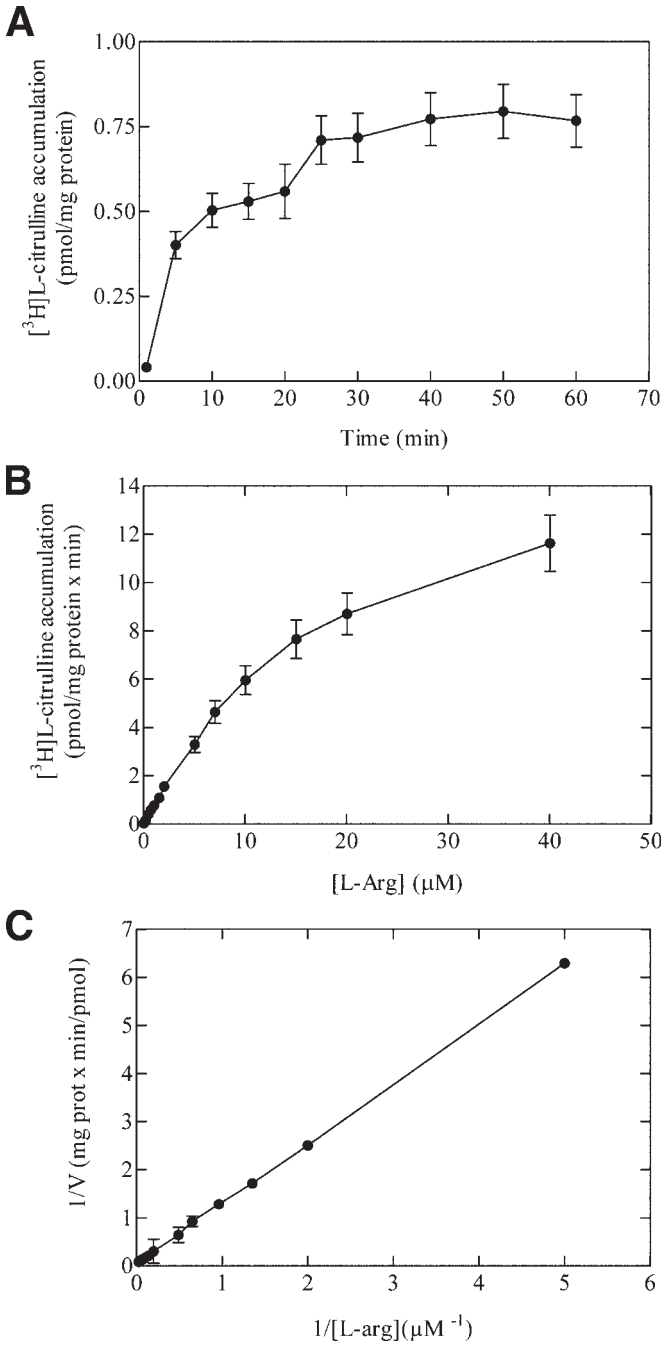

Fig. 5. Time course and kinetic parameters of NOS activity in zona glomerulosa cells from bovine adrenal gland. NOS activity was determined as described in *Methods*. Zona glomerulosa cell extracts were incubated for the indicated times, at 37°C, in the presence of 90 n*M* [^{3}H]L-arginine (**A**). Increasing concentrations of [^{3}H]L-arginine (0–40 μ*M*) were added in incubations for 30 min (**B**) and the data were processed by Lineweaver–Burk analysis to obtain K_m and V_{max} values (**C**). Kinetic parameters were calculated the least square method (r = 0.999). Results are expressed as mean ± SEM from three independent experiments, each performed in triplicate.

Discussion

In the present study, we showed that NO inhibits basal, ACTH- and AngII-stimulated aldosterone synthesis in primary cultures from bovine zona glomerulosa cells and that NO can be synthesized in these cells, because NOS activity was measured.

In order to elucidate the role of NO, two different NO donors, deta-NO and SNP, were used. Both NO donors showed similar inhibitory effects on bovine glomerulosa cells steroidogenesis. These effects were reverted in the presence of hemoglobin, thus confirming that the inhibitions caused by deta-NO and SNP were only mediated by the

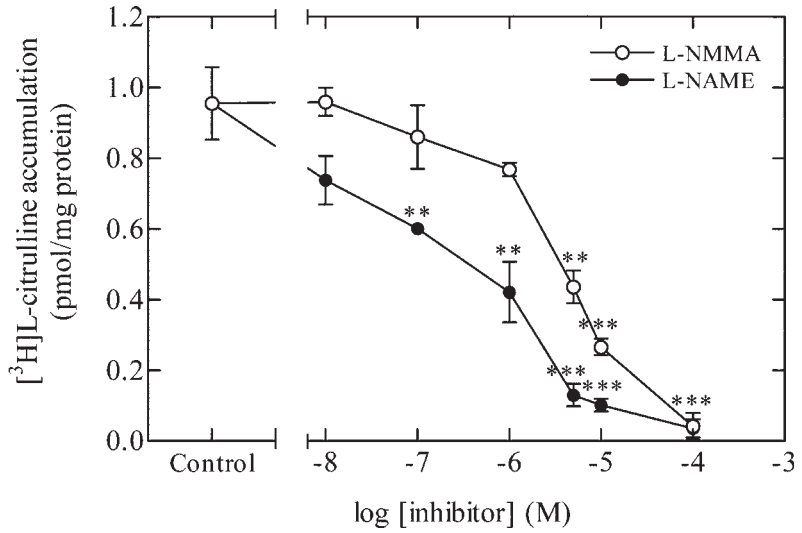

Fig. 6. Effects of L-NMMA and L-NAME on NOS activity in zona glomerulosa cells from bovine adrenal gland. NOS activity was determined as described in *Methods*. Zona glomerulosa cell extracts were incubated for 30 min, at 37°C, in the presence of 90 n*M* [^{3}H]L-arginine and increasing concentrations of L-NMMA and L-NAME (0–100 μ*M*). Results are expressed as mean ± SEM from three independent experiments, each performed in triplicate. $^{**}p < 0.01$; $^{***}p < 0.001$ vs control.

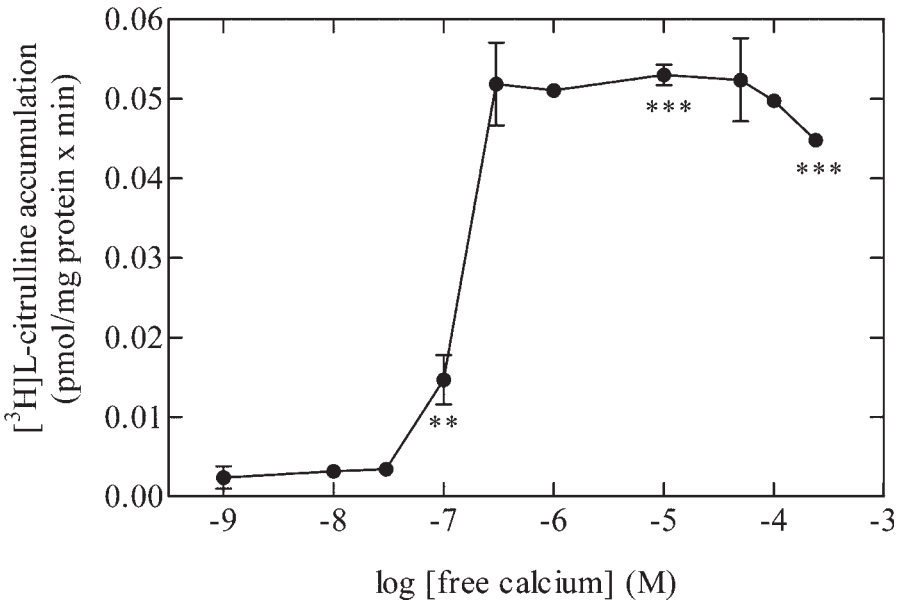

Fig. 7. Dose-dependent relationship of free [Ca^{2+}]-modulated NOS activity in zona glomerulosa cells from bovine adrenal gland. NOS activity was determined as described in *Methods*. Zona glomerulosa cell extracts were incubated for 30 min, at 37°C, in the presence of 90 n*M* [^{3}H]L-arginine and increasing concentrations of EGTA (0.01–10 m*M*). Each point of free Ca^{2+} concentration corresponds with each concentration of EGTA added. The free Ca^{2+} concentration was determined as described in *Methods*. Results are expressed as mean ± SEM from three independent experiments, each performed in triplicate. $^{**}p < 0.01$; $^{***}p < 0.001$ vs control.

released NO and not by nonspecific effects of these donors. The inhibition of basal aldosterone synthesis is in concordance with those results previously reporting basal corticosterone inhibition mediated by NO donors in rat adrenal zona fasciculata cells *(10)*, but disagree with studies indicating that NO is ineffective as a modulator of basal steroidogenesis *(13–17)*. This discrepancy could be due to different NO donor concentrations or protocols used by those authors. We suggest that the inhibition of basal aldosterone synthesis might be produced by a direct effect of NO on the steroidogenic pathway by affecting the activity of P450 cytochrome-dependent enzymes as was described by one of us *(6)* and others *(10)*.

Deta-NO and SNP inhibited AngII- and ACTH-stimulated aldosterone production in a concentration-dependent manner. This is the first study that shows that NO inhibits ACTH-stimulated aldosterone secretion in bovine zona glomerulosa cells. Natarajan et al. *(15)* reported that SNP and

S-nitroso-*N*-acetyl-penicillamine (SNAP) inhibited AngII- and ACTH-stimulated human aldosterone synthesis, but they did not mention whether the effect of SNP or SNAP was concentration-dependent in agonist-evoked rat and/or human zona glomerulosa cell steroidogenesis. NO-mediated inhibition of AngII-stimulated aldosterone synthesis is consistent with numerous previous studies using different NO donors in zona glomerulosa cells *(13–17)*.

Activation of a soluble guanylyl cyclase is the mechanism that mediates NO effects in many physiological systems. Deta-NO and SNP stimulated cGMP production in a concentration-dependent manner. Basal cGMP production was 20- and 16-fold increased by deta-NO and SNP, respectively. These data are consistent with the presence of soluble guanylyl cyclase in zona glomerulosa cells. We next examined the role of cGMP in NO-dependent aldosterone production inhibition at different steps. First, the effect of a soluble and not hydrolytic analog of cGMP, 8-Br-cGMP, on aldosterone secretion was analyzed. It had no effect on basal, AngII-, and ACTH-stimulated aldosterone synthesis. ODQ and methylene blue, both soluble guanylyl cyclase inhibitors, prevented NO donor-mediated stimulation of cGMP production but were ineffective to attenuate NO-inhibition on aldosterone production. Finally, Rp-8-Br-PET-cGMP, a protein kinase G inhibitor, was used to avoid cGMP-dependent phosphorylations. It had no effect on NO-dependent aldosterone inhibition. Taken together, these results confirm that the NO-inhibition mechanism of aldosterone secretion is independent of soluble guanylyl cyclase activation, cGMP levels, or PKG activity in bovine adrenal cortex zona glomerulosa cells. These results are in contrast with those that report that a cGMP analog could be an stimulator *(13)* or an inhibitor *(14)* of aldosterone synthesis. However, our conclusion is in agreement with previous results reported by Hanke et al. *(16)* and Kreklau et al. *(17)*.

Several publications show a complex relationship and many antagonistic effects between angiotensin II and NO *(23)*, but, to our knowledge, this is the first study about the possible role of NO on angiotensin II-linked signal transduction pathway. Both deta-NO and SNP had no effect on angiotensin II-stimulated PLC activity, measured as total IPs or IP_3 accumulation. These results suggest that the inhibitory effect of NO on AngII probably takes place after PLC activation, on one or more steps between PLC activation and aldosterone release.

Nitric oxide has been described as a modulator of the stress axis, limbic–hypothalamic–pituitary–adrenal *(24)*, although this role is not clear at all. In the present study we show that two NO-releasing agents inhibited ACTH-stimulated aldosterone secretion in a concentration-dependent manner. Thus, we examined the role of NO on ACTH as main transduction mechanism, the activation of adenylyl cyclase, and cAMP synthesis. Although low concentrations of ACTH can activate other transduction pathways with different second messengers, cAMP is the principal messenger at the concentrations of ACTH used in our assays (10 n*M*) *(25)*. Basal cAMP levels were 14-fold increased by 10 n*M* ACTH. Deta-NO or SNP had no effect on ACTH-stimulated cAMP production. However, the increase in cAMP production induced by ACTH is the result of a balance between activation of adenylyl cyclase and a direct inhibition of phosphodiesterase activity (PDE2), an effect mediated by inhibition of cGMP levels *(25,26)*. When we conducted our experiments to test if adenylyl cyclase activity was modulated by NO, IBMX was added to the incubation media to prevent cAMP degradation by PDE2. But in this way we probably masked the effect of NO donors. The large increase in cGMP levels in response to NO donors is enough to activate PDE2 in glomerulosa cells. For this reason, it is quite logical to expect that NO releasing agents in experimental conditions without IBMX would attenuate high cAMP levels produced in response to ACTH. Thus, PDE2 phosphodiesterase could be a target for NO in its inhibition of ACTH-stimulated aldosterone secretion in glomerulosa cells.

The cholesterol availability to cytochrome P450scc enzyme (responsible for the conversion of cholesterol into pregnenolone) is the regulatory step in steroidogenesis and the StAR (steroidogenic acute regulatory) protein plays a key role on the transfer of cholesterol from the outer to the inner mitochondrial membrane, where the P450scc enzyme is located; it is implicated in the rapid synthesis and secretion of steroids by adrenal cells under hormonal stimulation *(27)*.

Previous works have reported that, in different steroidogenic tissues, NO inhibits cytochrome P450-dependent enzymes by binding to the heme group of the protein *(6,10, 16,28)*. Consequently, we investigated the action of NO on the steroidogenic pathway leading to the production of aldosterone, by using a permeable cholesterol analog (22R-chol, that bypasses the action of StAR) and pregnenolone.

NO donors inhibited the 22R-chol and pregnenolone-stimulated aldosterone synthesis. Maximal inhibitions observed with 1 m*M* of NO donors were about 50% and 20%, respectively. The remarkable difference in the NO-mediated inhibition of aldosterone production in the presence of 22R-chol or pregnenolone suggests an inhibition of P450scc enzyme activity, in addition to other P450-dependent steroidogenic enzymes beyond progesterone ($P450_{C21}$, $p450_{C11\beta}$, and/or $P450_{C18}$) as was proposed by Hanke et al. *(16)*. Because the effects of NO on pregnenolone-stimulated aldosterone synthesis represent the summed inhibition of these latter three P450 enzyme activities, NO must inhibit P450scc activity to a far greater than anyone of the others. The NO-dependent inhibition of P450scc but not of 3β–hydroxysteroid dehydrogenase (that produces progesterone from pregnenolone and is not cytochrome P450-dependent enzyme) has also been described in MA-10 Leydig cells *(6)* and in rat adrenal fasciculata cells *(10)*.

Both ACTH and AngII have been shown to increase StAR expression and to stimulate the steroidogenesis *(29,30)*. NO-mediated inhibition on ACTH- or AngII-stimulated aldo-

sterone production was higher than that observed in the presence of 22R-chol, which would indicate another possible site of action for NO-provoked inhibition on aldosterone synthesis: on StAR protein. In this respect, a recent paper *(12)* has shown that NO inhibites StAR expression (both mRNA and protein levels) in ACTH-stimulated Y1 adrenal cells.

It is interesting to point out that both NO donors used in this study inhibited ACTH- and AngII-induced aldosterone synthesis to the same extent (80% for deta-NO and 60% for SNP). Thus, although ACTH and AngII might exert their stimulatory effects on StAR expression via different signal transduction pathways, our findings suggest NO must inhibit ACTH- and AngII-induced steroidogenesis independently on the signaling system leading to the increase in StAR expression.

In the adrenal gland there is a close association between steroidogenic cells and endothelial or neuronal cells. These cells release NO, which could be an effective means of regulating steroidogenesis. In addition, NO could also be produced by adrenal zona glomerulosa and/or fasciculata cells. This study shows that NO can be synthesized by bovine glomerulosa cells. We could demonstrate this by two different approaches, nitrite production and NOS activity. Nitrite accumulation was linear between 5 and 24 h. Nitrites were not detectable when incubating the cells for periods shorter than 5 h, probably because the NO endogenously produced is too low to produce detectable amounts of nitrite to be detected by Griess reaction. In this respect, Hanke and Campbell *(18)* could not detect nitrite production after 2-h incubation in primary culture from bovine glomerulosa cells.

Neither ACTH nor AngII modified nitrite production. The same results were observed for LPS or IL-1β, both of which have been used extensively to induce iNOS. In contrast, IL-1β stimulated nitrite accumulation and iNOS mRNA expression in rat Leydig cells in 12- and 24-h incubations *(7)*. However, two competitive NOS inhibitors, L-NMMA and 7-nitroindazole, inhibited nitrite accumulation. Particularly, 7-nitroindazole is considered more specific for nNOS isoform *(31)*. Consequently, these data suggest that bovine zona glomerulosa cells can synthetize NO, but the iNOS isoforms do not appear to be involved. Our results suggest the presence of one (or both) constitutive NOS isoforms, in agreement with previous results showing constitutive NOS in calf adrenal gland *(31)*, eNOS in rat glomerulosa cells *(15)*, and nNOS in rat adrenal cortex *(32)*.

Because most of the studies that have been published so far deal with mRNA or protein expression, we considered it pertinent to measure NOS activity in bovine glomerulosa cell extracts, as it is a more direct evidence for NO production. NOS activity was detected in zona glomerulosa cells. The enzyme activity was inhibited in the presence of two competitive inhibitors, L-NAME and L-NMMA, in a concentration-dependent manner, and was NADPH- and Ca^{2+}-dependent. In the presence of increasing concentrations of L-arginine, NOS showed a Michaelis–Menten kinetic (K_m = 24.2 μ*M* and V_{max} = 19.5 pmol/ mg protein × minute).

Calcium ion concentration had a biphasic effect on NOS activity. While an initial phase of concentration-dependent stimulation was observed between 0.01 and 0.5 μ*M* Ca^{2+}, NOS activity reached a plateau at concentrations between 0.5 μ*M* and 0.1 m*M*, followed by a concentration-dependent decrease in enzyme activity. These results are in agreement with the decline in nNOS activity reported by Bredt and Snyder *(19)* at high nonphysiological Ca^{2+} concentrations of (i.e., 1 m*M*). Moreover, Weissman et al. *(33)* have recently shown that calmodulin (CaM) binding to nNOS reached a maximal level at 1 μ*M* free Ca^{2+}. Further increase in free Ca^{2+} from 10 μ*M* to 1 m*M* resulted in a 24% reduction of maximal CaM binding. Our data show that in the presence of 1 m*M* Ca^{2+}, NOS activity was decreased by 25% (Fig. 7). The decrease of CaM binding to nNOS and the reduction of activity associated with high Ca^{2+} may involve binding of Ca^{2+} to low affinity binding sites on CaM that have recently been identified *(34)*.

It is well known that AngII activates PLC, increasing intracellular Ca^{2+} concentration to levels that we have shown to stimulate NOS activity *(35)*. It is reasonable then to speculate that AngII could elicit enzyme activity; however, our results show the nitrite production was not affected. It cannot be overlooked that AngII might regulate NOS through different stimulatory and inhibitory signals. In this respect, Hayashi et al. *(36)* have shown that calmodulin kinase–induced phosphorylation of nNOS inhibits enzyme activity.

In summary, this study shows that NO inhibits basal, ACTH- and AngII-induced aldosterone synthesis in bovine adrenal glomerulosa cells. This inhibition is independent of soluble guanylyl cyclase, cGMP levels, protein kinase G activity, cAMP production, or phospholipase C activity. NO inhibits aldosteronogenesis through, at least, a direct inhibition of cytochrome P450 steroidogenic enzymes, including P450scc enzyme activity. We present evidence that NO can be synthesized endogenously, because nitrites have been produced and NO synthase activity has been partially characterized in glomerulosa cells extracts.

In conclusion, our results support the hypothesis that NO can act as autocrine and/or paracrine modulator of aldosterone secretion from the adrenal gland, thus contributing to the regulation of aldosterone levels induced by hormones such as angiotensin II or ACTH.

Materials and Methods

Materials

Angiotensin II (AngII), aldosterone (Aldo), cultured medium (Dulbecco's modified medium), 22[R]hydroxycholesterol (22R-chol), pregnenolone, N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine monoacetate (L-NMMA), 7-nitroindazole, methylene blue, L-glutamine,

deoxyribonuclease I (DNAase), T-70 dextran, bacitracin, amphotericin B (fungizone), type II-S trypsin inhibitor, Hepes, EDTA, and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ACTH was obtained from Nuvacthén Devot. Bovine serum albumin (BSA, fraction V), collagenase A, and collagenase P were purchased from Boehringer Mannheim SA. (Darmstadt, Germany). Rp-8-bromo-PET-cyclic GMP and 1H-(1,2,4)-oxadiazolo[2,3-a]quinoxalin-1-one (ODQ) were from Biolog-Life Science Institute (Bremen, Germany). Streptomycin, penicillin G, and fetal calf serum were obtained from Seromed Biochrom KG (Berlin, Germany). Aldosterone antiserum was purchased from ICN Pharmaceuticals (USA). [^{3}H]Aldosterone (56 Ci/mmol), [2,3,4,5-^{3}H]L-arginine (49 Ci/mmol), myo[^{3}H] inositol (97 Ci/mmol), cAMP, and cGMP kits were purchased from Amersham International (Buckinghamshire, UK). Bovine ferrous hemoglobin was from Calbiochem-Novabiochem Co. (LA, USA). Sodium nitroprusside (SNP), 1-hydroxy-2-oxo-3, 3-bis[3-aminemetyl-(1-triecene)] (detanonoate, deta-NO, or NOC-18) and 8-bromo cyclic GMP was purchased from Alexis Co (Läufelfingen, Switzerland). Dowex AG 1-X8 (100–200 mesh, formate form) resin and AG 50 W-X4 (100–200 mesh, hydrogen form) ion-exchange resin was from Bio-Rad (CA, USA). All other chemicals were of A-grade purity.

Isolation and Culture of Glomerulosa Cells from Bovine Adrenal Gland

Bovine adrenal glomerulosa cells were isolated and cultured as previously described to use for patch clamp measurements *(37)*. Briefly, glomerulosa cell slices were prepared from 10–12-mo-old steers obtained from a local slaughterhouse. Thin tissue slices (0.5 mm slices of the outer position of bovine glands) were obtained using a Stadie–Riggs tissue microtome from Thomas Scientific (St. Laurent, Canada). Cell dissociation was achieved after four enzymatic periods of 30 min at 37°C in a humidified atmosphere with 5% of CO_2 in air. The zona glomerulosa was digested in DMEM medium containing collagenase P 1 mg/mL, collagenase A 0.5 mg/mL, and DNAse 25 mg/mL supplemented with L-glutamine 10 m*M*, penicillin 200 U/mL, streptomycin 200 mg/mL, HEPES 20 m*M*, and $NaHCO_3$ 75 mg/mL (serum-free DMEM). After each digestion period cells were dissociated by gentle aspiration with a sterile 10 mL pipet before being filtered and centrifuged for 10 min at 100*g*. Cells were resuspended in 5 mL of a 1.7 m*M* Tris, 140 m*M* NH_4Cl solution, pH 7.2 and incubated for 10 min at 37°C. Incubation was terminated by dilution with DMEM-0.1% BSA and centrifuged. This procedure eliminates any interference caused by the presence of red blood cells and does not affect the cell response to hormone stimulation *(6)*. Finally, cells were resuspended in DMEM supplemented with 10% fetal calf serum, L-glutamine 20 m*M*, penicillin 100 U/mL, streptomycin 100 mg/mL, fungizone 10 m*M*, and $NaHCO_3$ 75 mg/mL (FCS-DMEM). Glomerulosa cells were plated at a number of 800,000 cells/mL in 35-mm tissue culture dishes or 500,000 cells/mL in 12-well tissue culture plates. After 24 h at 37°C in a humidified atmosphere with 5% CO2 in air, the medium was replaced and cells maintained in culture for 3 d. Cell viability was assessed by the Trypan-blue dye exclusion test and determined by microscopy after each experiment. The relatively small size of glomerulosa cells compared to that of fasciculata cells, the presence of lipid droplets, as well as the responsiveness to AngII or ACTH confirmed than more 95% of the cell population was composed of glomerulosa cells.

Preparation of Zona Glomerulosa Cell Extracts

Glomerulosa cells obtained as described above were resuspended in ice-cold buffer containing 50 m*M* Tris-HCl, pH 7.4, 20 m*M* Hepes, 10 µg/µL leupeptin, 1 m*M* DTT, and homogenized with an Ultraturrax. The homogenate was centrifuged at 900*g* for 10 min at 4°C. The supernatant was decanted into a chilled 10 mL centrifuge tube and centrifuged at 9000*g* 10 min at 4°C. The supernatant of this last centrifugation was centrifuged again in the same conditions. The supernatant was aliquoted and stored at –70°C until later use for NOS activity and ion concentration assays.

Measurement of Aldosterone Secretion

After 3 d in culture, cells were washed and incubated 2 h in serum-free DMEM. Cells were washed again and incubated 15 min in HBS supplemented with $NaHCO_3$ 75 mg/mL, bacitracin 0.1 mg/mL, trypsin inhibitor 0.1 mg/mL, LiCl 10 m*M*, glucose 1 mg/mL, and BSA 0.1 mg/mL (assay medium). Cells were preincubated with NO donors (SNP or deta-NO) for 10 min before agonist addition. Agonists or corresponding vehicle control were then added, and the incubation was continued for 2 h at 37°C in a humidified atmosphere with 5% CO_2. Medium was removed and centrifuged at 4°C for 5 min at 20,800*g*, and the supernatant was stored at –20°C until aldosterone measurement was performed. Aldosterone concentrations were determined by radioimmunoassay (RIA) as previously described *(38)*.

cGMP and cAMP Assays

Treatment of the cells was similar to that used for aldosterone determination measurement, with the exception of the assay medium being supplemented with 0.5 m*M* of 3-isobutyl-1-methyl-xanthine (IBMX). The presence of IBMX, a cyclic nucleotide phosphodiesterases inhibitor, was necessary to increase the sensitivity of the method.

Agonists or vehicle were incubated for the times indicated in the text. After the incubation period, the plates were placed on ice and the medium was quickly aspirated; 0.5 mL of ice-cold trichloroacetic acid 70 mg/mL was then added for 30 min. After this, cells were scraped, disrupted, and centrifuged at 20,800*g* for 5 min at 4°C. The supernatant was washed with water-saturated diethyl ether and then water was evaporated in a speed vacuum samples concentrator (Savant AS 290). The pellets were stored at –20°C

until cGMP and cAMP measurements. cGMP and cAMP levels were determined using an enzyme immunoassay kit from Amersham International.

Measurement of Inositol Phosphates Accumulation

The PLC activity was determined as previously described for primary culture of rat glomerulosa cells *(39)*. Cells were grown the last 2 d in DMEM supplemented with 3 µCi/mL of myo-[^{3}H]inositol. The radioactive medium was discarded, and the cells were incubated in serum and inositol-free DMEM medium for 1 h. Glomerulosa cells were washed and incubated for 15 min in 1 mL HBS medium supplemented with 10 m*M* LiCl, BSA (1 mg/mL), and glucose (1 g/L). The medium was then changed, and cells were further incubated for 10 min at 37°C with 1 mL of the same medium supplemented with the hormones to be tested. After stopping the reaction with 5% (vol/vol) perchloric acid, total IPs (IP_1+IP_2+IP_3) were extracted and purified on an anion exchange chromatography column (Dowex AG1x8, formate form, 200–400 mesh; Bio-Rad). For each sample, a fraction containing total IPs was collected and counted. Radioactivity in the inositol phosphate fractions was measured by liquid scintillation in a Packard Tricarb 2700 TR model with a tritium efficiency of 65%. Results were corrected for quenching and expressed as DPM/10^6 cells.

Measurement of Nitrite Production

Nitrite production was determined by the method of Griess *(40)*. Briefly, glomerulosa cell culture medium was added to an equal volume of Griess reagent. Absorbance at 540 nm was measured, and the nitrite concentration was determined from a calibration curve of sodium nitrite standards.

Assay of NO Synthase Activity

NO synthase activity was performed by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline according to our modification of the Bredt and Snyder *(19)* method. Briefly, 50 µL of cellular extract were added to 90 µL of buffer containing 50 m*M* Tris-HCl, pH 7.4, 20 m*M* Hepes, 10 µg/µL leupeptin, 1 m*M* DTT, 1 m*M* NADPH, and 90 n*M* [^{3}H]L-arginine. After incubation at 37°C for the times indicated in the figures, samples were loaded onto 1 mL of Dowex AG50W X-4 (100–200 mesh, H^+ form) resin and eluted with 3 mL of water. The eluted [^{3}H]L-citrulline was quantified in a liquid scintillation counter in a Packard Tri-Carb 2700 TR liquid scintillation analyzer.

Measurement of Ca^{2+} by Atomic Absorption

Glomerulosa cell extracts were diluted in ultrapure water and filtered to eliminate particles larger than 0.22 µm. Knowing the exact Ca^{2+} concentration in the extracts was important to study the effect of calcium concentration on NOS activity. Consequently, Ca^{2+} and Mg^{2+} concentrations in the extracts were determined by atomic absorption in a Perkin Elmer 560 atomic absorption spectrophotometer using different standard solutions as reference. The results obtained were 0.68 and 0.76 m*M* for calcium and magnesium, respectively, and the final concentrations in the assay were of 0.24 m*M* for Ca^{2+} and 0.27 m*M* for Mg^{2+}. The dose–response curve of NOS activity in the presence of Ca^{2+} was obtained by adding different concentrations of EGTA (0.01–10 m*M*). Each point of free Ca^{2+} concentration in the figure corresponds with each concentration of EGTA added into the Ca^{2+} 21 assay. Free Ca^{2+} concentrations were determined using a software generously provided by Dr. Gilles Guillon (U469, INSERM, Centre de Pharmacologie-Endocrinologie, Montpellier, France).

Statistical Analysis

Results are expressed as mean ± SEM of three independent experiments performed in triplicate, unless indicated otherwise. Statistical analysis was done using ANOVA or Student's *t* test as appropriate, with level of significance set at $p < 0.05$.

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