Endothelial cell nitric oxide inhibits aldosterone synthesis in zona glomerulosa cells: modulation by oxygen

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Hanke, Craig J., and William B. Campbell. Endothelial cell nitric oxide inhibits aldosterone synthesis in zona glomerulosa cells: modulation by oxygen. Am J Physiol Endocrinol Metab 279: E846–E854, 2000.—The regulation of aldosterone synthesis by endogenous nitric oxide (NO) was examined in cultured cells of the adrenal cortex. Endothelial NO synthase (eNOS) was detected by Western blot in cultured adrenal endothelial cells (ECs) but not in zona glomerulosa (ZG) cells or adrenal fibroblasts. Neither inducible (iNOS) nor neuronal NOS (nNOS) isoforms were detected in the cells. Only ECs had NOS activity and converted [3H]L-arginine to [3H]L-citrulline. Angiotensin II (ANG II, 100 nM) increased EC production of nitrate/nitrite by 2.4-fold. Coincubation with ECs or treatment with DETA nonoate increased the fluorescence of ZG cells loaded with an NO-sensitive dye, diaminofluorescein 2 diacetate (DAF-2 DA). DETA nonoate inhibited ANG II (1 nM) and potassium (10 mM) -stimulated aldosterone release in a concentration-related manner. This inhibitory effect of NO was enhanced >10-fold by decreasing the oxygen concentration from 21 to 8%. Coincubation of EC and ZG cells in 8% oxygen inhibited ANG II-induced aldosterone release, and inhibition was reversed by blockade of NOS. These findings indicate that adrenal EC-derived NO inhibits aldosterone release by cultured ZG cells and that the sensitivity to NO inhibition is increased at low oxygen concentrations.

angiotsin II; potassium; fibroblasts; nitric oxide synthase;

Endothelial cells (ECs) release a number of soluble factors that regulate vascular tone, including nitric oxide (NO), endothelin, prostacyclin, and endothelium-derived hyperpolarization factor (7, 15, 22, 33, 35). The adrenal gland is highly vascularized, and morphological studies indicate a close association between steroidogenic zona glomerulosa (ZG) cells and ECs lining the adrenal capillaries and sinusoids (32). This anatomical relationship raises the possibility that ECs may release factors that regulate adrenal steroidogenesis. Along these lines, prostacyclin, endothelin-1, and endothelium-derived steroidogenic factor stimulate aldosterone production (8, 27, 28). There are no studies examining EC-mediated inhibition of aldosterone synthesis, and only a few studies have examined the effect of NO on steroidogenesis.

Previous in vitro studies have demonstrated that NO inhibits the synthesis of aldosterone and other steroid hormones (2, 10, 20). Natarajan et al. (19) reported that NO donors inhibited aldosterone synthesis in both rat and human ZG cells. The inhibition by NO occurred in ZG cells stimulated with both angiotensin II (ANG II) and adrenocorticotropic hormone. We reported that aldosterone synthesis stimulated by ANG II, 25-hydroxy-cholesterol, and progesterone was blocked by the addition of the NO donor ethanamine,2,2’-(hydroxynitrosohydrazono)bis (DETA) nonoate (13). The mechanism of inhibition was independent of guanylylcyclase activation and was due to binding of NO to the cytochrome P450 enzymes responsible for steroidogenesis. Several groups have suggested that NO mediates inhibition of corticosterone production by zona fasciculata cells (2, 10). Adams and co-workers (1, 2) found an increased testosterone production in rat Leydig cells after inhibition of NO production with Nω-nitro-L-arginine methyl ester.

Studies examining the steroidogenic effects of NO in intact adrenal glands have been less conclusive. Cameron and Hinson (5) demonstrated that NO increased perfusion of the intact adrenal, resulting in the stimulation of corticosterone synthesis but no change in aldosterone. Inhibition of NO synthase (NOS) in vivo in dogs and humans did not change serum aldosterone concentrations (14, 17). In contrast, Usui et al. (31) detected an increase in plasma aldosterone and increased adrenal expression of ANG II type 1 receptors in rats after chronic NOS inhibition. The steroidogenic response to adrenocorticotropic hormone is enhanced by NOS inhibition with Nω-monomethyl-L-arginine (12). This suggests that, in addition to its direct effects, NO may affect steroidogenesis indirectly by regulating ANG II receptor expression or the release of secretagogues.

Little is known about NO synthesis in the adrenal gland. Palacios et al. (21) demonstrated that homogenates of adrenal medulla and cortex contained NO enzymatic activity. Immunohistochemical studies indi-
cate the expression of neuronal NOS (nNOS) in neural cells of the adrenal medulla and cortex (3, 18). By use of in situ hybridization in the mouse adrenal, increased nNOS mRNA was detected in cortical tissue after immobilization stress (30). Cells of the zona fasciculata and ZG layers have recently been suggested to express NOS activity; however, the expression of NOS within adrenal ECs has not been investigated (9, 19). This study was designed to identify the source of NO production within the adrenal cortex and to test the hypothesis that ECs inhibit aldosterone synthesis by ZG cells through the release of NO. There are currently no reports of EC products inhibiting aldosterone synthesis; however, ECs are recognized as a common source of NO.

METHODS

Adrenal cell culture. Adrenal ZG cells were cultured as previously described by Rosolowsky and Campbell (27). ZG cells were plated at a density of 200,000 cells/well in 96-well, 24-well tissue culture-treated plates (Becton-Dickinson, Lincoln Park, NJ) and were maintained in culture for 3–6 days. Cells were fed daily with a modified Ham’s F-12 medium supplemented with 2% fetal bovine serum, 1% penicillin-streptomycin solution, 3 μg/ml gentamycin, 3 U/ml nystatin, 0.1% fungizone, and antioxidants.

Adrenal microvascular ECs and adrenal fibroblasts were isolated by removing a second slice from adrenal cortical tissue sections and digesting the cells for 30 min in collagenase solution (11, 29). Digested cells were washed in Earle’s balanced salt solution (EBSS) and resuspended in feed medium consisting of RPMI medium supplemented with a 1% penicillin-streptomycin solution and 15% fetal bovine serum. Cells were plated at a density of 50,000 cells/well on Corning 150-mm tissue culture plates and were allowed to adhere for 2 h. The culture dishes were then washed twice with warm balanced salt solution (EBSS) and resuspended in feed medium. After 2 days in culture, the dishes were examined for EC and fibroblast colonies, which were identified by their morphology. Cloning rings were used to isolate the desired colonies and transfer each to new culture dishes. Concentrations of fetal bovine serum were increased to 20% in suspected EC cultures and decreased to 5% in suspected fibroblast cultures. Most cell cultures were subcloned once more to obtain pure cultures, and then they were grown to confluence. The purity of the EC cultures was determined by their characteristic cobblestone morphology, as well as by uptake of di-acetylated low-density lipoprotein, and by the absence of smooth muscle α-actin. Adrenal fibroblasts were identified by characteristic morphology, a lack of contact inhibition at confluence, and their ability to thrive in low serum conditions. Cells were fed every 2 days and were used after reaching 80–100% confluence.

Western immunoblot. Cultures of adrenal fibroblasts, adrenal microvascular ECs, and ZG cells were grown in 225-cm² tissue culture flasks to 80–100% confluence. Cells were then incubated at 4°C for 10 min with rocking in 2 ml of l-citrulline buffer consisting of 11 mM HEPES buffer (pH 7.4) 350 mM sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 10% glycerol. After incubation, adherent cells were scraped, and lysate protein concentrations were determined by the Bio-Rad spectrophotometric method (Bio-Rad, Hercules, CA). Cell lysates were stored frozen at −80°C. Lysates were loaded at 50 μg protein/lane and were separated by polyacrylamide gel electrophoresis with a 10% resolving gel and a 4% stacking gel on a Bio-Rad minigel apparatus. Standards for endothelial NOS (eNOS), inducible NOS (iNOS), nNOS, and bovine brain lysates were included as controls. After electrophoresis, the protein bands were electrochemically transferred to nitrocellulose membranes (Bio-Rad). The nitrocellulose membranes were used immediately or stored frozen before incubation with primary antibody. Non-specific antibody binding was minimized by blocking the nitrocellulose membranes in 20 mM Tris base buffer, pH 7.5, containing 500 mM sodium chloride, 0.05% Tween 20, and 2% nonfat dry milk (blocking buffer) for 4 h at 4°C with gentle rocking. Membranes were exposed to anti-eNOS, anti-iNOS, or anti-nNOS mouse monoclonal primary antibodies (Transduction Laboratories, Lexington, KY) in blocking buffer for 1 h at 4°C. Anti-eNOS antibody was used at a 1:2,000 dilution, anti-iNOS antibody at a 1:1,000 dilution, and anti-nNOS antibody at a 1:500 dilution of the manufacturer’s original stock. Membranes were then washed three times with blocking buffer with gentle rocking and exposed to a goat anti-mouse secondary antibody coupled to horseradish peroxidase for 1 h at room temperature. After successive washes of the membrane, immunoreactive bands were identified with the Renaissance chemiluminescence detection kit (NEN, Boston, MA) and Kodak BioMax MR film.

NOS activity. Enzymatic activity of NOS in ZG cells, adrenal fibroblasts, and EC homogenates was determined as previously described (6). Cells were lysed in 50 mM Tris-HCl buffer, pH 7.5, supplemented with 0.1 mM EDTA, 0.1 mM EGTA, and 0.1% mercaptoethanol. Lysate protein concentrations were determined by the Bio-Rad spectrophotometric assay, and lysates were stored at −80°C. One hundred microliters of lysate protein were incubated in 100 μl of Tris buffer supplemented with 50 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 1 mM NADPH, 5 μM tetrahydrobiopterin, 10 μM L-arginine, 2.5 mM calcium chloride, and 0.2 μCi of [14C]L-arginine. After incubation at 37°C for 15 min, the reaction was stopped by the addition of 1 ml of ice-cold 20 mM HEPES, pH 5.5, containing 2 mM EDTA and 2 mM EGTA. NOS activity within intact cells was determined by incubation of confluent ECs in 25-cm² flasks with 0.2 μCi of [14C]L-arginine for 2 h at 37°C. The incubation was performed in 5 ml of 10 mM HEPES, pH 7.4, containing 155 mM sodium chloride, 5 mM potassium chloride, 1.8 mM calcium chloride, 1 mM magnesium chloride, and 5.5 mM glucose (HEPES buffer). At the end of the incubation, the buffer was removed to tubes, and the cells were frozen at −40°C. The cells were thawed, scraped, and sonicated in phosphate-buffered saline (pH 7.4) containing 2 mM EGTA. L-Citrulline was separated from unreacted L-arginine by Dowex 50Wx8 cation exchange resin in both whole cell and homogenate assays. The amount of L-[14C]citrulline in the flow through was determined by liquid scintillation spectrophotometry. Specific activity of NOS was expressed as pmol of [14C]citrulline (pm) after subtraction of the blank in homogenates and as a percentage of the conversion of L-[14C]arginine to L-[14C]citrulline in whole cell incubations.

Nitrate/nitrite determination. Measurement of nitrate and nitrite concentrations were used as an index of NO release in ZG cell and EC cultures. Measurement was made spectrophotometrically with a multichannel flow injection analyzer and Griess reagent, as previously described (24).

Fluorescence detection of NO. Diaminofluorescein 2 (DAF-2; Calbiochem, San Diego, CA) was used to determine the production of NO by donors in vitro. A solution of 5 μM DAF-2 was made in a plastic cuvette containing 3 ml of
HEPES buffer. The cuvette was placed in a Perkin-Elmer fluorometer and excited with 495 nm of light. Emission at 515 nm was monitored to establish a baseline. There was no detectable change in baseline fluorescence during the course of a 30-min incubation. Various concentrations of the short half-life NO donor 1-hexanamine,6-(2-hydroxy-1-methyl-2-nitrosohydrrazino)-N-methyl (MAHMA) nonoate were added and the fluorescence was monitored.

The cell-permeable form of the NO reactive dye, diaminofluorescein 2 diacetate (DAF-2 DA) was used to examine the intracellular accumulation of NO within ZG cells in culture. ZG cells were plated in 12-well tissue culture-treated plates at a density of 50,000 cells/well. Cells were grown as usual and were used for DAF-2 DA fluorescence studies before confluence at day 2 or day 3 in culture. ZG cells were washed three times with HEPES buffer before loading with DAF-2 DA. The dye was supplied by the manufacturer diluted to 5 mM in dimethyl sulfoxide. Immediately before ZG cells were loaded, 2 μl of this stock solution were mixed with 50 μl of 0.25% bovine serum albumin and then diluted to a final concentration of 5 μM DAF-2 DA in HEPES buffer. ZG cells were incubated with 1 ml/well of the dye solution in the dark at room temperature for 1 h. Cells were then rinsed three times in HEPES buffer and transferred to a Nikon diaphot inverted fluorescence microscope equipped with a Photometrics SenSYS CCD camera. Excitation light of 485 ± 22 nm was supplied, and emission at 530 ± 30 nm was detected through the use of an Omega Optical XF-22 filter cube. CCD camera exposure times were 4 s for all DAF-2 DA fluorescence studies. Sequential exposures were performed every 2–4 min for ~30 min. ZG fluorescence during coincubation with ECs was performed in a similar manner. ECs grown on beads (see below) (28) were removed from their original culture flask and washed three times in HEPES buffer. Beads were then resuspended in HEPES buffer supplemented with 100 μM L-arginine at a concentration of 5–10 mg beads/ml. The bead suspension was then carefully added to DAF-2 DA-loaded ZG cells and transferred to a fluorescence microscope. Intracellular fluorescence data were processed by subtracting a background fluorescence image acquired at time 0 of each incubation.

Controlled oxygen studies. Studies of the effect of oxygen concentration on aldosterone production were performed in a gas-tight anaerobic chamber equipped with an airlock and oxygen controller (Coy Laboratory Products, Grass Lake, MI). Internal oxygen concentrations were maintained within 0.5% of the indicated setpoint by injection of 95% nitrogen-5% carbon dioxide gas (Bentley Welding, Milwaukee, WI). Gas flow was automatically adjusted in response to oxygen concentrations detected by an oxygen sensor. ZG cells were cultured as described above. Cells were then transferred into the controlled oxygen environment and allowed to equilibrate for 2 h. Cells were then rinsed twice with 0.5 ml/well of preequilibrated Ham's F-12 medium supplemented with 1 mg/ml bovine serum albumin and were allowed to equilibrate for 2 h to remove antioxidants and inhibitors. Wash medium was removed and replaced with ZG steroidogenic medium, consisting of Ham's F-12 supplemented with 14 mM NaCl, 14 mM NaHCO₃, 2 mg/ml bovine serum albumin, and 1.8 mM CaCl₂. DETA nonoate and secretagogues were then added, and the incubation was continued for 2 h. The steroidogenic medium was removed and stored at −40°C until assayed.

EC/ZG cell coincubation experiments. ECs were grown on Corning 225-cm² tissue culture-treated flasks (Cambridge, MA), and 100 mg of Cytodex microcarrier beads were added while cells were subconfluent (28). After 24 h, the ECs had coated the microcarrier beads and were ready to be used in EC-ZG coincubation experiments. The EC-coated beads were dislodged from the underlying monolayer by spraying them with feed medium from a pipette and then transferring them to a sterile tube containing RPMI 1640 medium. The EC-coated beads were allowed to settle, and the medium was decanted. A second wash with RPMI 1640 medium was performed, and the medium was again decanted. The beads were then resuspended at 5–10 mg/ml in ZG steroidogenic medium. Bead suspensions were preequilibrated in controlled oxygen for 2 h before the start of coincubation. Suspensions of EC-coated or cell-free beads were transferred to multiwell plates of prewashed ZG cells, and ANG II was added. ECs used for whole cell NOS activity experiments were incubated in the same manner. In experiments involving NOS inhibition, EC-coated and cell-free beads were pre-treated with 30 μM nitro-L-arginine (LNA) for 15 min before addition to the ZG cells. Coincubations of EC-ZG cells were incubated at 37°C for 2 h. The medium was then removed and stored at −40°C for subsequent aldosterone assay.

Aldosterone assay. Aldosterone was measured by ELISA, as previously described (13). Statistical analysis was via Student's t-test. Data represent averages of multiple incubations from at least two cell preparations, or they are a representative experiment from multiple cell preparations. To control for variations in aldosterone production between ZG cell preparations, ANG II was included as a positive control in each experiment.

Materials. Reagents not listed above were as follows: modified low-sodium Ham's F-12 medium (Sigma Chemical, St. Louis, MO), EBSS and antibiotic-antimycotic solutions (GIBCO, Grand Island, NY), fetal bovine serum (Hyclone, Logan, UT), collagenase type I (Worthington Biochemical, Freehold, NJ) and dispase (Boehringer Mannheim Biochemicals, Indianapolis, IN), dial-acetylated LDL (Biomedical Technologies, Stoughton, MA), DETA nonoate and MAHMA nonoate (Cayman Chemical, Ann Arbor, MI), and ANG II (Beckman, Palo Alto, CA). All other reagents were purchased from Sigma Chemical.

RESULTS

Western immunoblot of cultured adrenal fibroblasts, ECs, and ZG cells revealed eNOS immunoreactive bands in the adrenal ECs (Fig. 1). Immunoreactive bands were not detectable in lanes containing adrenal fibroblast and ZG cell lysates. The molecular mass of the EC immunoreactive band was ~133 kDa and corresponds to the previously described molecular mass of eNOS enzyme. Western blots probed with iNOS and nNOS antibodies demonstrated immunoreactive bands in control lanes containing enzyme standards but did not reveal any immunoreactive bands in any of the three adrenal cell types (data not shown). A 24-h stimulation of all three cell types with 20 ng/ml interleukin-1β (IL-1β) before cell lysis had no discernible effect on the intensity of eNOS immunoreactive bands and did not produce any detectable bands in the iNOS western blot.

Enzymatic activity of NOS was determined as confirmation of the cellular localization of the enzyme in the adrenal cortex. Enzymatic conversion of L-[3H]arginine to L-[3H]citrulline was measured in lysates of adrenal cell cultures of fibroblasts, ECs, and ZG cells (Fig. 2). Specific cellular conversion of L-arginine to L-citrulline after blank subtraction was not detectable...
in adrenal fibroblasts and was not statistically significant in ZG cells. NOS activity was clearly identifiable in ECs in the presence and absence of IL-1β (20 ng/ml). Treatment of ECs with IL-1β caused a slight but significant increase in NOS activity (P < 0.02, IL-1β vs. control). Accumulation of nitrate/nitrite was used as an index of NO release in cultures of ZG cells and ECs (Fig. 3). ECs produced more nitrate/nitrite than did ZG cells in the presence and absence of 100 nM ANG II. Nitrate/nitrite production in ECs was increased 2.4-fold by stimulation with ANG II (P < 0.05). These data indicate that ECs, but not ZG cells or adrenal fibroblasts, contain NOS enzymatic activity, and that activity can be stimulated by ANG II.

In vitro studies with the NO-reactive dye DAF-2 were carried out to confirm the ability of the dye to detect NO release. Incubation of DAF-2 with the short-half-life NO donor MAHMA nonoate caused a concentration- and time-dependent increase in DAF-2 fluorescence (Fig. 4). Fluorescence was not increased by vehicle treatment (data not shown). DAF-2 was used in its cellular permeable form, DAF-2 DA, to determine whether NO released from NO donors or ECs entered ZG cells. ZG cells did not show any increased fluorescence after treatment with vehicles (data not shown). Fluorescence intensity was increased over 16 min after treatment of DAF-2-loaded ZG cells with DETA nonoate (1 μM) (Fig. 5, A-D). Similarly, treatment of ZG cells with ECs grown on beads resulted in increased fluorescence intensity over a 20-min exposure (Fig. 5, E-H). ZG cell fluorescence appeared more rapidly in cells directly covered by EC beads.

NO inhibits aldosterone synthesis by binding to the heme of adrenal cytochrome P450s (17). Thus NO must compete with oxygen for the cytochrome P450 heme site. We reasoned that changes in oxygen concentration of the ZG cell would likely change the sensitivity of aldosterone synthesis to NO inhibition. Aldosterone synthesis was stimulated by 1 nM ANG II in 21, 8, and 3% oxygen (Fig. 6A). In agreement with previous studies, maximal ANG II-stimulated aldosterone synthesis was decreased with decreasing oxygen concentration, and ANG II did not stimulate aldosterone in 1% oxygen (4, 26). DETA nonoate inhibited ANG II-stimulated aldosterone in a concentration-dependent manner at...
all oxygen concentrations; however, the IC₅₀ was decreased with decreasing oxygen concentration \( [P < 0.02 \text{ comparing ANG II-stimulated control to DETA nonoate (10}⁻⁴ \text{ M in 8% oxygen)}] \). The IC₅₀ for DETA nonoate inhibition of aldosterone in 21% oxygen was ~300 \( \mu \text{M} \), whereas the IC₅₀ at 8% or less oxygen was ~25 \( \mu \text{M} \). Potassium-stimulated aldosterone synthesis was also inhibited by decreasing oxygen concentrations (Fig. 6B). Similar to the ANG II stimulation, the IC₅₀ of DETA nonoate in potassium-stimulated ZG cells incubated in 21% oxygen was ~300 \( \mu \text{M} \). Decreasing oxygen concentrations resulted in a shift in the IC₅₀ of DETA nonoate to 25 \( \mu \text{M} \) after potassium stimulation of aldosterone concentration \( [P < 0.005, \text{potassium-stimulated control vs. DETA nonoate (10}⁻⁴ \text{ M in 8% oxygen)}] \). Trypan blue staining did not indicate a change in ZG cell viability after exposure to various oxygen concentrations. The experiment was repeated with various concentrations of ANG II in 21% oxygen to determine whether this effect was due to changes in oxygen concentration or to a decrease in the stimulation of aldosterone synthesis (Fig. 6C). The IC₅₀ of DETA nonoate was ~300 \( \mu \text{M} \) after stimulation with 10 and 100 \( \text{pM} \) ANG II. Thus the inhibitory potency of DETA nonoate was unchanged by varying amounts of aldosterone stimulation. These data suggest that the oxy-
gen concentration of the ZG cell in the intact adrenal gland may determine the inhibitory effect of NO.

The effect of EC-derived NO on ZG cell aldosterone synthesis was determined in coincubation studies in the presence or absence of a maximally inhibitory concentration of LNA (30 μM) (Fig. 7A). In 21% oxygen, aldosterone synthesis was stimulated by ANG II (100 nM) in ZG cells incubated with cell-free beads. Coincubation of ZG cells with ECs on beads decreased aldosterone synthesis in unstimulated and ANG II-stimulated ZG cells. However, inhibition of NO production by LNA did not increase aldosterone synthesis in EC coincubations. Although coincubation with ECs resulted in a decrease in aldosterone synthesis, the causative factor did not appear to be NO. The experiment was then repeated in an 8% oxygen environment. EC-derived NO inhibited ZG cell aldosterone synthesis in the decreased oxygen environment (Fig. 7B). ANG II stimulated an increase in aldosterone synthesis from ZG cells coincubated with cell-free beads. As in 21% oxygen, coincubation of ZG cells with ECs on beads inhibited the ANG II-stimulated aldosterone release. Inhibition of NO production with LNA had no effect on aldosterone synthesis by ZG cells coincubated with cell-free beads but reversed the EC-mediated inhibition of aldosterone release. Coincubation of ECs and ZG cells in the presence of NOS inhibition resulted in an increase in ANG II-stimulated aldosterone synthesis beyond that of cell-free controls \( P < 0.01 \). The aldosterone-stimulatory effects of EC products have been previously described (28). The effect of oxygen concentration on NOS activity in cultured ECs was determined by conversion of L-[\(^3\)H]arginine to L-[\(^3\)H]citrulline in intact cells. Conversion of L-[\(^3\)H]arginine to L-[\(^3\)H]citrulline was slightly reduced in ECs incubated in 8% oxygen compared with 21% oxygen (76 ± 0.2% conversion in 21% oxygen vs. 71 ± 0.2% conversion in 8% oxygen, \( P < 0.004 \); data not shown). Therefore, the enhanced inhibition of aldosterone by EC-derived NO is not due to an increase in NO synthesis.

**DISCUSSION**

The inhibition of steroid hormone synthesis by NO has been demonstrated in a variety of tissues (2, 10, 13, 19, 23). Studies using purified or semipurified cytochrome P450 enzymes indicate that inhibition by NO occurs through a direct interaction of NO with the heme group of cytochrome P450 enzymes responsible for steroidogenesis (13, 16, 34). The reactive nature of NO results in a short half-life and necessitates that the source of NO production be close to the target cells for inhibition to occur. The localization of NOS within the adrenal gland thus becomes critical to determining the physiological role of NO in adrenal steroid regulation.
Expression of NOS within the cells of the adrenal cortex remains a subject of debate. A number of studies found NOS activity or immunoreactive proteins in acutely isolated adrenal cortical tissue (3, 18). A recent study by Cymeryng et al. (9) indicates that endogenous production of NO inhibits zona fasciculata cell steroidogenesis. Natarajan et al. (19) demonstrated eNOS expression within acutely isolated rat ZG cells. However, the results of our investigation indicate that cultured bovine adrenal ZG cells do not contain detectable NOS by Western immunoblot or enzymatic activity assay. Although NOS was not detected in ZG cells or adrenal fibroblasts, adrenal ECs demonstrated both immunoreactive and enzymatically active eNOS. The differences between our results and those of Natarajan et al. may be explained by a species-specific variance in adrenal NOS expression or by effects of culture on the expression of cortical NOS. It appears that cortical NOS expression may be regulated, because immobilization stress increased nNOS mRNA in the adrenal cortex of rats (30). Clearly, other studies are required to characterize adrenal NOS expression and regulation.

Regardless of the specific cellular sources of NO production within the adrenal cortex, the ZG cell is likely to be exposed to NO. Although free radical generation by steroidogenic cytochrome P450 enzymes should increase degradation of NO, it does not appear sufficient to protect the cell from NO exposure. Even the relatively low concentrations of NO synthesized and released by cultured ECs resulted in ZG cell NO accumulation, as detected by DAF-2 DA fluorescence. The EC-ZG cell coincubation studies indicate that ANG II stimulation of ECs resulted in increased ZG cell NO accumulation. The stimulatory effect of ANG II on aldosterone release from ZG cells may therefore be partially inhibited by increased NO release from the adjacent ECs.

Aldosterone synthesis is regulated by ZG cell oxygenation. Brickner et al. (4) and Raff et al. (26) have demonstrated that decreasing oxygen concentrations in ZG cell cultures result in decreased aldosterone synthesis. The ability of NO to compete with carbon monoxide at the heme site of cytochrome P450 enzymes suggests a competition between NO and oxygen for occupation of the heme site (34). This was further suggested by the present data. The leftward shift in the DETA nonoate concentration response curve with decreasing oxygen concentration can be explained as a decrease in the oxygen available to compete with NO for heme binding. It is also possible that decreased oxygen concentration results in a decreased generation of reactive oxygen species and extends the half-life of NO. The results of this study cannot determine which of these mechanisms is more pronounced; however, it is clear that decreasing ZG cell oxygenation is directly inhibitory and enhances any inhibitory effects of NO. The enhanced inhibition of aldosterone release by NO was specifically caused by decreasing the oxygen concentration and was not due to a decrease in the stimulation of aldosterone synthesis, because the IC_{50} of DETA nonoate was not changed in the presence of various amounts of ANG II stimulation of aldosterone synthesis. Decreasing the oxygen concentration from 21 to 8% enhanced the EC-mediated inhibition of ZG cell aldosterone synthesis. It appears that NO release from cultured ECs is not sufficient to inhibit aldosterone release in a 21% oxygen environment. Cultured EC production of NO may be below the threshold for NO inhibition of aldosterone in 21% oxygen; however, the adrenal cortex is certainly not exposed to 21% oxygen concentrations in vivo. Decreasing oxygen concentration resulted in a lower threshold for NO-mediated inhibition and allowed EC-derived NO to inhibit
aldosterone synthesis. Thus the ability of endothelial NO to inhibit aldosterone release in 8% oxygen can be explained by the decrease in the IC_{50} for NO with the decreased oxygen concentration. This study provides evidence that EC-derived NO inhibits ZG cell aldosterone synthesis in culture. Whether NO has a physiological or pathological role in the regulation of aldosterone synthesis remains to be determined. The physiological relevance of these findings will depend on accurate determination of the partial pressures of oxygen and NO at the ZG cell layer in vivo. Although this study examines the possibility of physiological regulation of aldosterone release by EC-derived NO, there is also the potential for the inhibition of aldosterone synthesis by activated macrophages, which are capable of producing large amounts of NO. A recent study has demonstrated NO-mediated inhibition of steroid hormone synthesis with coincubations of macrophage and Leydig cells (23). Although the effects of EC-derived NO appear to be dependent on local oxygen concentration, the large amounts of NO generated by macrophages may inhibit steroidogenesis regardless of local oxygen concentration and could result in pathological changes in aldosterone release.

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