Inhibition of Adrenal Cell Aldosterone Synthesis by Endogenous Nitric Oxide Release

Craig J. Hanke, Timothy O’Brien, Kirkwood A. Pritchard, Jr, William B. Campbell

Abstract—Adrenal zona glomerulosa (ZG) cells do not contain nitric oxide (NO) synthase (NOS). We conferred endothelial NOS activity onto adrenal ZG cells through transduction with a recombinant adenosine encoding the endothelial NOS gene (AdeNOS) to determine the effect of endogenous NO on aldosterone synthesis. A 135-kDa protein band immunoreactive to anti–endothelial NOS antibody was observed in Western blots of AdeNOS-transduced ZG cells but not in control cells or cells transduced with adenovirus encoding the β-galactosidase gene (AdβGal). Nitrate/nitrite production in AdeNOS-transduced ZG cells increased from 0.15±0.01 to 0.27±0.01 μmol/L after stimulation with 1 mmol/L angiotensin II. The treatment of AdeNOS-transduced cells with 30 μmol/L l-nitro-arginine decreased angiotensin II–stimulated nitrite production from 0.27±0.01 to 0.17±0.01 μmol/L. Basal and angiotensin II–stimulated nitrite production was not increased in AdβGal-transduced or control cells. AdeNOS-transduced cells demonstrated diaminofluorescein-2 diacetate fluorescence, which was blocked by pretreatment with l-nitro-arginine. Angiotensin II–stimulated aldosterone synthesis decreased from 5123±177 pg/mL in AdβGal-transduced ZG cells to 72±27 pg/mL in AdeNOS-transduced cells. Treatment with the NOS inhibitor thioctitrulline (30 μmol/L) increased angiotensin II–stimulated aldosterone synthesis to 2158±45 pg/mL after AdeNOS transduction. These data demonstrate that adenovirus-mediated gene transfer of eNOS in ZG cells results in the expression of active endothelial NOS enzyme and that this endogenous NO production by ZG cells decreases aldosterone synthesis. (Hypertension. 2000;35[part 2]:324-328.)

Key Words: nitric oxide ■ aldosterone ■ adrenal glands ■ adenosine ■ nitric oxide synthase ■ zona glomerulosa

Nitric oxide (NO) derived from NO donors inhibits aldosterone synthesis in adrenal zona glomerulosa (ZG) cells from bovine, rat, and human adrenal glands.1–3 NO-mediated inhibition occurs in ZG cells stimulated with angiotensin II (Ang II), adrenocorticotropic hormone, and potassium ion, as well as with the steroidogenic substrates 25-hydroxycholesterol and progesterone.2,3 NO stimulates the accumulation of cGMP in adrenal ZG cells; however, this appears to be unrelated to NO-mediated inhibition of aldosterone release.3 The mechanism of inhibition appears to occur primarily via direct interaction of NO with cytochrome P450 enzymes of the steroidogenic pathway.3 The results of studies of the effects of NO on other steroidogenic cell types indicate that the inhibitory effects are not limited to aldosterone. NO inhibits the synthesis of testosterone in rat Leydig cells and the synthesis of corticosterone in rat adrenal zona fasciculata cells.4–5 Thus, it appears likely that the inhibitory effects of NO may be seen in a variety of cells containing steroidogenic cytochrome P450 enzymes. Studies of the biochemical interactions between NO and various cytochrome P450 enzymes have clearly demonstrated the ability of NO to rapidly bind the heme portion of the cytochrome P450 enzyme.9,10 This binding process inactivates the enzyme by preventing binding to oxygen.11 Raman spectrophotometric analysis of the cytochrome P450/NO interaction suggests that there may be 2 phases to the inhibition by NO. The first phase involves a rapid occupation of the heme group by NO and results in a reversible inhibition of enzymatic activity. After a 2-hour exposure to NO, a second phase of inhibition appears, which likely involves the nitrosylation of a tyrosine of the cytochrome P450 enzyme.9,10 This second phase results in irreversible inhibition of enzymatic activity.

Determination of the physiological role of NO in aldosterone synthesis is complicated by the interactions of vasodilatory effects of NO with the regulatory pathways that stimulate aldosterone synthesis. The administration of NO donors such as sodium nitroprusside and nitroglycerin has long been recognized to dramatically lower blood pressure. Conversely, the inhibition of endogenous NO production through the use of NO synthase (NOS) inhibitors increases blood pressure.12 The effects of these manipulations on plasma aldosterone concentrations have produced conflicting results. Usui et al13 demonstrated an increase in plasma aldosterone concentrations and ZG cell angiotensin II type 1 (AT1) receptor expression in rats after NOS inhibition.
with N^o-nitro-L-arginine methyl ester. In contrast, studies of the effects of NOS inhibition in dogs and humans in vivo found no change in serum aldosterone concentrations. The impact of systemic NO inhibition on aldosterone synthesis is difficult to analyze. Increased blood pressure from NOS inhibition results in decreased activation of the renin-angiotensin-aldosterone system and decreases aldosterone synthesis. Thus, to determine the effects of NO on aldosterone synthesis in vivo, it is necessary to isolate the ZG cell from reflex responses to changes in blood pressure or to selectively administer the NOS inhibitor to the adrenal gland.

The adrenal cortex has been shown to contain NOS activity and endothelial NOS (eNOS) protein. Although the cellular localization of NOS within the adrenal gland remains controversial, there clearly are sources of NO within close proximity to the ZG cell. Adrenal capillary endothelial cells have been shown with the use of Western immunoblotting and enzymatic activity to contain eNOS (C.J. Hanke and W.B. Campbell, unpublished observations, 1999). The close association of these cells with ZG cells in the intact adrenal gland suggests that they may be a prominent source of ZG cell NO. The results of recent studies have indicated the presence of eNOS in ZG cells of the rat and humans and a currently unidentified form of NOS in rat zona fasciculata cells. However, workers at our laboratory have not been able to detect eNOS in bovine ZG cells through the use of Western immunoblotting or enzymatic activity assays. In the present study, we confer NOS activity on bovine ZG cells with the use of an adenovirus containing the eNOS gene and investigate the effects of endogenous NO on aldosterone synthesis, and we test the hypothesis that the generation of NO by eNOS in ZG cells will result in the inhibition of aldosterone synthesis.

Methods

Gene Transfer

Before infection, ZG cells were cultured and maintained as previously described. ZG cells were grown onto 24-well plates and infected through incubation with 250 µL of feed medium per well containing 25 plaque-forming units (PFU) per cell of adenovirus. During the initial transduction period, the plates were gently rocked every 15 minutes for 1 hour to allow optimal distribution of viral particles. After 1 hour of incubation, feed medium was added to bring the final volume to 1 mL. Cells were then allowed to incubate as usual for 18 to 24 hours. ZG cells grown in 12-well plates and 75-cm² flasks were infected at the same multiplicity of infection in 300 µL feed medium per well and 2 mL feed medium per flask, respectively. Optimal multiplicity of infection for ZG cells was determined with the use of a replication-defective adenoviral vector encoding the Escherichia coli β-galactosidase gene driven by the cytomegaloivirus promoter (AdβGal). ZG cells were exposed to multiplicity of infection ranging from 5 to 200 PFU/cell. For the determination of transduction efficiency, transduced cells were washed 3 times in PBS, pH 7.4, and fixed in PBS containing 1% glutaraldehyde and 1 mM MgCl₂, for 15 minutes at room temperature. ZG cells expressing the β-galactosidase gene product were incubated with a solution of 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, and 0.2% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside. Transgene-expressing cells were determined with the use of a replication-defective adenoviral vector (AdENoS). Cultures of ZG cells were grown in 75-cm² tissue culture flasks to 80% to 100% confluency. Transduction with adenovirus was carried out as described earlier. Cells were then incubated at 4°C for 10 minutes with rocking in 2 mL lysate buffer consisting of 11 mM/ L HEPES, pH 7.4, with 350 mM/ L sucrose, 0.1 mM/ L EDTA, 1 mM/ L dithiothreitol, 10 µg/mL leupeptin, 2 µg/mL aprotinin, 10 µg/mL soybean trypsin inhibitor, 10 µg/mL PMSF, 1% Nonidet P-40, and 10% glycerol. After this incubation, adherent cells were scraped, and lysate protein concentrations were determined according to spectrophotometric assay (Bio-Rad). Cells lysates were stored frozen at −80°C. Lysates were loaded at 50 µg protein/lane and separated through PAGE with a 10% resolving gel and 4% stacking gel on a Bio-Rad minigel apparatus. After electrophoresis, the protein bands were electrophotorectified to nitrocellulose membranes (Bio-Rad). The nitrocellulose membranes were used immediately or stored frozen before incubation with primary antibody. Nonspecific binding was minimized by blocking nitrocellulose membranes in 20 mM/ L Tris buffer, pH 7.5, containing 500 mM/ L sodium chloride, 0.05% Tween 20, and 2% nonfat dry milk (blocking buffer) for 4 hours at 4°C with gentle rocking. Membranes were exposed to anti-eNOS mouse monoclonal primary antibody (Transduction Laboratories) at a 1:2000 dilution of the manufacturer’s stock in blocking buffer for 1 hour at 4°C. Membranes were then incubated with goat anti-mouse secondary antibody coupled to horseradish peroxidase for 1 hour at room temperature. After successive washes of the membrane, immunoreactive bands were identified with use of the Renaissance chemiluminescence detection kit (New England Nuclear Research Products) and Kodak BioMax MR film.

NITRATE/NITRITE ASSAY

Cultured ZG cells were incubated for 2 hours in 10 mM/ L HEPES buffer, pH 7.4, containing 155 mM/ L sodium chloride, 5 mM/ L potassium chloride, 1.8 mM/ L calcium chloride, 1 mM/ L magnesium chloride, and 5.5 mM/ L glucose (HEPES buffer). The measurement of total nitrates was made after reduction of nitrate with cadmium. Automatic sample injection and nitrite quantification were carried out with the use of a multichannel flow injection analyzer (Automated QuikChem Ion Analyzer; Lachat Instruments Inc.) and the Griess reagent for spectrophotometric determination of nitrate and nitrite concentrations as previously described.

Intracellular NO Fluorescence Imaging

The cell-permeable form of the NO reactive dye dianisidofluorescein-2 diacetate (DAF-2 DA) was used to examine the accumulation of NO within ZG cells in culture. ZG cells were plated onto 12-well tissue culture-treated plates at a density of 50 000 cells per well. Cells were grown as usual and used for DAF-2 DA fluorescence studies before confluence at day 3 in culture. Adenoviral transduction was performed as described earlier. ZG cells were washed 3 times with HEPES buffer before loading with DAF-2 DA. The dye was supplied by the manufacturer diluted to 5 mM/ L in DMSO. Immediately before the loading of ZG cells, 2 µL of this stock solution was mixed with 50 µL of 0.25% BSA and then diluted to a final concentration of 5 µmol/ L 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 hour. Cells were then rinsed 3 times in HEPES buffer and transferred to a Nikon Diaphot inverted fluorescence microscope equipped with a Photometrics SenSYS CCD camera. Excitation and emission light was detected at 485 ± 22 and 530 ± 30 nm respectively through the use of an Omega Optical XF-22 filter cube. CCD camera exposure times were 4 seconds for all DAF-2 DA fluorescence studies. Sequential exposures were performed every 2 to 4 minutes for ~>30 minutes. Intracellular fluores-
Stimulation of ZG cells with 1 nmol/L Ang II resulted in the transduced with AdeNOS but not with Ad Gal.

Increased production of total nitrites from $0.15 \pm 0.01$ to $0.27 \pm 0.01$ μmol/L ($P<0.05$) in AdeNOS-transduced ZG cells. Nitrite production was not significantly increased above basal concentrations by Ang II stimulation of nontransduced or AdβGal-transduced ZG cells. Treatment with 30 μmol/L L-nitro-arginine (LNA) decreased AdeNOS-transduced ZG cell total nitrites production from $0.27 \pm 0.01$ to $0.17 \pm 0.01$ μmol/L ($P<0.01$) but had no significant effect on nontransduced or AdβGal-transduced nitrite production.

NO production in infected ZG cells was determined with the NO-sensitive fluorescent dye DAF-2 DA. Digital fluorescent imaging demonstrated an accumulation of NO-mediated fluorescence in AdeNOS-transduced ZG cells 14 minutes after stimulation with 10 μmol/L A23187 (Figure 3A). Fluorescence was decreased during the same time period in AdeNOS-transduced ZG cells treated with 30 μmol/L LNA and 10 μmol/L A23187 (Figure 3B). DAF-2 DA fluorescence from AdβGal-transduced ZG cells stimulated with A23187 was almost undetectable (Figure 3C). Uninfected ZG cells stimulated with A23187 did not demonstrate detectable fluorescence (data not shown). Based on these data, ZG cells express eNOS enzyme and actively synthesize NO after transduction with AdeNOS but not with AdβGal.

Adenovirus-mediated overexpression of eNOS in ZG cells decreased basal and Ang II–stimulated aldosterone synthesis ($P<0.001$) (Figure 4). Basal and Ang II–stimulated (1 nmol/L) aldosterone synthesis from AdeNOS-transduced ZG cells was $24 \pm 1$ and $72 \pm 27$ pg/mL, respectively. Basal and Ang II–stimulated (1 nmol/L) aldosterone synthesis in AdβGal-transduced ZG cells was $839 \pm 273$ and $5123 \pm 177$ pg/mL, respectively. Thus, aldosterone synthesis in AdeNOS-transduced ZG cells was 1% to 3% of the aldosterone synthesis in AdβGal-transduced ZG cells. In a separate experiment, treatment of infected ZG cells with thiocitrulline (30 μmol/L), an NOS inhibitor, during the transduction period resulted in a decreased basal aldosterone synthesis but an increase in Ang II–stimulated aldosterone synthesis (Figure 5). After thiocitrulline treatment, basal aldosterone synthesis was $199 \pm 18$ pg/mL.
in AdβGal-transduced ZG cells and 19±4 pg/mL in AdeNOS-infected cells. However, thiocitrulline treatment resulted in the recovery of Ang II–stimulated aldosterone synthesis in AdeNOS-transduced ZG cells (1379±80 pg/mL in AdβGal-transduced ZG cells versus 2158±45 pg/mL in AdeNOS-transduced ZG cells). Therefore, the inhibition of eNOS activity with thiocitrulline allows the recovery of Ang II–stimulated aldosterone from ZG cells.

Discussion

Donors of NO, including sodium nitroprusside, nonoates, and S-nitroso-N-acetyl-penicillamine, inhibit aldosterone synthesis in ZG cells from the adrenal glands of cows, rats, and humans.1–3 Current evidence indicates that NO is produced within the adrenal cortex and that the ZG cell is likely to be exposed to NO in vivo.2,17 In the present study, we examined the adenovirus-mediated expression and enzymatic activity of eNOS within the ZG cell and the effects of endogenous NO production on aldosterone synthesis.

ZG cells were receptive to adenoviral transduction and readily synthesized adenoviral gene products. The transduction of ZG cells with AdeNOS resulted in the detection of an immunoreactive band corresponding to a molecular mass of 135 kDa, which is identical to that previously described for eNOS.23 Control ZG cells did not contain detectable eNOS enzyme, which is in contrast to the results of Natarajan et al.2 It is possible that the discrepancy between the 2 studies represents a species-specific variance, because the present study was performed in bovine ZG cells and that of Natarajan et al was performed in rat and human ZG cells. Regardless of the specific expression of NOS within the ZG cell, the close association of microvascular and capillary endothelial cells and other potential NO-producing cell types suggests that NO may be produced in close proximity to ZG cells and may affect ZG steroidogenesis.18

Measurements of nitrate/nitrite as stable metabolites of NO22 were used to confirm eNOS enzymatic activity after AdeNOS gene transfer. DAF-2 was recently described as a selective fluorescent probe for the detection of NO.24 Reaction of NO with DAF-2 causes N-nitrosation of the dye to the highly fluorescent triazole form. The reaction is irreversible and results in a ~180-fold increase in DAF-2 fluorescence. Increases in nitrate/nitrite and DAF-2 fluorescence were detected in AdeNOS-

Figure 3. DAF-2 DA fluorescence in AdeNOS-transduced or AdβGal-transduced ZG cells. A, Fluorescent images of AdeNOS-transduced ZG cells after a 14-minute stimulation with 10 μmol/L A23187. B, Fluorescent images of 30 μmol/L LNA-treated AdeNOS-transduced ZG cells after a 14-minute stimulation with 10 μmol/L A23187. C, Fluorescent images of AdβGal-transduced ZG cells after a 14-minute stimulation with 10 μmol/L A23187. (Magnification ×100, bars 100 μm.) Images were processed through subtraction of a background fluorescence image acquired at time 0 and colorized to facilitate viewing.

Figure 4. Effect of AdeNOS and AdβGal transduction of ZG cells on basal and Ang II–stimulated aldosterone synthesis. Transduced ZG cells were incubated for 2 hours at 37°C in presence or absence of 1 nmol/L Ang II (AII). Samples were stored at −40°C for subsequent aldosterone assay. Results are mean±SEM (n=4). *P<0.001 compared with control. †P<0.001 compared with AdeNOS transduced.

Figure 5. Effect of thiocitrulline treatment on basal and Ang II–stimulated aldosterone synthesis in AdeNOS- and AdβGal-transduced ZG cells. ZG cells were treated with 30 μmol/L thiocitrulline during transduction with AdeNOS and AdβGal. Cells were incubated for 2 hours at 37°C in presence or absence of 1 nmol/L Ang II (AII). Samples were stored at −40°C for subsequent aldosterone assay. Results are mean±SEM (n=4). *P<0.001 compared with control. †P<0.01 compared with AdeNOS transduced.
transduced ZG cells but not in AdβGal-transduced ZG cells. Increased eNOS activity was stimulated by the previously described NOS activator A23187 and by Ang II, a peptide known to stimulate calcium flux in ZG cells.25-26 These results indicate that AdeNOs transduction results in a catalytically active eNOS enzyme that can be stimulated by factors known to increase intracellular calcium concentrations. The calcium-sensitive activation of eNOS enzyme has been previously described.27 In both nitrate/nitrite measurements and fluorescence studies, eNOS enzymatic activity was inhibited with LNA. These data suggest that eNOS enzymatic activity can be regulated within the ZG cell through the use of previously described stimulators and inhibitors.

The ability of various NO donors to inhibit aldosterone synthesis in previous studies argues that NO may be an important intra-adrenal regulator of steroidogenesis.1-3 However, the generation of endogenous NO via enzymatic processes in the ZG cell represents a more physiological setting. The expression of eNOS enzyme within the ZG cell allows cells to be exposed to NO for long time periods without the accumulation of NO donor byproducts. This experimental protocol may be useful to examine the effects of long-term NO exposure on cytochrome P450 enzymatic function. The effects of adenosine-mediated gene transfer of eNOS in this study suggest that the long-term exposure of ZG cells to NO results in a significant decrease in aldosterone synthesis. Long-term treatment with the NOS inhibitor thioctirulline restored aldosterone synthesis in AdeNOs-treated ZG cells. A comparison of nitrate/nitrite concentrations after AdeNOs transduction to nitrite concentrations that were seen in NO donors suggests that ZG cells were exposed to lower concentrations of NO in the present study than in studies with NO donors. However, NO-mediated inhibition of aldosterone synthesis was as effective in the present study as in those with NO donors. Although NO production in the present study may be less than that in studies with NO donors, ZG cell exposure times were dramatically longer. It is possible that the effects of long-term NO exposure cause structural modifications and irreversible inhibition of cytochrome P450 enzymes, as suggested by previous investigators.9,10 The kinetics and time-sensitive nature of NO interaction with steroidogenic cytochrome P450 enzymes remain to be determined.

In summary, the present results indicate that bovine ZG cells do not express eNOS normally but that the exposure to an adenosine encoding eNOS can be used to confer NOS activity and NO production on the cells. Furthermore, endogenous NO synthesis in ZG cells inhibits aldosterone production.

Acknowledgments

This work was supported by grants from the National Institutes of Health (HL-52159) and the American Heart Association, Wisconsin Affiliate. This research was conducted during the tenure of a Student/Predoctoral Fellowship from the American Heart Association, Wisconsin Affiliate. The authors thank Gretchen Barg for her secretarial assistance.

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Hypertension. 2000;35:324-328
doi: 10.1161/01.HYP.35.1.324
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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