Decreased Renal Expression of Nitric Oxide Synthase Isoforms in Adrenocorticotropin-Induced and Corticosterone-Induced Hypertension

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Abstract—Administration of adrenocorticotrophic hormone (ACTH) leads to the development of hypertension. Because glucocorticoids can affect the nitric oxide system at several sites, the present study tested the hypothesis that nitric oxide synthase (NOS) expression may be altered in ACTH-induced and corticosterone-induced hypertension in the rat. This was addressed by measuring Nos1, Nos2, and Nos3 mRNA in the kidney, adrenal gland, heart, and hypothalamus of 16 ACTH-treated and 16 vehicle-treated rats as well as in 10 corticosterone-treated and 10 control rats. In addition, in situ hybridization and immunohistochemistry were used to confirm changes by detection of Nos mRNA in RNA and NOS protein in tissues. Systolic blood pressure of ACTH and corticosterone rats was elevated (165±6 and 162±11 mm Hg; P<0.001 versus control). Each Nos isoform mRNA was measured by reverse transcriptase-polymerase chain reaction technique. In ACTH rats, mRNA for Nos2 was reduced in renal cortex by 58±5% and in medulla by 68±7%; for Nos3, mRNA reductions of 59±6% and 51±11% were seen (P<0.001 after Hochberg correction for multiple comparisons). In corticosterone rats, Nos2 mRNA decreased in cortex by 68±5% and in medulla by 62±6%; Nos3 mRNA by 50±8% in cortex, and Nos1 by 29±7% in medulla (all P<0.001 after Hochberg correction). Reductions seen in kidney were supported by in situ hybridization and immunohistochemistry. Apart from a 62±2% decrease in Nos2 mRNA in adrenal of ACTH rats (corrected P<0.05), no significant changes were seen in the other nonrenal tissues for any isoform. In conclusion, we have shown for the first time that the physiological components of glucocorticoid action (ACTH and corticosterone) when given chronically in vivo reduce Nos2 and Nos3 expression in the kidney. Such changes are consistent with a role in hypertension for ACTH and corticosterone. (Hypertension. 2001;37:1164-1170.)

Key Words: immunohistochemistry ■ hybridization ■ RNA ■ nitric oxide ■ isoenzymes ■ reverse transcriptase–polymerase chain reaction

Administration of adrenocorticotropin (ACTH) produces hypertension in humans, rats, dogs, and sheep, and excess endogenous secretion is associated with Cushing’s syndrome.1 The elevation in blood pressure (BP) is explicable by the action of cortisol in humans2 and corticosterone in the rat,3 but the mechanism by which these steroids raise BP is still not well understood. Studies of ACTH-induced and corticosterone-induced hypertension in the rat and cortisol-induced hypertension in humans suggest a role for the nitric oxide (NO) system in these forms of hypertension.4-5 Given that glucocorticoids are known to have several potential sites of action in the NO system, including an inhibitory effect on transmembrane l-arginine transport6 and suppression of nitric oxide synthase (NOS)27 as well as tetrahydrobiopterin synthesis,6 we wanted to define whether elements of the NO system are altered in ACTH-induced hypertension. Contrary to early views, reflected in their nomenclature, each NOS has varying degrees of constitutive and inducible capabilities.5,9 The aim of the present study was to examine NOS expression in ACTH, corticosterone, and vehicle-treated rats. Because dexamethasone can reduce human NOS3 promoter activity and mRNA stability10 and NOS2 control is at the level of transcription and protein stability,11 we looked for changes in Nos mRNA levels, and alterations were verified by examination of NOS protein changes in tissue sections.

Methods

Rats

Male 8-week-old Sprague-Dawley rats (250 to 280 g) from Animal Resources Center, Perth, Western Australia, were housed (4 rats per plastic cage) at 21°C to 23°C in the St George Animal House, which has separate rooms for surgery, BP monitoring, and metabolic

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studies. Rats were fed a commercial diet (Gordon’s Specialty Stock Feeds, Yanderra, NSW, Australia), and given free access to tap water. All procedures were ethically approved.

**Determination of Physiological Parameters**

Every other day, body weight, 24-hour food and water intake, urine volume, and excreted Na⁺ were measured in separate 18×23×43-cm wire metabolic cages. Systolic BP was recorded on alternating days by tail cuff (Narco Biosystems, Inc). At least 5 consecutive cycles of inflation/deflation were performed on each rat while it was conscious, and the mean of the last 3 recordings, which showed no more than a 10-mm Hg difference, was taken to be systolic BP.

**ACTH and Corticosterone Treatment Protocols**

Six days of control measurements were followed by 10 further days of measurements, during which ACTH or vehicle (0.9% NaCl) was administered. Sixteen rats were injected (at 10 AM and 6 PM) with 0.25 mL/kg (0.5 mg · kg⁻¹ · d⁻¹) SC synthetic ACTH (Synacthen Depot, Novartis) BID. Concurrently, another 16 rats received 0.25 mL/kg SC of vehicle BID. At the end of the experiment, rats were euthanatized by use of pentobarbital (60 mg/kg IP). The corticosterone protocol was similar, except that 10 rats were injected subcutaneously twice daily with corticosterone (Sigma) 120 µmol · kg⁻¹ · d⁻¹ in ethanol and 10 were given ethanol vehicle.

**Biochemical Measurements**

Blood samples of 6 to 8 mL were taken from the cannulated carotid artery under anesthesia and stored at −80°C. The right kidney was fixed in 4% paraformaldehyde/10% phosphate buffer, pH 7.4, for immunohistochemical studies. Extraction of total RNA from left kidney was done with RNaZol B solution (Brescatex) in a Polytron homogenizer. RNA samples were stored in diethylpyrocarbonate-treated water with 2 µg of tRNA at −80°C for <4 months. Integrity of RNA was assessed by gel electrophoresis. RNA concentrations were estimated from absorption at 260 nm.

**Nos mRNA Measurements**

Nos mRNA isofoms were semiquantified by a reverse-transcription–polymerase chain reaction (RT-PCR) method. For the RT step, a 3-µL aliquot (3 µg) of total RNA was dissolved in 20 µL of reaction mixture that contained 1 mmol/L dNTP, 1 U RNAsin (Promega), 100 pmol/L random hexamers (Promega), reaction buffer (final concentration contained 50 mmol/L KCl, 20 mmol/L Tris-HCl [pH 8.4], 2.5 mmol/L MgCl₂, and 10 µg/L nuclease-free BSA), and 200 U of murine leukemia virus reverse-transcriptase (Gibco BRL) and kept at 42°C for 60 minutes. The enzyme was inactivated by increasing the temperature to 96°C for 5 minutes. Samples then were cooled to 4°C. For the PCR step, 3 µL of the resulting RT mixture was transferred into 30 µL of reaction buffer (see above) that contained 50 pmol/L specific primer and 5 U of Taq polymerase (Gibco BRL). A separate PCR mixture was made for each Nos isoform mRNA and β-actin mRNA internal control, to give 4 tubes for each sample. To reduce cross-hybridization and enhance specificity, primers for Nos1, Nos2, and Nos3 cDNAs were chosen for minimum interisoform homology. Primer and PCR product sizes are shown in the Table. PCR involved 34 cycles of 95°C, 60°C, and 72°C for 1 minute each in a Perkin-Elmer/Cetus model 480 thermal cycler, β-actin PCR comprised 24 cycles of 95°C, 62°C, and 72°C for 1 minute each.

**PCR product size** was seen by electrophoresis on a 3% agarose gel of 3 µL of each of the 4 PCR product mixtures combined. Specificity of RT-PCR products was confirmed by Southern blot transfer onto Hybond-N⁺ nylon membrane (Amersham) and probing with oligonucleotide (Table) end-labeled with [γ⁻³²P]ATP (Amersham) by use of T4 polynucleotide kinase (New England Biolabs). Hybridization was performed in 6×SSC, 5×Denhardt’s, and 0.1% SDS at 42°C. After high-stringency washing of the product, autoradiography was performed at −80°C overnight.

Quantity of PCR product was assessed by dot-blot hybridization. Briefly, 5 µL of each PCR product mixture was denatured at 22°C.
for 30 minutes with 500 μL of 0.4 mol/L NaOH and 10 mmol/L EDTA to give 2-fold dilutions. Samples were then transferred onto Hybond-N+ membranes by use of a dot-blot apparatus (BioRad). DNA then was immobilized with GS Gene Linker (BioRad) and hybridized at 42°C for 4 hours with internal oligonucleotide probes (see above). After a high-stringency wash was completed, membranes were exposed to Kodak X-ray film at 22°C for 4 to 8 hours. Optical density of spots on autoradiograms was measured with a Molecular Dynamics personal densitometer (Qune Corp). For each tissue sample with each probe, 3 measurements of signal intensity at different dilutions were obtained.

Nos mRNA concentration was expressed relative to β-actin signal. Within-assay and between-assay variation was 5.9% and 8.4%, respectively. For each experiment, a set of RNA preparations for each tissue underwent RT-PCR through to quantification together, to avoid time-dependent degradation of RNA or cDNA and to reduce run-to-run differences in amplification efficiency.

In Situ Hybridization Histochemistry
Nos2 and Nos3 PCR products were subcloned into pGEM-Teasy (Promega). Antisense and sense riboprobes that incorporated digoxigenin-11-UTP (Boehringer Mannheim) were generated with T7 and SP6 polymerases, respectively. Tissue was collected into 10% neutral buffered formalin, and sections were applied to slides prepared under RNase-free conditions. After dewaxing and rehydration, sections were treated with proteinase K, fixed in 4% paraformaldehyde, and acetylated with 0.1 mol/L triethanolamine HCl and 0.25% acetic anhydride (pH 8.0). Prehybridization was performed in a 42°C humidified chamber with buffers supplied in the Boehringer in situ hybridization kit. Slides were drained, incubated overnight at 42°C in hybridization buffer with 10 to 20 ng/μL of riboprobe, and washed to remove unbound probe as described in the kit instructions. After being blocked, sections were incubated for 4 hours at 22°C with 1:500 antidigoxigenin antiserum conjugated with alkaline phosphatase. RNA-RNA hybrids were then detected colorimetrically with nitro blue tetrazolium. Slides were counterstained with hematoxylin and dehydrated. Coverslips were applied, and slides were examined and photographed with a light photomicroscope.

Immunohistochemistry
Longitudinal (coronal) slices of 3 to 4 mm of fixed right kidney were embedded, and 5-μm sections were mounted on silane-coated slides, deparaffinized in xylene, and rehydrated through graded alcohols. Next, antigen retrieval solution (Dako Corp) was applied and endogenous peroxide activity blocked with 3% H2O2 for 10 minutes. Nonspecific staining was blocked with 2% skim milk powder in 50 mmol/L Tris-buffered saline. Anti-mouse macrophage-derived NOS2 and anti-mouse NOS3 monoclonal antibodies (Transduction Laboratories) were applied, and slides were incubated overnight at 4°C in a humidified chamber, washed, and treated with goat anti-mouse IgG conjugated with biotin. The secondary antibody was detected with a peroxidase-conjugated streptavidin system. Slides were counterstained with hematoxylin and dehydrated. Coverslips were applied, and slides were examined and photographed with a light photomicroscope.

Figure 1. A, Detection of Nos2, Nos3, and β-actin mRNA in control and ACTH kidney tissue. Shown are RT-PCR products after electrophoresis on 3% agarose gel stained with ethidium bromide. Lanes 1 to 5 show control samples; lanes 6 to 10, ACTH samples; and M is a size marker (pUC19 cut with HpaII). Bands are 693 bp, Nos3; 395 bp, Nos2; and 240 bp, β-actin mRNA. B, Example of similar result for corticosterone (lanes 6 to 10) and control (lanes 1 to 5).

Figure 2. Nos isoform mRNA in kidney of ACTH and control rats. Top, Cortex; bottom, medulla. Results are expressed relative to mRNA for a constantly expressed gene (β-actin). Probability values are from t test of ACTH vs control. After Hochberg correction, all differences remained significant at the P<0.001 level, except Nos2 in medulla (P<0.01), nNOS (Nos1) indicates neural NOS; iNOS (Nos2), inducible NOS; and eNOS (Nos3), constitutive, Ca2+-dependent NOS.

Figure 3. Nos isoform mRNA in kidney of corticosterone and control rats. Top, Cortex; bottom, medulla. After Hochberg correction, P was <0.001 for Nos2 and Nos3 mRNA in cortex, and in medulla, P<0.05 for Nos1 mRNA and P<0.001 for Nos2 but probability was not significant for Nos3.
Laboratories), each at 1:200, were applied, and sections were incubated in a humidity chamber for 1 hour at 22°C. An LSAB 2 kit (Dako) was then used to detect immunoreactivity. Sections were incubated sequentially with biotinylated-link antibody, peroxidase-labeled streptavidin, and DAB-substrated chromogen and counterstained or not with hematoxylin. Positive staining gave a brown product. Negative control omitted antibody.

Statistical Analysis
A Microsoft Excel Statistical Analysis Package was used for $t$ tests. Hochberg correction was applied to adjust for multiple comparisons.13

Results

BP and Body Weight
Systolic BP of the 16 ACTH-treated rats was (mean±SE) 165±6 mm Hg and of the 16 control rats was 127±2 mm Hg ($P<0.001$). For the 10 corticosterone-treated (corticosterone) and 10 control rats, BPs were 162±11 and 111±6 mm Hg ($P<0.001$). ACTH reduced body weight (272±3 versus 214±4 g; $P<0.001$), as did corticosterone (261±11 versus 226±20 g; $P<0.001$). ACTH increased water intake (32±1 versus 71±4 mL/d; $P<0.001$), but corticosterone did not (31±1 versus 35±3 mL/d). Both increased urine volume: 6±1 versus 41±2 mL/d ($P<0.001$) and 5±1 versus 13±1 (P<0.01), respectively. Food intake was 28±1 g/d before ACTH treatment and >24±1 g/d during treatment. For corticosterone, food intake was 31±1 g/d before and >24±1 during treatment. Urinary Na$^+$ (mmol/d) was increased on treatment days 1 (control versus ACTH, 0.95±0.04 versus 1.55±0.15; $P<0.01$), 5 (0.95±0.04 versus 1.44±0.09, $P<0.01$), and 7 (0.95±0.04 versus 1.32±0.10; $P<0.01$), with no change in urinary K$^+$ (2.30±0.08 versus 2.16±0.30 mmol/d on day 7). Na$^+$ and K$^+$ were not measured in corticosterone rats. Serum corticosterone at euthanatization was 4.0±0.3 in ACTH versus 1.1±0.05 nmol/mL in control rats ($P<0.001$) and 1.5±0.06 in corticosterone versus 1.0±0.07 nmol/mL in control rats ($P<0.01$).

Confirmation of Identity of PCR Products
RT-PCR products of the expected sizes were found for each of the 3 Nos isoform mRNAs in all tissues examined. A typical ethidium bromide–stained gel of RT-PCR products is shown in Figure 1. Southern blotting detected a signal of expected size in each case (not shown).

Nos Isoform mRNA Concentrations in ACTH and Control Rats
In kidney, Nos2 and Nos3 mRNA were reduced by 58±5% and 59±6%, respectively, in cortex and 68±7% and 51±11% in medulla, but no change occurred in Nos1 mRNA (Figure 2). Decreases remained significant after correction for multiple comparisons ($P<0.01$). Nos2 mRNA in adrenal (3.08±0.49) was higher than kidney and was reduced by 62±2% with ACTH to 1.16±0.07 ($P=0.005$), which remained significant ($P<0.05$) after Hochberg correction. A 46% decrease in Nos1 mRNA was seen in hypothalamus (0.91±0.09 versus 1.68±0.23; $P=0.03$), but significance was lost after Hochberg correction. No other changes were seen.

Figure 4. In situ hybridization of Nos2 mRNA in rat kidney. Dark purple color is digoxigenin-labeled Nos2 antisense riboprobe after high-stringency washing; this was apparent in medullary distal convoluted tubule and outer medullary collecting duct in control rat (top) and ACTH rat (middle). No signal was seen in glomeruli, proximal convoluted tubule, vasculature, and papillary surface epithelium. Bottom, Sense probe control showing absence of labeling; this applied to all regions of kidney in both control and ACTH rats. Magnification ×400.
negative control gave no signal, which supported specificity. A similar pattern was seen with immunohistochemistry (Figure 5): in control rats, medulla showed strongest immunoreactivity, with brown cytoplasmic staining seen in late distal convoluted tubule and collecting duct. In ACTH rats, immunostaining was markedly weaker. In contrast, NOS2 immunostaining was not detected in renal arterial vasculature, glomerular capillaries, or proximal tubule of control or ACTH rats, consistent with an absence of basal expression in these tissues. No staining was seen in negative control in which preimmune serum was substituted for NOS2 antibody (result not shown). Results for corticosterone rats were similar (Figure 6). In the case of NOS3, signal seen in vascular structures of kidney was decreased by ACTH and corticosterone (Figure 7).

**Discussion**

We found that the rise in BP and metabolic changes in response to ACTH and corticosterone are accompanied by reductions in Nos2 and Nos3 isoform mRNAs and encoded proteins in kidney. A decrease in Nos1 mRNA was seen in renal medulla in response to corticosterone. Relative concentrations we saw were similar to those reported by others. Not only Nos3, but also Nos2 is expressed constitutively in the kidney, and the decrease in each of these mRNAs we saw by RT-PCR and by in situ hybridization was confirmed by immunohistochemistry of NOS2 and NOS3 proteins. Our findings thus indicate an action of ACTH and corticosterone to suppress Nos mRNAs. Moreover, the localization that we saw was similar to that which others have found in unstimulated rat kidney. NOS2 is found primarily in intercalated cells of the inner medullary collecting duct and distal convoluted tubule. Moreover, the inner medullary collecting duct is the site of highest NOS activity in the kidney and contains mRNA for each isoform. Although others have found evidence for low basal expression of NOS2 in the afferent arteriole and S3 segment of proximal convoluted tubule, this has not been observed consistently and was not seen by us. In the case of NOS3 protein and Nos3 mRNA, localization has
and NOS3 expression in kidney. Because the major ACTH-stimulated adrenal corticosteroid in man, cortisol, might also be involved in essential hypertension, decreases in NOS in the origin of the latter merits further investigation.

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