Increased Nitric Oxide Synthase-3 Expression in Kidneys of Deoxycorticosterone Acetate-Salt Hypertensive Rats

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Abstract. In addition to its hemodynamic effects, nitric oxide (NO) may play a role in the renal tubular handling of sodium. Experiments were conducted to determine possible changes in renal nitric oxide synthase-3 (NOS3) expression in rats treated with deoxycorticosterone acetate (DOCA) and high salt. All rats were uninephrectomized, and either a placebo or DOCA pellet was implanted subcutaneously. Placebo-treated rats were then given tap water to drink and DOCA-treated rats received a 0.9% NaCl solution to drink. Once a week, rats were placed in metabolic cages so that a 24-h urine sample could be collected. After 3 wk, the animals were sacrificed and the kidneys removed and prepared for subsequent immunohistochemical or Western blot analysis. Urinary excretion of nitrate and nitrite (NOx) was measured to provide an indication of intrarenal production of NO. DOCA-salt hypertensive rats exhibited increased urinary NOx excretion (2.43 ± 0.48 μmol NOx/mg creatinine) compared with the placebo control animals (1.17 ± 0.06 μmol NOx/mg creatinine). Western blot analysis revealed that NOS3 protein levels in both the cortex and medulla were greater in DOCA-salt rats compared with placebo-treated animals. Immunohistochemical analysis of kidneys revealed that NOS3 expression in placebo rats was localized in vascular endothelial cells with slight, but detectable, immunoreactivity in medullary collecting ducts. In DOCA-salt rats, a very large increase in the intensity of immunostaining was detected in tubular epithelia of the proximal tubule, thick ascending limb of Henle’s loop, and cortical and medullary collecting duct; immunoreactivity in endothelial cells appeared unchanged. These data suggest that increased tubular expression of NOS3 is responsible, at least in part, for the increased renal production of NO in DOCA-salt hypertension, and are consistent with a role for NO in the renal tubular response to salt loading.

The three major isoforms of nitric oxide synthase (NOS), NOS1, NOS2, and NOS3, are all constitutively expressed in the kidney (1–4). In general, renal NOS is considered to be responsible for production of nitric oxide (NO), which may play an important role in the regulation of renal hemodynamics including tubuloglomerular feedback and renin release (5–7). In addition, NO is thought to influence tubular sodium reabsorption through changes in renal medullary blood flow and tubular sodium reabsorption (8–10).

NOS1, sometimes referred to as brain NOS or neuronal NOS, is found in neurons throughout the central and peripheral nervous system, but has also been identified in other cells within the kidney. Using immunostaining techniques, Wilcox et al. first reported that the macula densa showed a very strong signal for NOS1 (11). Bachmann et al. confirmed this observation and went on to identify NOS1 expression in the efferent arteriole, macula densa, and other “distal tubular NOS-positive cells” (1). Terada et al. used reverse transcription-PCR methods on microdissected nephron segments to identify NOS1 mRNA expression throughout the entire nephron, including the proximal tubule, loop of Henle, and collecting ducts (12). NOS2 is often referred to as inducible NOS because its production can be induced in macrophages, smooth muscle, and other cells by cytokines; however, it appears to be constitutively expressed in renal tubules (13). NOS2 mRNA is expressed in proximal tubule, medullary thick ascending limb, and cortical, outer and inner medullary collecting ducts in normal rat kidneys (14,15). Using Western blots, Mattson and Higgins have reported significantly greater NOS2 expression in medulla compared with cortex (16). NOS3 is the isoform found primarily in vascular endothelial cells, thus referred to as endothelial NOS, but has also been identified within the renal epithelium. Bachmann et al. used immunohistochemical techniques to localize NOS3 protein principally to the endothelium of the pre- and postglomerular vessels and vasa recta (1). Ujije et al. used reverse transcription-PCR to detect NOS3 mRNA in glomeruli, preglomerular vasculature, proximal tubules, thick ascending limb, and collecting duct in the normal rat (4). Increases in salt intake have been shown to increase urinary NO excretion and NOS3 expression in the kidney (16,17). Therefore, it is possible that NO produced by NOS3 within the
renal tubules may be a mechanism for inhibiting sodium reabsorption during conditions of salt loading.

The vasodilator action of NO has led to numerous studies investigating the importance of NO in the development and maintenance of hypertension. Many of these studies have focused on the role of NO derived from the vascular endothelium, because of its well-characterized effects on vascular tone. However, the kidney plays an important role in the regulation of BP through regulation of extracellular fluid volume. Since NO has profound effects on renal hemodynamics and sodium reabsorption, we investigated whether the kidney’s ability to produce NO is increased in rats treated with deoxycorticosterone acetate (DOCA) and high salt, *i.e.*, the DOCA-salt hypertensive rat.

**Materials and Methods**

**Animal Experiments**

Experiments were performed using male Sprague Dawley rats (200 to 220 g body wt; Harlan Laboratories, Indianapolis, IN) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved and monitored by the Medical College of Georgia Committee for Animal Use in Research and Education. Rats were housed under conditions of constant temperature and humidity, and exposed to a 12-h light/dark cycle. A right flank incision was used to remove the right kidney under methohexital sodium (50 mg/kg; Brevital®, Eli Lilly, Indianapolis, IN) anesthesia. A time-release 200-mg pellet of DOCA was implanted subcutaneously. After recovery from anesthesia, rats were given saline (0.9% NaCl) to drink *ad libitum*. In control rats, a placebo pellet was implanted and tap water was given to drink. Once a week, rats were maintained in metabolic cages for a 24-h period so that urine could be collected.

After 3 wk, animals were anesthetized with Brevital and a carotid catheter was implanted and exteriorized at the back of the neck. One day later, arterial BP was recorded in conscious unrestrained rats using a MacLab 8e data acquisition system (ADInstruments, Milford, MA). Rats were then exsanguinated and the left kidney was removed, dissected into cortex and medulla, and immediately frozen in liquid nitrogen. Tissues were stored at −80°C until protein was extracted. Alternatively, the kidney was fixed in neutral-buffered formalin for 1 h and then paraffin-embedded in preparation for the immunohistochemical study.

Urine samples were diluted with distilled deionized H₂O and then assayed for total nitrate and nitrite concentration (NOₓ), using a previously reported modified Griess reaction utilizing nitrate reductase to reduce nitrate to nitrite (18,19). Sodium nitrate was used for the standard curve. Urine creatinine concentrations were measured by the picric acid colorimetric method. Briefly, saturated picric acid was diluted 10-fold with 1% NaOH. An aliquot of diluted urine was added to 200 μl of the picric acid/NaOH solution in a microtiter plate for a 1 h and then paraffin-embedded in preparation for the immunohistochemical study.

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Protein isolation and Western blot analysis

Samples of renal cortex or medulla were pulverized while still frozen and placed into TRIzol (Life Technologies, Grand Island, NY) at a ratio of 100 mg of tissue to 1 ml of reagent. Briefly, proteins were precipitated with isopropanol from the organic phase, and the pellet was washed three times with guanidinium HCl followed by a final wash with ethanol. The protein pellet was solubilized with 1% sodium dodecyl sulfate (SDS), and protein was quantified by a modified Lowry assay (BioRad DC), using bovine serum albumin as a standard.

For partial purification of NOX, samples of renal cortex or medulla were pulverized while still frozen and then placed into homogenization buffer (5 to 10 ml buffer/1 g tissue) containing 50 mM Tris–HCl, pH 7.4, 0.1 mM ethylenediaminetetra-acetic acid, 0.1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetra-acetic acid, 0.1% vol/vol β-mercaptoethanol, 10% vol/vol glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin A. The pulverized tissue was homogenized in a glass/Teflon Dounce homogenizer for 20 strokes. To enrich the protein purification for NOX proteins, the crude homogenates were solubilized and purified on 2′5′-ADP-Sepharose. The detergent, 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS), was added to a final concentration of 20 mM and gently rocked for 30 min at 4°C. The preparation was centrifuged at 10,000 × g at 4°C for 30 min. The solubilized protein fraction was further enriched by batch affinity chromatography on 2′5′-ADP-Sepharose and gently rocked for 30 min before it was transferred to a poly prep column. The column was washed with 4 to 5 column volumes of homogenization buffer containing CHAPS (20 mM) and NaCl (1 M) and further washed with 2 to 3 column volumes of homogenization buffer containing CHAPS (20 mM). NADPH-dependent proteins were then eluted from the column by homogenization buffer containing CHAPS (20 mM) and NADPH (10 mM). The eluate from these NADPH washes was collected and assayed for protein (Bradford method), using bovine serum albumin as a standard.

Equal amounts of protein from placebo and DOCA-salt-treated rats were loaded and run on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. The proteins were blotted onto nitrocellulose by wet blotting for 50 min in a Tris/glycine buffer. Blots were allowed to air dry for 15 min and then blocked for 1 h at room temperature in the presence of 5% nonfat dry milk in a Tris/glycine buffer. Blots were allowed to air dry for 15 min and then blocked for 1 h at room temperature in the presence of 5% nonfat dry milk in a Tris/glycine buffer. Blots were then incubated with the primary antibodies (H32 or Transduction Laboratories; 1:1000) diluted in 5% nonfat dry milk solution overnight at 4°C. The sections were washed twice for 10 min with 5% milk/TTBS before incubating with the horseradish peroxidase-linked secondary antibody diluted in 5% milk/TTBS. The blots were then washed in TTBS (1 × 15 min and 4 × 5 min) before being incubated with enhanced chemiluminescence (ECL) solution for 1 min and then exposed to autoradiographic film for detection of chemiluminescence.

**Immunohistochemistry**

Paraffin-embedded sections of 3 μm were cut and captured onto 3′-aminopropyltriethoxysilane-coated slides and dewaxed, and endogenous peroxidase activity was quenched by incubation in methanol with 0.3% hydrogen peroxide for 20 min at room temperature. The sections were washed in phosphate-buffered saline (PBS), pH 7.4, for 3 × 5 min, and incubated with normal goat serum for 30 min at room temperature. Primary anti-NOS3 antibodies (H32 or Transduction Laboratories) were diluted (1:1000) and allowed to incubate overnight at 4°C. The sections were washed (PBS, 3 × 5 min, at room temperature) and incubated with biotinylated horse anti-mouse for 30 min at room temperature, and then washed (PBS, 3 × 5 min) and incubated with a peroxidase-labeled avidin-biotin complex (ABC) for 1 h at room temperature (ABC Elite kit, Vector Laboratories, Burlingame, CA). The immunoreactivity was visualized using a solution of 3,3′-diaminobenzidine as chromogen with 0.2% hydrogen peroxide in PBS for 5 min to provide a brown end reaction product and then counterstained with Harris’ hematoxylin (×200), dehydrated, cleared, and?
mounted. Both primary antibody incubations and the subsequent processing for immunostaining were done in parallel with negative controls for optimum comparisons. Negative controls were treated in an identical manner except without adding primary antibody.

**Materials**

Acrylamide, Tris, ethylenediaminetetra-acetic acid, blotting grade blocker nonfat dry milk, and the dye reagent concentrate for the protein assay were obtained from BioRad Laboratories (Hercules, CA). Sodium dodecyl sulfate, β-mercaptoethanol, Tween 20, NaCl, and glycerol were obtained from Fisher Scientific (Pittsburgh, PA). CHAPS, ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetra-acetic acid, pepstatin A, phenylmethylsulfonyl fluoride, and picric acid were obtained from Sigma Chemical Co. (St. Louis, MO). NADPH was obtained from Boehringer Mannheim (Indianapolis, IN), and the 2′-5′-ADP-Sepharose, ECL reagents, and autoradiographic (hyperfilm ECL) film were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). NOS3 antibodies were obtained from BIOMOL Laboratories (Plymouth Meeting, PA) (H32) (20) and Transduction Laboratories. The peroxidase-labeled avidin-biotin complex (ABC) was obtained from Vector Laboratories (ABC Elite kit).

**Statistical Analyses**

Statistical comparisons of the urinary NOx data were done by a two-way ANOVA with a Fisher protected least significant difference post hoc test. Densitometric analysis of Western blots were compared by the unpaired nonparametric Mann–Whitney U test. Values are reported as mean ± SEM. *P*, 0.05 was considered significant.

**Results**

Three weeks after initiating DOCA-salt treatment, BP was significantly higher in DOCA-salt rats compared with placebo-treated controls (197 ± 6 mmHg versus 133 ± 3 mmHg).

![Figure 1](image1.png)

**Figure 1.** Total urinary excretion of nitrate and nitrite (NOx) from deoxycorticosterone acetate (DOCA)-salt-treated (n = 6) and placebo-treated rats (n = 7). Values represent the mean ± SEM. *P < 0.05 compared with the placebo rats.

![Figure 2](image2.png)

**Figure 2.** Western blot of unpurified protein (16 μg/lane) from the cortex (top panel) and medulla (bottom panel) of two placebo (P) and two DOCA-salt (D)-treated rats as probed by a nitric oxide synthase-3 (NOS3)-specific primary antibody (H32; 1:1000).

![Figure 3A](image3A.png)

**Figure 3A.** Western blot (A) of purified protein (4 μg/lane) from the cortex of three placebo (P) and three DOCA-salt (D)-treated rats as probed by a NOS3-specific primary antibody (Transduction Laboratories; 1:1000). EC indicates the standard prepared from human endothelial cell lysate. (B) The mean values from densitometric analysis of the Western blots. Data represent the mean ± SEM of three experiments. *P < 0.05 compared with the placebo controls.

![Figure 3B](image3B.png)

**Figure 3.** Western blot (A) of purified protein (4 μg/lane) from the cortex of three placebo (P) and three DOCA-salt (D)-treated rats as probed by a NOS3-specific primary antibody (Transduction Laboratories; 1:1000). EC indicates the standard prepared from human endothelial cell lysate. (B) The mean values from densitometric analysis of the Western blots. Data represent the mean ± SEM of three experiments. *P < 0.05 compared with the placebo controls.

Urinary excretion of NOx was significantly higher in DOCA-salt rats compared with placebo controls after 1 wk and remained elevated for the entire 3-wk period (Figure 1).

Western blots using protein isolated from renal cortex and medulla indicated a greater amount of NOS3 expression in DOCA-salt compared with placebo-treated rats (Figure 2). Using ADP-purified protein extracted from cortical tissue, Western blots indicated very little NOS3 expression in placebo rats but strong immunoreactive bands were observed in DOCA-salt rats (Figure 3A). In renal medulla, placebo rats possessed small amounts of ADP-purified NOS3 protein that were greatly increased in the DOCA-salt hypertensive animals (Figure 4A). Densitometric scans of the Western blots indi-
cated that NOS3 protein expression in DOCA-salt rats was significantly greater than placebo (Figures 3B and 4B).

Immunohistochemical staining was completed with two different anti-NOS3 antibodies that demonstrated similar results. Figures 5 through 7 show results from staining with H32, a monoclonal anti-NOS3 antibody. A large amount of NOS3 immunoreactivity was noted throughout the tubular structures of DOCA-salt-treated animals, whereas relatively little could be discerned in tubules of placebo-treated rats (Figures 5 and 6). Positive immunoreactivity was noted throughout the kidney in large epithelia and was particularly strong in proximal tubules and inner medullary collecting ducts. In addition, staining appeared to be present in thick ascending limb and cortical collecting duct (not shown). With our current methods, we were unable to discern a difference in the degree of NOS3 expression in vascular endothelial cells from DOCA-salt and placebo rats (Figure 7).

Discussion

These studies provide information about the renal expression and localization of NOS3 as well as NO production in DOCA-salt hypertensive rats. We show evidence that DOCA-salt-treated rats have an increased production of NO compared with placebo animals and that NOS3 expression is increased in the cortex and medulla of DOCA-salt hypertensive rats. Immuno-

Figure 4. Western blot (A) of purified protein (4 μg/lane) from the medulla of three placebo (P) and three DOCA-salt (D)-treated rats as probed by a NOS3-specific primary antibody (Transduction Laboratories; 1:1000). EC indicates the standard prepared from human endothelial cell lysate. (B) The mean values from densitometric analysis of the Western blots. Data represent the mean ± SEM of three experiments. *P < 0.05 compared with the placebo controls.

Figure 5. Immunohistochemical analysis of NOS3 (H32; 1:1000) in renal cortex from placebo (A) and DOCA-salt (C)-treated rats. Panels B and D show negative stains of kidneys from placebo and DOCA-salt rats, respectively. Peroxidase staining for NOS3 is indicated by the brown color. Glomerular staining was similar in both groups, whereas proximal and distal tubules are heavily stained in DOCA-salt but not placebo rats. Magnification, ×200.
histochemical analysis of kidneys from these animals determined that the increase in NOS3 is localized to the renal tubular system and not the vascular endothelium. These findings are consistent with a role for NO in the control of salt and water excretion in volume-expanded hypertension.

Urinary excretion of NOx was elevated in the DOCA-salt rats, which provided our initial evidence that NO production is increased in this model. The correlation between increased tubular NOS3 and NOx excretion is consistent with the possibility that NOx excretion is a reflection of intrarenal NO synthesis (6,21,22). However, our studies did not determine whether NO production is increased in other organ systems or tissues. An increase in NO synthesis in the DOCA-salt rat is consistent with the observation of King et al. that the NOS inhibitor N\(^{G}\)-monomethyl-L-arginine produces a significantly larger increase in mean arterial pressure in DOCA-salt hypertensive rats compared with placebo control rats (23). Contrary to this finding are other studies in which it was observed that endothelial-derived NO release stimulated by endothelin-1 or acetylcholine was decreased in the perfusate of isolated kidneys from DOCA-salt rats (24,25). Since these experiments were presumably examining endothelial-derived NO release, it may be possible that NO production is increased in renal tubules at a time in which endothelial production is decreased. Furthermore, it is important to consider that the amount of NO released in response to a specific agonist may not reflect the degree to which NO determines arterial pressure, and that changes in NO production are not necessary to alter the relative contribution of NO to basal vascular tone in an in vivo setting.

In the present study, we were unable to detect any major changes in endothelial expression of NOS3 levels in the kidney using immunohistochemical techniques which would suggest that sources other than the endothelium, i.e., tubular epithelium, may be responsible for the elevated urinary excretion of NO during DOCA-salt hypertension.

Using different immunostaining techniques, Bachmann et al. demonstrated NOS3 localization within endothelial cells of the interlobular artery, afferent and efferent arterioles, and NOS1 within the macula densa of the juxtaglomerular apparatus and in “distal tubular locations” (1). These investigators used the same monoclonal antibody as in the current study, H32, to localize NOS3. Our data in placebo control animals generally agree with this description for the NOS3 localization and further identify a significantly elevated amount of NOS3 within tubular structures of the kidney in the DOCA-salt hypertensive rats. It is not clear why we did not see any NOS3 signal in glomeruli, but we suspect that our methods do not have sufficient sensitivity for such a high degree of localization. Using either Western blot analysis or immunohistochemistry, we observed a relatively small degree of expression of
NOS3 in the cortex or medulla of placebo animals. Mattson and Higgins recently showed fairly strong NOS3 expression in the medulla of normal rats (16). Regardless of the potential differences in basal NOS expression, when rats were treated with DOCA-salt, medullary and cortical NOS3 expression was clearly increased.

Others have shown that increased dietary sodium can increase excretion of NOx in the urine and increase the expression of NOS isoforms in the inner medulla of normal rats (16,17). Furthermore, rats maintained on a high salt diet respond to NOS inhibition with a greater increase in mean arterial pressure compared with rats on a normal diet, again indicating increased NOS activity associated with salt loading (26). Similarly, DOCA-treated rats on a normal to low sodium diet have a diminished pressor response to the NOS inhibitor N\textsuperscript{G}-monomethyl-L-arginine than did DOCA-rats on a high salt diet (23). Thus, the increase in NOS levels in the proximal tubule and collecting ducts in DOCA-salt hypertension would seem to be due to the high salt intake of these rats. The localization would suggest that this may be an adaptive mechanism to increase sodium excretion, since it has been shown that NO can inhibit sodium transport in collecting ducts (10). It remains possible, however, that hypertension regardless of salt intake may also increase NOS in the kidneys. Spontaneously hypertensive rats with a normal salt intake have been observed to have a higher activity of Ca\textsuperscript{2+}-dependent NOS in their medulla compared with normal rats (27). In DOCA-salt animals, the higher tubular flow rate may also be a factor in elevating NOS3 expression due to the associated increases in shear stress within the renal tubules.

We hypothesize that in addition to its adaptive effect on sodium loading, increased renal NO production may also play a renal protective role in DOCA-salt hypertension. NO production has been shown to ameliorate tubulointerstitial fibrosis of obstructive nephropathy (13). Inhibition of NO production can also accelerate formation of glomerular lesions in stroke-prone spontaneously hypertensive rats on a high salt diet (28). Furthermore, reduction of renal NO production has been proposed to be linked to an increase in glomerular sclerosis associated with the aging process (29). In the DOCA-salt rat, a large amount of interstitial fibrosis occurs that may be attenuated by NO, although this hypothesis has yet to be experimentally tested.

In summary, we observed an increased production of NO by the kidney in DOCA-salt hypertensive rats. Renal tubular epithelial cells appear to drive this production of NO through increased expression of NOS3 protein. These changes in DOCA-salt hypertension suggest that tubular production of NO
may be important in the regulation of sodium excretion, and, specifically, the inhibition of tubular sodium reabsorption.

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