Effect of renal injury-induced neurogenic hypertension on NO synthase, caveolin-1, AKt, calmodulin and soluble guanylate cyclase expressions in the kidney

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Bai Y, Ye S, Mortazavi R, Campese V, Vaziri ND. Effect of renal injury-induced neurogenic hypertension on NO synthase, caveolin-1, AKt, calmodulin and soluble guanylate cyclase expressions in the kidney. *Am J Physiol Renal Physiol* 292: F974–F980, 2007. First published November 22, 2006; doi:10.1152/ajprenal.00157.2006.—Single injection of a small quantity of phenol into the cortex of one kidney in rats results in development of persistent hypertension (HTN) which is thought to be mediated by activation of renal afferent and efferent sympathetic pathways and sodium retention. Nitric oxide (NO) plays a major role in regulation of renal vascular resistance, tubular Na+ reabsorption, pressure natriuresis, and thereby systemic arterial pressure. The present study was performed to test the hypothesis that chronic renal injury-induced HTN may be associated with dysregulation of NO system in the kidney. Accordingly, urinary NO metabolite (NOx) and cGMP excretions as well as renal cortical tissue (right kidney) expressions of NO synthase (NOS) isoforms [endothelial, neuronal, and inducible NOS, respectively (eNOS, nNOS, and iNOS)], NOS-regulatory factors (Caveolin-1, phospho-AKt, and calmodulin), and second-messenger system (soluble guanylate cyclase [sGC] and phosphodiesterase-5 [PDE-5]) were determined in male Sprague-Dawley rats 4 wk after injection of phenol (50 μl of 10% phenol) or saline into the lower pole of left kidney. The phenol-injected group exhibited a significant elevation of arterial pressure, marked reductions of urinary NOx and cGMP excretions, downregulations of renal tissue nNOS, eNOS, Phospho-eNOS, iNOS, and alpha chain of sGC. However, renal tissue AKt, phospho-AKt, Calmodulin, and PDE-5 proteins were unchanged in the phenol-injected animals. In conclusion, renal injury in this model results in significant downregulations of NOS isoforms and sGC and consequent reductions of NO production and cGMP generation by the kidney, events that may contribute to maintenance of HTN in this model.

sympathetic activity; salt retention; cardiovascular disease; L-arginine/nitric oxide system; cGMP; phosphodiesterase-5

SINGLE INJECTION of a small quantity of phenol into the cortex of one kidney results in development of neurogenic hypertension (HTN) in genetically normal rats (3, 32, 33). The associated HTN persists long after complete healing of the initial injury and recession of the lesion to a microscopic scar. The intrarenal lesion in this model results in activation of renal afferent sympathetic pathway, which integrates with central noradrenergic control systems, resulting in activation of renal efferent sympathetic pathway. The latter, in turn, raises arterial pressure by augmenting renal vascular resistance and tubular sodium reabsorption and by modulating pressure natriuresis (31). The role of activation of renal afferent sympathetic pathway in the pathogenesis of HTN in this model is enforced by the observations that renal afferent impulses are enhanced (33) and HTN is prevented by renal denervation before phenol injection (32).

Nitric oxide (NO) is abundantly produced and all NO synthases are constitutively expressed by the kidney (7). The NO produced in the kidney plays a major role in regulations of renal vascular resistance, renal blood flow, renal sodium handling, and tubuloglomerular feedback system and hence, arterial blood pressure (4, 11, 12, 22, 24, 34). Many of the biological actions of NO are mediated by the second messenger, cGMP, which is produced from GTP by soluble guanylate cyclase (sGC) and rapidly degraded by cGMP-specific phosphodiesterases (PDE). The critical role of NO in regulation of blood pressure is evidenced by the observation that pharmacological inhibition of NO synthases results in HTN in otherwise normal animals (2, 18). Similarly, induction of oxidative stress and the consequent inactivation of NO by reactive oxygen species promote HTN in genetically normal, otherwise intact, animals (25, 36). Moreover, elevation of arterial pressure in various models of hereditary and acquired HTN is associated with and, at least in part, mediated by diminished NO bioavailability. For instance, HTN is associated with reduced production of NO and diminished abundance of some or all NO synthase (NOS) isoforms in the kidneys of animals with certain forms of HTN. These include Dahl salt-sensitive rats (15), salt-loaded Sprague-Dawley rats (16), rats with streptozotocin-induced diabetes (8), obese Zucker rats (9), rats with diet-induced obesity and metabolic syndrome (19), and those with chronic renal failure (20, 26, 29). However, in a number of other models, HTN is accompanied by the reduction of biologically active NO, despite normal or frequently increased NOS abundance, primarily as a result of oxidative stress and inactivation of NO by ROS. Examples of the latter models include adult spontaneously hypertensive rats (6, 17, 27), rats with lead-induced HTN (28), and rats with abdominal aorta coarctation (1).

To our knowledge, the effect of renal injury-induced neurogenic HTN on NO system in the kidney has not been previously investigated and was studied here. To this end, urinary excretion of stable NO metabolites (NOx) and cGMP as well as renal tissue expressions of the endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) NOS isoforms were determined in rats 4 wk after phenol injection. Sham-injected rats served as

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A two-step process. The first step is the conversion of nitrate to nitrite using a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA).

Western blot analyses. Protein abundance of phospho- and nonphospho eNOS, nNOS, iNOS, caveolin-1, phospho AKt, nonphospho AKT, calmodulin, and soluble guanylate cyclase (sGC) and PDE-5 were measured by Western blot analysis as described in our earlier studies (9). Antibody against sGC alpha and beta (the antibody recognized both isoforms) was purchased from Alexis Biochemicals (San Diego, CA). Anti-caveolin-1 antibody was purchased from Affinity Bioreagents, golden antibodies to PDE-5, phospho Akt and nonphosphorylated Akt, eNOS, iNOS, and nNOS were purchased from BD Biosciences (San Diego, CA). Actin monoclonal antibody was purchased from Sigma (St. Louis, MO). Phospho antibody was first used and then the nonphospho antibody was used to reprobe the blot. On each occasion, Western blots were run in triplicate and student’s t-test was used to verify the uniformity of protein load and transfer efficiency across the test samples. Experiments failing this test were discarded.

Measurements of urine total NO$_2$ plus NO$_3$ (NOx). We measured NO$_2$ and NO$_3$ (NOx), the stable metabolites of NO, in the urine using the Microplate Manager Bio-Rad Laboratories kit. This assay involves a two-step process. The first step is the conversion of nitrate to nitrite utilizing nitrate reductase. The second step is the addition of the Griess Reagents, which convert nitrite into a deep purple azo compound that can be measured by photometric method (Shimadzu, Tokyo, Japan). Known concentrations of NaNO$_2$ and NaNO$_3$ were used as standards in each assay.

Creatinine, sodium, and cGMP measurement. Urinary and plasma creatinine concentrations were measured using Nephrat kit and Creatinine Companion kit purchased from Eoxcell (Philadelphia, PA). Urine GMP was measured by an Enzyme Immunoassay Kit (Sigma). Urinary and plasma Na concentrations were determined by flame photometry (FLM-3, Radiometer, Copenhagen, DK). Fractional excretion of Na was calculated using the standard formula.

Data presentation and analysis. Data are presented as means ± SD; n = 6 rats in each group. *P < 0.05.

RESULTS

General data. Data are shown in Table 1. Body weight, serum creatinine, and creatinine clearance were unchanged while arterial pressure was significantly elevated in the phenol-injected rats compared with the placebo-injected animals. Fractional excretion of sodium in the renal injury group was slightly lower than that found in the control animals (0.68 ± 0.10 vs. 1.02 ± 0.10, P > 0.05). HTN was associated with significant reduction of urinary excretions of NO metabolites (NO$_2$ and cGMP (Fig. 1) which points to reduced NO production and biological activity in phenol-injected rats.

Table 1. Serum creatinine and arterial blood pressure in the phenol-injected and control groups

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<th>Control</th>
<th>Phenol</th>
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<tr>
<td>Blood pressure, mmHg</td>
<td>120±3.5</td>
<td>123±4.6</td>
</tr>
<tr>
<td>Baseline</td>
<td>121±2.9</td>
<td>156±1.68*</td>
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<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.58±0.04</td>
<td>0.52±0.08</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.42±0.04</td>
<td>0.45±0.04</td>
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Values are means ± SE; n = 6 rats in each group. *P < 0.05.
NOS isoform data. Data are illustrated in Figs. 2 and 3. The phenol-injected animals exhibited a marked reduction of eNOS and phospho-eNOS protein abundance in the kidney cortex when compared with the corresponding values obtained in the control group. Similarly, nNOS and iNOS protein expressions in the kidney cortex were significantly reduced in the phenol-injected rats.

Caveolin-1, AKt, and calmodulin data. Data are depicted in Figs. 4 and 5. Caveolin-1 protein abundance in the renal cortex was significantly reduced, whereas calmodulin abundance was unchanged in the phenol-injected compared with the placebo-injected control group. While total AKt abundance was mildly reduced, the phospho-AKt abundance was unchanged in the renal cortex of the phenol-injected animals.

Soluble guanylate cyclase and PDE-5 data. Data are depicted in Figs. 6 and 7. The phenol-injected animals showed a marked reduction of protein abundance of the α-chain of soluble guanylate cyclase in renal cortex. In contrast, protein abundance was not significantly affected.

Fig. 2. Representative Western blots and group data depicting endothelial nitric oxide synthase (eNOS) and phospho-eNOS protein abundance in the kidney cortex of the normal CTL and HTN rats; \( n = 6 \) in each group. \(* P < 0.05\).

Fig. 3. Representative Western blots and group data depicting inducible NOS (iNOS) and neuronal NOS (nNOS) protein abundance in the kidney cortex of the normal CTL and HTN rats; \( n = 6 \) in each group. \(* P < 0.05\).

Fig. 4. Representative Western blots and group data depicting caveolin-1 protein abundance in the renal cortex of the normal CTL and HTN rats; \( n = 6 \) in each group. \(* P < 0.05\).

Fig. 5. Representative Western blots and group data depicting calmodulin protein abundance in the renal cortex of the normal CTL and HTN rats; \( n = 6 \) in each group. \(* P < 0.05\).

Fig. 6. Representative Western blots and group data depicting soluble guanylate cyclase protein abundance in the renal cortex of the normal CTL and HTN rats; \( n = 6 \) in each group. \(* P < 0.05\).

Fig. 7. Representative Western blots and group data depicting PDE-5 protein abundance in the renal cortex of the normal CTL and HTN rats; \( n = 6 \) in each group. \(* P < 0.05\).
abundance of the enzyme was unchanged in the study animals. Similarly, PDE-5 protein abundance in the renal cortex was comparable in the two groups.

**DISCUSSION**

Animals studied 4 wk after injection of a small amount of phenol in the left kidney cortex exhibited a significantly higher arterial pressure than the placebo-injected animals, confirming the results of previous studies in this model (3, 32, 33). The observed HTN was accompanied by diminished fractional excretion of sodium denoting the presence of a salt-sensitive state. In a previous study we showed that intrarenal injection of phenol in rat raises arterial pressure, augments norepinephrin secretion from posterior hypothalamic nuclei, and increases renal sympathetic nerve activity (32, 33). We subsequently demonstrated that intrarenal injection of phenol results in activation of sympathetic nervous system and an immediate increase in renal tubular sodium reabsorption. The latter was associated with and mediated by redistribution of Na⁺ transporters from intermicrovillar cleft/intracellular membrane pools to apical membranes in proximal tubules (31). Together, these observations suggest that sodium retention may contribute to maintenance of HTN in phenol-induced renal injury.

Fig. 4. Representative Western blots and group data depicting AKT1 and phospho-AKT1 protein abundance in the kidney cortex of the normal CTL and HTN rats; n = 6 in each group. *P < 0.05.

Fig. 5. Representative Western blots and group data depicting caveolin-1 and calmodulin protein abundance in the kidney cortex of normal CTL and HTN rats; n = 6 in each group. *P < 0.05.
Investigation of NO system in the study animals revealed a significant reduction in urinary NOx and cGMP excretions and downregulation of NOS isoforms in the renal cortex. These observations point to reduction of NO production and bioavailability in this organ, a phenomenon which may contribute to the maintenance of HTN. NO plays a pivotal role in regulation of arterial pressure and renal and systemic hemodynamics (7). For instance, NO produced by different NOS isoforms in the kidney contributes to regulation of renal vascular resistance, renal blood flow, glomerular filtration rate, and renin secretion (12, 22, 24, 34). In addition, NO facilitates sodium excretion by promoting pressure natriuresis (regulation of tubuloglomerular feedback), inhibiting Na reabsorption, and modulating renal medullary blood flow (4, 11).

Three different isoforms of NO synthases have been thus far identified including nNOS, eNOS, and iNOS types. All three isoforms are constitutively expressed in the normal kidney (7). Elevation of arterial pressure in rats with chronic renal injury-induced HTN was accompanied by significant downregulations of all NOS isoforms in the renal cortex. This was associated with significant reduction of urinary excretion of NO metabolites. The latter finding points to diminished production of NO which is consistent with the observed downregulation of NOS isoforms in the study animals.

Enzymatic activation of NO synthases depends on binding of calmodulin to the inactive enzyme. Calmodulin binding process is Ca\(^{2+}\)/H11001 dependent for eNOS and nNOS and Ca\(^{2+}\)/H11001 independent for iNOS. In addition, eNOS activity is inhibited by its binding to caveolin-1 and markedly enhanced by its phosphorylation by phospho-AKt (13). Since the above proteins play a central role in regulation of NO production by NO synthases, we sought to examine the effect of renal injury-induced HTN on expression of these proteins in the kidney. The study revealed a significant reduction of caveolin-1 but no significant change in the abundance of either calmodulin or phospho-AKt in rats with renal injury-induced HTN compared with the corresponding values in the normal control animals. Thus the reduction in NO production in this model appears to be largely due to downregulation of NO synthases as opposed to dysregulation of proteins involved in modulation of NOS activities. Binding of eNOS to CAV-1 on plasma membrane inhibits the activity of this NOS isoform. However, CAV-1 has no effect on cytosolic NOS isoforms (iNOS, nNOS). The underlying mechanism responsible for and the potential impact of the reduction of CAV-1 in the kidneys of phenol-injected animals are uncertain and require further investigation.

![Fig. 6](http://ajprenal.physiology.org/) Representative Western blots and group data depicting protein abundance of the alpha and beta subunits of soluble guanylate cyclase (sGC) in the kidney cortex of the normal CTL and HTN rats; n = 6 in each group. *P < 0.01.

![Fig. 7](http://ajprenal.physiology.org/) Representative Western blots and group data depicting cGMP phosphodiesterase-5 (PDE5) protein abundance in the kidney cortex of the normal CTL and HTN rats; n = 6 in each group. *P < 0.05.
Urinary excretion of cGMP was markedly reduced in animals with renal injury-induced HTN. This abnormality could be due either to reduced production and/or increased degradation of cGMP. Many of the biological actions of NO are mediated by cGMP, which is generated from GTP following the activation of soluble guanylate cyclase by NO. Soluble guanylate cyclase is a heme-containing heterodimeric cytosolic enzyme composed of alpha and beta subunits which are both necessary for catalytic activity of the enzyme (5, 21, 35). Given the critical role of soluble guanylate cyclase in generation of cGMP and cGMP-mediated actions of NO, we sought to explore expression of this enzyme in the kidney of the study animals. The results revealed a marked downregulation of the alpha-chain of soluble guanylate cyclase in animals with renal injury-induced HTN. Thus downregulations of NO synthase isoforms in the kidney and the consequent reduction in NO production are compounded by the reduction of cGMP production capacity in rats with renal injury-induced HTN.

cGMP produced in various tissues is rapidly degraded by the cGMP-specific phosphodiesterases (PDEs) which exist as several different isoforms. PDE-5 is the dominant isoform of the enzyme in the kidney, brain, erectile tissues, and aorta (10, 14, 23, 30) and as such plays a key role in regulation of cGMP level in these organs. We therefore sought to determine the abundance of this enzyme in the kidneys of the study animals. The results showed no significant difference in renal tissue PDE-5 abundance between rats with renal injury-induced HTN and the normal control animals. Thus the reduction in cGMP production in the study animals does not appear to be due to increased PDE-5-mediated cGMP degradation.

Given the critical role of renal NO system in regulation of arterial pressure, the observed downregulation of NO isoforms and soluble guanylate cyclase in the kidney may contribute to HTN in this model. It should be noted that a significant rise in arterial pressure is observed immediately after injection of phenol in this model (3, 32, 33). Since alteration of the tissue NOS abundance is a time-dependent process, it remains to be determined if the observed cGMP reductions of urinary NOx and cGMP excretion obtained 24 h after phenol injection were unchanged in animals employed in the present study (data not shown). Consequently, downregulation of NO isoforms in the kidney must represent a secondary as opposed to a primary phenomenon and as such may contribute to the maintenance but not the onset of HTN in this model. The mechanism(s) responsible for downregulation of the renal NO system in this model and the role, if any, of renal sympathetic activation, in this process is uncertain and remains to be explored.

In conclusion, rats with chronic renal injury-induced HTN exhibited marked reductions of urinary NOx and cGMP excretion pointing to deficient NO production and biological activity. This was accompanied by and primarily due to downregulations of eNOS, nNOS, iNOS, and soluble guanylate cyclase in the kidney. Given the critical role of renal NO system in volume/pressure homeostasis, the observed abnormalities may, in part, contribute to the pathogenesis and maintenance of renal injury-induced HTN.

**REFERENCES**