Effect of renal medullary H$_2$O$_2$ on salt-induced hypertension and renal injury

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Taylor, Norman E., and Allen W. Cowley, Jr. Effect of renal medullary H$_2$O$_2$ on salt-induced hypertension and renal injury. *Am J Physiol Regul Integr Comp Physiol* 289: R1573–R1579, 2005. First published August 18, 2005; doi:10.1152/ajpregu.00525.2005.—Dahl salt-sensitive (SS) and consomic SS-13BN rats possess substantial differences in blood pressure salt-sensitivity even with highly similar genetic backgrounds. The present study examined whether increased oxidative stress, particularly H$_2$O$_2$, in the renal medulla of SS rats contributes to these differences. Blood pressure was measured using femoral arterial catheters in three groups of rats: 1) 12-wk-old SS and consomic SS-13BN rats fed a 0.4% NaCl diet, 2) SS rats fed a 4% NaCl diet and chronically infused with saline or catalase (6.9 μg·kg$^{-1}$·min$^{-1}$) directly into the renal medulla, and 3) SS-13BN fed high salt (4%) and infused with saline or H$_2$O$_2$ (347 nmol·kg$^{-1}$·min$^{-1}$) into the renal medullary interstitium. After chronic blood pressure measurements, renal medullary interstitial H$_2$O$_2$ concentration ([H$_2$O$_2$]) was collected by microdialysis and analyzed with Amplex red. Blood pressure and [H$_2$O$_2$] were both significantly higher in SS (126 ± 3 mmHg and 145 ± 17 nM, respectively) vs. SS-13BN rats (116 ± 2 mmHg and 56 ± 14 nM) fed a 0.4% diet. Renal interstitial catalase infusion significantly decreased [H$_2$O$_2$] (96 ± 41 vs. 297 ± 52 nM) and attenuated the hypertension (146 ± 2 mmHg catalase vs. 163 ± 4 mmHg saline) in SS rats after 5 days of high salt (4%). H$_2$O$_2$ infused into the renal medulla of consomic SS-13BN fed high salt (4%) for 7 days accentuated the salt sensitivity ([H$_2$O$_2$] 17 nM, 94 vs. 41 nM saline). The data show that medullary production of H$_2$O$_2$ may contribute to salt-induced hypertension in SS rats and that chronic renal medullary oxidant stress. SS-13BN rats; salt sensitivity

There is growing evidence of increased arterial vascular oxidative stress in both genetic (32, 33) and induced forms of hypertension (2, 8, 37), suggesting that reactive oxygen species (ROS) may contribute to the pathogenesis of this disease. Although most of these studies have focused on the ability of superoxide (O$_2^-$) to scavenge and inactivate nitric oxide (NO), recent evidence suggests that ROS other than O$_2^-$ produce hypertension by mechanisms independent of NO. The present study examines the role of the ROS hydrogen peroxide (H$_2$O$_2$) in the development of salt-sensitive (SS) hypertension.

Evidence that H$_2$O$_2$ could participate in hypertension was obtained in transgenic mice that overexpressed the human catalase gene (38). These mice showed significantly blunted arterial pressure and aortic H$_2$O$_2$ compared with wild-type mice in response to chronic infusions of norepinephrine or angiotensin II. In addition to catalase, glutathione peroxidase plays a key role in scavenging H$_2$O$_2$ in vivo. When the substrate of this enzyme, glutathione, was depleted in Sprague-Dawley (SD) rats, mean arterial pressure (MAP) increased significantly (9). Conversely, n-acetyl-l-cysteine, a well-known antioxidant that can increase glutathione stores, prevented the salt-induced hypertension of Dahl S rats when it was supplemented in the chow (40).

Studies in our laboratory in SD rats have found that local excess production of H$_2$O$_2$ within the medulla of the kidney would produce hypertension. In one of these studies, chronic renal medullary infusion of the O$_2^-$ dismutase (SOD) inhibitor, diethyldithiocarbamic acid, into a single remaining kidney produced hypertension (21). Co-administration of the SOD mimetic, tempol, failed to prevent this hypertension and only with the addition of catalase was hypertension attenuated (22). Because tempol was shown to inhibit the diethyldithiocarbamic acid-induced elevations of medullary O$_2^-$, as determined by microdialysis, the anti-hypertensive actions of catalase indicated a role for H$_2$O$_2$ in maintaining hypertension in this model. Insight into the mechanisms of these H$_2$O$_2$ responses was provided in a second study when acute interstitial infusion of H$_2$O$_2$ reduced medullary blood flow and sodium excretion, and these dose-dependent responses were reversed by catalase treatment (3). A third study then showed that chronic renal medullary interstitial (ri) infusion of H$_2$O$_2$ (1.0–1.4 μmol·kg$^{-1}$·min$^{-1}$) produced chronic hypertension in SD rats. Microdialysis studies conducted after day 5 of infusion found that renal interstitial H$_2$O$_2$ was nearly five times higher than saline-infused rats (22).

Although these studies demonstrated that elevations of H$_2$O$_2$ in the renal medulla could induce hypertension, the significance of endogenously produced H$_2$O$_2$ in this region of the kidney that may occur in genetic forms of hypertension remains to be established. The present study examined the role H$_2$O$_2$ produced within the renal medulla of SS rats might play in the development of salt-induced hypertension and related renal end-organ damage. SS rats were compared with an inbred consomic control strain, in which chromosome 13 of the Brown Norway rat was introgressed onto the genetic background of the SS rat (SS-13BN). This strain exhibits only a 30% for the Dahl salt-resistant rat (R), yet exhibits more than...
a 50% reduction of salt-induced hypertension, proteinuria, and glomerular disease (4).

The aims of the present study were twofold: 1) to determine whether SS rats exhibit greater levels of oxidative stress, as reflected by H$_2$O$_2$ in the renal medulla, relative to consomic SS-13$^{BN}$ rats; and 2) to determine the role that H$_2$O$_2$ within the renal medulla plays in salt-induced hypertension and renal injury in SS rats. Catalase was therefore infused chronically into the ri of SS rats fed a high (4%) NaCl diet to examine its effects on MAP, medullary H$_2$O$_2$, and kidney damage. Conversely, H$_2$O$_2$ was infused ri into SS-13$^{BN}$ rats fed a 4% NaCl diet to determine whether the trait of SS hypertension could be exacerbated in this strain. Microdialysis was used to collect samples of renal medullary interstitial H$_2$O$_2$ in each of these studies.

METHODS

Animal preparation. Experiments were performed on inbred lines of male Dahl SS/J and SS-13BN rats maintained as inbred colonies at the MCW (4). The MCW Institutional Animal Care and Use Committee approved all experimental protocols. Rats were maintained ad libitum on tap water, and a purified AIN-76A rodent diet containing 0.4% or 4% NaCl (Dyets, Bethlehem, PA).

Male SS and SS-13BN rats fed a 0.4% NaCl diet were uninephrectomized at 9 wk of age. After a 7- to 10-day recovery period, medullary interstitial and femoral arterial catheters were implanted as previously described (24). Five to six days later, daily 3-h measurements of MAP were begun using an online data collection and analysis system (5).

After the last day of blood pressure recording, acute surgical preparation for in vivo microdialysis of the left kidney was performed as described previously (21). After a 2-h equilibration period, dialysate effluent was collected over two 30-min intervals and renal medullary interstitial H$_2$O$_2$ concentrations ([H$_2$O$_2$]) were determined by fluorescence spectrometry using the Amplex Red H$_2$O$_2$ Assay Kit (Molecular Probes, Eugene, OR).

Group 1. Eleven-week-old SS and SS-13BN rats were prepared as in Group 2, except that H$_2$O$_2$ (347 nmol·kg$^{-1}$·min$^{-1}$) was infused into the interstitium instead of catalase, and the experiment was extended to 7 days of 4.0% NaCl. A previous study demonstrated that ri infusions of three to four times this dose of H$_2$O$_2$ produced hypertension in SD rats (22). As we were trying to determine the role of H$_2$O$_2$ in salt-induced hypertension, a dose was selected that elevated blood pressure only after the change to a 4% NaCl diet.

Histology preparation and measurements. Rats were euthanized, and the left kidney was removed, immersion-fixed in 10% neutral buffered formalin, and paraffin embedded. Sections were prepared and stained with Gomori trichrome stain to highlight the fibrotic tissue and hematoxylin and eosin to quantify cast formation as an index of tubular dysfunction. Interstitial fibrosis was determined by immunostaining with antibodies for α-smooth muscle actin (SMA) (Dako Cytomation), and detected using an Envision/horseradish peroxidase detection kit (DAKO Cytomation). Cast and SMA staining were quantified in 20 random frames using the Metamorph analysis software, as previously described (29), and reported as the mean ± positive area per frame for cast or SMA staining.

Statistical methods. Data are presented as means ± SE. In cases where each animal served as its own control in the pre- and posttreatment periods, the data were analyzed using a one-way ANOVA for repeated measures followed by a Tukey’s multiple range test. A P value of <0.05 was considered significant. Between-group comparisons were performed using a two-way ANOVA followed by a Tukey’s multiple range test to compare individual points. A Pearson correlation was performed using the data from individual rats in all three experimental groups to assess whether MAP was correlated to medullary H$_2$O$_2$ levels and tubular cast formation.

RESULTS

Group 1: renal medullary interstitial H$_2$O$_2$ in rats fed a 0.4% NaCl diet. SS rats exhibited higher MAP (126 ± 3 mmHg, n = 7) than SS-13$^{BN}$ rats (116 ± 2 mmHg, n = 7), even when these rats were fed a 0.4% NaCl diet from weaning, as shown in Fig. 1. Renal medullary interstitial H$_2$O$_2$ was also found to be significantly higher in SS compared with SS-13$^{BN}$ rats (145 ± 17 nM, n = 7 vs. 56 ± 14 nM, n = 7).

[Graphs showing mean arterial pressure (MAP; top) and renal medullary H$_2$O$_2$ concentrations (bottom) in 12-wk-old salt-sensitive (SS) and SS-13$^{BN}$ rats maintained on a 0.4% NaCl diet. Values are means ± SE. *P < 0.05 vs. control.]
Group 2: effect of renal medullary interstitial infusion of catalase on salt-induced hypertension and medullary H$_2$O$_2$ concentration in SS rats. Catalase was infused chronically into the renal medulla of SS rats to determine the contribution of medullary H$_2$O$_2$ production to salt-induced hypertension. As summarized in Fig. 2, MAP of the saline-infused control group increased from 129 ± 2 to 163 ± 4 mmHg after 5 days of high salt (4%), whereas MAP in SS rats infused with catalase increased from 122 ± 1 to only 146 ± 2 mmHg after 5 days of high salt. At the end of 5 days, renal medullary [H$_2$O$_2$] in saline-infused rats averaged 297 ± 52 nM (n = 7) and was significantly reduced (96 ± 41 nM, n = 7) in catalase-infused rats. These data show that medullary production of H$_2$O$_2$ contributes importantly to the salt-induced hypertension in the SS rat.

Group 3: effect of renal medullary interstitial infusion of H$_2$O$_2$ on salt-induced hypertension and medullary [H$_2$O$_2$] in SS-13BN rats. To further evaluate the importance of H$_2$O$_2$ in salt-induced hypertension, renal medullary [H$_2$O$_2$] was increased in relatively salt-insensitive SS-13BN rats to determine whether the SS trait of salt sensitivity could be restored in this consomic strain (Fig. 3). H$_2$O$_2$ was infused at a dose that did not change MAP over 3 days on a 0.4% salt diet (347 nmol·kg$^{-1}$·min$^{-1}$). When the salt in the diet was increased from 0.4 to 4%, MAP increased 25 mmHg (from 120 ± 2 to 145 ± 2 mmHg). In saline vehicle-infused SS-13BN rats, MAP increased only 16 mmHg (118 ± 2 to 134 ± 1 mmHg) when fed the high-salt diet. As determined by microdialysis at the end of the experiment, saline-infused SS-13BN rats exhibited H$_2$O$_2$ concentrations of 44 ± 9 nM (n = 6) compared with 83 ± 11 nM for H$_2$O$_2$-infused rats (n = 6). Renal medullary [H$_2$O$_2$] levels were highly correlated with MAP in the three experimental groups (r = 0.602, P < 0.001). Of particular note, SS rats infused with catalase exhibited the same level of hypertension as the H$_2$O$_2$-infused SS-13BN rats (146 ± 2 vs. 145 ± 2 mmHg) and also had similar levels of renal medullary [H$_2$O$_2$] (96 ± 41 vs. 83 ± 11 nM). Blood pressure salt sensitivity was exacerbated in the consomic SS-13BN simply by elevating medullary H$_2$O$_2$, indicating that excess H$_2$O$_2$ production in this region of the kidney contributes to salt-induced hypertension in SS rats.

Histological quantification of tubular necrosis and interstitial fibrosis. The percentage of the outer medulla exhibiting protein casts was used as an index of tubular necrosis. As shown in Fig. 4, cast formation in SS rats receiving catalase infusions (7.5 ± 1.0%, n = 6) was significantly less than saline-infused controls (16.0 ± 2.0%, n = 6). Despite considerable reduction of tubular damage, catalase-infused SS rats continued to exhibit significantly more tubular casts than the
saline-infused SS-13<sup>BN</sup> rats. There was no significant difference in cast staining between saline and H<sub>2</sub>O<sub>2</sub>-infused SS-13<sup>BN</sup> rats (4.7 ± 0.6%, n = 6 vs. 6.0 ± 1.3%, n = 6). Tubular necrosis was significantly correlated to MAP (r = 0.83, P < 0.05).

The percentage of tissue that stained positive for α-SMA in the outer medulla was used as an early index of interstitial fibrosis. SS rats receiving the medullary infusion of catalase exhibited significantly reduced SMA staining (0.6 ± 0.2%, n = 6) compared with saline-infused SS rats (2.3 ± 0.6%, n = 6), as shown in Fig. 5. Conversely, SS-13<sup>BN</sup> rats that were infused with H<sub>2</sub>O<sub>2</sub> and fed a 4% NaCl diet exhibited an elevated α-SMA staining compared with saline-infused controls (2.8 ± 0.7%, n = 6 vs. 0.7 ± 0.2%, n = 6). Taken together, these observations indicate that elevations of H<sub>2</sub>O<sub>2</sub> contribute to the development of medullary interstitial fibrosis.

**DISCUSSION**

The present study examined whether H<sub>2</sub>O<sub>2</sub> levels are elevated in SS rats and tested the hypothesis that increased renal medullary H<sub>2</sub>O<sub>2</sub> in this strain contributes to salt-induced hypertension and renal injury. The results indicate that H<sub>2</sub>O<sub>2</sub> is elevated in the renal interstitium of SS rats compared with the consomic SS-13<sup>BN</sup> control strain and that arterial pressures were significantly elevated in the SS, even before the introduction of a high-salt diet. Renal interstitial infusion of catalase in the SS significantly reduced H<sub>2</sub>O<sub>2</sub> levels and greatly attenuated the salt-induced increase in arterial pressure. Conversely, increasing renal medullary H<sub>2</sub>O<sub>2</sub> in SS-13<sup>BN</sup> rats increased the salt sensitivity. This suggests that the gene(s) protecting the SS-13<sup>BN</sup> from increased oxidative stress may also be responsible for the protection from salt-induced hypertension.
Reduction of medullary H$_2$O$_2$ in SS rats also significantly reduced renal injury as measured by a reduction in protein casts and α-SMA staining in the outer medulla. Tubulointerstitial fibrosis and capillary injury occur first in the outer medulla and juxtamedullary glomeruli in many forms of experimental hypertension, including Dahl S rats (16), and is considered a final common pathway of progressive kidney disease. Myofibroblasts, which contain α-SMA (20), are rarely found in the normal kidney, and when they become detectable in the interstitium, collagen deposition accumulates in the areas in which they reside (1), making α-SMA staining an important early index of interstitial fibrosis. α-SMA staining was significantly increased in SS-13BN rats infused with H$_2$O$_2$, suggesting that this ROS could play a significant role in initiating interstitial fibrosis in the kidney. These data support a role for H$_2$O$_2$ in the maintenance of salt-induced arterial pressure and renal injury in the SS rat.

Consomic SS-13$^{BN}$ rats as a control for SS rats. The selection of appropriate controls for the Dahl S and other hypertensive strains has been constrained by the absence of available methods to carry out extensive whole genome scans. Control strains for hypertension have been generally selected on the basis of the ancestral origins of the strains, even though all of the traditional “control” strains (Wistar-Kyoto, Dahl R, etc.) are now known to exhibit considerable genetic divergence from the hypertensive strains. This results in hundreds of phenotypic differences between the control and hypertensive strains, most of which are unrelated to the pathogenesis of hypertension. In support of this argument, there is a 77% allelic difference between Brown-Norway/MCW and SS/MCW rats.
a 48% difference between spontaneously hypertensive and Wistar-Kyoto rats, a 52% difference between SD and SS/MCW rats, and a 30% difference between Dahl R and SS/MCW rats as assessed by whole genome scans using 1,541 microsatellite markers (13). In contrast, the consomic SS-13BN rat exhibits only a 1.95% allelic difference from SS/MCW rats over the entire genome, and yet salt-induced hypertension, proteinuria, and glomerular disease is greatly reduced (4). Based on our genetic analysis, we believe that the SS-13BN rat is currently the best available control strain for phenotypic comparison with the SS/MCW rats because it possesses less genetic difference than any other available strain.

Salt sensitivity and renal medullary oxidative stress. SS rats have been shown to be deficient in NO (34) and 20-HETE (15), while also exhibiting increased sympathetic tone (14) and oxidative stress (25, 26, 33), all of which contribute to the salt sensitivity of this strain. This study provides direct evidence of increased oxidative stress within the renal medulla of the SS rat and supports indirect measurements made by others. Studies where tempol attenuated salt-induced hypertension in SS rats (12, 25) support a role for O2- in maintaining elevated pressure in this strain. However, just as in our diethyldithiocarbamic acid model of hypertension, administration of tempol was not fully protective. Indeed, Williams et al. (36) observed that tempol administered in the drinking water of SD rats was unable to sustain the inhibition of hypertension produced by ETB receptor blockade plus a high-salt diet and that this was accompanied by increased urinary H2O2 excretion. The purpose of this study was to determine whether H2O2 within the renal medulla of SS rats plays a role in salt-induced hypertension and renal injury separate from the direct effects of other ROS.

Increased H2O2 concentrations in the renal medulla of SS rats may be due to increased production or decreased scavenging of this ROS. H2O2 is a relatively stable species that diffuses freely into cells and is thought to be generated predominately from the SOD dismutation of O2-, although it can also be produced directly by certain oxidases, such as glucose oxidase, through a two-electron reduction of O2 (31). Evidence suggests that NADPH oxidase is an important source of ROS in hypertensive hypertension produced by ETB receptor blockade plus a high-salt diet and that this was accompanied by increased urinary H2O2 excretion. The purpose of this study was to determine whether H2O2 within the renal medulla of SS rats plays a role in salt-induced hypertension and renal injury separate from the direct effects of other ROS.

H2O2 has been shown to be both a vasodilator and a vasoconstrictor. It has been implicated as an endothelial-derived dilating factor in both animal and human studies based on observations that catalase inhibited dilation of mesenteric arteries from endothelial NO synthase knockout mice (23) and in human coronary arterial microvessels (27). This response has been attributed to membrane hyperpolarization through the activation of calcium-dependent K+ channels (27). In contrast, studies in several different vascular beds have reported that H2O2 acts as a vasoconstrictor (28, 30, 39). Aortic contraction was found to be Ca2+- dependent with evidence that hydroxyl radicals, cyclooxygenase products, protein kinase C, and products of protein tyrosine phosphorylation may play some role in these responses (39).

Recent studies in mesenteric arteries (10) and rat gracilus arterioles (6) provide some insight into these discrepancies by showing that responses to H2O2 are dose dependent in these vessels. Vasoconstriction occurred at lower doses (10–100 μM in mesenteric arteries) via a PGH2/TxA2 receptor-dependent mechanism, whereas vasodilation predominated at concentrations above 0.3 mM via a K+ channel-mediated hyperpolarization (10). The present study shows that interstitial [H2O2] ranges between 50 and 300 nM. Even after accounting for the 20–30% probe efficiency, levels are still well within the dose range that purportedly constricts vessels in other tissues. Indeed, acute studies in our laboratory have shown that i) H2O2 infusion in SD rats at a dose that increased H2O2 in medullarydialysates from 116 to 211 nM decreased medullary blood flow, urinary sodium excretion, and urine volume, suggesting that a physiological increase of H2O2 produces vasoconstriction in this region of the kidney (3).

We conclude that elevation of H2O2 in the renal medulla of SS rats plays an important role in salt-induced hypertension and renal injury, and therapies that reduce both O2- and H2O2 should prove to be more effective in the long-term treatment of hypertension.

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