Salt Intake, Oxidative Stress, and Renal Expression of NADPH Oxidase and Superoxide Dismutase

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Abstract. The hypothesis that a high salt (HS) intake increases oxidative stress was investigated and was related to renal cortical expression of NAD(P)H oxidase and superoxide dismutase (SOD). 8-Isoprostane PGF2α and malondialdehyde were measured in groups (n = 6 to 8) of conscious rats during low-salt, normal-salt, or HS diets. NADPH- and NADH-stimulated superoxide anion (O2·−) generation was assessed by chemiluminescence, and expression of NAD(P)H oxidase and SOD were assessed with real-time PCR. Excretion of 8-isoprostane and malondialdehyde increased incrementally two- to threefold with salt intake (P < 0.001), whereas prostaglandin E2 was unchanged. Renal cortical NADH- and NADPH-stimulable O2·− generation increased (P < 0.05) 30 to 40% with salt intake. Compared with low-salt diet, HS significantly (P < 0.005) increased renal cortical mRNA expression of gp91phox and p47phox and decreased expression of intracellular CuZn (IC)-SOD and mitochondrial (Mn)-SOD. Despite suppression of the renin-angiotensin system, salt loading enhances oxidative stress. This is accompanied by increased renal cortical NADH and NADPH oxidase activity and increased expression of gp91phox and p47phox and decreased IC- and Mn-SOD. Thus, salt intake enhances generation of O2·− accompanied by enhanced renal expression and activity of NAD(P)H oxidase with diminished renal expression of IC- and Mn-SOD.

Reactive oxygen species (ROS) have been implicated in growth factor signaling, mitogenic responses, apoptosis, and oxygen sensing (1). Oxidative stress is determined by the balance between the generation of ROS such as superoxide anion (O2·−) and the antioxidant defense systems such as superoxide dismutase (SOD). Oxidative stress has been implicated in many forms of angiotensin II (Ang II)-related hypertension (1–3). However, there is also substantial oxidative stress in the tissues or blood vessels of rats with desoxycorticosterone acetate–salt hypertension in which the circulating renin-angiotensin-aldosterone system is suppressed profoundly (4,5) and in models of salt-sensitive hypertension (6,7) and in angiotensin II (Ang II) (12,16) and is implicated in the pathogenesis of several models of hypertension (2,14). Phagocytic NAD(P)H oxidase (2) is composed of a glycosylated flavoprotein, gp91phox, associated with p22phox, and three cytosolic components, p40phox, p47phox, and p67phox, and a small guanosine triphosphate (GTP)-binding protein rac1. The catalytic core of NAD(P)H oxidase in phagocytes is the membrane-integrated flavocytochrome b558, comprising p22phox and gp91phox, which transfers electrons from NAD(P)H to O2 (11). An NAD(P)H oxidase has been characterized in vascular smooth muscle cells (VSMC) (2), vascular endothelium, and kidney (14), although there are some differences in component parts. Homologues of gp91phox include Mox-1/NOH-1 (now referred to as Nox-1), which is expressed in the colon and VSMC (17), and RENOX (now referred to as Nox-4), which is expressed in the kidney and VSMC. We detected the protein and/or mRNA for all of the phagocyte-type Nox isoforms in the normal rat kidney (14). We tested the hypothesis that salt loading, per se, induces oxidative stress in the kidneys of normal rats and related this to the activity and expression of NAD(P)H oxidase and to expression of SOD in the renal cortex.

Materials and Methods

Animal Preparation

Studies were approved by the Georgetown University Animal Care and Use Committee. They were performed according to the Guide for...
the Care and Use of Laboratory Animals (NIH publication No. 93-23, revised 1985) and the Guidelines of the Animal Welfare Act.

Experiments were performed on male Sprague-Dawley rats that weighed 210 to 300 g. Rats (n = 7 to 10 per group) were maintained on a standard rat diet (Na+ content, 0.3 g 100 g) for 8 to 10 d before being assigned randomly to synthetic, casein-based diets with precisely regulated NaCl contents (Teckland Inc., Madison, WI).

Rats were housed in individual cages under conditions of constant temperature and humidity. They were exposed to 12-h cycles of light and dark. They had unrestricted water intake. They were assigned to diets with a low-salt (LS), normal-salt (NS), or high-salt (HS) content for 7 d. Diets were identical apart from salt, for which sucrose was substituted. They contained (g/kg) 200 casein (high protein), 661 sucrose, 20 nonnutritive fiber (cellulose), 70 corn oil, 40 mineral mix, and 9.08 vitamin mix. For the HS diet, 6 g/kg NaCl was added and sucrose was decreased to 655 g/kg. For the NS and LS diets, NaCl was added, with corresponding decreases in sucrose contents. The Na+ content (g 100 g) of the diets were as follows: HS, 6.0; NS, 0.3; LS, 0.03.

On the last day of study, rats were placed in individual metabolism cages. A 24-h urine was collected into containers with streptomycin (2000 IU), penicillin G (2000 IU), and amphotericin B (5 μg) to prevent microbial overgrowth. The urine was centrifuged, separated from the sediments, and stored at −70°C until analyzed for volume, 8-isoprostane prostaglandin F2α (8-Iso), and malondialdehyde (MDA). Urine from LS, NS, and HS rats (n = 8 per group) was analyzed for PGE2 in a separate series of rats. At completion of the urine collections, groups of LS and HS rats were anesthetized, and the kidneys were flushed with ice-cold PBS and removed immediately. The cortex of each kidney was separated for extraction of mRNA and stored at −70°C. An additional set (n = 6 per group) of LS and HS rats were prepared for chemiluminescence measurements.

Chemiluminescence Measurements

Animals were anesthetized, and the right external jugular vein was cannulated. The abdomen was opened through a midline incision. Ice-cold PBS was infused via the catheter after the left renal vein was transected. The perfusion was continued until the fluid draining from the open renal vein became clear (usually after 40 to 50 ml of PBS in 15 to 20 min). The left kidney was removed and cleaned of connective tissues, and the renal cortex was separated on ice and immediately frozen using dry ice.

For measurements of oxidative activity, samples were transferred into test tubes containing PBS. They were homogenized on ice at a low speed and centrifuged at 3,000 rpm for 20 min. The supernatant was used for further analysis. Lucigenin (final concentration 5 μM)-enhanced chemiluminescence was used to determine the superoxide anion (O2·−) generation. The increase in chemiluminescence with specific substrate addition was used to assess the oxidant enzyme activities. The oxidative reactions were started by addition of 200 μM (final concentration) of xanthine, NADH, or NADPH. The background-adjusted chemiluminescence signal was sampled every second for 15 min and recorded with a 2-s integration using a tube luminometer with automated injector (AutoLumat Plus LB 953; EG&G Berthold, Germany). The average peak intensity units were converted to pmol O2·−/mg protein per min by comparison with cytochrome c reduction (18). The specificity for NADPH oxidase was tested using diphenyleneiodonium (DPI; 10−5 M). The final values were related to the sample (whole homogenate solution) protein concentration (Bio-Rad Protein Assay, Hercules, CA).

mRNA Isolation and Real-Time Quantitative RT-PCR

RNA isolation and RT were performed using oligonucleotide methods described previously (14,19). Briefly, total RNA was isolated from the kidney cortex with guanidinium isothiocyanate (Qiagen, Valencia, CA) and treated with DNase. RT reactions were performed using the SuperScript Preamplification System for the first-strand cDNA synthesis (Life Technologies BRL, Rockville, MD). Real-time quantitative PCR was done using an ABI Prism 7700 Sequence detection system. Primers and probes for the NADPH oxidase subunits p22phox, gp91phox, p47phox, p67phox, Nox-4, Nox-1, IC-SOD, Mn-SOD, and EC-SOD were designed using Primer Express software 101 (19) (Table 1). All primers and probes are designed using the gene-specific sequences deposited in the gene bank. The exception was p67phox, which was designed using the previously sequenced rat partial sequence (14). It was compared with mouse counterpart, and homologous sequence was used as primers and a probe. The probes were labeled with 6-carboxy-fluorescein phosphoramidite as a reporter at the 5’ end and with 6-carboxy-tetramethylrhodamine as a quencher at the 3’ end. ROX was used as a passive reference in each sample to normalize for non-PCR-related fluctuations in fluorescence signal. As an active reference, endogenous 18S ribosomal RNA (r18S) was amplified using specific primers and probes labeled with VIC (ABI) at the 5’ end as a reporter and 6-carboxy-tetramethylrhodamine at the 3’ end as a quencher. The sensitivity and specificity of the assays were assessed from serial dilutions of reference and target templates in separate PCR reactions. Optimal primer concentrations were determined as the minimum primer concentration giving maximum change in emission intensity (R0) and minimum cycle number of fluorescence signal of the product that crosses an arbitrary threshold set within the exponential phase of the PCR (ΔCt). Optimal probe concentrations were chosen as those that gave minimum ΔCt. The goal was to develop a fast assay system with the same geometric phase efficiency of approximately 100%. By the guidelines of PCR and real-time PCR, the correlation coefficient of approximately 0.995 and the standard curve slope of approximately −3.32 are considered suitable for these requirements. In our experiments, the efficiency and the accuracy of the PCR were judged by the slope value of the standard curve and correlation coefficient. To use the ΔCt method, we determined the relative efficiency of the gene-specific and internal control primers and probes. For this reason, the log of input amount of template versus ΔCt was plotted and the absolute value of the slope <0.1 was allowed and was considered as a proof that the efficiencies of target and reference were approximately equal and that they were reliable for usage of the ΔCt method. When the primers and the probes did not fulfill the requirements, they were redesigned. After the indicated validations, the comparative ΔCt method was used for relative quantification and statistical analysis (ABI Prism 7700 Sequence Detection System User’s Manual) (19).

Chemical Methods

8-Iso PGF2α. Urine for 8-Iso was extracted, purified, and analyzed with an enzyme immunoassay kit (Cayman Chemical) using methods described in detail and validated in a previous study (9). Our assay has a limit of detection of 1 pg/ml, an intra-assay coefficient of variation of 8% (n = 10), and an interassay coefficient of variation of 10% (n = 6). A blinded comparison of 12 rat urine samples analyzed both with our RIA method and with gas chromatography mass spectrometry (GCMS) (undertaken by Jack Roberts, M.D., University of Vanderbilt) showed a good correlation (r = 0.85; P < 0.001) without systematic bias. The variation includes that due to RIA and to GCMS. Briefly, samples were diluted to fall in the middle portion of the
standard curve (10 to 100 pg/ml). They were extracted using a polyboronic acid column, eluted with ethyl acetate containing 1% methanol, and evaporated under nitrogen. 8-Iso was assayed using an enzyme-linked competitive binding assay (ELISA) with mouse anti-rabbit IgG monoclonal antibody in a 96-well plate. Concentrations of 8-Iso were determined from absorbency at 405 nm using a standard curve. Samples were assayed in duplicate. Recovery of [3 H]8-Iso averaged 76% (n = 12).

MDA. MDA in the urine was measured by a commercial kit (Oxi-Tek TBARS assay kit; Zeptometrix Corp., Buffalo, NY) that uses the measurement of thiobarbituric acid reactive substances (TBARS). Briefly, urine (100 μl) was mixed with 100 μl of 8.1% SDS. The TBA/buffer reagent was prepared by mixing 0.5 g of polyboronic acid column, eluted with ethyl acetate containing 1% methanol, and evaporated under nitrogen. 8-Iso was assayed using an enzyme-linked competitive binding assay (ELISA) with mouse anti-rabbit IgG monoclonal antibody in a 96-well plate. Concentrations of the reaction product were determined from absorbency at 405 nm using a standard curve. Samples were assayed in duplicate. Recovery of [3 H]8-Iso averaged 76% (n = 12).

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### Statistical Analyses

Results are reported as mean ± SEM. Comparison between multiple groups was by the rigorous ANOVA using Welch’s correction. When appropriate, post hoc comparisons between groups were made by Dunnett’s t test. A trend test (Spearman correlation) was used to determine the graded effects of salt. For correcting for the chance of a type 1 error arising from multiple comparisons of mRNA expression, Bonferroni corrections to P values were applied. In the examination of nine subunits, P < 0.0056 (= 0.05/9) was required for a decisive conclusion of significance. Only results that remained significant at this level are presented in Figure 1. Data were analyzed using SPSS, v11.5 (SPSS, Inc., Chicago, IL).

### Results

The body weight, urine volume, and excretion of sodium and potassium are shown in Table 2. As anticipated, urine flow and Na+ excretion increased with salt intake. The excretions of 8-Iso and MDA increased 2.3-fold and 2.1-fold with salt intake (Figure 2, A and B). There were

### Table 1. Oligonucleotides and related products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequences for forward (F) and reverse (R) primers (5’-3’)</th>
<th>Location of product (bp)</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox-1</td>
<td>F 5’-GGAGTTGCAGAGTTCCTCATTTT-3’ R 5’-TTCTGGCCGAGCAGATAA-3’</td>
<td>888-910 bp</td>
<td>AF152963</td>
</tr>
<tr>
<td></td>
<td>Probe: 5’-6FAM-CAACCCCTAGTCTTGAGTGATCT-3’</td>
<td>1005-988 bp</td>
<td></td>
</tr>
<tr>
<td>Nox-4</td>
<td>F 5’-AGAATGGAGATCCCAAGAACTTT-3’ R 5’-ATGAGGAACATCGACACCATC-3’</td>
<td>489-511 bp</td>
<td>AY027527</td>
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<tr>
<td></td>
<td>Probe: 5’-6FAM-CTTCAACACGAGTCTGAGTCTCAGT-3’</td>
<td>570-548 bp</td>
<td></td>
</tr>
<tr>
<td>gp91phox</td>
<td>F 5’-AAAGGATGAGCCGATCAACAAGT-3’ R 3’-TACAGGAAATGGACCCACAT-3’</td>
<td>762-784 bp</td>
<td>AF298656</td>
</tr>
<tr>
<td></td>
<td>Probe: 5’-6FAM-CGGGAAACCTCCTCAGTACGTGAAATG-TAMRA-3’</td>
<td>839-817 bp</td>
<td></td>
</tr>
<tr>
<td>p22phox</td>
<td>F 5’-ACCTGACCGCTGTTGTTGAA-3’ R 5’-GTG GAG GAC AGC CCG GA-3’</td>
<td>259-277 bp</td>
<td>U18729</td>
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<td></td>
<td>Probe: 5’-6FAM-CTTCAAGGCCCTACCAAAATTAC T-TAMRA-3’</td>
<td>326-310 bp</td>
<td></td>
</tr>
<tr>
<td>p47phox</td>
<td>F 5’-ACGCTCACCAGGTACTTCAACA-3’ R 5’-TCATCGGGCCGCACTTT-3’</td>
<td>291-312 bp</td>
<td>AY029167</td>
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<td>Probe: 5’-6FAM-CGGTCCGGGACACCTTGAACCT-3’</td>
<td>385-369 bp</td>
<td></td>
</tr>
<tr>
<td>p67phox</td>
<td>F 5’-GCTTGGAGACATGGTGTTCAAGA-3’ R 5’-AGAATCGAGCAGCAGTGTCTTGC-3’</td>
<td>1215-1237 bp</td>
<td>AB002664</td>
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<td></td>
<td>Probe: 5’-FAM6-ACACACTAAACTGAGCTACCGCGCCGTCG-TAMRA-3’</td>
<td>1364-1339 bp</td>
<td></td>
</tr>
<tr>
<td>IC-SOD</td>
<td>F 5’-TGTTGCAATTTGAAGATCCTAG-3’ R 5’-CTTGTGTTTCTGTTGACACC-3’</td>
<td>384-406 bp</td>
<td>NM-017050</td>
</tr>
<tr>
<td></td>
<td>Probe: 5’-FAM6-CTTCAAGAGACTTTCATTTGCAC-TAMRA-3’</td>
<td>467-447 bp</td>
<td></td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>F 5’-TTAACCGCGACATGCTGCA-3’ R 5’-CCTGCGTACGCTTACAGTGT-3’</td>
<td>193-211 bp</td>
<td>NM-017051</td>
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<tr>
<td></td>
<td>Probe: 5’-FAM6-CAACGAGACCGACCGCAGCTAC-TAMRA-5’</td>
<td>267-247 bp</td>
<td></td>
</tr>
<tr>
<td>EC-SOD</td>
<td>F 5’-GCCCAGCTCCAGAATTGA-3’ R 5’-CTCAGTTCCCCGAACTCATG-3’</td>
<td>385-403 bp</td>
<td>X94371</td>
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<tr>
<td></td>
<td>Probe: 5’-FAM6-CAGCGAGCAGACACACCTCACC-TAMRA-3’</td>
<td>487-468 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: 5’-FAM6-CAGCGAGCAGACACACCTCACC-TAMRA-3’</td>
<td>430-453 bp</td>
<td></td>
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</tbody>
</table>
significant (P < 0.05) differences for excretion of both markers between LS and NS, and NS and HS. By contrast, there were no significant effects of salt on PGE2 excretion (Figure 2C). We conclude that dietary salt causes a graded increase in oxidative stress as reflected in excretion of lipid peroxidation products.

The basal O_2^- generation by renal cortical tissue was low and did not differ among groups (LS, 1.22 ± 0.06; NS, 1.15 ± 0.073; HS, 1.30 ± 0.12 pmol O_2^-/mg protein per min; not significant). The NADPH-induced signal from the tissue homogenate was reduced by 75 to 95% by DPI (10^-5 M). The NADPH-stimulated O_2^- generation was increased significantly in the kidney cortex from rats that received HS, compared with NS or LS (Figure 3). In contrast, xanthine (LS, 2.48 ± 0.16; NS, 2.06 ± 0.17; HS, 2.81 ± 0.25 pmol O_2^-/mg protein per min; not significant) was not significantly affected by salt intake. NADPH-stimulated O_2^- generation (LS, 78.27 ± 9.94; NS, 86.56 ± 13.17; HS, 108.91 ± 11.21 pmol O_2^-/mg protein per min; not significant) increased significantly with salt intake as assessed by the trend test.

Salt-induced changes in mRNA expression in the kidney cortex are presented as ΔC_T values in Tables 3 and 4 and as a fold changes in Figure 1. A unit increase in cycle value represents a twofold changed mRNA abundance. Because this figure represents changes compared with the mean values for the LS group, only mean changes can be calculated. The slope and the correlation coefficient for each gene are presented in Table 5. A positive trend test (Spearman correlation) indicated significant graded effects of salt to increase renal cortical expression of gp91phox and p47phox and to decrease the expression of IC-SOD and Mn-SOD. A unit increase in cycle value represents a twofold changed mRNA abundance. Compared with LS, HS significantly (P < 0.0056) increased the renal cortical mRNA expression of gp91phox by threefold and p47phox by 2.4-fold and decreased the expression of IC-SOD by 2.2-fold and Mn-SOD by 2.6-fold (Figure 1).

Discussion

The main new findings of this study are that normal rats have a graded increase in the excretion of 8-Iso with salt intake. This is mirrored by MDA but not by PGE2. The increased oxidative stress during HS is accompanied by increased NADPH and NADH oxidase activity in the kidney cortex and increased expression of the mRNA in the kidney cortex for the NAD(P)H oxidase components gp91phox and p47phox and decreased expression of the mRNA for IC- and Mn-SOD.

O_2^- interacts with arachidonate that is free or esterified into membrane phospholipids to yield isoprostanes. The steady-state excretion of 8-Isos has been used as an index of bodily oxidative stress (21). Comparing rats of HS with NS, the excretion of 8-Isos doubled, whereas urine flow increased fourfold. By contrast, comparing rats of LS with NS, the excretion of 8-Isos was reduced significantly without a change in urine flow. Changes in 8-Isos were paralleled by MDA, which is not a cyclooxygenase product and is another widely used marker of lipid peroxidation. The excretion of PGE2 did not increase with salt. Taken together, these findings suggest that oxidative stress increases with dietary salt in normal rats.

Salt loading increases the excretion of isoprostanes in the hypertensive Dahl salt-sensitive rat (7). It is interesting that in

Table 2. Body weights and data from metabolism cage studies

<table>
<thead>
<tr>
<th>Groups</th>
<th>Salt</th>
<th>n</th>
<th>Body wt (g)</th>
<th>UV (ml/24 h per 100 g)</th>
<th>U_{Na}V (μmol/24 h per 100 g)</th>
<th>U_{K}V (μmol/24 h per 100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HS</td>
<td>9</td>
<td>248 ± 4</td>
<td>22.5 ± 0.4</td>
<td>6694 ± 261</td>
<td>1391 ± 65</td>
</tr>
<tr>
<td>2</td>
<td>NS</td>
<td>7</td>
<td>251 ± 9</td>
<td>5.6 ± 1.1</td>
<td>636 ± 130</td>
<td>1391 ± 180</td>
</tr>
<tr>
<td>3</td>
<td>LS</td>
<td>10</td>
<td>254 ± 6</td>
<td>5.0 ± 0.5</td>
<td>1.3 ± 0.6</td>
<td>1404 ± 67</td>
</tr>
<tr>
<td>Effect of salt</td>
<td>ns</td>
<td></td>
<td></td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>ns</td>
</tr>
</tbody>
</table>

* Mean ± SEM values. HS, high salt; NS, normal salt; LS, low salt; UV, urine volume; U_{Na}V, renal sodium excretion; U_{K}V, renal potassium excretion; P values from ANOVA.
this low renin model, there is evidence of an active intrarenal angiotensin system that therefore might contribute to regulation of NADPH oxidase in the kidney (22). Arteriolar and venular generation of O$_2^·$/OH$^·$ in Sprague-Dawley rats increases with HS (23), accompanied by a diminished vasodilatory response to nitric oxide (NO), consistent with increased bioactivation of NO by O$_2^·$/OH$^·$. Normal rats fed HS do not develop hypertension (23,24), perhaps because of increased generation and action of NO within the juxtaglomerular apparatus of the kidney (24–26). Thus, the increased oxidative stress in these studies and our own likely occurs as a consequence of the increase in dietary salt intake rather than an increase in BP, but this was not assessed in the present study.

NAD(P)H oxidase has been assigned a major role in ROS generation in vascular and cardiac tissue with contributions from other sources such as xanthine oxidase and mitochondrial respiration (8). In the kidney, NAD(P)H oxidase mediates production of O$_2^·$ in mesangial cells (27), thick ascending limb cells (16), and rat renal medullary homogenates (15). Thus, this oxidase may play a critical role in producing ROS in the kidney. Indeed, the present study demonstrates that the enhanced oxidative stress associated with salt loading was accompanied by upregulation of NADPH and NADH oxidase activity in the kidney cortex. A more than twofold increase in the excretion of 8-Iso and MDA and in the mRNA expression of gp91phox and p47phox with high salt was accompanied by only an approximately 40% increase in O$_2^·$ generation from NADPH oxidase in the kidney. The O$_2^·$ generated in the kidney during NADPH addition likely derives mainly from NADPH oxidase because it was reduced by 75 to 95% by DPI, which is an effective, albeit not highly specific, inhibitor of NADPH oxidase (28). A discrepancy between structural and functional changes in NADPH oxidase has been shown previously. Hsich et al. (29) showed that even complete knockout of the gene for p47 phox had no effect on O$_2^·$ generation in aortic rings in the basal condition but resulted in a significant 50% decrease in O$_2^·$ in aortic rings that were treated with an SOD inhibitor. Several isoforms of nonphagocyte NADPH oxidase, containing gp91phox, p47phox, or p67phox homologues, are described (30–32).

Previously, we found that the normal kidney expressed the mRNA and protein for all of the subunits for phagocyte NAD(P)H oxidase and the mRNA for Nox-1 and Nox-4 (14) and for EC-, IC-, and Mn-SOD (19). Prolonged infusion of Ang II activates NAD(P)H oxidase in blood vessels (1,2,10,12), where it increases expression of the p22phox, Nox-1, and Nox-4 components in VSMC from conduit vessels (33–35) and p67phox in vascular adventitial cells (36). Small
resistance vessels express gp91phox, which also is upregulated by Ang II (37), and p67phox and xanthine oxidase, which are unaffected by HS intake (8). We found that prolonged Ang II infusion increased the expression of p22phox and Nox-1 but decreased the expression of Nox-4 in the renal cortex (19). This was accompanied by increased excretion of 8-Iso and decreased the expression of Nox-4 in the renal cortex (19). Mn-SOD in the kidney cortex of the rat, although this effect is not prevented by blocking AT1 or AT2 receptors and therefore may be mediated via another mechanism such as Ang (1

Previously we have found that the quantitative mRNA approach corresponds to changes in protein expression of NAD(P)H oxidase components in the kidney (14). The specificity of the PCR permits discrimination between the Nox isoforms, which could not be achieved reliably with our antibodies. The finding that the cortical NADPH and NADH isoforms, which could not be achieved reliably with our antibodies. The finding that the cortical NADPH and NADH isoforms may impair the effects of NO on tubuloglomerular feedback responses (13). However, it is not clear whether salt loading enhances ROS generation in the thick ascending limb or the juxtaglomerular apparatus and what physiologic consequences would follow from this.

Table 3. The mRNA expression in the kidney cortex (expressed as ΔC_T) for NADPH oxidase subunits and SOD normalized to r18s: Effects of dietary salt intake

<table>
<thead>
<tr>
<th>Condition</th>
<th>p22phox</th>
<th>gp91phox</th>
<th>Nox-1</th>
<th>Nox-4</th>
<th>p47phox</th>
<th>p67phox</th>
<th>IC-SOD</th>
<th>Mn-SOD</th>
<th>EC-SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td>2.6 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>4.5 ± 0.3</td>
<td>7.2 ± 0.3</td>
<td>5.4 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>NS</td>
<td>2.5 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>4.8 ± 0.3</td>
<td>5.8 ± 0.3</td>
<td>6.2 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>LS</td>
<td>2.7 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>5.0 ± 0.2</td>
<td>6.7 ± 0.4</td>
<td>6.7 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Rigorous ANOVA (Welch)</td>
<td>0.4953</td>
<td>0.0000</td>
<td>0.2466</td>
<td>0.0327</td>
<td>0.0001</td>
<td>0.6033</td>
<td>0.0329</td>
<td>0.0026</td>
<td>0.0613</td>
</tr>
<tr>
<td>Trend test (Spearman correlation), P value</td>
<td>0.7110</td>
<td>0.0000</td>
<td>0.0749</td>
<td>0.3092</td>
<td>0.0000</td>
<td>0.3820</td>
<td>0.0005</td>
<td>0.0000</td>
<td>0.2117</td>
</tr>
</tbody>
</table>

*a mRNA ΔC_T expression summaries are shown as mean ± SEM values. SOD, superoxide dismutase.

Table 4. The mRNA expression in the kidney cortex (expressed as ΔC_T and a fold difference) for NADPH oxidase subunits and SOD normalized to r18s: Effects of dietary salt intake

<table>
<thead>
<tr>
<th>Condition</th>
<th>p22phox</th>
<th>gp91phox</th>
<th>Nox-1</th>
<th>Nox-4</th>
<th>p47phox</th>
<th>p67phox</th>
<th>IC-SOD</th>
<th>Mn-SOD</th>
<th>EC-SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔC_T for HS</td>
<td>2.6 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>4.5 ± 0.3</td>
<td>7.2 ± 0.3</td>
<td>5.4 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>ΔC_T for LS</td>
<td>2.7 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>5.0 ± 0.2</td>
<td>6.7 ± 0.4</td>
<td>6.7 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Fold difference between LS and HS</td>
<td>1.07</td>
<td>3.03</td>
<td>1.41</td>
<td>1.41</td>
<td>2.46</td>
<td>1.14</td>
<td>2.29</td>
<td>2.63</td>
<td>1.14</td>
</tr>
</tbody>
</table>

*a The fold difference is calculated according to the notion that a unit increase in cycle value represents a twofold change in mRNA abundance, because all assays had the same geometric phase efficiency: nearly 100%.

Table 5. The slope and the correlation coefficient for the each gene expression data using real-time PCR

<table>
<thead>
<tr>
<th>Condition</th>
<th>GAPDH</th>
<th>p22phox</th>
<th>gp91phox</th>
<th>Nox-1</th>
<th>Nox-4</th>
<th>p47phox</th>
<th>p67phox</th>
<th>IC-SOD</th>
<th>Mn-SOD</th>
<th>EC-SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation coefficient</td>
<td>0.998</td>
<td>0.996</td>
<td>0.997</td>
<td>0.996</td>
<td>0.997</td>
<td>0.996</td>
<td>0.998</td>
<td>0.997</td>
<td>0.998</td>
<td></td>
</tr>
</tbody>
</table>
of the hypertensive Dahl salt-sensitive rat (7). This result was confirmed in the present study in normal rats. A downregulation of Mn-SOD may contribute to oxidative stress in the kidneys of animals challenged with salt loading. Our previous findings (19) suggest that this downregulation of Mn-SOD with salt loading may be a consequence of a reduction in Ang II, but this was not tested in the present study.

ROS activate mitogen-activated protein kinase in renal tubule cells (38), contribute to hypertrophic responses to Ang II (38) and to cellular injury (39), and increase fibrogenic matrix protein synthesis in mesangial cells (40). A high salt intake enhances oxidative stress in rat skeletal muscle arterioles and vessels (23) and increases BP, protein excretion, and renal fibrosis and worsens renal function in several models of chronic renal failure (7,38,41–43) and accelerates the decline of renal function in patients with chronic renal failure (44). Conversely, salt restriction ameliorates renal disease progression in animal models (42) and is recommended for patients with chronic renal failure (45). Although high BP surely contributes to the detrimental effects of high salt intake on renal function in these conditions, an increase in oxidative stress may have an important additional role (1). It is remarkable that rats fed a high salt intake had the highest ROS generation despite the established effects of salt to suppress renin release from the kidneys. This could indicate that salt intake modifies the effects of Ang II on the ROS-generating systems. The results of this and a previous study (19) suggest that high salt and Ang II both regulate the expression of key components of NADPH oxidase and SOD but that these effects can be distinct.

This observation provides a hypothesis to explain the synergistic effects of salt loading and Ang II on BP and renal function in these models of chronic renal failure (7,38,41–43) and accelerates the decline of renal function in patients with chronic renal failure (44). Indeed, renin angiotensin II, but this was not tested in the present study.

References


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