The SOD Mimetic Tempol Ameliorates Glomerular Injury and Reduces Mitogen-Activated Protein Kinase Activity in Dahl Salt-Sensitive Rats

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Abstract. It was shown recently that renal injury in Dahl salt-sensitive (DS) hypertensive rats is accompanied by mitogen-activated protein kinase (MAPK) activation. The present study was conducted to elucidate the contribution of reactive oxygen species to MAPK activities and renal injury in DS rats. DS rats were maintained on high salt (H; 8.0% NaCl; n = 7) or low salt (L; 0.3% NaCl; n = 6) diets; H + a superoxide dismutase mimic, tempol (3 mmol/L in drinking water; n = 8); or H + hydralazine (0.5 mmol/L in drinking water; n = 8) for 4 wk. Mean BP (MBP) in DS/H and DS/L rats was 185 ± 7 and 113 ± 3 mmHg, respectively. DS/H rats showed a higher ratio of urinary protein excretion and creatinine (Uprotein U/ 

Increasing evidence supports the role of reactive oxygen species (ROS) in the pathophysiology of hypertension and organ damage. Clinical studies have shown that vitamins and other antioxidants have BP-lowering effects in hypertensive patients (1, 2). Furthermore, exaggerated vascular superoxide anion (O2−) production has been observed in different animal models of hypertension, including spontaneously hypertensive rats (SHR) (3), stroke-prone SHR (4), deoxycorticosterone acetate (DOCA)-salt hypertensive rats (3), and angiotensin II (Ang II)-induced hypertensive rats (5). The Dahl salt-sensitive (DS) rat is a genetic model of salt-sensitive hypertension and develops renal damage, characterized by glomerular injury (6). Previous studies showed that the nephrosclerosis was associated with elevated renal cortical contents of malondialdehyde, an index of lipid peroxidation in DS hypertensive rats (7). Furthermore, Swei et al. (8) demonstrated that O2− production in the mesenteric microvessels and plasma hydrogen peroxide levels were significantly elevated in DS hypertensive rats. Recent studies by Trolliet et al. (9) showed that hypertensive nephropathy in DS rats is associated with increases in the renal tissue O2− levels, as measured by the lucigenin chemiluminescence method. These observations suggest that salt-dependent hypertension and nephropathy are accompanied by increases in ROS. However, the precise mechanisms responsible for ROS-dependent progression of renal injury in DS hypertensive rats remain to be elucidated.

NAD(P)H oxidase is one of the major sources of O2− in a variety of cells (10). Rajagoplan et al. (11) showed that O2− generation in response to NAD(P)H was increased in vascular homogenates of Ang II–infused hypertensive rats. It has also...
been shown that increased vascular O$_2^-$ is associated with increased NAD(P)H oxidase activity in DOCA-salt hypertensive rats (3) and renovascular hypertension in 2K1C Goldblatt rats (12) or SHR (13), indicating enhanced O$_2^-$ generation by the activation of NAD(P)H oxidase in hypertension. It has also been indicated that ROS serve as second messengers in signal transduction pathways (14–16). Among many intracellular signaling molecules, ROS-induced cellular events have been implicated, in part, in the activation of mitogen-activated protein kinases (MAPK), including the extracellular signal-regulated kinases (ERK) I/ERK2, c-Jun NH$_2$-terminal kinases (JNK), and p38 MAPK (10, 14–16). Studies have shown that MAPK are activated in experimental glomerular diseases, such as hypertension (17), diabetes (18), and glomerulonephritis (19). Furthermore, MAPK inhibitors or recombinant adenoviruses that contain dominant negative mutants of MAPK prevent mesangioliproliferative glomerulonephritis (19, 20). Jaimes et al. (21) showed that Ang II increased O$_2^-$ production and caused cell proliferation and hypertrophy in mesangial cells. It is interesting that the authors also showed that the latter effects were completely prevented by an ERK inhibitor, PD98059. These data suggest that MAPK is involved in the pathophysiology of O$_2^-$-dependent mesangial cellular changes.

We recently demonstrated that ERK1/ERK2 and JNK activities are significantly elevated in the renal cortical tissues of DS hypertensive rats (17). The present study was conducted to elucidate the contribution of ROS to MAPK activities and renal injury in DS hypertensive rats. Therefore, studies were performed to investigate whether 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (tempol) ameliorates renal injury and influences renal tissue ERK1/ERK2, JNK, and p38 MAPK activities in DS hypertensive rats. Tempol is a membrane-permeable, metal-independent SOD mimic that has been shown to be specific for O$_2^-$ (22, 23). To evaluate the possible contribution of NAD(P)H oxidase to ROS generation, we measured renal cortical mRNA expression of p22-phox and Nox-1, essential components of NAD(P)H oxidase (10, 24). We also examined the effects of a nonspecific vasodilator, hydralazine, on renal injury and MAPK activities in DS hypertensive rats.

**Materials and Methods**

**Animal Preparation**

All experimental procedures were performed according to the guidelines for the care and use of animals established by the Kagawa Medical University. Male DS and Dahl salt-resistant (DR) rats (Seac Yoshitomi, Fukuoka, Japan), weighing 205 to 220 g at the beginning of the experiments, were randomly selected to receive rat diet containing high salt (H; 8% NaCl; Oriental Yeast, Osaka, Japan) or low salt (L; 0.3% NaCl; Oriental Yeast) for 4 wk. The number of animals used is as follows: 7, 6, 6, and 6 for DS/H, DS/L, DR/H, and DR/L rats, respectively. In a separate experimental series, DS/H rats were treated with tempol (Sigma Co., St. Louis, MO; 3 mmol/L in drinking water; $n = 8$) or hydralazine (Wako Co., Tokyo, Japan; 0.5 mmol/L in drinking water; $n = 8$). The dose of hydralazine was determined on the basis of results from previous studies in DS rats (25). Preliminary data showed that hydralazine (0.5 mmol/L) and tempol (3 mmol/L) resulted in similar reductions in BP of DS/H rats.

Mean BP (MBP) was measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan) every week. Urine samples were collected for 24 h 1 d before harvesting. Blood and kidney samples were harvested at the end of the fourth week. After decapsulation, the left kidney was removed, snap-frozen in liquid nitrogen, and stored at $-80^\circ$C until processing for protein or RNA extraction and analysis of the collagen and thioarbituric acid reactive substances (TBARS) contents. The right kidney was perfused with chilled saline solution and fixed in 10% buffered paraformaldehyde for histologic examination. The heart was also excised, and the left ventricle was removed and weighed.

**Analysis of Kidney Samples**

p22-phox and Nox-1 mRNA expression levels in the renal cortical tissues were quantitatively analyzed by Northern blot analysis as described previously (26–28). The p22-phox probe used was the full-length cDNA as described previously in detail (26, 27). The cDNA used to prepare the Nox-1 probe was obtained by PCR amplification and subclone (28). Autoradiographic bands were quantified by densitometry and normalized by the expression of glyceraldehyde-3-phosphate dehydrogenase.

Previously, we found that the activation of ERK1/ERK2 and p38 MAPK by an in-gel kinase assay with specific substrates and immunoblotting for phospho-ERK1/ERK2 and p38 MAPK were highly correlated ($R^2 = 0.90$) in various cell types (15, 16). Therefore, we used immunoblotting for phospho-ERK1/ERK2 and phospho-p38 MAPK to evaluate ERK1/ERK2 and p38 MAPK activation as described previously (16, 29). JNK activity was measured using a commercially available kit based on the phosphorylation of recombinant c-Jun. Immunoblotting was performed with antibodies against phospho-ERK1/ERK2, phospho-c-Jun, and p38 MAPK (Cell Signaling Technology Inc., Beverly, MA) (16, 29). We also evaluated total ERK1/ERK2, JNK, and p38 MAPK protein expression using pan-ERK1/ERK2, JNK, and p38 MAPK antibodies (Cell Signaling Technology Inc.).

**Histologic Examination**

The excised kidneys were fixed with 10% formalin (pH 7.4), embedded in paraffin, sectioned into 4-μm slices, and stained with hematoxylin-eosin (HE) or Azan reagent. Using light microscopy, we evaluated the severity of the glomerular injury score according to previously described methods (6, 30). A minimum of 50 glomeruli in each specimen were examined. Proliferative lesions were scored into five grades using the specimens with HE staining as follows: 0 = no proliferation, 1 = minor (segmental lesion <25%), 2 = mild (segmental lesion 25 to 50%), 3 = moderate (diffuse proliferation without severe sclerotic change), and 4 = severe (diffuse proliferation with nearly complete sclerosis). Scoring of the mesangial matrix expansion was evaluated using the specimens with Azan staining as follows: 0 = no matrix expansion, 1 = minor, 2 = weak, 3 = moderate, and 4 = strong. The severity of the interlobular arteriolar injury was evaluated according to previously described methods (7). In each specimen, a minimum of six interlobular arteries were examined. Interlobular arteriolar injury was scored into five grades using the specimens with HE staining as follows: 0 = no lesions, 1 = mild muscular hypertrophy, 2 = twice normal muscle thickness, 3 = greater than twice normal muscle thickness, and 4 = fibrin deposition and almost complete obliteration of lumen.

Kidney slices were also processed for immunohistochemistry as described previously (31). Formalin-fixed tissue sections were depar-
affinized with xylene and rehydrated with graded ethanol. Endogenous peroxidase was blocked with hydrogen peroxide, and the samples were then rinsed in PBS. To yield adequate signals with the respective antibodies, we heated the slides at 121°C for 10 min in 0.01 mol/L citrate buffer (pH 6.0). The sections were incubated for 24 h at 4°C with polyclonal antibodies against p22-phox (Santa Cruz Biotechnology, Santa Cruz, CA) and diluted in PBS containing 1% BSA (1:50). After washing with PBS, the sections were incubated with biotinylated secondary antibody, an avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories, Burlingame, CA), and then 3,3’-diaminobenzidine (Dojindo Co., Kumamoto, Japan). Each section was counterstained with Mayer’s hematoxylin (Wako Co.), dehydrated, and cover-slipped.

Analytical Procedures
Urinary excretions of protein and creatinine were determined using assay kits (Wako Co.). We determined the degree of lipid peroxidation using biochemical assays of TBARS in the renal cortical tissues, as described previously (27). Renal cortical tissue collagen content was determined on the basis of the concentration of hydroxyproline (32). Urinary excretion of NOx was also determined as described previously (33).

Statistical Analyses
The values are presented as means ± SEM. Statistical comparisons of the differences were performed using one-way or two-way ANOVA combined with the Newman-Keuls post hoc test. P < 0.05 was considered statistically significant.

Results
BP, Left Ventricular Weight, Urine Volume, and Urinary Excretion of Sodium, Potassium, and NOx
The temporal profile of MBP is depicted in Figure 1A. MBP was identical among the six groups at the beginning of the protocol and unaltered during the protocol in DR/L (105 ± 4 mmHg), DR/H (102 ± 2 mmHg), and DS/L rats (113 ± 3 mmHg). However, DS/H rats progressively developed hypertension (185 ± 7 mmHg at Week 4). Concurrent administration of tempol significantly decreased MBP in DS/H rats (128 ± 3 mmHg at Week 4). Furthermore, administration of hydralazine resulted in a similar decrease in MBP of DS/H rats (127 ± 5 mmHg at Week 4). The mean left ventricular weight of DS/H rats was higher than that of DR/L, DR/H, and DS/L rats. The left ventricular weight in DS/H rats was significantly reduced by tempol or hydralazine treatment (Table 1). Table 1 summarizes the urine volume and urinary excretion of sodium and potassium. After 4 wk of treatment with a high-salt diet, the kidney weights and kidney weight-to-body weight ratios of DS/H rats were significantly higher than those of DR/L, DR/H, and DS/L rats, respectively (Table 1). Tempol- and hydralazine-treated DS/H rats showed significantly lower urine volume than untreated DS/H rats (P < 0.05). Urinary excretion of sodium and potassium was not affected by tempol or hydralazine in DS/H rats (Table 1). A high-salt diet significantly increased urinary excretion of NOx (U NOx V) in DR rats. However, U NOx V was not increased by a high-salt diet in DS rats (Table 1). U NOx V was significantly lower in DS/H rats than in DR/H rats. Treatment with tempol significantly increased U NOx V in DS/H rats, whereas it tended to be reduced by hydralazine treatment.

Urinary Protein Excretion and Collagen Content
After 4 wk of treatment with a high-salt diet, DS/H rats showed a markedly higher urinary protein excretion rate (348 ± 13 mg/d) as compared with DS/L (31 ± 1 mg/d), DR/H (22 ± 22 mg/d), and DR/L rats (8 ± 2 mg/d). Both tempol and hydralazine significantly reduced the urinary protein excretion rate in DS/H rats (97 ± 10 and 198 ± 24 mg/d, respectively). On the basis of the group comparisons, however, the urinary protein excretion rate of tempol-treated DS/H rats was significantly lower than that of hydralazine-treated DS/H rats (P < 0.05). The temporal profile ratio of urinary protein excretion and creatinine (U protein V/U cr V) is depicted in Figure 1B. After 4 wk of treatment with a high-salt diet, DS/H rats showed a higher U protein V/U cr V (20.3 ± 1.1) as compared with DS/L (2.4 ± 0.1), DR/L (0.7 ± 0.1), and DR/H rats (1.5 ± 0.1). Tempol markedly reduced U protein V/U cr V to 4.8 ± 0.4 in DS/H rats. Hydralazine slightly but significantly decreased U protein V/U cr V to 16.2 ± 1.5 in DS/H rats. However, U protein V/U cr V in tempol-treated rats was significantly lower than that of hydralazine-treated rats (P < 0.05; Figure 1B). The hydroxyproline concentration in the renal cortical tissue of DS/H rats was 24 ± 1 nmol/mg. The calculated cortical collagen content in DS/H rats was 23 ± 1 μg/mg, which was significantly higher than that of DR/L, DR/H, and DS/L rats (Table 1). In DS/H rats, both tempol and hydralazine significantly decreased the collagen content in the renal cortex. However, the renal cortical collagen content of tempol-treated DS/H rats was significantly lower than that of hydralazine-treated DS/H rats (P < 0.05; Table 1).

Histologic Findings
The glomerular histologic findings with HE and Azan staining are summarized in Figure 2. DR/L and DR/H rats showed...
Table 1. Effects of 4 weeks of a high-salt diet on body weight, left ventricular weight, and urine volume, urinary excretion of sodium, potassium, and NOx; and renal cortical collagen and thiobarbituric acid reactive substances contents in Dahl salt-resistant and Dahl salt-sensitive rats.

<table>
<thead>
<tr>
<th></th>
<th>DR Rats</th>
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<th>DS Rats</th>
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<tbody>
<tr>
<td></td>
<td>Low Salt</td>
<td>High Salt</td>
<td>Low Salt</td>
<td>High Salt</td>
<td>High Salt + Tempol</td>
<td>High Salt + Hydralazine</td>
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<tr>
<td>Body weight (g)</td>
<td>327 ± 2</td>
<td>326 ± 4</td>
<td>339 ± 2</td>
<td>336 ± 3</td>
<td>341 ± 2</td>
<td>340 ± 4</td>
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<tr>
<td>LVW (g/100 g body wt)</td>
<td>0.19 ± 0.02</td>
<td>0.20 ± 0.04</td>
<td>0.22 ± 0.03</td>
<td>0.38 ± 0.03b</td>
<td>0.26 ± 0.04c</td>
<td>0.27 ± 0.06c</td>
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<tr>
<td>UV (ml/d)</td>
<td>10 ± 1</td>
<td>66 ± 3b</td>
<td>13 ± 2</td>
<td>90 ± 6b</td>
<td>68 ± 5b,c</td>
<td>78 ± 3b,c</td>
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<tr>
<td>U(_{Na})V (mEq/d)</td>
<td>1.3 ± 0.4</td>
<td>26.7 ± 1.2b</td>
<td>0.9 ± 0.1</td>
<td>24.3 ± 1.4b</td>
<td>24.0 ± 1.9b</td>
<td>27.7 ± 0.7b</td>
</tr>
<tr>
<td>U(_{K})V (mEq/d)</td>
<td>1.0 ± 0.1</td>
<td>4.1 ± 0.1b</td>
<td>1.2 ± 0.1</td>
<td>4.2 ± 0.2b</td>
<td>3.7 ± 0.2b</td>
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</tr>
<tr>
<td>U(_{NOx})V (μmol/d)</td>
<td>0.22 ± 0.01</td>
<td>0.45 ± 0.02b</td>
<td>0.18 ± 0.02</td>
<td>0.21 ± 0.03</td>
<td>0.37 ± 0.02b,c</td>
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</tr>
<tr>
<td>Collagen content (μg/g)</td>
<td>12 ± 2</td>
<td>15 ± 1</td>
<td>13 ± 1</td>
<td>23 ± 1b</td>
<td>14 ± 1c</td>
<td>19 ± 1b,c</td>
</tr>
<tr>
<td>TBARS (nmol/g)</td>
<td>31 ± 3</td>
<td>30 ± 4</td>
<td>41 ± 6</td>
<td>108 ± 9b,c</td>
<td>48 ± 8c</td>
<td>72 ± 6b,c</td>
</tr>
</tbody>
</table>

a Values are means ± SE. DR, Dahl salt-resistant; DS, Dahl salt-sensitive; LVW, left ventricular weight; UV, urine volume; U\(_{Na}\)V, urinary excretion of sodium; U\(_{K}\)V, urinary excretion of potassium; U\(_{NOx}\)V, urinary excretion of NOx; TBARS, thiobarbituric acid reactive substances.

b \(P < 0.05\) versus same strain on low-salt diet.

c \(P < 0.05\): DS rats/high-salt diet versus DS rats/high-salt diet + tempol or hydralazine.

Figure 2. (A and B) Glomeruli in Dahl salt-sensitive rats. Scores of glomerular proliferation (C) and matrix expansion (D). A minimum of 50 glomeruli in each specimen were examined. *\(P < 0.05\) versus DS/L rats. Magnification, ×400 in A (hematoxylin-eosin [HE] stain) and B (Azan).
normal glomeruli or very slight glomerular damage (data not shown). DS/L rats also showed normal glomeruli or very slight glomerular damage; however, DS/H rats exhibited severely damaged glomeruli, characterized by cell proliferation (Figure 2A) and mesangial matrix expansion (Figure 2B). These findings were confirmed by scoring the glomerular proliferative lesions and mesangial matrix expansion (Figure 2C and D). Both of these parameters were markedly elevated in untreated DS/H rats. Concurrent administration of tempol or hydralazine ameliorated these glomerular changes and improved both indicators of glomerular injury. However, tempol lowered matrix scores to a greater extent than hydralazine (Figure 2D). These results indicate that the renoprotective effects of tempol are greater than those of hydralazine.

DR/L and DR/H rats showed normal interlobular arteries (data not shown). DS/L rats showed normal interlobular arteries or very slight interlobular arterial injury. Conversely, DS/H rats exhibited severely injured interlobular arteries with marked mural thickening because of muscular hypertrophy and fibrin accumulation (Figure 3). Concurrent administration of tempol or hydralazine markedly ameliorated interlobular arterial injury in DS/H rats (Figure 3). In DR/L, DR/H, and DS/L rats, averaged arterial injury scores were 0.0 ± 0.0, 0.0 ± 0.0, and 0.1 ± 0.1, respectively. In all DS/H rats, interlobular arteries showed varying degrees of injury (from 1 or 2 to 4; Figure 3). The average vascular injury score in DS/H rats was 2.4 ± 0.4. The arterial injury scores in tempol- and hydralazine-treated DS/H rats were significantly lower than those in untreated DS/H rats (0.8 ± 0.2 and 1.1 ± 0.3, respectively).

Renal Cortical TBARS Contents and mRNA Expression of NAD(P)H Oxidase Components

DS/H rats showed significantly higher renal cortical tissue TBARS contents than DR/L, DR/H, or DS/L rats (Table 1). Concurrent administration of tempol prevented the increases in TBARS contents of DS/H rats. Hydralazine also reduced renal cortical TBARS contents in DS/H rats, but these values were significantly higher than those observed in tempol-treated DS/H rats (Table 1).

In DR rats, a high-salt diet did not alter the expression of p22-phox mRNA in renal cortical tissues. Conversely, DS/H rats showed a higher expression of p22-phox mRNA than DS/L rats (Figure 4A). Similarly, the renal cortical Nox-1 mRNA expression of DS/H rats was significantly higher than that of DR/L, DR/H, or DS/L rats (Figure 4B). Treatment of tempol significantly decreased p22-phox mRNA expression in the renal cortical tissues of DS/H rats. Similarly, tempol tended to decrease Nox-1 expression; however, these changes are not statistically significant. Renal cortical p22-phox and Nox-1 expression were not statistically different between untreated and hydralazine-treated DS/H rats. Immunohistochemical studies showed that p22-phox was increased in damaged glomeruli with prominent expression in visceral glomerular epithelial cells (Figure 5). Furthermore, the glomerular staining level for p22-phox seemed to be stronger in DS/H rats than in DR/L, DR/H, DS/L, and tempol-treated rats.

Renal Cortical ERK1/ERK2, JNK, and p38 MAPK Activities

In concordance with previous studies (17, 34), ERK1/ERK2 and JNK activities in the renal cortical tissues were similar.

Figure 3. Interlobular arteries in Dahl salt-sensitive rats (A, HE stain; B, Azan stain). A minimum of six interlobular arteries in each specimen were examined. Magnification, ×400.
among DR/L, DR/H, and DS/L rats (data not shown). However, ERK1/ERK2 and JNK activities in the renal cortical tissues of DS/H rats were 7.0 ± 0.7- and 4.3 ± 0.2-fold higher, respectively, than those of DS/L rats. In DS/H rats, concurrent administration of tempol normalized both ERK1/ERK2 and JNK activities (Figure 6, A and B). Hydralazine-treated DS/H rats also showed reduced JNK activity (by 56 ± 3%), but ERK1/ERK2 activity was unaffected by hydralazine treatment. However, p38 MAPK activity was not different between DR/L, DR/H, DS/L, and DS/H rats. Furthermore, neither tempol nor hydralazine altered p38 MAPK activity in DS/H rats (Figure 6C). Among these animals, no differences in the amounts of ERK1/ERK2, JNK, and p38 MAPK were observed in samples by Western blot analysis with pan-ERK1/ERK2, JNK, and p38 MAPK antibodies (data not shown).

**Figure 4.** p22-phox (A) and Nox-1 mRNA (B) expression in renal cortical tissues. Representative autoradiographs of Northern blotting are shown. Data are expressed as the relative differences in DR/L, DR/H, DS/H, tempol-, or hydralazine-treated DS/H compared with DS/L rats after normalization to the expression of glyceraldehyde-3 phosphate dehydrogenase. *P < 0.05 versus DS/L rats; †P < 0.05, DS/H rats versus DS/H rats + tempol or hydralazine.

**Figure 5.** Immunohistochemical staining for glomerular p22-phox in DS/L, DS/H, tempol-treated DS/H, and hydralazine-treated DS/H rats. Increased staining for p22-phox is observed mainly in the glomerular visceral epithelial cells in DS/H rats. Furthermore, glomerular staining for p22-phox seems to be stronger in DS/H rats than in DS/L or tempol-treated DS/H rats. Magnification, ×400.
Discussion

The results from the present study show that nephropathy in DS/H hypertensive rats is associated with increases in renal cortical TBARS levels and the upregulation of mRNA of p22-phox and Nox-1, essential components of NAD(P)H oxidase. Furthermore, treatment with a SOD mimetic, tempol, markedly reduced renal cortical TBARS contents and ameliorated glomerular injury in these animals. In agreement with previous studies (17, 34), increased ERK1/ERK2 and JNK activities were observed in the renal cortical tissues of DS hypertensive rats. The present study provides further evidence that tempol treatment prevents increases in renal tissue ERK1/ERK2 and JNK activities. These data suggest that elevated ROS generation by the activation of NAD(P)H oxidase in DS hypertensive rats participates in the progression of renal injury through MAPK activation.

p22-phox and gp91-phox are heterodimers of cytochrome b558 and function as the final electron transporter from NAD(P)H to oxygen for the generation of $O_2^-$ (10, 24). Recent studies have also identified the existence of several gp91-phox homologues, such as Nox-1 and Nox-4 (10, 24, 35). It has been shown that the expression of p22-phox and Nox-1 is increased in atherosclerotic coronary arteries (36, 37). Furthermore, Fukui et al. (27) showed that the expression levels of p22-phox and gp91-phox in the left ventricle after myocardial infarction were significantly increased at the sites of infarction. Thus, these observations support a critical role for p22-phox and Nox-1 in cardiovascular disease states. Consistent with recent studies using reverse transcription–PCR analysis (35), we were able to detect the mRNA for p22-phox and Nox-1 (MOX1) in the kidney using Northern blot analysis. We also found that the expression levels of p22-phox and Nox-1 mRNA in the renal cortical tissues were upregulated in DS hypertensive rats. Furthermore, tempol markedly reduced renal cortical TBARS levels and ameliorated glomerular injury in DS hypertensive rats. These data suggest that NAD(P)H oxidase-dependent $O_2^-$ generation contributes to the progression of renal injury in salt-induced hypertension. In the present study, we also found that immunocytochemical staining for glomerular p22-phox seemed to be stronger in DS hypertensive rats than in normo-

Figure 6. Renal cortical extracellular signal-regulated kinase (ERK) 1/ERK2 (A), c-Jun NH₂-terminal kinase (JNK; B), and p38 mitogen-activated protein kinase (MAPK; C) activities in DS rats. Representative blots are shown in top figures. Densitometric analysis of ERK1/ERK2, JNK, and p38 MAPK activities are shown in bottom figures. Values were normalized by arbitrarily setting the densitometry of DS/L rats to 1.0. No differences in the amounts of ERK1/ERK2, JNK, and p38 MAPK were observed in the samples by Western blot analysis with pan-ERK1/ERK2, JNK, and p38 MAPK antibodies (data not shown). *P < 0.05 versus DS/L rats.
tensive rats. However, we were not able to address the immunocytochemical staining for Nox-1, because antibody specificity could not be ensured. Treatment with tempol significantly reduced p22-phox mRNA expression in the renal cortical tissues of DS rats. Nox-1 mRNA expression also tended to be decreased by tempol. These changes, however, were not statistically significant. Renal cortical p22-phox and Nox-1 expression was not statistically different between untreated and hydralazine-treated DS/H rats. At this time, we have no satisfactory explanation as to why the expression of p22-phox was decreased by tempol in DS rats. Further in vitro studies are required to determine the precise mechanisms responsible for tempol-induced alterations in the expression of NAD(P)H components.

Studies have indicated that tempol treatment reduces the $O_2^-$ levels and ameliorates $O_2^-$-related injury in inflammation (38), ischemia/reperfusion (39), and radiation (40). Consistent with previous observations in hypertensive animals (5, 23, 41), the present study showed that administration of tempol significantly decreased arterial pressure in DS rats. Therefore, the possibility exists that the renoprotective effects of tempol are dependent on arterial pressure changes. However, the results from the present study also demonstrate that although treatment with hydralazine results in similar reductions in arterial pressure and left ventricular weight, it actually causes less improvement in proteinuria and glomerular injury compared with tempol. Furthermore, decreases in renal cortical tissue TBARS levels in tempol-treated rats are significantly greater than in hydralazine-treated rats. These data suggest that the renoprotective effects of tempol are not simply associated with the reduction in arterial pressure. Previously, we reported that DS hypertensive rats showed reduced renal blood flow and urinary excretion of cGMP, which were restored by dietary L-arginine supplementation (42). These data suggest that reduced intrarenal nitric oxide (NO) levels are involved in renal vasoconstriction in DS hypertensive rats. Because $O_2^-$ reacts with NO, scavenging of $O_2^-$ by tempol may enhance the activity of the NO system. Indeed, previous studies showed that tempol-induced reductions in arterial pressure were markedly attenuated by NO synthase inhibition in SHR (41) or Ang II–infused hypertensive rats (5). In the present study, we observed that $U_{\text{NOx,V}}$ in DS/H rats was significantly lower than in DR/H rats. In addition, treatment with tempol significantly increased $U_{\text{NOx,V}}$ in DS/H rats, suggesting the possibility that the renoprotective effects of tempol in DS hypertensive rats are mediated through action on the NO system. We also observed that $U_{\text{NOx,V}}$ tended to be reduced by hydralazine treatment in DS/H rats. It has been shown that several antihypertensive agents, including angiotensin-converting enzyme inhibitors, calcium channel blockers, and AT$_1$ receptor antagonists, could increase endothelial NO production (43, 44). Thus, it seems likely that there are fewer similarities between tempol and hydralazine that affect NO production than between tempol and other antihypertensive agents.

ROS have been shown to activate MAPK, which are important mediators of the intracellular signal transduction pathway (10, 14–16, 29). Baas et al. (45) showed that $O_2^-$ activated ERK1/ERK2 in rat vascular smooth muscle cells. Further studies by Yamakawa et al. (46) showed that lysophosphatidylcholine-induced ROS generation and ERK1/ERK2 activation are prevented by treatment with an NAD(P)H oxidase inhibitor, diphenylene iodonium, or overexpression of dominant-negative p47-phox (a cytosolic component of NAD(P)H oxidase), suggesting that NAD(P)H-dependent $O_2^-$ production participates in the activation of ERK1/ERK2. In this study, both ERK1/ERK2 and JNK activities were markedly increased in the renal cortical tissues of DS hypertensive rats. We also found that renal p38 MAPK activity was unchanged in these animals, suggesting different activation of each MAPK sub-family. The results from the present study also demonstrated that tempol treatment normalizes renal cortical ERK1/ERK2 and JNK activities in DS rats. In vivo studies by Xu et al. (47) showed that intravenous injection of Ang II and phenylephrine resulted in increases in vascular MAPK activities along with an elevation in systemic BP, suggesting that BP changes increase MAPK activity. Recently, we also examined the effects of Ang II and phenylephrine on ROS production and MAPK activity in conscious rats. The results showed that Ang II (200 ng/kg per min, intravenously) or phenylephrine (40 µg/kg per min, intravenously) increased systemic BP and TBARS levels in plasma and left ventricular tissues, accompanied by increases in MAPK activities in the aorta and left ventricular tissues. It is interesting that pretreatment with tempol did not affect Ang II– or phenylephrine-induced elevation in arterial pressure. However, increases in TBARS levels and MAPK activities induced by Ang II or phenylephrine were completely prevented by tempol (L. Zhang and Y. Abe, unpublished data). These results indicate that ROS production and MAPK activation induced by Ang II or phenylephrine are not simply caused by arterial pressure changes. It also should be noted that activation of glomerular MAPK may not be caused by sustained hypertension. Hamaguchi et al. (34) reported that glomerular ERK1/ERK2 and JNK activities were similar between spontaneously hypertensive rats and normotensive Wistar-Kyoto rats, whereas SHR showed similar hypertension to DS hypertensive rats. Importantly, the results from the present study showed that although hydralazine prevented the elevation of renal cortical JNK activity, renal cortical ERK1/ERK2 activity was not affected by hydralazine treatment. These data suggest that $O_2^-$ contributes to the progression of renal injury in DS hypertensive rats through ERK1/ERK2 activation. On the basis of the finding that both arterial pressure and JNK activity were reduced by tempol and hydralazine to a similar extent, it can be speculated that arterial pressure changes directly influence JNK activity in the renal cortical tissues of DS rats. Nevertheless, studies also indicate that ROS activate JNK in vascular smooth muscle cells (15, 16). Clearly, further studies are required to determine the contributions of other ROS (e.g., hydrogen peroxide and hydroxyl radicals) to MAPK activation and the progression of renal injury in DS hypertensive rats. It is also possible that ROS would cause glomerular injury through MAPK-independent mechanisms. As already mentioned, $O_2^-$ reacts with NO (48). Therefore, scavenging of NO by $O_2^-$ may directly contribute to the development of hyper-
tension and renal injury. Furthermore, peroxynitrite, which is the chemical combination of $O_2^-$ with NO, oxidizes arachidonic acid and thus stimulates the formation of a potent vasoconstrictor isoprostane (48).

In summary, the present study provides evidence that nephropathy is associated with increases in renal cortical TBARS levels and the activation of MAPK in DS hypertensive rats. It was also shown that both p22-phox and Nox-1 mRNA were upregulated in renal cortical tissues of DS rats. Furthermore, tempol treatment prevents the elevation of TBARS levels and was also shown that both p22-phox and Nox-1 mRNA were levels and the activation of MAPK in DS hypertensive rats. It propathy is associated with increases in renal cortical TBARS 

donic acid and thus stimulates the formation of a potent vaso-

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