Tempol Attenuates the Development of Hypertensive Renal Injury in Dahl Salt-Sensitive Rats

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Background: Dahl salt-sensitive (DS) rats given a high-salt diet develop renal lesions that are virtually identical to those in human hypertensive nephrosclerosis and are associated with increased oxidative stress. This study looks at the effects of a superoxide scavenger in preventing of hypertensive renal damage in high-salt–treated DS rats.

Methods: The DS rats (n = 5 per group) were treated with 0.3% NaCl diets (LS), 8% NaCl diets (HS), and 8% NaCl diets plus 10 mmol/L tempol in drinking water (HS+T) for 5 weeks. Systolic blood pressure (SBP) was measured by the tail-cuff method. As markers of renal damage, we measured serum creatinine, creatinine clearance, histopathologic indices, and transforming growth factor–β1 (TGF-β1; a mediator for renal fibrosis) expression. In addition, 8-hydroxy-2′-deoxyguanosine (8-OHdG)–positive cells and expression of heme oxygenase–1 (HO-1) were quantified as markers of oxidative stress.

Results: We found that a high-salt diet (8% NaCl) led to the development of hypertension, increased oxidative stress in the renal tissue (8-OHdG immunoreactive staining and HO-1 protein expression), increased renal histopathologic damage (arteriosclerosis index, matrix score, and interstitial volume) accompanied by accumulation of TGF-β1, and decreased creatinine clearance in the DS rats. These adverse effects of salt were prevented by the tempol supplementation.

Conclusions: Histopathologic and biochemical findings indicate that, in the DS rat, salt-induced hypertensive nephropathy is associated with increased oxidative stress. Superoxide mimetic tempol can reduce this detrimental effect of salt feeding through TGF-β1 suppression and consequently prevent the development of hypertension and hypertensive nephropathy.

Key Words: Oxidative stress, renal injury, hypertension, tempol.

Increased oxidative stress has been reported in several animal models of hypertension and in humans with essential hypertension.1,2 Dahl salt-sensitive (DS) rats comprise the most studied hereditary model of increased oxidative stress and salt-sensitive hypertension.3,4 We have previously reported increased urinary excretion of 8-isoprostane in high-salt–treated DS (H-DS) rats.5

Reactive oxygen species (ROS) have various roles in the development and maintenance of hypertension. Increased production of ROS promotes the conversion of NO to peroxynitrate, reducing the bioavailability of NO and in turn increasing vascular tone and reducing sodium excretion.6,7 In another mechanism for high blood pressure (BP) in DS rats, elevated oxidative stress may contribute to 20-hydroxyeicosatetraenoic acid (20-HETE) deficiencies, increasing chloride transport in the thick ascending limb of the loop of Henle and the resetting of pressure–natriuresis.8 The ROS are therefore important in the pathogenesis of hypertension.

Although hypertension is a risk factor for the development of end-stage renal disease, not all patients go on to develop renal dysfunction. The mechanisms underlying hypertensive nephropathy are poorly understood. However, several investigators have demonstrated a relationship between ROS and organ damage.3,8 Increased formation of hydrogen peroxide (H2O2) and superoxide (O2−) within tissue and blood is seen in inflammatory conditions as a result of activation of neutrophils, other phagocytes, vascular wall cells, and renal cells.9,10 Accumulation of O2− has also been implicated in the activation of nuclear factor–κB (NF-κB), which transcriptionally regulates many cellular genes involved in the early immune, acute phase. Oxidative stress may then exacerbate...
inflammatory responses, including raised levels of interleukin (IL)–1β, tumor necrosis factor–α (TNF-α), IL-2, IL-6, IL-8, cyclooxygenase-2, intracellular adhesion molecules, and transforming growth factor–β (TGF-β). These proteins may contribute to organ damage through functional disorder, organ destruction, and fibrous change. In fact, antioxidants have been reported to both renoprotective and antihypertensive effects. For instance, membrane-permeable superoxide dismutase (SOD) mimetic 4-hydroxy-2,2,6,6-tetramethyl piperidinol (tempol) and other antioxidants such as α-tocopherol reduce BP and urinary protein excretion. These studies found associations between increased oxidative stress and hypertensive organ damage in experimental animals. In particular, several investigators stated that ROS participates in transforming growth factor–β (TGF-β) expression and that TGF-β promotes renal fibrosi change that leads to renal damage.

The association between local ROS and histopathologic changes in renal tissue, in which TGF-β is likely to be involved, is not fully studied; and we hypothesize that antioxidant therapy reduces not only BP but also directly reduces hypertensive renal damage through suppression of TGF-β. The present study examines the effect of antioxidant therapy with tempol on markers of oxidative stress such as 8-hydroxy-2′-deoxyguanosine (8-OHdG) and expression of heme oxygenase–1 (HO-1). We also examine the effect of tempol on histopathologic indices that indicate the degree of renal damage such as glomerulosclerosis, arteriosclerosis index, and interstitial volume, and on the expression of TGF-β, which is a mediator of renal fibrosis, in the kidney of DS rats.

**Methods**

**Animal Preparation**

Experiments were performed on 5-week-old male DS rats (Sankyo Labo Service Corp., Tokyo, Japan) weighing 164 to 180 g. The rats were housed in our animal research facility under controlled temperature conditions (24°C), humidity (45% to 50%), and lighting (12:12 h light–dark cycle). Protocols were approved by the Institutional Animal Care and Use Committee of Nihon University School of Medicine and were performed according to the rules and regulations set out by the Japanese Council for Animal Care.

**Study Protocol**

The rats were fed a low-salt diet (0.3% NaCl) for 1 week before the start of the experiment. Water was allowed ad libitum throughout the study.

At age 5 weeks, DS rats were divided into three groups and observed for 5 weeks. Rats in the first group (n = 5) were fed a low-salt diet and vehicle. Those in the second group (n = 5) were fed a high-salt diet and vehicle. Those in the third group (n = 5) received the high-salt diet and 10 mmol/L of tempol purchased from Sigma (St. Louis, MO). Tempol was readily soluble in water and was given in the drinking water. Systolic blood pressure (SBP) was measured weekly by the tail-cuff method. The dose of tempol used in this study was comparable to that in previous reports and was high enough to reduce oxidative stress–mediated tissue damage in these studies.

The rats were observed for 5 weeks and then killed. Before the rats were killed, serum and 24-h urine samples were collected for creatinine clearance. The kidneys were preserved for histologic analysis and immunohistochemical examination by fixation in methyl Carnoy, and the renal cortices were kept for protein extraction by snap-freezing in liquid nitrogen.

**Histologic Examination**

The left kidneys were used for the optical microscope study; sections 2 μm thick were stained with periodic acid-Schiff (PAS) stain, hematoxylin and eosin stain, and Masson trichrome stain. A histologic examination was made independently by two observers without prior knowledge of the experimental groups. To quantify the glomerular matrix, 50 glomeruli were selected at random, and the degree of glomerular matrix expansion was determined as described by Raij et al. To quantify the arteriosclerotic change, 50 arterioles were selected at random, and the degree of arteriosclerosis was determined as described by Bader et al. Moreover, to estimate the relative interstitial volume of the kidney, tissue sections were examined with a 121-point (100 square) eyepiece micrometer as described by Bennett et al.

**Immunohistochemical Study**

Immunohistochemical study for TGF-β and 8-OHdG was carried out essentially as previously described in detail. Briefly, the sections for polyclonal rabbit antibody to TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA) were immersed in 10 mmol/L citrate acid (pH 6) and then microwaved four times for 3 min each. The sections for mouse monoclonal antibody to 8-OHdG (Japan Institute for the Control of Aging, Shizuoka, Japan) were immersed in methanol containing 0.3% H2O2 to deactivate the endogenous peroxidase. Both sections were then incubated in PBS containing 5% goat serum and incubated with the first antibodies at 4°C overnight. The sections were then incubated with anti-rabbit IgG (DAKO, Carpenteria, CA) and anti-mouse IgG (DAKO) for TGF-β and 8-OHdG, respectively. Subsequently, the slides of anti–TGF-β and anti–8-OHdG were developed in a solution of 3,3′-diamino-benzidine tetrahydrochloride solution. Nonspecific binding of secondary antibodies or detection complex was excluded by omitting the primary antibodies.
Computer Image Analysis

Immunoreactivity of TGF-β1 is observed in the cytosolic compartment, and that of 8-OHdG in the nuclear compartment. It is therefore possible to quantify the immunoreaction by mathematical integration of the staining density. To quantify the immunohistologic data for TGF-β1 and 8-OHdG, 20 appropriate fields at a magnification 40 × 10 were randomly selected for each type of immunostain. Color images were obtained as picture file format (PICT) files by a scanner (Photograb; Fuji Film, Tokyo, Japan) and PICT image files were opened in gray-scale mode using Image Pro Plus (Media Cybernetics, Silver Spring, MD). We then calculated the average immunoreactive percentage area for each immunostain and used them as TGF-β1-positive and 8-OHdG-positive indices.

Sodium Doceyl Sulfate–Polyacrylalnde Gel Electrophoresis and Immunoblotting

For sodium doceyl sulfate–polyacrylalnde gel electrophoresis, frozen kidney tissue was mixed in extraction buffer. The samples were sonicated and boiled for 5 min. Protein concentration was estimated by the Bradford method. A 30-μg quantity of total protein was loaded on a 5% to 20% gradient polyacrylamide gel and stained with Coomassie Blue. Immunoblot for HO-1 was performed as previously described.22,23 A 20-μg quantity of total protein was separated by electrophoresis on a 10% to 20% gradient polyacrylamide gel and stained with horseradish peroxidase (American Qualex, La Mirada, CA). Enhanced chemiluminescence (Amersham, Buckinghamshire, UK) was used for detection. Densitometric analysis was performed with Image Pro Plus (Media Cybernetics). The HO-1 protein bands were normalized using the respective β-actin protein bands.

Statistical Analysis

Values are expressed as mean ± SE and were analyzed for statistical differences by analysis of variance with multiple comparisons by the method of Bonferroni and Dunn. Values of P < .05 were considered to be significant.

Results

SBP and Renal Function

A significant attenuation of SBP was found in the HS+T rats compared with the HS rats after 5-week administration of 10 mmol/L tempol (LS: 144 ± 13, HS: 224 ± 7, HS+T: 186 ± 5) (Fig. 1). Also, tempol significantly improved serum creatinine and creatinine clearance (Table 1).

Histopathologic Study

Optical microscopy revealed that glomeruli, arterioles, and the interstitium of LS rat kidneys were mostly intact. In HS rats, small arteries or arterioles showed intimal thickening and onion-skin–like lesions with deposition of fibrinoid material in the intima, accompanied by ischemic glomerulus (Figs. 2a, 2b). No such pathologic findings were observed in HS+T rats (Fig. 2e). Focal segmental or global glomerulosclerosis was more frequently observed in HS rats (Fig. 2a) than in HS+T rats (Fig. 2e). In HS rats, some glomeruli were larger and there was increased PAS-positive material in the glomerular capillary walls or in Bowman’s space compared with that in HS+T rats (Figs. 2c, 2f). The tubulointerstitium of HS rats showed patchy changes with round cell infiltration and fibrosis around the sclerosing glomeruli and arterioles to a greater extent than in HS+T rats (Figs. 2d, 2g). Increases in the degree of glomerulosclerosis (LS: 80.4 ± 2.5, HS: 188.8 ± 16.1, HS+T: 133.2 ± 8.3) (Fig. 3A), arteriosclerosis (LS: 1.31 ± 0.05, HS: 2.36 ± 0.14, HS+T: 1.54 ± 0.06) (Fig. 3B), and interstitial changes (LS: 10.8 ± 1.3, HS: 21.2 ± 0.7, HS+T: 15.8 ± 0.6, Fig. 3C) were seen in HS rats compared with LS rats, and a significant suppression of these indices was seen in HS+T rats compared with HS rats.

Immunohistochemical Study

Cells expressing TGF-β1 were identified in tubules and glomeruli in the rats in each group. The TGF-β1 staining area was present to a lesser extent in HS+T and LS rats than in HS rats (Figs. 4a, 4c). The TGF-β1 staining area was less in HS+T rats than in HS rats (LS: 0.27 ± 0.06, HS: 0.90 ± 0.28, HS+T: 0.16 ± 0.03) (Fig. 5A). Cells with anti–8-OHdG staining nuclei displayed a diffuse distribution in the kidneys (Figs. 4b, 4d). The 8-OHdG–positive cell per area was higher in HS rats than in HS+T or LS rats (LS: 159 ± 7, HS: 347 ± 9, HS+T: 259 ± 20) (Fig. 5B).
Expression of HO-1 Protein in the Kidney

We sought evidence for altered levels of oxidative stress in the kidney tissue of tempol-treated DS rats. Immunoblotting showed that levels of HO-1 protein at euthanasia were significantly reduced in kidneys retrieved from animals that were treated versus control animals given the untreated high-salt diet.

For HO-1, an immunoreactive band was seen more clearly in HS rats than in LS or HS/T rats, and tempol significantly reduced expression of HO-1 protein compared with that in HS rats (LS: 0.1 ± 0.0, HS: 2.7 ± 0.2, HS/T: 1.5 ± 0.2) (Fig. 6).

Table 1. Body weight and renal function of Dahl salt-sensitive (DS) rats in each group at 5 weeks

<table>
<thead>
<tr>
<th></th>
<th>LS</th>
<th>HS</th>
<th>HS+T</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>339.58 ± 4.24</td>
<td>309.80 ± 12.42</td>
<td>314.52 ± 6.45</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.44 ± 0.02</td>
<td>1.02 ± 0.27*</td>
<td>0.50 ± 0.03</td>
</tr>
<tr>
<td>Clearance of creatinine (mL/min)</td>
<td>1.71 ± 0.09</td>
<td>0.72 ± 0.16*</td>
<td>1.382 ± 0.21</td>
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HS = high-salt diet (8% NaCl); HS+T = high-salt plus tempol diet (8% NaCl + 10 mmol/L tempol); LS = low-salt diet (0.3% NaCl).

* P < .05 v all other groups.

FIG. 2. Photomicrographs of renal tissue specimens in HS rats (a: ×200, b: ×200, c: ×200, d: ×100) or HS+T (e: ×200, f: ×200, g: ×100). (a) Severe arteriosclerosis with ischemic glomerular and interstitial damage in kidney section of HS rat; (b) small artery showing onion-skin-like lesion with fibrinoid material in kidney section of HS rats; (c) increase in size and increased periodic acid-Schiff (PAS)-positive material in glomerular capillary walls or Bowman’s space in kidney section of HS rats (PAS stain); (d) patchy changes with round cell infiltration and fibrosis in renal tubulointerstitium in kidney of HS rats; (e, f, g) almost intact glomeruli small arteries, and tubulointestitium in kidney of HS+T rat. (a, b, e) Hematoxylin and eosin stain; (c, f) PAS stain; (d, g) Masson trichrome stain. Abbreviations as in Fig. 1.

FIG. 3. Extent of glomerulosclerosis (A), arteriosclerosis (B), and interstitial change (C) in kidney sections from LS, HS, and HS+T rats. Values are semiquantitative scores from five rats and are expressed as the means ± SE. *Significant differences in HS rats versus LS and HS+T rats (P < .05). Abbreviations as in Fig. 1.
Discussion

In this study, we examined whether $O_2^{-}$ directly influences pathologic indices of the kidney and also whether tempol reduces BP and renal damage in DS rats. Markers of renal functions such as serum creatinine and creatinine clearance were significantly worse in HS rats than in LS rats. Also, histopathologic indices such as the arteriosclerosis index, matrix score, interstitial volume, and immunoreactivity for 8-OHdG and TGF-$\beta_1$ were all significantly higher in HS rats than in LS rats. Tempol successfully suppressed all indices in HS rats relative to HS rats and had an antihypertensive action. High salt consumption can therefore induce high BP and increasing generation of $O_2^{-}$, which directly causes functional and pathologic changes in the kidneys of DS rats.

We have demonstrated that high salt intake increases SBP, markers of the production of ROS and renal injury in DS rats. Meng et al. showed a decrease in renal Mn and Cu/Zn SOD protein expression in DS rats, even during low salt intake, accompanied by increased renal $O_2^{-}$ production. From this result, these investigators speculated that low-salt–treated DS (L-DS) rats might have a deficient production or stability of renal medullary Mn SOD, and salt-induced hypertension was associated with a further decrease in medullary Mn SOD protein expression. Their report agrees with the present study because the SOD mimetic tempol decreased BP and oxidant stress in the kidney in H-DS rats. On the other hand, we did not clearly evaluate the amount of SOD in the kidney of L-DS rats because we did not give tempol to L-DS rats. In our previous study, there are no significant difference of BP and urine 8-isoprostane between DS and Dahl salt-resistant (DR) rats given a low-salt diet. Moreover, in H-DS rats, we recognized an increase in BP and urine 8-isoprostane that which was not seen in high salt loaded DR rats.
Meng et al demonstrated that high salt increased urine 8-isoprostane excretion on both DS and DR rats. This difference between our study and that of Meng et al may be derived from the age of rats or from the salt content of the low-salt diet (our study, 0.3%; Meng et al study, 0.03%). In any event, we suggest that BP increase may be more important than salt loading for production of ROS. Furthermore, we recognized a correlation between urine 8-isoprostane and renal expression of 8-OHdG and HO-1 in DS rats (unpublished data). Therefore, we suspect that loss of SOD in L-DS rats that do not show high BP is too small to consider. However, we need to measure local O$_2^-$ production and SOD value in the future. On the other hand, superoxide inhibits prostacyclin synthese$^{25}$ and reacts with NO to form peroxynitrite, depleting NO in vascular endothelial cells and blunting the vasodilation pathway.$^{6}$ Peroxynitrite is itself a powerful cytotoxic agent and can generate toxic hydroxyl radicals. Also, NO synthase activity and production of NO in the outer medulla of DS rats were found to be significantly lower than in DR rats.$^{3,25}$

The O$_2^-$ generated acts both extracellularly and intracellularly, where it can have harmful effects including lipid peroxidation,$^{26,27}$ protein aggregation, and DNA destruction.$^{28}$ Several antioxidants including tempol, α-tocopherol, and glutathione reportedly reduce BP and adverse effects of O$_2^-$ in vivo.$^{1–4,11,29}$ Of these antioxidants, tempol has a low molecular weight; it is a stable SOD mimetic that is both metal independent and cell membrane permeable. Several investigators have suggested that tempol prevents the elevation of BP and reduces O$_2^-$-induced damage during inflammation, radiation, and ischemic/reperfusion injury.$^{30,31}$

Both 8-OHdG and HO-1 are markers of oxidative stress that are decreased by tempol in H-DS rats. The substance 8-OHdG, which is the DNA base-modified product and the most popular marker for evaluating oxidative DNA damage, has the biologic significance of including G:C to T:A base pairs.$^{32}$ Renolipid biliverdin are powerful antioxidants, and HO-1 has a renoprotective role.$^{33,34}$ Haugen et al stated that HO-1 is an oxidative sensitive protein and is a useful marker for oxidative stress.$^{33}$

Transforming growth factor–β$_1$ promotes tissue fibrosis by upregulating the genes encoding extracellular matrix proteins. Jiang noted that cellular ROS mediates TGF-β-induced plasminogen activator inhibitor-1 upregulation in mesangial cells.$^{15}$ Iglesias-De La Cruz et al reported that H$_2$O$_2$ induces TGF-β$_1$ synthesis and thereby increases extracellular matrix gene expression in cultured human mesangial cells.$^{12}$ The present study also shows that tempol attenuates immunoreactivity for TGF-β$_1$ in the kidney of H-DS rats. Also, renal interstitial fibrosis was reduced in the tempol-treated group. Therefore, TGF-β may be central to the deterioration of renal function. As a result, tempol may lower renal fibrosis caused by ROS, through TGF-β suppression. As recently reported by Nishiyama et al,$^{35}$ ROS contribute to the progression of renal injury through ERK1/ERK2 activation in DS hypertensive rats. In the present study, we evaluated neither mitogen-activated protein kinase (MAPK) activity nor that downstream of MAPK cascade and TGF-β-Smad signaling.

Because tempol prevents the development of hypertension and renal dysfunction while reducing renal O$_2^-$ production and oxidative stress markers such as 8-OHdG and HO-1, our data suggest (but do not prove) that oxidative stress contributes not only to the development of hypertension but also to the development of renal dysfunction in H-DS rats. Of course, it is possible that the effect of tempol on ROS levels depends on arterial BP reduction. However, several investigators stated that tempol suppresses not only ROS levels but also ROS-related tissue damages in the absence of arterial BP changes in vivo.$^{14,16}$ We therefore infer that the effects of tempol on oxidative stress markers are not solely the result of arterial BP reduction.

Although we successfully demonstrated that tempol inhibits improvement of hypertensive organ damage in rats, the number of animals in each group was small. In addition, for BP measurement, we did not use telemetry. Because the tail-cuff method is a temporary BP measurement method, we could not investigate average 24-h BP and BP during the sleep period, which are mostly associated with hypertensive organ damage. Measurement of BP by telemetry produces reproducible, non-stressed, and continuous BP values in the rodent hypertensive model. Therefore, telemetry may be a more useful method to investigate relation of hypertension and hypertensive organ damage.

In conclusion, long-term administration of tempol, which is a stable, membrane-permeable SOD mimetic, significantly reduced SBP in H-DS rats. Tempol also significantly reduced renal TGF-β$_1$ expression and damage in H-DS rats. Reduction of renal damage is important in suppressing the development of hypertension. This is the first study to demonstrate, using histochemical and immunopathologic methods, that scavenging of O$_2^-$, both extracellularly and intracellularly, with tempol suppresses renal damage in H-DS rats. The antihypertensive actions of tempol depend partly on reducing renal damage caused by oxidative stress.

References


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