Contributions of 20-HETE to the Antihypertensive Effects of Tempol in Dahl Salt-Sensitive Rats

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Abstract—The present study evaluated whether reactive oxygen species–induced alterations in bioavailability of 20-HETE in the kidney contribute to the antihypertensive and renoprotective actions of antioxidant therapy with Tempol in the Dahl salt-sensitive (DS) rat. Superoxide inhibited the synthesis of 20-HETE by renal cortical microsomes and enhanced breakdown of 20-HETE to a more polar product. Addition of Tempol (1 mmol/L) to the drinking water reduced mean arterial pressure from 187 ± 9 to 160 ± 3 mm Hg in DS rats fed an 8%-NaCl diet for 2 weeks. 20-HETE excretion rose from 117 ± 11 to 430 ± 45 ng/day, and 8-isoprostane excretion fell from 14 ± 1 to 8 ± 1 ng/day. Tempol also increased creatinine clearance and reduced the severity of renal damage in DS rats fed a high-salt diet. Blockade of NO synthase with Nω-nitro-L-arginine methyl ester (25 mg/kg per day) did not attenuate the antihypertensive or renoprotective actions of Tempol in DS rats. However, chronic blockade of the formation of 20-HETE with N-hydroxy-N′-(4-butyl-2 methylphenyl) formamidine (HET0016, 10 mg/kg per day) blunted the antihypertensive and renoprotective effects of Tempol. These findings indicate that the antihypertensive and renoprotective effects of reducing oxidative stress with Tempol depends in part on increasing the bioavailability of 20-HETE in the kidney. (Hypertension. 2003;41[part 2]: 697-702.)

Key Words: free radicals ■ reactive oxygen species ■ superoxide ■ blood pressure ■ sodium ■ cytochrome P450

Increased oxidative stress has been reported in renovascular,1,2 angiotensin II–induced,3,4 mineralocorticoid,5,6 and lead-induced hypertension,7 as well as in the spontaneously hypertensive rat8-12 and Dahl salt-sensitive (DS)13-15 genetic models of hypertension. Antioxidant therapy with the membrane-permeable superoxide dismutase (SOD) mimetic Tempol2,3,5,11,12 or with other antioxidants such as vitamin E16 increases oxidative stress, induced by glutathione depletion17 or renal medullary infusion of an SOD inhibitor,18 has been reported to increase blood pressure and/or end-organ damage in some of these models. Similarly, increased oxidative stress, induced by glutathione depletion17 or renal medullary infusion of an SOD inhibitor,18 has been reported to increase blood pressure in normotensive Sprague-Dawley rats. Alterations in redox status and oxidative stress have also been observed in human essential hypertensives19,20 and in women with preeclampsia.21 Together, these studies document that there is an association between increased oxidative stress and hypertension in man and experimental animals, but the mechanisms by which oxidative stress contribute to the development and maintenance of hypertension remain to be established.

Most investigators have concluded that increased oxidative stress contributes to the development of hypertension by increasing vascular tone and reducing sodium excretion. These actions have been attributed to increased conversion of NO to peroxynitrite by reactive oxygen species (ROS), leading to decreased bioavailability of NO. However, no studies have confirmed that chronic blockade of the formation of NO attenuates the antihypertensive effects of antioxidants, or that NO donors or L-arginine (L-Arg) supplementation blocks the hypertensive actions of elevated production of ROS. Thus, it remains possible that ROS may influence renal and vascular function by some other mechanism, such as altering levels of CYP450 metabolites of arachidonic acid (AA) in the kidney or via direct effects on ion channels. In this regard, epoxycisatrienoic acids (EETs) and 20-HETE are the primary CYP450 metabolites of AA produced in the kidney, where they inhibit sodium transport in the proximal tubule and/or thick ascending limb of the loop of Henle (TALH).22 Because these compounds are unsaturated fatty acids, they may directly interact with ROS via lipid peroxidation, as has been reported for AA,23 to form more polar inactive metabolites or even vasoactive metabolites, like isoprostanes. It is also possible that ROS may inhibit the formation of EETs and/or 20-HETE by oxidizing the heme moiety of CYP450 enzymes in a manner similar to that described for NO.24 Thus, elevated oxidative stress may contribute to the deficiency in the renal formation of 20-HETE previously observed in DS rats, which contributes to elevated chloride transport in the TALH25 and the resetting of pressure-natriuresis in this model.26

Thus, the aim of the present study was to examine the effects of antioxidant therapy with Tempol on the urinary...
excretion of 20-HETE in DS rats and the contribution of changes in 20-HETE bioavailability in mediating the antihypertensive and renoprotective effects of Tempol.

Methods

Effect of O2− on Renal Formation of 20-HETE and Metabolism of 20-HETE

Microsomes from the renal cortex of rats were prepared by differential centrifugation as previously described.17 The metabolism of AA to 20-HETE was determined by incubating microsomal protein (0.5 mg) with [14C]-AA (0.1 µCi, 40 µmol/L) for 15 minutes at 37°C. To evaluate the effect of O2− on 20-HETE production, a xanthine/xanthine oxidase (X/XO, 10−6 M/150 µU) O2−-generating system or an O2− donor (K2O2, 1 mmol/L) was added to the microsomal incubations. Products of AA were extracted with ethyl acetate and separated by reverse-phase high-performance liquid chromatography (HPLC) and monitored with a radioactive flow detector, as previously described.18

The effects of O2− on stability of [14C]-20-HETE in aqueous solutions were examined. 20-HETE was prepared by incubating [14C]-AA with human recombinant CYP4F3 enzyme purchased from Gentest Corp and purified by reverse-phase HPLC. [14C]-20-HETE (0.1 µCi, 40 µmol/L) was incubated in a 0.1 mol/L KPO2 buffer for 15 minutes with vehicle or 1 mmol/L K2O2. The samples were extracted with ethyl acetate, and the products were separated by reverse-phase HPLC.

Preparation of Animals for Chronic Study

These experiments were conducted on 45 male DS/Jr rats obtained from a colony derived from rats obtained from John Rapp (Medical College of Ohio) and maintained at the Medical College of Wisconsin since 1997. The rats were housed in an Animal Care Facility approved by the American Association for the Accreditation of Laboratory Animal Care, and all protocols were approved by the Medical College of Wisconsin’s Animal Welfare Committee. The rats were maintained on a low-salt (0.4% NaCl) diet purchased from Dyets Inc (No. 113755) until they were 10 weeks of age. Canulas were implanted in the femoral artery and vein using aseptic surgical techniques. After a 1-week recovery period, baseline mean arterial pressure (MAP) was recorded for 3 consecutive days. Catheters were connected to transducers interfaced with a computerized data acquisition system, and blood pressure and heart rate were recorded at a frequency of 300 Hz for 5 hours/day from 10:00 AM and 3:00 PM in conscious unrestrained rats housed in their home cages. During a recording session, MAP was averaged over 1-minute periods, and a mean daily MAP was calculated for each recording session. The rats were then switched to a high-salt (8% NaCl) diet for 2 weeks and assigned to 1 of 6 treatment groups. Group 1 (n=9) received the SOD mimetic Tempol in drinking water (1 mmol/L). Rats in group 2 (n=10) were treated with Tempol (1 mmol/L) and N-nitro-L-arginine methyl ester (L-NAME; 75 mg/L) alone in drinking water. The rats in group 4 (n=7) received Tempol (1 mmol/L) in drinking water and daily intravenous injections of HET0016 (N-hydroxy-N’-[4-butyl-2 methylphenyl] formamidine, 10 mg/kg), a selective inhibitor of the formation of 20-HETE, to investigate the possible contribution of 20-HETE to the effects of Tempol.20 The vehicle for HET0016 was 10% lecithin in 0.9% NaCl. Rats in group 5 were treated with daily intravenous injections of HET0016 (10 mg/kg) alone. Group 6 served as the control group (n=7) and drank regular tap water during the dietary salt load. Blood pressure was measured on days 3, 5, 7, 10, 12, and 14 of the high-salt diet.

During the control period and after 2 weeks on a high-salt diet, a 24-hour urine sample was collected into glass bottles that were packed in ice and that contained 25 mg triphenyl phosphine to measure urinary excretion of 20-HETE, 8-isoprostane, proteinuria, and creatinine. Blood samples were collected on the same days to measure plasma creatinine levels. Kidneys were collected, sectioned, and stained by the periodic acid–Schiff method to assess the degree of glomerulosclerosis as previously described.50

Analytical Determinations

The concentration of 20-HETE in urine samples was measured via a fluoroscent HPLC assay, as previously described.28 Urinary 8-isoprostane concentrations were measured via an enzymatic immunoassay kit (Cayman Chemical, No. 416358), after extraction from the samples by immunoaffinity chromatography. Urinary protein concentration was determined using the Bradford method and bovine serum albumin as a standard.

Measurement of NO Synthesis in Rats Treated With L-NAME

We compared NOS activity in the brain and renal papilla of DS rats fed a high-salt diet for 2 weeks and treated chronically with either L-NAME (25 mg/kg per day in drinking water; n=6) or vehicle (n=9) by measuring the conversion of L-[14C]-arginine to l-citrulline, as previously described.97 Kidneys and brains were removed and homogenized in a minimum volume (1 mL for brain, 100 µL for papilla) of 10 mmol/L potassium buffer (pH 7.7) containing 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 7.5 µg/mL protease inhibitor cocktail (P-8340, Sigma). Homogenates were centrifuged at 3000g for 10 minutes and 9000g for 20 minutes. Total protein concentration of the homogenates was determined using the Bradford method and γ-globulin as the standard; 300 µg of papilla homogenate or 100 µg of brain homogenate was incubated with L-[14C]-arginine and the appropriate cofactors for 45 minutes at 37°C, and the conversion of L-Arg to l-citrulline was determined by reverse-phase HPLC equipped with a radioactive flow detector.

Statistical Analysis

Mean values±SE are presented. The significance of difference in mean values between groups was evaluated by ANOVA followed by the Student-Newman-Keuls test. A value of P<0.05 for a 2-tailed test was considered significant.

Results

Effect of O2− on Renal Formation of 20-HETE and the Metabolism Of 20-HETE

The effects of O2− on synthesis of 20-HETE by renal microsomes are presented in Figure 1A. Addition of 1 mmol/L K2O2 to the incubations reduced the formation of 20-HETE by 97%. Addition of the X/XO O2−-generating system, which has previously been shown to produce O2− in aqueous solutions,31 inhibited the reaction by 40%.

The effect of K2O2 on the stability of 20-HETE in aqueous solution is illustrated in the representative chromatogram presented in Figure 1B. Addition of K2O2 oxidized [14C]-20-HETE to more polar products. Using liquid chromatography–mass spectrometry (LC-MS), we isolated the large peak and determined that it had a molecular weight of 353, which is identical to that of isoprostanes and is consistent with the addition of 2 hydroxyl groups across the double bonds of 20-HETE.

Contribution of NO or 20-HETE to the Antihypertensive Action of Chronic Antioxidant Therapy With Tempol in DS Rats

The effects of inhibition of NO or 20-HETE formation on the effects of Tempol on MAP in DS rats fed an 8%-salt diet for 2 weeks are summarized in Figure 2. Baseline MAP was not significantly different in all groups studied and averaged 122±3 mm Hg. MAP rose by 62 mm Hg in control DS rats
fed a high-salt diet for 2 weeks. Tempol blunted the hypertensive effect of the high-salt diet in DS rats by \( \approx 50\% \). Blockade of NOS activity with L-NAME had no significant effect on the ability of Tempol to lower blood pressure. In contrast, chronic blockade of 20-HETE formation with HET0016 reduced the antihypertensive effect of Tempol by 67%. MAP in DS rats treated with HET0016 alone was not significantly different from that measured in control DS rats after 2 weeks of the high-salt diet. L-NAME accelerated the rate of development of hypertension in DS rats during the first week of the high-salt diet; however, the final blood pressure achieved was not different from that seen in the control DS rats. NOS activity in renal papilla and brain of DS rats fed a high-salt diet (\( n = 9 \)) averaged 1.38 \( \pm \) 0.2 and 5.54 \( \pm \) 0.5 nmol/L per hour per milligram of protein, respectively. Chronic treatment of the rats with L-NAME (\( n = 6 \)) reduced NOS activity by 63% in the papilla and 50% in the brain.

**Effect of Chronic Antioxidant Therapy With Tempol on Excretion of 8-Isoprostane and 20-HETE in DS Rats**

The effects of Tempol on the excretion of 8-isoprostane and 20-HETE are presented in Figure 3. Baseline excretion of 8-isoprostane in prehypertensive DS rats (panel A) was similar in all groups studied and averaged 7.4 \( \pm \) 0.3 ng/day. This value is 3 to 4 times higher than what we typically measure in normotensive Sprague-Dawley, Lewis, and Brown-Norway rats under the same experimental conditions (data not shown). 8-Isoprostane excretion doubled in DS rats fed a high-salt diet for 2 weeks. Chronic treatment of the rats with Tempol reduced 8-isoprostane levels by 50% in the rats fed a high-salt diet. 8-Isoprostane excretion was not altered by chronic treatment with L-NAME or HET0016, nor did either drug affect the ability of Tempol to reduce 8-isoprostane excretion in the DS rats fed a high-salt diet.

Baseline excretion of 20-HETE in DS rats (Figure 3B) fed a low-salt diet was similar in all experimental groups and averaged 174 \( \pm \) 11 ng/day. 20-HETE excretion was not altered by 2 weeks of a high-salt diet. In contrast, the excretion of 20-HETE rose to 430 \( \pm \) 45 ng/day in rats treated with Tempol. Chronic blockade of NO synthesis with L-NAME did not prevent the increase in 20-HETE excretion in rats given Tempol. However, chronic treatment of DS rats with HET0016 blocked the ability of Tempol to increase 20-HETE excretion (88 \( \pm \) 12 ng/day). L-NAME alone had no affect on 20-HETE excretion in DS rats fed a high-salt diet. In contrast,
The effects of Tempol on renal function, hypertrophy, and end-organ damage in DS rats fed a high-salt diet for 2 weeks exhibited significant hypertrophy and glomerulosclerosis relative to normotensive Lewis, Brown-Norway, and Sprague-Dawley rat strains. This finding confirms previous reports of increased O2 levels, by using either a O2 donor or a O2-generating system, inhibits CYP450-dependent metabolism of AA in renal cortical microsomes. In addition to inhibiting the formation of 20-HETE, O2 also promotes the conversion of 20-HETE to a more polar product through lipid peroxidation.

The present study examined whether O2 influences the synthesis and/or breakdown of 20-HETE in the kidney, and whether an elevation in renal 20-HETE levels contributes to the antihypertensive actions of Tempol in DS rats. The results indicate that increasing O2 levels, by using either a O2 donor or a O2-generating system, inhibits CYP450-dependent metabolism of AA in renal cortical microsomes. In addition to inhibiting the formation of 20-HETE, O2 also promotes the conversion of 20-HETE to a more polar product with a molecular weight of 353, which is consistent with the formation of an ω-hydroxylation metabolite of isoprostane. Thus, raising O2 levels in vitro can inhibit the synthesis of 20-HETE by renal tissues and enhance the breakdown and metabolism of 20-HETE by lipid peroxidation.

Because we previously reported that a deficit in 20-HETE appears to contribute to an elevation in loop chloride transport20 and resetting of pressure-natriuresis20 in DS rats, we evaluated whether oxidative stress might contribute to this deficiency. We found that baseline 8-isoprostane excretion, a marker for oxidative stress, was 4 times higher in DS rats relative to normotensive Lewis, Brown-Norway, and Sprague-Dawley rat strains. This finding confirms previous observations that the DS rat is a model of elevated oxidative stress, as evidenced by increased vascular and renal produc-

**Effect of Chronic Antioxidant Therapy With Tempol on Renal Function and End-Organ Damage in DS Rats**

The effects of Tempol on renal function, hypertrophy, and injury are presented in the Table. The kidneys of DS rats fed a high-salt diet for 2 weeks exhibited significant hypertrophy and end-organ damage, as indexed by a significant increase in left kidney weight and glomerular diameter (normal, 90 μm), histological evidence of severe glomerulosclerosis, proteinuria, elevated plasma creatinine concentration (normal, 0.3 mg/dL), and reduced endogenous creatinine clearance (normal, ≥2 mL/minute). Chronic treatment of the rats with Tempol reduced the degree of renal hypertrophy, proteinuria, and glomerulosclerosis and improved creatinine clearance in DS rats fed a high-salt diet. Chronic treatment of rats with L-NAME did not prevent the renoprotective actions of Tempol. Administration of L-NAME or HET0016 alone had no significant affect on proteinuria, renal hypertrophy, glomerulosclerosis or creatinine clearance in DS rats fed a high-salt diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>Creatinine Clearance, ml/min</th>
<th>Plasma Creatinine, mg/dL</th>
<th>Protein Excretion, mg/day</th>
<th>Glomerular Injury Score</th>
<th>Left Kidney Weight, g</th>
<th>Glomerular Diameter, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>0.30±0.08</td>
<td>0.75±0.06</td>
<td>211±30</td>
<td>3.12±0.07</td>
<td>2.09±0.08</td>
<td>156±3.0</td>
</tr>
<tr>
<td>Tempol (n=9)</td>
<td>0.88±0.13*</td>
<td>0.56±0.03*</td>
<td>91±3*</td>
<td>2.36±0.03*</td>
<td>1.66±0.06*</td>
<td>107±3.1*</td>
</tr>
<tr>
<td>Tempol+L-NAME (n=7)</td>
<td>0.76±0.06*</td>
<td>0.66±0.01</td>
<td>110±6*</td>
<td>2.41±0.05*</td>
<td>1.64±0.05*</td>
<td>108±3.2*</td>
</tr>
<tr>
<td>Tempol+HET0016 (n=6)</td>
<td>0.43±0.11†</td>
<td>0.77±0.04†</td>
<td>227±31†</td>
<td>2.84±0.07†</td>
<td>2.18±0.11†</td>
<td>145±3.4†</td>
</tr>
<tr>
<td>L-NAME (n=7)</td>
<td>0.44±0.03</td>
<td>0.81±0.05</td>
<td>190±18</td>
<td>3.35±0.06</td>
<td>1.90±0.08</td>
<td>150±3.1</td>
</tr>
<tr>
<td>HET0016 (n=5)</td>
<td>0.41±0.11</td>
<td>0.79±0.05</td>
<td>272±36</td>
<td>3.39±0.04</td>
<td>2.17±0.04</td>
<td>151±3.8</td>
</tr>
</tbody>
</table>

All values are mean±SE.

*P<0.05 vs control DS rats fed a high-salt diet (8% NaCl) for 2 weeks; †P<0.05 vs Tempol-treated DS rats.

Discussion

The present study examined whether O2 influences the synthesis and/or breakdown of 20-HETE in the kidney, and whether an elevation in renal 20-HETE levels contributes to the antihypertensive actions of Tempol in DS rats. The results indicate that increasing O2 levels, by using either a O2 donor or a O2-generating system, inhibits CYP450-dependent metabolism of AA in renal cortical microsomes. In addition to inhibiting the formation of 20-HETE, O2 also promotes the conversion of 20-HETE to a more polar product with a molecular weight of 353, which is consistent with the formation of an ω-hydroxylation metabolite of isoprostane. Thus, raising O2 levels in vitro can inhibit the synthesis of 20-HETE by renal tissues and enhance the breakdown and metabolism of 20-HETE by lipid peroxidation.

Because we previously reported that a deficit in 20-HETE appears to contribute to an elevation in loop chloride transport20 and resetting of pressure-natriuresis20 in DS rats, we evaluated whether oxidative stress might contribute to this deficiency. We found that baseline 8-isoprostane excretion, a marker for oxidative stress, was 4 times higher in DS rats relative to normotensive Lewis, Brown-Norway, and Sprague-Dawley rat strains. This finding confirms previous observations that the DS rat is a model of elevated oxidative stress, as evidenced by increased vascular and renal produc-
tion of $\text{O}_2^{-}$, elevated plasma levels of $\text{F}_2$-isoprostanes, higher urinary excretion of $\text{F}_2$-isoprostanes, and reduced SOD levels in the kidney relative to Dahl salt-resistant rats. We also found that the baseline excretion of 20-HETE was 3- to 4-fold lower than we have previously reported in salt-sensitive Lewis, Brown-Norway, and Sprague-Dawley rats. Consistent with the findings of Meng et al., we found that 8-isoprostane excretion increased after development of hypertension in DS rats fed a high-salt diet for 2 weeks. Tempol prevented the increase in 8-isoprostane excretion, increased 20-HETE excretion by a factor of 4, and reduced the rise in arterial pressure by >50%. Tempol was also renoprotective and reduced proteinuria, renal hypertrophy, and the degree of glomerulosclerosis in DS rats fed a high-salt diet. These findings indicate that $\text{O}_2^{-}$ production increases in DS rats fed a high-salt diet, and this contributes to the reduced excretion of 20-HETE. It also indicates that scavenging of $\text{O}_2^{-}$ increases renal 20-HETE levels, and that this contributes to the antihypertensive effects of Tempol by promoting sodium excretion.

We evaluated the effect of chronic blockade of the formation of 20-HETE on the ability of Tempol to reduce blood pressure and renal damage in DS rats and found that the effects of Tempol were largely reversed by chronic inhibition of the formation of 20-HETE with HET0016, which effectively lowered 20-HETE excretion. This finding indicates that increased ROS formation may contribute to the deficiency in renal 20-HETE formation that we previously reported in DS rats and is consistent with other reports that oxidative stress is elevated in the kidney and vasculature of DS rats. Overall, the present study provides further evidence that decreased renal production and increased breakdown of 20-HETE contributes to the sodium retaining and prohypertensive actions of ROS in the kidney.

The majority of previous studies examining the antihypertensive actions of Tempol have focused on the reaction of $\text{O}_2^{-}$ with NO and concluded that Tempol likely lowers blood pressure by increasing NO bioavailability by promoting vasodilation and natriuresis. However, in the present study, we found that blockade of NO synthesis with L-NAME did not affect the antihypertensive and renoprotective actions of Tempol in DS rats. In other studies, we confirmed that this dose of L-NAME reduced NO synthase activity in the renal papilla by 63±3% (n=7). In vivo, the inhibition is likely to be much greater when one considers the fact that L-NAME is a competitive inhibitor and that preparation of tissues for the NOS assay involves a dilution factor of >100. Thus, it is unlikely that the lack of L-NAME to prevent the antihypertensive effects of Tempol was due to incomplete inhibition of renal NO synthase activity. The fact that NO inhibition did not affect the ability of Tempol to reduce blood pressure and renal damage may not be entirely surprising, given that several recent studies have emphasized that there is a NO deficit in the DS rat. In this regard, Yuan and Cowley and Szentivanyi et al. have reported that synthesis of NO and expression of NO synthase protein are reduced in the medulla of DS rats. These findings are also consistent with the observation that systemic injection of L-Arg into the renal medulla normalizes pressure natriuresis and prevents salt-induced hypertension in DS rats. In the present study, we also found that chronic treatment of the rats significantly increased the rate of development of hypertension on the first week of the high-salt diet. This finding is consistent with the results of previous studies, demonstrating that part of the antihypertensive effect of Tempol is due to increases in NO bioavailability. However, chronic treatment of DS rats did not affect the final blood pressure outcome after 2 weeks of a high-salt diet relative to the control rats. Furthermore, L-NAME treatment did affect the renoprotective effects of Tempol treatment after 2 weeks of a high-salt diet. Thus, our findings suggest that the long-term (2-week) beneficial effects of reducing oxidative stress with Tempol in the DS model depend on a mechanism that is independent of NO.

**Perspectives**

The results indicate that $\text{O}_2^{-}$ inhibits the formation of 20-HETE in renal cortical microsomes and enhances the degradation of 20-HETE in vitro and provides convincing evidence that increased renal 20-HETE bioavailability contributes to the antihypertensive and renoprotective actions of Tempol in DS rats. These findings confirm that elevated oxidative stress is an important contributor to the development and progression of hypertension and renal disease in the DS rat, and antioxidants and other agents that enhance the renal production of 20-HETE may have therapeutic implications in the treatment of salt-sensitive hypertension and/or associated end-organ damage.

**Acknowledgments**

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**References**


