Arterial Pressure Response to the Antioxidant Tempol and ET_B Receptor Blockade in Rats on a High-Salt Diet

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Abstract—We hypothesized that increased superoxide contributes to mean arterial pressure (MAP) regulation in male Sprague-Dawley rats fed a high-salt diet and/or during endothelin (ET_B) receptor blockade. Four groups on either a normal- or high-salt diet were studied for 1 week: (1) control; (2) tempol, a superoxide dismutase mimetic, in their drinking water (1 mmol/L); (3) A-192621, an ET_B antagonist, in their food (10 mg/kg daily); or (4) both tempol and A-192621. Without ET_B blockade, tempol had no effect on MAP (telemetry) in rats on the normal-salt diet but significantly reduced MAP in rats on the high-salt diet (100±3 vs 112±2 mm Hg, P<0.05). On the normal-salt diet, A-192621 increased MAP with or without tempol. Under high-salt conditions, tempol attenuated the increase in MAP produced by A-192621, but only during the initial days of treatment. Plasma 8-isoprostanes were increased in all rats on the high-salt diet and were further increased after 3 days of A-192621 but not after 7 days; tempol inhibited the increase produced by A-192621 but had no influence on the increase produced by high salt. H_2O_2 excretion was significantly higher in rats on a high-salt diet for the 7-day drug treatment compared with those on a normal-salt diet. Tempol further increased H_2O_2 excretion in rats on a high-salt diet, an effect accelerated in A-192621–treated rats. These data suggest that blood pressure lowering by tempol in rats on a high-salt diet may be unrelated to reductions in superoxide and that renal H_2O_2 may account for the limited ability of tempol to attenuate hypertension produced by ET_B receptor blockade. (Hypertension. 2004;44:770-775.)

Key Words: endothelin ■ oxidative stress ■ blood pressure monitoring ■ prostaglandins ■ hypertension, sodium dependent ■ free radicals

Reactive oxygen species (ROS) have been implicated in the development of hypertension and other pathologic processes. There is evidence that increased ROS may contribute to the elevated blood pressure in the spontaneously hypertensive rat; the 2-kidney, 1-clip hypertensive rat; the angiotensin II–infused rat, the doxycorticosterone acetate–salt rat, and the Dahl salt-sensitive rat.1–10 Tempol, a superoxide dismutase (SOD) mimetic, has been shown to lower ET-1 by removing it from the systemic circulation, thereby limiting ETA receptor activation.13,14 Chronic ETB receptor blockade increases plasma ET-1 levels and produces salt-dependent hypertension.15 Similarly, in the ET_B-deficient rat, both plasma ET-1 concentrations and basal arterial pressure are significantly elevated and are further increased by high salt intake.16,17 Animals on a high-salt diet have an increase in urine ET-1 levels as well as higher indices of oxidative stress.1–10,18–20

Recently, our laboratory has provided evidence that renal ET_B receptors function to increase medullary blood flow in rats on a high-salt diet, consistent with a role for ET_B receptors in controlling renal salt and water balance.21

In vitro studies have demonstrated that ET-1 can directly stimulate superoxide production through the ETA receptor.19,20 More recently, it was shown that elevations in arterial pressure produced by long-term infusion of exogenous ET-1 can be reduced by treatment with tempol.10 Therefore, the purpose of the current study was to determine the role of superoxide in blood pressure regulation under conditions of elevated endogenous ET-1. Experiments determined the effect of the SOD mimetic tempol on arterial pressure in rats on a high-salt diet and during ET_B receptor blockade.

Methods

Telemetry Blood Pressure Measurements

Telemetry transmitters (Data Sciences) were implanted according to the manufacturer’s specifications into male Sprague-Dawley rats (175 to 200 g; Harlan Laboratories, Indianapolis, Ind) as previously described.15 In brief, a midline incision was used to expose the...
abdominal aorta that was momentarily occluded to allow implantation of the transmitter catheter, which was secured in place with tissue glue. The transmitter body was sutured to the abdominal wall while closing the incision. The skin was closed with staples that were removed 7 to 10 days later, after the incision had healed. Rats were returned to their individual cages and allowed to recover for at least 1 week before being used in experiments. The cages were placed on top of the telemetry receivers, and arterial pressure measurements were continuously recorded except on days when rats were placed in metabolic cages.

Protocol
Rats were given free access to chow that contained either normal (0.8%) or high (10%) salt (NaCl) for 2 weeks. During the second week, separate groups of rats on the normal- or high-salt diet were given the ET<sub>B</sub> receptor antagonist A-192621 at a dose of 10 mg/kg daily in the food with or without tempol in the drinking water (1 mmol/L). This dose of A-192621 has been shown to be effective at blocking ET<sub>B</sub>-mediated hemodynamic and renal responses. Also, administration of tempol at this concentration in the drinking water has been shown by several laboratories to reduce superoxide levels in kidney and vascular tissue. At the end of each week, rats were placed in metabolic cages for 24-hour urine collection. After 3 or 7 days of treatment with A-192621 and/or tempol, rats were anesthetized with sodium pentobarbital (65 mg/kg IP), and blood was withdrawn from the abdominal aorta for analysis.

Assays and Chemicals
Plasma concentrations of ET and 8-iso-prostaglandin F<sub>2α</sub> (8-isoprostane) were analyzed by enzyme immunoassay (R&D Systems and Cayman Chemical, respectively). Urine H<sub>2</sub>O<sub>2</sub> was determined by the amplex red fluorescent dye assay (Molecular Probes). All normal- and high-salt rat chow was obtained from Harlan Teklad. A-192621 was kindly provided by Abbott Laboratories (Abbott Park, Ill.). Tempol was obtained from Sigma Chemical.

Statistical Analysis
ANOVA for repeated measures combined with post hoc contrasts was used for statistical evaluation of mean values each week for telemetry measurements (SuperANOVA, Abacus Concepts Inc). Two-way ANOVA with Bonferroni post tests was used for renal function data and the biochemical assays (Graph Pad Prism 4, Graph Pad Software, Inc). Values are reported as mean±SE with *P<0.05 being considered significant.

Results
On a normal-salt diet, all groups of rats had similar baseline mean arterial pressure (MAP) measurements, with the overall 24-hour average being 98±1 mm Hg (Figure 1). Treatment with the ET<sub>B</sub>-selective antagonist A-192621 (10 mg/kg daily) significantly increased MAP compared with control (114±4 and 101±3 mm Hg, respectively). Tempol had no effect on MAP in either control (97±5 mm Hg) or A-192621 (111±3 mm Hg)–treated rats. Rats on a high-salt diet had a baseline 24-hour average MAP of 109±1 before tempol or A-192621 treatment (Figure 2) that was significantly greater than rats on a normal-salt diet (P<0.05). As previously reported, ET<sub>B</sub> receptor blockade caused MAP to increase even further in rats on a high-salt diet (140±5 mm Hg) compared with rats on a normal-salt diet. Tempol significantly lowered MAP in rats on the high-salt diet compared with rats on high salt alone (100±3 and 112±2 mm Hg, respectively, P<0.05). During the final 3 days of tempol treatment, tempol had no sustained effect on MAP in A-192621–treated rats (138±5 mm Hg) but significantly attenuated the rate at which MAP increased during A-192621 treatment under high-salt conditions.

The high-salt diet had no significant effect on plasma ET concentrations (Figure 3). Tempol had no effect on plasma ET levels in rats on a normal- or a high-salt diet. Rats treated with A-192621 had significantly higher plasma ET levels compared with control on both normal- and high-salt diets, whereas tempol had no effect on the increase in plasma ET level compared with rats treated with A-192621 alone on either diet.

Plasma concentrations of 8-isoprostane, often used as an index of oxidative stress, were no different among the groups of rats after 7 days of treatment with tempol and/or A-192621, regardless of dietary salt content (Figure 4). However, the high-salt diet itself produced a significant
increase in plasma 8-isoprostane levels ($P<0.005$). A-192621 significantly increased plasma 8-isoprostanes compared with those in control rats after 3 days but not after 7 days of treatment. Although tempol had no effect on plasma 8-isoprostane levels in rats treated with high salt alone, tempol significantly inhibited the increase in 8-isoprostane levels produced by A-192621 after 3 days of treatment.

H$_2$O$_2$ excretion was significantly higher in all groups of rats on the high-salt diet at the end of the drug treatment period compared with rats on either a normal-salt diet or a high-salt diet with 3 days of drug treatment (Figure 5). In rats on a high-salt diet and 7 days of A-192621 treatment, tempol significantly increased H$_2$O$_2$ excretion compared with control and A-192621–treated rats.

**Discussion**

Li et al$^{19}$ have demonstrated that ET-1 stimulates superoxide production via ET$_A$ receptor–mediated activation of NADPH oxidase in the rat carotid artery. Furthermore, Sedeek et al$^{10}$ recently reported that tempol lowers arterial pressure during long-term ET infusion. This led us to hypothesize that superoxide may contribute to the hypertension produced by ET$_B$ receptor blockade, a condition wherein endogenous ET levels are increased. The main findings from the current study indicate that treatment with the SOD mimetic tempol attenuates the development of hypertension in rats on a high-salt diet given an ET$_B$ receptor antagonist, but only in the initial days of treatment. Therefore, superoxide appears to contribute, at least partially, to the hypertension associated with long-term ET$_B$ receptor blockade. We also observed that long-term treatment with tempol lowered MAP in rats given a high-salt diet alone. Tempol had no effect on MAP in rats on a normal-salt diet with or without ET$_B$ receptor blockade. Given the numerous reports that tempol effectively reduces superoxide levels with this dosing regimen,$^{2,6}$ these data support a role for superoxide in the maintenance of arterial pressure in rats on a high-salt diet.

Our laboratory has recently provided evidence that blocking the ET$_B$ receptor leads to elevations in plasma ET levels and MAP that are exacerbated in rats on a high-salt diet.$^{15}$ Thus, a lack of ET$_B$ receptor function results in a salt-sensitive form of hypertension. Administering an ET$_A$ antagonist will attenuate the hypertension whether rats are on a normal- or a high-salt diet.$^{15}$ Therefore, the hypertension may
be the result of reduced ET B-mediated vasodilation and diuretic/natriuretic activity in combination with increased ETA receptor activation. The current study expands on these initial findings to demonstrate only a minor role for superoxide in regulating arterial pressure during long-term ETA receptor activation.

We found it somewhat surprising that tempol had no effect on the increase in MAP produced by chronic ETB receptor blockade in rats on a normal-salt diet. If it is assumed that the hypertension produced by ETB blockade is a result of increased ET levels and ETA-mediated vasoconstriction, then this result is in direct contrast to studies that used a long-term infusion of exogenous ET-1. Therefore, our results lead us to speculate that the elevations in MAP produced by ETB blockade are more a function of a lack of ETB-induced vasodilation and diuretic/natriuretic activity than simply a reduction in ET clearance with an associated increase in ETA activation.

In rats on a high-salt diet, it is unclear why tempol was unable to sustain the inhibition of hypertension produced by ETB receptor blockade. Although it is possible that the influence of superoxide wanes over time, there are alternative explanations. As an SOD mimetic, tempol converts superoxide to H2O2, which is acted on by endogenous catalase to produce O2 and H2O. The combination of tempol and ETB receptor blockade during high-salt conditions may result in more H2O2 production than can be handled by the endogenous H2O2-scavenging systems. After 7 days of treatment, tempol increased H2O2 excretion with or without ETB receptor blockade. Increased renal H2O2 may limit the ability of tempol to lower MAP under these circumstances, because chronic increases in H2O2 in the renal medulla produce hypertension. Alternatively, because ETB receptors appear to play an important role in the renal response to a high-salt diet, scavenging superoxide may not be sufficient to restore sodium balance without an accompanying increase in MAP. Overall, these findings support a role for superoxide in contributing to salt-dependent hypertension; however, the mechanisms and conditions still require further definition, because dismutating superoxide is insufficient to produce a sustained normalization of blood pressure.

The ability of tempol to significantly decrease MAP in rats on a high-salt diet indicates that high salt alone increases oxidative stress. In the current study, a high-salt diet significantly raised plasma 8-isoprostane levels and H2O2 excretion compared with rats on a normal-salt diet. High salt intake is also associated with increased intrarenal production of ET in normal rats and in several models of salt-dependent hypertension. These models are also uniquely sensitive to blood pressure lowering during ETA receptor blockade. Antioxidant treatment with tempol also lowers arterial pressure in these models. In vitro and in vivo studies have shown that both angiotensin II and ET-1 are important stimulators of superoxide production. Because angiotensin II production is low during increased salt intake, our data are consistent with the possibility that ET-1 could contribute to elevated oxidative stress during increased dietary salt intake.

Measuring lipid peroxidation is frequently used as an indirect assessment of oxidative stress in various diseases, such as hypertension. The oxidation of arachidonic acid to form 8-iso-prostaglandin F2α (8-isoprostane) is often used as an index of oxidative stress in vivo. Consistent with the concept that high salt increases oxidative stress, we observed that plasma 8-isoprostane levels were significantly higher in normal Sprague-Dawley rats on a high-salt diet than in those on the normal-salt diet. Previous studies in Dahl salt-sensitive rats have shown that plasma 8-isoprostane levels are significantly elevated in the second week of high-salt treatment compared with Dahl salt-resistant rats. Even though tempol decreased MAP in rats treated with high salt, tempol did not attenuate the increase in plasma 8-isoprostane levels at the end of the 7-day drug treatment period. There are several potential explanations for these results. First, Xu et al recently observed that short-term
administration of tempol lowers blood pressure without reducing superoxide levels in the aorta or vena cava. The effect of tempol was associated with decreases in renal nerve activity, so the authors proposed that tempol may have actions other than decreasing vascular superoxide. It is possible that tempol may lower superoxide in a local environment sufficient to lower arterial pressure but not enough to reduce measurable plasma levels of oxidized lipids, such as 8-isoprostane. Other explanations for our findings may be that 8-isoprostane is simply not a very sensitive measure of oxidative stress or that other factors beside superoxide may be contributing to 8-isoprostane formation.

Although all animals on a high-salt diet had significantly higher plasma 8-isoprostane levels, tempol had no effect on this increase. However, 8-isoprostane was significantly increased in rats on a high-salt diet treated with A-192621 after 3 days of treatment. Tempol inhibited the increase in 8-isoprostane produced by A-192621 at the same time MAP was reduced. However, by the end of the 1-week protocol, A-192621 did not increase 8-isoprostane above untreated levels, and tempol had no effect on either blood pressure or plasma 8-isoprostane values. These observations suggest that high salt induces oxidative stress in normal rats but that the initial phase of A-192621–induced hypertension is dependent on superoxide.

Under normal- and high-salt conditions, ET<sub>B</sub> receptor blockade significantly increased plasma ET levels compared with their control counterparts, which as discussed earlier, could be contributing to the elevation in MAP. A-192621 treatment tended to stimulate a greater increase in plasma ET in rats on high-salt compared with rats on a normal-salt diet (P=0.06). We have previously shown that high salt significantly increases urinary ET, so a slightly greater effect of A-192621 in rats on a high-salt diet is consistent with a greater increase in ET production under high-salt conditions. Increasing dietary salt alone had no significant effect on plasma ET levels in untreated animals, but this is not surprising, because plasma ET levels are not always a reliable indicator of local production. ET levels are determined by a balance between synthesis, metabolism, and clearance, which can all vary according to location. Unfortunately, these factors limit our ability to measure local ET production in a reliable fashion.

**Perspectives**

This study provides further confirmation that superoxide contributes to the maintenance of MAP in rats on a high-salt diet by using a highly sensitive method for measuring MAP. When endogenous ET is elevated by ET<sub>B</sub> receptor blockade, treatment with a SOD mimetic attenuated the development of hypertension, although this effect was not sustained and was evident only during high salt intake. These findings indicate that either tempol has a limited ability to reduce oxidative stress under these conditions or that superoxide has a limited role in the hypertension produced by the absence of ET<sub>B</sub> receptor function. A number of studies have shown that tempol at the same dose as used in the current study will reduce superoxide production. 8-isoprostane data suggest that tempol did not reduce superoxide levels despite having significant effects on MAP. These observations suggest that tempol may be reducing MAP by a means other than scavenging superoxide, as suggested by Xu et al. and/or that 8-isoprostane measurements are not a very reliable indicator of oxidative stress. The current study also demonstrated that SOD mimetics may have limited efficacy in salt-sensitive hypertension, and we speculate that these drugs may allow the accumulation of H<sub>2</sub>O<sub>2</sub> in the kidney, where H<sub>2</sub>O<sub>2</sub> can induce hypertension. This possibility is supported by the observed increase of H<sub>2</sub>O<sub>2</sub> excretion during tempol administration.

In hypertension, ROS have been shown to have chronic proinflammatory actions in which the formation of superoxide and H<sub>2</sub>O<sub>2</sub> are significantly increased. The augmented superoxide levels are able to increase inflammatory cytokines indirectly by activating nuclear factor-κB (NF-κB). The administration of pyrrolidine dithiocarbamate, a potent antioxidant and NF-κB blocker, has been demonstrated to decrease blood pressure and inflammation in hypertensive rats. In vivo studies have shown that overexpressing the human ET-1 gene causes chronic kidney inflammation. In vitro, ET-1 stimulates secretion of interleukin-6 (IL-6), an inflammatory cytokine, from human vascular smooth muscle cells via activation of NF-κB. In that same study, pyrrolidine dithiocarbamate inhibited the ET-1–mediated IL-6 secretion from these cells, which suggests that ET-1 stimulates ROS production. IL-6 has also been shown to potentiate angiotensin II–mediated superoxide production, but whether or not IL-6 potentiates ET-1–mediated superoxide production still has to be thoroughly investigated. Future studies will be needed to explore the involvement of other ROS and inflammatory mediators in the hypertension produced by ET<sub>B</sub> receptor blockade.

**Acknowledgments**

This study was supported by a Predoctoral Fellowship from the American Heart Association, Southeast Affiliate (J.M.W.), an Established Investigator Award (D.M.P.), and grants from the National Institutes of Health HL64776 (D.M.P.) and HL60653 (J.S.P.). The authors would also like to thank Hiram Ocasio and Janet Hobbs for their excellent technical assistance.

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Hypertension. 2004;44:770-775; originally published online September 27, 2004;
doi: 10.1161/01.HYP.0000144073.42537.06
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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