NADPH Oxidase Inhibition Attenuates Oxidative Stress but Not Hypertension Produced by Chronic ET-1

Ahmed A. Elmarakby, E. Dabbs Loomis, Jennifer S. Pollock, David M. Pollock

Abstract—Experiments were conducted to test the hypothesis that hypertension produced by chronic ET-1 infusion is mediated by NADPH oxidase-dependent superoxide production. Mean arterial pressure (MAP) was continuously monitored in male Sprague Dawley rats by telemetry. After baseline measurements, rats were placed on a high-salt diet (8% NaCl) and osmotic minipumps were implanted to infuse ET-1 (5 pmol/kg per minute intravenous) for 12 days. Control rats were maintained on the high-salt diet only. Separate groups of rats were also infused with ET-1 and given the superoxide dismutase mimetic, tempol (1 mmol/L), or the NADPH oxidase inhibitor, apocynin (1.5 mmol/L), in the drinking water. Infusion of ET-1 significantly increased MAP when compared with baseline values (132±3 versus 114±2 mm Hg, P<0.05). Neither tempol nor apocynin treatment had any effect on the increase in MAP produced by ET-1 when compared with baseline values (127±5 versus 113±2 and 130±3 versus 115±2 mm Hg, respectively). Plasma 8-isoprostane, an indicator of oxidative stress, was significantly increased in ET-1–infused rats compared with rats on a high-salt diet alone (128±33 versus 51±5 pg/mL; P<0.05). Both tempol and apocynin treatment significantly attenuated the ET-1–induced increase in plasma 8-isoprostane (72±10 and 61±6 pg/mL, respectively). Similarly, ET-1 infusion also significantly increased aortic superoxide production (chemiluminescence and dihydroethidium staining techniques), which was prevented by both tempol and apocynin. These data provide evidence that chronic ET-1 infusion increases vascular NADPH oxidase-dependent superoxide production but does not account for chronic ET-1–induced hypertension. (Hypertension. 2005;45:283-287.)

Key Words: endothelin ■ oxidative stress ■ sodium

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide that can produce vasoconstriction primarily through ETA receptor activation.1 Salt-dependent models of hypertension such as DOCA-salt rats2,3 and chronic angiotensin II–infused rats4 display a high level of ET-1 mRNA and peptide expression. Furthermore, ETA receptor antagonists attenuated the increase in blood pressure in these models, suggesting an important role of ET-1 in the pathogenesis of salt-dependent hypertension.2,5,6 Additionally, ET-1 is believed to contribute to hypertension in the black population that is known to be predominantly salt-sensitive.7,8

Considerable recent attention has been paid to the possibility that ET-induced vasoconstriction may be dependent, at least in part, on the production of superoxide anion.9–11 In cultured pulmonary artery smooth muscle cells, Wedgwood et al have shown that exposure to ET-1 significantly increases superoxide production, and this effect can be blocked by pre-incubation with the ETα receptor antagonist PD-156707.11 Additionally, exogenously applied ET-1 can stimulate superoxide anion formation in rat aortic rings.9,12 In conscious rats, Sedeek et al observed that 9 days of ET-1 infusion significantly increased blood pressure that was completely abolished when rats were treated with the superoxide dismutase (SOD) mimetic, tempol.13 In isolated carotid arteries from DOCA-salt hypertensive rats, blockade of ETα receptors and NADPH oxidase will inhibit superoxide production.14 Furthermore, our laboratory has also shown that ETα receptor blockade can prevent the increase in blood pressure and oxidative stress in salt-sensitive ETβ-deficient hypertensive rats, an example of a high-endothelin model of hypertension.15

Despite ample evidence to support the hypothesis that ET-1 stimulates the formation of superoxide, the source of superoxide in the chronic ET-1 infusion model of hypertension has yet to be determined. Therefore, the purpose of the present study was first to confirm that chronic ET-1 infusion increases arterial pressure and oxidative stress, and second to determine whether ET-dependent increases in blood pressure and oxidative stress are dependent on superoxide and NADPH oxidase.

Methods

Animal Protocols
Experiments used male Sprague-Dawley rats with an initial body weight of 200 to 250 grams (Harlan Laboratories). Animal protocols were in accordance with National Institutes of Health guidelines and

Received October 1, 2004; first decision October 21, 2004; revision accepted December 1, 2004.
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Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000153051.56460.6a
approved and monitored by the Medical College of Georgia Institutional Animal Care and Use Committee. Rats were housed under conditions of constant temperature and humidity and exposed to a 12:12-hour light–dark cycle. Telemetry transmitters (Data Sciences, Inc) were implanted according to manufacturer’s specifications while under Na pentobarbital anesthesia (65 mg/kg intraperitoneal) as previously described.16 Rats were allowed to recover from implant surgery for 7 to 10 days before obtaining 3 to 4 days of basal blood pressure measurements. Preliminary experiments indicated that chronic ET-1 infusion did not produce consistent increases in blood pressure when on a normal salt diet, similar to that recently reported by Wang and Wang.17 Therefore, all rats were placed on a high-salt diet (8% NaCl). Three days later, rats were anesthetized with ketamine (100 mg/kg intraperitoneal) and osmotic minipumps (Alza Scientific, Palo Alto, Calif) were implanted subcutaneously to deliver ET-1 (5 pmol/kg per minute; Phoenix Pharmaceuticals) through a catheter in the jugular vein for 12 days. Rats were then divided into 3 groups (n=7 to 10): (1) ET-1 only; (2) ET-1 infusion plus the SOD mimetic, tempol (1 mmol/L; Sigma-Aldrich Co) in the drinking water; and (3) ET-1 infusion plus the NADPH oxidase inhibitor, apocynin (1.5 mmol/L; Sigma-Aldrich Co) in the drinking water. Tempol and apocynin were started at the same time as ET-1 infusion was initiated and given for the duration of the experiment. Previous studies have shown that these concentrations of tempol and apocynin placed in the drinking water are effective at reducing superoxide and oxidative stress in vivo.18 The four groups served as a high-salt diet control and drank tap water throughout the experiment (n=6). In a separate series of experiments, rats were given ET-1 infusion with or without tempol being administered via osmotic minipump implanted subcutaneously at a dose of 30 mg/kg per day (n=6 in both groups).

At the end of all experiments, rats were anesthetized using sodium pentobarbital anesthesia (65 mg/kg intraperitoneal) and a blood sample was taken from the abdominal aorta. Plasma was stored at −80°C for later measurement of 8-isoprostane using an enzyme-linked immunosorbent assay kit (Cayman Chemical Co). The thoracic aorta was carefully dissected for determination of superoxide production using lucigenin chemiluminescence and dihydroethidium staining techniques.

**Lucigenin Chemiluminescence**

Superoxide levels in vascular tissue was measured using lucigenin chemiluminescence.12,13 Following isolation, the thoracic aorta was immediately placed in ice-cold physiological saline solution of the following concentrations (mM): NaCl 130, KCl 4.7, KH2PO4 1.8, MgSO4·7H2O 1.17, NaHCO3 14.9, dextrose 5.5, EDTA 0.26, and CaCl2 1.6. The aorta was cleaned of adhering adventitial tissue and cut into 2-mm rings in length. Individual aortic rings were placed in a 96-well microplate (OptiPlate-96 polystyrene microplates; Packard Instruments) and sealed with Topseal-A. Both sample and background wells were in duplicate, with 200 μL of 5 μmol/L lucigenin per well. Background wells contained the lucigenin without tissue. Plates were dark-adapted for 30 minutes and then counted on a Top Count microplate scintillation and luminescence counter (Packard Instruments). The scintillation microplate counter was covered with blackout cloth to prevent interference from extraneous light. Background luminescence was subtracted from sample fluorescence and the counts per minute (cpm) were normalized to the dry weight of the tissue.

**Dihydroethidium Staining**

Dihydroethidium (DHE) is a lipophilic cell-permeable dye that is rapidly oxidized to ethidium in the presence of free radical superoxide. In theory, the produced ethidium is fixed by intercalation into nDNA, thus giving an indication of oxidant stress within cells undergoing investigation.18 Freshly isolated segments of the aorta were placed in optimum cutting temperature formulation made of water-soluble glycols and resins (Sakura, Torrance, Calif) and immediately frozen in liquid nitrogen. This provides a convenient specimen matrix for cryostat sectioning at temperatures of −10°C and below. The aorta segments were cut into sections using a Leica CM 1850 cryostat (10 to 20 μm) before being incubated with DHE (2 μmol/L) in phosphate-buffered saline at 37°C for 30 minutes. Fluorescence images were obtained with a Zeiss LSM 510 META confocal microscope using filters with an excitation of 488 nm and an emission range of 574 to 595 nm.

**Statistical Analysis**

Mean arterial pressure (MAP) data were analyzed by ANOVA with repeated measures (SuperANOVA, Abacus Concepts Inc).Statistical differences in the mean values for plasma 8-isoprostane and aortic superoxide measurements were determined by ANOVA and the Fisher protected least significant difference test was used to determine differences between individual means (StatView, Abacus Concepts Inc). Values are reported as mean±SEM, with P<0.05 being considered significant.

**Results**

Figure 1 shows the MAP in chronic ET-1–infused rats fed high-salt diet. ET-1 infusion increased MAP progressively over the first week of infusion and plateaued during the second week between 130 and 140 mm Hg. Unexpectedly, neither tempol nor apocynin treatment was able to attenuate the elevation in MAP produced by ET-1 infusion. Tempol treatment showed some tendency to lower MAP on days 4 and 5 of ET-1 infusion, but these changes were not sustained.

Plasma 8-isoprostane, an indicator of oxidative stress, was significantly increased by >2-fold in ET-1–infused rats compared with control rats on a high-salt diet alone (Figure 2A). Both tempol and apocynin treatment abolished the ET-1–induced increase in 8-isoprostane. Aortic superoxide production was also determined using lucigenin chemiluminescence. Consistent with 8-isoprostane data, ET-1 infusion significantly increased aortic superoxide production compared with control (Figure 2B). Tempol, as well as apocynin, significantly attenuated ET-1–induced aortic superoxide production; values in the tempol and apocynin groups were not different from untreated controls.

DHE staining was used as a qualitative approach to detect superoxide production in frozen aortic sections from chronic ET-1–infused rats. Confirming the lucigenin data, DHE fluorescence staining intensity for superoxide production was greater in aortic sections from ET-1–infused rats (Figure 3).
Both tempol and apocynin reduced fluorescence intensity in ET-1 infused rats.

In a separate series of experiments, we also determined the effect of a continuous subcutaneous administration of tempol in rats during chronic ET-1 infusion. Consistent with results with tempol in the drinking water, the persistent elevation in MAP produced by ET-1 infusion was unaffected by tempol (Figure 4A). Again, similar to oral dosing, tempol significantly attenuated the increase in aortic superoxide produced by ET-1 infusion (Figure 4B).

**Discussion**

In the present study, infusion of ET-1 for 12 days significantly increased MAP, plasma 8-isoprostane, and superoxide production in male Sprague Dawley rats maintained on a high-salt diet. Both tempol and apocynin inhibited these measures of oxidative stress induced by ET-1 infusion, yet to our surprise had no effect on the increase in MAP. These studies provide clear evidence that although ET-1 can increase vascular oxidative stress by activation of NADPH oxidase, these changes are not responsible for ET-1–induced hypertension.

In vitro studies have shown that ET-1 may be an important modulator of superoxide anion formation in vascular tissue.9,10 Blocking endogenous ET-1 action with the selective ET\(_A\) receptor antagonist, BQ123, prevented the increase in superoxide formation induced by cyclosporine and oxidized low-density lipoprotein.10 Also, ET\(_A\) receptor blockade inhibited superoxide production in isolated carotid arteries taken from DOCA-salt hypertensive rats.14 Moreover, exogenously applied ET-1 dose-dependently stimulated superoxide production in rat aortic rings.9,12

Previous studies discussed led to the hypothesis that the hypertensive actions of ET-1 may involve the formation of oxygen radicals. In support of this hypothesis, Sedeek et al recently showed that the increase in arterial pressure in response to ET-1 infusion for 9 days was attenuated by tempol.13 Obviously, these results conflict with findings in the current study, although the reason for the difference is not clear. Sedeek et al used the same dose of ET-1; however, tempol was administered intravenously in the same minipump as with ET-1. In our experiments, tempol was given either in the drinking water or via osmotic minipump delivered subcutaneously. In either case, we verified that tempol was reducing vascular oxidative stress by several measures. Likewise, Sedeek et al used plasma thiobarbituric acid–reacting substances (TBARS) and urinary 8-isoprostane to verify reduction of oxidative stress by tempol. Therefore, it is clear that tempol is able to demonstrate efficacy at reducing oxidative stress regardless of the route of administration. Our studies used continuous measurement of MAP by telemetry, whereas Sedeek et al measured MAP at a single time point at the end of the study with an acutely implanted catheter. It
would be surprising if this relatively minor technical difference could explain the rather clear differences in response to tempol. The previous studies were also conducted in rats on a normal salt diet, whereas our rats were given a high-salt diet. In a recent study by Wang and Wang,\textsuperscript{17} chronic ET-1 had no effect on MAP in rats on a normal salt diet, and in preliminary experiments, we were unable to produce ET-induced hypertension in rats on a normal salt diet. It is unclear why Sedeek et al were able to observe hypertension in rats on a normal salt diet. In a recent study by Wang and Wang,\textsuperscript{17} chronic ET-1 infusion in conscious rats. A separate group was also given tempol subcutaneously (30 mg/kg per day). Values are mean±SE (n=6 animals per group); \textsuperscript{*}P<0.05 vs ET-1 only.

Our laboratory has recently shown that ET\textsubscript{A} receptor blockade in the ET\textsubscript{A}-deficient rat will prevent salt-induced hypertension and reduces oxidative stress associated with this model.\textsuperscript{15} Because inhibiting ET\textsubscript{A} receptors can reverse the hypertension associated with ET\textsubscript{B} receptor deficiency or pharmacological blockade of ET\textsubscript{B} receptors, these results may first appear to be in contrast with the current findings. However, it is possible that lowering arterial pressure alone will reduce oxidative stress rather than oxidative stress causing an increase in arterial pressure. Furthermore, increases in oxidative stress are not common to all models of hypertension. Chronic epinephrine infusion increases MAP without any apparent elevation in reactive oxygen species.\textsuperscript{20} A number of studies have shown that the SOD mimic, tempol, will reduce arterial pressure in various models of hypertension while at the same time decreasing oxidative stress.\textsuperscript{19,21,22} We recently reported that tempol, in another ET-dependent model of hypertension, only attenuated the elevation in MAP during the first few days of ET\textsubscript{B} receptor blockade, i.e., an effect that was not sustained.\textsuperscript{23} At least in terms of ET-induced hypertension, a cause-and-effect relationship between superoxide and hypertension does not appear to exist.

Our study used 3 distinct measures of oxidative stress to verify that tempol and apocynin were capable of reducing superoxide. Because these inhibitors are considered to be permeable to the cell membranes, it is unlikely that tempol and apocynin were able to reduce oxidative stress in some tissue locations and not others. Nonetheless, it remains possible that tempol and apocynin are ineffective at reducing ET-induced increases in reactive oxygen species in a localized area or by a mechanism unaffected by these agents.

In the DOCA-salt model of hypertension, Li et al recently provided evidence that ET-1 increases superoxide by stimulation of NADPH oxidase.\textsuperscript{14} Our results using the NADPH oxidase inhibitor are consistent with the observation that ET-1 can directly stimulate NADPH oxidase in vivo. However, the ability of ET-1 to stimulate NADPH oxidase does not appear to play a major role in ET-dependent hypertension because inhibition of ET-induced increases in NADPH oxidase activity had no effect on MAP.

Although our results indicate that ET-1-induced hypertension is not dependent on NADPH oxidase-generated superoxide, it remains possible that other reactive oxygen species may contribute to hypertension in this model. For example, although tempol clearly reduces superoxide levels, it also increases hydrogen peroxide that has been shown to increase MAP if generated in sufficient quantities in the kidney.\textsuperscript{24} Also, it is possible that ET-1 can increase superoxide production or other reactive oxygen species independent of NADPH oxidase in a manner that is not detected by the 3 different measures of oxidative stress used in the current study. These findings underscore the reality that our knowledge of how reactive oxygen species behave in a complex animal model is limited and that future studies will have to use new approaches.

In conclusion, these studies indicate that ET-1-dependent hypertension does not require the generation of NADPH oxidase-dependent superoxide production. It would appear as though ET-1-dependent hypertension is a function of the well-established ability of ET-1 to produce prolonged vasoconstriction, endothelial dysfunction, and a shift in the renal pressure–natriuresis relationship independent of superoxide.

**Perspectives**

Despite considerable evidence that ET-1 can increase vascular oxidative stress, the current study suggests that this effect of ET-1 does not influence arterial pressure regulation. We observed a very clear dissociation between vascular superoxide and the level of arterial pressure. However, this does not preclude a role for ET-1–dependent changes in oxidative
stress on functional changes that may occur within the vascular wall during conditions such as atherosclerosis and inflammation. Perhaps the current study serves a reminder that chronic blood pressure regulation is more a function of changes in the pressure–natriuresis relationship that is known to be shifted toward higher blood pressures by ET-1.16,25

Acknowledgments
These studies were supported by grants from the National Heart Lung and Blood Institute (HL64776, HL60653, and HL74167), American Heart Association Established Investigator Awards to D.P. and J.P., and Pre-doctoral Fellowships from the American Heart Association Southeast Affiliate awarded to A.E. and E.D.L. The authors express their appreciation for the expert technical assistance provided by Hiram Ocasio, Vic Veguilla, and Janet Hobbs.

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Hypertension. 2005;45:283-287; originally published online December 27, 2004; doi: 10.1161/01.HYP.0000153051.56460.6a
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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