Role of Reactive Oxygen Species in Endothelin-Induced Hypertension

Mona H. Sedeek, Maria T. Llinas, Heather Drummond, Lourdes Fortepiani, Sean R. Abram, Barbara T. Alexander, Jane F. Reckelhoff, Joey P. Granger

Abstract—Recent reports have indicated that endothelin-induced vasoconstriction in isolated aortic vascular rings may be mediated by the production of superoxide anion. The purpose of this study was to determine the role of superoxide anion in mediating the chronic renal and hypertensive actions of endothelin. Endothelin-1 (5 pmol/kg per minute) was chronically infused into the jugular vein by use of mini-osmotic pump for 9 days in male Sprague-Dawley rats and in rats treated with the superoxide anion scavenger tempol (30 mg/kg per day). Mean arterial pressure in the endothelin-1–treated rats was 141 ± 3 mm Hg, compared with 125 ± 2 mm Hg in control rats. Endothelin-1 increased renal vascular resistance (15.3 ± 2.5 versus 10 ± 1.3 mm Hg/mL per minute) and decreased renal plasma flow (6.5 ± 0.9 versus 8.7 ± 0.7 mL/min) in control rats. Endothelin-1 also significantly increased TBARS in the kidney and urinary 8-isoprostaglandin F2α excretion. The increase in arterial pressure in response to endothelin-1 was completely abolished by tempol (127 ± 4 versus 127 ± 2 mm Hg). Tempol also markedly attenuated the renal plasma flow and renal vascular resistance response to endothelin-1. Tempol also significantly decreased the level of 8-isoprostaglandin F2α in the endothelin-1–treated rats. Tempol had no effect on arterial pressure or renal hemodynamics in control rats. These data indicate that formation of reactive oxygen species may play an important role in mediating hypertension induced by chronic elevations in endothelin. (Hypertension. 2003;42[part 2]:806-810.)

Key Words: endothelin • hypertension, chronic • vasoconstriction • anions • hemodynamics

Endothelin, a 21–amino acid peptide, has potent and sustained vasoconstrictive effects on vascular smooth muscle both in vivo and in vitro.1 Three mature (21-residue) endothelin isoforms (ET-1, ET-2, and ET-3) are encoded in human, rat, and pig genomes, of which the most potent isoform, endothelin-1, is synthesized by the endothelium.1 Endothelin-1 has been shown to result in an elevation of mean arterial pressure (MAP) in conscious animals.2–3 Additionally, endothelin-induced hypertension has been associated with an increase in total peripheral resistance and renal vascular resistance (RVR), an effect that is dose-dependent in dogs and rats.2,3 Endothelin-1 also decreases both renal plasma flow (RPF) and glomerular filtration rate (GFR) through vasoconstriction of the glomerular afferent and efferent arterioles.4 A role for endogenous endothelin in mediating the renal and systemic cardiovascular alteration in various forms of hypertension has also been suggested.1 Recent reports have indicated that endothelin-induced vasoconstriction may be mediated by the production of superoxide anion.5–6 In vitro studies have shown that the increased superoxide anion production observed in cells treated with cyclosporine is completely blunted by endothelin-1 receptor blockade.3 Furthermore, inhibition of superoxide anion production abolished endothelin-induced contraction of isolated vascular rings treated with cyclosporine,6 suggesting that superoxide anion may play a role in mediating endothelin-induced vasoconstriction. Although these studies support the notion that the hypertensive actions of endothelin may involve the formation of oxygen radicals, the importance of reactive oxygen species in mediating the chronic hypertension and renal vasoconstriction induced by endothelin in vivo is still unknown. Thus, the purpose of this study was to determine whether endothelin-induced hypertension and changes in renal hemodynamics is mediated by increased production of reactive oxygen species.

Methods

All studies were done in 225 to 250 g male Sprague-Dawley rats purchased from Harlan Sprague-Dawley Inc. Animals were housed 3 to a cage in a temperature-controlled room (23°C) with a 12:12-hour light/dark cycle. All experimental procedures executed in this study were in accordance with National Institutes of Health Guidelines for the Use and Care of Animals and with approval by the Animal Care and Use Committee at the University of Mississippi Medical Center.

Experimental Design

Animals were divided into 4 groups: (1) control group, (2) endothelin-treated group (5 pmol/kg per minute, Alexis), (3) tempol-
treated group (30 mg/kg per day, Sigma), and (4) endothelin-1 (5 pmol/kg per minute) plus tempol (30 mg/kg per day) treated group. Drugs were chronically infused for 9 days through the jugular vein by use of a mini-osmotic pump (Alzet model 2002). Catheters (PE-60) connected to a mini-osmotic pump were inserted into the jugular veins under isoflurane anesthesia as previously described. Animals were anesthetized with isoflurane on day 7 of the experiment for insertion of femoral arterial and venous catheters (PE-50). Bladder catheters were inserted into the bladder through a midline lower abdominal incision. Renal hemodynamics and arterial pressure were measured in conscious, chronically instrumented rats on day 9 of the protocol.

Measurement of Renal Hemodynamics and Arterial Pressure in Conscious Rats
Renal hemodynamics and arterial pressure were determined in conscious control (n = 12), tempol-treated rats (n = 9), endothelin-1–treated (n = 9) rats, and rats treated with endothelin-1 plus tempol (n = 6). Measurement of renal hemodynamics was performed in conscious rats with GFR and effective renal plasma flow (ERPF) determined by the clearance of [125I] iothalamate and paraaminohippurate, as described previously. MAP was monitored with a pressure transducer connected to a blood pressure monitoring system.

Assessment of Oxidative Stress
Oxidative stress was determined in a separate group of animals: control group (n = 7), endothelin-1–treated group (n = 5), tempol-treated group (n = 5), and endothelin-1 plus tempol–treated group (n = 5). On day 5, rats were placed in metabolic cages, and 24-hour urine was collected on ice and centrifuged for 10 minutes at 1500g, then kept at −80°C. On the 9th day of the experiment, rats were euthanized under inhalational anesthesia. Kidneys were washed with heparinized saline, excised, placed in liquid nitrogen and kept at −80°C until assays were performed.

TBARS Assay
Lipid peroxidation within the kidney was assessed by measuring malondialdehyde, as described previously. Kidneys were homogenized in buffer containing 20 mmol/L Tris-HCl, pH 7.4, containing 5 mmol/L butylated hydroxy toluene. Samples were centrifuged at 5000g at 4°C for 20 minutes, with storage of the supernatant at −80°C. Supernatant was treated with TCA 40% molar in HCL and 0.1 mol/L thiothiobarbituric acid and incubated at 90°C for 30 minutes. Samples were diluted with water, and the mixtures were centrifuged at 1500g for 10 minutes. Absorbance of the supernatant was read at 525 nm. Total protein concentration was determined with the use of a Sigma protein determination kit (P5656, Sigma Chemical Co).

Determination of Urinary 8-Isoprostan e PGF2α
8-Isoprostan e PGF2α (8-ISO PGF2α) was measured according to method previously described. We used an enzyme immunoassay kit to measure 8-ISO PGF2α in urine (Cayman chemical kit catalog, No.516351). Urine samples were diluted with EIA buffer supplied with the kits and assayed without purification.

Detection of Superoxide Production in Isolated Vascular Smooth Muscle Cells
The direct effect of endothelin-1 on superoxide anion production in rat vascular smooth muscle cells was also determined. A10 cells, passage 3 to 6, were seeded on glass slides, incubated for 24 hours in growth media, and then washed with PBS. These cells were derived from the thoracic aorta of DB1X embryonic rats and have many of the characteristics of vascular smooth muscle cells. Cells were incubated in serum-free DMEM and endothelin-1 (10−8, 10−9, 10−7, and 10−6 mol/L) for 24 hours. After incubation, hydroethidine (5 μmol/L) was added to the medium and incubated for 15 minutes at room temperature in the dark. Fluorescence caused by conversion of hydroethidine to ethidium was used as an index of production of superoxide anion, with a Leica TCS-SP2 laser scanning confocal microscopy used as described. The average fluorescence intensities were quantified by dividing the nucleus fluorescence by its area with the use of Leica confocal software.

Statistical Analysis
All data are expressed as mean±SEM. Comparisons between groups were analyzed by means of factorial ANOVA followed by the Fisher test. A value of P<0.05 was considered statistically significant.

Results

Effect of Tempol on Endothelin-Induced Hypertension in Conscious Rats
Chronic infusion of endothelin-1 at the rate of 5 pm/kg per minute for 9 days in conscious rats significantly increased MAP (141±3 mm Hg versus 125±2 mm Hg in the control group, P<0.01). Tempol completely blocked the effect of endothelin-1, as MAP in the endothelin-1 plus tempol–treated group was 127±4 mm Hg. Tempol alone has no effect on MAP in control rats, as illustrated in Figure 1.

Effect of Tempol on Endothelin-Induced Changes in Renal Hemodynamics in Conscious Rats
The renal hemodynamic changes in response to endothelin-1 and tempol are illustrated in Figure 2. Endothelin-1 significantly increased RVR, as RVR averaged 15±2.5 mm Hg/mL.
per minute in the endothelin-1–treated group and 10±1.3 mm Hg/mL per minute in control rats. Tempol treatment significantly decreased RVR in endothelin-1–treated rats (8.6±1 mm Hg/mL per minute). Endothelin-1 decreased ERPF by 26%, as ERPF was 6.5±0.9 mL/min in endothelin-1–treated rats versus 8.7±0.7 mL/min in control rats. Tempol virtually abolished the effect of endothelin-1 on ERPF. ERPF in endothelin plus tempol was 8.5±0.75 mL/min, as compared with 8.7±0.7 mL/min in control rats. Tempol had no effect on ERPF in control rats. Endothelin-1 also had no effect on GFR in control or tempol-treated groups, as shown in Figure 3.

### Increased Renal Levels of TBARS and Urinary 8-ISO PGF2α Excretion in Rats Chronically Treated With Endothelin-1

TBARS, within the kidney, were measured to assess lipid oxidation. TBARS levels in kidney homogenates were significantly increased in endothelin-1–treated rats (462±142 ng/µg protein) versus (48±13 ng/µg protein), as compared with control rats (P<0.01). Tempol tended to reduce TBARS level (287±52 versus 462±142 ng/µg protein) in endothelin-1–treated rats; however, statistical significance was not reached.

Urinary 8-ISO PGF2α excretion was assayed to measure the effects of ET-1 on phospholipid oxidation. Figure 4 illustrates a significant increase in urinary 8-ISO PGF2α excretion in endothelin-1–treated rats (11±1 ng/d), as compared with control rats (7.5±1 ng/d) (P<0.01). Tempol treatment significantly blocked the effect of endothelin-1 on urinary 8-ISO PGF2α excretion (8.9±0.8 versus 11±1 ng/d, P<0.05). Tempol alone significantly decreased urinary 8-ISO PGF2α (4.0±0.6 versus 7.5±1.0 ng/d) in control rats (P<0.05).

### Direct Effect of Endothelin-1 on Superoxide Production in Cultured Rat Vascular Smooth Muscle Cells

As shown in Figure 5, endothelin-1 caused a significant (P<0.01) and dose-dependent increase in fluorescence (Emax=52±3.5, 55.6±4, and 98±4 for 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/L, respectively, versus 38.5±2.6 relative fluorescence arbitrary units in control cells that received no treatment).

### Discussion

A role of endothelin in mediating the renal and cardiovascular alterations in various forms of hypertension has accumulated over past decade. Results from recent in vitro studies have suggested that endothelin-1 may also be an important regulator of superoxide anion formation in vascular tissue. Administration of BQ123, a selective ETₐ receptor antagonist, prevented stimulation of superoxide anion formation induced by cyclosporine and oxidized LDL. Moreover, exogenously applied endothelin-1 dose-dependently stimulated superoxide anion formation in rat aortic rings. Collectively, these studies support the notion that the renal and hypertensive actions of endothelin may involve the formation of oxygen radicals.

To determine whether endothelin-induced hypertension and changes in renal hemodynamics are mediated by increased reactive oxygen species production, we assessed the formation of reactive oxygen species in response to long-term infusion of endothelin-1 for 9 days in conscious, chronically instrumented rats. We found that endothelin-induced hypertension in rats was associated with significant increases in the urinary excretion of 8-ISO PG F₂α, which is one of the stable byproducts of phospholipid oxidation. Moreover, lipid peroxides, measured as TBARS in kidney tissues, were markedly elevated (>9-fold) in rats chronically treated with endothelin. 8-ISO PG F₂α and TBARS are also elevated in other forms of hypertension such as angiotensin II–induced hypertension, renovascular, obesity, and pregnancy-induced hypertension. Interestingly, we and others have recently reported these forms of hypertension are also associated with an increase in the endogenous formation of endothelin. The importance of endogenous endothelin in mediating enhanced oxidative stress in various animal models of hypertension remains to be determined.
To determine the importance of reactive oxygen species in mediating the chronic hypertension induced by endothelin, we examined the effects of tempol, a stable, membrane-permeable superoxide dismutase (SOD) mimic, in rats with endothelin-induced hypertension. Chronic infusion of endothelin-1 over 9 days increased blood pressure by ≈18 to 20 mm Hg. In sharp contrast, the hypertensive response to endothelin was completely abolished in rats treated with tempol. It is unlikely that the antihypertensive effect of tempol was a nonspecific effect, since tempol had no effect in normotensive control rats. Moreover, the antihypertensive effects of tempol have been reported in some but not all animal models of hypertension.11,21 We also believe the effects of tempol in our study were through inhibition of oxidative stress, since our biomarkers of reactive oxygen species, 8-ISO PG F₂α, was significantly reduced and TBARS were attenuated by tempol treatment in rats with endothelin-induced hypertension.

We previously reported that endothelin-induced hypertension is associated with an increase in RVR and reduced pressure natriuresis.3 Endothelin also decreased renal plasma flow through vasoconstriction of the glomerular afferent and efferent arterioles.2 Since chronic endothelin-induced renal vasoconstriction may be mediated by the production of superoxide, we were also interested in whether tempol improves renal function. In the present study, we mentioned earlier, these changes in renal hemodynamics were associated with a marked increase in lipid peroxidation within the kidney. Tempol administration significantly decreased the RVR response to endothelin. Thus, it appears that the chronic renal vasoconstriction in response to endothelin may in part be mediated through the production of reactive oxygen species.

The effect of endothelin to stimulate reactive oxygen species production may be due to direct and indirect mechanisms. Although the elevation in arterial pressure and subsequent endothelial dysfunction may be a stimulus for endothelin-induced superoxide anion formation, several studies have shown that the production of reactive oxygen species is not elevated in certain rat models of experimental hypertension such as noradrenergic-induced hypertension. To assess the direct actions of endothelin on superoxide anion production, we performed an additional in vitro study to examine the direct effects of endothelin-1 in rat vascular smooth muscle cells. Using fluorescence resulting from conversion of hydroethidine to ethidium as an indicator of superoxide anion production, we found that endothelin-1 produced a dose-dependent and significant increase in superoxide anion production in rat vascular smooth muscle cells. These findings are consistent with growing evidence for a direct effect of endothelin to stimulate reactive oxygen species production.12

We did not examine the potential mechanism whereby endothelin-1 stimulates superoxide anion formation or the cellular origin of superoxide anion produced by endothelin-1. It is well known that endothelial and/or smooth muscle cell NAD(P)H oxidase is an important superoxide anion–generating system.22 Recent studies have also indicated that rat smooth muscle production of superoxide anion involves a protein kinase C (PKC)-dependent pathway.23 Since endothelin-1 is a potent stimulator of PKC in vascular smooth muscle cell, it is possible that PKC-mediated stimulation of NAD(P)H oxidase is responsible for endothelin-1–induced superoxide anion production. Further studies are necessary to examine the potential mechanism whereby endothelin causes oxidative stress.

**Perspectives**

We report that chronic endothelin-1–induced hypertension in rats is associated with decreases in RPF and increases in RVR and increases in TBARS and urinary 8-ISO PG F₂α, excretion, markers of oxidative stress. The increase in MAP in response to endothelin-1 was completely abolished by tempol, a SOD mimetic. Tempol also markedly attenuated the RPF response to endothelin-1 and significantly decreased the RVR response to endothelin-1. Tempol also significantly decreased the level of 8-ISO PGF₂α in the endothelin-1–treated rats. Conversely, tempol had no effect on MAP or renal hemodynamics in control rats. These data indicate that formation of reactive oxygen species may play an important role in mediating the changes in renal function and hypertension induced by chronic elevations in endothelin.

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**References**

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