Endothelin Mediates Superoxide Production and Vasoconstriction through Activation of NADPH Oxidase and Uncoupled Nitric-Oxide Synthase in the Rat Aorta

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ABSTRACT

Experiments were designed to test the hypothesis that elevated levels of endothelin 1 (ET-1) in the vasculature activate NADPH oxidase and/or uncoupled nitric-oxide synthase (NOS), resulting in O$_2^-$ production, and mediate increased constriction. Rat aortic rings were incubated with ET-1 or vehicle in the presence and absence of superoxide dismutase (SOD), ebselen (glutathione peroxidase mimetic), apocynin (NADPH oxidase inhibitor), l-NNAME (N$^\omega$-nitro-l-arginine methyl ester) (NOS inhibitor), tetrahydrobiopterin (BH$_4$) (NOS cofactor), or selective ETA and ETB receptor antagonists (BQ-123 [cyclo(D-Asp-Pro-D-Val-Leu-D-Trp)] and A-192621 [2R-(4-propoxyphenyl)-4S-(1,3-benzodioxol-5-yl)-1-N-(2,6-diethylphenyl)aminocarbonyl-methyl]-pyrrolidine-3R-carboxylic acid, respectively). O$_2^-$ production was monitored by oxidized dihydroethidine staining and/or lucigenin chemiluminescence. ET-1 significantly increased O$_2^-$ production compared with vehicle. SOD, ebselen, and apocynin inhibited the ET-1-induced increase in O$_2^-$ in intact and endothelium-denuded aorta. l-NNAME and BH$_4$ inhibited the ET-1-induced increase in O$_2^-$ in intact tissue, whereas these two compounds had no effect on ET-1-induced O$_2^-$ in endothelium-denuded aorta. Preincubation with BQ-123 or A-192621, individually, had no effect on ET-1-induced O$_2^-$; however combining both antagonists inhibited the ET-1-stimulated increase in O$_2^-$.

Rat aortic rings were incubated with ET-1 or vehicle in the presence or absence of sepiapterin (BH$_4$ synthesis substrate) or apocynin and mounted on wire myographs to determine isometric force generation in response to increasing KCl concentrations. ET-1 increased the contractile response to KCl compared with vehicle. Treatment with either sepiapterin or apocynin attenuated the ET-1-mediated increase without effect of sepiapterin or apocynin alone. These data support the hypothesis that ET-1 increases vascular tone, in part, through ETA/ETB receptor activation of O$_2^-$ production from NADPH oxidase and NOS uncoupling.

Not long ago, O$_2^-$ was considered simply a destructive byproduct of cellular reactions. However, it is becoming clear that O$_2^-$ and other reactive oxygen species (ROS) play a role in maintaining cellular homeostasis and cell signaling (Wolin, 2000). O$_2^-$ is generated by virtually every cell type within the vascular wall (Madamanchi et al., 2005). Furthermore, experimental animal models and clinical studies of atherosclerosis, hypertension, and diabetes have demonstrated increased vascular O$_2^-$ and/or ROS production (for review see Griendling and FitzGerald, 2003; Madamanchi et al., 2005; Munzel et al., 2005).

Endothelial dysfunction and the bioavailability of nitric oxide (NO) have been documented as one of the many risk factors for cardiovascular disease. Several possible abnormalities may account for the dysfunction, including 1) reduced NO synthesis, 2) uncoupling of NO synthase (NOS) to produce O$_2^-$, and 3) degradation of NO via its interaction with O$_2^-$ from other enzymatic sources. NOS uncoupling has been shown to occur when the NOS cofactor tetrahydrobiopterin (BH$_4$) is oxidized or in limited quantities (Vasquez-Vivar et al., 1998; Milstein and Katusic, 1999; Laursen et al., 2001). It has been reported recently (Landmesser et al., 2003) that BH$_4$ oxidation and subsequent uncoupling of NOS contrib-

**ABBREVIATIONS:** ROS, reactive oxygen species; ET-1, endothelin; NO, nitric oxide; NOS, nitric-oxide synthase; BH$_4$, tetrahydrobiopterin; PSS, physiological saline solution; PEG, polyethylene glycol; SOD, superoxide dismutase; l-NNAME, N$^\omega$-nitro-l-arginine methyl ester; DHE, dihydroethidine; l-NNA, N$^\omega$-nitro-l-arginine; DOCA, deoxycorticosterone acetate; BQ-123, cyclo(D-Asp-Pro-D-Val-Leu-D-Trp); A-192621, 2R-(4-propoxyphenyl)-4S-(1,3-benzodioxol-5-yl)-1-N-(2,6-diethylphenyl)aminocarbonyl-methyl]-pyrrolidine-3R-carboxylic acid.
utes to blood pressure elevation in a model of low-renin hypertension.

The most widely studied enzymatic source of O$_2^-$ in the vascular wall is NADPH oxidase, but uncoupled NOS, xanthine oxidase, cytochrome P450, and enzymes of the mitochondrial electron transport chain are also possible sources of O$_2^-$ and NO react to form another oxidant, peroxynitrite (ONOO$^-$) (Beckman and Koppenol, 1996). Recently, Kuzkaya et al. (2003) have reported that BH$_4$ is more sensitive to oxidation by ONOO$^-$ than O$_2^-$, suggesting that there is increased NOS uncoupling in the presence of ONOO$^-$. Landmesser et al. (2003) suggested that activated NADPH oxidase is critical for producing ROS, which leads to the oxidation of BH$_4$. Recently, it has been postulated that activated NADPH oxidase provides the priming event (“kindling” radicals), which may lead to NOS uncoupling and production of O$_2^-$ or “bonfire” radicals within the endothelium and vascular wall (Munzel et al., 2005).

Endothelin (ET-1) is a potent endothelial-derived vasoconstrictor peptide. ET-1 is generally known to stimulate ETA receptors in vascular smooth muscle cells to produce vasoconstriction and ET$_B$ receptors on endothelial cells to produce vasodilation. The balance between ETA and ET$_B$ receptor activity may contribute to the pathogenesis of vascular disease (Ram, 2003; Kirchengast and Luz, 2005). Some but not all experimental models of hypertension, atherosclerosis, and diabetes display high levels of circulating ET-1 and are associated with endothelial dysfunction (for review, see Boussoude and Giaid, 2003; Cernacek et al., 2003; Muller et al., 2003; Schiffrin, 2005). Both ET-1 and O$_2^-$ have been the subjects of investigation within the cardiovascular field over the past decade, yet little is known about the precise relationship between these important modulators of vascular function. Wedgwood et al. (2001a) demonstrated that ET-1-stimulated O$_2^-$ production in isolated pulmonary arterial smooth muscle cells is blocked by the actions of an ETA receptor antagonist. Li et al. (2003) have shown that an ETA receptor antagonist blocks the O$_2^-$ production in rat carotid arteries. The ET$_B$ receptor has been shown to stimulate the production of ROS in human umbilical endothelial cells and sympathetic neurons (Dai et al., 2004; Dong et al., 2005).

Although ET-1 has been linked to the production of O$_2^-$ in the vasculature, thus far, no studies have examined the role of NOS in ET-1-mediated O$_2^-$ production and its functional consequences on vasoconstriction. Therefore, we hypothesized that elevated levels of ET-1 in the vasculature activate NADPH oxidase and/or uncoupled NOS, resulting in O$_2^-$ production and increased vasoconstriction. Both ETA and ET$_B$ receptors have been reported to mediate O$_2^-$ production; thus, we also sought to determine whether ET-1-stimulated O$_2^-$ production is receptor subtype-dependent.

**Materials and Methods**

**Materials.** All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Optimum cutting temperature compound was purchased from VWR (West Chester, PA). NaCl, KCl, EDTA, and apocynin were purchased from Fisher Scientific Co. (Pittsburgh, PA). Endothelin-1 was purchased from American Peptide Co., Inc. (Sunnyvale, CA). Tetrahydro-L-biopterin was purchased from Cayman Chemical (Ann Arbor, MI). ET-1 receptor antagonists were kindly provided by Abbott Laboratories (Abbott Park, IL).

**Isolation and Incubation of Rat Aortic Rings.** Experiments were conducted in male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 200 to 250 g. The institutional animal care and use committee approved all of the procedures. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.; Abbott Laboratories), and thoracic aorta was removed, cleaned of adhering tissue in ice-cold physiological saline solution (PSS), and cut into 3- to 4-mm segments. Composition of PSS was as follows: 130 mM NaCl, 4.7 mM KCl, 1.8 mM KH$_2$PO$_4$, 1.17 mM MgSO$_4$, 14.9 mM NaHCO$_3$, 5.5 mM dextrose, 0.26 mM EDTA, and 1.6 mM CaCl$_2$.

**Measurement of ET-1-Mediated O$_2^-$ Production with Lucigenin-Enhanced Chemiluminescence.** Vessels were left intact or denuded of endothelium and incubated for 4 h at 37°C in either PSS (basal) or ET-1 (0.001–1 μM) in siliconized polypropylene microcentrifuge tubes. Denudation of the aorta was accomplished by rubbing the tissue with blunt forceps. The denudation technique was verified in a subset of aortic tissue by testing the vascular reactivity of the aortic ring to constrict to phenylephrine (10 μM) followed by testing the relaxation of the ring to acetylcholine (100 μM). The denudation technique used demonstrated constriction to phenylephrine followed by a total absence of relaxation to acetylcholine, whereas the endothelium-intact tissue was fully relaxed. A subset of rings were pre-incubated for 1 h with one of the following inhibitors: superoxide dismutase (SOD; 200 U/ml), L-NAME (100 μM), ebselen (50 μM), BH$_4$ (3 μM), BQ-123 (cyclo-[c-Trp-D-Asp-Pro-d-Val-Leu]); selective ETA antagonist, 100 nM; A-192621 [2-(4-propoxyphenyl)-4-(1,3-benzodioxol-5-yl)-1-(2,5-ethylphenyl)aminocarboxylmethyl]-pyrrolidine-3-carboxylic acid; selective ET$_B$ antagonist, 100 nM), or vehicle. Aortic rings were placed in duplicate wells containing 200 μl of lucigenin (5 μM) in a 96-well microplate (OptiPlate-96; PerkinElmer Life and Analytical Sciences, Boston, MA) and sealed with Topseal-A (PerkinElmer Life and Analytical Sciences). Background wells contained lucigenin solution without tissue. Lucigenin solutions were maintained in the dark. Plates were dark adapted for 30 min and counted on a TopCount scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences) set to single photon-counting mode for 3 s/well. The counts were repeated eight times to reduce variability, averaged, and normalized to per minute (counts per minute). Background luminescence was subtracted, and the specific counts per minute were normalized to the dry weight of the tissue. The lucigenin method utilized in this study is a modified high-throughput method, which accommodates comparisons of multiple aortic segments simultaneously. Validation of the high-throughput method utilized a purified substrate/enzyme source of O$_2^-$ xanthine/xanthine oxidase, in the presence and absence of SOD.

**In Situ Detection of ET-1-Mediated O$_2^-$ Production.** Vessels were incubated for 4 h at 37°C in either PSS (basal) or ET-1 (1 μM) in siliconized polypropylene microcentrifuge tubes. A subset of rings was preincubated for 1 h with one of the following inhibitors: polyethylene glycol-SOD (PEG-SOD; 250 U/ml) or apocynin (300 μM). Aortic rings were frozen in optimum cutting temperature compound media (Tissue-Tek; Sakura Finetech, Tokyo, Japan), cut on a cryostat (Cryostat CM3000; Leica Microsystems, Inc., Deerfield, IL) into 30-μm thick sections, and mounted on glass slides. Slides were protected from light and incubated in dihydroethidium (DHE; 1 μM) for 30 min at 37°C. Images were obtained with a LSM 510 META confocal microscope (Carl Zeiss Inc., Thornwood, NY), with an excitation of 488 nm and emission of 574 to 595 nm or a 560-nm long pass filter.

**Vascular Reactivity.** Aortic rings were incubated as previously outlined. Rings were mounted between pins in a multichamber myograph for the measurement of isometric force generation. The chamber was filled with warmed (37°C) gassed (95% O$_2$, 5% CO$_2$) PSS. Tension was set to 25 mN, and the buffer was replaced with calcium-free PSS to determine the passive tension. ET-1 treatment resulted in the development of active tension, and in calcium-free buffer, passive tension was determined to be 10 ± 1 mN. Once passive tension was established, cumulative doses of KCl (8–100 mM) were
accomplished in calcium-replete PSS in the presence or absence of inhibitors. Force generation (in millinewtons) was monitored using a computer analysis program (Myodaq; Danish Myo Technology, Aarhus, Denmark).

**Statistics.** For O$_2^*$ production using lucigenin-enhanced chemiluminescence, statistical analysis was performed using an analysis of variance with Newman-Kuels post hoc test (Prism; GraphPad Software Inc., San Diego, CA). Maximum responses in vasoconstriction were compared by analysis of variance with Fisher’s protected least significance difference post hoc test (Statistica; StatSoft, Tulsa, OK).

**Results**

ET-1 increased O$_2^*$ production in both endothelium-intact and endothelium-denuded aortic tissue using lucigenin-enhanced chemiluminescent O$_2^*$ detection (Fig. 1, A and B). Specificity of the assay was demonstrated by a significant reduction of the signal in the presence of SOD (Fig. 1, A and B). ET-1 significantly increased O$_2^*$ at 0.1 and 1 μM in endothelium-intact and denuded tissue. The endothelium-denuded segments seem to have a higher O$_2^*$ production at 0.01 μM ET-1; however, this failed to reach statistical significance (Fig. 1B). Aortic tissue dissected from the same animal was used to directly compare the response to ET-1 in endothelium-intact and denuded rings. No significant difference was detected in the ET-1-stimulated O$_2^*$ production between endothelium-intact and endothelium-denuded aortic tissue (Fig. 1C).

ET-1-mediated O$_2^*$ production was verified with the oxidative fluorescent dye DHE. Increased nuclear staining, indicating enhanced O$_2^*$ production, is evident in the tissues incubated with ET-1 when compared with those incubated in PSS (Fig. 2, A and B). The NADPH oxidase inhibitor apocynin and the antioxidant PEG-SOD prevented the increase in DHE staining (Fig. 2, C and D).

ET-1-increased O$_2^*$ production in endothelium-intact aortic tissue was significantly inhibited in the presence of the NOS inhibitor l-NAME, the NOS cofactor BH$_4$, and antioxidant ebselen (Fig. 3, A, C, and E). To determine whether the endothelium was the source for the l-NAME-inhibitable O$_2^*$, the aortic tissue was endothelium-denuded before incubation with ET-1. ET-1-mediated O$_2^*$ production in the endothelium-denuded aortic tissue was inhibited only in the presence of ebselen (Fig. 3, B, D, and F). ET-1-stimulated O$_2^*$ production in endothelium-intact tissue was significantly inhibited in the presence of both specific ET-1 receptor antagonists (Fig. 4B), whereas individually, the antagonists did not inhibit O$_2^*$ production (Fig. 4A). l-NAME, BH$_4$, ebselen, BQ-123, or A-192621 had no effect on basal aortic tissue O$_2^*$ detection (data not shown).

Aortic vasoconstriction was assessed by monitoring the increase in force generation by increasing KCl concentrations. Incubation of endothelium-intact aortic tissue with ET-1 increased the vasoconstriction in comparison with in-
cubation with vehicle alone. The maximal increase in force to KCl in the vehicle-treated vessels was 90 ± 5%, whereas in the ET-1-treated vessels, the increase in force was 118 ± 11% (Fig. 5). The NADPH oxidase inhibitor apocynin significantly decreased the maximal ET-1-induced vasoconstriction (68 ± 16% increase in force). Since we observed that uncoupled NOS via oxidized BH4 was a source of the $O_2^-$ (Fig. 3), we determined contractile responses to KCl in vessels pretreated with sepiapterin, a precursor in the salvage pathway for the synthesis of BH4. Sepiapterin also significantly decreased the maximal ET-1-induced vasoconstriction (83 ± 18% increase in force). Apocynin and sepiapterin had no effect on vehicle-treated vessels (75 ± 8 and 87 ± 9% increase in force, respectively).

**Discussion**

The new findings of this study are that 1) ET-1 mediates $O_2^-$ production through uncoupled NOS in an endothelium-dependent manner, 2) endothelium-independent $O_2^-$ production stimulated by ET-1 does not seem to be via activation of uncoupled NOS, 3) ET-1 activation of $O_2^-$ production involves both $E_{TA}$ and $E_{TB}$ receptor subtypes in aortic tissue, and 4) two sources of $O_2^-$, NADPH oxidase and uncoupled NOS, contribute to increased vasoconstriction mediated by ET-1. Additionally, we have confirmed the previous findings that ET-1-stimulated $O_2^-$ production is dependent on NADPH oxidase. The present study supports the hypothesis that ET-1 mediates $O_2^-$ production from activated NADPH oxidase providing the kindling radicals to uncouple NOS and facilitate endothelial dysfunction in a mechanism similar to that proposed by Munzel et al. (2005).

**ET-1-Mediated $O_2^-$ Production and NOS Uncoupling.**

We used two methods of $O_2^-$ detection in this study, lucigenin-enhanced chemiluminescence and oxidized dihydroethidine staining. Specificity of both $O_2^-$ detection methods was verified by the inclusion of SOD, PEG-SOD, or ebselen. Ebselen...
is an antioxidant that has been reported to have thioredoxin reductase and/or glutathione peroxidase-like activity (Zhao et al., 2002). The lucigenin method for O$_2^*$ detection has been reported to reflect levels of O$_2^*$ extracellularly and intracellularly, because lucigenin is cell-permeable (Munzel et al., 2002). Interestingly, we found that both the endothelium-intact and endothelium-denuded aortic tissues were stimulated by ET-1 to generate O$_2^*$. No quantitative difference was detected between the O$_2^*$ production in endothelium-intact and denuded tissue from the same animal. This led us to speculate that, regardless of whether the endothelium is present, ET-1 maximally stimulated O$_2^*$ production.

The present study extended the findings of Li et al. (2003) that ET-1 increases O$_2^*$ via NADPH oxidase activation by showing that ET-1 also activates uncoupled NOS in an endothelium-dependent manner. Experimental evidence to determine whether NOS is uncoupled requires measurements of O$_2^*$ that is inhibited by a nonspecific NOS inhibitor L-NNA or L-NAME (Munzel et al., 2002). These inhibitors can block the transfer of electrons to either L-arginine or oxygen (Andrew and Mayer, 1999). In our experiments, L-NAME inhibited the ET-1-mediated O$_2^*$ production in the endothelium-intact but not denuded tissue. These results indicate that endothelial NOS is a source of ET-1-stimulated O$_2^*$. Landmesser et al. (2003) have suggested that uncoupled endothelial NOS, via oxidized BH$_4$, is a source of O$_2^*$ production in aortic tissue in the DOCA-salt treated rat. Therefore, exogenous BH$_4$ was added to the tissue to provide a source of reduced BH$_4$ for NOS (Cosentino and Luscher, 1999). These data revealed that exogenous BH$_4$ prevented the ET-1-stimulated O$_2^*$ production in the endothelium-intact tissue. BH$_4$ did not inhibit the ET-1-mediated O$_2^*$ production in the endothelium-denuded aorta, thus confirming that BH$_4$ is not acting as a nonspecific antioxidant.

**ET-1 Receptor-Mediated O$_2^*$ Production.** ET$_A$ receptors have been demonstrated to mediate O$_2^*$ production in vascular smooth muscle cell cultures as well as pulmonary vascular preparations (Galle et al., 2000; Wedgwood et al., 2001a; Wedgwood et al., 2001b; Sedeek et al., 2003; Laplante et al., 2005). ET$_B$ receptor-mediated ROS production in cultured human umbilical vein endothelial cells has also been reported recently (Dong et al., 2005). These authors demonstrated that apocynin inhibited the ET-1-mediated ROS production and endothelial cell proliferation, although the authors did not determine whether ET-1 stimulation also produced NOS uncoupling. The present study demonstrated that both ET$_A$ and ET$_B$ receptor signaling is critical for stimulation of O$_2^*$ production in aortic tissue, which was a puzzling finding. However, it is plausible that ET-1 may activate NADPH oxidase through both ET$_A$ and ET$_B$ receptors since both receptor subtypes have been reported to increase O$_2^*$ (Galle et al., 2000; Wedgwood et al., 2001a; Sedeek et al., 2003; Dai et al., 2004; Dong et al., 2005; Laplante et al., 2005). NADPH oxidase is expressed in all cell types in aortic tissue. Thus, we would speculate that ET-1 mediates stimulation of NADPH oxidase via both ET$_A$ and ET$_B$ receptors on smooth muscle and endothelial cells, which produce the so-called kindling radicals to further cause oxidation of BH$_4$. 

![Fig. 4](image4.png) ET-1 (1000 nM)-mediated O$_2^*$ production in endothelium-intact aortic tissue in the presence of specific ET-1 receptor antagonists. A and B, ET$_A$ receptor antagonist (BQ-123; 100 nM) and ET$_B$-receptor antagonist (A-192621; 100 nM) individually (n = 11) (A) or simultaneously (n = 7) (B). Values are means ± S.E. * p < 0.05 versus basal; ** p < 0.01 versus basal; *** p < 0.001 versus basal; †, p < 0.05 versus ET-1.

![Fig. 5](image5.png) ET-1-induced vasoconstriction of aortic rings in the absence or presence of sepiapterin BH$_4$ synthesis substrate (100 μM, n = 7) (upper panel) or the NADPH oxidase inhibitor apocynin (300 μM; n = 8) (lower panel). Values are means ± S.E. Maximal responses of ET-1-treated aortic segments (n = 8) are significantly increased compared with PSS-treated aortic segments (n = 8; p < 0.05), ET-1 + apocynin (p < 0.001), and ET-1 + sepiapterin (p < 0.05).
resulting in NOS uncoupling. We have hypothesized that this may result from some type of “cross-talk” between the ET receptor signaling pathways. Recently, Just et al. (2004) have suggested that both ET receptors function synergistically in renal vasoconstriction studies.

**ET-1-Mediated O2\textsuperscript{-} Production and Vasoconstriction.** ET-1 is a known vasoconstrictor; thus, we hypothesized that O2\textsuperscript{-} from NADPH oxidase and/or uncoupled NOS will mediate an increase in constriction. We found an increase in constriction with ET-1 treatment, and our results implicate at least two sources of O2\textsuperscript{-} that mediate the increased vasoconstriction, NADPH oxidase and uncoupled NOS. As previously discussed, BH4 is a critical factor in mediating O2\textsuperscript{-} production from uncoupled NOS in the present study as well as others (for review, see Channon, 2004). For vascular reactivity studies, we used sepiapterin to provide a source for intracellular production of BH4 in the aortic tissue. Sepiapterin is a substrate in the salvage pathway for the synthesis of BH4 (Alp and Channon, 2004). Neither apocynin nor sepiapterin affected the vasoconstriction in the absence of ET-1. Both apocynin and sepiapterin lowered maximal constriction when compared with ET-1 alone. Thus, functionally, these data indicate that ET-1-mediated increased constriction is facilitated through the products of both NADPH oxidase and uncoupled NOS.

**Perspectives.** In the present study, aortic tissue was incubated with exogenous ET-1 to model pathological states in which there are high levels of ET-1, such as salt-sensitive hypertension, atherosclerosis, and diabetes (Lariviere et al., 1993; White et al., 1994; Bousette and Giaid, 2003; Cernacek et al., 2003; Muller et al., 2003). ET-1 is known to be a paracrine factor and to act locally within a given tissue or cell type; thus, this ex vivo method models the paracrine effects of ET-1 within the vascular tissue. It is difficult to assess the local concentrations of ET-1 within any given tissue at different points in a pathological state. Elmarakby et al. (2004) reported that plasma ET-1 levels are increased approximately 10-fold in ET\textsubscript{A} receptor-deficient rats on a high-salt diet compared with wild-type rats on a high-salt diet. When the same rats are chronically treated with an ET\textsubscript{A} receptor antagonist, plasma ET-1 levels increase approximately 100-fold compared with wild-type rats on a high-salt diet. Under conditions of reduced receptor availability, we reasoned that the tissue ET-1 would be “dispelled” into the circulation. Thus, the plasma levels of ET-1 would be a reasonable approximation of the tissue levels, suggesting that tissue levels can become extremely high.

There are many pathological states in which the vasculature is exposed to high levels of ET-1 and endothelial dysfunction is present. These conditions may or may not be associated with chronic hypertension. Indeed, we reported recently that chronic ET-1 infusion in whole animals on a high-salt diet increased aortic O2\textsuperscript{-} production as well as produced a hypertensive state (Elmarakby et al., 2005). The NADPH oxidase inhibitor apocynin reduced vascular O2\textsuperscript{-} production without affecting hypertension. Although the mechanisms of endothelial dysfunction may be unknown, it appears that O2\textsuperscript{-} and/or ROS production from diverse enzymatic and cellular sources may be involved in many vascular pathologies (Munzel et al., 2005). Our data are consistent with the hypothesis that ET-1 stimulates NADPH oxidase in all cell types of the vasculature as well as the uncoupling of NOS in the endothelium via the oxidation of BH4. These events translate to enhanced vasoconstriction and may contribute to vascular disorders. Future studies will focus on additional ET-1-mediated dysfunction of NOS regulatory pathways in the vasculature.

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**References**


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