Increased $O_2^-$ Production and Upregulation of $ET_B$ Receptors by Sympathetic Neurons in DOCA-Salt Hypertensive Rats

Xiaoling Dai, James J. Galligan, Stephanie W. Watts, Gregory D. Fink, David L. Kreulen

Abstract—Superoxide anion ($O_2^-$) production is elevated in the vasculature of hypertensive animals but it is not known if $O_2^-$ production is also elevated in the sympathetic nervous system. We measured $O_2^-$ levels in prevertebral sympathetic ganglia of deoxycorticosterone acetate (DOCA)-salt hypertensive rats using the dihydroethidine (DHE) fluorescence method. $O_2^-$ was elevated in ganglia from DOCA-salt rats compared with normotensive sham rats. Treatment of ganglia with endothelin (ET)-1 (3×10^-8 mol/L) resulted in a 200% increase in fluorescence intensity in neurons, which was attenuated by the $ET_B$ receptor antagonist BQ788 (10^-7 mol/L). ET-1 also increased the $O_2^-$ induced fluorescence in dissociated sympathetic neurons and PC-12 cells via activation of $ET_B$ receptors, but not $ET_A$ receptors. To evaluate whether elevated ET-1 levels in the ganglia might contribute to the elevated $O_2^-$ found in ganglia we measured the amount of ET-1 using an ELISA assay. ET-1 levels in sham rat celiac ganglia were 695.6±40.9 picogram per gram; they were not different than ET-1 levels in ganglia from DOCA-salt rats. We then compared ET-1 receptor levels in ganglia from sham and DOCA-salt animals. ET-1 receptor mRNA levels were 32% higher and ET-1 receptor protein levels were 20% higher in celiac ganglia from DOCA-salt rats than from sham rats separately. In conclusion, $O_2^-$ is elevated in prevertebral sympathetic ganglia in DOCA-salt hypertension, and ET-1 is a potent stimulus for the elevation of $O_2^-$ levels in sympathetic ganglia, an effect that may be mediated by the upregulation of ET-1 receptors. (Hypertension. 2004;43:1048-1054.)

Key Words: endothelin receptors, endothelin hypertension sympathetic nervous system oxidative stress
Methods

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

Animals

Animal procedures were followed in accordance with the institutional guidelines of Michigan State University. DOCA-salt hypertensive and sham rats were prepared as previously described. The mean arterial pressures for the DOCA-salt and sham rats were 181.3±3.1 mm Hg and 111.8±4.3 mm Hg separately.

Sympathetic Ganglia Harvest and Cell Culture

Rats were euthanized with a lethal dose of sodium pentobarbital (65 mg/kg) and the CG or the IMG were removed. CG from normal rats were cultured as previously described. Rat pheochromocytoma PC-12 cells were differentiated with 50 ng/mL NGF for 1 week.

Measurement of Superoxide Anion Generation

O2·− levels were examined by measuring fluorescence signal intensity resulting from intracellular probe oxidization. IMG were from 4 groups: IMG of sham rats, IMG of DOCA-salt rats, or IMG of sham rats incubated with ET-1 or ET-1 plus the ETB receptor antagonist BQ788 (10−7 mol/L). The cells were divided into 2 treatment groups: 1 group using ET-1 (3×10−7 mol/L) or the ETB receptor antagonist BQ788 was tested against both agonists. The control received no treatment. IMG or cells were loaded with the oxidant-sensitive fluorogenic probe dihydroethidine (DHE) (2 μmol/L; Molecular Probes) for 45 or 30 minutes before measuring fluorescence (excitation: 514 nm; emission: 560 nm) with a confocal microscope. The intensity of the fluorescent signal is proportional to the O2·− levels. Images were analyzed using ImagePro Plus software (Media Cybernetics, Inc) to measure the fluorescence intensity of individual cells. Fluorescence intensity was normalized to IMG from sham rats or control group using the same parameters.

RNA Isolation and Reverse-Transcription

Polymerase Chain Reaction

Total RNA was isolated using TRizol procedures. The cDNA was synthesized, and polymerase chain reaction (PCR) was performed. PCR amplicons were analyzed on agarose gel. The sequences of PCR primers for ETB receptor (GenBank NM_017333) were 5'ATGGCCGCCCACCCACTAAGAC and 5'CACGAGGCATGATA- CACGAGGCATGATACAGAGGCATGATA-CAATCG, producing a 195bp amplicon.

Western Blotting

Tissues were homogenized and a membrane-enriched protein fraction was isolated by centrifugation. Protein concentrations were determined using the Lowry method. Protein was run in polyacrilamide gel followed by transferring to the polyvinylidine fluoride membrane. The membrane was incubated overnight with the primary ETB receptor antibody (Alomone Labs) and for 1 hour with the secondary antibody. Immunoreactivity was detected using a chemiluminescence kit.

Sympathetic ET-1 Content Measurement

CG were homogenized and immediately heated to 100°C for 10 minutes followed by chilling on ice. The homogenate was centrifuged at 14,000g for 30 minutes at 4°C. The supernatant was dried in a Speed-Vac and reconstituted in 250 μL calibrator diluent. ET-1 content was measured using the ET-1 QuantiGlo chemiluminescent assay kit (R&D Systems).

Data Analysis

Data are presented as mean±SE of the mean for the number of animals. Statistical significance was assessed by Student t test or 1-way ANOVA test with Dunnett multiple comparison post-test using Prism 3.0 software (GraphPad Software).

Drugs

ET-1, S6c, BQ610 and BQ788 were obtained from Peninsula Laboratories, and DOCA salt was purchased from Sigma Company.

Results

Fluorogenic Detection of O2·− Levels in Inferior Mesenteric Ganglia

Cells were grouped by diameter so that the fluorescent intensity of both neurons and glia were quantified. Cells with diameter ranging from 15 to 35 μm were identified as neurons and from 5 to 10 μm as glia. Representative optical slices through sham and DOCA-salt IMG are shown in Figure 1 (n=4 sham rats; n=4 DOCA-salt rats). O2·− levels were higher in the IMG from a DOCA-salt rat (Figure 1B) than from a sham rat (Figure 1A). Neurons displaying elevated O2·− levels were distributed throughout ganglia. Compared with sham ganglia, the fluorescent intensities of neurons and glia were 250% and 200% greater, respectively, in DOCA-salt ganglia (Figure 1C).

Effects of ET-1 Administration on O2·− Levels in Sympathetic Ganglia

O2·− levels were evaluated in IMG of sham rats incubated with ET-1 or ET-1 plus the ETB receptor antagonist BQ788 (n=4 rats in each group). Example optical slices of control and treated ganglia are shown in Figure 2.
IMG received no ET-1. O$_2^-$ levels in both neurons and glia were higher in the ET-1 treated IMG (Figure 2B) compared with the control (Figure 2A). ET-1 treatment resulted in 250% and 215% increase in fluorescent intensity in neurons and glia, respectively (Figure 2D). Ganglia pretreated with the ETB receptor antagonist BQ788 followed by ET-1 treatment showed no increase in O$_2^-$ fluorescence (Figure 2C) compared with the control (Figure 2A). This indicates that ET-1 is acting on ET$_B$ receptors to elevate O$_2^-$ levels in prevertebral sympathetic ganglia.

**Fluorogenic Detection of O$_2^-$ Level in Dissociated Celiac Ganglionic Neurons and PC-12 Cells**

To determine whether ET-1 acts on neurons directly to elevate O$_2^-$ levels, we incubated freshly dissociated celiac ganglionic neurons from normal rats and differentiated PC-12 cells with ET-1 and measured O$_2^-$ levels. Representative confocal images of DHE fluorescence in treated cells are shown in Figure 3 and Figure 4 (n=4 dishes of
cultured cells in each group). The changes in fluorescent intensity were quantified (Figure 5). Phase-contrast images of control cells are shown in Figure 3A and Figure 4A in parallel with the corresponding confocal images of control cells.

Compared with the control (Figure 3B, 38±4.5 arbitrary fluorescence units [AFUs]), sympathetic ganglionic cells incubated with ET-1 showed a 400% increase in fluorescence, indicating elevated O$_2^-$ levels (Figure 3C, 201±6.3 AFUs). The response was limited to cells with typical neuronal morphology with soma diameters ranging from 15 to 35 μm. Of 175 neurons counted over 4 sets of independent experiments, 174 (99.5%) were DHE positive when they were incubated with ET-1, whereas no control neurons (58 neurons counted) exhibited the fluorescence intensity above background levels. The ET-1-induced increase in the fluorescence intensity was attenuated to 45% by the pretreatment with the ET$_A$ receptor antagonist BQ610 (Figure 3G, 90±6.5 AFUs). Pretreatment of cells with the ET$_A$ receptor antagonist BQ610 did not reduce the ET-1-induced increase in the fluorescence intensity (Figure 3D, 213±9.5 AFUs). Likewise, cells treated with S6c showed a 400% increase in O$_2^-$ levels (Figure 3F, 203±5.7 AFUs). The S6c-induced increase was attenuated to 31% by the pretreatment with ET$_B$ receptor antagonist BQ788 (Figure 3H, 65±3.8 AFUs) but not by the pretreatment with BQ610 (Figure 3G, 210±8 AFUs). These experiments indicate that ET$_B$ receptors mediate superoxide anions production in sympathetic neurons.

The actions of ET receptors activation on O$_2^-$ generation were also evaluated in PC-12 cells, a catecholamine-secreting tumor cell line derived from chromaffin cells that have functional ET receptors.$^{17}$ ET-1 incubation induced a 225% increase in the fluorescence intensity (Figure 4C, 192±8 AFUs) compared with control cells (Figure 4B, 59±3 AFUs). The ET-1-induced increase in the fluorescence intensity was attenuated to 40% by pretreatment with ET$_B$ receptor antagonist BQ788 (Figure 4E, 78±6 AFUs). Pretreatment with the ET$_A$ receptor antagonist BQ610 did not change the effects of ET-1 (Figure 4D, 209±8 AFUs). Cells treated with S6c showed a 270% increase in fluorescence intensity (Figure 4F, 219±6 AFUs). This increase was not blocked by BQ610 (Figure 4G, 199±4.5 AFUs) but was attenuated to 34% by the ET$_B$ receptor antagonist BQ788 (Figure 4H, 74±10 AFUs). These results indicated that ET-1 acts on the ET$_B$ receptors to induce O$_2^-$ production in PC-12 cells.

**Measurement of ET-1 Levels**

In the crude protein fractions extracted from CG of sham and DOCA-salt rats (n=4 sham rats; n=4 DOCA-salt rats), there were 695.6±40.9 and 723.3±71.7 picograms of ET-1 per gram of wet weight of ganglia, respectively. They were not significantly different (P>0.05).

**ET$_B$ Receptor mRNA Level and Protein Expression in CG from Sham and DOCA-Salt Hypertensive Rats**

Using the primers designed stringently for the ET$_B$ receptor, reverse-transcription PCR was done in CG of sham and DOCA-salt rats (n=4 sham rats; n=4 DOCA-salt rats). The representative images are shown in Figure 6. The PCR amplicons for the ET$_B$ receptor and β-actin were detected in CG at the expected size (195 bp and 500 bp, respectively) in 1.5% ethidium-stained agarose gel (Figure 6A).
Elevated ET$_B$ receptor protein levels in celiac ganglia from DOCA-salt rats. Immunoblotting for the ET$_B$ receptor in CG from sham and DOCA-salt rats shows a single 40KDa band (A). Optical densities of the bands were quantified and the densities were normalized to Sham. The change in ETB receptor protein expression is upregulated in sympathetic ganglia of DOCA-salt rats. Similarly, ET-1 increases intracellular O$_2^-$ production in dissociated ganglionic neurons. This demonstrates that the mechanisms responsible for ET-1 induced O$_2^-$ generation are endogenous to neuron cell bodies and do not require the presence of vasculature or glia. Sympathetic IMG neurons projecting to mesenteric arteries are distinct from neurons projecting to mesenteric veins by their localization, neurochemical phenotypes, and electrophysiological properties. There was no evidence that subpopulations of neurons were affected differently because the increased superoxide anion signal was evenly distributed throughout the IMG, including both ganglionic neurons and glia. Likewise, when dissociated neurons were incubated with ET-1, O$_2^-$ levels increased in all neurons. By contrast, in the central nervous system, Ang II administration increases O$_2^-$ levels increased in all neurons. By contrast, in the central nervous system, Ang II administration increases O$_2^-$ in the vicinity of sympathetic preganglionic neurons projecting to the adrenal gland results in the activation of sympathetic preganglionic neurons innervating the adrenal gland and the subsequent release of catecholamine from adrenal medulla, which in turn elevates the blood pressure and heart rate. This opens the possibility that a change in the redox environment of sympathetic ganglionic neurons induced by O$_2^-$ may activate sympathetic neurons and result in the vasoconstriction and hypertension. O$_2^-$ may also indirectly modulate the excitability of sympathetic neurons by several mechanisms. One possible mechanism is the ability of O$_2^-$ to modulate the sympathetic excitability by quenching or inactivating nitric oxide (NO), which is known to exist in the sympathetic nervous system. We have shown previously that NO increases a Ca$^{2+}$-activated K$^+$ current in isolated sympathetic neurons, an effect that would reduce the firing rate. By causing a reduction in ganglionic NO levels, O$_2^-$ would eliminate this inhibitory effect of NO and this would result in an increased excitability of sympathetic neurons. Another possible mechanism is that O$_2^-$ may act as an intracellular second messenger and regulate the gene expression of antioxidant enzymes, such as SOD and catalase, in the sympathetic neurons as it does in blood vessels.

In sympathetic ganglia from sham rats, incubation with ET-1 increased O$_2^-$ production to the levels found in ganglia of DOCA-salt rats. Similarly, ET-1 increases intracellular O$_2^-$ production in dissociated ganglionic neurons. This demonstrates that the mechanisms responsible for ET-1 induced O$_2^-$ generation are endogenous to neuron cell bodies and do not require the presence of vasculature or glia. Sympathetic IMG neurons projecting to mesenteric arteries are distinct from neurons projecting to mesenteric veins by their localization, neurochemical phenotypes, and electrophysiological properties. There was no evidence that subpopulations of neurons were affected differently because the increased superoxide anion signal was evenly distributed throughout the IMG, including both ganglionic neurons and glia. Likewise, when dissociated neurons were incubated with ET-1, O$_2^-$ levels increased in all neurons. By contrast, in the central nervous system, Ang II administration increases O$_2^-$ in 40% of neurons in the lamina terminalis. Furthermore, it appears that the glia in sympathetic ganglia also have the capacity to generate O$_2^-$, which is similar to the increased superoxide production mediated through NAD(P)H oxidase in microglia of the ventral mesencephalon in Parkinson disease.

ET-1 levels in the protein extracts of celiac ganglia were the same in sham and DOCA-salt rats. In contrast, in the superior cervical ganglia of spontaneously hypertensive rats, there is an increased intracellular ET-1 immunoreactivity and mRNA. This may reflect different ganglia and/or different hypertensive models. ET-1 can be released from the cultured sympathetic neurons' analogous to
endothelial cells. The crude ganglionic protein extracts included intracellular and extracellular ET-1, and therefore did not differentiate between stored and released ET-1. Particularly the higher sympathetic outflow present in DOCA-salt hypertensive rats would result in elevated ET-1 release.

Two G-protein coupled ET-1 receptors, ET_A and ET_B, have been identified and cloned,27,28 both are widely distributed in vascular tissues,13 the central nervous system including neurons and glia,5,29 the sympathetic nervous system,7 and PC-12 cells.30 The activation of ET receptor subtypes may be tissue specific. Elevated O_2^- production in the vasculature of DOCA-salt hypertension is mediated by ET_A receptors;9 O_2^- production in sympathetic ganglia, which include neurons and glia, dissociated sympathetic neurons, and PC-12 cells, is elevated by the activation of ET_B receptors, but not by ET_A receptors. ET_A receptor knockout mice have developmental defects in the great vessels.31 By contrast, homozygous ET_B receptor gene knockout mice have lethal developmental defects in the enteric nervous system.31,32 In addition, the ET_B receptor is essential in neural crest development,33 from where neurons and glia of the sympathetic nervous system and the enteric nervous system are differentiated.

ET_B receptor mRNA levels and protein expression were upregulated in celiac ganglia from DOCA-salt rats when compared with sham rats. Upregulation of ET_B receptors also occurs in the vasculature in DOCA-salt hypertension,13 where it is thought to be important in mediating the enhanced contractile response to ET-1 that occurs in veins but not arteries. A similar mechanism may occur in ET-1-mediated increases in O_2^- production in sympathetic ganglia. In the face of similar ET-1 levels in normotensive and hypertensive ganglia, upregulated ET_B receptors may be mediating the enhanced O_2^- production. However, the possibility of upregulated activity or mRNA expression of NAD(P)H oxidase cannot be ruled out.33 For example, Ang II upregulates vascular NAD(P)H oxidase subunits nox1, nox4, gp91^phox, and p22^phox mRNA expression, an effect that is thought to mediate Ang II stimulated O_2^- production.34

**Perspectives**

This study demonstrates that O_2^- is elevated in prevertebral sympathetic ganglia in DOCA-salt hypertensive rats. Furthermore, we have identified ET-1 as a stimulus to increase O_2^- levels in sympathetic postganglionic neurons and PC-12 cells, and this increase can be attenuated by the specific ET_B receptor antagonist BQ788 pretreatment. Finally, ET-1 may be a potent stimulus for the elevation of O_2^- levels in sympathetic ganglia in DOCA-salt hypertension, an effect that is mediated by the upregulated ET_B receptor pathway. We propose that O_2^- production evoked by ET-1 may play roles in the increased sympathetic excitability and pathogenesis in the DOCA-salt hypertension. We further speculate that ROS in the sympathetic nervous system may be the important target for therapeutic treatment of hypertension.


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