Effect of Antioxidant Therapy on Blood Pressure and NO Synthase Expression in Hypertensive Rats
Nosratola D. Vaziri, Zhenmin Ni, Fariba Oveisi, Debra L. Trnavsky-Hobbs

Abstract—Earlier studies have demonstrated evidence for increased reactive oxygen species, enhanced NO synthase (NOS) expression, and elevated NO production in spontaneously hypertensive rats (SHR). Given the negative-feedback regulation of NOS by NO, we hypothesized that enhanced NO inactivation by ROS may contribute to compensatory upregulation of NOS in SHR. The present study was designed to test this hypothesis. Eight-week-old male SHR and Wistar-Kyoto rats were treated for 3 weeks with either a placebo or the potent antioxidant, lazaroid (desmethyltirilazad, 10 mg \cdot kg^{-1} \cdot d^{-1}, by gastric gavage). Tail arterial blood pressure, urinary excretion of NO metabolites (ie, nitrate and nitrite), and immunodetectable NOS isotype proteins in the vascular, renal, cardiac, and cerebral tissues were measured. The placebo-treated SHR group showed a marked elevation of blood pressure and a significant upregulation of aorta, kidney, and cardiac tissue endothelial and inducible NOS (eNOS and iNOS, respectively) proteins and of brain and renal tissue neuronal NOS. Lazaroid therapy ameliorated hypertension and mitigated the upregulation of eNOS and iNOS in vascular, renal, and cardiac tissues but had limited effect on the expression of renal and brain neuronal NOS. In contrast, lazaroid therapy had no effect on blood pressure, urinary nitrate and nitrite excretion, or tissue NOS isotype expressions in the Wistar-Kyoto group. These findings support the role of oxidative stress in the genesis and/or maintenance of hypertension and compensatory upregulation of the expression of eNOS and iNOS in SHR. (Hypertension. 2000;36:957-964.)

Key Words: stress \n free radicals \n hypertension, experimental \n antioxidants \n nitric oxide \n nitric oxide synthase

We have recently demonstrated a marked upregulation of renal and vascular expressions of endothelial and inducible NO synthase (eNOS and iNOS, respectively) proteins in young spontaneously hypertensive rats (SHR). These observations substantiated the results of several earlier studies,2–7 which provided evidence for enhanced NO systems in young and adult SHR. The presence of severe hypertension in the face of a marked increase in the NO system may be indicative of enhanced NO inactivation. Superoxide and other reactive oxygen species (ROS) can avidly react with and inactivate NO.8–11 It is of note that several recent studies have provided compelling evidence for increased ROS activity in SHR.12–15 Therefore, the associated increased ROS activity can enhance NO inactivation and reduce bioactive NO. This can, in turn, contribute to the maintenance of hypertension and cause a compensatory upregulation of NO synthase (NOS) isotype expression. In fact, administration of the cell-permeable superoxide dismutase mimetic, tempol, has been recently shown to lower blood pressure and increase NO availability in SHR.16 We have recently shown that biologically active NO exerts a negative-feedback influence on NOS expression in cultured human endothelial cells.17 Thus, increased ROS-mediated inactivation of NO can potentially contribute to a compensatory upregulation of NOS via a reduction in NO bioavailability, as recently shown in lead-induced hypertension.11,17 The present study was undertaken to test the hypothesis that antioxidant therapy may alleviate hypertension and reverse the compensatory upregulation of NOS isotypes in SHR.

Methods

Animals
Eight-week-old male SHR (Harlan Sprague Dawley Inc, Indianapolis, Ind) were housed in a temperature-controlled light-regulated space with 12-hour light and dark cycles. The animals were allowed free access to a low nitrate basic rat chow and water. The animals were randomly assigned to the lazaroid-treated and vehicle-treated control groups. The lazaroid-treated group received a potent antioxidant lazaroid compound, desmethyltirilazad (U7489G, UpJohn Inc), at 10 mg \cdot kg^{-1} \cdot d^{-1} by gastric gavage for 3 weeks. The placebo-treated SHR group received the inactive vehicle instead. A control group of age-matched male genetically normotensive Wistar-Kyoto rats (WKY) served as controls. To determine the possible effect of lazaroid therapy in genetically normotensive animals, the studies were repeated comparing 2 groups of WKY treated with lazaroid and placebo, as described for the SHR group. A minimum of 6 animals was included in each group. Timed urine collections were obtained by placing the rats in metabolic cages. Food was withdrawn, but water was provided the night before and during the collection period. At the conclusion of the 3-week treatment period, the animals were anesthetized with intraperitoneal injections of pentobarbital sodium (Nembutal, 50 mg/kg). Blood was obtained by cardiac puncture, and brain, heart (left ventricle), thoracic aorta, and kidneys were immedi-
ately harvested, cleaned, and promptly frozen in liquid nitrogen. The samples were then stored at −70°C until they were processed.

Measurement of Arterial Pressure
Arterial pressure was measured by tail plethysmography as described in our earlier studies. Briefly conscious rats were placed on a heated pad in a temperature-controlled quiet space. They were allowed to rest for 15 minutes with the tail placed inside a tail cuff. The cuff was inflated and released several times to condition the animal to the procedure. Thereafter, 4 consecutive measurements were taken by a rat-tail blood pressure monitor, recorded by a student oscillograph (Harvard Apparatus Inc), and averaged for presentation.

Measurement of Total NOX
Urine nitrate and nitrite (NOx) concentration was measured by using the purge system of a model 270B nitric oxide analyzer (NOA228, Sievers Instruments Inc) in a manner that was identical to that described in our earlier studies.

Tissue Preparation and Western Blot Analyses
Kidney, aorta, heart, and brain tissues were prepared for measurements of endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) protein abundance by Western blot analysis. The procedures were performed in a manner that was identical to that described in our previous studies using anti-eNOS, anti-iNOS, and anti-nNOS antibodies (Transduction Laboratories). Briefly, aorta, heart, brain, and kidney tissue protein preparations (50 μg for the aorta, heart, and brain; 100 μg for the kidney) were size-fractionated on 4% to 12% Tris-glycine gel (Novex) at 120 V for 3 hours. In preliminary experiments, we found that the given protein concentrations were within the linear range of detection for our Western blot technique. After electrophoresis, proteins were transferred onto Hybond-ECL membranes (Amersham Life Science Inc) at 400 mA for 120 minutes with the use of the Novex transfer system. The membrane was prehybridized in 10 mL buffer A (10 mmol/L Tris hydrochloride, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20, and 10% nonfat milk powder) for 1 hour and then hybridized for an additional 1-hour period in the same buffer containing 10 μL of the given anti-NOS monoclonal antibody (1:1000). The membrane was then washed for 30 minutes in a shaking bath, with the wash buffer (buffer A without nonfat milk) changed every 5 minutes before 1 hour of incubation in buffer A plus goat anti-mouse IgG-horseradish peroxidase at a final titer of 1:1000. Experiments were performed at room temperature. The washes were repeated before the membrane was developed with a light-emitting nonradioactive method using ECL reagent (Amersham Inc). The membrane was then subjected to autoradiography for 1 to 5 minutes. The autoradiograms were then scanned with a laser densitometer (model PD1211, Molecular Dynamics) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain before prehybridization. This step verified the uniformity of protein load and transfer efficiency across the test samples.

In an attempt to explore possible cross-reactivity between eNOS and iNOS in the vascular tissue, we probed cultured endothelial cell protein preparations for immunodetectable iNOS by Western blot. No detectable iNOS was found, whereas abundant eNOS was present in this preparation. This observation excludes discernible cross-reactivity with antibodies, accurate assessment of relative quantities of the 2 enzymes is not possible by the methods used in the present study.

Data Analysis
ANOVA was used in statistical analysis of the data, which are presented as mean±SEM. A value of P<0.05 was considered significant.

Results
Blood Pressure and NOx Excretion
As expected, the placebo-treated SHR group exhibited a marked elevation in arterial blood pressure compared with the WKY control group. Antioxidant therapy with the lazaroid compound resulted in a significant amelioration of hypertension. In confirmation of our earlier study, compared with the WKY control group, the placebo-treated SHR group showed a significant increase in urinary excretion of NOx. Antioxidant therapy lowered the urinary NOx excretion in the lazaroid-treated SHR group toward values seen in the control animals. In contrast to the data obtained in the SHR group, lazaroid therapy had no effect on either blood pressure or urinary NOx excretion in the WKY group (Figure 1).

Aorta NOS Isotypes
Compared with the WKY group, the placebo-treated SHR group showed a marked upregulation of eNOS and iNOS protein abundance in the aorta. Antioxidant therapy resulted in a significant attenuation of aorta eNOS and iNOS protein expressions in the lazaroid-treated SHR group (Figure 2). However, lazaroid therapy had no effect on either eNOS or iNOS expression in WKY aortas (Figure 3).

Kidney NOS Isotypes
Kidney tissue eNOS and iNOS protein abundance was markedly increased in the placebo-treated SHR group relative to the corresponding values found in the WKY control group. The upregulations of the kidney tissue eNOS and iNOS
protein expressions were significantly attenuated by antioxidant therapy in the lazaroid-treated SHR group. As with eNOS and iNOS proteins, renal tissue nNOS protein abundance was significantly increased in the placebo-treated SHR group. However, the magnitude of the lazaroid-induced fall in renal nNOS protein expression was far less than that seen with eNOS and iNOS proteins (Figure 4). In contrast to data obtained in the SHR group, lazaroid therapy had no effect on either eNOS, iNOS, or nNOS in the kidneys of the WKY group (Figures 3).

**Brain nNOS Protein**

Compared with the WKY control group, the placebo-treated SHR group showed a marked increase in brain tissue nNOS protein abundance. Lazaroid therapy caused a minimal reduction in immunodetectable nNOS protein abundance in the SHR brain and no effect in the WKY brain (Figures 3 and 5).

**Heart NOS Isotypes**

The placebo-treated SHR exhibited a marked upregulation of cardiac eNOS and iNOS protein expressions. Antioxidant therapy caused a significant but partial reversal of the cardiac eNOS and iNOS protein elevations in the lazaroid-treated SHR. However, lazaroid therapy had no effect on cardiac tissue NOS isotype expressions in WKY (Figures 3 and 6).

**Discussion**

Several recent studies have provided compelling evidence for increased ROS generation in the vascular tissues of SHR. For instance, using fluorescence microscopy, Suzuki et al have demonstrated enhanced superoxide production in mesenteric arterioles of SHR in vivo. Likewise Grunfeld et al have reported increased superoxide generation in cultured aortic endothelial cells from SHR compared with corresponding cells from WKY. Moreover, Cosentino et
al have shown increased superoxide and hydrogen peroxide release in aortic strips prepared from SHR. ROS are thought to contribute to the generation and/or maintenance of hypertension in SHR by several mechanisms. These include inactivation of endothelium-derived NO, nonenzymatic generation of vasoconstrictive F2-isoprostanes from arachidonic acid peroxidation, and depletion of the NOS cofactor tetrahydrobiopterin. The role of oxidative stress in the genesis and maintenance of hypertension in SHR is supported by amelioration of hypertension with antioxidant administration. In addition to SHR, oxidative stress has been implicated in a variety of other hypertensive disorders, including lead-induced hypertension, uremic hypertension, cyclosporine-induced hypertension, salt-sensitive hypertension, pre-eclampsia, essential hypertension, and diabetes. In addition, long-term consumption of high-fat and highly refined sugar diets, which are known to cause oxidative stress, has been shown to produce hypertension in normotensive animals. Finally, we have recently shown that induction of oxidative stress by glutathione depletion leads to severe sustained hypertension and depressed NO availability in genetically normotensive Sprague-Dawley rats. Thus, oxidative stress appears to be a common feature of hypertensive disorders of diverse origins.

ROS avidly react with and inactivate NO. ROS-mediated NO inactivation can contribute to hypertension and endothelial dysfunction by limiting the availability of biologically active NO. Earlier studies have revealed that NO can rapidly inhibit NOS enzymatic activity, presumably by interacting with the iron core of the hemeprotein of the enzyme. In addition, we have recently shown that NO exerts a negative-feedback role in the regulation of endothelial NOS expression. On the basis of these considerations, we hypothesized that upregulation of renal and vascular NOS isotypes found in our earlier study of SHR may be due to the ROS-mediated reduction of NO availability and, hence, diminished negative-feedback regulation of NOS expression. If true, amelioration of oxidative stress by antioxidant therapy should mitigate the upregulation of NOS isotypes in SHR.

In the present study, the untreated SHR group exhibited a significant elevation of arterial blood pressure, increased urinary NO metabolite excretion, and marked upregulation of renal, vascular, and cardiac eNOS and iNOS and of brain and kidney nNOS protein expressions. Administration of the potent antioxidant, desmethyltirilazad, for 3 weeks resulted in a significant amelioration of hypertension despite marked reductions in urinary NO excretion and renal, vascular, and cardiac NO protein expressions. These data suggest that alleviation of oxidative stress by antioxidant therapy diminishes ROS-mediated NO inactivation and, thereby, raises the availability of bioactive NO.
in the treated SHR. The rise in the bioactive NO availability, in turn, enhances NO-mediated vasodilatory tone, which could, in part, account for the observed amelioration of hypertension. In addition, improved NO availability restores the NO-mediated negative-feedback regulation of NOS activity and protein expression and, thereby, reverses the compensatory upregulation of NOS isotypes in the treated SHR. The observed effects of antioxidant therapy on blood pressure and NO metabolism in SHR were not due to a nonspecific action of the drug used. The latter assertion is substantiated by the lack of any effect of lazaroid therapy on blood pressure, urinary NO\textsubscript{x} excretion, or tissue NOS isotype expressions in the normotensive WKY. The latter findings parallel those of our recent studies demonstrating that in the absence of oxidative stress, antioxidant therapy has no effect on blood pressure, urinary NO\textsubscript{x} excretion, or NOS expression\textsuperscript{11,17} The results of the present study in SHR are consistent with those of our recent studies in rats with lead-induced hypertension, which is marked by oxidative stress and compensatory upregulation of renal and vascular NOS isotypes\textsuperscript{11,17,18} Administration of desmethyltirilazad in animals with lead-
induced hypertension reversed oxidative stress, improved NO availability, and ameliorated hypertension\(^27\) in a manner similar to that found in SHR in the present study. In a more recent series of studies, we found that amelioration of oxidative stress and hypertension with a vitamin E–fortified diet was coupled with enhanced NO availability and a reversal of compensatory up-regulation of renal and vascular NOS isotype expressions in rats with lead-induced hypertension,\(^{11,17}\) mirroring the findings of the present study in SHR.

The untreated SHR exhibited a marked increase in urinary total NO\(_x\) excretion despite avid ROS-mediated oxidation and sequestration of NO as peroxynitrite (ONOO\(^-\)) and nitrated tyrosine and other molecules. These events are expected to lower rather than raise urinary NO\(_x\) excretion. Although this is true for the unsteady-state phase, isomerization of ONOO\(^-\) and turnover of the nitrated molecules will eventually lead to formation of NO\(_3^-\) and NO\(_2^-\), which are excreted in the urine. Thus, during a steady-state condition, such as chronic hypertension, urinary NO\(_x\) reflects NO production despite ongoing ROS-mediated inactivation of NO.

The antioxidant used in the present study was desmethyl-tirilazad, which is a powerful scavenger of various ROS and a potent inhibitor of lipid peroxidation.\(^{46}\) This compound and its closely related derivatives have been widely used to study the effect of oxidative stress in a wide range of disorders.\(^{46}\) In addition, we have used this agent in our earlier studies demonstrating the role of oxidative stress in the pathogenesis of uremic and lead-induced hypertension.\(^{19,27}\)

Antioxidant therapy significantly ameliorated hypertension and partially reversed the upregulation of NOS isotypes in various tissues of SHR. However, it did not fully restore either blood pressure or NOS isotype expression to the levels found in the normal control animals. Increased shear stress and cyclic strain upregulated eNOS expression. In addition,
both systemic hypertension and regional cerebral arterial hypertension induced by simulated microgravity increase nNOS expression in the brain. Thus, the residual elevation of NOS isotype expression in the lazaroid-treated SHR may be due to the moderate hypertension observed in these animals. The partial role of elevated blood pressure in the upregulation of NOS isotypes is evidenced by significant but incomplete reversal of renal and vascular NOS isotype expressions with different antihypertensive agents (angiotensin type 1 receptor blockers and calcium channel blockers) in this model (X.Q. Wang, N.D. Vaziri, unpublished data, 2000). Thus, both ROS-mediated attenuation of negative-feedback regulation of NOS by NO and increased shear stress associated with hypertension contribute to the upregulation of NOS isotypes in SHR.

In conclusion, hypertension in untreated SHR was accompanied by increased urinary excretion of NO metabolites and marked upregulations of renal, vascular, and cardiac NOS isotype expression, confirming our earlier study. Administration of the potent antioxidant compound desmethyltirilazad ameliorated hypertension, lowered urinary NO metabolite excretion, and attenuated the compensatory upregulation of NOS isotypes in the tested organs. These findings point to the role of oxidative stress in the pathogenesis of hypertension and disordered NO metabolism in SHR.

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