Compensatory Up-Regulation of Nitric-Oxide Synthase Isoforms in Lead-Induced Hypertension; Reversal by a Superoxide Dismutase-Mimetic Drug

N. D. VAZIRI, Y. DING, and Z. NI
Division of Nephrology and Hypertension, Department of Medicine, University of California, Irvine, Irvine, California

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ABSTRACT
Chronic exposure to low levels of lead causes hypertension (HTN) that is, in part, due to increased inactivation of nitric oxide (NO) by reactive oxygen species (ROS). The latter results in functional NO deficiency and compensatory up-regulation of NO synthase (NOS). We have previously shown evidence for increased hydroxyl radical (OH) activity in rats with lead-induced HTN. Since in the biological systems OH is primarily derived from superoxide (O2-) we hypothesize that lead-induced oxidative stress and HTN must be due to increased O2- production and as such could be ameliorated by administration of a cell-permeable O2- scavenger. We, therefore, studied the effects of the superoxide dismutase (SOD)-mimetic drug tempol (15 mmol/kg/day i.p. x 2 weeks) and placebo in lead-exposed (given lead acetate, 100 ppm in the drinking water for 12 weeks) and normal control rats. Lead exposure resulted in a marked elevation of blood pressure, a significant reduction in urinary NO metabolites (NOx) excretion, and up-regulations of endothelial and inducible NOS abundance in the kidney, aorta, and heart and of neuronal NOS in the cerebral cortex and brain stem. Administration of tempol ameliorated HTN, increased urinary NOx excretion, and reversed the compensatory up-regulation of NOS isoforms in rats with lead-induced HTN. These abnormalities recurred within 2 wk after discontinuation of tempol. In contrast to the lead-exposed rats, the normal control rats showed no change in either blood pressure, urinary NOx excretion, or tissue NOS expression in response to either administration or discontinuation of tempol. Thus, the study supports the presence of increased O2- activity and its role in the pathogenesis of HTN and altered NO metabolism in lead-exposed animals.

Lead is a common industrial and environmental pollutant. Extended exposure to low levels of lead causes sustained hypertension (HTN) in humans and experimental animals (Harlan, 1988; Gonick et al., 1997; Vaziri et al., 1997, 1999a,b). The precise mechanism by which lead exposure causes HTN remains uncertain and several possible factors, including alterations of adrenergic system, renin-angiotensin pathway, endothelium-derived vasoregulatory factors, and signal transduction system have been considered. For instance, chronic lead exposure has been shown to raise plasma norepinephrine, suppress β-adrenergic receptor density, decrease cAMP production, and reduce vasodilatory response to β-adrenergic stimulation in the vascular tissue, while increasing renal tissue β-adrenergic receptor (Tsao et al., 2000). Moreover chronic exposure to lead has been reported to raise plasma angiotensin-converting enzyme and kininase II activities, events that can support a rise in blood pressure by elevating plasma angiotensin II and depressing plasma bradykinin levels (Carmignani et al., 1999). In addition altered prostaglandin production, enhanced endothelin generation, and increased protein kinase C activity have been implicated in the pathogenesis of lead-associated HTN (Khalil-Manesh et al., 1993; Watts et al., 1995; Gonick et al., 1998).

Earlier studies in our laboratory have revealed that lead-induced HTN in rats is associated with and is largely due to increased reactive oxygen species (ROS) and depressed NO availability. The latter is due to avid inactivation and sequestration of NO by ROS (Gonick et al., 1997; Vaziri et al., 1997, 1999a,b; Ding et al., 2001). We have further shown that the reduction in the biologically active NO in rats with lead-induced HTN is accompanied by a compensatory up-regulation of renal and vascular NO synthase (NOS) expression (Vaziri et al., 1999a). This phenomenon is consistent with our earlier studies, which demonstrated that NO exerts a negative feedback on regulation of NOS expression (Vaziri and Wang, 1999). In fact, we have shown that nonspecific antioxidant therapy with vitamin E can ameliorate HTN, enhance NO availability, and partially reverse the compensatory up-regulation of endothelial (eNOS) and inducible NOS abundance in the kidney, aorta, and heart and of neuronal NOS in the cerebral cortex and brain stem. Administration of tempol ameliorated HTN, increased urinary NOx excretion, and reversed the compensatory up-regulation of NOS isoforms in rats with lead-induced HTN. These abnormalities recurred within 2 wk after discontinuation of tempol. In contrast to the lead-exposed rats, the normal control rats showed no change in either blood pressure, urinary NOx excretion, or tissue NOS expression in response to either administration or discontinuation of tempol. Thus, the study supports the presence of increased O2- activity and its role in the pathogenesis of HTN and altered NO metabolism in lead-exposed animals.

ABBREVIATIONS: HTN, hypertension; ROS, reactive oxygen species; NO, nitric oxide; NOS, nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; SOD, superoxide dismutase; ‘OH, hydroxyl radical; O2-, superoxide; NOx, NO metabolites; nNOS, neuronal nitric-oxide synthase.
(iNOS) NOS isoforms in the kidney and vascular tissues of rats with lead-induced HTN (Vaziri et al., 1999a).

In an attempt to discern the nature of ROS involved in lead-induced HTN, we recently carried out a series of in vivo and in vitro experiments using salicylate trapping technique to quantify hydroxyl radical production (Ding et al., 2000, 2001). We further conducted infusions of superoxide dismutase (SOD) and hydroxyl radical scavenger dimethyldiotheria in this model (Ding et al., 1998, 2001). The results revealed strong evidence for increased hydroxyl radical (‘OH) activity in rats with lead-induced HTN and lead-treated cultured endothelial cells (Ding et al., 2000, 2001). No significant effect was observed with native SOD administration in lead-treated rats (Ding et al., 1998). However, native SOD, which is a peptide, cannot enter the intracellular space where the bulk of superoxide is generated in the mitochondria and cytoplasm.

‘OH is primarily produced from sequential reductions of superoxide (O$_2^-$) and hydrogen peroxide. Therefore, increased ‘OH activity in biological systems is most likely due either to increased O$_2^-$ production, reduced O$_2^-$ dismutation, or presence of an electron donor such as a transition metal catalyzing production of ‘OH from hydrogen peroxide. If the latter were the case, increased SOD activity that converts O$_2^-$ to hydrogen peroxide could theoretically augment ‘OH generation and oxidative stress. In contrast, if oxidative stress is due to excess O$_2^-$ activity, administration of a cell-permeable SOD should alleviate ‘OH production and oxidative stress. To explore these possibilities, we recently conducted an in vitro study in which lead-treated cultured endothelial cells and their vehicle-treated controls were incubated for 24 h with and without the cell-permeable SOD-mimetic agent tempol (Vaziri and Ding, 2001). The study showed complete reversal of lead-induced compensatory up-regulation of eNOS expression by tempol, thus pointing to increased O$_2^-$ activity in the lead-treated cells. In contrast, tempol had no effect on the control cells (Vaziri and Ding, 2001). Based on the results of the latter study, we hypothesize that administration of the cell-permeable SOD-mimetic should ameliorate HTN, enhance NO availability, and attenuate the compensatory up-regulation of NOS isoforms in animals with lead-induced HTN. The present study was undertaken to test this hypothesis.

**Materials and Methods**

**Animals.** Male Sprague-Dawley rats with an average weight of 200 g were housed in a climate controlled, light-regulated space with 12-h light (~500 lux) and dark cycles. They were fed a regular rat chow ad libitum. The animals were randomly assigned to the lead-exposed and normal control groups. Animals in the lead-exposed group were provided with a drinking water that contained 100 ppm lead acetate for 12 weeks. The control group was provided with regular H$_2$O. The lead-exposed and control animals were treated with daily intraperitoneal injections of tempol (15 mmol/kg; Sigma Chemical Co., St. Louis, MO) or placebo for 2 weeks beginning at week 10. Subgroups of six animals in each of the tempol- and placebo-treated groups were monitored for 2 weeks after the cessation of tempol or placebo therapies.

At weeks 10, 12, and 14, tail arterial pressure was measured and animals were placed in metabolic cages for a timed urine collection. Blood pressure was measured by tail plethysmography as described in our earlier studies (Genick et al., 1997). At the conclusion of the given observation periods, under general anesthesia (thiobutabarbital 100 mg/kg i.p.) animals were euthanized by exsanguination and brain, kidney, thoracic aorta, and heart were harvested. The tissues were immediately cleaned then frozen in liquid nitrogen and stored at −70°C until processed.

**Measurement of Total Nitrate and Nitrite (NO$_3^-$).** The concentration of NO$_3^-$ in the test samples was determined by means of the Sievers Instruments model 270B nitric oxide analyzer (NOA; Sievers Instruments, Boulder, CO) as described in our earlier studies (Vaziri et al., 1998d).

**Measurements of Tissue NOS Isoforms.** Frozen tissues were processed for determination of eNOS, iNOS, and neuronal NOS (nNOS) protein abundance using anti-eNOS, -iNOS, and -nNOS monoclonal antibodies (Transduction Laboratories, Lexington, KY) as described in our previous studies (Vaziri et al., 1998a; Ni et al., 1998, 1999). Briefly, thoracic aorta, kidney, left ventricle, cerebral cortex, and brain stem were homogenized (25% w/v) in 10 mM HEPES buffer, pH 7.4, containing 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, 10 mg/ml leupeptin, and 2 mg/ml aprotinin at 0–4°C with a tissue grinder fitted with a motor-driven ground glass pestle. Homogenates were centrifuged at 12,000g for 5 min at 4°C to remove tissue debris without precipitating plasma membrane fragments. The supernatant was used for determination of NOS proteins. Total protein concentration was determined with the use of a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA). The tissue extracts (50 μg of protein for aorta and heart and 100 μg of protein for kidney and brain) were size-fractionated on 4 to 12% Tris-glycine gel (Novex, Inc., San Diego, CA) at 120 V for 3 h. After electrophoresis, proteins were transferred onto Hybond-ECL membrane (Amersham Pharmacia Biotech, Arlington Heights, IL) at 400 mA for 120 min with the Novex transfer system. In preliminary experiments we had found that the given protein concentrations were within the linear range of detection for our Western blot technique. The membrane was prehybridized in 10 μl of buffer A (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, and 10% nonfat milk powder) for 1 h and then hybridized for an additional 1-h period in the same buffer containing 10 μl of the given anti-NOS monoclonal antibody (1:1000). The membrane was then washed for 30 min in a shaking bath, and the wash buffer (buffer A without nonfat milk) was changed every 5 min before 1 h of incubation in buffer A plus goat anti-mouse IgG/ horseradish peroxidase at the 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 with the use of ECL Western blot detection reagent (Amer sham Pharmacia Biotech). The membrane was then subjected to autoluminography for 10 s. The autoluminographs were scanned with a laser densitometer (model PD1211; Molecular Dynamics, Sunnyvale, CA) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain, which verified the uniformity of protein load and transfer efficiency across the test samples.

**Data Analysis.** Data are expressed as mean ± S.E.M. Analysis of variance, multiple range test, regression analysis, and Student’s $t$ test were used as appropriate. $P$ values less than 0.05 were considered significant.

**Results**

**Arterial Pressure and Urinary NO$_3^-$ Excretion**

Data are shown in Fig. 1. As expected lead exposure resulted in a marked increase in arterial blood pressure. Development of HTN in the lead-exposed animals was coupled with a significant fall in urinary NO$_3^-$ excretion. Administration of tempol resulted in a significant amelioration of HTN and normalization of urinary NO$_3^-$ excretion in rats with lead-induced HTN. Discontinuation of tempol resulted in a rise in blood pressure and a fall in urinary NO$_3^-$ excretion to
levels that were virtually identical to those obtained prior to institution of therapy with tempol. Blood pressure in the lead-treated animals was inversely related to urinary NO\textsubscript{x} excretion (r = -0.7, p < 0.01). In contrast to the lead-exposed animals, the control animals showed no significant change in either blood pressure or urinary NO\textsubscript{x} excretion in response to either administration or discontinuation of tempol.

No significant difference was found in body weight (424 ± 27 versus 422 ± 26 g), serum creatinine (0.49 ± 0.04 versus 0.48 ± 0.02 mg/dl) or creatinine clearance (2.3 ± 0.2 versus 2.1 ± 0.4 ml/min) between the lead-exposed and the control groups.

NOS Isoform Expressions

**Aorta.** Data are illustrated in Fig. 2. Lead exposure resulted in a significant increase in the aorta eNOS protein abundance compared with values obtained in the control group. Administration of tempol for 2 weeks resulted in a significant decline in the aorta eNOS protein abundance toward control values. Discontinuation of tempol led to the rise in the aorta eNOS abundance to the elevated values observed before tempol administration in rats with lead-induced HTN. As with eNOS, aorta iNOS was significantly elevated in rats with lead-induced HTN, declined to normal level with tempol administration, and rose to pretreatment values 2 weeks following cessation of tempol. In contrast to rats with lead-induced HTN, normal control rats showed no significant change in either eNOS or iNOS expression in the aorta in response to either administration or discontinuation of therapy with tempol.

**Left Ventricle.** Data are depicted in Fig. 3. As with the aorta, both eNOS and iNOS protein expressions were significantly increased at baseline and significantly fell with tempol administration. Within 2 weeks after cessation of tempol eNOS protein abundance in the left ventricle rose to the pretreatment level, whereas iNOS protein abundance increased to a level that was considerably above the baseline in rats with lead-induced HTN. The reason for the observed rebound in cardiac tissue iNOS abundance following discontinuation of tempol is not clear and awaits further investigation. Once again, neither administration nor discontinuation of tempol significantly altered cardiac eNOS or iNOS expressions in the control animals.

**Kidney.** Data are shown in Fig. 4. In the lead-exposed animals, eNOS, nNOS, and iNOS protein expressions in the renal tissue were significantly increased at baseline and declined with tempol administration. Discontinuation of ther-
apy with tempol resulted in a rise in iNOS, eNOS, and nNOS abundance to pretreatment levels within 2 weeks. In contrast to the lead-exposed animals, tempol had no significant effect on the renal tissue NOS isoform expressions in the normal control animals.

**Brain.** Data are illustrated in Fig. 5. In rats with lead-induced HTN, nNOS protein expression in both cerebral cortex and brain stem was significantly increased at baseline, fell with administration of tempol, and rose to pretreatment values 2 weeks after discontinuation of tempol (C2 and L2). n = 6 in each subgroup. *p < 0.05 versus no treatment or discontinued treatment; #p < 0.05 versus the control group.

The study animal used in the present study showed detectable iNOS protein in the aorta, kidney, and cardiac tissues. The iNOS expression in the given tissues is not due to classic immunologic induction of iNOS, which leads to the generation of massive quantities of NO, as exemplified by septic shock. Instead, low-level expression of iNOS occurs constitutively in the kidney, heart, blood vessels, and other tissues under normal conditions (Ahn et al., 1994; Mohaupt et al., 1994; Morrissey et al., 1994; Park et al., 1996) Moreover, dysregulation of constitutively expressed iNOS has been reported in various conditions associated with disturbances of blood pressure, fluid, and electrolytes (Vaziri et al., 1998a,b,c; Barton et al., 2001). The observed iNOS reactivity is not due to cross-reaction of anti-iNOS antibody with other NOS isotypes, because we found no detectable iNOS by Western blot using either cultured endothelial cells or positive eNOS or nNOS control preparations. We therefore believe that up-regulation of iNOS in the kidney and other tissues of the lead-exposed animals represents the modulation of constitutively expressed iNOS in these animals.

**Discussion**

NO is produced from L-arginine by a family of enzymes known as NO synthases in nearly all tissues where it serves as a biological modulator with diverse actions. For instance, NO plays a major role in regulation of blood pressure by promoting vasodilation, renal sodium, and water excretion.
(Dijkhorst-Oei and Koomans, 1998) and inhibition of central sympathetic activity (Harada et al., 1993). The pivotal role of NO in regulation of blood pressure is evidenced by the fact that inhibition of NO production by NOS inhibitors causes severe HTN. Superoxide and other ROS avidly oxidize and inactivate NO and in the process produce highly reactive and cytotoxic by-products, such as peroxynitrite (ONOO⁻), which can modify proteins, lipids, DNA, and other molecules (Halliwell, 1997). Thus, oxidative stress can potentially contribute to HTN by promoting avid ROS-mediated NO inactivation, leading to functional NO deficiency. In fact, numerous studies by our group and other investigators have demonstrated the presence of oxidative stress in animals and humans with different forms of HTN. These include rats with lead-induced HTN (Gonick et al., 1997; Vaziri et al., 1997, 1999a,b; Ding et al., 2001); uremic HTN (Vaziri et al., 1998d); spontaneous HTN (Schnackenberg et al., 1998; Schnackenberg and Wilcox, 1999; Roberts et al., 2001). Finally, we have recently demonstrated that induction of oxidative stress by glutathione peroxidase depletion can cause a severe antioxidant-remediable HTN marked by avid inactivation and sequestration of NO and pronounced reduction in NO availability in genetically normal, otherwise intact rats (Vaziri et al., 2000b). The latter study provided convincing evidence that oxidative stress, per se, can cause HTN.

Exposure to lead for 12 weeks resulted in marked elevation of blood pressure and significant reduction in urinary \( \text{NO}_x \) excretion in rats used in the present study. The observed rise in blood pressure and fall in urinary \( \text{NO}_x \) excretion were paradoxically accompanied by up-regulation of eNOS and iNOS in the heart, kidney, and vascular tissues and nNOS in the cerebral cortex and brain stem. The reduction in urinary \( \text{NO}_x \) excretion was not due to diminished \( \text{L}-\text{arginine} \) intake or impaired renal clearance of \( \text{NO}_x \), since food intake and body weight were similar and glomerular filtration rate as estimated by creatinine clearance was comparable in the lead-treated and control groups. Instead, as demonstrated in our earlier studies (Vaziri et al., 1999b), the reduction in urinary \( \text{NO}_x \) excretion was clearly substantiated by one of our recent studies (Vaziri et al., 1999a). The present study confirms the results of the latter investigation and expands the previous findings to brain nNOS and cardiac NOS isoform expressions.

NO has been shown to exert a negative feedback action on NOS enzymatic activity (Buga et al., 1993). In addition, we have recently shown that NOS expression is negatively regulated by NO (Vaziri and Wang, 1999). For instance, incubation with NO scavenger oxyhemoglobin up-regulates, whereas incubation with NO donor sodium nitroprusside down-regulates eNOS expression in cultured endothelial cells. Moreover, up-regulatory action of oxyhemoglobin is obviated by coincubation with an NO donor (Vaziri and Wang, 1999). Accordingly, reduction in bioactive NO due to its avid inactivation by ROS can, in part, account for the observed up-regulation of NOS isoforms in rats with lead-induced HTN. This contention is supported by the fact that administration of SOD-mimetic agent tempol to rats with lead-induced HTN reversed up-regulation of NOS isoforms and simultaneously raised urinary \( \text{NO}_x \) excretion, denoting improved NO availability. The validity of this supposition was clearly substantiated by one of our recent studies (Vaziri et al., 1999b) of rats with lead-induced HTN in which reduced urinary \( \text{NO}_x \) excretion was coupled with massive tissue accumulation of nitrotyrosine, which is a footprint of NO inactivation and sequestration by ROS. Antioxidant therapy with vitamin E administration ameliorated HTN, reduced tissue nitrotyrosine abundance, and raised urinary \( \text{NO}_x \) excretion.

The role of oxidative stress in the pathogenesis and maintenance of HTN is further enforced by the observation that alleviation of oxidative stress by a variety of antioxidants or dietary modifications enhances NO availability and ameliorates HTN in rats with lead-induced HTN, uremic HTN, diet-induced HTN, and spontaneous HTN (Gonick et al., 1997; Vaziri et al., 1997, 1998d, 1999a,b, 2000a; Ding et al., 1998, 2001; Schnackenberg et al., 1998; Schnackenberg and Wilcox, 1999; Roberts et al., 2001). Finally, we have recently demonstrated that induction of oxidative stress by glutathione peroxidase depletion can cause a severe antioxidant-remediable HTN marked by avid inactivation and sequestration of NO and pronounced reduction in NO availability in genetically normal, otherwise intact rats (Vaziri et al., 2000b). The latter study provided convincing evidence that oxidative stress, per se, can cause HTN.

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by limiting ROS-mediated NO sequestration in lead-exposed rats (Vaziri et al., 1999b).

In contrast to administration of cell-impermeable native SOD, which had no effect on either blood pressure or urinary NO\textsubscript{x} (Ding et al., 1998), administration of the cell-permeable SOD-mimetic drug tempol (Samuni et al., 1988; Mitchell et al., 1990) resulted in a dramatic improvement of HTN and normalization of urinary NO\textsubscript{x} excretion in rats with lead-induced HTN used in the present study. This observation suggests that oxidative stress and elevated \textit{OH} generation in lead-exposed animals (Ding et al., 2001) is primarily due to increased abundance of superoxide, which is the precursor of \textit{OH}.

Discontinuation of tempol resulted in recurrence of HTN, reduction of urinary NO\textsubscript{x} excretion, and reappearance of compensatory up-regulation of NOS isoforms in various tissues. These findings argue against possible reduction of lead burden as a potential mechanism of action of this drug. This is because if the latter were the case the benefits observed of therapy on blood pressure, urinary NO\textsubscript{x}, and NOS isoforms would have persisted indefinitely after discontinuation of tempol and lack of further lead exposure. In fact, tissue lead would have persisted indefinitely after discontinuation of the present in vivo results, we have recently found a similar reversal of lead-induced up-regulation of eNOS protein expression by both tempol and desmethyltirilazad in cultured endothelial cells in vitro where the amount of lead exposure and raised urinary NO\textsubscript{x} in this model (Vaziri et al., 1999a,b). Moreover, in conformity with the present in vivo results, we have recently found a similar reversal of lead-induced up-regulation of eNOS protein expression by both tempol and desmethyltirilazad in cultured endothelial cells in vitro where the amount of lead exposure was necessarily constant (Vaziri and Ding, 2001). These observations suggest that the effect of the given antioxidants on NOS expression was not mediated by a change in the lead burden. Likewise, the similarity of the results of the in vivo studies with the in vitro experiments wherein hemodynamic influences are necessarily absent, points to a pressure-independent direct action of lead and the given antioxidants on eNOS expression. It is of note that elevated arterial pressure up-regulates eNOS and nNOS but has no effect on iNOS expression in vascular or cardiac tissues (Barton et al., 2001). Thus, the reduction in blood pressure with administration and recurrence of HTN following discontinuation of tempol could have, in part, contributed to the observed changes of eNOS and nNOS expressions in the lead-exposed animals. However, alterations in blood pressure had no role in the genesis of the observed changes in the tissues’ iNOS protein expression, which is entirely independent of pressure and shear stress (Barton et al., 2001).

In contrast to the lead-exposed animals, normal control animals exhibited no significant change in either blood pressure, urinary NO\textsubscript{x} excretion, or tissue NOS isoforms in response to either administration or discontinuation of tempol. These observations indicate that in the absence of oxidative stress, natural antioxidant system is sufficient to contain ROS generated in the course of normal metabolism. Moreover, these data argue against an unrelated direct or indirect effect of tempol on the measured parameters.

In conclusion, lead-treated rats exhibited marked elevation of blood pressure, significant reduction in urinary NO\textsubscript{x} excretion, and compensatory up-regulations of renal, cardiac, and vascular tissue eNOS and iNOS, as well as brain and kidney tissue nNOS. These abnormalities nearly disappeared with administration and recurred with discontinuation of the cell-permeable SOD-mimetic drug tempol, which had no discernible effect in the control animals. Taken together, these findings point to the role of increased superoxide abundance in the pathogenesis of oxidative stress, altered NO metabolism, and HTN in rats with lead-induced HTN.

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References


Address correspondence to: N. D. Vaziri, M.D., MACP, Division of Nephrology and Hypertension, Department of Medicine, University of California Irvine Medical Center, Bldg. 53, Room 125, Route 81, 101 The City Dr., Orange, CA 92868. E-mail: ndvaziri@uci.edu