Nitric Oxide Synthase Expression in the Course of Lead-Induced Hypertension

Nosratola D. Vaziri, Yaoxian Ding, Zhenmin Ni

Abstract—We recently showed elevated reactive oxygen species (ROS), reduced urinary excretion of NO metabolites (NOx), and increased NO sequestration as nitrotyrosine in various tissues in rats with lead-induced hypertension. This study was designed to discern whether the reduction in urinary NOx in lead-induced hypertension is, in part, due to depressed NO synthase (NOS) expression. Male Sprague-Dawley rats were randomly assigned to a lead-treated group (given lead acetate, 100 ppm, in drinking water and regular rat chow), a group given lead and vitamin E–fortified chow, or a normal control group given either regular food and water or vitamin E–fortified food for 12 weeks. Tail blood pressure, urinary NOx excretion, plasma malondialdehyde (MDA), and endothelial and inducible NOS (eNOS and iNOS) isotypes in the aorta and kidney were measured. The lead-treated group exhibited a rise in blood pressure and plasma MDA concentration, a fall in urinary NOx excretion, and a paradoxical rise in vascular and renal tissue eNOS and iNOS expression. Vitamin E supplementation ameliorated hypertension, lowered plasma MDA concentration, and raised urinary NOx excretion while significantly lowering vascular, but not renal, tissue eNOS and iNOS expression. Vitamin E supplementation had no effect on either blood pressure, plasma MDA, or NOS expression in the control group. The study also revealed significant inhibition of NOS enzymatic activity by lead in cell-free preparations. In conclusion, lead-induced hypertension in this model was associated with a compensatory upregulation of renal and vascular eNOS and iNOS expression. This is, in part, due to ROS-mediated NO inactivation, lead-associated inhibition of NOS activity, and perhaps stimulatory actions of increased shear stress associated with hypertension. (Hypertension. 1999;34:558-562.)

Key Words: lead hypertension, lead-induced free radicals nitric oxide vitamins antioxidants lipids

Prolonged exposure to low levels of lead causes systemic hypertension in humans and laboratory animals.1–5 In a series of recent studies, we showed that elevation of blood pressure in rats with lead-induced hypertension is accompanied by a marked increase in plasma and tissue lipid peroxidation product, an increase in malondialdehyde (MDA), and a substantial reduction in urinary excretion of stable NO metabolites (NOx).4–6 On the basis of these findings, we hypothesized that lead-induced hypertension in this model may be due, in part, to enhanced NO inactivation by reactive oxygen species (ROS). In support of this proposition, we demonstrated marked amelioration of hypertension together with normalization of plasma MDA concentration and urinary NOx excretion with a variety of antioxidants, including des-methyltirilazad,4 dimercaptosuccinic acid,6 and vitamin E,7 with this model.

The reduction in urinary NOx excretion, which is consistently found in this model,4,6 may be due to either diminished NO production and/or enhanced NO sequestration. With respect to the latter possibility, we recently demonstrated a marked increase in nitrotyrosine abundance in the plasma, kidney, heart, brain, and liver of rats with lead-induced hypertension.7 Because nitrotyrosine is a secondary by-product of interactions of NO, ROS, and tyrosine residues of proteins,8–10 its accumulation in the tissues of animals with lead-induced hypertension reflects ROS–mediated NO inactivation and sequestration in this model. A second possible mechanism for depressed urinary NOx excretion in this model is diminished NO generation. This can, in turn, be due to diminished l-arginine availability, quantitative NO synthase (NOS) deficiency, or NOS inhibition. This study was intended to determine the possible effect of long-term lead exposure on NOS protein expression and NOS activity in rats with lead-induced hypertension. We also explored the effect of lead on NOS activity in a cell-free preparation in vitro.

Methods

Animals
Male Sprague-Dawley rats (Harlan Sprague Dawley) (average weight, 200 g) were used. Rats were randomly assigned to lead-treated (Pb; n=6) and control (CTL; n=6) groups. The drinking water in the Pb group was supplemented with lead acetate (100 ppm) for 12 weeks. In contrast, the CTL group was provided with distilled water throughout the observation period. The Pb and CTL groups were fed regular rat chow containing tocopherol (40 U/kg). To
discern the role of increased ROS, additional groups of 6 lead-treated and 6 control rats were fed a vitamin E–fortified diet (Pb+E and CTL+E groups), which consisted of regular rat chow containing 5000 U/kg tocopherol (prepared by Harlan Teklad). Subgroups of animals were anesthetized with Nembutal (50 mg/kg IP) and killed by exsanguination using cardiac puncture 4, 8, and 12 weeks after initiation of the study. Plasma was separated; kidneys and the thoracic aorta were harvested, cleaned in PBS, frozen in liquid nitrogen, and stored at −70°C until processing. Five-hour urine samples were obtained with individual metabolic cages. Plasma MDA was determined by high-performance liquid chromatography as previously described.4

Measurement of Blood Pressure

Conscious rats were placed in a restrainer on a heated pad and allowed to rest inside the cage for 15 minutes before blood pressure was measured. The procedure was performed in a climate-controlled room with an ambient temperature of 70°F. Rat tails were placed inside a tail cuff, and the cuff was inflated and released several times to allow the animal to become conditioned to the procedure. A minimum of 4 consecutive blood pressure measurements were obtained with use of a rat tail sphygmomanometer attached to a student oscillograph (Harvard Apparatus) and averaged.11

In Vitro Effect of Lead on eNOS Activity

These experiments were conducted to discern the possible effect of lead on eNOS enzymatic activity in a cell-free preparation. To this end, endothelial cells were cultured and cellular protein was extracted as previously described.12 Aliquots of this eNOS-containing preparation were assayed for calcium-dependent NOS activity in the absence of various concentrations of lead acetate (0, 1, 10, 30, and 100 μg/L).

NOS Activity and Protein Assays

Frozen renal and aortic tissues were processed for these assays as previously described.13 NOS activity of tissue preparations was determined from the rate of conversion of [3H] L-arginine to [3H] L-citrulline as previously described.13 eNOS and iNOS protein abundance were measured by Western blot analysis with anti-eNOS, anti-Mac NOS-I monoclonal antibodies (Transaction Laboratories) as previously described.11

Tissue Lead Determination

Slices of renal cortex were dried at 70°C for 24 hours. Samples were then weighed and processed for lead measurement by an atomic absorption spectrometer (model 305 with graphite furnace, Perkin-Elmer).

Measurement of Total Nitrite and Nitrate

Total nitrite and nitrate (ie, NOx) was measured with a Sievers Instruments NO analyzer (model 270B, NOATM) as previously described.14

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=6)</th>
<th>Pb (n=6)</th>
<th>Pb+E (n=6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>128±7</td>
<td>180±6</td>
<td>150±7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma MDA, μmol/L</td>
<td>2.05±0.16</td>
<td>4.63±0.13</td>
<td>2.52±0.44</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urinary NOx, μmol/5 h · 100 g−1 body weight</td>
<td>0.68±0.12</td>
<td>0.39±0.09</td>
<td>0.59±0.11</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>425±7</td>
<td>423±7</td>
<td>422±4</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.42±0.01</td>
<td>0.43±0.01</td>
<td>0.42±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>2.1±0.5</td>
<td>2.4±0.2</td>
<td>2.0±0.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data Presentation and Analysis

Data are presented as mean±SEM. ANOVA, a multiple-range test, and regression analysis were used as appropriate. P<0.05 was considered significant.

Results

Effects of Long-Term Lead Exposure

A significant rise in arterial blood pressure was noted 8 weeks after the onset of lead exposure in the Pb group. This was accompanied by a marked reduction in urinary NOx excretion when compared with the CTL group. The Pb group exhibited a marked rise in plasma lipid peroxidation product (MDA; Figure 1). No significant difference was found in either food intake, body weight, creatinine clearance, or hematocrit between the study groups (Table).

Longitudinal measurements of eNOS and iNOS revealed a significant rise in the abundance of these NOS isotypes in the aorta and kidney of lead-treated animals beginning 4 to 8 weeks after initiation of the study. Plasma was separated; kidneys and the thoracic aorta were harvested, cleaned in PBS, frozen in liquid nitrogen, and stored at −70°C until processing. Five-hour urine samples were obtained with individual metabolic cages. Plasma MDA was determined by high-performance liquid chromatography as previously described.4
weeks after lead exposure (Figures 2 and 3). The rise in aorta eNOS protein mass was accompanied by an increase in calcium-dependent NOS activity in the Pb group by 62% ± 13%, 62% ± 7%, and 91% ± 5% relative to values in the CTL group at weeks 4, 8, and 12, respectively (P < 0.05 for all comparisons). Likewise, the rise in iNOS protein abundance in the aorta was coupled with significant elevations of calcium-independent NOS activity by 53% ± 15%, 60% ± 7%, and 86% ± 6% of control values at weeks 4, 8, and 12, respectively (P < 0.05 for all comparisons).

**Effects of High-Dose Vitamin E**

Administration of high-dose vitamin E significantly ameliorated but did not completely abrogate lead-induced hypertension in the Pb+E group. In addition, vitamin E supplementation mitigated a lead-induced rise in plasma MDA concentration and prevented the fall in urinary NOx excretion (Table) and the rise in eNOS and iNOS protein abundance in the aorta (Figure 4). However, the change in kidney tissue eNOS and iNOS protein abundance with vitamin E supplementation did not reach statistical significance (Figure 5). No significant difference was found in kidney tissue lead concentration between the Pb and Pb+E groups (8.2 ± 0.88 versus 10.8 ± 1.00 μg/g dry tissue, respectively; P = NS). Interestingly, vitamin E supplementation had no effect on systolic blood pressure in normal control animals (120 ± 4 mm Hg in the CTL group versus 119 ± 3 mm Hg in the CTL+E group; P = NS). Likewise, vitamin E did not alter plasma MDA concentration (0.96 ± 0.15 μmol/L in the CTL group and 1.0 ± 0.14 μmol/L in the CTL+E group; P = NS) and had no effect on NOS isotype expression of either aortic or renal tissues in normal animals (data not shown).

**Effect of Lead on NOS Activity In Vitro**

These experiments were conducted to discern the possible effect of lead on NOS enzymatic activity. To this end, aliquots of an endothelial cell protein preparation were incubated in the presence of either vehicle or lead acetate at 1-, 10-, 30-, and 100-μg/dL concentrations before NOS activity was measured. At concentrations of 10, 30, and 100 μg/dL, lead acetate had no detectable effect on NOS activity. However, at 1 μg/dL, lead acetate had no detectable effect on NOS activity.

**Discussion**

The rise in blood pressure in lead-treated animals was associated with a marked reduction in urinary NOx excretion. This phenomenon could be due to either depressed NO production or enhanced NO sequestration. The reduction in urinary NOx excretion was not due to low-dietary L-arginine content because body weight and food intake were similar in the 2 groups. Likewise, it cannot be attributed to a quantita-
ative NOS deficiency because eNOS and iNOS proteins were elevated in lead-treated animals. A plausible explanation for the decline in urinary NOx excretion in the face of upregulation of NOS expression is the potential ROS-mediated oxidation and sequestration of NO. In this regard, superoxide (O$_2^-$) and other ROS avidly oxidize NO to form peroxynitrite (ONOO$^-$), which can interact with proteins, lipids, and DNA.8 For instance, peroxynitrite avidly reacts with tyrosine to form nitrotyrosine.

The supposition that hypertension and depressed urinary NOx excretion in the face of increased NOS expression is, at least in part, due to ROS-mediated NO inactivation and sequestration is supported by the results of vitamin E therapy. Concomitant administration of vitamin E abrogated elevation of MDA and resulted in a rise in urinary NOx excretion and a fall in vascular eNOS and iNOS expression. Together, these findings point to enhanced ROS-mediated NO inactivation and sequestration as contributory factors in the pathogenesis of hypertension and depressed urinary NOx excretion in lead-treated animals. Accordingly, antioxidant therapy with high-dose vitamin E mitigated excess ROS activity and, hence, NO inactivation and sequestration. This, in turn, increased NO availability, which ameliorated hypertension, and decreased NO sequestration, which increased urinary NOx excretion. This supposition is supported by results of our earlier studies, which demonstrated marked overabundance of nitrotyrosine in the plasma, kidney, heart, liver, and brain of rats with lead-induced hypertension and its normalization by high-dose vitamin E supplementation.

In contrast to the effect seen in lead-exposed animals, vitamin E supplementation had no effect on blood pressure, plasma MDA concentration, or expression of NOS isotypes in control animals. Thus, in the absence of oxidative stress, antioxidant therapy does not significantly influence this process.

Previous studies have shown inhibition of NOS activity by lead in vitro.15 These observations were confirmed by results of the present study, which demonstrated significant inhibition of NOS activity at lead acetate concentrations ≥10 μg/dL. It should be noted that according to our previous studies, plasma lead concentration in this model is 10 μg/dL and, as such, is sufficient to partially inhibit NOS activity in vivo. This observation might appear to be at variance with the results of NOS activity measurements obtained in the aorta of lead-treated rats, which demonstrate increased rather than decreased activity. However, the volume of tissue protein preparation used in this assay is 1/25 of that of the total reaction mixture. This degree of dilution is more than sufficient to obviate the inhibitory action of the original tissue lead content present in vivo.

The precise mechanism responsible for upregulation of renal and vascular eNOS and iNOS expression in animals with lead-induced hypertension is unclear. Increased blood pressure, shear stress, and blood flow have been shown to upregulate NOS expression.14,16–18 In addition, ROS-mediated inactivation of NO may have contributed to upregu-
lation of NOS by diminishing the negative feedback exerted by NO on NOS expression. In confirmation of the latter supposition, we recently demonstrated downregulation of eNOS protein expression by addition of NO donors and upregulation of eNOS expression by NO scavengers in cultured endothelial cells. The rats used in the present study consumed drinking water that contained 100 ppm lead acetate. We wish to point out that lower levels of lead exposure can also produce hypertension. The available data do not allow definitive conclusions about the possible effects of exposure to lower levels of lead on the study parameters. Additional studies are necessary to address this issue.

In summary, the lead-treated animals exhibited a significant rise in arterial blood pressure, a marked rise in plasma MDA concentration, and a fall in urinary NOx excretion. This was associated with a paradoxical upregulation of vascular and renal tissue eNOS and iNOS expression. High-dose vitamin E supplementation ameliorated hypertension and normalized plasma MDA concentration, urinary NOx excretion, and aortic, but not renal, tissue eNOS and iNOS expression. These findings point to the role of oxidative stress in the pathogenesis of lead-induced hypertension, reduced urinary NOx excretion, and compensatory upregulation of renal and vascular NOS expression in this model.

References
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