Lead-Induced Hypertension. III.
Increased Hydroxyl Radical Production

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Lead-induced hypertension has previously been shown to be closely associated with an increase in reactive oxygen species in low lead (100 ppm)-treated rats. The present study has attempted to define the specific moiety involved by noting the blood pressure (BP), reactive oxygen species (MDA-TBA), hydroxyl radical, and nitrotyrosine responses to infusion of the reactive oxygen species scavenger dimethylthiourea. Dimethylthiourea, a reputed scavenger of hydroxyl radical, normalized BP and MDA-TBA in the lead-treated rats but had no effect in normal control animals. MDA-TBA, hydroxyl radical, and nitrotyrosine, the tissue end product of peroxynitrite, were reduced to or toward normal by dimethylthiourea. The results, therefore, are consistent with the suggestion that either hydroxyl radical or peroxynitrite may be the reactive species affected by lead. Am J Hypertens 2001;14:169–173 © 2001 American Journal of Hypertension, Ltd.

Key Words: Lead, blood pressure, reactive oxygen species, hydroxyl radical, nitrotyrosine, dimethylthiourea.

The hypertensive response of Sprague-Dawley rats to administration of low, but not high, oral doses of lead has been well documented in previous publications.1–6 Studies of the mechanisms involved have implicated a diminution in the vasodilating endothelium-derived relaxing factor (EDRF), together with an increase in reactive oxygen species (ROS) (as manifested by increased plasma malondialdehyde [MDA] levels), and an increase in the vasoconstricting factors endothelin III and the sodium–potassium adenosine triphosphatase inhibitor-containing hypertension-associated protein.2,3,5 Administration of the lead-chelating reactive oxygen species scavenger 2,3-dimercaptosuccinic acid (DMSA), as well as the non–lead-chelating scavengers lazaroid and vitamin E, ameliorate the lead-induced hypertension and normalize urinary excretion of nitric oxide metabolites (NOx) and plasma MDA levels and tissue nitrotyrosine, which is the footprint of NO inactivation by reactive oxygen species.3,4,6–8

On the basis of these earlier studies, we speculated that one or more of the principal reactive oxygen species (superoxide anion [O2−], hydrogen peroxide [H2O2], or hydroxyl radical [OH−]) might be involved in the genesis of lead-related hypertension, through either a direct vasoconstrictive effect9,10 or by inactivation of EDRF.11 In this regard recently we have shown increased hydroxyl radical production in lead-treated endothelial cells in vitro.12 The present study was designed to determine whether or not lead exposure increases hydroxyl radical production in vivo. In addition, to explore the role of hydroxyl radical in the genesis of hypertension, in the present study we measured the effects of a scavenger of hydroxyl radical, dimethylthiourea (DMTU),13 on arterial pressure, on plasma MDA, plasma OH, and plasma nitrotyrosine abundance.

Methods

Animals

Commencing at the age of 2 months, male Sprague-Dawley rats weighing 200 g were fed a standard laboratory chow and water containing 0.01% lead acetate (100 ppm) for 12 weeks. A group of normal animals fed standard laboratory chow for the same time period served as controls. The work was carried out in accordance with the National Institutes of Health guidelines.

DMTU Infusion

Eight lead-treated and seven control rats were anesthetized with an intraperitoneal injection of 100 mg/kg thiobuterarbital (Research Biochemical International, Natick, MA) and were placed on a heated table to maintain body temperature at 37°C. Sodium salicylate was administered intraperitoneally as 100 mg/kg body weight before anesthesia. A tracheostomy was performed, and a PE-240 catheter was placed in the trachea. A PE-90 catheter with a flared tip was placed in the bladder for urine collection. PE-50...
catheters were placed in the left carotid artery for blood pressure (BP) measurement and blood collection and in both jugular veins for intravenous infusion. An intravenous infusion of 0.5 mL/100 g body weight per hour of a solution of 2.5% albumin was started and continued for the duration of the experiment. After the surgical procedure was completed, a priming dose of 50 μCi [3H]inulin was injected intravenously. This was followed by the infusion of 5 μCi of [3H]inulin in the albumin solution. Saline or DMTU was infused at a rate of 0.2 mL/100 g body weight per hour. After a 60-min equilibration, a 30-min baseline period with saline infusion was undertaken. At this time, rats received DMTU, which was infused at 8 mg/kg/min for 30 min, preceded by a bolus injection (250 mg/kg). Finally, a 30-min saline recovery period was instituted. Urine samples were collected during the last 15 min of each infusion period. Blood samples (0.4 mL) from the carotid artery were withdrawn at the middle of each 15-min clearance period. Hematocrit was monitored during the experiment. At the end of the experiment, whole blood was collected for the measurement of lead.

**Determination of Lead in Blood**

Lead content of whole blood was measured using an atomic absorption spectrophotometer (Varian, model 400Z with graphite furnace). Whole blood lead values were expressed as micrograms per deciliter.

**Determination of Lipoperoxides in Plasma**

Plasma lipoperoxides were determined by measuring malondialdehyde-thiobarbituric acid (MDA-TBA) according to the method of Wong et al.14 Briefly, 50 μL of plasma was mixed with 0.75 mL of 0.44 mol/L H3PO4, 0.25 mL of aqueous 42 mmol/L TBA, and 0.45 mL of H2O. The mixture was heated in a boiling water bath for 60 min. After cooling on ice, an equal volume of alkaline methanol (50 mL of methanol and 4.5 mL of 1 N NaOH) was added. Subsequently, 50 μL of the neutralized reaction mixture was injected into a 4.6 × 250-mm chromatographic column packed with Bondapak C18 (5-μm particle diameter). A guard column, 3.9 by 23 mm, packed with Bondapak Corasil C18 (37- to 50-μm particle diameter) was used. Mobile phase was a mixture of 50 mmol/L phosphate buffer pH 6.8 (600 mL) and methanol (400 mL). The flow was 1 mL/min and detection was done at 532 nm. The concentration of plasma lipoperoxides was determined from the calibration curve prepared with a tetramethoxyxpropane standard solution (0.61 to 4.86 μmol/L), processed exactly as the plasma samples.

**Determination of Hydroxyl Radical**

The technique for measuring 2,3 dihydroxybutyric acid (2,3 DHBA), 2,5 DHBA, and salicylate was modified from that described by McCabe et al.15 This method depends on the use of salicylate as a spin-trap for hydroxyl radical, with 2,3 DHBA and 2,5 DHBA as products; however, 2,3 DHBA does not occur endogenously and thus is most applicable as a measure of hydroxyl. Results are expressed as a ratio of 2,3 DHBA to salicylate to compensate for irregularities in salicylate absorption. Standards were purchased from Sigma (St. Louis, MO). Water was first purified as distilled water (Arrowhead) then further purified by an Ultrapure7 ion exchanger (Barnstead/Thermolyne, Dubriere, IA). The chromographometric system consisted of a dual piston pump (ESA model SP 8250) and a Coulouchem II (ESA model 5100S) dual potentiotstat electrochemical detector. Separation of analytes was achieved on a reversed-phase DHBA-250 column (5 μm, 250 by 3.0 mm). Analytes were detected on the dual electrode analytical cell (ESA model 5010) with the first electrode (E1) set to oxidize the DHBA at 250 mV and the second electrode (E2) set to oxidize salicylate at +750 mV. Detection was by a PC-based data station (model SP 4270 integrator). An all-PEEK filter (purchased from ESA; Chelmsford, MA) was used between the column and the analytical cell.

The mobile phase consisted of 50 mmol/L sodium acetate, 50 mmol/L sodium citrate, 25% methanol, and 5.0% isopropyl alcohol, adjusted to pH 2.50 with phosphoric acid. The mobile phase was passed through the system at 0.5 mL/min.

Retention time for 2,3 DHBA averaged 8.9 min, for 2,5 DHBA averaged 6.2 min, and for salicylate averaged 25.2 min. The area under the peak was compared to the area produced by injection of standards.

**Determination of Nitrotyrosine**

Plasma nitrotyrosine abundance was determined by Western blot using an antinitrotyrosine monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY) following the procedure of Vaziri et al.7 Briefly, plasma preparations containing ~100 μg of protein were size-fractionated on 4% to 20% tris-glycine gel (Novex Inc., San Diego, CA) at 120 V for 2 h. After electrophoresis, proteins were transferred onto PVDF membrane (Amersham Life Science Inc., Arlington Heights, IL) at 200 mA for 120 min using the Novex transfer system. The membrane was prehybridized in buffer A (10 mmol/L tris-hydrochloride, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20, and 5% nonfat milk powder) for 1 h and then hybridized overnight at 4°C in the same buffer containing 0.5 μg/mL antinitrotyrosine monoclonal antibody. The membrane was then washed for 30 min in a shaking bath, changing the wash buffer (buffer A without nonfat milk) every 5 min before 1 h of incubation in buffer A plus goat antimouse IgG horse radish peroxidase at the final titer of 1:2000. Experiments were carried out at room temperature. The washes were repeated before the membrane was developed with a light-emitting nonradioactive method using ECL reagent (Amersham Life Science Inc., Arlington Heights, IL). The membrane was then subjected to autoradiography for 20 sec to 5 min. The autoradiographs 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.

**Statistical Analysis**

Results were expressed as mean ± standard deviation. Paired or unpaired t tests were used in statistical analysis of the data as appropriate. Statistical significance for the tests was assessed using a two-tailed probability level of less than .05.

**Results**

**Blood Pressure and Blood Lead Concentration**

As expected, mean blood pressure (MBP) in the lead-treated group obtained at the end of the 12-week lead exposure period was significantly higher than that in the controls (Fig. 1). Blood lead determination averaged 12.4 ± 1.8 μg/dL in lead-treated animal and <1 μg/dL in controls.

**Effect of DMTU on MBP**

Baseline MBP was elevated in lead-treated rats, as measured by intraarterial measurements. Infusion of DMTU resulted in a normalization of MBP in lead-treated rats, although it had no effect in controls (Fig. 1). The effect of DMTU on MBP persisted for at least 1 h.

**Effect of DMTU on Plasma Lipoperoxides, Hydroxyl Radical, and Nitrotyrosine**

Lipoperoxides in plasma, represented by the MDA-TBA level, were significantly elevated at baseline in lead-treated rats. Infusion of DMTU resulted in a decrease in plasma MDA-TBA levels to normal in the lead-treated rats (Fig. 2). Hydroxyl radical in plasma was also elevated in the lead-treated rats (Fig. 3). DMTU infusion resulted in a decrease in hydroxyl radical to below control levels. Nitrotyrosine production in plasma or tissue may be considered to represent circulating peroxynitrite levels. Nitrotyrosine was elevated in the lead-treated group, was reduced by DMTU, and continued to be reduced after DMTU was replaced by saline (Figs. 4 and 5).

**Effects on Urine Flow Rate, Glomerular Flow Rate, and Hematocrit**

No significant changes in either urine or glomerular flow rate were observed after infusion of DMTU in the study groups (Table 1). Hematocrit ranged from 38% to 45%, with no consistent change produced by infusions.

**Discussion**

Animals with lead-induced hypertension used in the present study showed a significant increase in hydroxyl...
Dimethylthiourea is known to act as a potent scavenger of OH, peroxynitrite, and to a lesser extent, H₂O₂, and thus would be anticipated to reverse the effects of any preformed OH⁻ or peroxynitrite. Peroxynitrite formation results from the interaction of NO and O₂⁻, is known to induce membrane lipid peroxidation, and decomposes to hydroxyl radical. There is also evidence that peroxynitrite can be produced from NO, SOD, and H₂O₂.

The results of the present study showed that DMTU effectively returned the BP to normal in anesthetized lead-treated rats. In contrast, DMTU had no effect on the blood pressure of normal control animals. We had previously shown that blood pressure was elevated in low lead-treated animals in the unanesthetized state, and that administration of DMSA, a scavenger of reactive oxygen species, returned BP to normal. Both plasma MDA-TBA, a measure of lipid peroxidation and thus of reactive species formation, and hydroxyl radical were elevated in lead-treated animals and reduced to or below normal by DMTU infusion. In a separate study, when rat endothelial cells were incubated in vitro with varying concentrations of lead, a parallel set of observations was noted. At the highest concentration of lead acetate (1 ppm) both MDA-TBA and hydroxyl radical were significantly elevated above control levels.

Prior studies support the contention that ROS and either hydroxyl radical or superoxide may be vasoconstrictors. Nakazono et al found that an infusion of a fusion protein-bound form of SOD lowered the blood pressure of the spontaneously hypertensive rat, possibly by decreasing the formation of superoxide or hydroxyl radical. Katusic and VanHoutte demonstrated that superoxide anion, generated by xanthine and xanthine oxidase in the presence of catalase, caused contraction of canine basilar arteries in vitro. Auch-Schwelk et al demonstrated that aortic rings from spontaneously hypertensive rats had concentration-dependent contractions on exposure to xanthine plus xanthine oxidase-derived reactive oxygen species. Although SOD and catalase showed modest effects in reducing the contractile tension, deferoxamine, the iron chelator that prevents the generation of hydroxyl radical from hydrogen peroxide (and has also been shown to scavenge peroxynitrite), totally abolished the contractions.

Dreher and Junod demonstrated that hypoxanthine-xanthine oxidase exposure led to an increase in intracellular calcium in human umbilical vein endothelial cells, and this increase was inhibited by o-phenanthroline, a compound that blocks the iron-catalyzed formation of hydroxyl radical. In the converse direction, Pieper et al

Table 1. Glomerular filtration rate (mL/min/100 g body weight) after infusion with DMTU

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<th>Baseline</th>
<th>DMTU</th>
<th>Saline</th>
<th>Recovery</th>
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<td>Controls</td>
<td>0.71 ± 0.20</td>
<td>0.71 ± 0.21</td>
<td>0.71 ± 0.15</td>
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<tr>
<td>Lead</td>
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<td>0.62 ± 0.23</td>
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FIG. 4. Western blot representation of nitrotyrosine response to sequential infusions of dimethylthiourea (DMTU) and saline. Lane 1 = baseline control; lane 2 = baseline lead; lane 3 = DMTU control; lane 4 = DMTU lead; lane 5 = Saline control; lane 6 = Saline lead.

FIG. 5. Effects on plasma nitrotyrosine of sequential infusions of dimethylthiourea (DMTU) and saline. *P < .05, lead group at baseline vs DMTU or saline treatment; **P < .01, lead group vs control at baseline.
have demonstrated that the impaired relaxation response to acetylcholine seen in aortic strips removed from diabetic rats could be reversed by pretreatment with either DMTU or deferoxamine, implicating the hydroxyl radical.

Peroxynitrite has been demonstrated to act as a highly reactive nitrogen species inducing membrane lipid peroxidation. Although peroxynitrite is ordinarily thought of only as a short-acting vasodilator, in low doses it caused constriction of the middle cerebral arteries of the rat, and therefore may also act as a vasoconstrictor. Repetitive injections of peroxynitrite also have been shown to lead to sustained hypertension in the rat. Although the authors attributed the phenomenon to tachyphylaxis, it would appear more likely that a by-product of the reaction of peroxynitrite with tissue or plasma may be responsible. Therefore, either the reactive oxygen species, hydroxyl radical, or the reactive nitrogen species, peroxynitrite, are the most likely reactive species responsible for the hypertension produced by lead.

Acknowledgments
We gratefully acknowledge the technical advice of Fariba Oveis and the secretarial assistance of Lydian Reitz.

References


23. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–1624.


37. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–1624.


