Effect of Lead on Nitric Oxide Synthase Expression in Coronary Endothelial Cells
Role of Superoxide
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Abstract—Chronic exposure to low levels of lead causes hypertension (HTN) in humans and animals. We have previously shown that increased reactive oxygen species (ROS) leads to enhanced NO inactivation, depressed NO bioavailability, and compensatory upregulation of NO synthases (NOSs) in rats with lead-induced HTN. We have further demonstrated increased ROS generation with lead exposure in cultured endothelial cells. In the present study, we tested the effect of lead (medium containing lead acetate, 1 ppm) alone and with either the superoxide dismutase–mimetic agent tempol or a potent antioxidant lazaroid compound (both at $10^{-8}$ and $10^{-7}$ mol/L) on endothelial NOS expression and NO production in cultured human coronary endothelial cells. Lead-treated cells showed a significant upregulation of endothelial NOS (eNOS) protein abundance ($P<0.002$) and a significant increase in the production of NO metabolites ($NO_2^- + NO_3^- = NO_X$, $P<0.01$). Cotreatment with either tempol or lazaroid abrogated the lead-induced upregulation of eNOS protein and NO production. In contrast, tempol and lazaroid had no effect on either eNOS protein expression or NO production in the control cells. Thus, lead exposure upregulated eNOS expression in vitro, simulating the results of our previous in vivo studies. This phenomenon points to a direct as opposed to an indirect (eg, HTN-mediated) effect of lead on NO metabolism. The reversal of lead effect by lazaroid and the cell-permeable superoxide dismutase–mimetic agent tempol suggests that lead exposure increases generation and/or reduces dismutation of superoxide, which in turn promotes oxidative stress, enhances NO inactivation, and elicits a compensatory upregulation of eNOS whose expression is negatively regulated by NO. (Hypertension. 2001;37:223-226.)

Key Words: lead □ blood pressure □ endothelium □ free radicals □ nitric oxide □ arteries

In an earlier study, we found strong evidence for oxidative stress, depressed NO availability, and antioxidant-remediable hypertension (HTN) in rats exposed to low levels of lead.1,2 We subsequently showed that the elevated blood pressure and depressed urinary NO metabolite (NOx) excretion in this model were not due to reduced NO production capacity. On the contrary, renal and vascular expressions of NO synthase (NOS) isotypes were paradoxically increased in rats with lead-induced HTN.3 Interestingly, the upregulation of NOS isotypes was reversed, whereas HTN improved and urinary NOx excretion paradoxically rose with antioxidant therapy in lead-treated animals.

We hypothesized that the reduction in NO availability despite the paradoxical upregulation of renal and vascular NOS isotypes may be due to avid inactivation and sequestration of NO by reactive oxygen species (ROS). To test this hypothesis, we measured kidney, heart, aorta, brain, and liver abundance of nitrotyrosine, which is the footprint of NO interaction with ROS. The study revealed a marked increase in nitrotyrosine abundance in all tested tissues of animals with lead-induced HTN. Antioxidant therapy ameliorated HTN, lowered tissue nitrotyrosine burden, and enhanced NO availability despite a reduction in NOS isotype expressions.4 Together, these studies demonstrated that lead exposure in this model is associated with oxidative stress that leads to avid ROS-mediated inactivation, sequestration of NO, and reduced availability of bioactive NO and HTN. We then asked whether oxidative stress in this model is secondary to a systemic in vivo effect or a direct cellular action of lead. To explore this possibility, we carried out a series of in vitro experiments with lead-treated cultured endothelial cells. The study revealed a marked dose-dependent increase in the production of the lipid peroxidation product malondialdehyde (MDA) and an enhanced generation of hydroxyl radicals by lead-treated endothelial cells compared with the control cells.5 In a companion study, we showed increased hydroxyl radical generation in tissues obtained from animals with lead-induced HTN.6 Thus, these studies showed that lead promotes hydroxyl radical generation both in vivo and in vitro. We recently showed that both endogenous and exogenous NO exerts a negative feedback regulation on endothelial NOS (eNOS) expression in cultured human coronary endo-
thelial cells. Based on these observations, we believe that by lowering the availability of bioactive NO through an ROS-mediated inactivation of endogenous NO, lead causes a compensatory upregulation of NOS expression. If this is true, lead treatment, which causes oxidative stress in endothelial cells, should upregulate eNOS in cultured endothelial cells in vitro. The present study was undertaken to explore this possibility. Because hydroxyl radical is primarily derived from superoxide and hydrogen peroxide, we also sought to explore the effect of the superoxide dismutase (SOD)-mimetic agent tempol and a nonspecific antioxidant (desmethyltirilazad) on eNOS expression in lead-treated and control cultured human coronary endothelial cells.

**Methods**

**Cell Culture**

Human coronary artery endothelial cells (BioWhittaker Inc) were cultured in a manner that was precisely similar to that described in our earlier studies. Cells obtained on passages 3 and 4 were used. Once a 70% confluence was reached, the cells were incubated in a medium containing 1 ppm lead acetate for 24 hours and then incubated for an additional 24 hours in the same medium with tempol or lazairord at a concentration of $10^{-8}$ or $10^{-7}$ mol/L. Cells treated with inactive vehicles were used as controls. Cells were then harvested, and the culture media were collected and stored at $-70^\circ$C until processed. Cell viability was tested with trypan blue exclusion test and morphological examination and was found to be >95% in all experiments.

**Measurement of Total Nitrate and Nitrite**

NO production was assessed from total nitrate and nitrite (NO$_x$) recovered in the extracellular medium. NO$_x$ was measured with the purge system of the Sievers NO Analyzer (model 270B; Sievers Instruments Inc) as previously described. The amount of NO$_x$ produced was normalized against total cellular protein, which was measured with a kit from Bio-Rad.

**Measurement of eNOS Protein**

Endothelial cells were processed and eNOS protein abundance was determined by Western blot analysis with anti-eNOS antibody (Transduction Laboratory Inc) in a manner precisely similar to that described in our earlier studies.

**Data Analysis**

ANOVA and Student’s $t$ test were used in statistical evaluation of the data, which are presented as mean±SEM. A value of $P<0.05$ was considered to be significant.

**Results**

The lead-treated coronary endothelial cells exhibited a significant upregulation of eNOS protein expression compared with the control cells ($P<0.01$). Upregulation of eNOS protein expression in the lead-treated cells was accompanied by a significant increase in NO production ($P<0.01$) as discerned from NO$_x$ recovered in the incubation media (Figure 1).

The addition of the SOD-mimetic agent tempol dose-dependently mitigated upregulation of eNOS expression ($P<0.05$) and NO production ($P<0.01$) in the lead-treated coronary endothelial cells. However, tempol had no effect on either eNOS expression or NO production in the control cells ($P=NS$ for both) (Figure 2).

As with tempol, the addition of desmethyltirilazad, the potent antioxidant lazaroid compound, significantly mitigated upregulation of eNOS expression ($P<0.05$) and NO production ($P<0.01$) in lead-treated coronary endothelial cells. However, lazaroid had no effect on either eNOS expression or NO production in the control cells ($P=NS$ for both) (Figure 3).

**Discussion**

Exposure to lead resulted in a marked upregulation of eNOS protein expression and NO production in cultured human endothelial cells. The addition of tempol dose-dependently mitigated the upregulation of eNOS expression and NO production in lead-treated endothelial cells. However, the addition of lazaroid had no effect on either eNOS expression or NO production in control cells. These findings suggest that oxidative stress plays a role in the upregulation of eNOS expression in response to lead exposure.
coronary endothelial cells in vitro. These findings parallel the results of our earlier in vivo studies in lead-treated rats, which exhibited a significant upregulation of renal and vascular NOS isoforms together with avid NO inactivation by ROS. Increased shear stress and cyclic strain associated with HTN have been shown to upregulate eNOS expression in vivo. The observed upregulation of eNOS in endothelial cells in vitro, where baromechanical stresses that are inherent to intact animals were absent, substantiates the effect of lead at the cellular level. Moreover, the use of human endothelial cells here demonstrated the relevance of the findings to humans as opposed to the experimental animals used in our previous studies.

In several earlier studies, we have found evidence of oxidative stress and increased ROS activity leading to enhanced NO oxidation and depressed NO bioavailability in rats with lead-induced HTN. We have further shown that lead-induced oxidative stress is primarily due to increased hydroxyl radical generation in both intact animals and cultured endothelial cells. It should be noted that hydroxyl radicals are primarily produced from sequential reduction of superoxide to hydrogen peroxide and of hydrogen peroxide to hydroxyl radicals, as seen in the following equations:

\[
\text{SOD} \\
\text{O}_2^- + 2\text{H} \rightarrow \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + e^- (\text{Fe}^{2+}) \rightarrow \text{OH}^- + \text{OH}^- + \text{Fe}^{3+} \quad (\text{Fenton reaction}) \\
\text{H}_2\text{O}_2 + e^- (\text{O}_2^-) \rightarrow \cdot \text{OH}^- + \text{OH}^- + \text{O}_2 \quad (\text{Haber-Weiss reaction})
\]

Consequently, increased hydroxyl radical generation may be due to either enhanced superoxide production, its reduced dismutation by SOD, or the presence of an electron-donor transition metal, among other possibilities. In an earlier study with native SOD infusion, we found no significant improvement in either blood pressure or NO availability in rats with lead-induced HTN. However, being a peptide, native SOD is incapable of penetrating intracellular space where the bulk of superoxide is generated by the mitochondria and cytoplasmic oxidase enzymes. Consequently, a lack of demonstrable effect of intravenously administered native SOD does not preclude the possible role of excess superoxide production in animals with lead-induced HTN.

In an attempt to pursue the hypothesis that lead-induced oxidative stress may be due to enhanced superoxide production, in the present study we used the novel cell-permeable SOD-mimetic agent tempol, which had been shown to effectively inactivate \(\text{O}_2^\cdot\)\(^{10,11}\). The study demonstrated the efficacy of cell-permeable SOD-mimetic tempol in reversing the lead-induced compensatory upregulation of NOS expression and NO production in cultured human endothelial cells. Interestingly, tempol had no effect on either eNOS expression or NO production in the control cells. These findings conform with the results of our earlier in vivo studies, which revealed a reversal of compensatory regulations of renal and vascular NOS isoforms together with enhanced NO availability and amelioration of HTN by antioxidant therapy in rats with lead-induced HTN. In contrast, vitamin E supplementation had no effect on NOS expression, NO availability, or blood pressure in the normal control animals. Those observations demonstrated that in the absence of oxidative stress, the natural antioxidant system is sufficient to contain ROS generated in the course of normal metabolism and that as such, supplemental antioxidants provide no added effect.\(^3\) The absence of a discernible effect of tempol on the NO system in the control endothelial cells shown here mirrors our results in in vivo experiments with vitamin E supplementation in normal rats. In addition, the results exclude an unrelated direct effect of the drugs used in this system.

The effect of tempol on eNOS expression and NO production in the lead-treated cells was simulated by the potent nonspecific antioxidant desmethyltirilazad, which also had no effect on normal control cells. This observation suggests that the effect of tempol was based on its antioxidant action, as opposed to an unrelated action. Taken together, the study points to excess \(\text{O}_2^-\) generation and/or depressed \(\text{O}_2^-\) dismutation in the lead-treated endothelial cells as an underlying mechanism responsible for enhanced lipid peroxidation and hydroxyl radical generation shown in our earlier studies in lead-treated endothelial cells. Further studies are required to explore this possibility in animals with lead-induced HTN.

In conclusion, exposure to lead resulted in upregulation of eNOS expression in human coronary endothelial cells in vitro, paralleling the results of our in vivo experiments. Lead-induced upregulation of eNOS expression was prevented by the SOD-mimetic agent tempol, which had no effect on the control cells, thus pointing to an increase in net superoxide production by lead-treated cells. The effect of tempol was reproduced by the nonspecific antioxidant laz- aristad compound, indicating that the observed effect was due

![Figure 3. Representative Western blots and group data depicting eNOS protein abundance (top) and NO production (bottom) in lead-exposed cells (left) and normal cells (right) incubated for 24 hours in the presence of lazaroid at 0.0, 10^-5, and 10^-7 mol/L concentrations (n=4 separate experiments, **P<0.05, **P<0.01 vs no lazaroid).](http://hyper.ahajournals.org/issue)
to the antioxidant as opposed to an unrelated direct action of tempol.

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_Hypertension_. 2001;37:223-226
doi: 10.1161/01.HYP.37.2.223

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/37/2/223

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