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Lead exposure raises superoxide and hydrogen peroxide in human endothelial and vascular smooth muscle cells

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Lead exposure raises superoxide and hydrogen peroxide in human endothelial and vascular smooth muscle cells.

Background. Chronic lead exposure causes hypertension and cardiovascular disease, which are associated with, and, in part, due to oxidative stress. While occurrence of oxidative stress in lead-exposed animals and cultured endothelial cells has been well-established, direct and specific evidence on the type of the reactive oxygen species (ROS) produced by lead-exposed vascular cells is lacking and was investigated.

Methods. Human coronary endothelial (EC) and vascular smooth muscle cells (VSMC) were incubated in appropriate culture media in the presence of either 1 ppm or 10 ppm lead acetate or sodium acetate (control) for 1 to 30 minutes or 60 hours. Productions of superoxide and hydrogen peroxide in the cell populations were determined by flow cytometry using hydroethidine and dihydrorhodamine, respectively. Data from a minimum of 10,000 cells were collected and analyzed using Cell Quest software. In addition, Cu Zn superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), and NAD(P)H oxidase ($gp91^{phox}$) were measured.

Results. Short-term lead exposure resulted in a significant rise in both superoxide and hydrogen peroxide production by both EC and VSMC. After long-term exposure, detectable superoxide levels fell to near normal level, while hydrogen peroxide production remained high. This was associated with up-regulations of $gp91^{phox}$, elevation of superoxide dismutase, reduction of VSMC catalase, and no change in GPX levels. Together, these events can account for the observed decline in superoxide and the rise in hydrogen peroxide following long-term lead exposure.

Conclusion. Lead exposure promotes generation of superoxide and hydrogen peroxide in human EC and VSMC. This phenomenon can potentially contribute to the pathogenesis of the lead-associated hypertension and cardiovascular disease, and points to the potential benefit of lowering lead burden in the exposed populations.

Chronic exposure to low levels of lead causes sustained arterial hypertension (HTN), which persists indefinitely after the cessation of lead exposure in humans and experimental animals [1–9]. In a series of earlier studies [2–6], we demonstrated that lead-induced HTN is associated with oxidative stress and increased production of reactive oxygen species (ROS). We further showed that the associated oxidative stress plays a major role in the pathogenesis of HTN via avid inactivation of nitric oxide (NO) by ROS [2, 5]. In fact, alleviation of oxidative stress by a variety of antioxidants resulted in marked amelioration of HTN and enhanced NO availability in rats with lead-induced HTN [2, 4–6]. Avid inactivation of NO by ROS was associated with a compensatory up-regulation of NO synthase (NOS) isoforms that was largely reversed by antioxidant therapy in lead-treated animals [4–6].

By using a salicylate trapping technique, we recently found indirect evidence for increased hydroxyl radical (.OH) generation in the lead-treated rats and cultured endothelial cells [10, 11]. The role of excess .OH activity was further confirmed by the observations that intravenous administration of the reputed .OH scavenger, dimethythiourea (DMTU), ameliorates HTN and increases urinary excretion of NO metabolites (NOx) in rats with lead-induced HTN [11]. In contrast, intravenous administration of the native superoxide dismutase (SOD) alone or together with catalase had no discernible effect on either blood pressure or urinary NOx excretion [12]. However, as a peptide, native SOD is cell impermeable and, as such, its lack of demonstrable action does not necessarily preclude increased cellular production of superoxide. In an attempt to overcome this obstacle, we recently used the cell-permeable SOD-mimetic agent, tempol, in a study designed to explore the effect of lead exposure on superoxide production in cultured human coronary endothelial cells. The results revealed reversal of both lipid peroxidation and compensatory up-regulation of endothelial NO synthase (eNOS) expression by the SOD-mimetic drug, tempol, in lead-treated endothelial cells [13]. The latter study provided strong but indirect evidence for increased superoxide production in lead-treated endothelial cells. These in vitro findings were subsequently confirmed by our in vivo studies, which showed amelioration of HTN and oxidative stress

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together with enhanced NO availability and reversal of compensatory up-regulation of NOS isoforms by tempol administration in rats with lead-induced HTN [6]. In an attempt to explore the mechanism of oxidative stress induced by lead exposure, we recently determined protein abundance of several antioxidant enzymes, including SOD isoforms, catalase (CAT), and glutathione peroxidase (GPX), as well as superoxide-generating enzyme, NAD(P)H oxidase, in various tissues of rats with lead-induced hypertension. The results showed mild up-regulation of NAD(P)H oxidase and no evidence of quantitative deficiency of the measured antioxidant enzymes [14].

The present study was undertaken to determine directly the effect of lead exposure on production of superoxide and hydrogen peroxide in cultured human endothelial cells, as well as human vascular smooth muscle cells.

METHODS

Cell culture

Human coronary artery endothelial cells and human vascular smooth muscle cells (Cambrex Bio Science, Walkersville, Inc., Walkersville, MD, USA) were cultured in a manner that was described in our earlier studies [13]. Cells obtained on passages 3 and 4 were used. Once a 70% confluence was reached, the cells were incubated in a medium containing either 1 ppm or 10 ppm lead acetate for 5, 10, 30 minutes (short-term study), or for 60 hours (long-term study). Cells treated with the identical concentrations of sodium acetate were used as controls. The given time course was selected in order to dissect the rapid actions of lead on the vascular cells from the superimposed compensatory and genomic alterations that might occur with long-term exposure. The lead concentration range employed here is consistent with those used in previous in vitro studies [10]. Although plasma lead levels observed with chronic lead exposure in humans are usually below those used in the in vitro studies, tissue concentrations (kidney, bone, brain) are frequently very high. This supposition is based on animal studies that have revealed 10- to 140-fold higher lead concentrations in the kidney than in the blood following long-term lead exposure [15, 16]. In this context, the lead concentration range employed in the present tissue culture study is at or below those found in the kidneys of rats exposed to low levels of lead for extended periods. Thus, the lead concentration range employed in the tissue culture experiments here are biologically relevant.

Flow cytometry

At the conclusion of each incubation period, the cells were loaded with either 5 μmol/L hydroethidine (HE) or 2.5 μmol/L dihydrorhodamine 123 (DHR) (Molecular Probe, Inc., Eugene, OR, USA) for 3 hours for measurements of superoxide and hydrogen peroxide, respectively. Cells were then washed with phosphate-buffered saline (PBS) buffer, trypsinized, and maintained in PBS at 4°C before being analyzed by flow cytometry (FACSort, Becton Dickinson, San Jose, CA, USA). This instrument was equipped with an Argon laser for excitation at 488 nm. The emission for DHR was collected at FL1 detector (530/30 nm) and that of HE at FL2 detector (585/42 nm). Generally, data from 10,000 cells were collected and analyzed using Cell Quest software (Becton Dickinson).

Measurements of SOD, catalase, glutathione peroxidase, and NAD(P)H oxidase

Protein abundance of the above molecules in the cell preparations was determined by Western blot analysis using antibodies against CuZn SOD, catalase, glutathione peroxidase (Calbiochem, Inc., San Diego, CA, USA), and $gp91^{phox}$ subunit of NAD(P)H oxidase (Transduction Laboratories, Lexington, KY, USA) as described previously [17, 18].

CuZn SOD enzymatic activity was determined using the Bioxytech SOD-525™ Kit purchased from OXIS International (Portland, OR, USA).

Statistical analysis

Flow cytometry data were acquired and analyzed by Cell Quest Software (Becton Dickinson). Analysis of variance (ANOVA) and Student *t* test were used in the statistical evaluation of the data using Sigma Stat Software (Microsoft Group, Chicago, IL, USA). Data are presented as mean ± SEM. *P* values less than 0.05 were considered significant.

RESULTS

Short-term studies

Smooth muscle cells. Data are illustrated in Figure 1. Significant elevation of superoxide and hydrogen peroxide was observed in vascular smooth muscle cells treated with 1 ppm lead acetate for 5 to 10 minutes. Likewise, a significant increase in both superoxide and hydrogen peroxide was observed at 5, 10, and 30 minutes in cells incubated in the medium containing 10 ppm lead acetate.

Endothelial cells. Data are summarized in Figure 2. Exposure to 1 ppm lead acetate resulted in a significant rise in superoxide production at 5 to 10 minutes. Increased superoxide production was evident at 5, 10, and 30 minutes in endothelial cells exposed to 10 ppm lead acetate. Hydrogen peroxide production was significantly increased in the endothelial cells exposed to either 1 or 10 ppm lead throughout the 30-minute observation period.

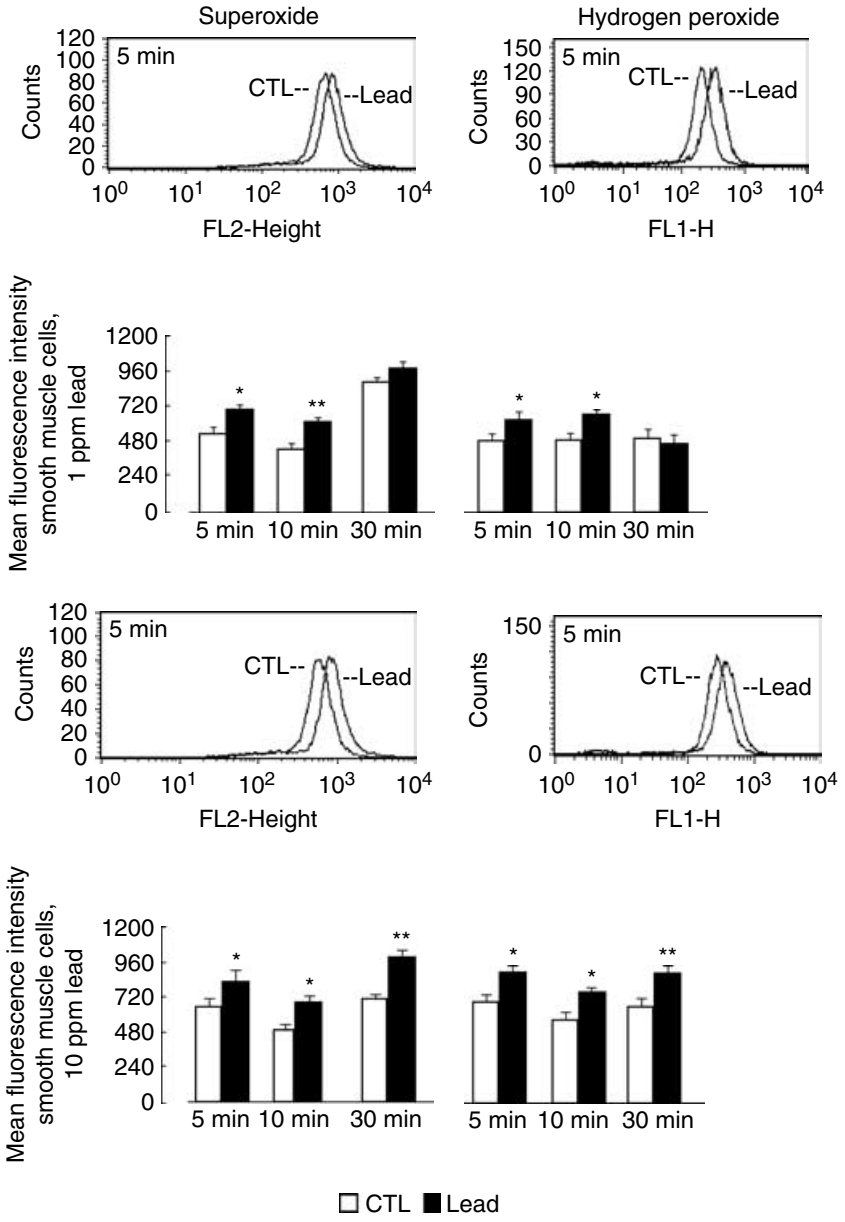

Fig. 1. Representative overlayed histograms and group data depicting superoxide and H_2O_2 production by human coronary artery smooth muscle cells incubated in lead acetate (at 1 ppm or 10 ppm), or equivalent concentrations of Na acetate (CTL) for 5 to 30 minutes. The increase in fluorescence intensity in the lead-treated cells is shown as a rightward shift on the x-axis. $^{*}P < 0.05$; $^{**}P < 0.01$, $N = 9$.

Long-term studies

Smooth muscle cells. Data are shown in Figure 3. The production of hydrogen peroxide was significantly increased in the cells incubated for 60 hours with either 1 ppm or 10 ppm lead acetate as compared with that found in the control cells. However, the generation of superoxide in the two lead-treated groups did not significantly differ from that found in the control groups.

Endothelial cells. Data are depicted in Figure 4. As with the vascular smooth muscle cells, a significant elevation of hydrogen peroxide production was observed in human coronary artery endothelial cells treated with either 1 ppm or 10 ppm lead acetate for 60 hours. No significant difference was found in superoxide production between the lead-treated and the control cells.

SOD data. Data are shown in Figure 5. Immunodetectable Cu Zn SOD abundance rose significantly both in endothelial and vascular smooth muscle cells incubated with either 1 ppm or 10 ppm lead acetate for 60 hours. This was accompanied by a significant but less pronounced rise in CuZn SOD activity in the vascular smooth muscle cells and an insignificant rise in the corresponding values in endothelial cells exposed to 10 ppm lead acetate for 60 hours.

Catalase data. Data are shown in Figure 6. Long-term exposure to lead acetate resulted in a significant concentration-dependent decline in immunodetectable

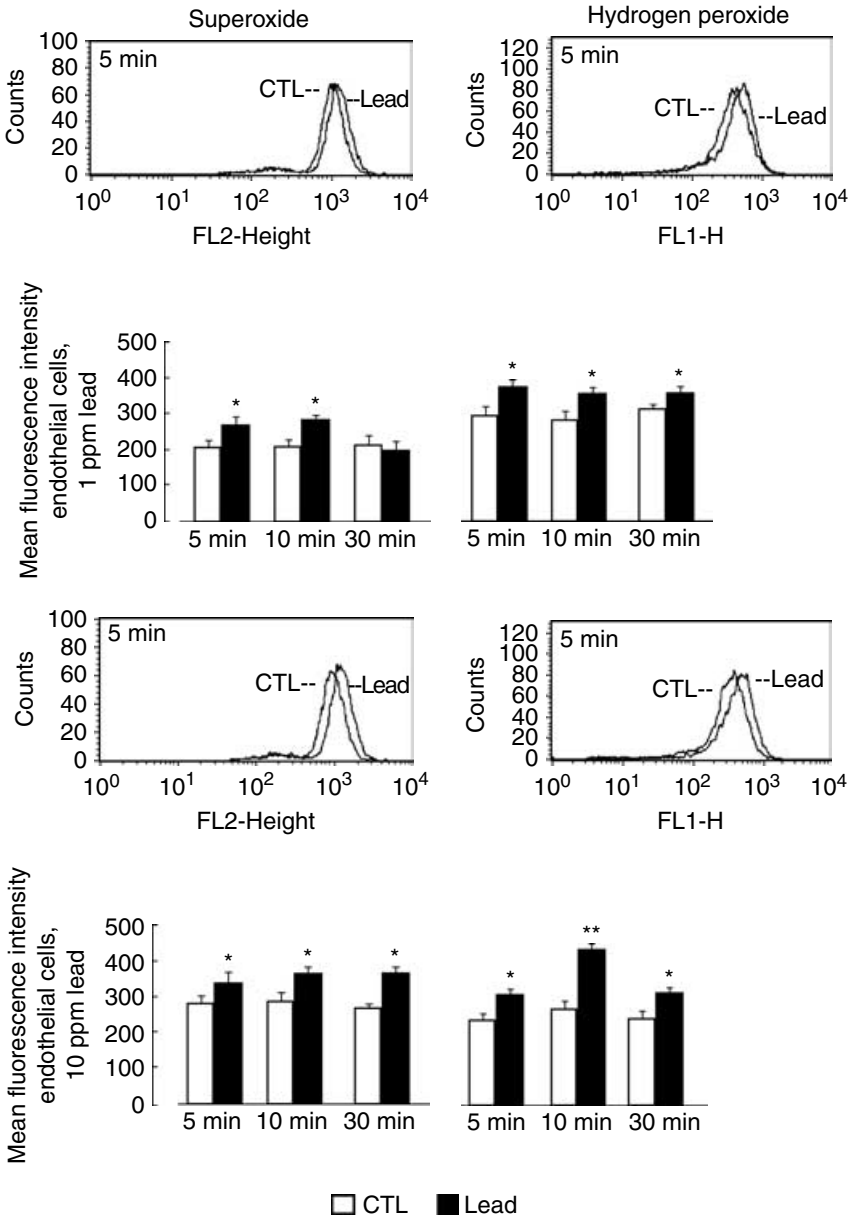

Fig. 2. Representative overlayed histograms and group data demonstrating superoxide and H_2O_2 production by human coronary artery endothelial cells incubated in lead acetate (at 1 ppm or 10 ppm), or equivalent concentrations of Na acetate (CTL) for 5 to 30 minutes. The increase in fluorescence intensity in the lead-treated cells is shown as a rightward shift on the x-axis. $^*P < 0.05$; $^{**}P < 0.01$, $N = 9$.

catalase in cultured vascular smooth muscle cells. However, long-term exposure to lead at the given concentrations did not significantly affect catalase abundance in cultured endothelial cells.

Glutathione peroxidase data. Data are given in Figure 6. Despite increased hydrogen peroxide production, GPX abundance remained virtually unchanged in both cultured endothelial and vascular smooth muscle cells exposed to lead for 60 hours.

gp91^{phox} data. Data are illustrated in Figure 7. Exposure of endothelial cells to lead resulted in a significant up-regulation of gp91^{phox} subunit of NAD(P)H oxidase, a well-known source of superoxide. Unlike endothelial cells, vascular smooth muscle cells do not express gp91^{phox} and tested negative for this protein.

DISCUSSION

The present study revealed that exposure to lead results in a rise in superoxide and hydrogen peroxide production directly measured by flow cytometry in human coronary endothelial cells. These observations are consistent with the indirect evidence found in our earlier studies in cultured endothelial cells [10, 13] and rats with lead-induced HTN [6, 12, 11]. In addition, lead exposure caused an increase in superoxide and hydrogen peroxide generation in human vascular smooth muscles. Accordingly, the study demonstrated the contribution of both endothelial and vascular smooth muscle cells to the pathogenesis of lead-induced oxidative stress in the vascular tissues. In addition to causing HTN, chronic exposure to lead results in a marked increase in the development of

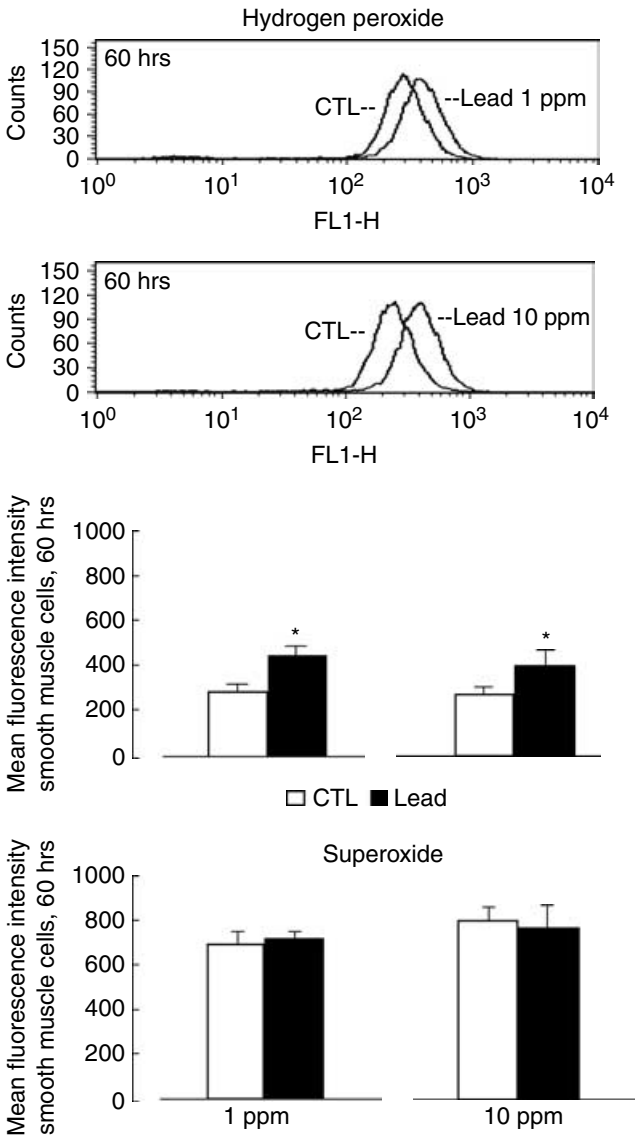

Fig. 3. Representative overlayed histograms and group data depicting superoxide and H_2O_2 production by human coronary artery smooth muscle cells incubated in lead acetate (at 1 ppm or 10 ppm), or equivalent concentrations of Na acetate (CTL) for 60 hours. The increase in fluorescence intensity in the lead-treated cells is shown as a rightward shift on the x-axis. $^*P < 0.05$, $N = 9$.

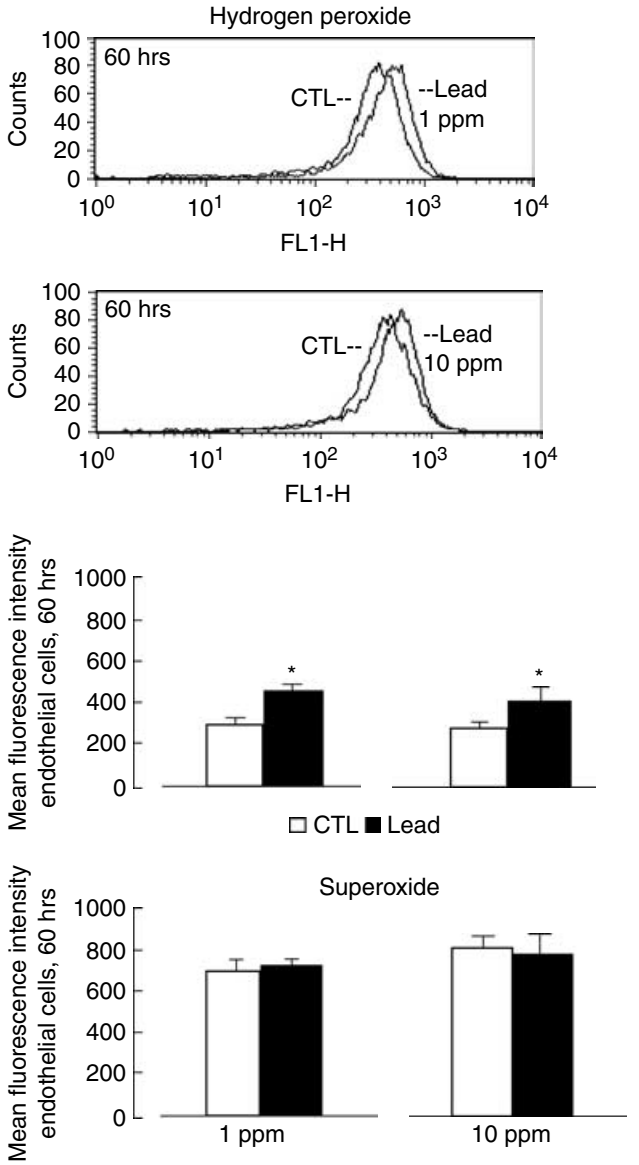

Fig. 4. Representative overlayed histograms and group data depicting superoxide and H_2O_2 production by human coronary artery endothelial cells incubated in lead acetate (at 1 ppm or 10 ppm), or equivalent concentrations of Na acetate (CTL) for 60 hours. The increase in fluorescence intensity in the lead-treated cells is shown as a rightward shift on the x-axis. $^*P < 0.05$; $^{**}P < 0.01$, $N = 9$.

arteriosclerotic cardiovascular disease [1, 19]. Oxidative stress plays a central role in the pathogenesis of atherosclerosis. As described in several recent reviews [20, 21], oxidative stress can lead to endothelial dysfunction, HTN, and altered vascular function and structure by several mechanisms. For instance, inactivation of nitric oxide by ROS can result in vasoconstriction, increased platelet/leukocyte adhesion, vascular smooth muscle cell migration/proliferation, and matrix accumulation leading to vascular remodeling. In addition, via activation of the redox-sensitive nuclear factor, NFκB, and consequent generation of proinflammatory and profibrotic cytokines, oxidative stress can promote inflammation, which is an important component of atherogenesis. Moreover, ROS-mediated endothelial injury and generation of atherogenic oxidized lipids/lipoproteins can contribute to cardiovascular consequences of oxidative stress. Thus, increased ROS generation by the vascular cells may be an important link in development of ischemic cardiovascular disease in the lead-exposed populations. Cells employed here were the product of primary culture of human coronary artery endothelial and vascular smooth muscle cells studied after only 3 to 4 passes

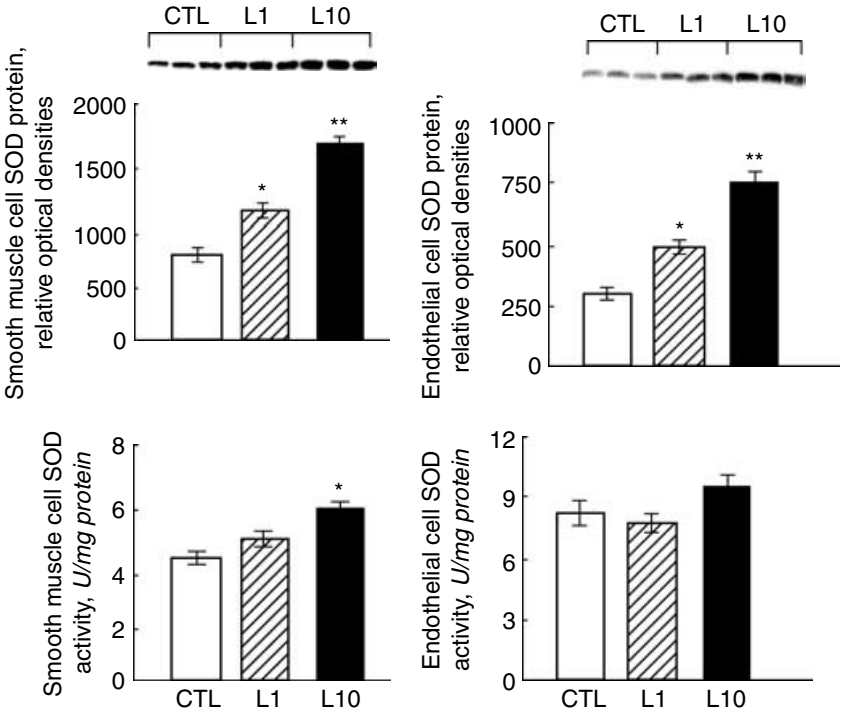

Fig. 5. Representative Western blot and group data depicting Cu-Zn SOD protein abundance and enzymatic activity in human coronary artery endothelial and vascular smooth muscle cells incubated in lead acetate (at 1 ppm or 10 ppm), or equivalent concentrations of Na acetate (CTL) for 60 hours. $^{*}P < 0.05$ vs CTL; $^{**}P < 0.01$ vs. 1 ppm and CTL, $N = 9$.

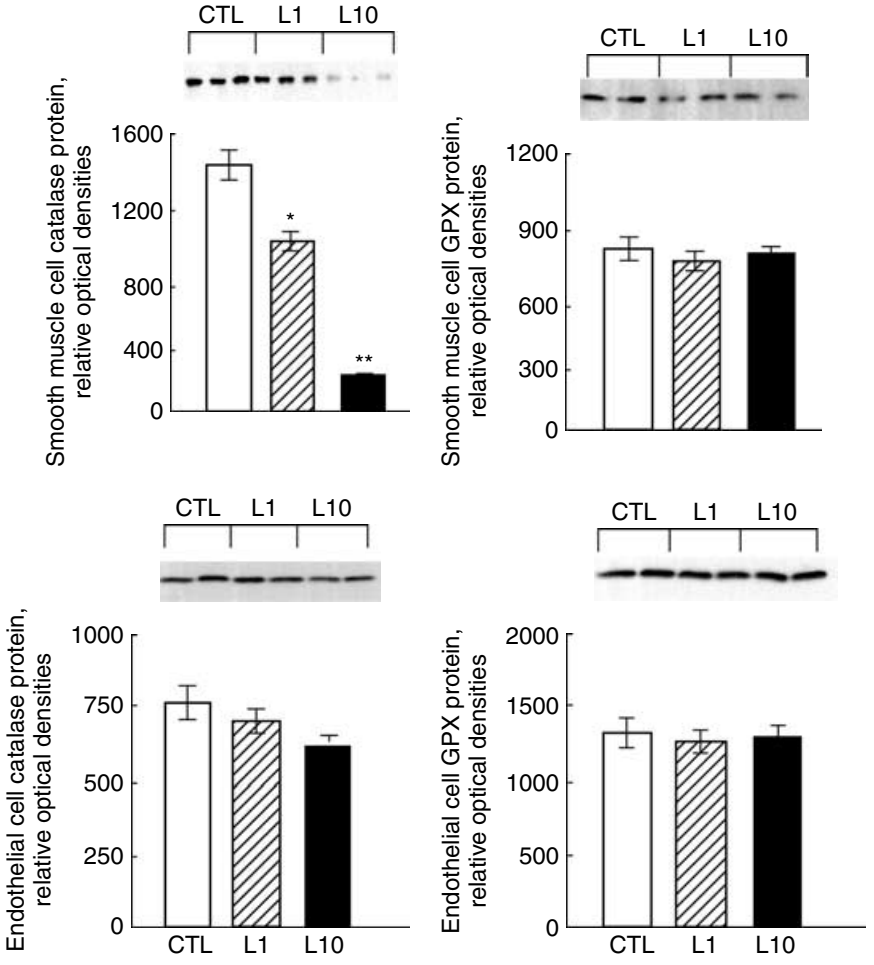

Fig. 6. Representative Western blots and group data depicting catalase and glutathione peroxidase (GPX) in human coronary artery endothelial and vascular smooth muscle cells incubated in lead acetate (at 1 ppm or 10 ppm), or equivalent concentrations of Na acetate (CTL) for 60 hours. $^{*}P < 0.05$ vs. CTL; $^{**}P < 0.01$ vs. 1 ppm and CTL, $N = 9$.

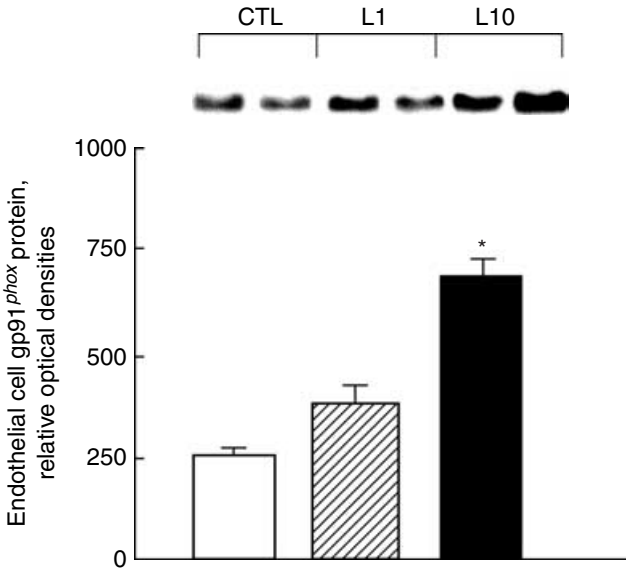

Fig. 7. Representative Western blot and group data depicting gp91^{phox} abundance in human coronary endothelial cells incubated in media containing lead acetate (1 or 10 ppm), or equivalent concentration of Na acetate (CTL) for 60 hours. $^{*}P < 0.05$.

to avoid transformation. For these reasons, the data obtained can have clinical relevance.

It is of note that short-term exposure to lead resulted in significant elevations of both superoxide and H_2O_2, whereas chronic lead exposure resulted in elevation of H_2O_2, but not superoxide, in both endothelial and vascular smooth muscle cells. This phenomenon can be explained by the compensatory up-regulation of superoxide dismutase (SOD), which catalyzes dismutation of superoxide to H_2O_2, and hence, reduction of superoxide and elevation of H_2O_2 in the chronic phase. In addition, concurrent rise in NO production, occasioned by up-regulation of endothelial NO synthase, shown in our earlier studies [13], may contribute to the decline in detectable superoxide. This is based on the great affinity of superoxide for reaction with NO. Since SOD expression is regulated by its substrate, superoxide, stimulation of superoxide production by lead can account for the compensatory up-regulation of SOD in cells exposed to lead for extended periods. Hydrogen peroxide is converted to water by catalase ($2\ H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$) and GPX, which utilizes glutathione (GSH) as electron donor ($H_2O_2 + 2GSH \xrightarrow{GPX} {}_2H_2O + GS\text{-}SG$). In contrast to the CuZn SOD, CAT abundance declined significantly in chronically lead-exposed vascular smooth muscle cells, and insignificantly in the endothelial cells, while GPX remained unchanged. Given the critical role of catalase and GPX in detoxification of hydrogen peroxide and lipoperoxides, the observed reduction or lack of a compensatory up-regulation of these enzymes undoubtedly contributes to oxidative stress and H_2O_2 accumulation in the lead-exposed endothelial and vascular smooth muscle cells.

NAD(P)H oxidase isoforms have emerged as a major source of ROS in the vascular tissue [22]. We therefore sought to assess the effect of long-term lead exposure on immunodetectable NAD(P)H oxidase in the human endothelial and vascular smooth muscle cells. The study showed a significant up-regulation of gp91^{phox} subunit of this enzyme in the lead exposed endothelial cells. This observation points to up-regulation of NAD(P)H oxidase as a potential source of ROS in lead-exposed endothelial cells. It is of note that gp91^{phox} is not expressed in the vascular smooth muscle cells and, as such, was not detected here.

Unlike the in vivo condition, wherein vascular cells are influenced by a myriad of hemodynamic forces, neuronal stimuli, humoral factors, and interaction with other cell types, such as leukocytes and platelets, these confounding factors are absent in vitro. Thus, increased ROS generation in lead-exposed endothelial and vascular smooth muscle cells in vitro points to the direct effect of lead in promoting oxidative stress in the vascular tissue. These observations support the potential benefit of the reduction in lead burden by preventing further exposure and promoting lead excretion in the affected persons.

CONCLUSION

Lead exposure promotes generation of superoxide and hydrogen peroxide in the cellular components of the vascular wall. This phenomenon can contribute to the pathogenesis of the associated hypertension and cardiovascular disease and points to the potential benefit of lowering lead burden in the exposed populations.

LIST OF ABBREVIATIONS

18-OH-THA	18-OH-tetrahydro-11-dehydrocorticosterone
5α-THB	5α-tetrahydro-corticosterone
5α-THF	5α-tetrahydro-cortisol
BMI	Body mass index
CMO	Corticosterone methyl oxidase
CMO-II	Corticosterone methyl oxidase type II
CYP11B1	11β-hydroxylase
CYP11B2	Aldosterone synthase
DOC	11-deoxycorticosterone
GC-MS	Gas chromatography-mass spectrometry
MO	Methyl oxidase
Substance B	Corticosterone
Substance S	11-deoxy-17-hydroxycorticosterone
THA	Tetrahydro-11-dehydrocorticosterone
THAldo	Tetrahydro-aldosterone
THB	Tetrahydro-corticosterone
THDOC	Tetrahydro-11-deoxycorticosterone
THE	Tetrahydro-cortisone
THF	Tetrahydro-cortisol

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REFERENCES

1. Vaziri ND: Pathogenesis of lead-induced hypertension: Role of oxidative stress. *J Hypertension* 20:S15–20, 2002
2. Vaziri ND, Ding Y, Ni Z, *et al*: Altered nitric oxide metabolism and increased oxygen free radical activity in lead-induced hypertension: Effect of lazaroid therapy. *Kidney Int* 52:1042–1046, 1997
3. Gonick HC, Ding Y, Bondy SC, *et al*: lead-induced hypertension: Interplay of nitric oxide and reactive oxygen species. *Hypertension* 30:1487–1492, 1997
4. Vaziri ND, Ding Y, Ni Z: Nitric oxide synthase expression in the course of lead induced hypertension. *Hypertension* 34:558–562, 1999
5. Vaziri ND, Liang K, Ding Y: Increased nitric oxide inactivation by reactive oxygen species in lead-induced hypertension. *Kidney Int* 56:1492–1498, 1999
6. Vaziri ND, Ding Y, Ni Z: Compensatory upregulation of NO synthase isoforms in lead-induced hypertension: Reversal by a SOD-mimetic drug. *J Pharmacol Exp Ther* 298: 679–685, 2001
7. Sharp DS, Osterloh J, Becker CE, *et al*: Blood pressure and blood lead concentration in bus drivers. *Environ Health Perspect* 78:131–137, 1988
8. Harlan WR: The relationship of blood lead levels to blood pressure in the U.S. population. *Environ health Perspect* 78:9–13, 1988
9. Khlil-Menesh F, Gonick HC, Weiler EW, *et al*: Effect of chelating treatment with dimercaptosuccinic acid (DMSA) on lead-related blood pressure changes. *Environs Res* 65:86–99, 1994
10. Ding Y, Gonick HC, Vaziri ND: Lead promotes hydroxyl radical generation and lipid peroxidation in cultured aortic endothelial cells. *Am J Hypertension* 13:552–555, 2000
11. Ding Y, Gonick HC, Vaziri ND, *et al*: Lead-induced hypertension: Increased hydroxyl radical production. *Am J Hypertension* 14:169–173, 2001
12. Ding Y, Vaziri ND, Gonick HC: Lead-induced hypertension. II. Response to sequential infusions of L-arginine, superoxide simutase, and nitroprusside. *Environ Res* 76:107–113, 1998
13. Vaziri ND, Ding Y: Effect of lead on NO synthase expression in coronary endothelial cells: Role of superoxide. *Hypertension* 37:223–226, 2001
14. Vaziri ND, Lin CY, Ding Y, Sindhu RK: Superoxide dismutase, catalase, glutathione peroxidase and NAD(P)H oxidase in lead-induced hypertension. *Kidney Int* 63:186–194, 2003
15. Malvezzi CK, Moreira EG, Vassilieff I: Effect of L-arginine, dimercaptosuccinic acid (DMSA) and the association of L-arginine and DMSA on tissue lead mobilization and blood pressure level in plumbism. *Braz J Med Biol Res* 34:1341–1346, 2001
16. Cory-Slechta DA: Mobilization of lead over the course of DMSA chelation therapy and long-term efficacy. *J Pharmacol Exp Ther* 246:84–91, 1988
17. Vaziri ND, Dicus M, Ho ND, *et al*: Oxidative stress and dysregulation of superoxide dismutase and NAD(P)H oxidase in renal insufficiency. *Kidney Int* 63:179–185, 2003
18. Lim CS, Vaziri ND: The effects of iron dextran on oxidative stress in cardiovascular tissues of rats with chronic renal failure. *Kidney Int* 65:1802–1809, 2004
19. Sokas RK, Simmens S, Sophar K, *et al*: Lead level in Maryland construction workers. *Am J Ind Med* 31:188–194, 1997
20. Vaziri ND: Role of oxidative stress and antioxidant therapy in chronic kidney disease and hypertension. *Curr Opin Nephrol Hyptens* 13:93–98, 2004
21. Rodriguez-Itrube B, Vaziri ND, Herrera-Acosta J, Johnson R: Oxidative stress, renal infiltration of immune cells and salt sensitive hypertension: All for one and one for all. *Am J Physiol, Renal Physiol* 286:F606–F616, 2004
22. Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: Role in cardiovascular biology and disease. *Circ Res* 86:494–501, 2000