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Abstract

Chronic lead exposure induces hypertension in humans and animals, affecting endothelial function. However, studies concerning acute cardiovascular effects are lacking. We investigated the effects of acute administration of a high concentration of lead acetate (100 µM) on the pressor response to phenylephrine (PHE) in the tail vascular bed of male Wistar rats. Animals were anesthetized with sodium pentobarbital and heparinized. The tail artery was dissected and cannulated for drug infusion and mean perfusion pressure measurements. Endothelium and vascular smooth muscle relaxation were tested with acetylcholine (5 µg/100 µL) and sodium nitroprusside (0.1 µg/100 µL), respectively, in arteries precontracted with 0.1 µM PHE. Concentration-response curves to PHE (0.001-300 µg/100 µL) were constructed before and after perfusion for 1 h with 100 µM lead acetate. In the presence of endothelium (E^+), lead acetate increased maximal response (E_{max}) (control: 364.4 ± 36, Pb^{2+}: 480.0 ± 27 mmHg; $P < 0.05$) and the sensitivity (pD_2; control: 1.98 ± 0.07, 2.38 ± 0.14 log mM) to PHE. In the absence of endothelium (E^-) lead had no effect but increased baseline perfusion pressure (E^+: 79.5 ± 2.4, E^-: 118 ± 2.2 mmHg; $P < 0.05$). To investigate the underlying mechanisms, this protocol was repeated after treatment with 100 µM L-NAME, 10 µM indomethacin and 1 µM tempol in the presence of lead. Lead actions on E_{max} and pD_2 were abolished in the presence of indomethacin, and partially abolished with L-NAME and tempol. Results suggest that acute lead administration affects the endothelium, releasing cyclooxygenase-derived vasoconstrictors and involving reactive oxygen species.

Key words: Lead acetate; Vascular reactivity; Endothelium; Hypertension

Introduction

Environmental exposure to pollutant metals, including lead, contributes to cardiovascular disease (1). Moreover, this metal is recognized as a common environmental and occupational health hazard (2). Lead is an important toxic agent that can exert adverse effects in humans given sufficient exposure and/or accumulation in the body (3). Experimental studies in animals and epidemiological reports suggest a close relationship between lead exposure and hypertension (4,5).

Lead is one of the metals most extensively used in the industrial sector in several countries including Brazil, a fact that contributes to its wide environmental distribution. Therefore, usually all humans have lead in their organism as a result of exposure to exogenous sources (6). Some workers are exposed to high levels of lead in their activities. This exposure occurs during the manufacture of ammuni-

tion, batteries, sheet lead, solder, ceramic glazes, caulking, some brass and bronze plumbing, circuit boards, military equipment, some surgical equipment, and some medicines (herbal remedies from China, India) (6). Barbosa Jr. et al. (7), studying 62 lead-exposed subjects, demonstrated a positive correlation between the increased inhibition of nitric oxide (NO) with increasing blood lead or plasma lead concentrations. These investigators suggested a significant inhibitory effect of lead on NO and provided clinical evidence for a mechanism involving the association between lead exposure and increased cardiovascular risk.

Previous reports have suggested that lead acts at multiple sites within the cardiovascular system. Additionally, chronic and acute lead poisoning causes cardiac and vascular damage with potentially lethal consequences (8,9). Lead also has other effects on the cardiovascular system,

including endothelial dysfunction (10) and inhibition of the sarcolemmal sodium potassium ATPase (11). These actions also include direct effects on the excitability and contractility of the heart (8,9), vascular actions on the compliance and contractility of smooth muscle (12-14), and tissue damage by free radicals (15). This metal also promotes reductions in vascular β-adrenoceptor density and cAMP levels, factors that contribute to increased blood pressure (16). In addition, harmful effects on other possible sites of action as within the central nervous system might occur affecting blood pressure regulation (17,18).

The actions of lead on the endothelium seem to involve the release of vasoactive factors, although the findings obtained to date remain controversial. Previous findings have shown that lead exposure might increase (19) or decrease (20) plasma endothelin levels, as well as increase or decrease urine NO concentration or endothelial NO synthase (eNOS) overexpression (21).

Although the chronic and acute effects of lead on living animals are well known, there are comparatively few studies concerning the acute effects of lead on vascular reactivity. Therefore, the aim of the present study was to investigate the acute effects of continuous perfusion with a high concentration of lead acetate (100 µM) on the pressor reactivity of the perfused rat tail artery.

Material and Methods

Animals

Studies were performed on male Wistar rats (250-300 g). All experiments were conducted in accordance with the guidelines for biomedical research as stated by the Brazilian Societies of Experimental Biology. The experimental protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences, EMESCAM (Protocol #003/2007). All rats had free access to water and rat chow *ad libitum*.

Isolated rat tail vascular bed preparation

Isolated rat tail arteries were perfused as previously reported (22). Rats were first anesthetized with sodium pentobarbital (65 mg/kg, *ip*), and heparin (500 IU, *ip*) was administered 10 min later. At the base of the tail 2 cm of the tail artery were dissected free and cannulated with an intracath (Nipro 24G ¾, Brazil). The whole tail (average length of 18 to 20 cm) was then severed from the body, placed in a tissue bath and perfused at a constant flow of 2.5 mL/min with a peristaltic pump (Milan, Brazil), with Tyrode solution (120 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 20 mM HEPES buffer, 11 mM glucose, and 3 µM EDTA), bubbled with 100% O_2, at 36 ± 0.5°C).

The HEPES buffer was carefully chosen to prevent lead acetate precipitation. After a 45-min equilibration period, the experimental protocol was initiated. The mean perfusion pressure (MPP) was measured using a pressure transducer (TSD104A, BIOPAC Systems, Inc., USA), and data were recorded using an interface and software for computer data acquisition (model MP100A, BIOPAC Systems, Inc.). As a constant flow was maintained, changes in MPP represent changes in vascular resistance.

Effects of the acute administration of lead on the actions of phenylephrine in the presence and absence of endothelium

After a 45-min stabilization period, endothelial integrity was evaluated using acetylcholine (Ach) administration (5 µg/100 µL) in the tail artery precontracted with 0.1 µM phenylephrine (PHE). Endothelial integrity was considered to be adequate when relaxation attained more than 50% of maximum contraction induced by a 0.1 µM PHE. Smooth muscle viability was tested using sodium nitroprusside (SNP: 0.1 mg/mL) in the tail artery, which had been previously contracted with PHE. This test was performed before and after each complete experiment.

The vasoconstrictor response to the stimulation of an α_1-adrenoceptor agonist was evaluated by administering PHE (0.001-300 µg/100 µL) to preparations (N = 10) with intact endothelium (control group). After the first concentration-response curve to PHE the preparations were perfused for 1 h with a buffer solution containing 100 µM lead acetate and a second concentration-response curve of PHE was constructed in the presence of 100 µM lead acetate. To ensure that the effects were not dependent on time, another group of rats was used under the same conditions in a time control experiment performed 1 h later, after *iv* administration of 0.9% saline. This lead concentration was selected because it is known to produce a clear negative inotropic effect and to reduce myosin ATPase activity in the rat myocardium (23).

The same protocol was performed as described above in preparations following endothelium damage (E⁻: N = 10) induced by {3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate} (CHAPS, 8 mg in 80 µL), as previously described (24). The absence of functional endothelium was confirmed by the inability of ACh (5 µg in 100 µL) to produce relaxation. The endothelium-independent vasodilator response to SNP (0.1 mg/mL) was also determined. Concentration-response curves to PHE were constructed at 30 min after endothelial damage and repeated after perfusion with a solution containing 100 µM lead acetate for a period of 1 h (E⁻ Pb^{2+}, N = 10).

Time-control concentration-response curves to PHE (0.001-300 µg in 100 µL) were constructed before and after an infusion period of 1 h with Tyrode solution in preparations with (N = 10) and without endothelium (N = 10).

The role of prostanoids and nitric oxide in the effects of lead on the phenylephrine-induced pressure response

To analyze the involvement of NO and prostanoids derived from cyclooxygenase (COX) activity in the effects

of lead acetate on the pressure responses to PHE, two protocols were performed. Preparations were perfused for 1 h with 100 µM lead acetate in both situations: with the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME 100 µM, Pb^{2+} L-NAME, N = 13) and with indomethacin (10 µM, Pb^{2+} indomethacin, N = 9), respectively. Basal NO release was evaluated by comparing the areas under the concentration-response curves for PHE, obtained in the absence and presence of L-NAME (25). The same protocol was also performed without lead acetate in the absence and in the presence of 10 µM indomethacin (N = 9) or 100 µM L-NAME (N = 8).

The role of free radicals in the effects of lead on the phenylephrine-induced pressure response

To investigate whether free radicals play a role in the effects of lead acetate on the pressure response to PHE, preparations were perfused for 1 h with 1 µM tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), an agent that mimics superoxide dismutase (SOD), in the absence and in the presence of lead acetate.

Drugs and reagents

Lead acetate, PHE, L-NAME, indomethacin, tempol, ACh hydrochloride, SNP, CHAPS, HEPES, EDTA, and sodium pentobarbital were purchased from Sigma (USA); and heparin was purchased from Roche (Brazil). NaCl, KCl, $MgCl_2$, $CaCl_2$, and glucose were purchased from Merck (Germany).

The drugs were dissolved in distilled water and all solutions were freshly prepared before use and protected from light. Indomethacin was dissolved in 0.1 M Tris buffer, pH 7.4.

Data analysis

Results regarding perfusion pressure measurements are presented as changes in MPP by subtracting peak pressure from baseline pressure (ΔMPP, mmHg). The relaxation responses to ACh and SNP are reported as percent relaxation in preparations precontracted with 0.1 µM PHE.

For each PHE concentration-response curve, the maximum effect and the bolus dose (µg) that produced one-half E_{max} (-log EC_{50}) were estimated using non-linear regression analyses (Graph Pad Prism Software, USA). The sensitivity of the agonist is reported as pD_2 (-log EC_{50}) and the maximum effect as maximal response (E_{max}). To compare the effects of different drugs on the response to PHE, results were reported as "differences of area under the concentration-response curves" (dAUC) in control and experimental situations. AUCs were calculated from the individual dose-response curves and the differences were reported as a percent of AUC (%dAUC) of the corresponding control situation (26).

Data are reported as means ± SEM and were analyzed by the Student *t*-test (paired or unpaired) and by one-way analysis of variance (ANOVA). When ANOVA showed a significant effect of treatment, the Tukey *post hoc* test was used to compare means. A P value <0.05 was considered to be significant.

Results

Effects of the acute administration of lead on the actions of phenylephrine in the presence and absence of endothelium

To evaluate the interference of the duration of the experiments with the stability of the preparations, concentration-response curves to PHE were constructed before and after 1 h of continuous perfusion with Tyrode solution. This protocol showed that the duration of the experiments did not interfere with the E_{max} (before: 522 ± 19.6, after: 506 ± 13.2 mmHg) or the pD_2 (before: 1.6 ± 0.04, after: 1.7 ± 0.03 log mM). On the other hand, 1 h of perfusion with lead acetate (100 µM) increased E_{max} and pD_2 (Figure 1A).

Figure 1B demonstrates that, in the absence of endothelium, lead acetate increased the baseline perfusion pressure (E^+: 79.5 ± 2.4 *vs* E^-: 118 ± 2.2 mmHg; P < 0.05, *t*-test) but did not change E_{max} or pD_2 (Figure 1B).

To investigate how acute lead acetate treatment affected the endothelium or smooth muscle integrity, single doses of ACh, an endothelial-dependent vasodilator, and SNP, an endothelial-independent vasodilator, were used. Treatment with lead damaged the endothelium since the relaxation produced by ACh was significantly decreased (control: 60%; Pb^{2+}: 20%; P < 0.05). The integrity of smooth muscle was preserved since SNP-induced vasorelaxation was unchanged (control: 81%; Pb^{2+}: 91%).

Role of endothelial factors in the effects of lead

L-NAME was used to investigate the putative role of NO in the effects of lead acetate on the pressor response to PHE. In control groups, L-NAME produced the expected increase in E_{max} and pD_2 (Figure 2A). However, after perfusion with lead acetate plus L-NAME, a significant increase in E_{max} was observed when compared with the separate effect of L-NAME on the untreated preparations (Figure 2B).

When the COX pathway-derived prostanoids were investigated with the use of indomethacin, just a small increase of E_{max} to PHE was observed before lead administration (control: 427 ± 35 *vs* indomethacin: 528 ± 37 mmHg; P < 0.05). After lead plus indomethacin administration, the increased pressor response to PHE, previously observed after lead treatment alone, was abolished (Figure 2C). However, under indomethacin treatment, NO production was still present. Then, the next step in our study was to evaluate whether the inhibition of NO production by L-NAME could demonstrate the participation of another vasoconstrictor agent. For this, we performed a protocol using the association of the two drugs, i.e., L-NAME and indomethacin in the presence of lead acetate. After this
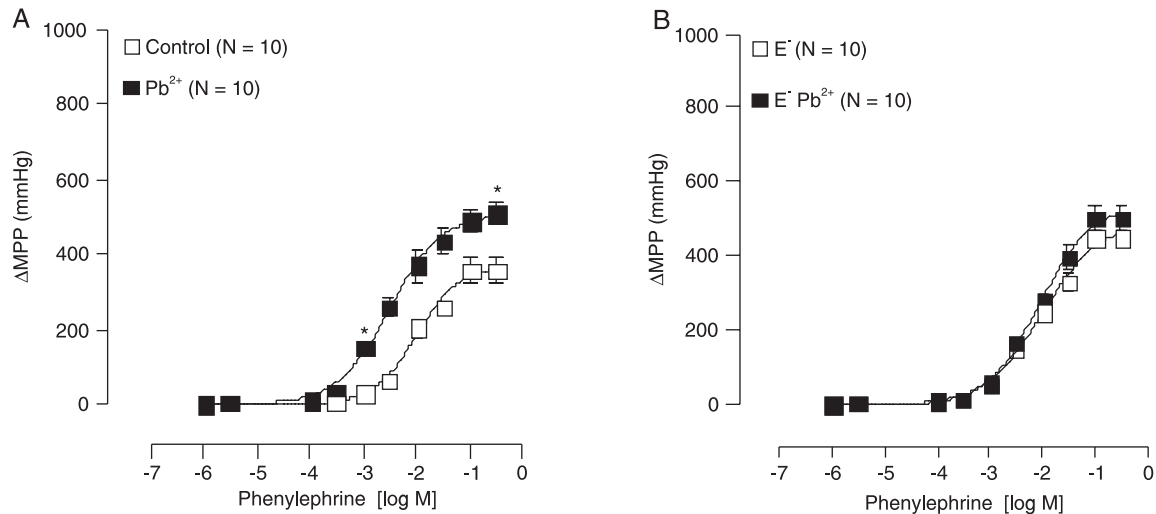
Figure 1. Changes in mean perfusion pressure (ΔMPP; mmHg) of the tail artery in response to increasing doses of phenylephrine in Wistar rats. *A*, With endothelium, before (control, open squares) and after (filled squares, Pb^{2+}) infusion of 100 µM lead acetate for 1 h. *B*, Without endothelium before (open squares, E^-) and after (filled squares, Pb^{2+}) infusion of 100 µM lead acetate for 1 h. Data are reported as means ± SEM. *P < 0.05 for maximal response (E_{max}) and sensitivity (pD_2): Pb^{2+} *vs* control (paired *t*-test).

procedure, it was observed that the Pb^{2+} L-NAME/indomethacin treatment showed a leftward displacement of the concentration-response curve to PHE (Figure 2D); however, the effects of lead on the E_{max} to PHE were abolished. The magnitude of the response of these groups was evaluated using the areas under the curves (Figure 2E). This analysis suggested the involvement of a COX-derived vasoconstrictor in the effect of lead.

The role of free radicals on the effects of lead acetate

We also investigated the role of free radicals in the effect of lead. Tempol, an SOD mimetic, was used to investigate whether free radicals play a role in the action of 100 µM lead acetate on the pressor responses to PHE. Tempol promoted a small increase in the sensitivity to PHE (Figure 3B). Moreover, in the presence of tempol, the effects of lead acetate were partially abolished (Figure 3C). Figure 3D shows the magnitude of these responses.

Discussion

The main finding of the present study shows that acute exposure to 100 µM lead acetate increases the reactivity of the tail artery to PHE. This increased reactivity is endothelium-dependent, since the effects of lead were abolished in the absence of endothelium. Additionally, this study demonstrated that COX inhibition by indomethacin prevented vasoconstriction, suggesting that the increased reactivity of the tail artery depends on the participation of a COX-derived vasoconstrictor. Results also showed that the blockade of the superoxide anion by tempol partially

prevented the effects of lead, suggesting the involvement of free radicals in the effects of this metal.

It is well established that chronic lead exposure induces the production or release of endothelin, a powerful vasoconstrictor (19), inhibits the production or release of a hyperpolarizing factor (26) and reduces the release or production of NO (21,27). The combination of these lead-induced effects might increase vascular tonus and consequently blood pressure.

These effects appeared to be related to an imbalance of endothelial-derived vasoconstrictor and vasodilator compounds (19). In addition, other investigators have demonstrated that chronic exposure to moderate levels of lead increases blood pressure (14,28), probably due to an increased vascular reactivity to α-adrenergic agonists. Previous studies have shown that chronic administration of this metal can act directly on vascular smooth muscle (29), also increasing the pressor response to norepinephrine in isolated arteries (20). In addition, Heydari et al. (30) reported that lead was able to increase E_{max} and pD_2 in the presence of PHE in thoracic aortic rings of rats treated with lead for 8 to 12 weeks. Furthermore, it is known that this metal increases vascular responses to endogenous compounds such as norepinephrine (14) and enhances sympathetic activity, modifying the baroreflex sensitivity and inducing alterations in the signal transduction system present in cell membranes, such as cAMP, Ca^{2+} and NO (31).

To further understand the vasoconstrictor effect promoted by lead acetate and its capacity to harm the vascular endothelium and the smooth muscle cells we investigated the actions of lead on the vascular reactivity of the tail artery.
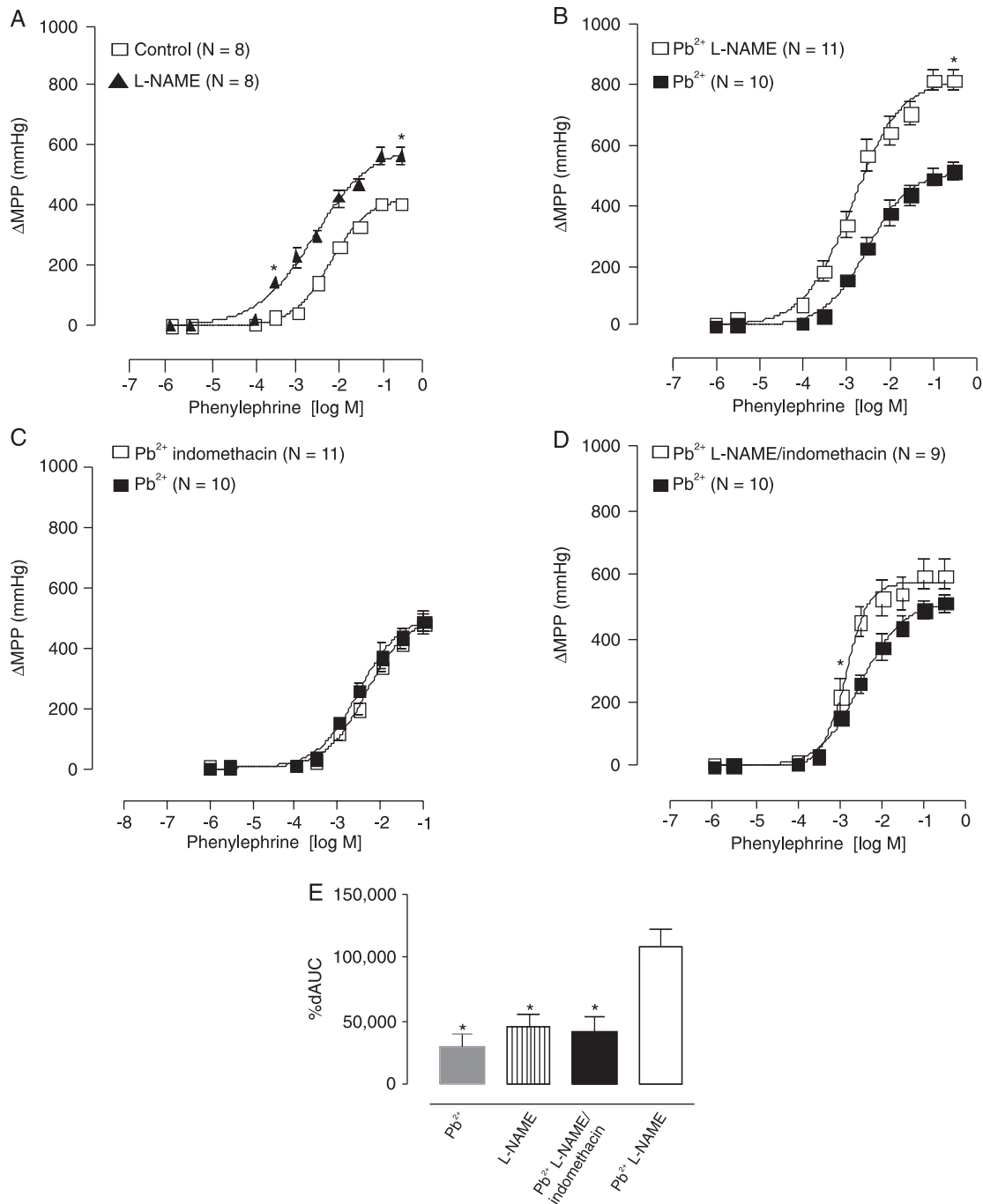
Figure 2. Changes in mean perfusion pressure (ΔMPP; mmHg) in response to increasing doses of phenylephrine in the tail artery of Wistar rats. *A*, Before (open squares, control) and after (filled triangles, L-NAME) perfusion with L-NAME. *B*, Effect of lead acetate in the absence (filled squares, Pb^{2+}) and in the presence of L-NAME (open squares, Pb^{2+} L-NAME). *C*, Effect of lead acetate in the absence (filled squares, Pb^{2+}) and in the presence of indomethacin (open squares, Pb^{2+} indomethacin). *D*, Effect of lead acetate in the absence (filled squares, Pb^{2+}) and in the presence of L-NAME and indomethacin (open squares, Pb^{2+} L-NAME/indomethacin). *E*, Difference of areas under the concentration-response curves (dAUC) to phenylephrine for the following groups: Pb^{2+} *vs* control, L-NAME *vs* control, and Pb^{2+} L-NAME/indomethacin *vs* Pb^{2+}. Data are reported as means ± SEM. *P < 0.05 for maximal response (E_{max}) and sensitivity (pD_2): L-NAME *vs* control (paired *t*-test). *P < 0.05 for E_{max}: Pb^{2+} L-NAME *vs* Pb^{2+}; Pb^{2+} L-NAME/indomethacin *vs* Pb^{2+} (unpaired *t*-test). *Panel E*, *P < 0.05 *vs* Pb^{2+} L-NAME (one-way ANOVA).
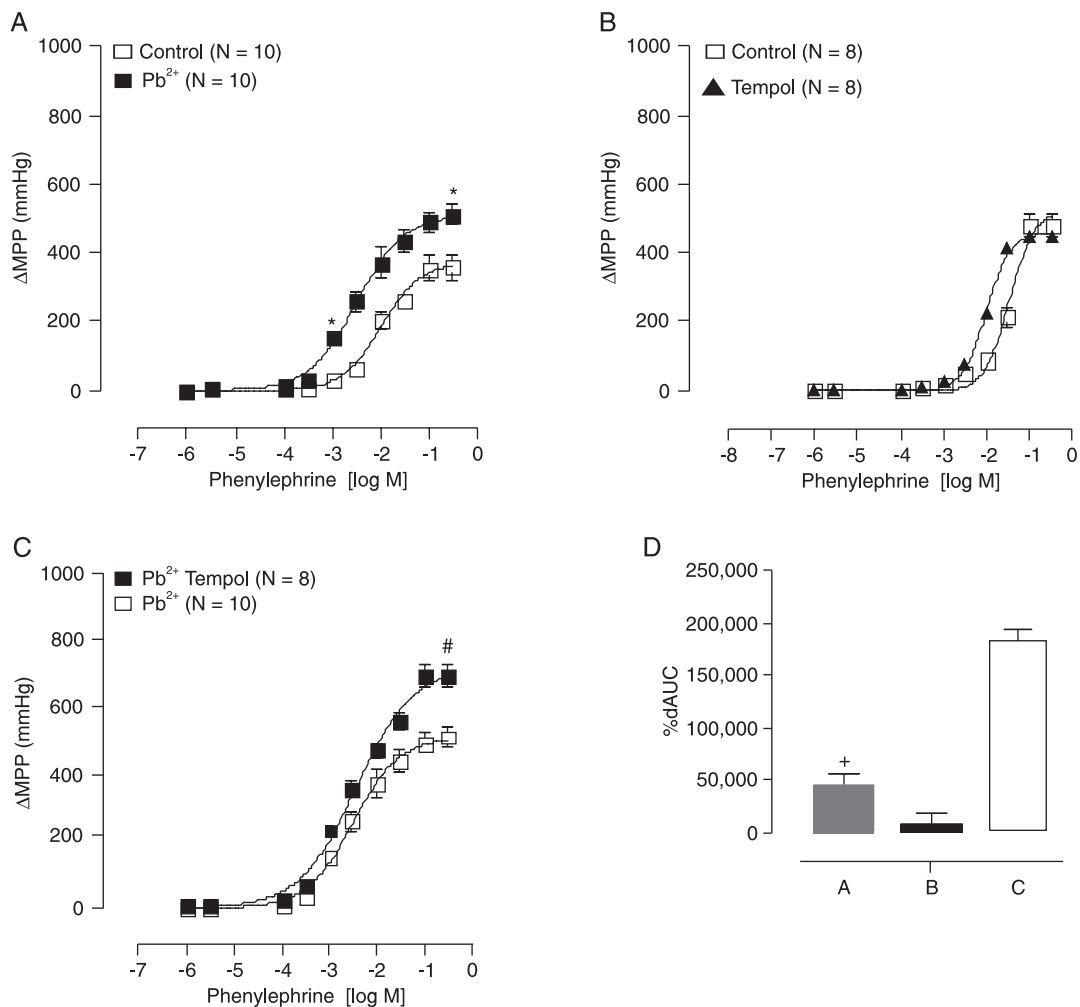
Figure 3. Changes in mean perfusion pressure (ΔMPP; mmHg) in response to increasing doses of phenylephrine in the tail artery of Wistar rats. *A*, Before (control, open squares) and after (Pb^{2+}, filled squares) infusion of 100 µM lead acetate for 1 h. *B*, Before (control, open squares) and after (filled triangles) infusion of tempol. *C*, Effect of lead acetate in the absence (Pb^{2+}, open squares) and presence of Tempol (Pb^{2+} Tempol, filled squares). *D*, Difference of areas under the concentration-response curves (dAUC) to phenylephrine between: (A: Control *vs* Pb^{2+}; B: Control *vs* Tempol; C: Pb^{2+} Tempol *vs* Pb^{2+}). Data are reported as means ± SEM. *P < 0.05 for maximal response (E_{max}) and sensitivity (pD_2): Pb^{2+} *vs* control; #P < 0.05 for E_{max}: Pb^{2+} *vs* Pb^{2+} Tempol; +P < 0.05 for %dAUC: Pb^{2+} *vs* Pb^{2+} Tempol and Tempol (one-way ANOVA).

Our findings showed that the action of lead in the tail artery with intact endothelium increased the vasoconstrictor response to PHE, the maximal response (E_{max}) and the sensitivity (pD_2). These actions were endothelium-dependent, since lead effects were abolished after endothelium removal. Considering that a significant impairment of endothelial function was suggested, lead could be inhibiting the release of endothelium-derived vasodilators or stimulating the release of endothelial vasoconstrictors. Our findings seem to corroborate those of Marques et al. (32), as they reported that lead-induced hypertension damaged both endothelium-

dependent and -independent relaxation. This response was accompanied by increased eNOS protein expression and down-regulation of soluble guanylate cyclase (sGC). Marques et al. (32) also suggested that these responses seem to involve reactive oxygen species (ROS) acting on the relaxation mechanisms of NO/cyclic guanosine monophosphate in the vascular wall.

We observed a significant increase of the E_{max} promoted by lead acetate in the presence of L-NAME. However, L-NAME was not able to completely abolish the response promoted by lead acetate. This result suggests that the

effects on the pressor response to PHE cannot be totally attributed to NO. To better clarify this issue, our next step was to evaluate the pathway of COX-derived prostanoids. For this, preparations were treated with indomethacin, a COX inhibitor. We observed that the lead-induced vasoconstrictor effects on the tail arterial bed were abolished by indomethacin. These results suggest that lead acetate might stimulate the release of COX-derived vasoconstrictor prostanoids. Although suggesting the production of a vasoconstrictor prostanoid, our findings did not enable us to distinguish which compound caused this. However, Courtois et al. (33) found an increase in the expression of COX-2 protein in aortic rings perfused with lead and suggested that this response was associated with a deregulation of the β_1-sGC subunit and an increased production of ROS as the vasoconstrictor mechanism.

Given that NO production was still present after inhibition by indomethacin because of the intact endothelium, the production of another endothelium-derived vasoconstrictor had to be taken into account. To better clarify this issue we also carried out a protocol associating two drugs, L-NAME and indomethacin, in the presence of lead. In this protocol, we observed a leftward displacement of the concentration-response curve to PHE, which confirms that the effects of lead on the vascular tail bed are probably mediated by the release of a COX-derived vasoconstrictor plus another constrictor component.

There is growing evidence that the chronic effects of lead are also associated with oxidative stress (15,19,21). In contrast, previous studies have shown that ROS up-regulates the expression of COX-2 isoforms, which is primarily responsible for the synthesis of prostaglandins involved in pathological processes, including hypertension (34). Therefore, the ability of lead acetate to generate ROS, specifically the superoxide anion ($O_2^{\bullet-}$), was evaluated. For this, the preparations were perfused with tempol. This treatment partially abolished the actions of lead acetate on the pressor response to phenylephrine, suggesting that acute treatment with lead probably involved the pathway of $O_2^{\bullet-}$ production and/or liberation. Our findings showing that COX is also involved reinforce the importance of ROS-induced generation by lead. However, this response does not rule out the possibility that the chronic action of this metal can induce the production of other kinds of free radicals, such as the radical hydroxyl ($OH^{\bullet-}$) and hydrogen peroxide (H_2O_2), as previously suggested (35,36).

It is important to emphasize that the present findings reinforce the biological significance of lead as an environmental contaminant that damages the human organism, producing harmful effects on the cardiovascular system. Although significant progress has occurred regarding environmental contamination, there are still serious problems produced by heavy metals. The positive correlation between the concentration of plasma lead and hypertension (37,38), one of the most prominent cardiovascular problems in many countries, and the increase in all causes of lead-related deaths, including circulatory and cancer mortality (39) reinforce the biological significance of lead as an important hazard.

The results obtained thus far might be interpreted considering two limitations. The first limitation regards the lead concentration we used in this study, which is clearly toxic and far above safety values for human beings. We used a high lead concentration to begin to understand its effects. In the future, we will use smaller concentrations. The second limitation is that it is not possible to compare the present results with those for other vascular beds such as resistance vessels or the greater conductance vessels such as the aorta. However, the results of the present study provide guidance for further studies using much lower lead concentrations to better elucidate the cardiovascular effects of the metal.

In conclusion, the present study raises the possibility of a new mechanism of action of lead, i.e., an endothelial COX-derived vasoconstrictor mechanism, reinforcing the importance of ROS-induced generation by lead.

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