

# Lead-Induced Downregulation of Soluble Guanylate Cyclase in Isolated Rat Aortic Segments Mediated by Reactive Oxygen Species and Cyclooxygenase-2

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**Abstract.** Lead exposure is a known cause of hypertension. Although most studies have focused on lead-induced endothelial dysfunction and on the involvement of reactive oxygen species (ROS), it has been recently demonstrated that the vascular wall of lead-exposed rats has both an altered the endothelium-independent relaxing response and a reduced expression of soluble guanylate cyclase (sGC). The aim of the present study was to determine in *in vitro* incubated rat isolated aortic segments if lead downregulates sGC expression, analyzing the involvement of ROS and cyclooxygenase-2 (COX-2). The experiments were performed in isolated aortic segments from Wistar rats that were incubated with lead for 24 h. Lead significantly reduced sGC- $\beta_1$  subunit expression in a concentration-dependent manner. The maximal reduction in sGC- $\beta_1$

subunit expression was achieved with 1 ppm lead. Vitamin C (30  $\mu\text{mol/L}$ ) partially restored sGC- $\beta_1$  subunit expression in lead (1 ppm)-exposed aortic segments. A similar protection of sGC- $\beta_1$  subunit expression was obtained with both a protein kinase A inhibitor, H89 (1  $\mu\text{mol/L}$ ) and with rofecoxib (1  $\mu\text{mol/L}$ ), an inhibitor of COX-2 activity. Moreover, lead exposure increased COX-2 expression in the arterial wall. While vitamin C reduced both COX-2 expression and superoxide anion production related to lead exposure, rofecoxib failed to modify superoxide anion generation in lead-incubated aortic segments. In conclusion, the present results suggest the involvement of ROS and COX-2 in the downexpression of sGC- $\beta_1$  subunit induced by lead in the rat vascular wall.

Lead exposure is a well-known cause of hypertension in humans and experimental animals (1,2). Although different considerations have been raised to explain the pathogenesis of lead-induced hypertension, several studies have suggested the primary involvement of the increased production of reactive oxygen species (ROS) observed in lead-exposed animals (3,4). Elevated levels of ROS reduce the bioavailability of nitric oxide (NO), thus inducing an impaired endothelium-dependent vasorelaxation (5).

NO is generated in the endothelium and induces vasodilatation by stimulating soluble guanylate cyclase (sGC) in the adjacent smooth muscle cells (6). Exogenous nitrovasodilators also cause vasodilatation of the vascular wall, independently of the endothelium, by activating the formation of cyclic GMP (cGMP) in the adjacent smooth muscle cells (7).

sGC is a heterodimer composed of a large ( $\alpha_1$ ) and a small

( $\beta_1$ ) subunit (8). Recent cloning and expression experiments have revealed that, although both the  $\alpha_1$  and  $\beta_1$  subunits seem to contain a catalytic domain, the sGC enzymatic activity requires the presence of both subunits (9). In this regard, we recently demonstrated an impaired endothelium-independent vasodilating response associated with downexpression of the sGC  $\beta_1$ -subunit (10). Moreover, although we are aware of no data in the literature about a possible role for ROS in the regulation of sGC expression, our previously mentioned study demonstrated that administration of vitamin C, an antioxidant, prevented the downexpression of sGC observed in the vascular wall of lead-exposed rats (10).

Although the intracellular signal regulators of sGC expression are still not completely established, different studies have demonstrated that in addition to nerve growth factor, endotoxin and interleukin-1 $\beta$ , cyclic adenosin monophosphate (cAMP)-stimulating agents, *i.e.*, prostaglandins, downregulate sGC expression (11–13). Therefore, the inhibition of the cAMP-coupled signaling pathway, such as protein kinase A activation, could protect the expression of sGC. Interestingly, it has been also demonstrated that ROS upregulate cyclooxygenase-2 (COX-2) expression, a COX isoform primarily responsible for the synthesis of prostaglandins involved in pathologic processes including hypertension (14,15). Therefore, on the basis of the above-mentioned findings, the aim of the present study was to determine in *in vitro* incubated rat isolated aortic segments if lead downregulates sGC expression in the vascular

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wall of rats, analyzing the involvement of ROS generation and COX-2 expression.

## Materials and Methods

### Incubation of Isolated Aortic Segments

Studies were approved by the Institutional Ethics Committee for animal studies and were performed in accordance with the international convention on animal experimentation. The experiments were carried out on male 300-g Wistar Kyoto rats. The animals were anesthetized with pentobarbital (30 mg/kg intramuscularly), and the descending thoracic aorta was removed and cut into 5-mm-long segments and individually suspended into culture solution (RPMI medium containing 5% fetal calf serum) at 37°C. Increasing concentrations of lead acetate (0 to 1 ppm) were added to the culture solution, and the aortic segments were incubated for 24 h in the presence and in the absence of vitamin C (30  $\mu\text{mol/L}$ ), rofecoxib (1  $\mu\text{mol/L}$ ), a COX-2 inhibitor (16), and an inhibitor of protein kinase A activity, H-89 (1  $\mu\text{mol/L}$ ) (17). The aortic segments were then immediately frozen in liquid nitrogen for molecular biology determinations. The lead concentrations in the present study were chosen on the basis of previous *in vitro* studies from Vaziri *et al.* (18,19), who elegantly demonstrated that 0.1 ppm to 1 ppm lead promoted lipid peroxidation and 1 ppm lead upregulated endothelial nitric oxide synthase expression in rat and human cultured endothelial cells, respectively.

### Determination of sGC- $\beta_1$ Subunit and COX-2

#### Expression by Western Blot Analysis

The expression of sGC- $\beta_1$  subunit and COX-2 proteins was analyzed by Western blot as described elsewhere (20). In brief, the aortic rings were pulverized and solubilized in Laemmli buffer containing 2-mercaptoethanol (21). The proteins obtained were separated in denaturing SDS/10% polyacrylamide gels. Equal amounts of proteins (20  $\mu\text{g}$  per lane) estimated by bicinchoninic acid reagent (Pierce, Rockford, IL) were loaded. To verify that equal amounts of proteins were loaded in the gel, a parallel gel was run and stained with Coomassie, and the intensities of the protein bands were examined. The proteins were then blotted into nitrocellulose (Immobilion-P; Millipore Ibérica, S.A.). The blots were blocked overnight at 4°C with 5% nonfat dry milk in TBS-T (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1% Tween 20). Western blot analyses were performed with monoclonal antibodies against sGC- $\beta_1$  subunit and COX-2, respectively. The blots were incubated with the first antibody (1:2500) for 1 h at room temperature and, after extensive washing, with the second antibody (horseradish peroxidase-conjugated anti-rabbit Ig antibody) at a dilution of 1:1500 for another hour. Specific sGC- $\beta_1$  subunit and COX-2 proteins were detected by enhanced chemoluminescence (ECL, Amersham Corp) and evaluated by densitometry (Molecular Dynamics). Prestained protein markers (Sigma) were used for molecular mass determinations. To compare the sGC- $\beta_1$  subunit and COX-2 isoform expression with the expression of another protein, we analyzed the expression of  $\beta$ -actin by Western blot, using a  $\beta$ -actin monoclonal antibody (Sigma Aldrich). For this purpose, a parallel gel with identical samples was run; after blotting onto nitrocellulose, Western blot analysis was then performed with the  $\beta$ -actin monoclonal antibody (1:2000).

### Measurement of cGMP

cGMP levels in the vascular wall were determined as described previously (10). In brief, aortic segments were incubated for 24 h in the presence and in the absence of 1 ppm lead in the culture solution (RPMI medium containing 5% fetal calf serum at 37°C). The vessels

were then washed and incubated in the same medium that contained the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (2 mmol/L) to prevent breakdown of cGMP for 20 min at 37°C. Similar experiments were performed incubating the different aortic segments with the nitric oxide donor, sodium nitroprusside (SNP,  $10^{-4}$  mol/L). The vessels were then immediately stored in liquid nitrogen. The frozen segments were extracted at 4°C with a 49:1 (vol/vol) mixture of 0.1 mol/L ethanol/HCl. The extracts were evaporated using a speed vac evaporator (Model VR-1/120/240; Heto Lab-Equipment A/S, Denmark). cGMP concentrations were measured in acetylated samples by means of a RIA kit (Amersham International). The sensitivity of the assay was 0.5 fmol. The intraassay and interassay variations were <8.9% and <16%, respectively.

### Superoxide Anion Generation in the Aortic Segments

The amount of superoxide anion generated by the vascular wall was determined by measuring the SOD-inhibitable reduction of ferricytochrome C, as described (22). In brief, aortic segments were incubated in culture RPMI-1640 medium containing 1% FCS and 0.1 mmol/L ferricytochrome C at 37°C for 30 min, as reported. The supernatants were collected, and the generation of superoxide anion was calculated as the difference in absorbance between aortic segments incubated with and without SOD 100  $\mu\text{g/mL}$ . The difference was then divided by the molar extinction coefficient change between ferricytochrome C and ferrocyanochrome C to determine the amount of superoxide radicals produced over 30 min. All observations were made in triplicate and the data averaged. The absorbance was measured in a spectrophotometer at 550 nm.

In addition to this quantitative method, we detected the generation of superoxide anion by confocal microscopy. The oxidative fluorescent dye dihydroethidium (DHE, 5  $\mu\text{mol/L}$ ) was used to evaluate *in situ* production of superoxide. DHE is freely permeable to cells and in the presence of superoxide anion is oxidized to ethidium, and trapped by intercalating with the DNA (23). Ethidium is excited at 488 nm with an emission spectrum of 610 nm. For confocal experiments, aortic segments incubated in the presence and in the absence of 1 ppm lead were cut into 30- $\mu\text{m}$ -thick sections and placed on a glass. DHE (2  $\mu\text{mol/L}$ ) was added to each tissue section. The slices were incubated in a light-protected humidified chamber at 37°C for 30 min. Images were obtained with a Leica Confocal Microscope (Leica TCS SP2 Microsystems, Heidelberg, Germany).

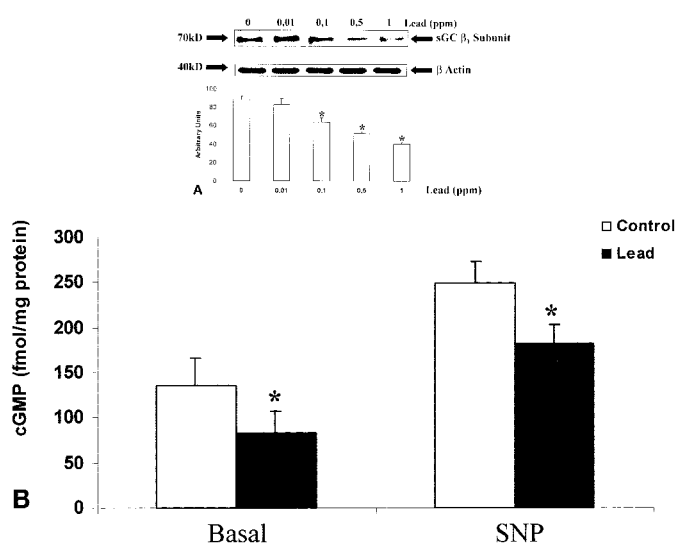
### Statistical Analyses

Results are expressed as mean  $\pm$  SEM. Each of the above-mentioned studies was performed in at least a minimum of five different aortic segments. Comparisons were performed by ANOVA or paired and unpaired *t* test when appropriate. Bonferroni correction for multiple comparisons was used to determine the level of significance of *P*. *P* < 0.05 was considered significant.

## Results

### Effect of Lead Exposure on sGC Expression

The Western blot experiments showed that the *in vitro* exposure of rat vascular wall to lead induced a dose-dependent decrease in sGC- $\beta_1$  subunit protein expression (Figure 1A). The maximal reduction in sGC- $\beta_1$  subunit expression was achieved with 1 ppm lead (Figure 1A). This lead concentration was used in most of the following experiments. No changes in  $\beta$ -actin expression were observed in control and lead-incubated aortic segments (Figure 1A).



**Figure 1.** (A) Representative Western blot showing soluble guanylate cyclase (sGC)  $\beta_1$ -subunit expression in isolated rat aortic segments incubated in the absence and in the presence of increasing concentrations of lead. The expression of the constitutive protein  $\beta$ -actin is also shown. The bottom shows the densitometric analysis of the Western blot. Results are represented as mean  $\pm$  SEM of five different aortic segments. \*  $P < 0.05$  with respect to control. (B) Bar graph showing cyclic GMP (cGMP) accumulation in the vascular wall of control and 1 ppm lead-incubated rat aortic segments before and after the addition of the nitric oxide donor, sodium nitropruside (SNP,  $10^{-4}$  mol/L). Results are represented as mean  $\pm$  SEM of four different aortic segments. \*  $P < 0.05$  with respect to each corresponding control aortic segment.

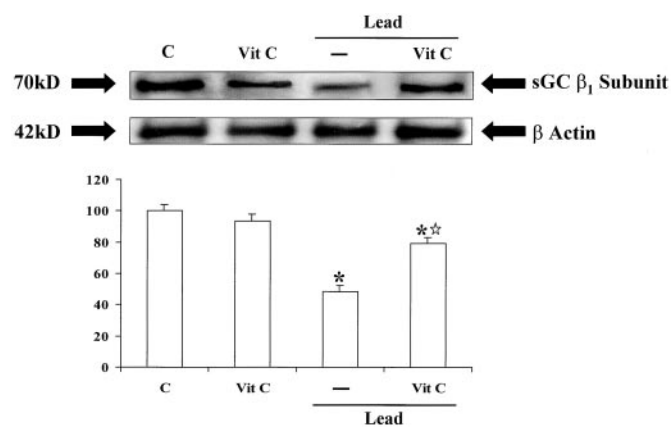
The decreased expression of sGC- $\beta_1$  subunit observed in the vascular wall after lead exposure was associated with a reduced content of cGMP both under basal conditions and after stimulation with an exogenous nitric oxide donor,  $10^{-4}$  mol/L SNP (Figure 1B).

#### sGC expression, Vitamin C, and Superoxide Anion

In a previous work, we demonstrated *in vivo* that antioxidant therapy with vitamin C inhibited the downexpression of sGC- $\beta$  subunit protein elicited by lead administration and restored NO-dependent vascular relaxation (10). We thus analyzed *in vitro* whether vitamin C modified sGC expression in lead-incubated aortic segments.

Vitamin C (30  $\mu$ mol/L) partially although significantly protected sGC- $\beta_1$  subunit expression in 1 ppm lead-exposed aortic segments; however, the expression of sGC- $\beta_1$  subunit remained reduced with respect to that detected in control aortic segments (Figure 2). Vitamin C concentrations greater than 30  $\mu$ mol/L failed to preserve sGC- $\beta_1$  subunit expression to a higher magnitude than that observed with 30  $\mu$ mol/L vitamin C (data not shown). In the absence of lead, sGC- $\beta_1$  subunit expression was not significantly modified by the addition of 30  $\mu$ mol/L vitamin C (Figure 2).

Incubation of the rat aortic segments with lead stimulated the release of superoxide anion (control,  $3.15 \pm 0.84 \mu$ g  $O_2^-$ /mg



**Figure 2.** Representative Western blot showing sGC  $\beta_1$ -subunit expression in isolated rat aortic segments exposed to 1 ppm lead for 24 h in the presence and in the absence of vitamin C (30  $\mu$ mol/L). Similar experiments were performed in isolated non-lead-exposed aortic segments. The bottom shows the densitometric analysis of the Western blot. Results are represented as mean  $\pm$  SEM of six different experiments. \*  $P < 0.05$  with respect to control. \*  $P < 0.05$  with respect to lead exposure alone.

protein; lead,  $16.87 \pm 1.53 \mu$ g  $O_2^-$ /mg protein;  $P < 0.05$ ). Vitamin C (30  $\mu$ mol/L) reduced the amount of superoxide anion released from lead-exposed rat aortic segments ( $8.03 \pm 1.12 \mu$ g  $O_2^-$ /mg protein;  $P < 0.05$  with respect to lead-exposed aortic segments).

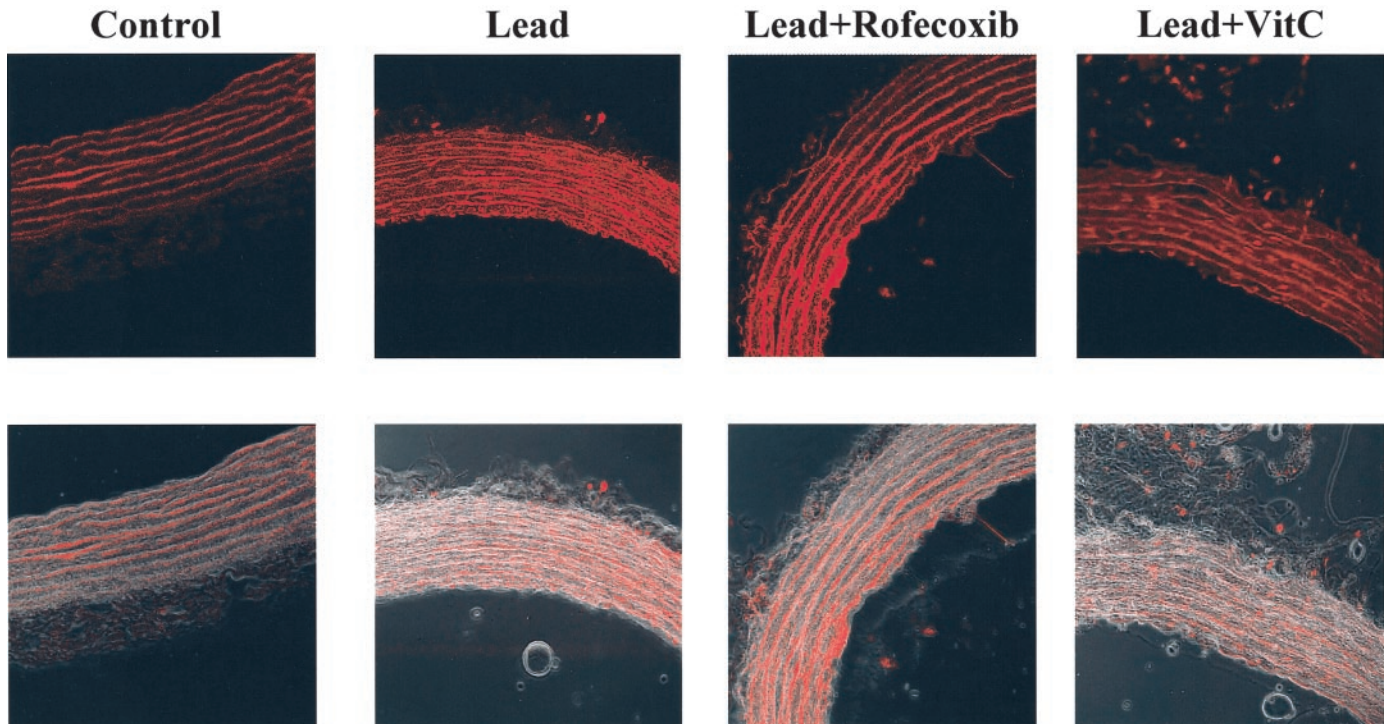
Confocal microscopy experiments confirmed these findings. A greater superoxide anion content was observed in the vascular wall of the segments incubated in the presence of 1 ppm lead when compared with control rings (Figure 3). Moreover, the addition of vitamin C reduced the superoxide anion content in the vascular segments exposed to 1 ppm lead (Figure 3).

The expression of gp<sup>91</sup>phox-containing NADPH oxidase, a NADPH oxidase subtype considered as one of the major vascular superoxide anion sources, was not different between control and lead-exposed aortic segments (Figure 4). Moreover, vitamin C failed to modify gp<sup>91</sup>phox-containing NADPH expression in lead-incubated aortic segments (Figure 4).

#### Relationship among sGC, COX-2 Expression, and Superoxide Anion Production

A protein kinase A inhibitor, H-89 (1  $\mu$ mol/L), partially restored the downexpression of sGC- $\beta_1$  subunit induced by lead exposure of the aortic segments (Figure 5). Moreover, lead-exposed aortic segments showed a marked upexpression of COX-2 protein, an effect that was prevented by 30  $\mu$ mol/L vitamin C (Figure 6). Rofecoxib (1  $\mu$ mol/L), an inhibitor of COX-2 activity, partially protected sGC- $\beta_1$  subunit expression in lead-incubated aortic segments (Figure 5). However, rofecoxib did not significantly modify the increased release of superoxide anion induced by lead exposure of the aortic segments ( $15.84 \pm 2.21 \mu$ g  $O_2^-$ /mg protein;  $P = NS$  with respect to lead-exposed aortic segments). This was further confirmed in confocal microscopic sections of lead + rofecoxib-incubated aortic segments labeled with the oxidative dye DHE in





**Figure 3.** *In situ* detection of superoxide anion in rat aorta. Fluorescent photomicrographs of confocal microscopic sections of the aorta labeled with the oxidative dye DHE (red fluorescence when oxidized to ethidium by superoxide anion). Isolated aortic segments were incubated in the absence and in the presence of 1 ppm lead for 24 h. Additional experiments were performed in lead-exposed aortic segments in the presence of vitamin C (30  $\mu\text{mol/L}$ ) and rofecoxib (1  $\mu\text{mol/L}$ ). In the upper panels are shown the immunofluorescence picture, whereas the bottom panels show the combination of the immunofluorescence pictures with the optical microscopy photomicrographs.

which we did not observe any significant differences in the content of superoxide anion with respect to aortic segments exposed to lead alone (Figure 3).

## Discussion

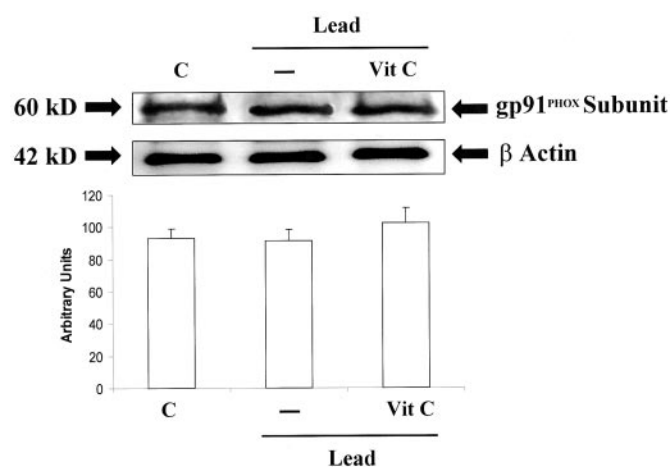
In this study, we have shown that (1) lead exposure decreased sGC  $\beta_1$ -subunit protein expression in *in vitro* incubated rat aortic segments; (2) this effect was associated with an increased content of superoxide anion in the vascular wall after lead exposure, and it was prevented by the use of an antioxidant agent such as vitamin C; (3) lead-exposure upregulated COX-2 expression which was prevented by vitamin C; and (4) COX-2 activity was involved in the downregulation of sGC- $\beta_1$  subunit elicited by lead exposure.

Lead-induced hypertension is the element of chronic lead intoxication that has received more attention in the literature. Different considerations have been raised to explain the pathogenesis of lead-induced hypertension. In this regard, several studies have focused on the ability of the endothelium to generate NO in this model of hypertension. These studies demonstrated the existence of an altered NO synthesis, probably because of a diminished eNOS activity and/or increased NO catabolism by oxygen free radicals (3,5). However, we recently demonstrated that, independently of the NO-generating system, lead administration downregulated sGC, the main receptor of NO, in the vascular wall, an effect that was prevented by the co-administration of the antioxidant vitamin C,

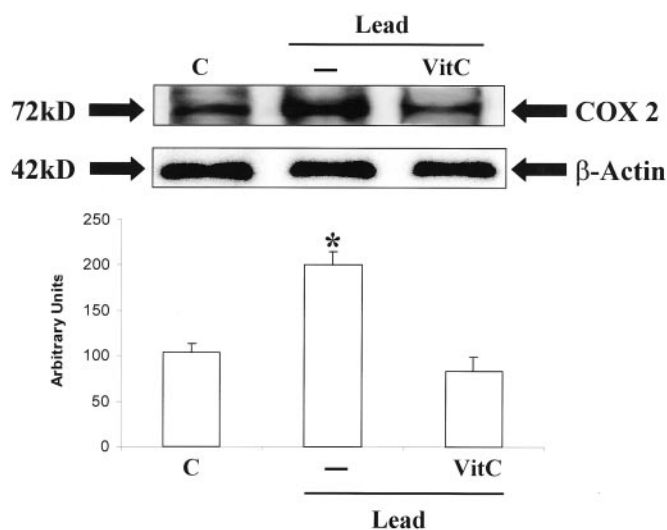
suggesting the involvement of reactive oxygen species (10). In the present study, we have confirmed *in vitro* that lead induces a downexpression of sGC  $\beta_1$ -subunit in the vascular wall. In this regard, it is well established that the presence of both  $\alpha_1$  and  $\beta_1$  subunits is required for sGC activity and the reduction in one of these subunits is enough to decrease the ability of sGC to generate cGMP in response to NO (9). In the same line of evidence, lead-exposed aortic segments showed a reduced content of cGMP under basal conditions and after stimulation with the exogenous nitric oxide donor, sodium nitroprusside.

The fact that vitamin C protected sGC  $\beta_1$ -subunit expression in lead-exposed aortic segments suggested the involvement of ROS in the modulation of sGC expression. In our experiments, lead exposure stimulated the ability of the vascular wall to release superoxide anion, which is in accordance with previous works from Vaziri *et al.* (3) and Ding *et al.* (18,24), which demonstrated an increased lipid peroxidation and enhanced hydroxyl radical generation in rats and cultured endothelial cells after lead exposure. In this regard, ROS stimulation by lead has been recently associated with inactivation of nitric oxide, which was correlated with a compensatory up-expression of endothelial nitric oxide synthase in rat aortic segments and cultured human coronary endothelial cells (19,25).

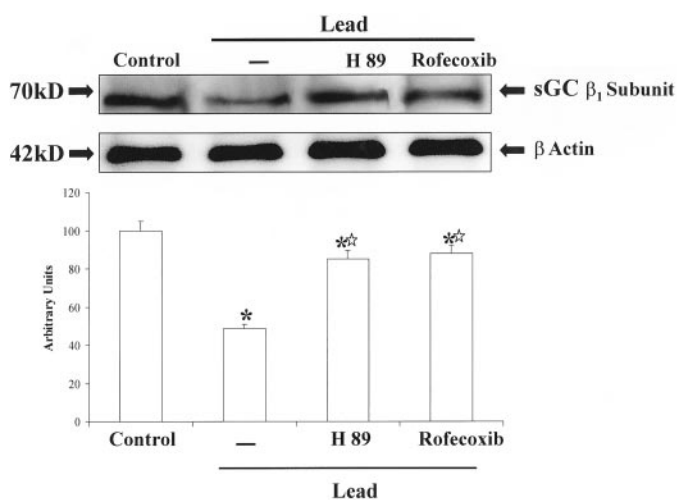
It has been recently postulated that gp<sup>91</sup>phox-containing NADH oxidase is one of the main sources of superoxide anion in the arterial wall (26). However, the increased ability of the arterial wall to release superoxide anion after lead exposure



**Figure 4.** Representative Western blot showing the expression of gp<sup>91</sup>phox-containing NADPH in control, 1 ppm lead-exposed, and 30 μmol/L vitamin C + 1 ppm lead-exposed isolated aortic segments. The bottom shows the densitometric analysis of the Western blot. The Western blot was performed by using a goat polyclonal antibody against the carboxy terminal of gp<sup>91</sup>phox of human origin (1:500; Santa Cruz Biotechnology Inc.). The immunoblot shows a typical band for gp<sup>91</sup>phox at ≈ 60 kDa. The gp<sup>91</sup>phox refers to the molecular weight in human tissues where the subunit is heavily glycosylated. Results are represented as mean ± SEM of five different experiments.



**Figure 6.** Representative Western blot showing the expression of the COX-2 isoform in control, lead (1 ppm)-exposed and 1 ppm lead + 30 μmol/L vitamin C-exposed isolated aortic segments. Results are represented as mean ± SEM of five different experiments. \*  $P < 0.05$  with respect to control.



**Figure 5.** Representative Western blot showing the expression of the sGC β<sub>1</sub> subunit in control and 24-h lead (1 ppm)-exposed aortic segments. Additional experiments were performed in 1 ppm lead-exposed aortic segments in the presence of the protein kinase A inhibitor, H89 (1 μmol/L), and an inhibitor of COX-2 activity, rofecoxib (1 μmol/L). Results are represented as mean ± SEM of five different experiments. \*  $P < 0.05$  with respect to control; ☆  $P < 0.05$  with respect to lead exposure alone.

was not associated with an increased expression of gp<sup>91</sup>phox-containing NADH oxidase. The present experimental design did not allow us to determine whether lead stimulated the activity of gp<sup>91</sup>phox-containing NADH and/or modified the

expression of other enzymes related with superoxide anion generation in the arterial wall such as nox-1 (27). Further investigations are needed to elucidate these issues.

Cyclic AMP is a second messenger of the products of COX-2 pathway (28). Interestingly, attenuation of sGC expression has been recently demonstrated to be induced by cyclic AMP-elevating mediators (13,29). In this regard, we observed that lead exposure not only reduced the expression of sGC-β<sub>1</sub> subunit but also increased the expression of the COX-2 isoform. Moreover, lead-induced up-expression of COX-2 protein was associated with the downregulation of sGC-β<sub>1</sub> subunit expression because rofecoxib, an inhibitor of COX-2 activity, attenuated the reduction of sGC-β<sub>1</sub> subunit expression. This was further supported by the fact that an inhibitor of protein kinase A activity, H-89 (17), also attenuated the down-expression of sGC-β<sub>1</sub> subunit to a similar level as rofecoxib.

The question then raised was if COX-2 enhanced superoxide anion production by lead or the increased superoxide anion generation induced by lead stimulated the expression of COX-2 protein. In our experiments, while the addition of vitamin C prevented lead-induced COX-2 expression, rofecoxib failed to modify the enhanced superoxide anion production induced by lead exposure of the vascular wall, suggesting that superoxide anion was involved in the upregulation of COX-2 expression elicited by lead. In this regard, increased ROS activity has been demonstrated to exert an upregulation of COX-2 expression in luteal cells (30). Moreover, Fang *et al.* (31) have demonstrated that transfection of catalase-encoding cDNA induces the transcriptional activation of the COX-2 gene and increases mRNA stability, thus supporting that peroxides upregulate COX-2 expression.

It is noteworthy that both vitamin C and rofecoxib only partially restored the downexpression of the sGC β<sub>1</sub> subunit by

lead, suggesting that other signaling pathways stimulated by lead and/or lead itself may be involved in the downregulation of the sGC- $\beta_1$  subunit expression in the vascular wall. In this regard, it has been demonstrated that heavy metals, including lead, may modulate the expression of several other proteins (32,33). Further experiments are warranted to test these hypotheses.

In conclusion, our data have shown for the first time the involvement of ROS via COX-2 expression in the downregulation of sGC- $\beta_1$  subunit expression induced by lead in the rat vascular wall. These results suggest a role for ROS and COX-2 in the pathogenesis of lead-induced hypertension.

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