Elsevier Editorial System(tm) for Toxicology and Applied Pharmacology
Manuscript Draft

Manuscript Number: TAAP-D-14-00992R1

Title: MAPK pathway activation by chronic lead-exposure increases vascular reactivity through oxidative stress/cyclooxygenase-2-dependent pathways

Article Type: Full Length Article

Keywords: Lead exposure; MAPK pathway; oxidative stress; cyclooxygenase-2; vascular reactivity; blood pressure.

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Abstract: Chronic exposure to low lead concentration produces hypertension; however, the underlying mechanisms remain unclear. We analyzed the role of oxidative stress, cyclooxygenase-2-dependent pathways and MAPK in the vascular alterations induced by chronic lead exposure. Aortas from lead-treated Wistar rats (1st dose: 10 μg/100 g; subsequent doses: 0.125 μg/100 g, intramuscular, 30 days) and cultured aortic vascular smooth muscle cells (VSMCs) from Sprague Dawley rats stimulated with lead (20 μg/dL) were used. Lead blood levels of treated rats attained 21.7 ± 2.38 μg/dL. Lead exposure increased systolic blood pressure and aortic ring contractile response to phenylephrine, reduced acetylcholine-induced relaxation and did not affect sodium nitroprusside relaxation. Endothelium removal and L-NAME left-shifted the response to phenylephrine more in untreated than in lead-treated rats. Apocynin and indomethacin decreased more the response to phenylephrine in treated than in untreated rats. Aortic protein expression of gp91(phox), Cu/Zn-SOD, Mn-SOD and COX-2 increased after lead exposure. In cultured VSMCs lead 1) increased superoxide anion production, NADPH oxidase activity and gene and/or protein levels of NOX-1, NOX-4, Mn-SOD, EC-SOD and COX-2 and 2) activated ERK1/2 and p38 MAPK. Both antioxidants and COX-2 inhibitors normalized superoxide anion production, NADPH oxidase activity and mRNA levels of NOX-1, NOX-4 and COX-2. Blockade of the ERK1/2 and p38 signaling pathways abolished lead-induced NOX-1, NOX-4 and COX-2 expression. Results show that lead activation of the MAPK signaling pathways activates inflammatory proteins such as NADPH oxidase and COX-2, suggesting a reciprocal interplay and contribution to vascular dysfunction as an underlying mechanisms for lead-induced hypertension.

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Vitória, October 05, 2014.

The Editorial Office

Toxicology and Applied Pharmacology

Dr. Yongxiao Cao

Dear Editor.

I hereby submit for publication in Toxicology and Applied Pharmacology the manuscript "MAPK pathway activation by chronic lead-exposure increases vascular reactivity through oxidative stress/cyclooxygenase-2-dependent pathways".

This study showed that low lead concentration after 30 days of exposure attained a blood content of 21,7 μg/dL. This value, which is much lower than the reference value (60 μg/dL) considered being the limit exposure in humans occupationally exposed, was sufficient to increase systolic blood pressure and phenylephrine-induced contractility and to decrease endothelium-dependent vasodilator responses in rat aortas. Our results also suggest that p38 and ERK1/2 MAPK are involved in the increase in vascular COX-2 levels and ROS production after lead exposure that act in concert to produce the vascular changes that could contribute to the occurrence of arterial hypertension. Therefore, our results suggest the need of a revision of the blood values of lead content considered to be safe in humans.

Please reply to the following address:

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Sincerely Yours
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***Detailed Response to Reviewers**

Dr. Yongxiao Cao, Ph.D
Associate Editor
Toxicology and Applied Pharmacology

Dear Dr Cao,

It was a pleasure to learn that our manuscript entitled “MAPK pathway activation by chronic lead-exposure increases vascular reactivity through oxidative stress/cyclooxygenase-2-dependent pathways” (**TAAP-D-14-00992**) by Simões et al. was found interesting although a revision was required. The manuscript has been revised taking the reviewers’ comments into account. We have included in Figure 5 new data about protein expression of EC-SOD, as suggested by Reviewer 1. Some little mistakes now observed in other figures and in the text have been corrected.

We are very grateful to the reviewers and believe that the manuscript has been improved by considering their constructive comments. We hope that the new version of the manuscript fulfils all your requirements for its publication in Toxicology and Applied Pharmacology. Nevertheless, if anything is not clear, please do not hesitate to contact us.

Sincerely yours,

Maylla Ronacher Simões

Dr. Mercedes Salaices

Answer to Reviewers' comments:

Reviewer #1: This article examines the effect of chronic lead exposure on vascular smooth muscle cell (VSMC) function and hypertension. The authors showed that treating Wistar and Sprague-Dawley rats contributes to vascular dysfunction by enhancing vasoconstriction in response to phenylephrine treatment, impairing the acetylcholine-induced vasodilator response, and increasing systolic blood pressure. According to the findings, lead increases the generation of superoxide production via MAPK activity and downstream activation of pro-oxidant enzymes such as NADPH and COX-2. While this paper contains several strengths, including the identification of a molecular mechanism behind lead-induced hypertension, there are several concerns that need to be addressed:

- The use of two different rat strains was puzzling. While spontaneously hypertensive rats were developed from the Wistar strain, the article did not specify that the Wistars in question were "spontaneously hypertensive." Therefore, it would make more sense to use either SD rats or Wistars but not both.

Answer: **Thank you for the suggestion because this item deserves an explanation. One of our goals was to define if Pb could induce an increase in arterial pressure. Then, we used Wistar rats that were not hypertensive. We have a great experience using this rat strain to study the effects of other metals like mercury and cadmium on vascular reactivity experiments. You are right that it would make more sense to use the same rat strain for all the experiments. The use of Sprague Dawley rats to obtain VSMC for culture was related to the fact that they were available at the time of the experiments. We did that to avoid using a large number of rats because the SDs were being used as controls for other experiments and the aortas were available.**

- The authors looked at the expression of Cu/Zn-SOD and Mn-SOD but not EC-SOD. Considering that EC-SOD expression is extremely high in VSMCs, it seems likely that EC-SOD would be upregulated in response to increased superoxide anion production as well.

Answer. **We agree with the referee, EC-SOD is a major extracellular antioxidant enzyme highly expressed in the vasculature. Then it was possible that EC-SOD would be upregulated in response to lead as occur with Cu/Zn-SOD and Mn-SOD. In addition, in a previous work we have found that EC-SOD was increased in VSMC in response mercury (Aguado**

et al., 2012). Thus, new measurements made in VSMC exposed to lead show that EC-SOD protein expression was increased. These new results have been included in the Fig 5 and in the manuscript.
(Abstract Page 3 line 18; Materials and methods – Page 13, line 18; Results – page 20, lines 13-14 and Discussion - Page 25, line 9 to 15).

- In the discussion, the authors mentioned that NO bioavailability is reduced by the production of ROS. Traditionally, this would indicate the formation of peroxynitrite (-ONOO) from free NO, however, the authors did not have a method for indicating that this was occurring (for example, western blotting for nitrotyrosine on VSMC proteins).

Answer: This is an important issue. We apologize for not reinforce this aspect in the manuscript. As the referee points NO react with superoxide anions forming peroxynitrite, thereby promoting decrease in NO bioavailability but also nitrosative stress that contributes to the endothelial dysfunction. For example, ONOO- caused PGIS nitration and TxA2 activation in several diseased conditions such as atherosclerotic vessels, hypoxia-reperfusion injury, cytokines-treated cells, diabetes, as well as hypertension (Zhou, 2007). We have tried to measure peroxynitrite formation by western blotting for nitrotyrosine on VSMC proteins. Unfortunately the available antibodies in our laboratory were nonselective and therefore we could not obtain reliable data of the formation of peroxynitrite. In spite of this, a paragraph about the role that peroxinitrite might have in the altered vascular responses after lead exposure was added to the discussion section and is highlighted in yellow. *(Page 24, lines 17 to 22).*

Reviewer #2: This is a well-conducted study an important topic.

The authors mention that the blood lead levels in the chronically treated rats was 1.7 +/- 2.38 micrograms/dL. How does this compare to blood lead levels in humans, and what types of effects are detected at this blood lead level in humans?

Answer: This is an important comment. The effects of lead on human health depend on blood levels and on the duration of the exposure. Several studies have supported the association between high blood lead levels and hypertension in humans (Andrzejak et al., 2004; Patrick, 2006; Kosnett et al., 2007). The Agency for Toxic Substances and Disease Registry (ATSDR) considered the reference blood lead concentration level to be 60 µg/dL, and concentrations that exceed these values require

removal from lead exposure (Patrick, 2006; Kosnett et al., 2007). Nevertheless, individuals with baseline blood lead levels of 46.8 µg/dL or 67.8 µg/dL have also shown increases in arterial pressure (Andrzejak et al., 2004; Malvezzi et al., 2001) suggesting that even lower than recommended maximal doses might have cardiovascular deleterious consequences. In support of this, a similar association was also reported in treated rats with lead blood concentrations between 31.8 µg/dL and 42,5 µg/dL (Gonick et al., 1997; Marques et al., 2001). Moreover, in the present study, treated rats attained a blood lead concentration of 21.7 ± 2.38 µg/dL that increased systolic blood pressure. A paragraph was added to the discussion section and is highlighted in yellow. *(Page 22, lines 13 to 24 and page 23, lines 1 to 2).*

Highlights

- Lead-exposure increases oxidative stress, COX-2 expression and vascular reactivity.
- Lead exposure activates MAPK signaling pathway.
- ROS and COX-2 activation by MAPK in lead exposure.
- Relationship between vascular ROS and COX-2 products in lead exposure.

MAPK pathway activation by chronic lead-exposure increases vascular reactivity through oxidative stress/cyclooxygenase-2-dependent pathways

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Abstract

Chronic exposure to low lead concentration produces hypertension; however, the underlying mechanisms remain unclear. We analyzed the role of oxidative stress, cyclooxygenase-2-dependent pathways and MAPK in the vascular alterations induced by chronic lead exposure. Aortas from lead-treated Wistar rats (1st dose: 10 µg/100 g; subsequent doses: 0.125 µg/100 g, intramuscular, 30 days) and cultured aortic vascular smooth muscle cells (VSMCs) from Sprague Dawley rats stimulated with lead (20 µg/dL) were used. Lead blood levels of treated rats attained 21.7 ± 2.38 µg/dL. Lead exposure increased systolic blood pressure and aortic ring contractile response to phenylephrine, reduced acetylcholine-induced relaxation and did not affect sodium nitroprusside relaxation. Endothelium removal and L-NAME left-shifted the response to phenylephrine more in untreated than in lead-treated rats. Apocynin and indomethacin decreased more the response to phenylephrine in treated than in untreated rats. Aortic protein expression of gp91(phox), Cu/Zn-SOD, Mn-SOD and COX-2 increased after lead exposure. In cultured VSMCs lead 1) increased superoxide anion production, NADPH oxidase activity and gene and/or protein levels of NOX-1, NOX-4, Mn-SOD, EC-SOD and COX-2 and 2) activated ERK1/2 and p38 MAPK. Both antioxidants and COX-2 inhibitors normalized superoxide anion production, NADPH oxidase activity and mRNA levels of NOX-1, NOX-4 and COX-2. Blockade of the ERK1/2 and p38 signaling pathways abolished lead-induced NOX-1, NOX-4 and COX-2 expression. Results show that lead activation of the MAPK signaling pathways activates inflammatory proteins such as NADPH oxidase and COX-2, suggesting a

reciprocal interplay and contribution to vascular dysfunction as an underlying mechanisms for lead-induced hypertension.

Keywords: lead exposure; MAPK pathway; oxidative stress; cyclooxygenase-2; vascular reactivity; blood pressure.

Introduction

Lead is an environmental pollutant that has emerged as a potential risk factor related to the development of cardiovascular complications. It has been extensively reported that chronic exposure to low levels of lead causes hypertension (Gonick et al., 1997; Vaziri et al., 1997; Vaziri et al., 1999). Several mechanisms have been implicated in lead-induced hypertension. Thus, alterations of the muscular and endothelial layers of blood vessels induced by direct interaction with lead causes disturbances in the renin-angiotensin-aldosterone system, stimulation of the sympathetic system and an excessive synthesis of reactive oxygen species (ROS) (Zawadzki et al., 2006). Moreover, alterations in calcium exchangeability (Goldstein, 1993), inhibition of the Na^+/K^+-ATPase (Weiler et al., 1990), direct activation of smooth muscle protein kinase C (Watts et al., 1995) and endothelial dysfunction (Silveira et al., 2014) have also been reported.

It has been demonstrated that alterations in vascular tone are possibly involved in lead-induced hypertension (Marques et al., 2001). ROS have a key role in the pathogenesis of cardiovascular disease, which results in the disturbance of the structure of biological cell membranes in many organs within an organism, the impairment of cellular function, alterations of NO synthase activity and increased concentrations of inflammatory markers (Cai and Harrison, 2000; Elahi et al., 2009). COX-2, a pro-inflammatory enzyme, has been described as a major source of free radicals (Virdis et al., 2013, Martínez-Revelles et al., 2013). It has been established that COX-2-derived prostanoids contribute to the endothelial dysfunction in hypertensive animals (Wong et al., 2010). A recent study by our group demonstrated that low levels of blood lead increased

vascular reactivity. This increase was associated with reduced NO bioavailability, increased ROS, increased participation of COX-2-derivated prostanoids and increased renin-angiotensin system activity (Silveira et al., 2014).

Mitogen-activated protein kinases (MAPK) are a family of enzymes that comprise global groups of signaling proteins that play critical regulatory roles in cell physiology (Chang et al., 2001; Chen et al., 2001). The activation of extracellular signal-regulated protein kinases (ERK1/2) is mainly associated with cell survival and proliferation (Hetman et al., 2004), while c-Jun N-terminal kinases (JNK) and p38 MAPK cascades are associated with the promotion of inflammation and programmed cell death (Tibbles et al., 1999; Chen et al., 2001). Some studies have linked the prostanoid pathway to the activation of the MAPK signaling cascade and to the induction of oxidative stress (Chen et al., 2005; Kim et al., 2005). In addition, MAPK activation by heavy metals may modulate mechanisms that induce oxidative stress (Leonard et al., 2004). Despite the extensive documentation of the toxic effects of lead in the cardiovascular system, a complete and detailed elucidation of the cell target and mechanisms by which lead exerts its effects remains to be defined. This study investigates the role of oxidative stress and cyclooxygenase-2 in the altered vascular reactivity induced by a 30-day treatment with a low concentration of lead. In addition, we analyze the ability of lead to activate ERK1/2, p38MAPK and JNK1/2 in VSMCs and its implication on the oxidative stress and cyclooxygenase-2 pathways involved in vascular alterations induced by lead.

Materials and methods

Ethics statement and animals

Male Wistar (250-300 g) and Sprague Dawley (SD) (4 months old) rats were used. All experimental procedures were approved by the Institutional Ethics Committee of the Federal University of Espirito Santo (CEUA-UFES 063/2011) and by the Ethical Committee of Research of the Universidad Autónoma de Madrid, Spain (CEI-UAM 31-759). This study was carried out in strict accordance with the guidelines for biomedical research as stated by the Brazilian Societies of Experimental Biology, the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH publication No. 85-23, revised 1996) and with the current Spanish and European laws (RD 223/88 Ministerio de Administraciones Públicas, MAPA, and 609/86).

Rats were housed under a 12-h light /12-h dark cycle, had free access to water and were fed with rat chow *ad libitum*. In one set of experiments, Wistar rats were divided into two groups: control (vehicle-saline, intramuscular) or treated with lead acetate for 30 days (1st dose: 10 μg/100 g; subsequent doses: 0.125 μg/100 g, intramuscular, to cover daily loss). At the end of the treatment, rats were anesthetized and killed by exsanguination. The thoracic aortas were carefully dissected out, and the fat and connective tissue removed. For the vascular reactivity experiments, the aortas were divided into cylindrical segments (4 mm in length). In another set of experiments, thoracic aortas from SD rats were isolated and processed to obtain primary cultures of smooth muscle cells (VSMCs) for cell culture experiments.

Blood pressure measurements

Indirect systolic blood pressure was measured at the beginning and at the end of treatment using tail-cuff plethysmography (IITC Life Science, Inc.). Conscious rats were restrained for 5–10 min in a warm and quiet room and conditioned to numerous cuff inflation-deflation cycles by a trained operator. Systolic blood pressure was measured, and the mean of ten measurements was recorded.

Blood lead level measurements

Blood lead levels were measured by inductively coupled plasma mass spectrometry (NexIon 300D, PerkinElmer, USA) after acid decomposition of the whole blood samples as previously described (Liu et al., 1996). The samples were measured in triplicate.

Vascular function

Vascular function was studied in aortic segments by isometric tension recording using an isometric force transducer (TSD 125C, CA, USA) connected to an acquisition system (Biopac Systems, Inc., Santa Barbara, USA). Segments were mounted between two parallel wires in organ baths containing Krebs–Henseleit solution (KHS, in mM: 124 NaCl, 4.6 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 0.01 EDTA, 23 $NaHCO_3$, 11.1 glucose) at 37 C and gassed with 95% O_2–5% CO_2 (pH 7.4). After a 45-min equilibration period, aortic rings were initially exposed twice to 75 mM KCl to test their functional integrity and to assess the maximal developed tension. The presence of endothelium was confirmed by the effect of 10 µM acetylcholine in segments previously contracted with 1 µM phenylephrine. A relaxation equal to or greater than 90%

was considered demonstrative of the functional integrity of the endothelium. After a washout period, single concentration-response curves to phenylephrine (0.1 nM - 0.3 mM) or acetylcholine (ACh, 0.01 nM - 0.3 mM) were performed. In some experiments, concentration–response curves to sodium nitroprusside (SNP, 0.01 nM–0.3 mM) were performed in segments contracted with phenylephrine (1 μM).

Parallel experiments with aortic segments from the same animal were performed in the absence (control) and presence of the nonspecific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), the cyclooxygenase inhibitor indomethacin (10 μM) and the NADPH oxidase inhibitor apocynin (30 μM). These drugs were added to the bath 30 min before the phenylephrine curves. The influence of the endothelium on the response to phenylephrine was investigated after mechanical removal of this vascular component by rubbing the lumen with a needle. The absence of endothelium was confirmed by the inability of 10 μM acetylcholine to produce relaxation.

In situ detection of vascular $O_2^{\bullet-}$ production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate $O_2^{\bullet-}$ production in situ, as previously described (Wiggers et al., 2008). Hydroethidine freely permeates the cell membrane and is oxidized in the presence of $O_2^{\bullet-}$ to ethidium bromide, which is trapped by intercalation with DNA. Ethidium bromide is excited at 546 nm and has an emission spectrum of 610 nm. Frozen tissue segments were cut into 10-μm-thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37 C in Krebs–HEPES buffer (in mM: 130 NaCl, 5.6 KCl, 2 $CaCl_2$, 0.24 $MgCl_2$, 8.3

HEPES, and 11.1 glucose, pH = 7.4). Fresh buffer containing DHE (2 μM) was applied topically to each tissue section, covered with a cover slip, incubated for 30 min in a light-protected humidified chamber at 37 C and then viewed with an inverted fluorescence microscope (NIKON Eclipse Ti-S, x40 objective; N.Y., U.S.A.) using the same imaging settings in the untreated and lead-treated rats. Fluorescence was detected with a 568-nm long-pass filter. For quantification, eight frozen tissue segments per animal were sampled for each experimental condition and averaged. The mean fluorescence densities in the target region were calculated.

Cell culture

Primary cultures of VSMCs were obtained from SD rats as previously described (Aguado et al., 2013). Rat thoracic aortas were aseptically removed, cleaned of fat tissue and blood cells and placed in cold (4ºC) Dulbecco´s Modified Eagle´s Medium (DMEM)/F-12 (HAM) medium (Sigma Chemical Co., St. Louis, MO, USA) containing 0.1% BSA, 200 U/ml penicillin, and 200 μg/ml streptomycin (Gibco, Invitrogen, Paisley, UK). The aortas were digested in the same medium containing 2 mg/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, New Jersey, USA) and incubated for 30 min at 37ºC in a humidified atmosphere of CO_2 (5%). Then, after peeling off the adventitia using forceps, the medial smooth muscle layer was cut into small pieces and placed on 60 x 15-mm tissue dishes in DMEM/F-12 (HAM) medium supplemented with 10% fetal calf serum (Biological Industries, Kibbutz, Israel) containing 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were allowed to reach confluence (10-12 days). Confluent cells were trypsinized with PBS/trypsin-EDTA (Sigma),

washed and plated at a density of 30% in DMEM medium. Cells were identified as smooth cells by immunocytochemical staining with specific monoclonal anti-α-actin antibody (Sigma Chemical Co). We should emphasize that the cells employed here were the product of primary culture of VSMCs studied after only 5 passes to avoid transformation.

VSMCs were plated onto 6-well plates and cultured until 80% confluence. DMEM/F-12 (HAM) medium supplemented with serum was replaced with fresh medium containing 100 U/mL penicillin and 100 µg/mL of streptomycin for 24 h before treatment. For the experiments, quiescent cells were incubated with vehicle (control) or with lead acetate (20 µg/dL, during 48 h) in the absence or presence (30 min before and throughout lead incubation) of the ROS scavenger tempol (10 µM), the NOX-1 inhibitor ML 171 (0.5 µM), the specific scavenger of mitochondrial superoxide mito-TEMPO (5 µM) or the COX-2 inhibitors celecoxib (10 µM) and rofecoxib (10 µM). In another set of experiments, cells were incubated with lead acetate for different stimulation times (5 min-24 h) to evaluate the effect on the activation of p38, ERK1/2, JNKs MAPK and Akt. Finally, the effects of the ERK1/2 inhibitor U0126 (10 µM) and the p38 MAPK inhibitor SB203580 (10 µM) on COX-2, NOX-1 and NOX-4 mRNA levels were evaluated in cells exposed to 20 µg/dL lead acetate for 48 h.

Cell Viability Assay

We used the MTT reduction assay following the procedure previously described (Mosmann, 1983). In brief, after incubation with lead acetate at different concentrations, 0.5 mg/ml MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was added to each well, and incubation was

performed at 37 °C for 2 h. The formazan salt formed was dissolved in DMSO, and colorimetric determination was performed at 540 nm. Control cells without lead were considered to have 100% viability. VCMS viability after exposure to lead was expressed as the percent of control within each individual experiment.

Detection of superoxide anion production by fluorescence microscopy

VSMCs were incubated with lead for 48 h, and intracellular $O_2^{\bullet-}$ production was measured by fluorescence imaging analysis using DHE as described above. Briefly, VSMCs were plated onto glass coverslips inserted into 6-well plates and cultured and preincubated with tempol (10 µM) and mito-TEMPO (5 µM) 30 min before lead exposure. Afterwards, cells were loaded with DHE (10 µM) in cell culture medium (DMEM/F-12 HAM, serum free) for 30 min at 37ºC. Images were then acquired with a Leica TCS SP2 confocal system (x40) and processed using Metamorph image analysis software. Non-stimulated VSMCs were imaged daily in parallel using the same image settings during the course of the study. DHE fluorescence was quantified in individual cell nuclei (10-20 nuclei/ image/experimental condition). At least 5 independent experiments were performed. Then, we expressed the effects of the different drugs as fold increases over the control.

NADPH oxidase activity

Cells were grown on 6-well culture plates and incubated with lead (20 µg/dL, during 48 h). The $O_2^{\bullet-}$ production generated by NADPH oxidase activity was determined by a chemiluminescence assay using lucigenin (5 µM) and NADPH

(100 µM). The reaction was started by the addition of a mixture of lucigenin and NADPH to the protein sample in a final volume of 250 µL. Chemiluminescence was determined every 2.4 seconds for 5 min in a plate luminometer (AUTO-Lumat LB953, Berthold Technologies GmbH & Co. KG, Bad Wildbadzz Germany). Buffer blank was subtracted from each reading. Luminescence was normalized by protein concentration measured by the Lowry assay and data were expressed as fold increase over the control.

Western blot analysis

Proteins from homogenized aortas and cells were separated on a 10% SDS-polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham, GE Healthcare, Buckinghamshire, UK) that were incubated overnight at 4º C with monoclonal antibodies for COX-2 (1:200; Cayman Chemical, Ann Arbor, MI, USA), COX-1 (1:500, Cayman Chemical), and gp91(phox) (1:1000, Transduction Laboratories, Lexington, UK) and polyclonal antibodies for Mn-SOD (0.025 µg/ml; StressGen Bioreagent Corp., Victoria, Canada), Cu/Zn-SOD (1:10000; Nventa Biopharmaceuticals, Victoria, BC, Canada), EC-SOD (1:4000; Enzo life Science), phospho ERK1/2 and ERK1/2, phospho p38 and p38 MAPK, phospho JNK and JNK, phospho Akt and Akt (1:1000; Cell Signaling, Boston, MA). Membranes were thoroughly washed and incubated with horseradish peroxidase-coupled anti-rabbit (1:2000; Bio-Rad, USA) or anti-mouse (1:5000; StressGen Bioreagent Corp., Victoria, Canada) antibodies for 1 h at room temperature. After thorough washing, the bands were detected using an ECL plus Western Blotting detection system (GE Healthcare) after exposure to X-Ray AX film (Konica Minolta, Tokyo, Japan and

Hyperfilm ECL International). Signals on the immunoblot were quantified using the Image J computer program. α-Actin (aorta) (1:5000, Sigma Chemical Co) and β-actin (cells) (1:10000; Transduction Laboratories) expressions were used as loading controls.

Quantitative real time PCR assay

Total RNA was obtained using TRI Reagent (Sigma Chemical Co) according to the manufacturer's recommendations and was reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) with random hexamers. qRT-PCR for COX-2, NOX-1 and NOX-4 was performed using the fluorescent dye SyBRGreen (iTaq FAST SyBRGreen Supermix with ROX, *Bio-Rad*, USA). To normalize, we amplified β_2-microglobulin as a housekeeping gene. All qRT-PCRs were performed in duplicate. Primers sequences were as follows: COX-2 (FW: AAGGGAGTCTGGAACATTGTGAAC; RV: CAAATGTGATCTGGACGTCAACA), NOX-1 (FW: CGGCAGAAGGTCGTGATTA; RV: TGGAGCAGAGGTCAGAGT), NOX-4 (FW: GCCTCCATCAAGCCAAGA; RV: CCAGTCATCCAGTAGAGTGTT) and β_2-microglobulin (FW: ACCCTGGTCTTTCTGGTGCTT; RV: TAGCAGTTCAGTATGTTCGGCTT). Quantification was performed on a 7500 Fast (Applied Biosystems). PCR cycles proceeded as follows: initial denaturation for 30 s at 95°C followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. At the end of the PCR, a melting curve analysis was performed to show the specificity of the product detected. To calculate the relative index of gene expression, we employed the $2^{-\Delta\Delta Ct}$ method using untreated samples as a calibrator (Livak et al., 2001).

In vitro wound healing assay

To verify if lead induced cell migration, a wound healing assay was performed. For this assay, cells were seeded and cultured to confluence in a 24-well plate. Then, the cells were switched to serum-free medium for 24 h before the initiation of the experiments. A wound was made with a P10 pipette tip (CRP, with a filter). The medium was changed twice (5 mL/well) to wash away any cell debris remaining in the wound area. A line was drawn through the center of the wells, perpendicular to the wound. A picture was taken at time zero at the site of intersection of the line and the wound. Then, the cells were treated for 24 h with lead (20 µg/dL). At 24 h, we took a picture in the same location. Adobe Photoshop CS2 was used to determine the area of wound closure compared to time 0 for the stimulus and with respect to the control situation.

Cell proliferation assay

Cell proliferation was assessed using a CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). VSMCs were seeded on 96-well plates (20×10^3 cells/well) in DMEM/F12 (HAM) medium and were allowed to attach for 24-36 h. Afterwards, cells were switched to serum-free medium for 24-h. Cells were then treated with lead (20 µg/dL) or vehicle for 48 h. The proliferative response was quantified by adding MTS tetrazolium solution (20 µL/well). After 2-3 h of incubation, absorbance was measured at 490 nm in a microplate reader (ASYS Hitech GmbH, Austria). Different assays were each performed in triplicate.

Drugs and reagents

L-phenylephrine hydrochloride, acetylcholine chloride, sodium nitroprusside, apocynin, lucigenin, mito-TEMPO, ML 171, tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), salts and other reagents were purchased from Sigma Chemical Co. and Merck (Darmstadt, Germany). DHE, streptomycin and penicillin were obtained from Invitrogen (Carlsbad, CA, USA). Celecoxib and rofecoxib were obtained from Pfizer (New York, NY, USA) or LKT Laboratories (St. Paul, Mn, USA), respectively. U0126 and SB203580 were obtained from Calbiochem (Darmstadt, Germany). Lead acetate was obtained from Vetec (Rio de Janeiro, RJ, Brasil). All drugs were dissolved in distilled water except celecoxib, rofecoxib, U0126 and SB203580, which were dissolved in DMSO. DMSO did not have any effect on the parameters evaluated in VSMCs.

Data analysis and statistics

Contractile responses were expressed as a percentage of the maximal response induced by 75 mM KCl. Relaxation responses to ACh or SNP were expressed as the percentage of the previous contraction. For each concentration-response curve, the maximal effect (R_{max}) and the concentration of agonist that produced 50% of the maximal response (EC_{50}) were calculated using non-linear regression analysis (GraphPad Prism, GraphPad Software, Inc., San Diego, CA). The sensitivities of the agonists were expressed as pD_2 ($-\log EC_{50}$). To compare the effects of endothelium denudation or L-NAME on the contractile responses to phenylephrine, some results were expressed as differences in the area under the concentration response curves (dAUC) for the control and experimental groups. AUCs were calculated from the individual

concentration-response plots using a computer program (GraphPad Prism, GraphPad Software, Inc., San Diego, CA). The differences were expressed as the percentage of the AUC of the corresponding control situation.

All values are expressed as the mean ± SEM of the number of animals or independent experiments in the case of cell cultures used in each experiment. The results were analyzed using Student's *t* test or one- or two-way ANOVA, followed by the Bonferroni post hoc test or *Tukey* test by using GraphPad Prism Software. Differences were considered statistically significant at $P < 0.05$.

Results

Rats exposed to lead for 30 days had similar body weight [Control: before 218 ± 3.08 g and after 325 ± 5.80 g (n = 9); lead-treated: before 217 ± 2.57 g and after 328 ± 7.27 g (n = 9) P>0.05] and tibia length [Control: 3.8 ± 0.1 cm (n = 6); Lead-treated: 3.4 ± 0.1 cm (n = 9) P>0.05]. The left ventricular mass:tibia length ratio was not affected in lead-treated animals (0.173 ± 0.003 g/cm) when compared with controls (0.178 ± 0.006 g/cm). The blood lead concentration attained was 21.7 ± 2.38 µg/dL (n = 6) and the systolic blood pressure (SBP) was increased by treatment (Control: 127 ± 0.57 mmHg, n = 7; lead treated: 144 ± 1.67 mmHg, n = 7, P<0.05).

Effects of lead treatment on vascular reactivity

Lead treatment did not affect the response to KCl (untreated: 3.75 ± 0.13 g, n = 9; lead-treated: 3.52 ± 0.19 g, n = 9; P>0.05). However, treatment increased vasoconstrictor responses to phenylephrine and decreased the endothelium-dependent responses induced by ACh (Figure 1 A-B, Table 1). The vasodilator

responses induced by SNP were unaffected by lead treatment (Figure 1 C). These results suggest that lead treatment affects endothelial function in aortic rings.

Effects of lead treatment on endothelial modulation of vasoconstrictor responses

To evaluate if lead treatment alters NO modulation in aortic segment responses, the effect of endothelium removal and incubation with the NOS inhibitor L-NAME (100 µM) on vasoconstrictor responses were investigated. Both endothelium removal and NOS inhibitor addition left-shifted the concentration-response curves to phenylephrine in aortic segments from both groups, but this effect was smaller in preparations from lead-treated rats than in those from control rats, as shown by the dAUC values (Figures 2 A-B). These findings suggest that NO production and/or bioavailability is reduced after lead treatment.

Role of oxidative stress and prostanoids on the effect of lead on vasoconstrictor responses

Reduction in NO bioavailability caused by increased ROS production is associated with endothelial dysfunction accompanying hypertension (Cai and Harrison, 2000). The basal $O_2^{\bullet-}$ production in the aortas from lead-treated rats was greater than that from the untreated rats (Figure 3 A). The participation of ROS in the vascular responses was evaluated using the NADPH oxidase inhibitor apocynin (30 µM). Apocynin reduced the vascular response to phenylephrine in both experimental groups (Figure 3 B, Table 1); however, this

effect was greater in preparations from lead-treated rats than in those from control rats as demonstrated by the dAUC (Figure 3 B). gp91(phox), Cu/Zn-SOD and Mn-SOD protein expression was increased in the aorta from the lead treated group compared to controls (Figure 3 C).

The cyclooxygenase inhibitor indomethacin (10 µM) was used to investigate the role of prostanoids on the increased response to phenylephrine in lead-treated rats. Indomethacin did not alter phenylephrine responses in control aortic segments. However, in arteries from lead-treated rats, indomethacin reduced phenylephrine contraction (Figure 4 A, Table 1). We investigated COX-2 protein expression and observed an increase in this enzyme in the lead-treated group (Figure 4 B).

Altogether, these results show that chronic treatment with low concentrations of lead increase oxidative stress and prostanoid pathways and could contribute to the impaired vascular function observed in the aortas from lead-treated rats.

Effect of lead exposure on oxidative stress and COX-2 expression in VSMCs

In an attempt to investigate the underlying mechanism of lead effects on oxidation and COX-2 expression observed in vascular reactivity, we used VSMCs. First, we evaluated cell viability with 10, 20 and 100 µg/dL lead acetate during 48 h using a MTT cell viability assay. No differences were observed in cell viability (data not shown). Therefore, we chose the concentration of 20 µg/dL because this is close to the concentration attained in blood from rats treated for 30 days with lead acetate.

Incubation of VSMCs with lead (20 µg/dL, 48 h) increased $O_2^{\bullet-}$ production, as determined by increased DHE-induced fluorescence (Figure 5A). This increased production of $O_2^{\bullet-}$ was diminished by the SOD mimetic tempol and by the specific scavenger of mitochondrial superoxide mito-TEMPO (Figure 5 A), suggesting a role for mitochondrial oxidative stress in the effects of lead. Lead also increased NADPH oxidase activity as well as the mRNA levels of the NADPH oxidase subunits NOX-1 and NOX-4 (Figure 5 B-D). The increased NADPH activity was normalized by ML 171 (0.5 µM) and tempol (10 µM). In addition, the NADPH oxidase activity and NOX mRNA levels were reduced by the COX-2 inhibitors celecoxib (10 µM) and rofecoxib (10 µM) (Figure 5 B-D), which implies a role for COX-2-derived products in NADPH oxidase activation. We also studied whether lead-treatment might alter the expression of the superoxide detoxificant enzyme SOD. Mn-SOD and EC-SOD protein expression was augmented in VSMCs treated with lead (Figure 5 E and G). However, Cu/Zn-SOD protein expression was not affected (Figure 5 F).

VSMCs treated with lead for 48 h also increased COX-2 mRNA levels (Figure 6 A). This increase was accompanied by an increase in COX-2 protein expression, without changes in COX-1 protein expression (Figure 6 B-C). In addition, we observed that pre-treatment of VSMCs with the antioxidant tempol prevented lead-induced COX-2 gene expression, suggesting a relationship between ROS and COX-2 products (Figure 6 A).

MAPK signaling pathways involved in the lead-induced NOX and COX-2 mRNA levels

The MAPK pathway plays a role in modulating COX-2 (expression and activity) and ROS production (Ohnaka et al., 2000; Wang et al., 2013; Guo et al., 2014).

Thus, we investigated whether the MAPK signaling pathway was involved in the effects induced by lead on NOX and COX-2 mRNA levels. We observed a time-dependent stimulation of ERK1/2 by lead at 30-60 minutes but not at long exposure times (3 and 24 h) (Figure 7 A). In contrast, p38 was activated by lead only after long exposure times (24 h) (Figure 7 B). However, neither JNK nor Akt were activated by lead at any time points (Figure 7 C-D). The p38 MAPK inhibitor SB203580 (10 µM) but not the ERK1/2 MAPK inhibitor U0126 (10 µM) normalized lead-induced NOX-1 gene expression (Figure 8 A); both U0126 and SB203580 normalized lead-induced NOX-4 and COX-2 gene expression (Figure 8 B-C). These findings suggest that the activation of these kinases by lead are involved in events that induce NADPH oxidase and COX-2 activation. However, lead did not induce either cell migration or proliferation (Figure 9 A-B).

Table 1.
pD_2 and the maximum response to phenylephrine in aortic segments from untreated rats and rats treated with lead with or without endothelium, L-NAME, apocynin, or indomethacin.

	Untreated		*Lead treated*	
	R_{max}	***pD_2***	***R_{max}***	***pD_2***
Control	99±3.5	-6.37±0,20	127±5.2*	-7.26±0.22*
E-	137±9.6*	-7.11±0.07*	148±7.9†	-7.87±0.30†
L-NAME	133±8.2*	-6.95±0.12	146±2.9†	-7.91±0.16†
Apocynin	84±5.5*	-6.22±0.18	89±6.7†	-7.39±0.25
Indomethacin	89±3.4	-6.73±0.28	90±4.5†	-7.65±0.25

Data are expressed as the mean ± SEM. R_{max} values were expressed as a percentage of the maximal response induced by 75 mM KCl. *P<0.05 vs Control untreated. † P<0.05 vs Control Lead treated. R_{max}: Maximal response.

Discussion

The exposure to environmental chemicals, including lead, is emerging as a potential cardiovascular risk factor (Weinhold, 2004; Mamtani et al., 2011; Simões et al., 2011). The main finding of the present study is that 30-days treatment with a low dose of lead increases blood lead concentrations to values (21.7 µg/dL) lower than the reference value (60 µg/dL), but these concentrations are sufficient to increase systolic blood pressure and phenylephrine-induced contractility and to decrease endothelium-dependent vasodilator responses in rat aortas. Our results also suggest that p38 and ERK1/2 MAPK are involved in the increase in vascular COX-2 levels and ROS production after lead exposure that act in concert to produce the vascular changes that could contribute to the occurrence of arterial hypertension.

The effects of lead on human health depend on blood levels and on the duration of the exposure. Several studies have supported the association between high blood lead levels and hypertension in humans (Andrzejak et al., 2004; Patrick, 2006; Kosnett et al., 2007). The Agency for Toxic Substances and Disease Registry (ATSDR) considered the reference blood lead concentration level to be 60 µg/dL (Patrick, 2006; Kosnett et al., 2007). Nevertheless, individuals with baseline blood lead levels of 46.8 µg/dL or 67.8 µg/dL have also shown increases in arterial pressure (Malvezzi et al., 2001; Andrzejak et al., 2004) suggesting that even lower than recommended maximal doses might have cardiovascular deleterious consequences. A similar association was reported in treated rats with lead blood concentrations between 31.8 µg/dL and 42.5 µg/dL (Gonick et al., 1997; Marques et al., 2001). Moreover, in the present study,

treated rats attained a blood lead concentration of 21.7 ± 2.38 µg/dL that increased systolic blood pressure.

The mechanisms by which lead may cause hypertension are not entirely clear (Heydari et al., 2006). Different studies have identified several candidates, including oxidative stress, impairment of the NO system, inflammation, dysregulation of vasoactive hormones, and alterations of cellular Ca^{2+} transport and intracellular Ca^{2+} distribution (Goldstein, 1993; Feng et al., 1995; Heydari et al., 2006). Using animal models, our group has already successfully documented that chronic exposure to lead exerts detrimental effects on the function of the heart and aorta (Simões et al., 2011; Fiorim et al., 2011; Silveira et al., 2014; Fioresi et al., 2014). In the present study, aortic reactivity to phenylephrine was increased, whereas relaxation in response to acetylcholine was reduced. Similar findings were recently reported in aortas from rats with blood lead levels smaller than those in the present study (Silveira et al., 2014). Alterations in the reactivity of the aorta after lead treatment were endothelium dependent, as evidenced by the magnitude of lead-induced effects being reduced in aortic rings without endothelium. In addition, the NOS inhibitor L-NAME increased the reactivity to phenylephrine to a lower extent in arteries from lead-treated rats. These results suggest that lead decreased endothelial NO bioavailability, consequently increasing the reactivity to phenylephrine in aortic rings. In agreement, a previous study showed that treatment with 100 ppm lead acetate for 28 days reduces NO bioavailability in the rat aorta (Karimi et al., 2002). However, our group has reported that low concentrations of lead increases the NO bioavailability in the initial stages of lead exposure (7 days) (Fiorim et al., 2011), but this effect might be changed after a long exposure, as

we observed in the present study. However, the endothelium-independent relaxation induced by the NO donor sodium nitroprusside was not altered after 30 days of lead exposure, in agreement with previous reports (Silveira et al., 2014).

Oxidative stress can lead to endothelial dysfunction, vascular structural alterations and hypertension, (Rodriguez-Iturbe et al., 2004; Vaziri, 2004). The inactivation of NO by ROS can result in vasoconstriction, increased platelet/leukocyte adhesion, vascular smooth muscle cell migration/proliferation, and matrix accumulation leading to vascular remodeling (Touyz et al., 2011; Drummond et al., 2011). It is known that increased ROS production contributes to the inflammatory process associated with lead-induced hypertension (Vaziri et al., 1997; Silveira et al., 2014). Herein, we observed increased superoxide anion production in the aorta from lead-treated rats and in VSMCs exposed to lead. In addition, the antioxidant apocynin reduced the vasoconstrictor response induced by phenylephrine, more in aortas from lead-treated rats, suggesting the involvement of superoxide anions in the vascular effects of lead, in accordance with the results recently described (Silveira et al., 2014). On the other hand, the formed peroxynitrite by the reaction of NO with superoxide anion would also contribute to the altered vascular responses observed after lead exposure, as it has been described by other investigators in vascular diseases such as atherosclerosis, hypertension, ischemia, endotoxic shock, and diabetes (Zou, 2007).

NADPH oxidase isoforms have been described as a major source of ROS in vascular tissue (Griendling et al., 2000). Our results point to the up-regulation of

NADPH oxidase as the potential source of ROS in lead-exposed vascular cells. Thus, we found that 1) the protein expression of the gp91(phox) NADPH oxidase subunit was increased in arteries from treated rats; and 2) the mRNA levels of NOX-1 and NOX-4 subunits of NADPH oxidase and NADPH activity were increased in lead-treated VSMCs. However, the participation of other sources of ROS, such as the mitochondria, cannot be discarded. Thus, the increased $O_2^{\bullet-}$ production observed in VSMCs treated with lead was diminished by the mitochondria-targeted SOD-2 mimetic mito-TEMPO. Decreased antioxidant defenses would also contribute to the increased oxidative stress. A major antioxidant defense system against $O_2^{\bullet-}$ are the superoxide dismutases which plays an important role in regulating blood pressure and endothelial function by reducing extracellular $O_2^{\bullet-}$ level, thereby preventing oxidative inactivation of NO released from endothelium (Oury et al., 1994; Oury et al., 1996; Stralin et al., 1995). However, the protein expression of Mn-SOD, Cu/Zn-SOD and EC-SOD was increased in lead-treated arteries or VSMCs, suggesting that antioxidant mechanisms are activated in lead-exposed cells probably to protect against increased oxidative stress. In agreement, Ni et al. (2004) demonstrated an increase in superoxide and hydrogen peroxide in human endothelium and VSMCs from human coronary arteries after lead acetate (1 ppm) treatment for 60 h, accompanied by an increase in Cu/Zn-SOD protein expression. In the same study, the authors demonstrated a significant up-regulation of the gp91(phox) subunit of NADPH oxidase in lead-exposed endothelial cells.

COX-2, the source of the prostaglandins that mediate inflammation, is rapidly induced in response to different stimuli, including growth factors,

proinflammatory cytokines and oxidative stress (Feng et al., 1995; Martínez-Revelles et al., 2013; Wang et al., 2013). Increased vascular COX-2 expression is usually associated with hypertension (Álvarez et al., 2007). COX-2 derived prostanoids were also implicated in the vascular effects of lead (Silveira et al., 2014) and other heavy metals, such as mercury (Pecanha et al., 2010). Herein, we observed an increase in COX-2, but not in COX-1, protein or mRNA levels in aortas from lead-treated rats and/or in VSMCs exposed to lead. After COX blockade with indomethacin, a reduction in the phenylephrine-induced vasoconstrictor responses in aortic segments from lead-treated rats was observed, but not in control rats. These findings suggest the participation of COX-2-derived prostanoids in the increased vasoconstrictor responses induced by lead treatment.

Some studies have reported that oxidative stress upregulates COX-2 expression (Feng et al., 1995; Garcia-Cohen et al., 2000; Álvarez et al., 2007) and that antioxidant treatment reduces COX-2 expression (Feng et al., 1995; Martínez-Revelles et al., 2013). In fact, increased ROS production is hypothesized as one possible mechanism for the increased vasomotor COX-2 activity in the setting of hypertension (Garcia-Cohen et al., 2000; Álvarez et al., 2007; Martínez-Revelles et al., 2013). As discussed above, we show that lead stimulated COX-2 expression and ROS production. More importantly, our results also suggest an interaction between COX-2 and ROS in VSMCs exposed to lead. Thus, the ROS scavenger tempol reduced the increased COX-2 mRNA levels, whereas the COX-2 inhibitors celecoxib and rofecoxib reduced the increased NADPH activity and NOX-1 and NOX-4 mRNA levels. This reciprocal interaction between both pathways would increase the harmful

effects of lead at the vascular level. In aortas from angiotensin II-infused mice, the existence of a reciprocal relationship between ROS and COX-2-derived products has been described as responsible for the vascular dysfunction observed in this hypertension model (Martínez-Revelles et al., 2013). Sancho et al. (2011) also showed a reciprocal regulation of NADPH oxidase and the COX-2 pathway in liver cells under inflammatory conditions.

Previous reports indicate that MAPK pathways play an important role in modulating COX-2 (expression and activity) and ROS production (Ohnaka et al., 2000; Wang et al., 2013; Guo et al., 2014). However, dissection of the specific molecular mechanisms and signaling cascades continue to be the focus of intense research. Evidence shows that MAPK cascades are the major signaling pathway that regulates cell proliferation, migration, differentiation, inflammation and apoptosis (Yang et al., 2003). Moreover, Aguado et al., (2013) reported that mercury, another heavy metal, induced MAPK activation in VSMCs. Therefore, we speculated that MAPK signaling pathways might be involved in the lead-induced alterations in VSMCs. Our current data show that lead induces an early activation of ERK1/2 and a delayed activation of p38 MAPKs without effects on JNK. Posser et al. (2007) showed the toxic effect of lead on C6 glioma cells and a significant activation of p38 and JNK MAPK. In addition, we found that lead did not stimulate Akt phosphorylation, which corroborates results published by Lu et al. (2001) in human astrocytoma cells. To investigate if the p38 and ERK1/2 MAPK pathways were involved in COX-2 and NADPH oxidase activation, the effect of specific inhibitors on COX-2, NOX-1 and NOX-4 expression was investigated. U0126 (inhibitor of ERK1/2) and SB203580 (inhibitor of p38) abrogated lead-induced COX-2, NOX-1 and NOX-4

mRNA expression in cultured VSMC. These findings suggest that the activation of inflammatory proteins such as NADPH oxidase and COX-2 in response to lead exposure is mediated through p38 and ERK1/2 signaling pathways. However, when we analyzed the effects of lead on migration and proliferation in VSMCs, no differences were observed in this model of exposure. There is evidence that lead can increase the proliferation of rat liver cells (Liu et al., 1997), VSMC (Fujiwara et al., 1995) and spleen cells (Razani-Boroujerdi et al., 1999). Lu et al., (2001) demonstrated that lead induces proliferation in human 1321N1 astrocytoma cells that is mediated by the activation of the MEK1/2 and ERK1/2 signal transduction pathways in a PKC□-dependent manner. The discrepancies with our data could be due to differences in the exposure protocols related to doses and/or duration of lead exposure.

In summary, the present study demonstrated for the first time that treatment with low doses of lead increased systolic arterial blood pressure, promoted vascular dysfunction and activated MAPK signaling pathways. These effects are associated with the activation of inflammatory proteins such as NADPH oxidase and COX-2 that act in concert to contribute to vascular dysfunction. These findings strongly support that lead exposure should be considered an environmental risk factor for cardiovascular disease.

Conflict of Interest Statement

None declared.

Acknowledgements

This work was supported by MINECO (SAF2012-36400), ISCIII (RD12/0042/0024), PRONEX-CNPq/FAPES (48511935/2009). MRS was a fellow of CAPES and CNPq. AMB was supported by the Ramon y Cajal Program (RyC2010-06473). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Figure legends

Fig.1. Chronic lead treatment affects aortic reactivity. Effects of 30-day exposure to lead on the concentration-response curves to (A) phenylephrine, (B) acetylcholine and (C) sodium nitroprusside (SNP) in aortic rings. Data are expressed as the mean ± SEM. *P<0.05 versus untreated using two-way ANOVA and Bonferroni post-test. Number of animals used is indicated in parentheses.

Fig.2. Effects of chronic lead treatment on NO-mediated vascular response in aortic rings. Effects of (A) endothelium removal (E^-) and (B) L-NAME (100 µM) on the concentration-response curve to phenylephrine in aortic rings from untreated and treated rats. *P < 0.05 versus E+ or control using two-way ANOVA and Bonferroni post-test. The insert shows differences in the area under the concentration-response curves (dAUC) in (A) endothelium–denuded and intact segments and (B) in the presence and absence of L-NAME. *P<0.05 versus untreated by Student's *t*-test. Data are expressed as the mean ± SEM. Number of animals used is indicated in parentheses.

Fig.3. Lead induces vascular oxidative stress. (A) Representative fluorescent photomicrographs of arterial sections labeled with the oxidative dye hydroethidine and vascular superoxide anion quantification. *P < 0.05 versus untreated by Student's *t*-test. (B) Effects of the NADPH oxidase inhibitor apocynin on the concentration-response curve to phenylephrine in aortic rings from untreated and treated rats; insert shows the difference in the area under the concentration-response curve (dAUC) of the respective group. *P<0.05 versus control using two-way ANOVA and Bonferroni post-test or versus

untreated by Student's *t*-test. (C) Densitometry analyses of western blots for gp91(phox), Cu-Zn/SOD and Mn/SOD protein expression in aortas from untreated and treated rats. Representative blots are also shown. Data are expressed as the mean ± SEM. *P<0.05 versus untreated by Student's *t*-test. Number of animals used is indicated in parentheses.

Fig.4. Role of prostanoids in aortic segments from untreated and treated rats. (A) Effect of the nonselective COX inhibitor indomethacin on the concentration-response curve to phenylephrine in aortic rings from untreated and treated rats. *P < 0.05 versus control using two-way ANOVA and Bonferroni post-test. (B) Densitometric analysis of western blots for COX-2 protein expression in aortas from untreated and treated rats. Representative blots are also shown. *P<0.05 versus untreated by Student's *t*-test. Data are expressed as the mean ± SEM. Number of animals used is indicated in parentheses.

Fig.5. Lead induces oxidative stress in vascular smooth muscle cells. Effect of lead (Pb) on VSMC superoxide anion production in the absence or the presence of mito-TEMPO (5 µM) and Tempol (10 µM) (A); NADPH Oxidase activity in the absence or the presence of ML 171 (0.5 µM), Tempol (10 µM), Celecoxib (10 µM) and Rofecoxib (10 µM) (B); NOX-1 and NOX-4 gene expression in the absence or the presence of Celecoxib and Rofecoxib (C,D); Mn-SOD, Cu/Zn-SOD and EC-SOD protein expression (E-G). Representative images of cells stained with dihydroethidium and representative blots are also shown. Data are expressed as the mean ± SEM. *P<0.05 versus untreated cells (Ct or untreated), &P < 0.05 versus Pb using one-way ANOVA followed by

Tukey test or Student's *t*-test. Number of different cultures is indicated in parentheses.

Fig.6. Effect of lead on vascular cycloxygenase-2 (COX-2) expression and the relationship with oxidative stress. (A) Effects of lead (Pb) on COX-2 mRNA levels in the absence or the presence of Tempol (10 µM). Effect of lead on COX-2 (B) and COX-1 (C) protein expression. Representative blots are also shown. Data are expressed as the mean ± SEM. *P<0.05 versus untreated cells (Ct), &P<0.05 versus Pb using one-way ANOVA followed by *Tukey* test or Student's *t*-test. Number of cell cultures is indicated in parentheses.

Fig.7. Lead induces ERK1/2 and p38 MAPK phosphorylation. Effects of lead on (A) ERK1/2, (B) p-38 MAPK, (C) JNK and (D) Akt activation. Representative blots are also shown. The results are expressed as the ratio between phospho-MAPK and total MAPK and normalized to values obtained for unstimulated control (0) cells. Data are expressed as the mean ± SEM. *P<0.05 versus unstimulated cells (0) by one-way ANOVA followed by *Tukey* test. Number of cell cultures is indicated in parentheses.

Fig.8. Role of ERK1/2 and p38 MAPK pathways on lead-induced NOX-1, NOX-4 and COX-2 expression. Effects of U0126 (10 µM) and SB203580 (10 µM) on lead (Pb)-induced (A) NOX-1, (B) NOX-4 and (C) COX-2 gene expression in smooth muscle cells. Data are expressed as the mean ± SEM. *P<0.05 versus untreated (Ct), &P<0.05 versus Pb by one-way ANOVA followed by *Tukey* test. Number of cell cultures is indicated in parentheses.

Fig.9. Effects of lead on cell migration and proliferation. Data are expressed as the mean ± SEM. Number of cell cultures is indicated in parentheses.

1 **Abbreviation:** ROS, reactive oxygen species; COX-2, cyclooxygenase; MAPK,
2 Mitogen-activated protein kinases; DHE, dihydroethidium; ERK, extracellular
3 signal-regulated protein kinases; JNK, c-Jun N-terminal kinases.

4

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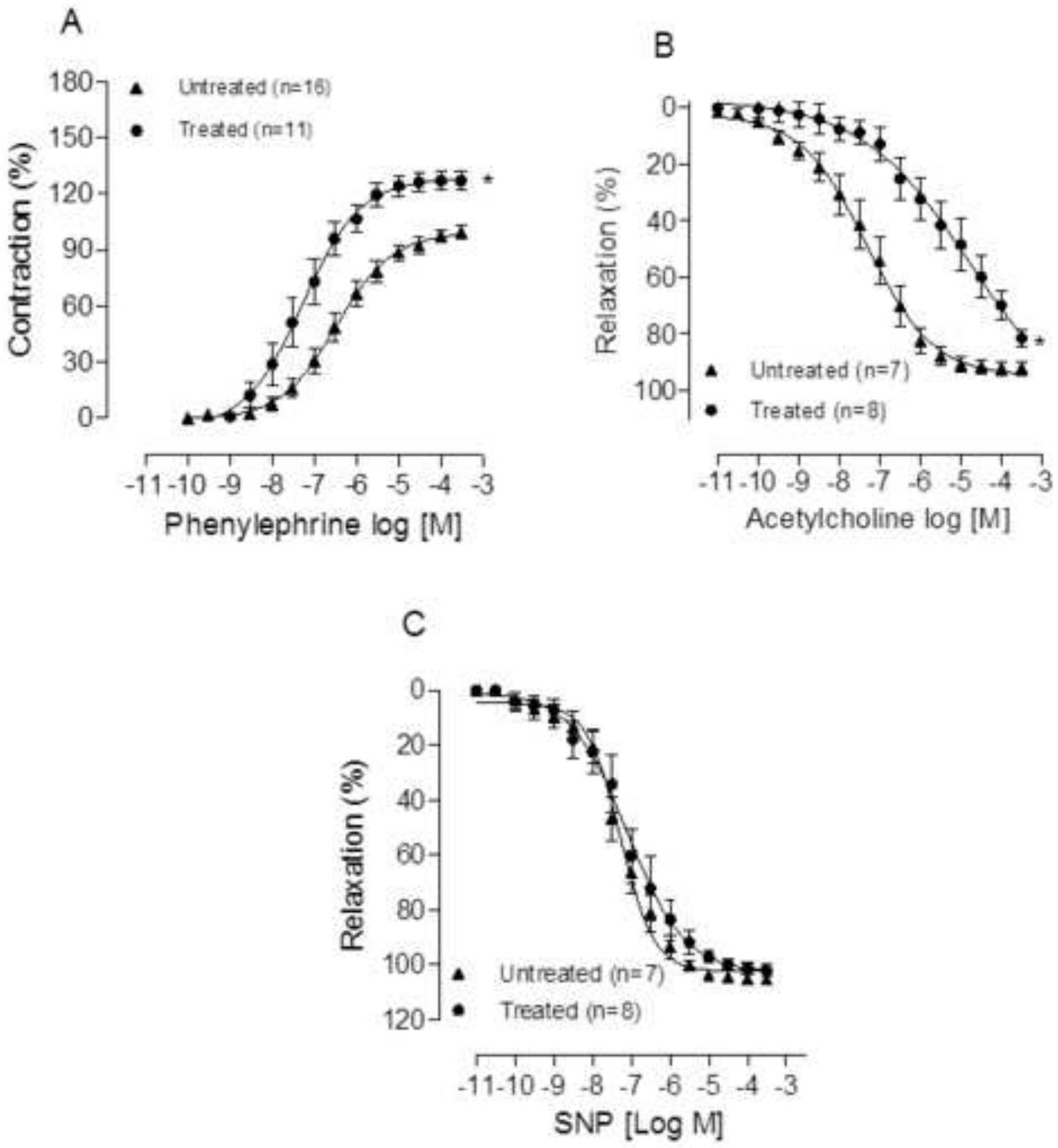

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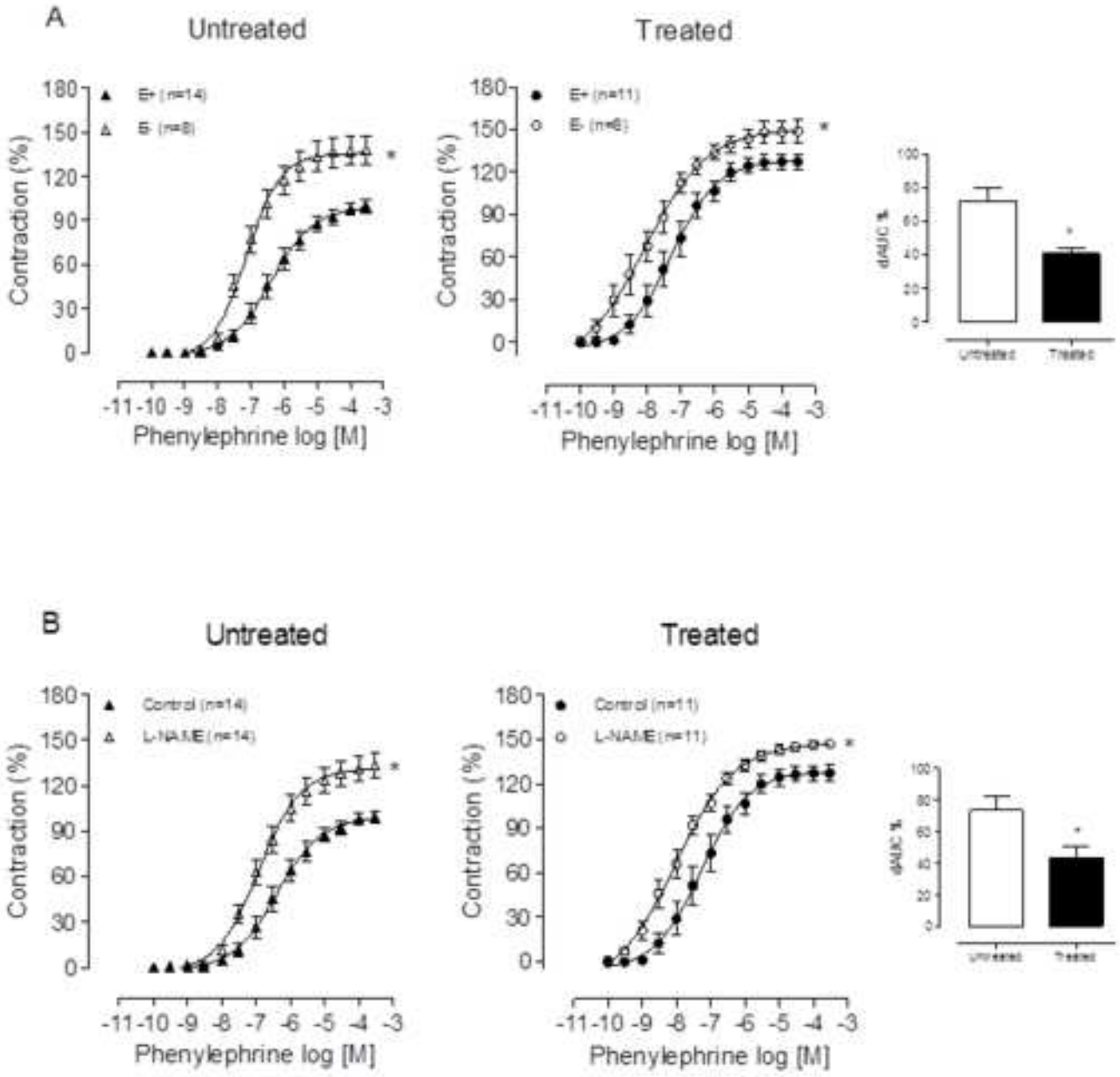

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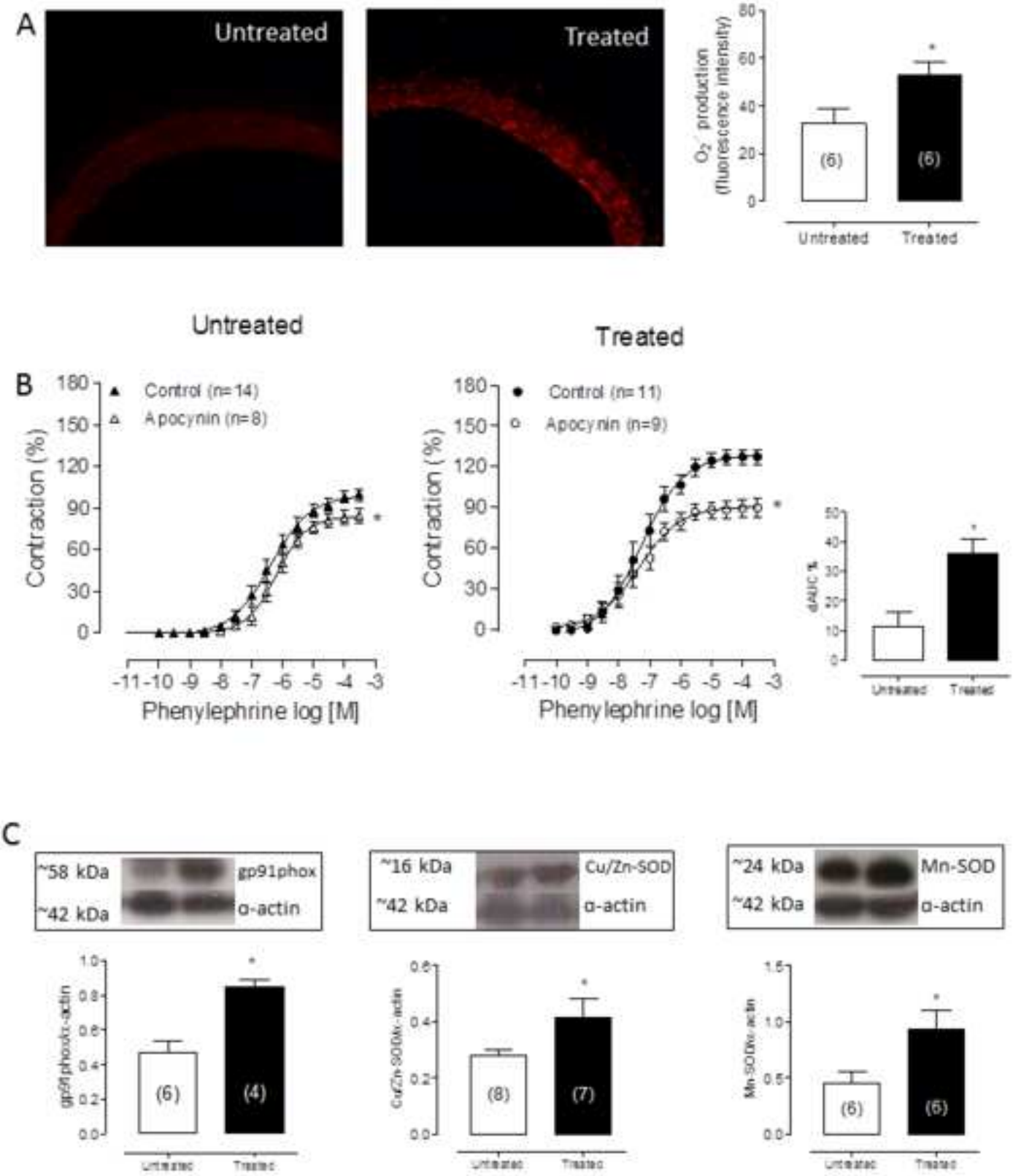

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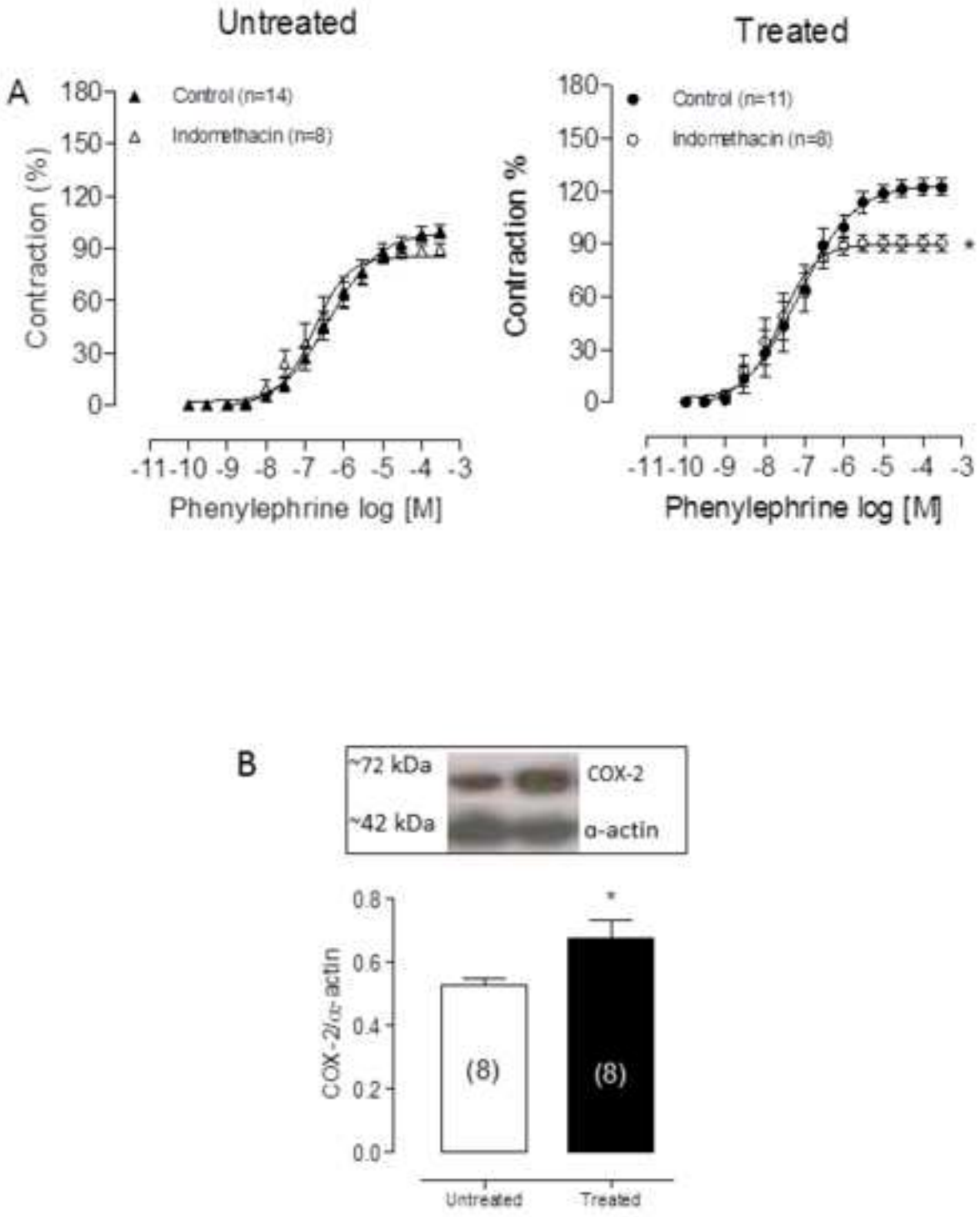

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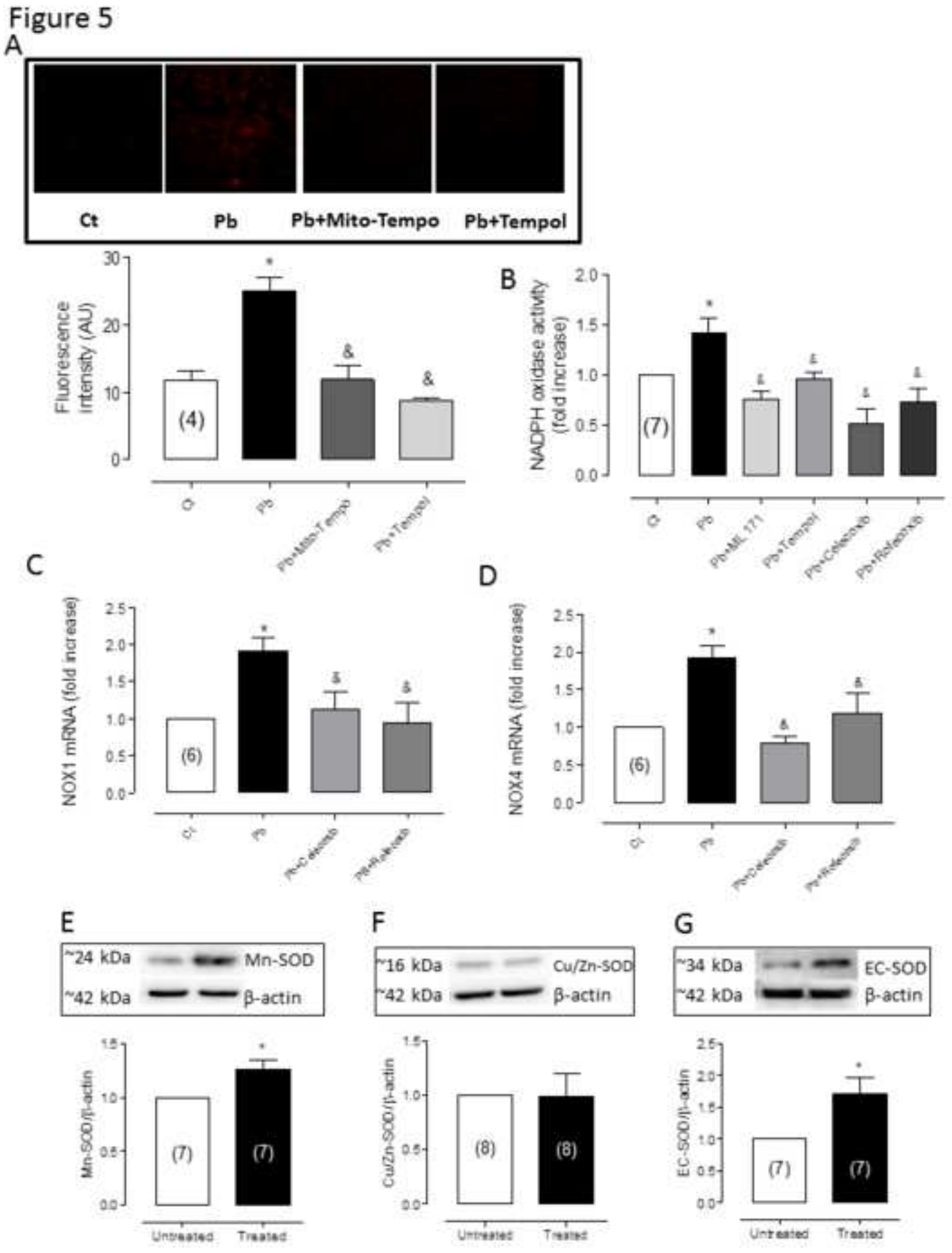

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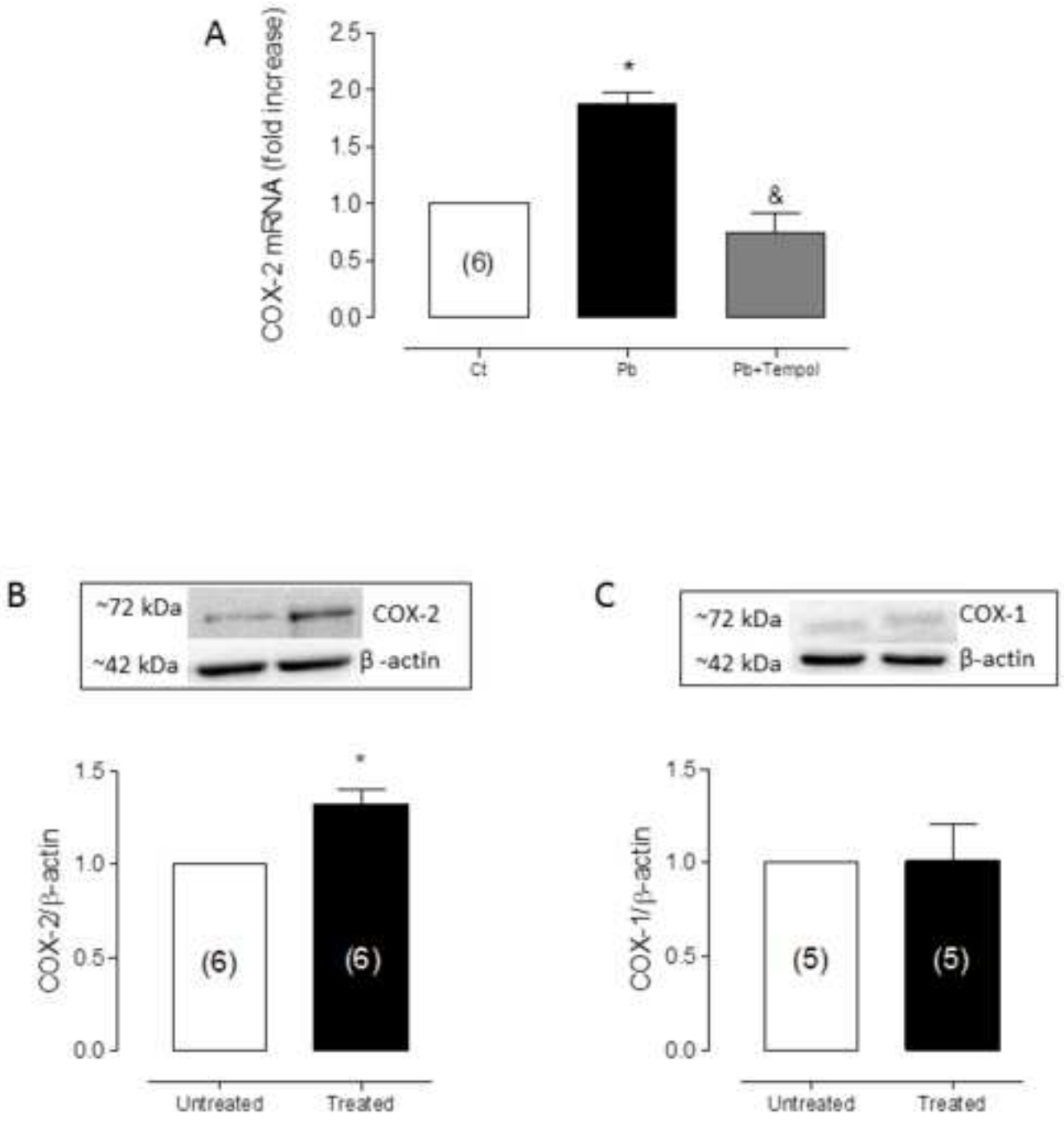

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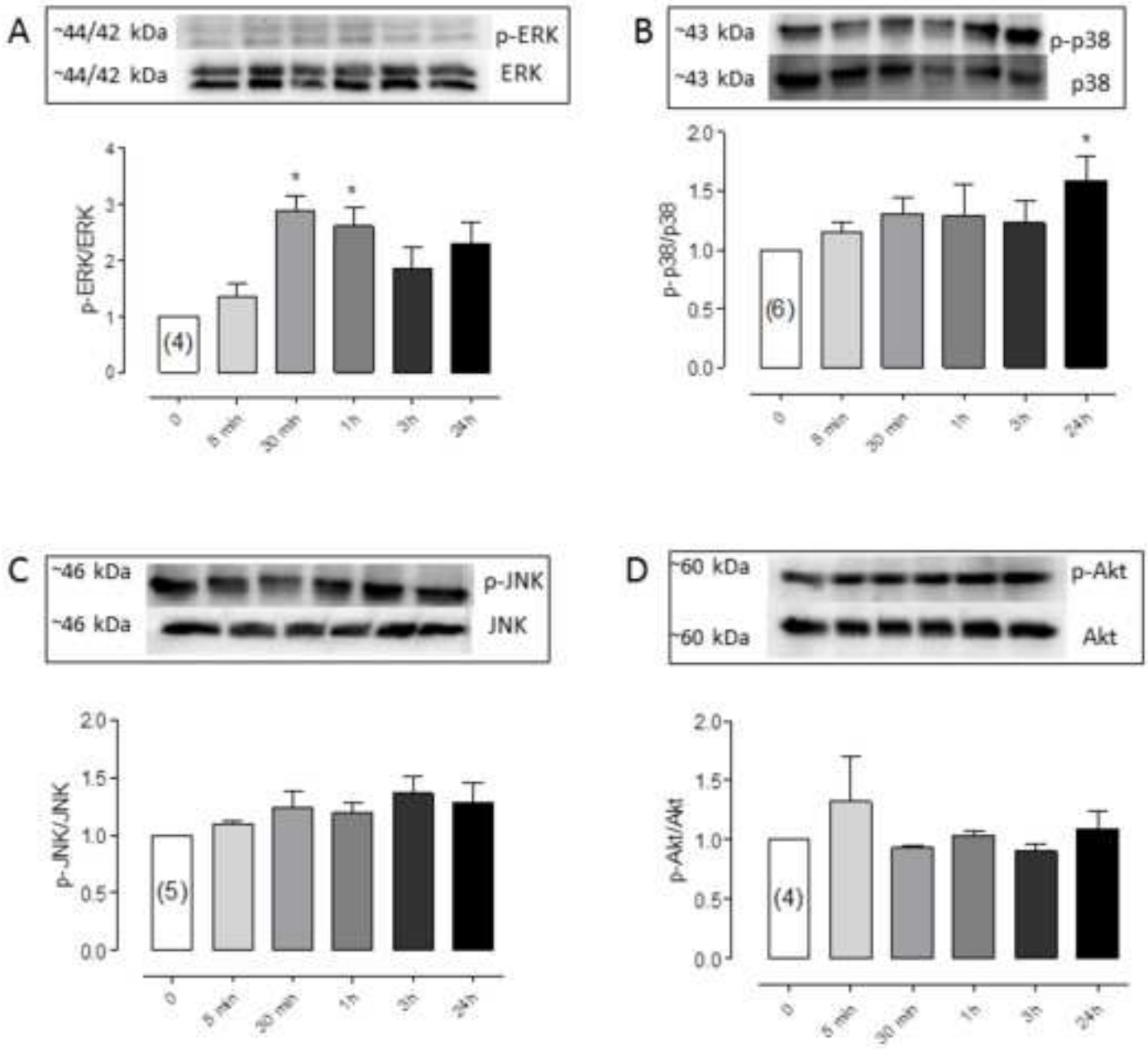

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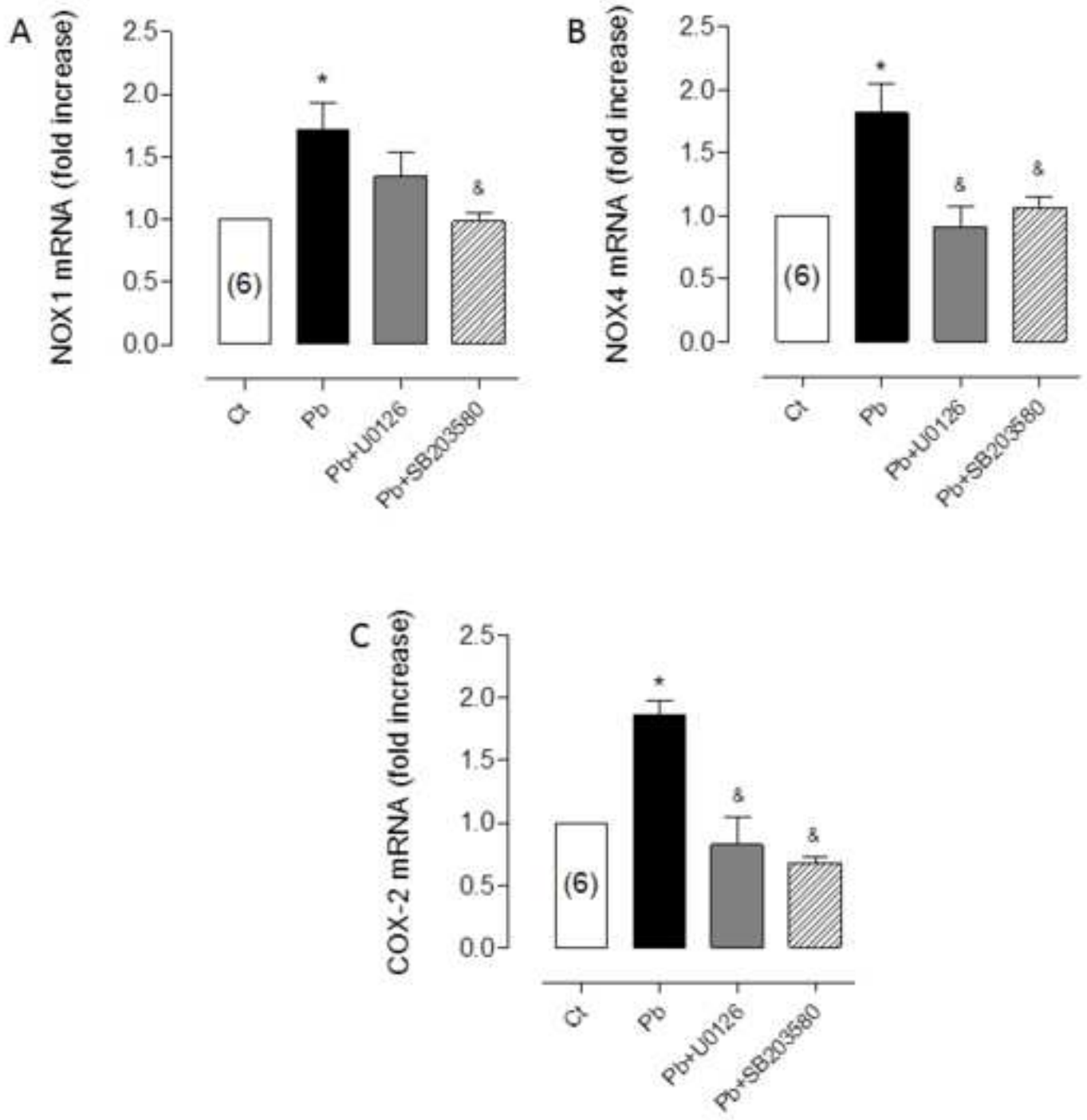

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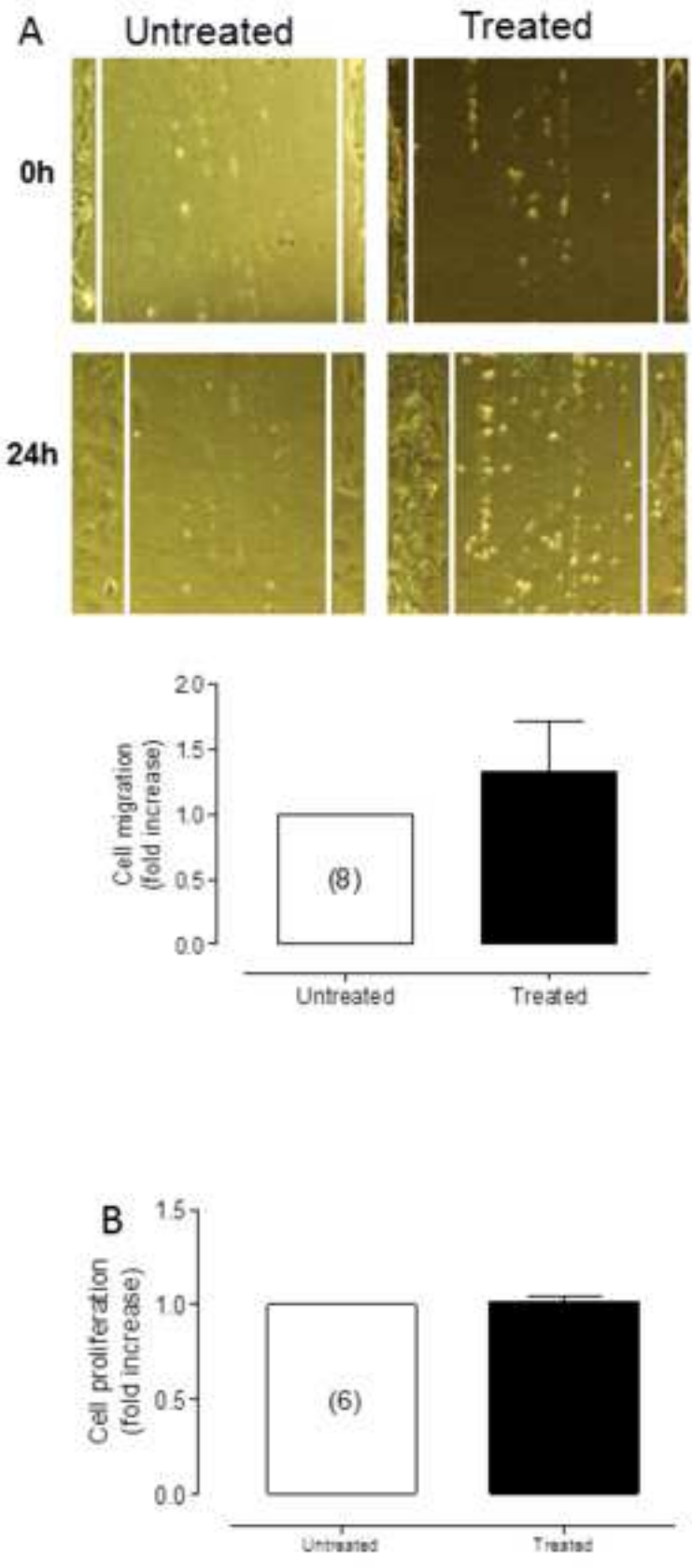

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TOXICOLOGY AND APPLIED PHARMACOLOGY

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