

## Original Article

# Effects of hydrogen peroxide on phenylephrine-induced contraction of abdominal or thoracic aorta from adult rats

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**Abstract:** Background: The effect of oxidative stress promotes activity enzymatic in vascular wall that produces hydrogen peroxide with the effect on endothelium and the development of cardiovascular diseases. Aims: We evaluated the effect of hydrogen peroxide on vascular function in thoracic and abdominal aorta. Methods: Under basal conditions, 3- and 6-month-old rats had similar blood pressure, but age- and aortic segment-dependent differences in other parameters. Results: In abdominal aorta of 6-month-old rats, superoxide dismutase activity was unchanged but there was a decrease in catalase activity, nitric oxide concentration and the phenylephrine-induced contraction. This diminished phenylephrine response was similar when adding H<sub>2</sub>O<sub>2</sub>, later restored by L-NAME or Wortmannin. Moreover, p-Akt expression increased in 6-month-old rats after adding H<sub>2</sub>O<sub>2</sub>. In thoracic aorta of 6-month-old rats, antioxidant activity increased. No differences were found in 3- or 6-month-old rats regarding the response to Ach or Phe in this aortic segment. In conclusion, in this study we found that H<sub>2</sub>O<sub>2</sub> plays a key role in the modified endothelium-dependent response to phenylephrine (through PI3K/Akt/NO mechanisms) in the abdominal aorta of normotensive rats. The results were different in thoracic aorta.

**Keywords:** Abdominal aorta, hydrogen peroxide, nitric oxide, PI3K/Akt, endothelium, aging

## Introduction

The endothelium plays a fundamental role in the regulation of vascular tone through the release of vasoactive substances, specifically nitric oxide (NO) and reactive oxygen species (ROS). NO is produced in a controlled manner from the amino acid L-arginine by the action of a family of enzymes called nitric oxide synthases (NOS) [1]. The ROS released by the endothelium include the superoxide anion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the hydroxyl radical (OH<sup>-</sup>) [2]. Together NO and ROS play a key role in modulating the tone of vascular smooth muscle cells under basal conditions. The diminished production of NO and/or the increasing production of ROS in the vasculature are associated with vascular contraction and hypertension [3-5].

On the other hand, it is well-known that endothelium-dependent control of vascular tone is

distinct during different stages of life: early postnatal, juvenile growth [6-8] and aging [9-14]. In fact, endothelial cells are under the influence of fluctuating stimuli that modulate vascular reactivity. During aging, higher endothelial oxidative stress associated with ROS is a result of several factors, such as the augmented activity of NADPH oxidase and the uncoupling of endothelial nitric oxide synthase (eNOS), as well as from increased mitochondrial respiration in the absence of appropriate compensation by means of increasing antioxidant mechanisms [8-11]. Impaired endothelial function, developed in the elderly, has been suggested as an important cause of the increased risk for cardiovascular disease [15].

Regarding the role of ROS in the vascular response, studies about the action of H<sub>2</sub>O<sub>2</sub> are particularly attractive since this molecule has different and important effects on various vascular segments explored. Experimental eviden-

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ce shows that in the skeletal muscle arterioles of young rats,  $H_2O_2$  releases thromboxane (TXA<sub>2</sub>) and generates the contraction of vascular smooth muscle [16]. In coronary arterioles of pig,  $H_2O_2$  induces dilation that is dependent and independent of the endothelium. These actions take place through the release of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and the activation of calcium channels by potassium ( $K_{Ca}$ ), respectively [17]. Other studies have demonstrated that  $H_2O_2$  produces vasodilation by activating eNOS, evidenced by the fact that the presence of L-NAME reduces this vasodilation [18]. It has been proposed that  $H_2O_2$  modulates relaxation through endothelium-dependent and -independent mechanisms in the vascular beds [17, 19]. This  $H_2O_2$ -induced vasodilation is attributed to the synergistic effect of the phosphorylation of PI3-kinase/Akt-dependent eNOS serine 1179 and the activation of MEK/ERK1/2 [19].

In addition, it has been described that an increased  $H_2O_2$  production in endothelial cells contributes to diminished contractile response in the thoracic aorta of rats with renovascular hypertension (2K-1C) [20]. Moreover, recent research has shown that in the thoracic aorta of rats (2K-1C);  $H_2O_2$  produced by the endothelium cells contributes to a reduction in the contractile response through the activation of adrenoceptor  $\alpha_1$  ( $\alpha_1$ ). Apparently, this reduction in contractile tone is attributable to an increased production of  $H_2O_2$  by phenylephrine (Phe, an  $\alpha_1$ -adrenoceptor agonist) in endothelial cells [20]. Also, it has been shown that in the thoracic aorta of rats with genetic predisposition to the development of hypertension, the increased activity of superoxide dismutase (SOD) and the decreased activity of catalase (CAT) are responsible for the production and the release of  $H_2O_2$  in the arterial wall [21, 22].

Hence, there have been advances in the understanding of the pathophysiological processes involved in the generation of oxidative stress following an increase in ROS, as well as insights into the association of these processes with the risk of cardiovascular disease. Nevertheless, the role of oxidative stress has yet to be fully elucidated in some contexts, such as its effect on endothelial cells under basal conditions and its association with changes in vascular reactivity along the aorta of normotensive rats during early adulthood during *in vitro* experiments.

Therefore, the aim of the present study was to evaluate: a) the possible difference in the effect of oxidative stress on endothelium cells of young (3 months old) rats and those in early adulthood (6 months old) under basal conditions, b) the role of  $H_2O_2$  (and its underlying mechanisms) in the modulation of vasomotor tone in thoracic and abdominal aortic rings of normotensive rats, and c) the involvement of the PI3K/Akt/NO pathway in these effects.

### Materials and methods

#### Drugs

The drugs employed were purchased from Sigma Chemical Company: L-phenylephrine hydrochloride, acetylcholine chloride, L-NAME and Wortmannin. These were dissolved in distilled water and subsequent dilutions were obtained according to protocol requirements.

#### Animals

A total of 76 adult male Sprague Dawley rats (3 or 6 months old) were obtained from the animal facilities of the Escuela Superior de Medicina, Instituto Politécnico Nacional, Mexico-City. All the animals were housed under the same environmental conditions ( $22\pm 2^\circ\text{C}$ , 60% humidity, and artificial light from 08:00 to 18:00 h). Normal chow (Rat Chow 5012) and tap water were provided *ad libitum*. The handling of animals and procedures performed on them comply with the requirements specified by the Mexican norm (NOM-062-ZOO-1999, "Specifications for reproduction techniques, care and use of laboratory animals", SAGARPA) and the "Guide for the care and use of laboratory animals" (National Research Council). The animals were separated according to age (3 and 6 months old) and then randomly separated into groups.

#### *In vivo* experiments and acquisition of aortic rings

Rats were anesthetized intraperitoneally (*i.p.*) with a combination of xylazine (5-10 mg/Kg) and Ketamine (50-90 mg/Kg), and the trachea of each animal was cannulated. Tracheal cannula is sterile plastic material (2.5 mm in diameter), highly transparent, atoxic and apyrogenic. The rats were allowed to breathe room air spontaneously and placed on heated pads to maintain their temperature at  $37^\circ\text{C}$ , measured by

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rectal probes. The right carotid artery was catheterized with PE-80 tubing. For the measurement of systemic pressure, the carotid cannula was filled with heparinized saline (50 U/ml) and connected to a pressure transducer (TSD 104, Biopac Systems Inc., Santa Barbara, CA, USA). Blood pressure was recorded 30 minutes after the stabilization period on a computer with the software (MP100WSW, Biopac System Inc.). At the conclusion of the experimental protocols, animals were sacrificed by decapitation. The thoracic and abdominal aorta was dissected, removing fatty and connective tissue with care to avoid injury to the endothelial layer. The tissues were used immediately for *in vitro* assays or stored at  $-70^{\circ}\text{C}$  to await additional analyses.

### *Arterial preparations for reactivity studies*

Rats were anesthetized and exsanguinated by decapitation. The thoracic and abdominal aortae were cleaned of fat and connective tissue, and then cut into ring segments (4-5 mm in length). Aortic rings were mounted in 10 ml tissue baths filled with physiological saline solution containing (mM): NaCl 118, KCl 4.7,  $\text{KH}_2\text{PO}_4$  1.2,  $\text{MgSO}_4$  1.2,  $\text{CaCl}_2$  2.5,  $\text{NaHCO}_3$  25, EDTA 0.03, and Dextrose 11.7. The medium was maintained at  $37^{\circ}\text{C}$ , pH 7.4, and gassed continuously with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Each tissue was placed under an initial resting tension of 2 g and equilibrated for 60 min prior to the execution of experimental protocols. Contractions were measured isometrically and recorded with Acknowledge software (MP100WSW, Biopac Systems, Inc., Santa Barbara, CA, USA). Tissue samples were primed by the addition of Phe ( $1 \times 10^{-6}$  M) to the organ bath. After a steady-state contraction was achieved, bath contents were replaced with drug-free buffer several times. Tissues were then allowed to reach baseline tension and the priming procedure was repeated twice before conducting the experimental protocols. Functional endothelium was checked by the presence of at least 80% or 60% (3- and 6-month-old rats, respectively) relaxation in response to acetylcholine (Ach,  $1 \times 10^{-6}$  M) after pre-contracting the tissues with Phe ( $1 \times 10^{-6}$  M) or KCl (40 mmol/L).

### *The endothelium-dependent relaxation induced by Ach and the contractile responses to Phe, using thoracic and abdominal aortic rings*

After equilibration, the rings of 3- and 6-month-old rats were pre-contracted with Phe ( $1 \times 10^{-6}$

M) or  $\text{K}^+$  (40 mmol/L). When the plateau response was observed, cumulative concentration-response curves for Ach ( $1 \times 10^{-9}$  to  $1 \times 10^{-4}$  M) were constructed using endothelium-intact aortic ring preparations. Afterwards, the rings were rinsed four times and re-equilibrated for 1 h. A series of concentration-response curves for Ach and Phe ( $1 \times 10^{-9}$  to  $1 \times 10^{-4}$  M) were then built.

### *Endothelium cells mediated NO production in the thoracic and abdominal aortic rings*

In endothelium-intact thoracic and abdominal aortic rings from 3- or 6-month-old rats pre-contracted with Phe, cumulative concentration-response curves to Ach and to Phe were constructed after 30 min of incubation with the NO synthase inhibitor, NG-nitro-L-arginine methyl ester (L-NAME, 10 or 100  $\mu\text{M}$ ), and then compared with L-NAME-free preparations.

### *Role of $\text{H}_2\text{O}_2$ in the Phe-induced contractile response of thoracic and abdominal aortic rings*

After equilibration, cumulative concentration-response curves for Phe ( $1 \times 10^{-9}$  to  $1 \times 10^{-4}$  M) were constructed for endothelium-intact aortic rings from 6-month-old rats. Afterwards, the rings were rinsed four times and re-equilibrated for 1 h. A series of concentration-response curves for Phe ( $1 \times 10^{-9}$  to  $1 \times 10^{-4}$  M) was then built in presence of hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 1  $\mu\text{M}$ ). Afterwards, the rings were rinsed four times and re-equilibrated for 1 h, and cumulative concentration-response curves for Phe were elaborated again in presence of  $\text{H}_2\text{O}_2$  (10  $\mu\text{M}$ ).

### *Endothelium cells mediated NO production by $\text{H}_2\text{O}_2$ through the PI3K pathway in thoracic and abdominal aortic rings*

After equilibration, cumulative concentration-response curves for Phe ( $1 \times 10^{-9}$  to  $1 \times 10^{-4}$  M) were constructed using endothelium-intact aortic rings from 6-month-old rats. Afterwards, the rings were rinsed four times and re-equilibrated for 1 h. A series of concentration-response curves for Phe was then built in presence of hydrogen peroxide ( $\text{H}_2\text{O}_2$  1  $\mu\text{M}$ ). Afterwards, the rings were rinsed four times and re-equilibrated for 1 h, and cumulative concentration-response curves for Phe were elaborated again in presence of  $\text{H}_2\text{O}_2$  (10  $\mu\text{M}$ ) and Wortmannin (0.31  $\mu\text{M}$ ), a PI3K inhibitor.

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*Measurement of basal production of NO by the levels of nitrite (NOS2-) and nitrate (NOS3-) or activity of SOD and CAT in aortic rings*

The tissue was homogenized with 250 µl of buffer (Tris HCl 20 mM, NaCl 150 mM, EDTA 5 mM, glycerol 10% NP 40% at pH 7.4, with protease inhibitors; aprotinin, leupeptin 1:1000 sodium vanadate) for 15-20 seconds, then centrifuged to 10000 rpm for 10 min at 4°C. The supernatant was recovered and an aliquot was taken to quantify the concentration of total protein by the Bradford method. NO, SOD and CAT activity were estimated using the colorimetric assay.

Measurement was made of nitrate and nitrite in aortic tissues. Total NO content was measured after the thoracic and abdominal aorta were incubated with Nitrate Reductase and NADPH. After a 20-minute incubation at room temperature, color reagents are added and incubated at room temperature for 5 minutes. The concentration of nitrate in the sample was calculated by taking the measured nitrite concentration and subtracting it from the total NO concentration in the sample. The colored product was read at 550-570 nm. The activity of vascular SOD and CAT was assayed by fluorometric/spectrophotometric methods as previously described by Sousa et al., 2008.

### *Western blot analysis*

Samples randomly chosen from six animals from each group were processed by Western blot. Tissue samples were homogenized in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 10% glycerol, 5 mM EDTA, 1% NP-40; Roche, Mannheim, Germany) supplemented with protease and phosphatase inhibitors (50 µg/ml of phenyl methyl sulfonyl fluoride, 10 µg/ml aprotinin, 25 µg/ml leupeptin and 100 nM orthovanadate, all from Sigma). Proteins were obtained by centrifugation for 15 min at 15000 rpm at 4°C, and the supernatant was quantified by a modified Bradford assay (BioRad, Munchen, Germany). Proteins (30 µg) were resolved using sodium dodecylsulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE) with a Mini-Protean system (BioRad) and electrophoretically transferred to nitrocellulose membranes (GE Healthcare, formerly Amersham Bioscience, Piscataway, NJ, USA). The membranes were blocked with 5% non-fat dry milk diluted in 0.05% Tween-20 Tris-buffered saline and incu-

bated overnight with the primary antibodies. Pre-stained markers (BioRad) were included for size determination.

The antibodies used were rabbit polyclonal antibody against phosphorylated-Akt (p-Akt) (Millipore-AB143, diluted 1:1000), and mouse monoclonal antibody against  $\alpha$ -tubulin (Millipore, ABO5-82, diluted 1:1000). After incubation with the primary antibody, the membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (Santa Cruz, diluted 1:10000). Immunoreactive bands were detected with an enhanced chemiluminescence system (GE Healthcare-Amersham). When necessary, membranes were stripped by using a commercial solution (Chemicon). Films were analyzed with the Chemilmager 4400\_v3.2.1\_W2000\_XP program. To minimize inter-assay variations, samples from all animal groups in each experiment were processed in parallel.

### *Statistical analysis*

Contractions are expressed in grams and percent of developed force. Relaxations are expressed as the reduction of the maximum increment in tension (contraction) obtained after administering Phe or K<sup>+</sup>. The negative logarithms of the molar concentrations of Ach and Phe required to produce 50% of the maximal response (-log EC<sub>50</sub>) were calculated (by non-linear regression analysis) for each individual concentration-response curve.

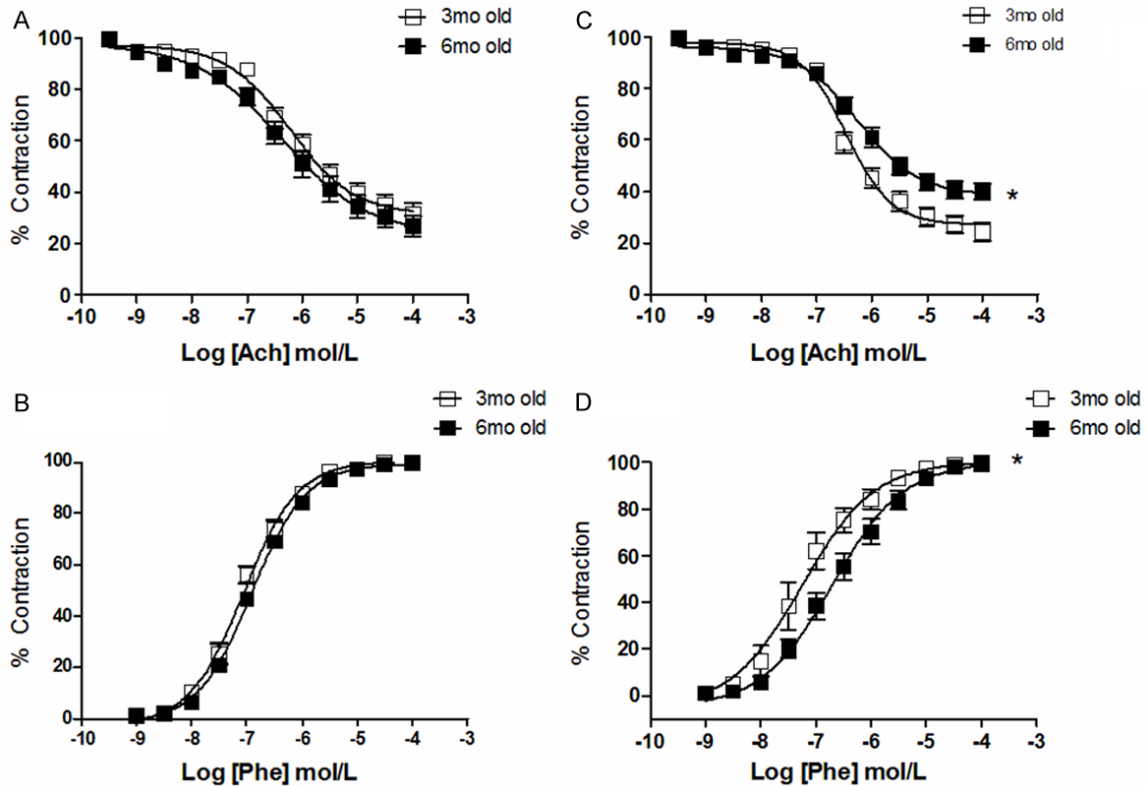
Data are presented as mean  $\pm$  standard error (S.E.M.). Statistical analysis was determined using comparisons between two groups which were assessed by Student's t-test. Comparisons between three or more groups were performed using one-way and two-way analysis of variance (ANOVA), followed by the Bonferroni or Tukey post-hoc tests. All comparisons the values of  $P \leq 0.05$  were considered statistically significant. For statistical analysis was used the Graph Pad Prism version 5.0 (Prism Graph Pad Software; San Diego, CA, USA).

## **Results**

### *The mean arterial pressure (MAP)*

MAP levels (94.25 $\pm$ 1.82 mmHg vs 95.25 $\pm$ 1.25 mmHg) and the arterial pulse pressure (PP;

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**Figure 1.** The endothelium-dependent relaxation by Ach and the contractile responses to Phe on thoracic and abdominal aortic rings. Concentration response by Ach (A) and Phe (B) in thoracic rings from rats of 3 and 6 mo old (NS). Concentration response in abdominal rings from rats of 3 and 6 mo old by Ach (C) and by Phe (D) were statistically significant. \* $P < 0.05$ .

41.75±0.92 vs 42.72±0.95) were not different from each other in 3- and 6-month-old rats.

*The endothelium-dependent relaxation by Ach and the contractile response to Phe on thoracic and abdominal aortic rings from 3- and 6-month-old rats*

The endothelium-dependent relaxant response to Ach was similar in thoracic aortic rings from 3- and 6-month-old rats (**Figure 1A**). However, in abdominal rings the relaxant response to Ach was substantially impaired in 6-month-old rats (**Figure 1C**). The impaired ability of endothelial cells to release NO was correlated with a reduced quantity of functional endothelium in abdominal aortic tissue of 6-month-old rats compared to thoracic aortic tissue of 6-month-old rats as well as the abdominal and thoracic aortic tissue of 3-month-old rats.

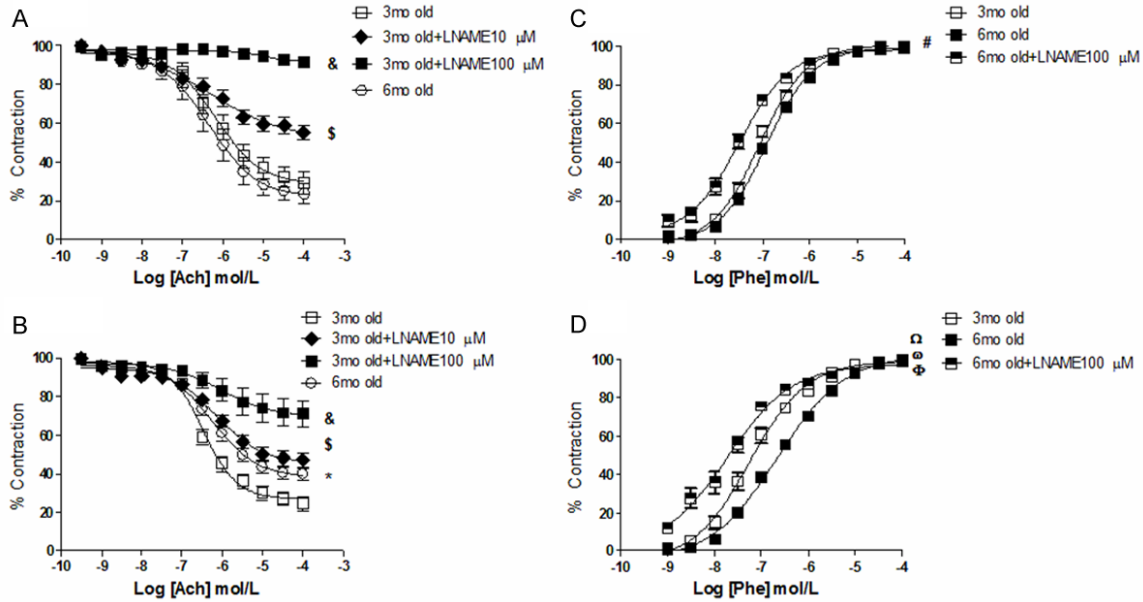
Phe caused concentration-dependent contractions in endothelium-intact thoracic and abdo-

minal aortic rings from rats. Phe-induced contractions were not different for the thoracic rings from 3- or 6-month-old rats (**Figure 1B**), but were indeed different for the abdominal rings from the rats of these two age groups. Abdominal rings from 6-month-old rats exhibited a lower sensitivity compared to the abdominal rings from 3-month-old rats (**Figure 1D**).

*The role of endothelium cells in mediating NO production by thoracic and abdominal aortic rings*

Incubation with two distinct concentrations of L-NAME, a NOS inhibitor, attenuated Ach-induced relaxation in 3-month-old rats. This relaxant effect was reduced by the 10  $\mu$ M concentration and abolished by the 100  $\mu$ M concentration of L-NAME in both thoracic (**Figure 2A**) and abdominal (**Figure 2B**) aortic rings. However, incubation of abdominal aortic rings from 3-month-old rats with L-NAME at 10  $\mu$ M resulted in a significantly attenuated Ach-in-

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**Figure 2.** Results are expressed as a percentage of contraction with phenylephrine (Phe). Data are expressed as the means  $\pm$  S.E.M. Concentration-response curves to ACh on thoracic (A) and abdominal (B) aortic rings from rats of 3 and 6 mo old with (3 mo old + L-NAME 10  $\mu$ M or 3 mo old + L-NAME 100  $\mu$ M) or without NG-nitro-L-arginine methyl ester (L-NAME). Concentration-response curves to Phenylephrine (Phe) on thoracic (C) and abdominal (D) aortic rings from rats of 3 and 6 mo with or without L-NAME 100  $\mu$ M. <sup>§</sup>P<0.05: 3 mo old vs 3 mo old + L-NAME 10  $\mu$ M; <sup>¶</sup>P<0.05: 3 mo old vs 3 mo old + L-NAME 100  $\mu$ M; \*P<0.05: 3 mo old vs 6 mo old; #P<0.001: 3 mo old vs 6 mo + L-NAME 100  $\mu$ M; <sup>°</sup>P<0.05: 6 mo old vs 6 mo + L-NAME 100  $\mu$ M; <sup>°°</sup>P<0.05: 6 mo old vs 6 mo. Two-way ANOVA test.

duced relaxation, similar to that found with the abdominal aortic rings from 6-month-old rats without L-NAME (Figure 2B).

The maximal contractile response to Phe was increased after incubation with L-NAME (100  $\mu$ M) in thoracic aortic rings from 6-month-old rats (Figure 2C). However, in abdominal aortic rings from 6-month-old rats, the incubation with L-NAME (100  $\mu$ M) restored the maximum contractile response to Phe (Figure 2D). The magnitude of contractile tone in these abdominal rings treated with L-NAME was similar to that observed in abdominal rings from 3-month-old rats treated with L-NAME (data not shown).

*The effects of H<sub>2</sub>O<sub>2</sub> on NO production through the PI3K pathway, attenuating the Phe-induced contraction in thoracic and abdominal aortic rings*

Regarding the Phe-induced contraction in endothelium-intact thoracic (Figure 3A) and abdominal (Figure 3B) aortic rings from 6-month-old rats, incubation with H<sub>2</sub>O<sub>2</sub> (1 or 10  $\mu$ M for 30 minutes) in the organ bath shifted the cumulative concentration-response curves to the right in a concentration-dependent manner.

However, addition of Wortmannin at a low concentration (0.31  $\mu$ M for 1 hour, Figure 3C, 3D) [23] totally reversed the lower sensitivity of the rings to Phe that was induced by H<sub>2</sub>O<sub>2</sub> (1  $\mu$ M).

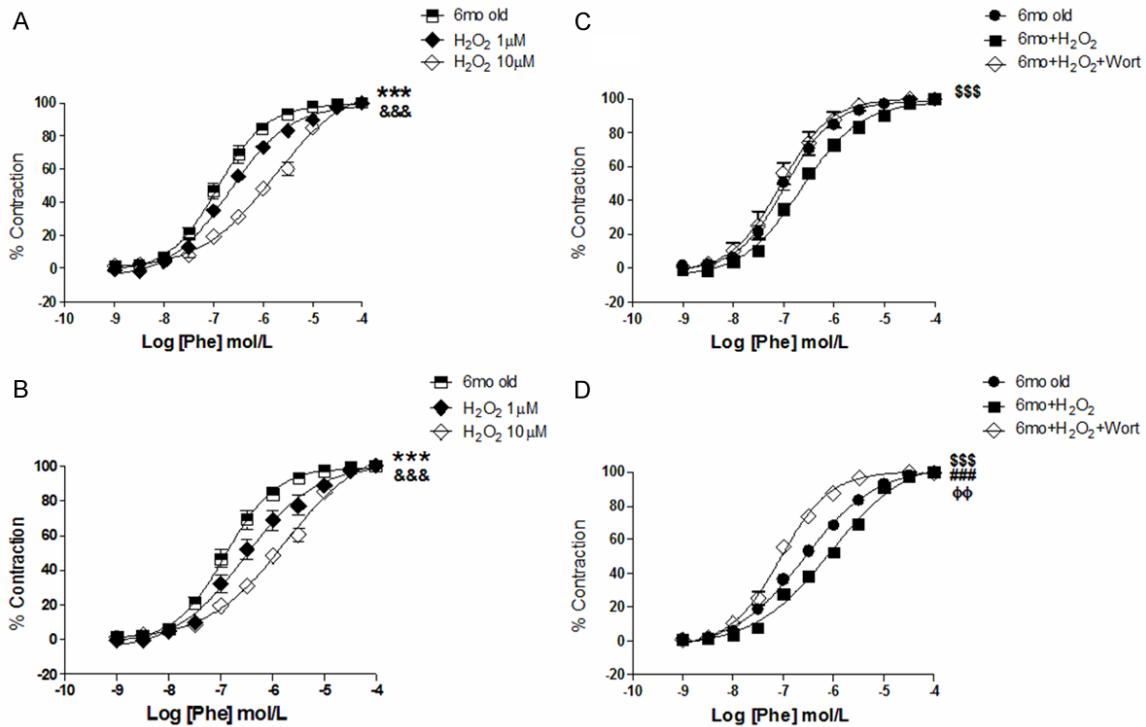
*The balance of oxidative stress in the thoracic and abdominal aorta*

The total NO concentration as well as the activity of SOD and CAT were measured under basal conditions in the thoracic and abdominal aorta. The results show that vascular NO bioavailability was reduced in thoracic (Figure 4A) and abdominal (Figure 4D) aorta of 6-month-old rats. Additionally, the enzymatic activity of SOD (Figure 4B) and CAT (Figure 4C) was higher in thoracic aortic rings from 6-month than 3-month-old rats. In the abdominal aortic rings, contrarily, the SOD activity was the same for 3- and 6-month-old rats (Figure 4E), while the CAT activity was substantially lower (Figure 4F) in 6-versus 3-month-old rats.

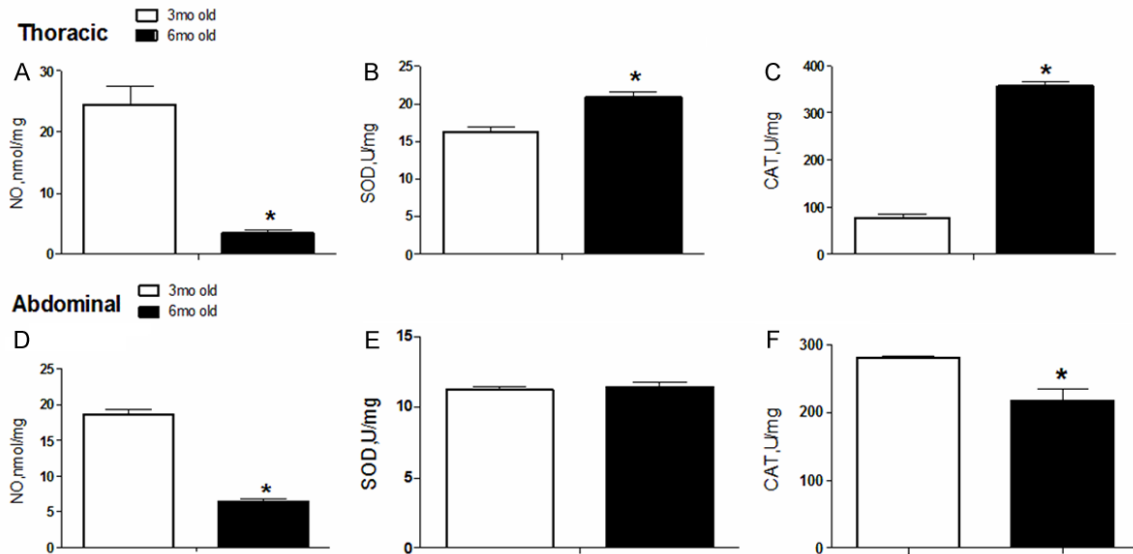
*Akt-phosphorylation in the abdominal aorta*

The expression of Akt-phosphorylation (p-Akt) was significantly enhanced in the abdominal aorta from 6-versus 3-month-old rats (Figure 5). In addition, H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) enhanced the

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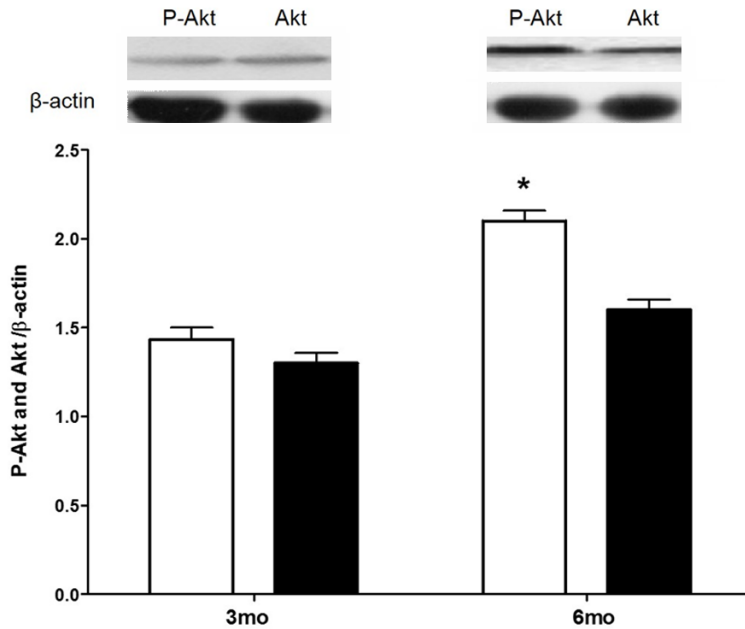


**Figure 3.** The response contractile by Phe with or without hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or H<sub>2</sub>O<sub>2</sub> + Wortmannine, an inhibitor of PI3K, on thoracic and abdominal aortic rings from rats of 6 mo old. Results are expressed as a percentage of contraction of Phe. Accumulative response by Phe in thoracic (A) and abdominal (B) aortic rings from rats of 6 mo old with H<sub>2</sub>O<sub>2</sub> (6 mo old vs H<sub>2</sub>O<sub>2</sub> 1 μM or 6 mo old vs H<sub>2</sub>O<sub>2</sub> 10 μM). Accumulative response by Phe on thoracic (C) and abdominal (D) aortic rings from rats of 6 mo old with H<sub>2</sub>O<sub>2</sub> (6 mo + H<sub>2</sub>O<sub>2</sub>; 1 μM) or H<sub>2</sub>O<sub>2</sub> + Wort (6 mo + H<sub>2</sub>O<sub>2</sub> + Wort; 1 μM + 0.31 μM, respectively). Data are expressed as the means ± S.E.M. \*\*\*P<0.001: 6 mo old vs H<sub>2</sub>O<sub>2</sub> 1 μM; &&&P<0.001: 6 mo old vs H<sub>2</sub>O<sub>2</sub> 10 μM; \$\$\$P<0.001: 6 mo old vs 6 mo + H<sub>2</sub>O<sub>2</sub>; ###P<0.001: 6 mo old vs 6 mo + H<sub>2</sub>O<sub>2</sub> + Wort; φφP<0.001: 6 mo + H<sub>2</sub>O<sub>2</sub> vs 6 mo + H<sub>2</sub>O<sub>2</sub> + Wort. Two-way ANOVA test.

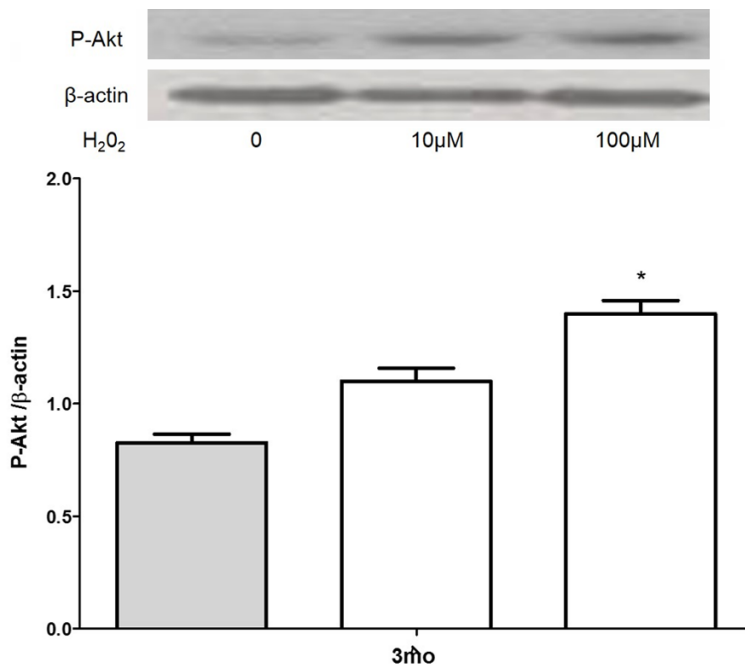


**Figure 4.** Measurement of basal production of NO by levels the nitrite (NO<sup>2-</sup>) and Nitrate (NO<sup>3-</sup>) and activity of SOD and CAT under basal conditions in thoracic and abdominal aortic rings from 3 and 6 mo old rats. Concentration of NO (A), the activity of SOD (B) and CAT (C) in thoracic aortic rings. Concentration of NO (D), the activity of SOD (E) and CAT (F) in abdominal aortic rings. Data are expressed as the means ± S.E.M. \*P<0.05 3 mo old vs 6 mo old. Student's t-test.

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**Figure 5.** The abdominal aortic content of Akt and p-Akt under basal conditions in rats of 3 and 6 mo old. Figure shows a representative blot proteins detected by Western blot were quantified by densitometric analysis. The results are expressed as the mean  $\pm$  S.E.M. (n=10). \*P<0.05. ANOVA test.



**Figure 6.** The abdominal aortic content of Akt and p-Akt at 3 mo old with 0, 10 and 100 H<sub>2</sub>O<sub>2</sub> ( $\mu$ M). Figure shows a representative blot Proteins detected by Western blot were quantified by densitometric analysis. The results are expressed as the mean  $\pm$  S.E.M. (n=10). \*P $\leq$ 0.05, ANOVA test.

p-Akt expression in the abdominal aortic rings from 3-month-old rats (**Figure 6**).

ferent vascular beds under physiological [16, 17, 19] and pathological [20, 28] conditions.

## Discussion

Diverse biological studies have been performed in different vascular beds under both physiological and pathological conditions. From these studies, it is well-known that increased production and/or bioavailability of ROS, a process known as oxidative stress, are related to the development of endothelial dysfunction. The impaired functionality of endothelial cells promotes the development of cardiovascular diseases such as hypertension [20, 21, 24, 25]. In the arterial system, the increased bioavailability of ROS associated with age has been linked to a functional remodeling of endothelial cells manifested in various ways, including a decreased production and/or increased breakdown of NO [12-14, 21], an attenuation of endothelium-dependent relaxation, and an increased contractile response to adrenergic agonists [26-28].

Several reports have shown that increased ROS is related to diminished production and increased consumption of NO, leading to an imbalance in pro- and anti-oxidant enzymatic activity [12-14, 21, 29-31]. For this reason, we examined the antioxidant-related enzymatic activity of SOD and CAT in the aorta. As a ROS marker, we analyzed the role of H<sub>2</sub>O<sub>2</sub> in the modulation of vascular tone in aortic rings from normotensive young rats. This decision was based on several studies that have shown a modulatory effect of H<sub>2</sub>O<sub>2</sub> on the contraction and the relaxation in different vascular beds under physiological [16, 17, 19] and pathological [20, 28] conditions.



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The results of the present study demonstrate that in rat aorta, the age-related increase in oxidative stress was related to functional changes in the endothelium. Accordingly, we observed an age-related decrease in the NO concentration under basal conditions. The age-related changes found presently involve the ability of the endothelium to produce and release NO, in part based on the modulating activity of SOD and CAT. However, such changes were distinct in thoracic and abdominal aortic segments, which seem to be a factor that predisposes endothelial tissue to dysfunction. These changes could be key for explaining the contrasting effects in thoracic and abdominal segments of Ach or Phe.

Compared to 3-month-old rats, with 6-month-old animals the contractile response to Phe and the endothelium-dependent relaxant response to Ach were unmodified in thoracic aorta but attenuated in abdominal aorta. This attenuation can be related to contrasting enzymatic activity in aortic segments. That is, in thoracic segments the enzymatic activity was increased, which may represent a protective mechanism for endothelium cells, maintaining the Ach-induced release of NO and an integral response to Phe. Contrarily, in the abdominal segments the SOD activity was unchanged and the CAT activity reduced, which could be associated with increase ROS obtained and therefore endothelium dysfunction, resulting in an attenuated response to Ach (relaxation) or Phe (contraction).

The key role of ROS in these phenomena was tested by the addition of H<sub>2</sub>O<sub>2</sub>. The presence of H<sub>2</sub>O<sub>2</sub> (at 1 and 10 μM) in thoracic and abdominal aortic rings of 6-month-old rats reduced the Phe-induced contraction in a concentration-dependent manner.

Moreover, the functional studies of the current contribution support the importance of NO and the PI3K pathway in the changes induced by H<sub>2</sub>O<sub>2</sub>. In this sense, the presence of L-NAME (a NOS inhibitor) or Wortmannin (an inhibitor of the PI3K pathway) restored the Phe-induced contractile response in abdominal aortic rings of 6-month-old rats to values that are similar to those observed in the aortic rings of 3-month-old rats. Hence, we can infer that H<sub>2</sub>O<sub>2</sub> (and other ROS) modulated the Phe-induced response in the abdominal aorta of 6-month-

old rats by releasing NO. This modulation is endothelium-dependent and based on the activation of the PI3K-pathway. The latter pathway exerts functional changes by regulating the functions of several systems and enzymes such as eNOS [19-21].

Molecular biology studies have shown that under basal conditions there is an increase in the content of p-Akt at the abdominal level in 6-month-old rats. Moreover, it was observed that the addition of H<sub>2</sub>O<sub>2</sub> (100 μM) increased phosphorylation-Akt expression in 3 month-old rats, as certain studies have shown [19, 29]. Thus, in the abdominal aorta of 6-month-old rats under basal conditions, the attenuation of the enzymatic activity of CAT is related to an increased bioavailability of H<sub>2</sub>O<sub>2</sub>, which plays a key role in the attenuation of the endothelium-dependent contractile response induced by the H<sub>2</sub>O<sub>2</sub>/PI3K/Akt/NO pathway (therefore, the vasomotor tone in aorta).

Finally, age is considered as a factor that significantly decreases aortic blood compliance. In vivo studies have reported that the values of the differential pressure (DP) and mean arterial pressure (MAP) were maintained at similar values in 3- and 6-month-old rats. Apparently, the aortic compliance is dependent of mechanisms of local adaptation. For instance, increasing the enzymatic activity of thoracic SOD and CAT ensures a constant supply of NO in the vascular wall by basal and induced mechanisms. This occurs despite the increase in its degradation due to increased ROS. Additionally, other endothelium-derived mediators produced under basal conditions participate in determining the level of NO. For example, hydrogen peroxide acts through PI3K/Akt to release NO through a pathway independent of calcium, which attenuates the contractile response to Phe in the abdomen and thus avoids overloading the smaller caliber vascular beds.

### Conclusions

The aortic arterial system acts as a hydraulic filter, transforming an intermittent to a laminar continuous flow. From an early age, an imbalance occurs in the generation of ROS and consequently NO availability decreases. However, local control mechanisms underlie the ability of aorta to maintain compliance.

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The current results showed differences according to the aortic segment tested (thoracic or abdominal) and the age of the rats (3 or 6 months old). The diminished response to Phe was related to age and abdominal segment, but restored by the addition of L-NAME or Wortmannin. On the other hand, the p-Akt expression increased with age or the addition of H<sub>2</sub>O<sub>2</sub>.

Overall, these results support the idea that in the aorta of young rats, the generation of oxidative stress under basal conditions triggers underlying mechanisms that maintain vascular tone. These mechanisms are not expressed homogeneously throughout the aortic segments, differentially involving changes in functional endothelium. For example, there is modulation of the activity of the antioxidant enzyme system as well as the availability of metabolites such as hydrogen peroxide. Furthermore, the current results support the role of the endothelium as a source of modulatory vasoactive substances. As previously it was mentioned, we found that H<sub>2</sub>O<sub>2</sub> is involved as a modulator of the contractile response under basal conditions, acting through the PI3K/Akt pathway at the abdominal level of normotensive rats.

Moreover, we speculate that increased enzymatic activity of SOD and CAT at the thoracic level prevents harmful effects to endothelial cells that could otherwise result from oxidative stress. This increase in enzymatic activity would allow for the maintenance of eNOS activity under basal conditions and induced stimuli, and thus sustain the role of the endothelium in the attenuation of the contractile response. Complementary studies could help to support or discard this idea.

In summary, despite the increase in the decomposition of NO and the decrease in the induced release of this compound in the abdominal aorta, a constant supply of NO in the vascular wall is maintained by the PI3K/Akt/eNOS pathway. In this way, an endothelium-dependent modulation is exerted on the contractile response to Phe in young normotensive rats.

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### Disclosure of conflict of interest

None.

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