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LONG-TERM PORTAL HYPERTENSION INCREASES THE VASODILATOR RESPONSE TO ACETYLCHOLINE IN RAT AORTA. ROLE OF PROSTAGLANDIN I₂

Running Title: Prostaglandin I₂ in long-term portal hypertension

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Abstract

We analyzed both the effect of long-term portal hypertension on the vasomotor response to acetylcholine in rat aorta, and the mechanism involved in this response. For this purpose, sham-operated and prehepatic portal hypertension rats (triple partial portal vein ligation) were used at 21 months after the surgery. The participation of nitric oxide and cyclooxygenase-derivates in the vasodilator response elicited by acetylcholine, after incubation with N^ω-nitro-l-arginine methyl ester, indomethacin, SC-560, NS-398, tranylcypromine and furegrelate was analyzed. Nitric oxide, thromboxane B₂ and 6-keto prostaglandin F_{1α} releases were measured, and sodium nitroprusside, U-46619, prostaglandin I₂ and forskolin vasomotor responses were also analyzed. Cyclooxygenase-1 and cyclooxygenase-2 expression was also analyzed. The acetylcholine vasodilating response was higher in portal hypertensive rats. Thromboxane A₂ and nitric oxide release and sodium nitroprusside and U-46619 sensitivity were similar in both groups. Prostaglandin I₂ release was not modified by portal hypertension, but vasodilator responses to this prostanoid and to forskolin were higher in portal hypertensive rats. Cyclooxygenase-1 and cyclooxygenase-2 expression remained unmodified by surgery. In conclusion, the increased vasodilation to acetylcholine is maintained in long-term portal hypertension. While the participation of endothelial NO remained unmodified, the COX-2 derivate PGI₂ does participate through an increased vasodilator response.

Keywords: Long-term portal hypertension; Aging; Acetylcholine; Nitric Oxide; Prostaglandin I₂.

Introduction

Portal hypertension (PH) is a clinical syndrome defined by a pathological increase in portal system blood pressure^{1,2}; it is the most frequent and serious complication in chronic liver disease^{1,2}. In prehepatic PH there is no underlying liver disease³ and, thus, its complications are due to the effects of its associated vasodilation on the splanchnic and systemic circulatory beds^{1,2}.

In experimental short-term PH (one month or less) systemic and splanchnic vasodilation is the factor behind all the vascular changes. The main factor behind this effect is nitric oxide (NO)⁴. But hyporesponsiveness to vasoconstrictors like noradrenalin (NA) has also been reported^{4,5}, and it seems to be associated to increased endothelial NO production^{6,7}. Prolonged PH (6 months) has been associated to a diminished vasoconstrictor response to NA, but it is less pronounced than in short-term PH. This NA effect is also modulated by the NO pathway⁶, indicating that the role of NO in the effect of PH is modified with time. Additionally, the participation of prostaglandin I₂ (PGI₂) has also been reported to change with time⁸. These results suggest a time-dependent change in the mechanisms implicated in increased vasodilation in PH.

In contrast to the situation in short-term PH, endothelial dysfunction and an increase in endothelium-dependent contraction have been reported in aging. This effect of aging is mainly mediated by an increase in vasoconstrictor prostanoids^{9,10} and/or a decrease of vasodilator factors such as PGI₂ or NO^{11,12}. The physiological aging process would thus be also involved in the effects of long-term PH.

Since PH is maintained for several years in humans, we analyzed the impact of long-term PH on vascular aging in rat aorta. The current study was designed to analyze two points: endothelial function in aorta from rats 21 months after portal ligation, and the mechanisms involved in the alterations if any were found.

Methods

Animals

Male Wistar rats, (≈ 250 g) were used. The animals were fed rat chow and water *ad libitum*. They were housed in groups of three to four animals, in a light/dark-controlled room, with an average temperature ($22 \pm 2^\circ\text{C}$) and humidity (65-70%). The animals were randomly divided into two groups: sham-operated rats (SO), and rats with prehepatic PH produced by triple partial portal vein ligation (TPVL). All the studies conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Surgical Techniques

The animals were anesthetized by i.m. injection of Ketamine (100 mg/kg) and Xylazine (12 mg/kg). The surgical procedure to produce PH by triple partial ligation of the portal vein has been described previously¹³. In brief, a midline abdominal incision was made, the portal vein was isolated and three ligatures, fixed on a sylactic guide, were performed in its superior, middle and inferior portions. The stenoses were calibrated by a simultaneous ligation (4-0 silk) around the portal vein and a 20-gauge blunt-tipped needle. The midline incision was closed in two layers with an absorbable suture (Polyglycolic acid) and 3-0 silk. Analgesia was maintained during 24 hours with Buprenorphine (0.05 mg/8 h s.c.). In SO rats, the procedure was the same except for ligation in the portal vein.

All the animals were sacrificed by exsanguination through the inferior vena cava 21 months after the operation. Previously, the rats of both groups were anesthetized by i.m. injection of

Ketamine (100 mg/kg) and Xylazine (12 mg/kg), a midline abdominal incision was made and portal pressure was registered and mesenteric venous vasculopathy and collateral circulation were studied. The thoracic aorta was carefully dissected out, cleaned of connective tissue, cut into 4 mm-long segments and placed in Krebs–Henseleit solution (KHS) (containing, in mmol/L: NaCl 115, CaCl₂ 2.5, KCl 4.6, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, glucose 11.1, Na₂EDTA 0.03) at 4°C.

Portal Pressure Measurement

Splenic pulp pressure, an effective indirect measurement of portal pressure (PP), was measured by inserting a 20-gauge fluid-filled needle into the spleen parenchyma. The needle was joined to a PE-50 tube, then connected to a pressure recorder (PowerLab 200 ML 201) and a transducer (Sensor SN-844) with a Chart V4.0 computer program (ADI Instruments); the recorder was re-calibrated before each experiment.

Mesenteric venous vasculopathy and portosystemic collateral circulation study methods

The existence of portal hyperpressure in the long-term (21 months) was confirmed by the development of mesenteric venous vasculopathy and portal-systemic collateral circulation. First, a midline abdominal incision with a large bilateral subcostal extension was performed. Mesenteric venous vasculopathy, a characteristic feature of splanchnic venous congestion, is shown as dilation and tortuosity of the superior mesenteric vein branches¹⁴.

Portosystemic collateral circulation was studied by macroscopic examination of the areas in which the collateral venous circulation was developed, i.e. the splenorenal, gastroesophageal, colorectal and hepatic hilum, were carefully studied for the presence of increased collateral veins¹⁵.

Vascular reactivity

We used the method described by Nielsen and Owman¹⁶. Briefly, two parallel stainless steel pins were introduced through the lumen of the aortic segment: one was fixed to the bath wall and the other connected to a force transducer (Grass FTO3C; Grass Instruments Co., Quincy, MA, USA); this was connected in turn to a model 7D Grass polygraph. Segments were suspended in an organ bath containing 5 mL KHS at 37° C continuously bubbled with a 95% O₂–5% CO₂ mixture (pH 7.4). The segments were subjected to a tension of 1 g, which was readjusted every 15 min during a 90-min equilibration period before drug administration. After the equilibration period, each aortic ring was exposed twice to KCl (75 mmol/L), to assess its maximum contractility. After a washout period, the presence of vascular endothelium was confirmed by the ability of 10 µmol/L acetylcholine (ACh) to relax segments precontracted with 0.1 µmol/L noradrenaline (NA).

Vasodilator response to ACh (0.1 nmol/L- 10 µmol/L) in 0.1 µmol/L NA-precontracted segments, and vasoconstrictor response to NA (0.1 nmol/L- 10 µmol/L) were performed in aortic segments from SO and PH rats.

To determine the participation of NO in response to ACh, arteries from both experimental groups were incubated with *N*^ω-nitro-L-arginine methyl ester (L-NAME, 0.1 mmol/L, a non-selective NOS inhibitor). This drug was added 30 min before a concentration–response curve to ACh was performed.

To assay the participation of endothelial prostanoid factors on the response to ACh, segments from both experimental groups were incubated with the unspecific inhibitor of cyclooxygenase (COX)-derived prostanoids indomethacin (10 µmol/L). This drug was added 30 min before the concentration–response curve to ACh was performed. To clarify the participation of COX isoforms in the ACh-induced relaxation, segments from both groups of

rats were incubated with the specific COX-1 inhibitor SC-560 (50 nmol/L) or the specific COX-2 inhibitor, NS-398 (10 μ mol/L). In another set of experiments, segments were incubated with the prostaglandin I₂ (PGI₂) synthesis inhibitor tranylecypromine (TCP, 10 μ mol/L) or with the thromboxane A₂ (TXA₂) synthase inhibitor, furegrelate (1 μ mol/L), to analyze the possible involvement of these vasoactive factors in either experimental group.

To assess possible differences in the responses induced by TXA₂, PGI₂ and NO in arteries from both groups, vasoconstrictor concentration-response curves to the TXA₂ mimetic U-46619, (1 nmol/L -10 μ mol/L), and vasodilator concentration-response curves to exogenous PGI₂ (1 nmol/L-5 μ mol/L) or to the NO donor sodium nitroprusside (SNP, 0.1 nmol/L-10 μ mol/L) in 0.1 μ mol/L NA-precontracted segments, were performed in arteries from both groups of rats. To assess the participation of superoxide anions (O₂⁻) on the vasodilator response to SNP, 0.1 mmol/L tempol (a O₂⁻ scavenger) was added to the chamber 30 minutes before the concentration-response curve to SNP was performed.

Concentration-response curves to forskolin (0.1 nmol/L-10 μ mol/L) were performed in both experimental groups to assay differences in adenylyl cyclase activation.

Nitric oxide release

Nitric oxide release was measured as previously described¹⁷. Briefly, aortic segments from SO and PH rats were subjected to a resting tension of 1 g similarly to vascular reactivity experiments. After an equilibration period of 60 min in HEPES buffer (in mmol/L: NaCl 119; HEPES 20; CaCl₂ 1.2; KCl 4.6; MgSO₄ 1; KH₂PO₄ 0.4; NaHCO₃ 5; glucose 5.5; Na₂PHO₄ 0.15; pH 7.4) at 37° C, arteries were incubated with the fluorescent probe 4,5-diaminofluorescein (2 μ mol/L) for 45 min and medium was collected to measure basal NO release. Once the organ bath was refilled, an ACh concentration-response curve (0.1 nmol/L - 10 μ mol/L) was applied at 2-min intervals. The fluorescence of the medium was measured at room temperature using a spectrofluorimeter (LS50 Perkin Elmer instruments, FL WINLAB Software) with excitation wavelength set at 492 nm and emission wavelength at 515 nm. Blank measurement samples were collected in the same manner but without aorta segments in order to subtract background emission. Some assays were performed in the presence of L-NAME (0.1 mmol/L) to ensure the specificity of the method. The amount of NO released was expressed as arbitrary units/mg tissue.

Western blot analysis for eNOS, iNOS, COX-1 and COX-2

Aortic segments from SO and PH rats were homogenized in a boiling buffer composed of 1 mmol/L sodium vanadate (Sigma-Aldrich), 1% SDS and pH 7.4, 0.01 mol/L Tris-HCl, the protein content was measured with a DC protein assay kit (Bio-Rad). Homogenates containing 30 μ g protein were electrophoretically separated on a 7.5% (for eNOS and iNOS) or 10% (for COX-1/2) SDS-PAGE, then transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked in 5% nonfat milk in Tris-HCl buffered saline 0.1% Tween 20 (TBS-T). Subsequent washes were done in TBS-T, and the membranes were incubated with antibody against eNOS (1:1000, Transduction Laboratories), iNOS (1:1000, Transduction Laboratories), COX-1 (1:500, Cayman Chemical) and COX-2 (1:200; Cayman Chemical), and individual horseradish peroxidase-conjugated secondary antibodies in blocking buffer. Immunoreactive proteins were detected by chemiluminescence with ECL Plus (Amersham). Endothelial cells, platelets and activated macrophages were used as positive controls in eNOS, COX-1 and iNOS/COX-2 blots, respectively. Signals on the immunoblot were quantified using a computer program (NIH Image V1.56). The same membrane was used to determine actin expression, and

the content of the latter was used to correct protein expression in each sample, by means of a monoclonal anti- α -actin antibody (1: 3000 dilution, Sigma).

Superoxide anion formation

Hydroethidine (HE), an oxidative fluorescent dye, was used to evaluate superoxide anion levels in situ, as previously described¹⁷. Briefly, aorta segments from SO and PH rats were cryoprotected with 30%w/v sucrose in PBS, embedded in optimum cutting temperature compound, OCT Tissue Tek, and 20 μ m cryostat sections were placed on a glass slide. HE (5 μ M) was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected, humidified chamber at 37°C for 30 min. Images were obtained with a LEICA (TCS ST2 DM IRE2) laser scanning confocal microscope (excitation 488 nm, emission 610 nm). Laser and image settings were fixed for the acquisition of images from both control and orchidectomized rats. The photomicrographs show the intensity and location of the oxidized HE, which reflects superoxide anion production, so that comparison of these groups could be made.

Prostanoid, endothelin (ET) and c-reactive protein (CRP) production

Segments of rat aorta were pre-incubated for 30 min in 5 mL of KHS at 37 C and continuously gassed with a 95% O₂–5% CO₂ mixture (stabilization period). After several 10-min washout periods in a bath of 0.2 mL KHS, the medium was collected to measure basal production. Once the chamber was refilled, the ACh concentration–dependent curve (0.1 nmol/L to 10 μ mol/L) was applied at 2-min intervals. Some samples were collected in presence of 0.1 mmol/L L-NAME or 1 μ mol/L furegrelate to assay the effect of these drugs on the production of the different vasoactive substances.

To measure the release of CRP, ET and the stable metabolites of, TXA₂ and PGI₂, we used TXB₂ and 6-keto PGF_{1 α} , respectively, we used Rat C-Reactive Protein ELISA kit (BD Biosciences), Endothelin EIA kit, Thromboxane B₂ EIA Kit, and 6-keto-Prostaglandin F_{1 α} EIA Kit (Cayman Chemical, USA). Different assays were made following the manufacturer's instructions. Results were expressed as ng CRP/mL mg tissue, pg ET/mL mg tissue, and pg prostanoid /mL mg tissue.

Drugs

L-NA hydrochloride, HE, SNP, ACh chloride, L-NAME hydrochloride, indomethacin, TCP, NS-398, SC-560, furegrelate, PGI₂ sodium salt, U-46619 and forskolin. Stock solutions (10 mmol/L) of drugs were made in distilled water; except for noradrenaline, dissolved in a NaCl (0.9%)–ascorbic acid (0.01% w/v) solution; indomethacin, PGI₂, forskolin, U-46619 and SC-560, were solubilized in ethanol and administered from a prepared stock in such a way that the maximal ethanol concentration of the medium was less than 0.001% (vol./vol.); NS-398 was solubilized in DMSO. All stock solutions were kept at –20°C, and appropriate dilutions were made in KHS on the day of the experiment.

Statistical analysis

The results are expressed as mean \pm S.E.M. for the number of rats indicated. Statistical analysis compared the curve obtained in the presence of the different substances with the control curve by means of repeated-measure two-way analysis of variance (ANOVA) using Graph Pad Prism 4.0 Software (San Diego, CA). For the experiments on NO and prostanoids release, the

statistical analysis employed was Student's *t* test for unpaired experiments. A *P* value of less than 0.05 was considered significant.

Results

Portal pressure

The portal pressure (PP) was higher in PH rats than in SO rats (SO: 9.07 ± 1.57 mmHg; PH: 12.07 ± 1.45 mmHg; $P < 0.05$).

Mesenteric venous vasculopathy

Rats with PH showed macroscopic mesenteric vasculopathy, with dilation and tortuosity of the superior mesenteric vein and of its branches in 62.5 % ($n = 5$) of the animals. In 2 rats it was spontaneous (Grade II), while in 3 animals it was related to the superior mesenteric vein clamping (Grade I)

Splenomegaly

Spleen weight (1.04 ± 0.27 g vs. 1.32 ± 0.34 g) and spleen weight/body weight ratio (0.22 ± 0.06 vs. 0.18 ± 0.07) were similar in both experimental groups.

Collateral circulation

In long-term PH, rats presented portosystemic collateral circulation (pararectal and paraesophageal types in 100% and splenorenal in 75%). One animal developed portohepatic collateral vessels through the hepatic accessory vein.

Liver weight

Liver weight (15.59 ± 1.89 g vs. 15.82 ± 2.92 g) and the liver weight/body weight ratio (2.56 ± 0.17 vs. 2.53 ± 0.25) were similar in both experimental groups.

Body Weight

Body weight gain was similar in SO rats (367.31 ± 83.50 g) and PH rats (378.85 ± 86.38 g).

Vascular reactivity experiments

The maximum responses elicited by 75 mol/L KCl (SO: 1590 ± 99.3 mg; 1728 ± 105.5 mg; $P > 0.05$), and 0.1 μ mol/L NA (SO: 1483 ± 92.34 mg; 1554 ± 143.6 mg; $P > 0.05$) were similar in aorta of both experimental groups.

The concentration-response curves to NA (0.1 nmol/L-10 μ mol/L) were similar in both groups of rats (data not shown). The vasodilator response to ACh (0.1 nmol/L-10 μ mol/L) was higher in PH rats compared to SO rats (Figure 1). Preincubation with 0.1 mM L-NAME practically abolished this response in both groups of rats (Figure 1).

Preincubation with the unspecific COX inhibitor indomethacin (10 μ mol/L) or with the specific COX-2 inhibitor NS-398 (10 μ mol/L) increased the vasodilator response to ACh in aortic segments from SO rats (Figure 2), but decreased it in segments from PH rats (Figure 2). Preincubation with the specific COX-1 inhibitor SC-560 (50 nmol/L) did not modify vasodilator response to ACh in either rat groups (Figure 2).

The vasodilator response to ACh was augmented in aortic rings from SO rats when they were preincubated with the TXA₂ synthesis inhibitor furegrelate (1 μ mol/L), while it was not modified in segments from PH rats (Figure 3). Preincubation with the PGI₂ synthesis inhibitor

TCP (10 $\mu\text{mol/L}$) did not modify the vasodilator response elicited by ACh in segments from SO rats, and decreased it in aortic rings from PH rats (Figure 3).

The vasoconstrictor response to the TXA₂ analogue U-46619 (0.1 nmol/L-10 $\mu\text{mol/L}$) was similar in aortic segments from both groups of rats (Figure 4D), as was the vasodilator response to the NO donor SNP (0.1 nmol/L-10 $\mu\text{mol/L}$) (Figure 4A). Preincubation with 0.1 mmol/L tempol did not modify vasodilator response to SNP in both groups of rats (Figure 4A). The vasodilator responses to exogenous PGI₂ (0.1 nmol/L-5 $\mu\text{mol/L}$) and to the adenylate cyclase activator forskolin (0.1 nmol/L-10 $\mu\text{mol/L}$) were significantly higher in segments from PH rats (Figures 4B and C).

NO release

Basal NO release was similar in both groups of rats, and ACh (0.1 nmol/L-10 $\mu\text{mol/L}$) increased NO release to a similar degree (Figure 5A). Preincubation with L-NAME (0.1 mmol/L) abolished NO release in arteries from both groups (data not shown).

CRP release

Basal CRP release was similar in both groups of rats (13.33 ± 1.2 ng/mL mg tissue vs. 11.75 ± 0.72 ng /mL mg tissue; $P > 0.05$; $n = 4$ each group). ACh stimulated increased CRP release in both groups in a similar way (20.67 ± 4.63 ng /mL mg tissue vs. 17.25 ± 2.61 ng/mL mg tissue; $P > 0.05$; $n = 4$ each group).

ET release

Basal ET release was similar in both groups of rats (4.6 ± 0.21 pg/mL mg tissue vs. 4.9 ± 0.44 pg /mL mg tissue; $P > 0.05$; $n = 4$ each group). ACh stimulated increased ET release in both groups in a similar way (5.7 ± 0.25 pg /mL mg tissue vs. 6.3 ± 0.63 pg/mL mg tissue; $P > 0.05$; $n = 4$ each group).

Prostanoid production

PH did not modify either basal or ACh-stimulated production of TXB₂ or 6-ketoPGF_{1 α} (Figures 5 B and C). TXB₂ production was increased by 0.1 mM L-NAME (Figure 5B). 1 $\mu\text{mol/L}$ furegrelate or 0.1 mmol/L L-NAME decreased 6-keto PGF_{1 α} production in PH (Figure 5C).

Superoxide anion formation

Superoxide anion formation was similar in both groups of rats (Figure 6).

Western Blot

Expression of eNOS, iNOS, COX-1 and COX-2 was similar in both experimental groups (Figure 7)

Discussion

Long-term PH rats show persistent splanchnic alterations and changes in aortic reactivity related to their portal hyperpressure. In the current study we demonstrated for the first time that in rats with a very long evolutive postoperative period (21 months), the ACh vasodilator response was higher than in SO animals. The increased response to COX-2 derivate PGI₂ seems to play a main role in this response. The operated rats showed portal hyperpressure, splenomegaly and systemic collateral vessels. The persistence of changes related to long-term PH indicates that triple partial portal vein ligation in the rat is an appropriate experimental model and that it maintains its validity even in very late states, making it possible to establish a correlation with human PH.

The hemodynamic alterations of PH do not involve only of just the porto-hepatic system hemodynamics but also affect splanchnic and systemic circulation^{4,18}. Increased arterial

vasodilation is the pathophysiological hallmark of the development of the hyperdynamic circulatory syndrome in PH¹⁹. Thus, arterial vasodilation leads to central hypovolemia, sodium retention and increased intravascular volume^{18,19}. This syndrome induces the most common and potentially lethal complications of liver cirrhosis, namely gastroesophageal varices, spontaneous bacterial peritonitis and portosystemic encephalopathy²⁰.

The vasodilatory state observed in short-term PVL rats (2-4 weeks) has been basically attributed to two mechanisms: increased circulating vasodilators and decreased response to vasoconstrictors^{19,21,22}. The involved vasodilators include mainly NO, but PGI₂ has also been proposed¹⁸. In turn, hyporeactivity to vasoconstrictors such as NA also contributes to the increased vasodilation²³.

It is well-established that short-term PH in rats induces an increase in vasodilator response to ACh in the splanchnic and peripheral vascular beds^{18,24,25}. Our first objective was to analyze whether the vasodilator response to ACh remained increased in long-term PH. We observed a higher response to ACh in segments from PH rats compared to SO rats, indicating the persistence of the increased vasodilator response.

The higher vasodilator response observed in short-term PH has been principally attributed to the increased release of circulating vasodilators with the higher NO release being the principal underlying mechanism¹⁸. Therefore, we analyzed the vasodilator response to ACh in the presence of the NO synthesis inhibitor L-NAME in aortic segments from both experimental groups. The response to ACh was practically abolished in segments from both SO and PH rats, confirming that NO is the main vasodilator factor implicated in the response to ACh, and suggesting that NO release and/or vasodilating response could be increased in PH. Our results show that eNOS and iNOS expression, and NO release were similar in both groups of rats. The vasodilator response to the NO donor SNP was also similar in both groups of rats. Altogether, these results seem to discount this hypothesis.

Since in short-term PH rats the vascular alterations could be a consequence of oxidative stress^{2,26}, we analyzed both the release of superoxide anions and their participation on the vasodilator response to NO donor SNP in our experimental conditions. Neither vasodilator response to SNP in presence of the superoxide anion scavenger tempol nor superoxide anion release were modified in our experimental conditions. These results seem to indicate that long-term PH can produce a rearrangement in oxidative stress, in contrast to results observed in short-term PH.

One of the most commonly used inflammation markers is CRP, which can predict risk for coronary events^{27,28}. This inflammatory marker has been reported to rise in short-time PH with and without cirrhosis²⁹. In our experimental model, PCR levels were not modified by PH. This observation suggests a possible re-balancing of inflammatory markers over time in PH.

Prostanoids derived from COX-1/2 also play a central role in this inflammatory process⁵. To analyze the possible participation of these enzymes and their derived products in the increased vasodilator response to ACh induced by PH, the effect of the non selective COX inhibitor indomethacin was tested. The results obtained indicate that vasoactive products from both COX-1 and/or COX-2 isoforms could participate in the vasomotor response to ACh in both cases. The differential effect of indomethacin, which increases vasodilator response in SO rats and decreases it in PH animals, indicates that the COX derivatives participate in both cases but with opposite effects that are apparently mediated by different mechanism/s: in segments from PH rats indomethacin inhibits the vasodilator response while in SO animals it facilitates this response.

The expression of COX-1 was similar in segments from both experimental groups. The presence of COX-1 inhibitor SC-560 did not modify the vasodilator response to ACh in any

case. This result, in contrast with previous results reported in aging³⁰, indicates that the participation of COX-1 derivatives in vasomotor response to ACh is very limited. Vasomotor function is the result of contractile and relaxing mediators which can both be released by COX-1 activation. Therefore, some compensatory effect between these derivatives could explain the absence of a net effect observed in aorta from the old rats. In any case, these results indicate that COX-1 derivatives do not participate in the altered vasomotor response to ACh in long-term PH, as they also do not in short-term PH²⁴.

COX-2 protein expression was also similar in arteries from both experimental groups. The presence of the selective COX-2 inhibitor NS-398 caused a similar effect and of the same level as the one described with indomethacin: increasing and decreasing the vasodilator response to ACh in segments from SO and PH rats, respectively. This result reinforces the lack of participation by the COX-1 derivatives in the vasodilator response to ACh in aged and aged plus long-term PH, and, consequently, supports the hypothesis that the effect is mediated by COX-2 derivatives. PGI₂ is the most important vasodilator COX-2 derivative, but its participation in short-term PH is controversial, since both participation and no participation have been reported³¹⁻³⁵. On the other hand, the role of PGI₂ in PH has been reported to be different depending on the evolution of the PH⁸. Therefore, we analyzed the possible participation of this vasodilating prostanoid in the increased vasodilation observed in long-term PH. Preincubation with the PGI₂ synthesis inhibitor TCP decreased the vasodilator response to ACh in segments from PH rats, but did not modify it in segments from SO rats, indicating a role for this vasodilator prostanoid only in segments from PH rats. This effect could be mediated by an increase in PGI₂ release and/or sensitivity. Our results indicate that a greater vasodilator response would be the implicated mechanism, because the release of 6-ketoPGF_{1α} is similar in both experimental groups, while the vasodilator response to exogenous PGI₂ is increased in PH rats.

The increased response to PGI₂ could be due to differences in signalling pathways. The vasodilator response to PGI₂ is mediated by adenylyl cyclase activation, which results in the production of nucleotide second messenger cAMP. This nucleotide in turn causes smooth muscle relaxation by different mechanisms³⁶. The fact that the vasodilator response to the adenylyl cyclase activator forskolin was also higher in segments from PH animals indicates that the higher vascular response of PGI₂ in PH is attributable to c-AMP-dependent events, as has previously been described in short-term PH^{37,38}. Taken together, these results show that the increased vasodilatory response to ACh in aorta from aged PH rats is mediated by an increase in the response to PGI₂ but not by NO dependent events. Additionally, these results indicate differences in the factors and mechanisms involved in the augmented vasodilator response in PH over the time.

Since NS-398 inhibition of COX-2 only increases vasodilator response to ACh in segments from SO rats, the possibility that COX-2 vasoconstrictor derivatives have different roles in this response cannot be ruled out. TXA₂ is the main vasoconstrictor COX derivative. The exact involvement of TXA₂ in the development of PH is still unclear, since both an increase and non participation of TXA₂ in hyperdynamic circulation have been reported^{39,40}. Incubation with the TXA₂ synthesis inhibitor furegrelate induced a decreased relaxation to ACh in SO segments, and this seems to confirm the participation of this prostanoid in aging, as has been previously described⁹. However, the relaxation remained unmodified in segments from PH rats, suggesting that release and/or the sensitivity to TXA₂ could be diminished in long-term PH. Further analysis indicates that both TXB₂ release and the vasoconstrictor response to the TXA₂ analogue U-46619 were similar in segments from both experimental groups. Complex interactions have been reported between COX derivatives; thus the synthesis of one prostanoid is usually accompanied by changes in the synthesis of the others. This imbalance can be shifted toward hypo, hyper or compensatory responses⁴¹. Therefore, we analyzed the release of the PGI₂ metabolite 6-keto PGF_{1α} after preincubation with furegrelate. Our results show that after furegrelate inhibition of TXA₂, there is a decrease in 6-ketoPGF_{1α} release in long term PH rats, indicating a new equilibrium between prostanoid synthesis and probably explaining the lack of modification in the vasodilator response to ACh in PH rats after preincubation with furegrelate.

Our observations demonstrate functional PGI₂ participation in long-term PH. This participation should have been demonstrated by a remnant vasodilator response to ACh in presence of the NO synthesis inhibitor L-NAME, but this did not occur, as we report above. In fact, in aortic rings from long-term PH rats, L-NAME practically abolished the vasodilator response to ACh, suggesting that NO is the only endothelial factor participating in this response. Taking into account that evidences for a considerable “cross-talk” between NO and PG biosynthesis pathway are increasing⁴², it could be reasonable to hypothesize that the total inhibition of vasodilator response to ACh observed in long-term PH could be due to some modulation of the biosynthesis of prostanoids associated to the inhibition of NO synthesis. The results obtained show increased TXB₂ release and decreased 6-keto PGF_{1α} release in presence of L-NAME, confirming our hypothesis.

Additionally, a hyporesponsiveness to NA has been reported in short-term PH, and it was associated with increased endothelial NO release⁶. The fact that NO response were similar in both groups of rats, and NO levels were not modified in our long-term experimental conditions, explains the similar NA response in PH rats and SO rats.

ET is known to play a role in the pathogenesis of PH through a decrease in circulating ET in short-term PH rats⁴³. In our long-term PH experimental model, ET levels were similar to those in SO rats, newly indicating that the mechanism/s implicated in vascular tone in long-term PH change with time, constituting the main finding of this study.

In conclusion, our results show an increased vasodilation to ACh in long-term PH. While the participation of endothelial NO remained unmodified, the COX-2 derivate PGI₂ does participate through an increased vasodilator response.

Perspectives

It is remarkable that in humans, PH presents a long term chronic evolution. However, to the best of our knowledge, there are no studies analyzing the effect of long-term PH on systemic vasculature, and this confers relevance on our study. For the first time, we have demonstrated that an increased PGI₂ vasodilator response seems to contribute to the decreased systemic vascular resistance, constituting a novel target for pharmacological therapies in long-term PH.

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Conflict of interests

No conflict of interests exist

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Figure Legends

Figure 1: Effect of preincubation with 0.1 mmol/L L-NAME on the vasodilator response to ACh in aortic segments from SO and PH rats. Results (mean \pm SEM) were expressed as a percentage of the contraction of the previous tone elicited by 0.1 μ mol/L NA. n= number of animals.

Figure 2: Effect of preincubation with 10 μ mol/L indomethacin, 50 nmol/L SC-560 and 10 μ mol/L NS-398 on the vasodilator response to ACh in aortic segments from SO (A) and PH (B) rats. Results (mean \pm SEM) were expressed as a percentage of the contraction of the previous tone elicited by 0.1 μ mol/L NA. n= number of animals.

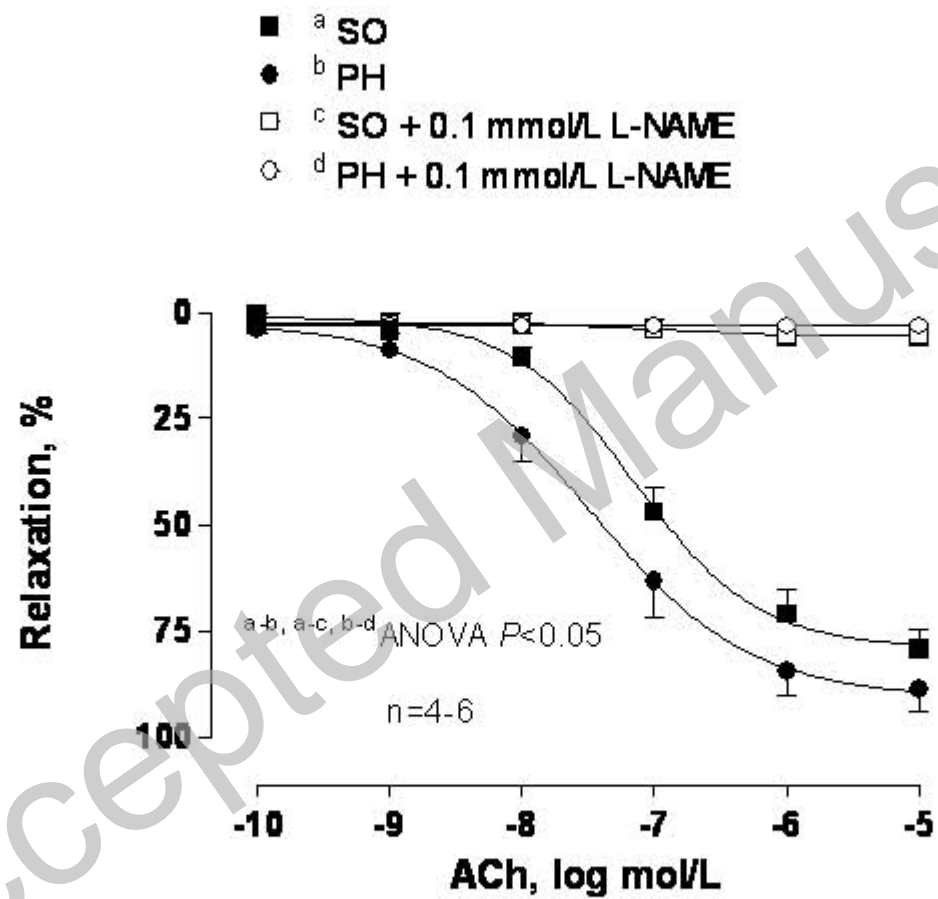
Figure 3: Effect of preincubation with 10 μ mol/L TCP and 1 μ mol/L furegrelate on the vasodilator response to ACh in aortic segments from SO (A) and PH (B) rats. Results (mean \pm SEM) were expressed as a percentage of the contraction of the previous tone elicited by 0.1 μ mol/L NA. n= number of animals.

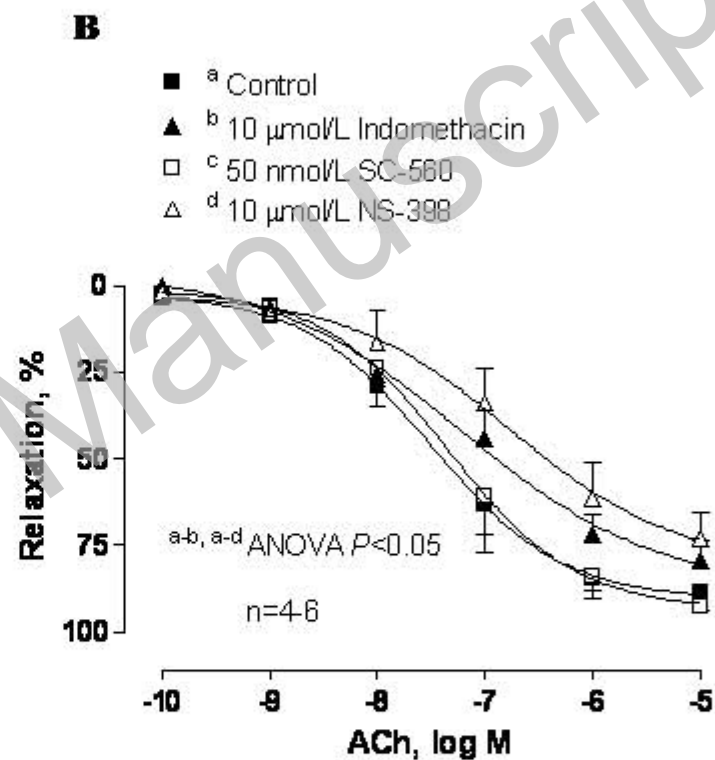
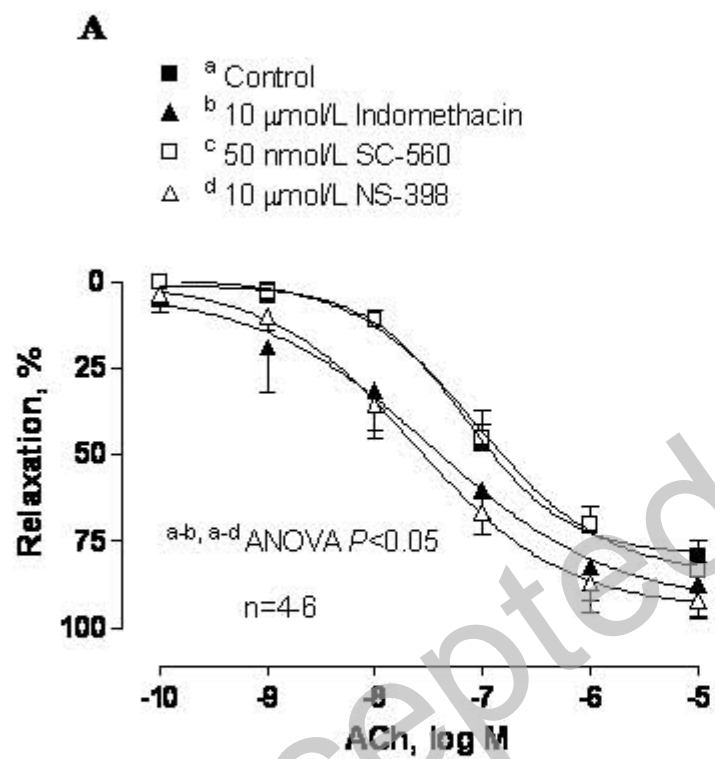
Figure 4: Vasoconstrictor response to U-46619 (B) and vasodilator responses to SNP (A), PGI₂ (C) and forskolin (D) in aortic segments from SO and PH rats. Vasoconstrictor results (mean \pm SEM) were expressed as a percentage of the contraction of the previous tone elicited by 75 mmol/L KCl. Vasodilator results (mean \pm SEM) were expressed as a percentage of the contraction of the previous tone elicited by 0.1 μ mol/L NA. n= number of animals.

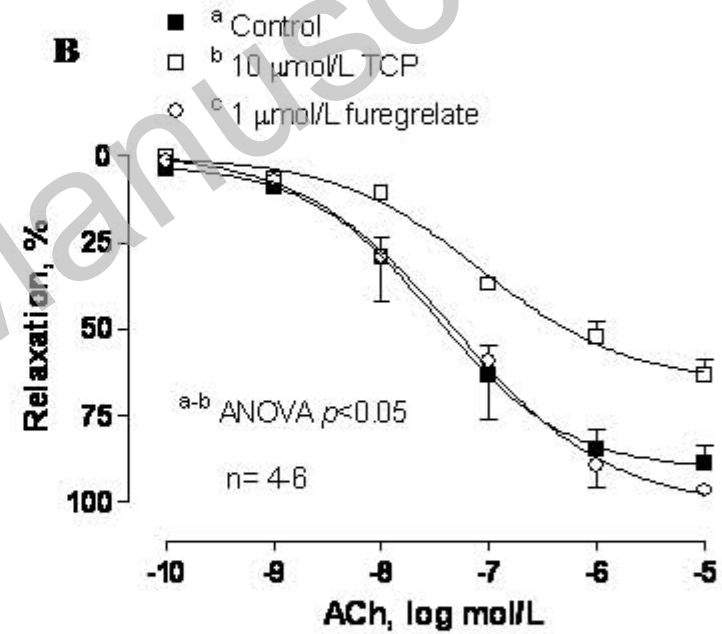
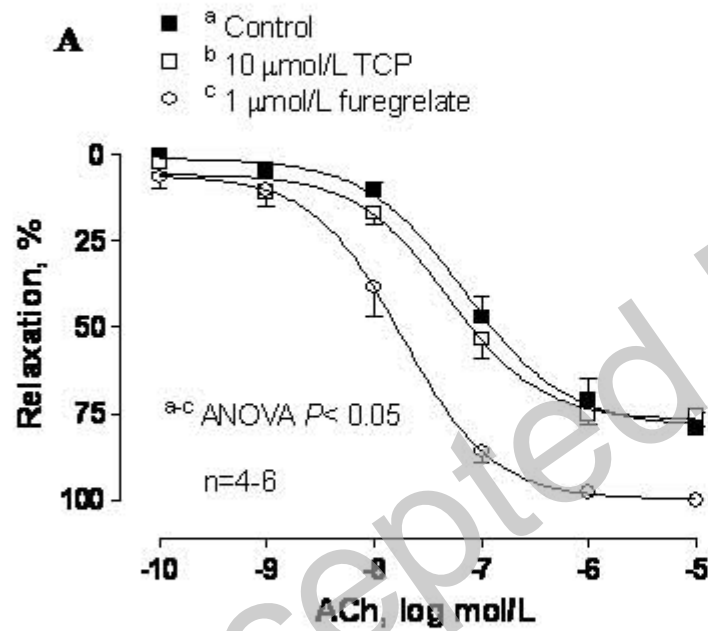
Figure 5: (A) Effect of PH on basal and ACh-stimulated NO release. Results were expressed as arbitrary fluorescence units per tissue mg. (B) Effect of 0.1 mmol/L L-NAME on TXB₂ release. (C). Effect of 0.1 mmol/L L-NAME and 1 μ mol/L furegrelate on 6 keto PGF_{1 α} release. n= number of animals. Results were expressed as pg prostanoid/mL mg tissue. **P* < 0.05 vs basal. # *P* < 0.05 vs control.

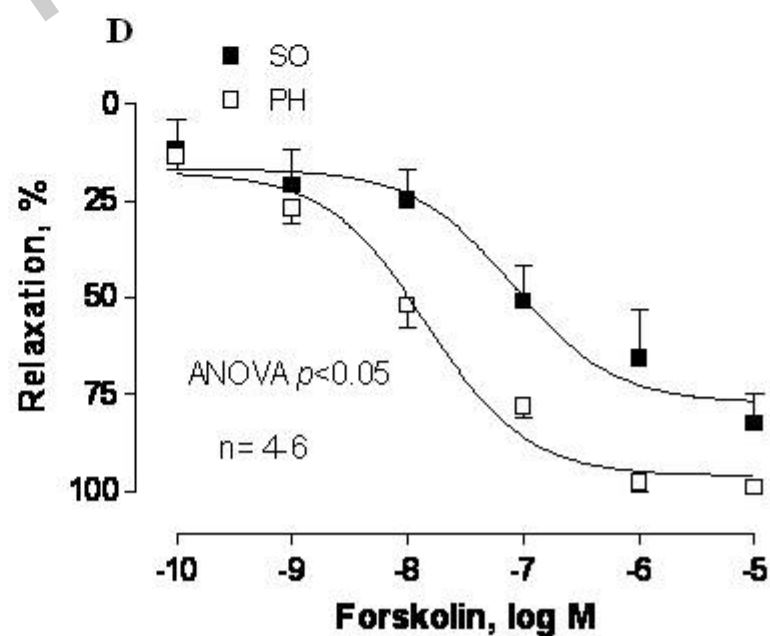
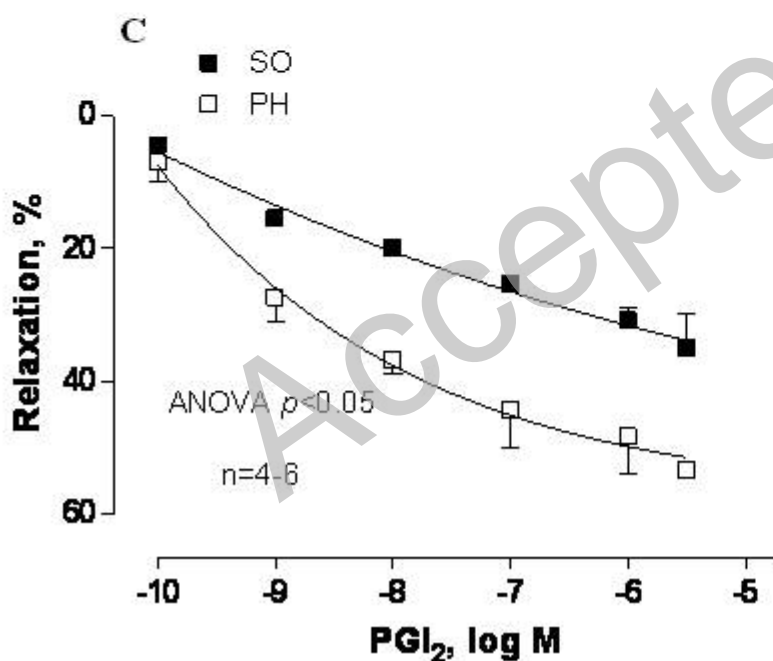
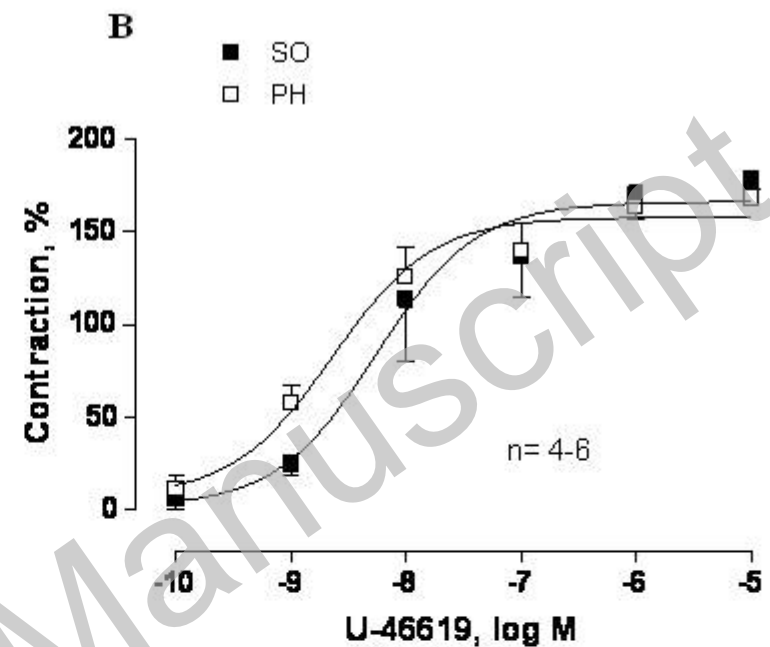
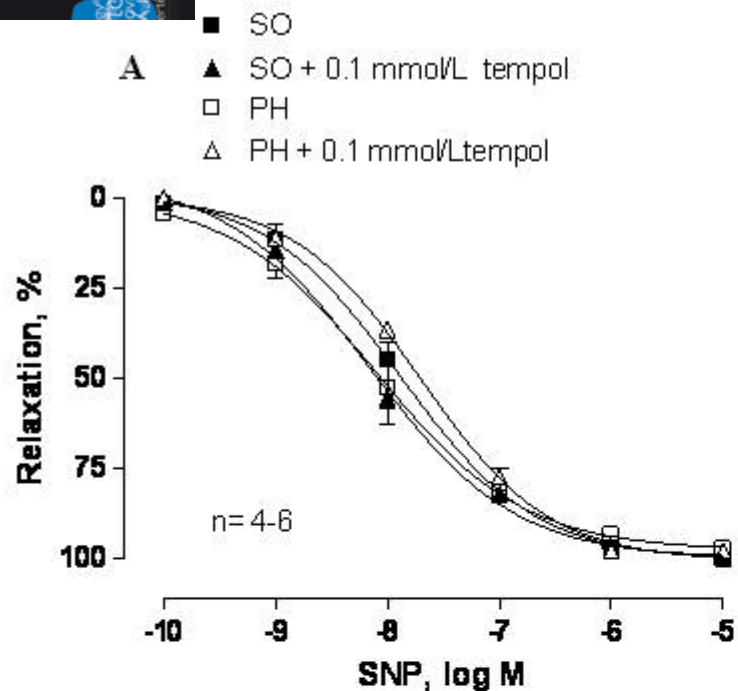
Figure 6: Fluorescent confocal micrographs showing *in situ* detection of superoxide anion in aorta segments from SO and PH rats. The sections shown are typical of preparations from four rats.

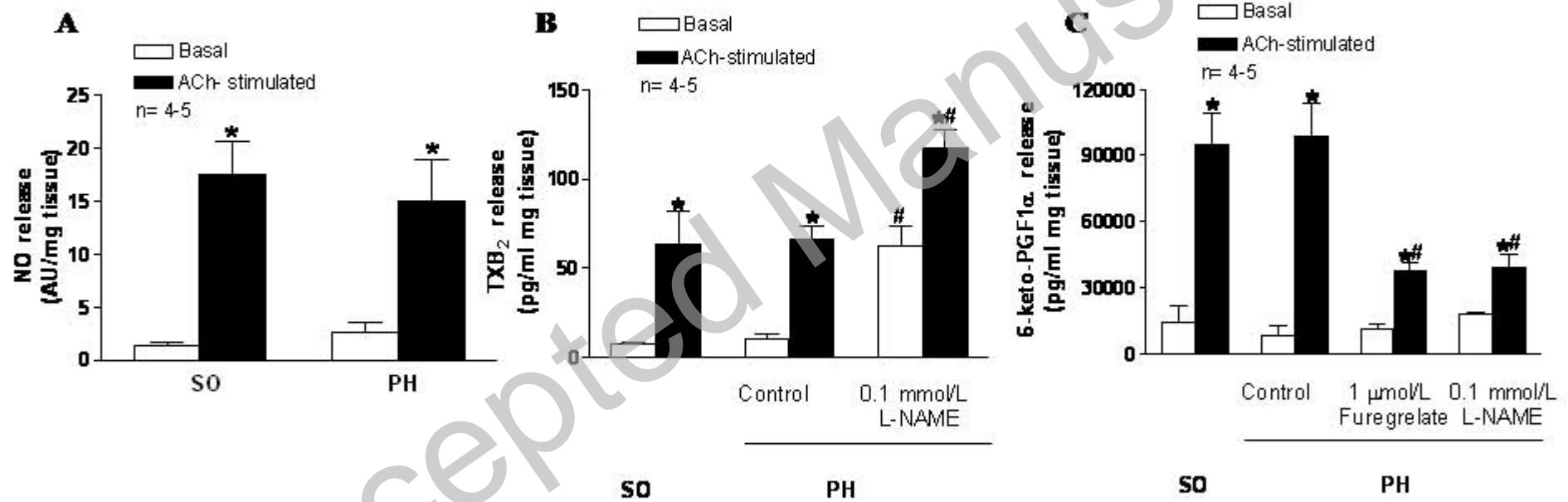
Figure 7: Representative western blot for eNOS, iNOS, COX-1 and COX-2 expression in aortic segments from SO and PH rats (A). Figure is representative of four separate segments in each group. Panel B shows densitometric analysis for eNOS, iNOS, COX-1 and COX-2 expression. Results (mean \pm SEM) were expressed as the ratio of the signal obtained for each protein, and the signal obtained for α -actin.

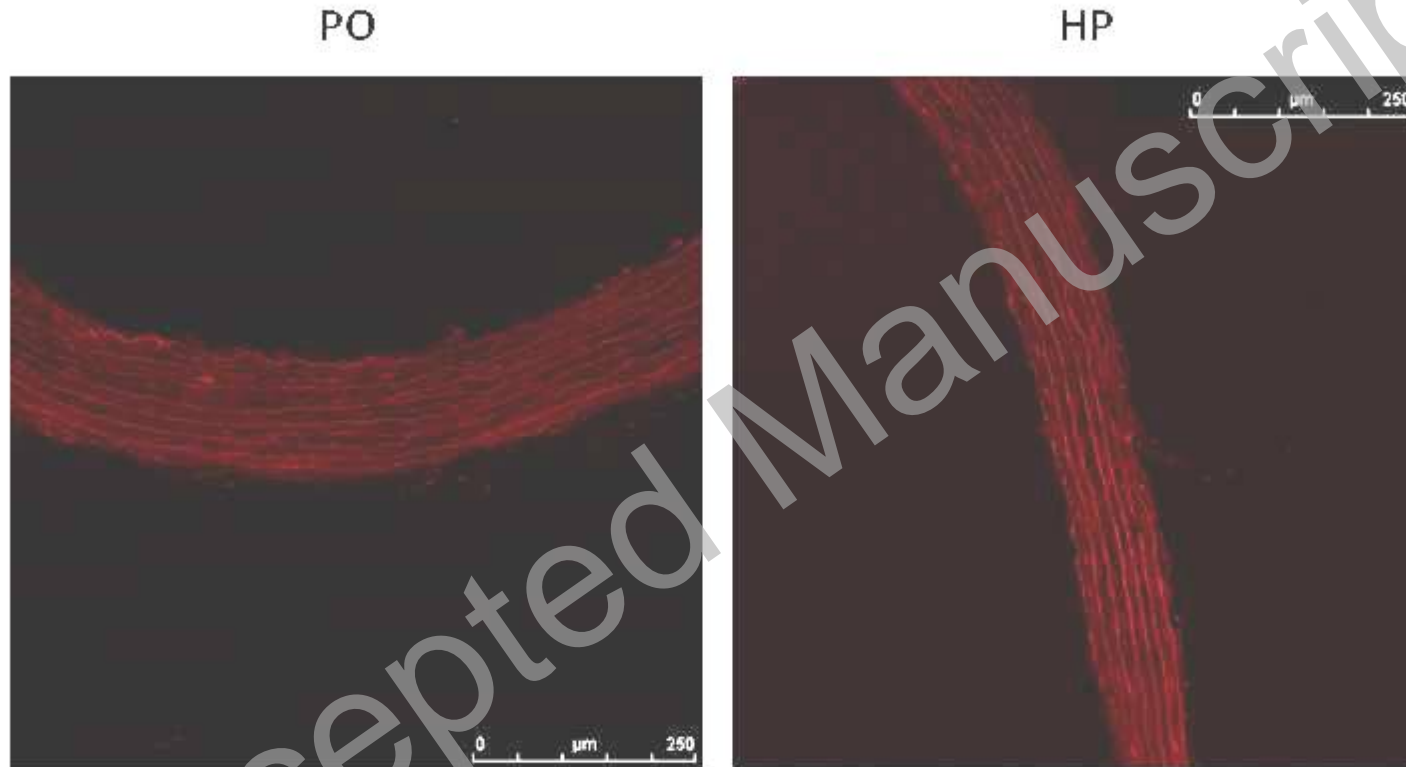












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