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Title Page

Losartan reduces the increased participation of COX-2 derived products in vascular responses of hypertensive rats

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Running title page

AT_1 receptors and COX-2 in hypertension

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List of non-standard abbreviations:

Ang II: angiotensin II; COX: cyclooxygenase; COX-2: inducible cyclooxygenase; DMEM: Dulbecco's modified Eagle's medium; DOCA: deoxycorticosterone acetate; HH: hydralazine-hydrochlorothiazide; MDA: malondialdehyde; $O_2^{\cdot-}$: superoxide anion; PG: prostaglandin; ROS: reactive oxygen species; RT-PCR: retro transcription polymerase chain reaction; SHR: spontaneously hypertensive rats; TAS: total antioxidant status; VSMC: vascular smooth muscle cells; WKY: Wistar Kyoto rat

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Abstract

This study analyzes the role of angiotensin II (Ang II), via AT_1 receptors, in the involvement of cyclooxygenase-2 (COX-2)-derived prostanoids in phenylephrine responses in normotensive (WKY) and spontaneously hypertensive (SHR) rats. Aorta from rats untreated or treated for 12 weeks with losartan (15 mg/kg·day) or hydralazine plus hydrochlorothiazide (44 and 9.4 mg/kg·day, respectively) and vascular smooth muscle cells (VSMC) from SHR were used. Vascular reactivity was analyzed by isometric recording, COX-2 expression by Western blot and RT-PCR, PGI_2, $PGF_{2\alpha}$, 8-isoprostane and total antioxidant status (TAS) by commercial kits, superoxide anion ($O_2^{-\cdot}$) using lucigenin chemiluminiscence and plasmatic malondialdehyde (MDA) by thiobarbituric acid assay. The COX-2 inhibitor NS 398 (1 μM) reduced phenylephrine responses more in SHR than in WKY. COX-2 protein and mRNA expressions, $PGF_{2\alpha}$, PGI_2, 8-isoprostane and $O_2^{-\cdot}$ production and MDA levels were higher in SHR but TAS was similar in both strains. Losartan, but not hydralazine-hydrochlorothiazide treatment, reduced COX-2 expression and the effect of NS 398 in SHR. Losartan also increased TAS and reduced $PGF_{2\alpha}$, PGI_2, 8-isoprostane and $O_2^{-\cdot}$ production and MDA levels in SHR. Ang II (0.1 μM) induced COX-2 expression in VSMC from SHR, that was reduced by apocynin (30 μM) and allopurinol (100 μM), NADPH oxidase and xanthine oxidase inhibitors, respectively. In conclusion, AT_1 receptor activation by Ang II could be involved in the increased participation of COX-2-derived contractile prostanoids in vasoconstriction to phenylephrine with hypertension, probably through COX-2 expression regulation. The increased oxidative stress seems to be one of the mechanisms involved.

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Introduction

Prostaglandins are important mediators in the regulation of cardiovascular functions. In healthy blood vessels most prostanoids are formed by the constitutive isoform of cyclooxygenase (COX-1). However, these mediators may also be synthesized by the inducible cyclooxygenase isoform, COX-2, that is not normally expressed in vascular cells but can be induced by agents such as lipopolysaccharide or cytokines (Vagnoni et al., 1999; Hernanz et al., 2003). Moreover, in the last years, it has become evident that prostanoid production from constitutively expressed COX-2 is also involved in the modulation of vascular responses (Henrion et al., 1997; García-Cohen et al., 2000; Alvarez et al., 2005; Adeagbo et al., 2005).

Hypertension, now considered a chronic inflammatory disease with elevated proinflammatory cytokine blood levels (Pauletto and Rattazzi, 2006, Ruiz-Ortega et al., 2006) and increased vascular COX-2 expression (Henrion et al., 1997; García-Cohen et al., 2000; Alvarez et al., 2005; Adeagbo et al., 2005), has been associated with changes in vascular responses such as impairment of endothelium-dependent vasodilator responses or enhancement of vasoconstrictor responses to different agonists (Schiffrin and Touyz, 2004). Angiotensin II (Ang II), the effector peptide of the renin-angiotensin system, physiologically regulates vascular tone and maintains normal vessel structure and function, although elevated levels of Ang II have been implicated in the pathophysiological processes that occur in hypertension. Recent studies have shown that Ang II has significant proinflammatory actions in the vascular wall including the production of reactive oxygen species (ROS), inflammatory cytokines and adhesion molecules (Schiffrin and Touyz, 2004; Cheng et al.,

2005; Pauletto and Rattazzi, 2006) all of which are known to contribute to the inflammatory responses occurring in hypertension. Ang II also stimulates the release of prostaglandins in a variety of cells including smooth muscle cells through the activation of phospholipase A_2 (Freeman et al., 1998). In addition, Ang II regulates COX-2 expression and prostanoids production in vascular smooth muscle cells (VSMC) from normotensive rats through the activation of AT_1 receptors (Ohnaka et al., 2000; Hu et al., 2002).

Increased oxidative stress has also been described in human and different models of hypertension, including spontaneously hypertensive rats (SHR) (McIntyre et al., 1999; Cai and Harrison, 2000; Griendling et al., 2000). Humoral factors, such as Ang II, may be responsible for the altered superoxide anion generation in hypertension. In fact, Ang II has been shown to stimulate superoxide generation by increasing the activity of NAD(P)H oxidase in cultured rat VSMC (Griendling et al., 1994), and this increase is higher in cells from SHR than in normotensive Wistar Kyoto (WKY) rats (Cruzado et al., 2005). In addition, increased vascular smooth muscle superoxide anion production via NADH/NADPH oxidase activation has been described in the hypertensive model induced by Ang II infusion (Rajagopalan et al., 1994). On the other hand, ROS like superoxide anion or hydrogen peroxide also induce COX-2 (Feng et al., 1995; Kiritoshi 2003) and these mediators have been implicated in the upregulation of COX-2 expression and prostanoids release induced by Ang II in mesangial cells (Jaimes et al., 2005).

In a previous report, we have described that COX-2 expression is higher in aortic segments from SHR than in WKY; the increase in COX-2 levels raises contractile prostanoid production in SHR and thereby, its participation in the

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vasoconstrictor response to phenylephrine (Alvarez et al., 2005). Similar results have been found by Adeagbo et al. (2005) using the deoxicorticosterone acetate (DOCA)-salt hypertensive model. The aim of the present study was to extend our previous results and to analyze whether Ang II, through the activation of AT_1 receptors, is implicated in the increased participation of COX-2-derived contractile mediators in phenylephrine responses observed in aorta from hypertensive rats.

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Methods

Animals. The investigation conforms to 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) and with the current Spanish and European laws (RD 223/88 MAPA and 609/86). Six-month old male WKY and SHR rats were used. Rats from both strains were divided into two groups: control and rats treated with the AT_1 receptor antagonist losartan (15 mg/kg·day, *v.o.*, 12 weeks; generously supply by Merck & Co., Inc, Rahway, NY, USA). An additional group of SHR (HH) treated *v.o.* by 12 weeks with the combination of hydralazine (44 mg/kg·day, Sigma Chemical, Co., St. Louis, MO, USA) and hydrochlorothiazide (9.4 mg/kg·day, Sigma Chemical, Co.) was used in some experiments. Systolic blood pressure was measured weekly using tail cuff plethysmography. At the end of the losartan and HH treatments, systolic blood pressure was reduced in SHR (control: 188.7±2.1, n=11; losartan: 124.0±1.4, n=15, p<0.01; HH: 132.6±6.0 mmHg, n=9, p<0.01). Losartan treatment also reduced systolic blood pressure in WKY rats (control: 131.1±2.8, n=13; losartan: 120.3±2.2 mmHg, n=15, p<0.05). Both treatments did not affect the body weight in either strain (results not shown). Rats were euthanized by decapitation and the thoracic aorta was removed and placed in Krebs-Henseleit solution at 4ºC.

Blood samples were collected in tubes containing 15% K_3EDTA as anticoagulant (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK) and placed in ice. Blood samples were centrifuged at 1500xg for 15 min at 4ºC. The obtained plasma was frozen at -20ºC and kept at -80ºC until used to

determine malondialdehyde (MDA) concentration and total antioxidant status (TAS).

Reactivity experiments. Vascular function was studied in aortic segments from WKY and SHR by isometric tension recording (Alvarez et al., 2005). The presence of endothelium was confirmed by the effect of 10 μM acetylcholine (Sigma Chemical Co.) on segments contracted with phenylephrine at a concentration that produces close to 50% of the contraction induced by 75 mM KCl. Afterwards, concentration-response curves to acetylcholine or phenylephrine were performed. The effect of the selective COX-2 inhibitor N-(2-Cyclohexyloxy-4-nitrophenyl)-Methanesulfonamide (NS 398, 1 μM) (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) in phenylephrine responses was investigated by its addition 30 min before phenylephrine. In some experiments, segments from WKY were incubated with Ang II (0.1 μM, Sigma Chemical Co.), which was added from the time the aorta was removed from the animal and maintained through the experiment (6h). In these vessels, the effect of NS 398 in phenylephrine responses was also evaluated.

Vasoconstrictor responses were expressed as a percentage of the tone generated by 75 mM KCl and vasodilator responses as a percentage of the previous tone generated by phenylephrine. To compare the effect of NS 398 on the response to phenylephrine in segments from both strains and from all treatment groups, some results were expressed as a percentage of inhibition of the maximum response to phenylephrine induced by NS 398 in each artery.

Cell cultures. VSMC were isolated from aortas of 5-month-old SHR. Arteries were treated during 20 min with collagenase (2 mg/ml, Worthington, NJ, USA) and adventitia was carefully removed. After that, VSMC were

obtained from segments by the explant method. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For experiments, cells from passages 3 to 8 were made quiescent by incubation in serum-free DMEM supplemented with 0.1% bovine serum albumin for 24 h. Cells were stimulated with Ang II for 2 h with or without pretreatment for 1 h with the specific inhibitors for xanthine oxidase (allopurinol, 100 µM) (Research Biochemicals Incorporated, Natick, MA, USA) and NADP(H) oxidase (apocynin, 30 µM) (Fluka-Sigma Chemical, Seelze, Germany).

Western blot analysis. COX-2 protein expression was determined in cellular lysates (20 µg protein) and in homogenates from the aortic segments (30 µg protein) used for reactivity experiments (approximately 6 h after extraction from the animal) and from segments obtained immediately upon removal from the animal (basal conditions), as previously described (Alvarez et al., 2005). Briefly, proteins were separated by 7.5% SDS-PAGE and then transferred to polyvinyl difluoride membranes overnight. Membranes were incubated with rabbit polyclonal antibody for COX-2 (1:1,000; Cayman Chemical; Ann Arbor, MI, USA). After washing, membranes were incubated with anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:2,000, Bio-Rad, Laboratories, Hercules, CA, USA). The immunocomplexes were detected using an enhanced horseradish peroxidase-luminol chemiluminiscence system (ECL Plus, Amersham International; Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, Amersham International). Signals on the immunoblot were quantified using the NIH Image computer program (US, version 1.56).

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To compare the results for protein expression within the same experiment and with others, we assigned a value of 1 in each gel to the expression of VSMC stimulated with Ang II or of arteries from losartan-untreated WKY rats in basal conditions.

RT-PCR assay. COX-2 mRNA was determined in aortic segments in similar conditions to those used for protein expression determination. Total RNA was obtained by using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). A total of 1 µg of DNAse I treated RNA was reverse-transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) in a 50 µl reaction. PCR was performed in duplicate for each sample using 0.5 µl of cDNA as template for COX-2, 1x TaqMan Universal PCR Master Mix (Applied Biosystems) and 10x of Taqman Gene Expression Assays (Applied Biosystems, Rn00568225_m1) in a 20 µl reaction. For quantification, quantitative RT-PCR was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following conditions: 2 min 50°C, 10 min 95°C and 40 cycles: 15 s 95 ºC, 1 min 60º. As a normalizing internal control we amplified β_2 microglobulin (Rn00560865_m1). To calculate the relative index of gene expression, we employed the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the untreated samples as calibrator.

Measurement of Prostaglandin $F_{2\alpha}$, Prostacyclin and 8-isoprostane production. The measurements of the metabolite of $PGF_{2\alpha}$, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, the metabolite of prostacyclin (PGI_2), 6-keto-$PGF_{1\alpha}$, and 8-isoprostane were determined in the incubation medium after completion of the phenylephrine concentration-response curves, using enzyme immunoassay commercial kits (Cayman Chemical for $PGF_{2\alpha}$ and 8-isoprostane and R&D

Systems Europe for PGI_2). The medium was frozen in liquid nitrogen, kept at -70ºC until analysis and processed following the manufacturer instructions.

Measurement of malondialdehyde (MDA) production. Plasmatic MDA levels were measured by a modified thiobarbituric acid (TBA) assay (Rodríguez-Martínez and Ruiz-Torres, 1992). Plasma was mixed with 20% trichloracetic acid in 0.6 M HCl (1:1, v/v), and the tubes were kept in ice for 20 min to precipitate plasma components to avoid possible interferences. Samples were centrifuged at 1500xg for 15 min before adding TBA (120 mM in Tris 260 mM, pH 7) to the supernatant in a proportion of 1:5 (v/v); then, the mixture was boiled at 97ºC for 30 min. Spectrophotometric measurements at 535 nm were made at 20ºC.

Plasmatic total antioxidant status (TAS) measurement. TAS was measured using the Calbiochem total antioxidant status assay kit (Calbiochem-Novabiochem Co.), according to the manufacturer's instructions.

Detection of superoxide anion. Superoxide anion production in aortic segments was determined with lucigenin chemiluminescence. Briefly, thoracic aortic rings (10-15 mm) were cleaned and placed in modified Krebs-HEPES (in mM: 119 NaCl, 20 HEPES, 1 $MgSO_4$, 4.6 KCl, 0.4 KH_2PO_4, 5 $NaHCO_3$, 1.2 $CaCl_2$, 11.1 glucose, pH 7.4) for 30 min at 37 ºC. Aortic rings were placed in Krebs-HEPES buffer containing lucigenin (20 µM). The chemiluminescence was then recorded every 30 s during 10 min with a luminometer (Optocom I BG-1, MGM Instruments Inc, Hamden, CT, USA). The differences between the values obtained before and after adding the rings to the buffer medium were calculated.

Data analysis and statistics. Results are expressed as mean ± S.E.M. of the number of rats indicated (*n*); differences were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni test or by the Mann-Whitney non-parametric test. A *p* value below 0.05 was considered significant.

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Results

Vasoactive responses. Acetylcholine (1 nM-30 µM) induced endothelium-dependent vasodilator responses that were greater in segments from normotensive than hypertensive rats. Losartan treatment increased responses to acetylcholine in aortic segments from both strains (Fig. 1).

The contractile response to phenylephrine (1 nM-30 µM) was slightly greater in segments from SHR than WKY, as previously reported (Alvarez et al., 2005). Losartan treatment did not affect contractile responses to phenylephrine both in WKY and SHR (Fig. 1). Thus, maximum response (WKY control: 110.9 $\pm$ 3.8, *n*=32; WKY losartan: 102.8 $\pm$ 6.6, *n*=16; SHR control: 122.2 $\pm$ 4.1, *n*=36; SHR losartan: 135.3 $\pm$ 6.5, *n*=21) and pD_2 values (WKY control: 6.84 $\pm$ 0.06; WKY losartan: 6.84 $\pm$ 0.08; SHR control: 7.04 $\pm$ 0.07; SHR losartan: 7.21 $\pm$ 0.11) were similar in control and losartan treated rats.

The selective COX-2 inhibitor NS 398 (1 µM), inhibited the phenylephrine response to a greater extent in segments from SHR than from WKY (Table 1), as previously described (Alvarez et al., 2005). Losartan treatment decreased, but not abolished, the inhibitory effect of this drug only in segments from SHR (Fig. 2 and Table 1).To evaluate the effect of lowering blood pressure *vs* AT_1 blockade, SHR were treated with a combination of hydralazine plus hydrochlorotiazide. This treatment did not modify the inhibitory effect of NS 398 in segments from SHR (Fig. 2 and Table 1).

To further analyze the modulatory effect of Ang II on COX-2 derived prostanoids, vessels from WKY were preincubated with Ang II (0.1 µM) from the dissection of the artery. As shown in Table 1, NS 398 induced an inhibitory

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effect of phenylephrine response that was greater in Ang II incubated segments than in the untreated vessels.

COX-2 expression. In freshly isolated segments (basal) COX-2 protein expression was greater in SHR than WKY (Fig. 3); this higher expression was accompanied by increased mRNA expression in SHR (Fig. 4A). At the end of the reactivity experiments (6 h), COX-2 protein (Fig. 3) and mRNA expressions showed increased levels in both strains (relative mRNA expression 6 h *vs* basal: 14.4 ± 4.4, n=8 for WKY and 24.1 ± 6.3, n=9 for SHR, p<0.05). In these conditions, the expression of COX-2 protein (Fig. 3) and mRNA (Fig. 4A) was also greater in SHR than WKY.

Losartan treatment did not affect the expression of either COX-2 protein (Fig. 3) or mRNA (data not shown) in WKY and reduced the increase in COX-2 protein and mRNA expression observed in SHR both in basal conditions and after 6 h incubation (Figs. 3 and 4B). However, HH treatment did not affect COX-2 mRNA expression in aortic segments from SHR either in basal conditions or after 6 h incubation (Fig. 4B). These results suggest that the effect of losartan in COX-2 expression is independent on its effect on blood pressure.

Prostaglandin $F_{2\alpha}$, PGI_2 and 8-isoprostanes production. At the end of the reactivity experiments, aortic segments from both strains released detectable amounts of the metabolite of $PGF_{2\alpha}$, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, of the metabolite of PGI_2, 6-keto-$PGF_{1\alpha}$, and 8-isoprostanes to the incubation medium (Fig. 5); the levels of the three compounds were higher in samples from SHR (Fig. 5). NS 398 (1 μM) reduced 13,14-dihydro-15-keto-$PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and 8-isoprostanes levels only in the medium with SHR segments. Losartan treatment decreased the release of these compounds into the

incubation medium from SHR but did not modify these levels in WKY (Fig. 5). After losartan treatment, NS 398 (1 µM) did not further inhibit the release of 13,14-dihydro-15-keto-$PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and 8-isoprostanes by SHR segments (Fig. 5). These results suggest that AT_1 receptors activation participates in the increased prostanoids release from COX-2 in SHR.

Vascular superoxide anion production and plasmatic MDA levels and TAS. Basal superoxide anion production was higher in segments from SHR than from WKY. Losartan treatment prevented the rise in superoxide production in aorta from SHR (Fig. 6). Plasmatic MDA levels were also higher in SHR than in WKY rats, whereas no differences in TAS were observed in plasma samples from WKY and SHR. Losartan treatment reduced MDA levels in both strains, abolishing the differences between strains, and increased TAS only in SHR (Fig. 6). These results suggest that AT_1 receptors activation participates in the increased oxidative stress and the decreased TAS observed in SHR.

Effect of Ang II on COX-2 expression. In quiescent VSMC from SHR, Ang II (0.1 µM) increased COX-2 expression. Both apocynin (30 µM) and allopurinol (100 µM) reduced COX-2 expression in Ang II-stimulated cells (Fig. 7), thus demonstrating the participation of reactive oxygen species in this effect.

Discussion

The main finding of this study is that endogenous Ang II, acting through AT_1 receptors, is involved in the increased participation of vasoconstrictor prostanoids from COX-2 in the response to phenylephrine in aortic segments from hypertensive rats, probably by regulating COX-2 expression.

The renin angiotensin system plays an important role in the pathogenesis of several cardiovascular diseases, including hypertension. In the present study, in keeping with findings obtained elsewhere, the AT_1 receptor antagonist losartan significantly lowered blood pressure in hypertensive rats. Several mechanisms have been proposed as responsible for the hypertensinogenic effect of Ang II. Thus, Ang II has been shown to stimulate both hyperplasia and hypertrophy in VSMC (Touyz et al., 1999) and it plays a role in the profibrotic processes (Laviades et al., 1998) as well as in the altered vasoconstrictor or vasodilator responses described in hypertension (Schiffrin and Touyz, 2004). In addition, it is now accepted that an inflammatory reaction may be recorded in association with the hypertensive process and the role of endogenous Ang II in vascular inflammation has also been suggested (Schiffrin and Touyz, 2004).

The present and previous results (Alvarez et al., 2005) demonstrate that the participation of contractile prostanoids from COX-2 in vasoactive responses is increased with hypertension. This is supported by the fact that phenylephrine-induced responses were inhibited by the selective COX-2 inhibitor NS 398 as well as by the protein synthesis inhibitor dexamethasone and the TXA_2/PGH_2 receptor antagonist SQ 29,548 to a greater extent in aorta from SHR (Alvarez et al., 2005). In different hypertension models, other investigators have also

described increased vascular contractility associated with increased production of contractile prostanoids from COX-2 (Zerrouk et al., 1998; Adeagbo et al., 2005). We have found that basal COX-2 mRNA and protein expressions were greater in aortic segments from hypertensive rats; such expressions were increased during the course of the experiment, being these expressions also greater in SHR than WKY. In addition, the NS 398-sensitive release of $PGF_{2\alpha}$, and 8-isoprostane was greater in the hypertensive animals. In different models of hypertension it has been described increased vascular basal COX-2 protein expression (Henrion et al., 1997; García-Cohen et al., 2000; Alvarez et al., 2005; Adeagbo et al., 2005) and prostanoids production from COX-2 (Zerrouk et al., 1998; Adeagbo et al., 2005) as well as serum and urinary 8-isoprostane levels (Schnackenberg and Wilcox, 1999; Adeagbo et al., 2005).

It has been suggested that although COX-1 primarily contributes to basal prostanoids production in the kidney and aorta, Ang II increases prostanoids *via* COX-2 activity (Qi et al., 2006) and/or expression, but different results have been reported depending on the cell type or stimulus. Thus, in VSMC (Ohnaka et al., 2000; Hu et al., 2002), ventricular cardiomyocytes (Rebsamen et al., 2003), coronary vascular wall (Rocha et al., 2002) and mesangial cells and glomerulus (Jaimes et al., 2005) from normotensive rats, Ang II upregulates COX-2 mRNA expression through the activation of AT_1 receptors (Ohnaka et al., 2000; Hu et al., 2002). However, there are also evidences showing that Ang II attenuates COX-2 expression in kidney (Harris et al., 2004) and VSMC stimulated with IL-1β (Jiang et al., 2004). To our knowledge, there are no reports analyzing the influence of hypertension in Ang II induced prostaglandin production from COX-2. Our results suggest that AT_1 receptors activation by

Ang II plays a role in the increased participation of contractile prostanoids in phenylephrine responses in aorta from hypertensive rats through the up-regulation of COX-2 expression and the subsequent increase of contractile prostanoids production. This is based in several observations. 1) In VSMC from SHR, Ang II increased COX-2 expression. 2) Losartan treatment reduced the increased COX-2 protein and mRNA expressions and the increased release of $PGF_{2\alpha}$ and 8-isoprostane found in aortic segments from SHR, without further effects by the COX-2 inhibitor NS 398 in this release. In this line of evidence, losartan treatment is reported to decrease the increased inducible nitric oxide synthase and COX-2 expression observed after experimental renal interstitial fibrosis (Manucha et al., 2004). However, the AT_1 receptor blocker candesartan has also been reported to increase the renocortical COX-2 mRNA expression in 5 week-old SHR but has no effect in older rats (Hocherl et al., 2001). 3) Losartan treatment reduced the inhibitory effects of NS 398 in phenylephrine responses in aortic segments from SHR but not in WKY. 4) The incubation of aortic segments from WKY with Ang II increased the inhibitory effect of NS 398 in phenylephrine responses. 5) The treatment with hydralazine plus hydrochlorothiazide diminished blood pressure but did not modify the increase in both COX-2 mRNA expression and the inhibitory effect of NS 398 observed in segments from hypertensive rats without treatment, suggesting that the observed effects of losartan treatment were probably independent of its lowering blood pressure properties.

After losartan treatment no change in phenylephrine response in SHR was observed despite of downregulation of COX-2 pathway. As mentioned, losartan treatment decreased the production of the contractile prostanoid $PGF_{2\alpha}$ and 8-

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isoprostane as well as the vasodilator PGI_2. Therefore, it is possible that the final effect of these mediators in phenylephrine contraction could be counterbalanced although other contractile mediators or signalling pathways might be upregulated after AT_1 receptors blockade to maintain contractile responses. Furthermore, Cediel et al., (2003) demonstrated improvement of smooth muscle cell function by AT_1 receptor antagonists associated to the reversion of medial hypertrophy that could improve contractile machinery of smooth muscle cells.

Ang II mediates many of its cellular actions by stimulating formation of intracellular ROS, thereby participating in the increased oxidative stress observed in hypertension (Schiffrin and Touyz, 2004; Cheng et al., 2005). Ang II stimulates superoxide anion generation in vascular rings through AT_1 receptor activation (Rajagopalan et al., 1996). Similarly, in cultured rat VSMC, Ang II stimulates superoxide anion production by increasing the activity of NAD(P)H oxidase (Griendling et al., 1994) greatly in SHR rats (Cruzado et al., 2005). Losartan treatment abolished the increased $O_2^{\cdot -}$ production observed in aorta from SHR. Moreover, losartan normalized the increased plasmatic MDA levels and increased TAS, thus suggesting that losartan may prevent oxidative stress in hypertension by inhibiting lipid peroxidation. Accordingly, losartan improved the impaired endothelium-dependent relaxation to acetylcholine observed in aorta from SHR, as previously reported (Schiffrin and Touyz, 2004). These results confirm the participation of the renin-angiotensin system in the endothelial dysfunction associated to hypertension, probably trough its ability to generate ROS. Other investigators have also found reduction of malondialdehyde levels and plasmatic and urinary 8-isoprostrane levels, as well

as increase of antioxidant systems, after inhibition of the renin-angiotensin system (Mervaala et al., 2001; Donmez et al., 2002; Chamorro et al., 2004).

It has been reported induction of COX-2 expression after oxidative stress stimulus in mesangial cells (Feng et al., 1995; Kiritosi et al., 2003) and reduction of COX-2 expression in renal cortex after antioxidant treatments (Li et al., 2005). Our group has described that oxidative stress induces higher COX-2 expression in aorta from hypertensive than from normotensive rats (García-Cohen et al., 2000). In agreement, we observed here that the increased COX-2 expression found in VSMC from SHR after Ang II stimulation was decreased by apocynin and allopurinol, inhibitors of superoxide anion production by NADPH oxidase and xanthine oxidase, respectively. As discussed above, since losartan is diminishing the oxidative stress both at vascular and systemic levels, all together these findings point to the excess of ROS as one possible mechanism for the increased COX-2 expression and activity observed in SHR. In agreement, in DOCA-salt hypertension (Adeagbo et al., 2005) and in diabetic rats (Li et al., 2005), it has also been suggested that oxidative stress is a component of the mechanism involved in the production of contractile prostanoids from COX-2, that are responsible for the increased vascular reactivity to vasoconstrictors in aorta and kidney. In addition, ROS have been implicated in the upregulation of COX-2 expression and prostanoids release induced by Ang II in mesangial cells (Jaimes et al., 2005).

In conclusion, endogenous Ang II activation of AT_1 receptors seems to participate in the increased COX-2 expression and contractile prostanoids production from this isoform that are involved in phenylephrine responses with

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hypertension. Our findings point to oxidative stress as one of the mechanism involved in these effects.

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Footnotes

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Legends for Figures

Fig. 1. Concentration-response curves to acetylcholine and phenylephrine in aortic segments from WKY and SHR untreated or treated with losartan (15 mg/kg·day). Number of animals (*n*) used is indicated in parenthesis. **p*<0.01 *vs* WKY and ^{+}p<0.05 *vs* untreated rats by two-way ANOVA.

Fig. 2. Effect of NS 398 (1 μM) on the concentration-response curve to phenylephrine in aortic segments from SHR untreated or treated with losartan (15 mg/kg·day) or hydralazine plus hydrochlorothiazide (HH, 44 and 9.4 mg/kg·day, respectively). Number of animals (*n*) used is indicated in parenthesis.

Fig. 3. Densitometric analysis of the western blot for the inducible isoform of cyclooxygenase (COX-2) protein in aortic segments from WKY and SHR untreated and treated with losartan (15 mg/kg·day) in two experimental conditions: basal (freshly isolated arteries) and 6 h (after concentration-response curves to phenylephrine were performed). Representative western blot is represented above. Number of animals (*n*) used is indicated in parenthesis. **p*<0.05 *vs* WKY; $^{\#}p$<0.05 *vs* basal and $^{\$}p$<0.05 *vs* untreated rats by one-way ANOVA.

Fig. 4. A) Quantitative RT-PCR assessment of COX-2 mRNA expression in basal (freshly isolated arteries) or 6h (incubated for 6h in similar conditions to those used for reactivity experiments) conditions. The results are expressed as the relative expression using the $2^{-\Delta\Delta Ct}$ method using the WKY samples (either basal or 6 h) as calibrator. The ΔCT values for untreated WKY were: basal, 6.39 ± 0.48; 6 h, 2.95 ± 0.24. B) Effect of losartan (15 mg/kg·day) or hydralazine plus

hydrochlorotiazide (44 and 9.4 mg/kg·day, respectively) treatments on quantitative RT-PCR assessment of cyclooxygenase-2 (COX-2) mRNA expression in rat aorta from WKY and SHR in two experimental conditions: basal (freshly isolated arteries) and 6 h (incubated for 6 h in similar conditions to those used for reactivity experiments). The results are expressed as the relative expression using the $2^{-\Delta\Delta Ct}$ method using the SHR untreated samples (either basal or 6 h) as calibrator. Number of animals (*n*) used is indicated in parenthesis. **p*<0.05 by a Mann-Whitney non parametric test.

Fig. 5. Effect of losartan treatment (15 mg/kg·day) on the release of 13,14-dihydro-15-keto-$PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and 8-isoprostane to the incubation medium after concentration-response curves to phenylephrine in aortic segments from WKY and SHR. The effect of NS 398 (1 µM) on this release is also shown. Number of animals (*n*) used is indicated in parenthesis. **p*<0.05 *vs* WKY; $^{\#}p$<0.05 *vs* Control and $^{\$}p$<0.05 *vs* untreated rats by one-way ANOVA.

Fig. 6. Effect of losartan treatment (15 mg/kg·day) on vascular superoxide anion production and plasmatic malondialdehyde (MDA) levels and total antioxidant status (TAS) in WKY and SHR. Number of animals (*n*) used is indicated in parenthesis. **p*<0.05 *vs* WKY; and $^{\#}p$<0.05 *vs* untreated rats by one-way ANOVA.

Fig. 7. Effects of angiotensin II (0.1 µM) on cyclooxygenase-2 (COX-2) protein expression in VSMC from SHR in the absence and the presence of apocynin (30 µM) or allopurinol (100 µM). Representative western blot is represented above. Number of animals (*n*) used is indicated in parenthesis. **p*<0.05 *vs* untreated cells and $^{\#}p$<0.05 *vs* angiotensin II determined by one-way ANOVA. Activated macrophages were used as positive control (C+).

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Table 1. Inhibitory effect of NS 398 (1 μM) on the vasoconstrictor response to phenylephrine in aortic segments from normotensive (WKY) and hypertensive rats (SHR) untreated or treated for 12 weeks with losartan (15 mg/Kg·day) or hydralazine-hydrochlorothiazide (HH, 44 and 9.4 mg/kg·day, respectively). The effect of NS 398 in aortic segments from WKY preincubated with Angiotensin II (Ang II, 0.1 μM, 6h) is also shown.

	WKY	SHR
Untreated	33.5 ± 2.2 (12)	58.3 ± 6.4 (14) *
Losartan	25.7 ± 6.9 (10)	9.9 ± 4.4 (17) #
HH	-	53.2 ± 7.7 (10)
Ang II preincubation	45.2 ± 5.9 (7) #	-

Results are expressed as percentage of inhibition of the maximum response to phenylephrine induced by NS 398 in each case. Number of animals (*n*) used is in parentheses. *$p<0.05$ *vs* WKY; #$p<0.05$ *vs* untreated by one way ANOVA.

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Fig 1

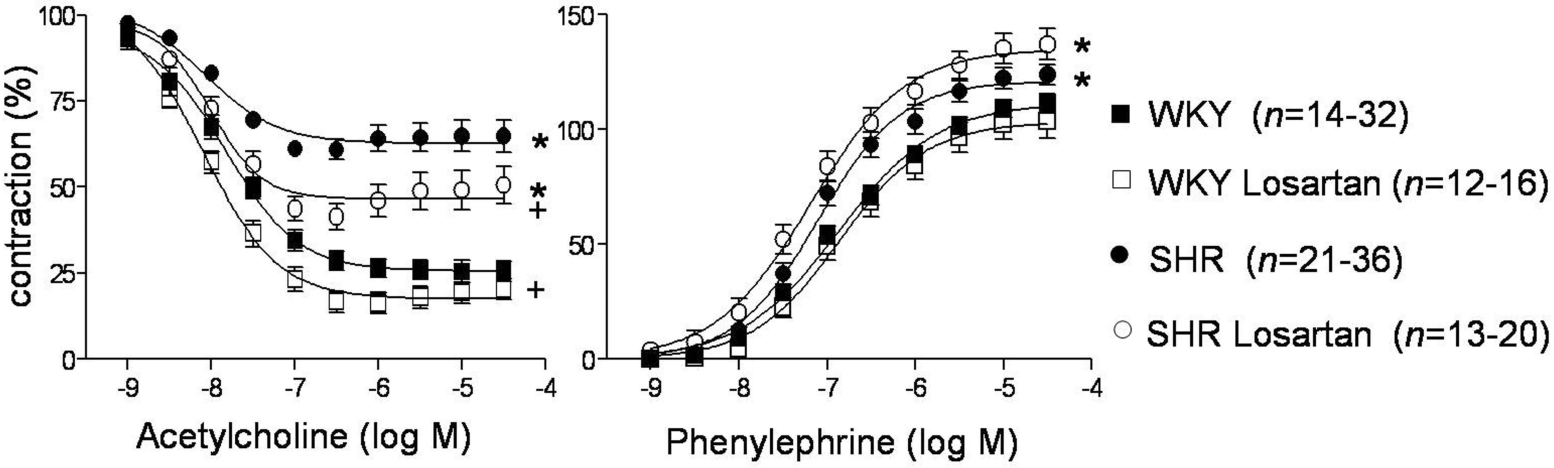

Fig 2

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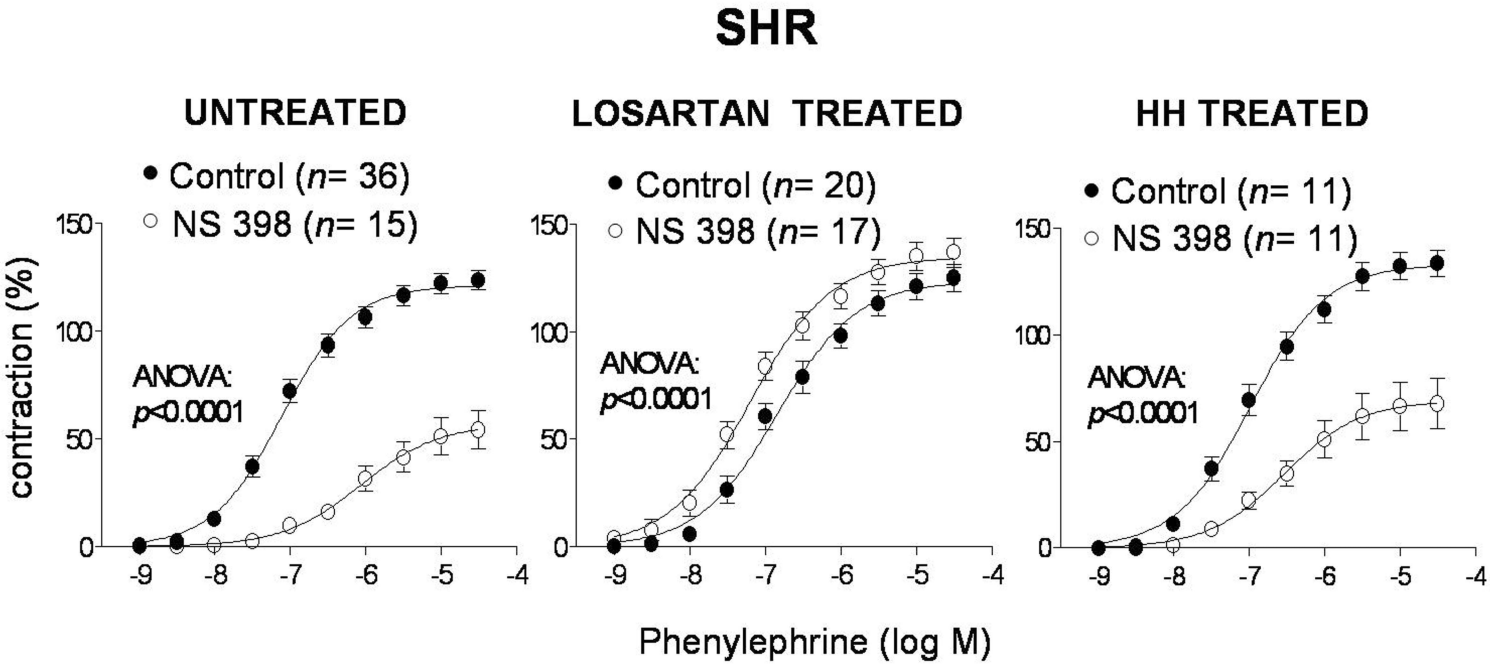

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Fig 3

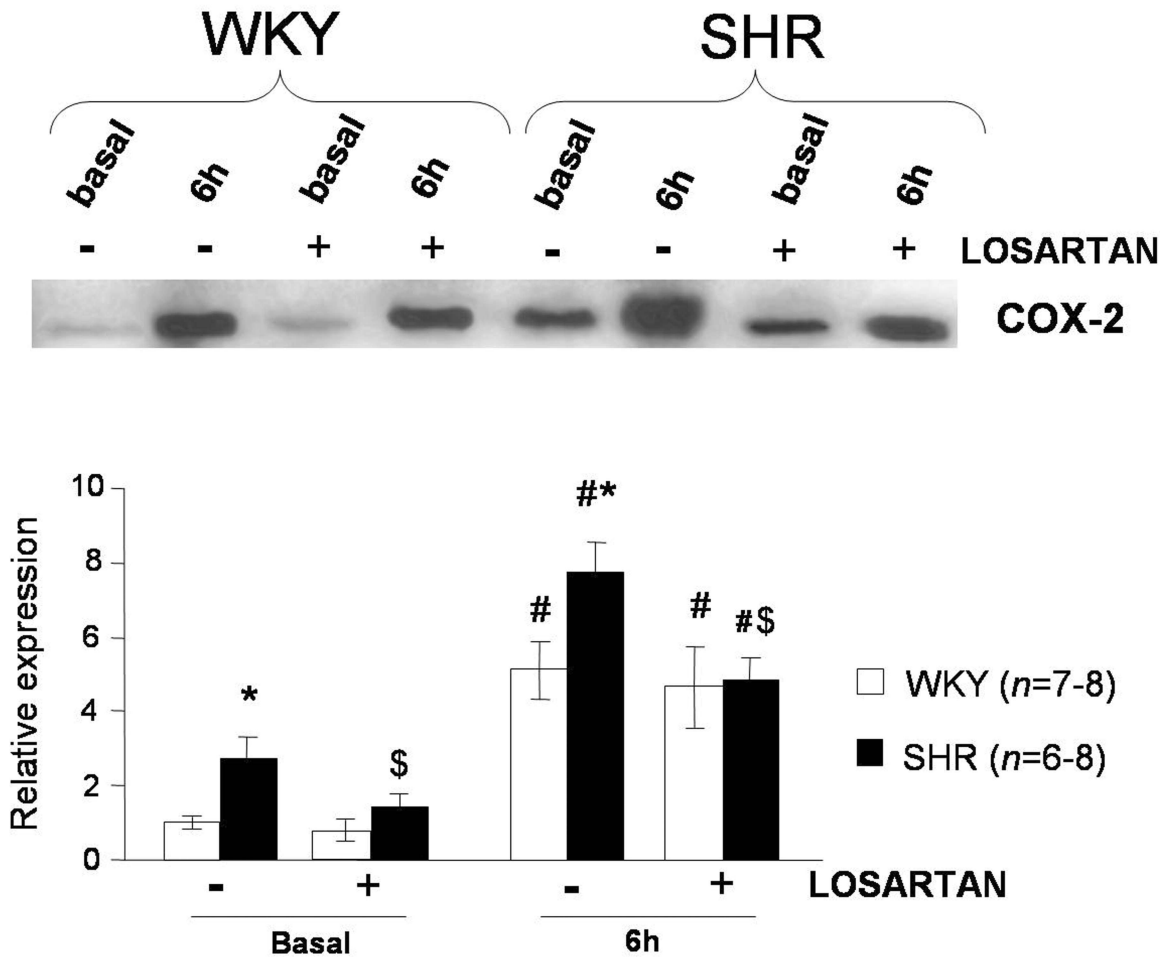

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Fig 4

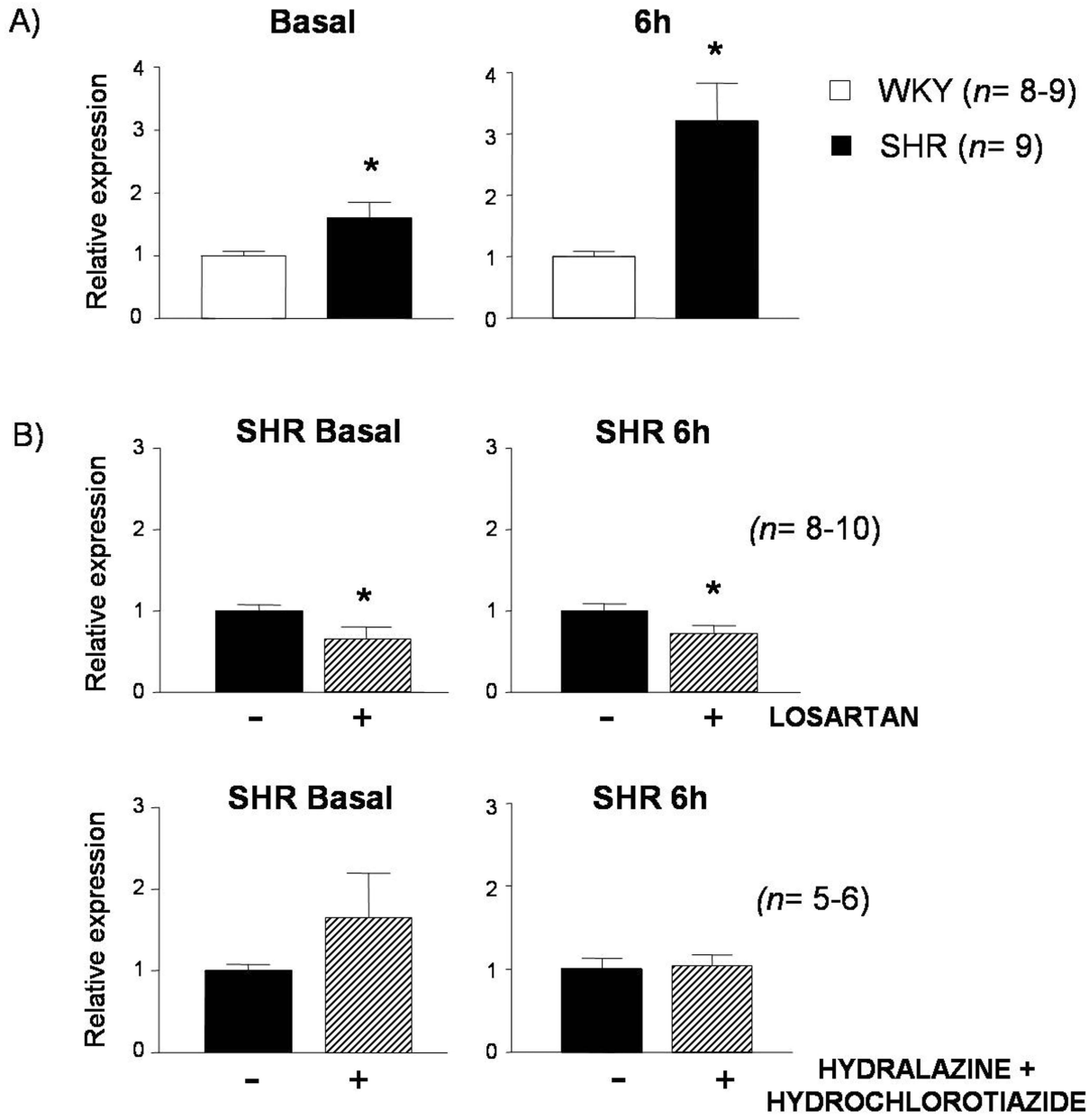

Fig 5

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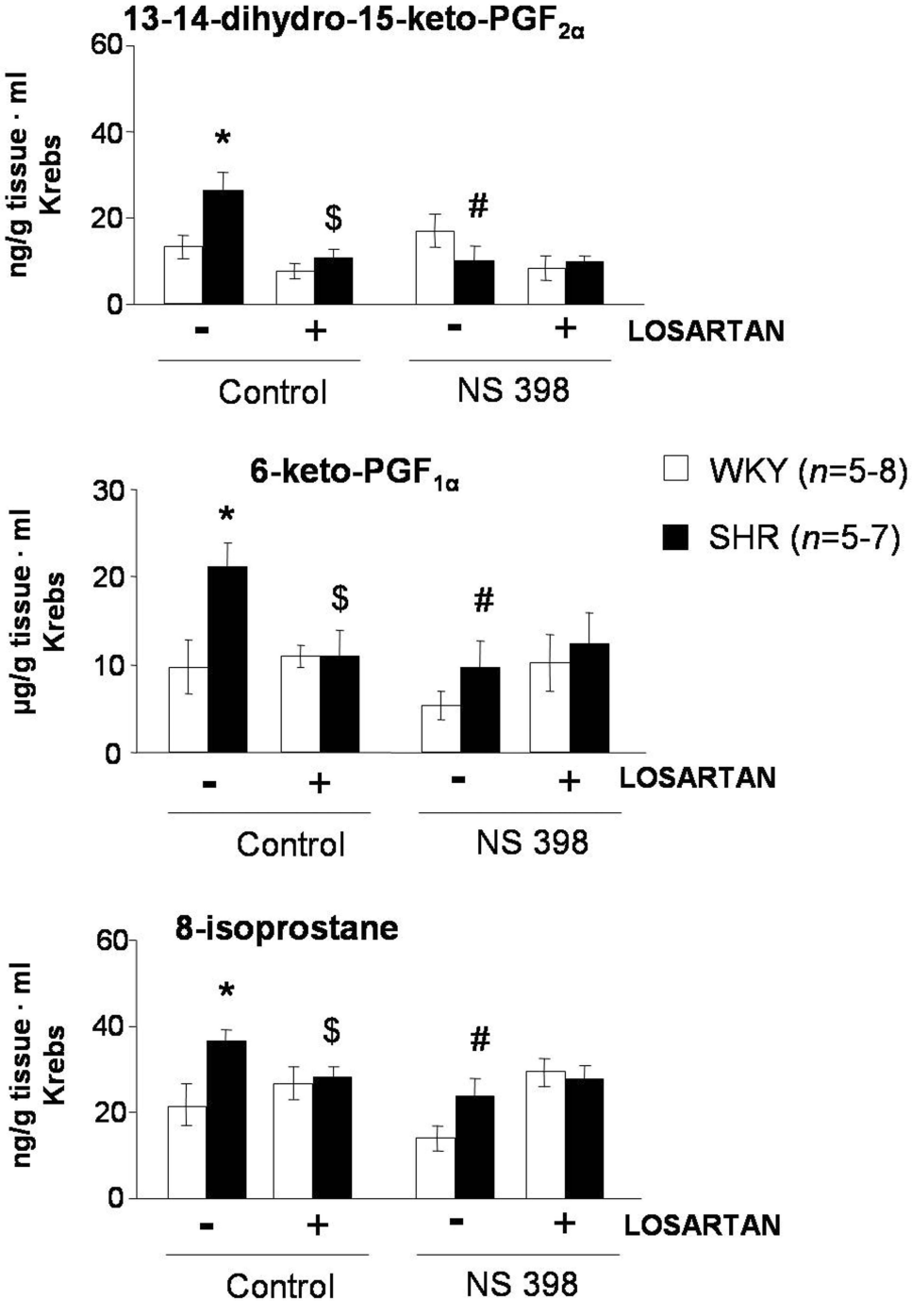

Fig 6

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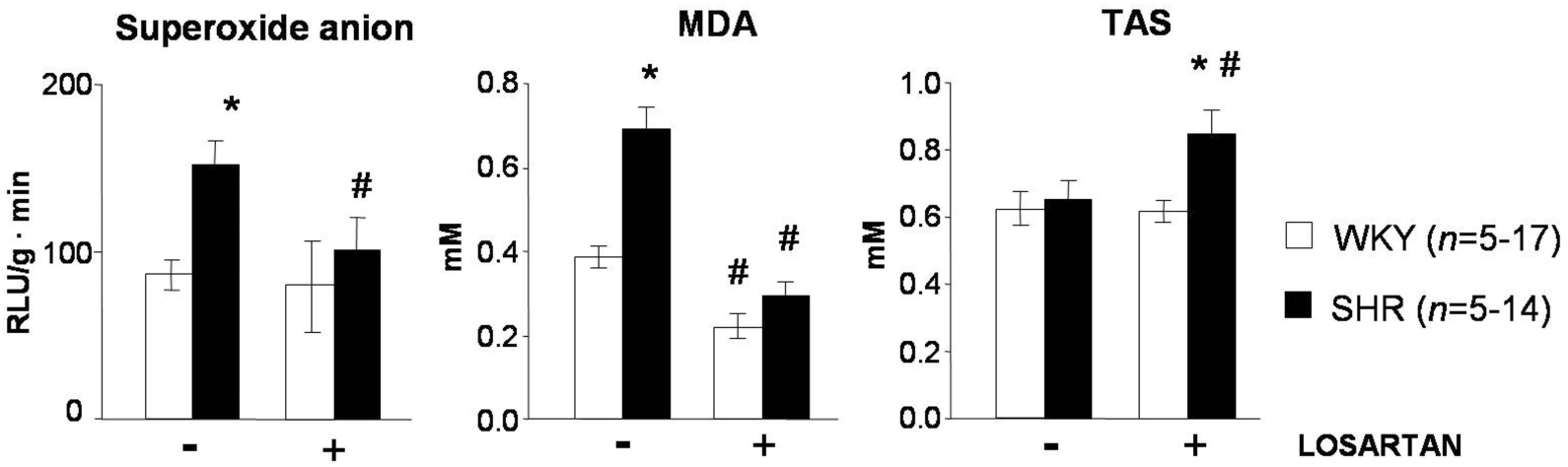

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Fig 7