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Vascular mechanisms involved in angiotensin II-induced venoconstriction in hypertensive rats

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ABSTRACT

To investigate the venoconstrictor effect of angiotensin II (Ang II) in spontaneously hypertensive rats (SHR), we used preparations of mesenteric venular beds and the circular muscle of the portal veins. Vessels were tested with Ang II in the presence or absence of losartan, PD 123319, HOE 140, L-NAME, indomethacin, or celecoxib. In the mesenteric venular bed of SHR, the effect of Ang II (0.1 nmol) was nearly abolished by losartan and enhanced by HOE 140, indomethacin, and celecoxib, while PD123319 and L-NAME had no effect. In portal vein preparations, cumulative-concentration response curves (CCRC) to Ang II (0.1–100 nmol/L) exhibited a lower maximal response (E_{max}) in SHR compared to Wistar rats. AT_1 receptor expression was similar in the two strains, while AT_2 receptor levels were lower in SHR portal veins when compared to Wistar. In SHR portal veins, losartan shifted the CCRC to Ang II to the right, while indomethacin and HOE 140 increased the E_{max} to Ang II. PD 123319, celecoxib, and L-NAME had no effect. Taken together, our results suggest that Ang II-induced venoconstriction in SHR is mediated by activation of AT_1 receptors and this effect may be counterbalanced by kinin B_2 receptor and COX metabolites. Furthermore, our data indicate that there are different cellular and molecular mechanisms involved in the regulation of venous tonus of normotensive and hypertensive rats. These differences probably reflect distinct factors that influence arterial and venous bed in hypertension.

1. Introduction

The venous system plays an important role in cardiovascular homeostasis since it contains about 65% of the total blood volume [25]. The capacitance properties of the cardiovascular system are primarily determined by veins and venules [24]. Alterations in venous tonus induced by hormones, peptides or drugs influence directly the cardiac output, right atrial pressure, and, therefore, cardiac performance [32,37].

Increase in venous tonus and reduction in venous compliance have been described in various models of experimental hypertension [10,16,27,30]; however, data are not conclusive and some aspects remain to be elucidated. Moreover, methodological problems involved in isolation of veins and venules commit study of this vascular bed. In spite of this, isolated portal vein and perfused mesenteric venular bed preparations have been used in biological research to asses venous function in view of the fact that these preparations respond to a variety of vasoactive agents [32,37]. Since splanchnic venous bed accommodates about 25% of the total blood volume [32] and mesenteric vascular bed can be destination for 10% of cardiac output [37], investigation of venous responses at these vascular regions could yield important information about circulatory function and control of blood pressure.

The renin-angiotensin system (RAS) is a coordinated hormonal cascade important to the regulation of renal sodium excretion and blood pressure. Angiotensin II (Ang II), the main effector peptide of RAS, binds two major receptors, AT_1 and AT_2 (AT_1R and AT_2R) [38]. The vast majority of Ang II actions occur via the AT_1R binding, including vasoconstriction, cellular proliferation, and activation of the sympathetic nervous system [35]. The actions of Ang II mediated by AT_2R are less well understood; however, it is known that AT_2R stimulation includes vasodilation, inhibition of cell proliferation and modulation of growth and remodeling in fetal vasculature [3].

Ang II promotes vasoconstriction in isolated mesenteric venules [8,37] and portal vein preparations [8,12,18,23] of normotensive rats; however, to our knowledge, the vascular effects of Ang II either in veins or venules from hypertensive rats have not been evaluated. Thus, the aim of the present study was to investigate the effects of Ang II in the mesenteric venular bed and in the

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circular muscle of portal veins from spontaneously hypertensive rats (SHR) by evaluating the participation of AT_1R and AT_2R on Ang II response. In addition, we analyzed the role of cyclooxygenase (COX) metabolites, nitric oxide (NO), and the kinin B_2R in modulating Ang II-mediated constriction in SHR.

2. Methods

2.1. Animals

Male Wistar and SHRs weighing 200–300 g were obtained from the Institute of Biomedical Sciences of the University of São Paulo (ICB-USP). All of the animal experiments were conducted in accordance with the guidelines of the Ethic Committee for Research of ICB-USP and conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication No. 85-23, revised 1996). Animals were kept in a temperature-controlled room on a 12 h light/12 h dark cycle with 60% humidity, standard rat chow, and water *ad libitum.*

2.2. Mesenteric venular bed preparation

Isolated perfused mesenteric venular bed preparations were performed according to the method previously described [37]. After rats were anesthetized with chloral hydrate (450 mg/kg, s.c.), a cannula (PE 50) was inserted retrogradely (1.0 cm) into the portal vein and the vascular mesenteric bed was dissected out at its border with the intestine. The mesenteric venular bed was perfused at a constant rate of 2 mL/min using a peristaltic pump (Miniplus 3, Gilson, France) with Krebs-Henseleit solution, pH 7.4, at 37 °C in the presence of 95% O_2 and 5% CO_2. To confirm the viability of tissues, preparations were exposed to 90 mmol/L KCl for 5 min. After 30 min of washing out the KCl with Krebs solution, Ang II (0.1 nmol) was administered *in bolus* in a final volume of 100 μL and vascular responses were evaluated as changes in the perfusion pressure (mmHg) (PowerLab 4S; ADInstruments, Australia).

2.3. Portal vein preparation

Isolated portal vein ring preparations were performed according to the method previously described [2]. Rats were anesthetized with chloral hydrate (450 mg/kg, s.c.), the portal vein was excised and connective tissue was removed. Rings of portal veins (3–4 mm length) were mounted under 0.5 g of passive tension in an organ bath (15 mL) containing Krebs-Henseleit solution, pH 7.4, at 37 °C with 95% O_2 and 5% CO_2. Preparations were allowed to equilibrate for 60 min; during this time, the bath solution was changed every 20 min. To confirm the viability of tissues, the preparations were exposed to 90 mmol/L KCl for 5 min. After 30 min of washing out the KCl with normal Krebs solution, a cumulative-concentration response curve (CCRC) to Ang II (0.1–100 nmol/L) was performed and changes in isometric tension (grams) were recorded (PowerLab 4S, ADInstruments, Australia). CCRC were analyzed by a data analyses program (Prism3, GraphPad) to evaluate the EC_{50} (the concentration of Ang II required to produce 50% maximum response) and maximum response (E_{max}). Efficacy and sensitivity of portal vein rings preparations in response to Ang II was determined as E_{max} and pEC50 (−log EC50), respectively.

2.4. Treatments of Mesenteric venous bed and portal vein ring preparations

To investigate the mechanisms involved in Ang II-mediated contraction, preparations of mesenteric venous beds and portal vein rings were incubated with Krebs-Henseleit solution containing losartan (specific AT_1R antagonist, 0.1 μmol/L), PD 123319 (specific AT_2R antagonist, 0.1 μmol/L), HOE 140 (specific B_2R antagonist, 20 nmol/L) [13], indomethacin (COX inhibitor, 10 μmol/L), or L-NAME (inhibitor of NO synthesis, 10 μmol/L) 30 min before Ang II injection. In addition, a group of SHR were treated with celecoxib (specific COX2 inhibitor, 10 mg/kg) [20] administered by gavage 3 h before were killed and the mesenteric venular beds and portal vein rings were prepared. All the concentrations of antagonists/inhibitors used in experiments were based in preliminary studies performed in our laboratory or in the literature, when specified.

2.5. RNA isolation and cDNA synthesis

Total RNA from the portal veins of SHR and Wistar rats was extracted using Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's protocol. Genomic DNA was digested with DNase I and first-strand cDNA was synthesized from 2 μg of total RNA in a volume of 20 μL, using SuperScript II enzyme (Invitrogen); RNAse Out (Invitrogen) was added to protect RNA during this process. Samples were incubated at 42 °C for 50 min, and the reaction was stopped by heating the samples at 70 °C for 15 min. Samples were cooled at 4 °C for 10 min.

2.6. Real-time PCR

Diluted samples from the reverse transcriptase reaction (1:10) underwent real-time PCR amplification using Platinum SYBR QPCR Supermix-UDG and specific primers for AT_1R (forward, CACTTTCCTGGATGTGCTGA; reverse, CCCAGAAAGCCGTAGAACAG; 141 bp) and AT_2R (forward, CTGCTGGGATTGCCTTAATGA; reverse, AGCAGATGTTTTCTGATTCCAAAGT; 94 bp). Gene expression of GAPDH mRNA was used for normalization (forward, GGTGCTGAGTATGTCGTGGA; reverse, ACTGTGGTCATGAGCCCTTC; 262 bp). Real-time PCRs were performed, recorded, and analyzed using the Corbett Research system (Corbett Life Sciences, Australia). The conditions for PCR were as follows: 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 15 s. The specificity of the SYBR Green assay was confirmed by melting point analysis. Expression data were calculated from the cycle threshold (C_t) value using the ΔC_t method for quantification [22]. Results were expressed as fold increases. All of the reagents utilized in this method were purchased from Invitrogen (USA).

2.7. Immunohistochemical assay for AT_1R and AT_2R

Immunohistochemical assay for detection of angiotensin receptors in portal vein was realized according with the method previously described [4]. The portal vein was fixed with 4% paraformaldehyde (PFA) solution for 6 h and immersed in sucrose solution (30%) for 12 h. After that, portal vein was embedded in medium for cryosectioning, cut into 8 μm thick sections with a Leica CM 1850 cryostat (Leica Instruments, Germany), and placed on slides. The vessels were incubated with rabbit anti-goat AT_1R or AT_2R antibody (IgG; Santa Cruz Biotechnology, USA) at 1:25 and 1:10 dilutions, respectively, at 4 °C for 18 h. Slides were washed with phosphate buffered saline (PBS) and incubated with biotin-conjugated secondary antibody (diluted 1:1000; Vector Laboratory, USA) at room temperature for 2 h. After incubation, slides were washed with PBS and incubated with the ABC Vectastain kit (Vector Laboratory, USA) at room temperature for 2 h. The signal was revealed by incubating the slides with 3,3-diaminobenzidine (DAB) (Sigma–Aldrich, USA) and 0.06% H_2O_2 for 30 and 60 s for the AT_1R and AT_2R antibodies, respectively. The semi-quantitative analysis of staining to AT_1R and AT_2R antibodies was determined at least in five portal vein slides from each animal and protein expression levels were expressed as arbitrary units

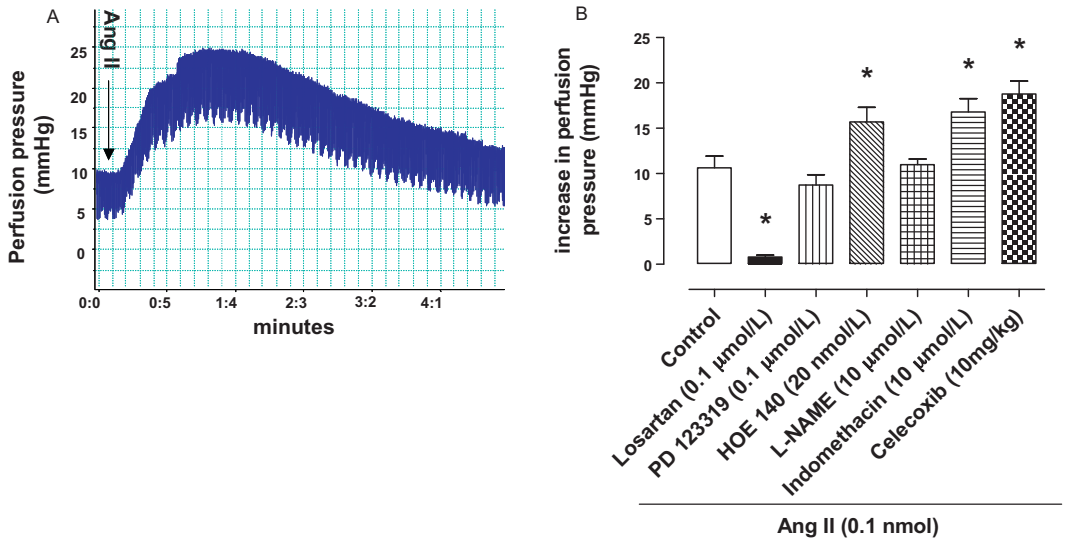

Fig. 1. (A) Representative recording of perfusion pressure alteration elicited by in bolus injection of Ang II in the rat isolated mesenteric venular bed. The arrow indicates drug application. (B) Effect of different drug treatments on Ang II-induced venoconstriction. Bar graph showing the effect of 0.1 nmol Ang II in isolated mesenteric venular beds from SHR, expressed as an increase in perfusion pressure (mmHg). Results represent mean ± SEM, $n = 5–8$ for each group. $^{*}P < 0.05$ *versus* control.

determined by optic densitometry with the KS-300 image program (Carl-Zeiss, Germany).

2.8. Drugs and reagents

Ang II was from Bachem-CA; HOE 140, PD 123319, L-NAME, and indomethacin were from Sigma–Aldrich; losartan was from Merck Sharp & Döhme and celecoxib was from Pfizer.

2.9. Statistical analysis

Comparisons were made by ANOVA followed by Tukey-Kramer test or Student's *t*-test when appropriate. Values were reported as mean ± standard error of mean (SEM). Statistical significance was set as $P < 0.05$.

3. Results

3.1. Effect of Ang II on mesenteric venous beds

Ang II injection induced a slight but consistent constriction in isolated mesenteric venules (Fig. 1A). No significant differences were observed between the responses of Wistar rats (10.6 ± 1.1 mmHg; $n = 6$) and SHR (10.6 ± 1.3 mmHg; $n = 8$).

Basal perfusion pressure in mesenteric venous bed was not modified by pre-incubation with different antagonists. In SHR preparations, the constriction induced by Ang II was nearly

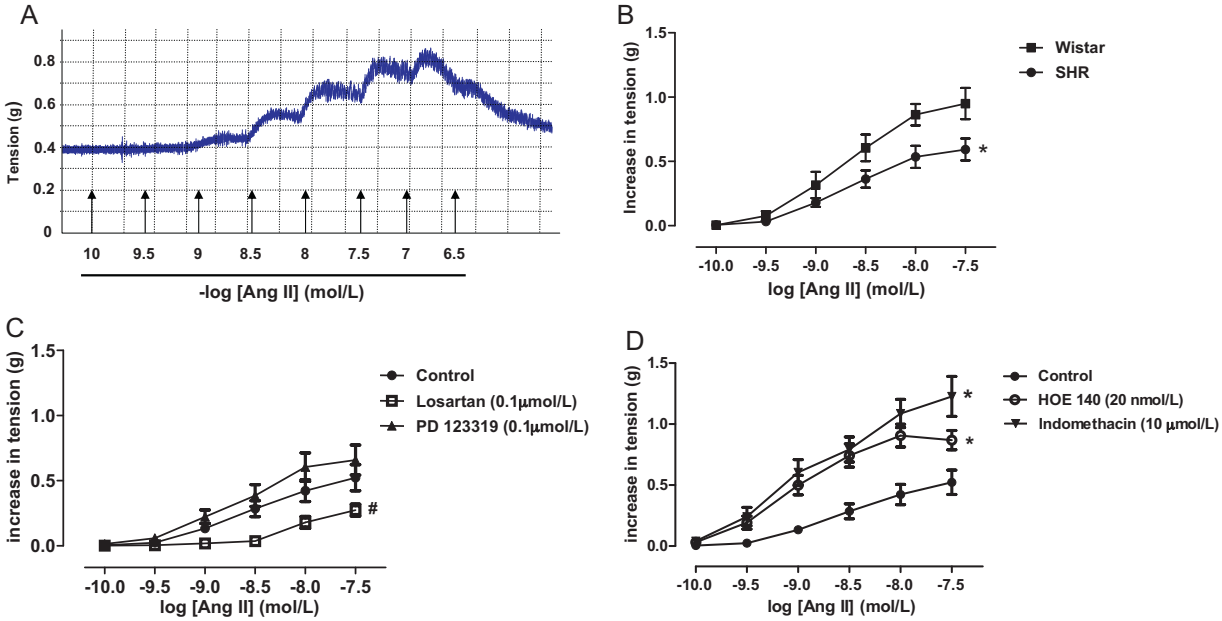

Fig. 2. Effect of Ang II on isolated portal vein rings. (A) Representative recording of a CCRC to Ang II on portal vein ring. Arrow indicates drug application. (B) CCRC to Ang II on isolated portal vein rings from SHR and Wistar rats, expressed as an increase in tension (g). Results represent mean ± SEM, $n = 6–8$ for each group. $^{*}P < 0.05$ *versus* Wistar rats. (C) CCRC to Ang II in isolated portal vein rings from SHR in the absence (●) or presence of losartan (■) or PD123319 (▲); (D) the absence (●) or presence of indomethacin (▼) or HOE 140 (○). Results represent mean ± SEM, $n = 6–8$ for each group. $^{*}P < 0.05$ *versus* control (E_{max}); $^{\#}P < 0.05$ *versus* control (pEC_{50}).

abolished ($P < 0.05$) by perfusion with losartan (0.8 ± 0.2 mmHg; $n = 7$), while PD123319 and L-NAME had no effect at all. In contrast, Ang II venoconstriction increased ($P < 0.05$) after B_2R blockade with HOE 140 (15.7 ± 1.6 mmHg; $n = 8$), and also after COX inhibition with indomethacin (16.8 ± 1.5 mmHg, $n = 6$) or celecoxib (18.8 ± 1.4 mmHg, $n = 5$). The results are shown in Fig. 1B.

3.2. Effect of Ang II on portal vein rings

Starting at 1 nmol/L, Ang II contracted rings of portal vein in a concentration-dependent manner. The E_{max} was reached at 50 nmol/L. At concentrations higher than 100 nmol/L, Ang II induces rapid desensitization (tachyphylaxis) in this preparation (Fig. 2A).

Fig. 2B shows the CCRCs to Ang II in both Wistar and SHR portal vein preparations. The E_{max} to Ang II was significantly reduced ($P < 0.05$) in SHR (0.62 ± 0.09; $n = 6$) compared to Wistar rats (1.00 ± 0.15; $n = 6$). No changes were detected in response to KCl (Wistar: 0.43 ± 0.07 g; $n = 7$ *versus* SHR: 0.31 ± 0.06 g; $n = 8$).

Pre-incubation of portal vein rings from SHR with losartan shifted to the right the CCRC to Ang II [Control: pEC_{50}: 8.62 ± 0.05 mol/L; $n = 6$ *versus* Losartan: 7.95 ± 0.06 mol/L; $n = 4$ ($P < 0.05$)], whereas PD 123319 treatment had no effect (Fig. 2C). Pre-incubation with indomethacin and HOE 140 increased the E_{max} to Ang II [Control: 0.57 ± 0.09 g, $n = 8$ *versus* Indomethacin: 1.21 ± 0.14 g, $n = 7$ and HOE 140: 1.01 ± 0.08 g, $n = 11$ ($P < 0.05$)], as demonstrated in Fig. 2D. L-NAME and celecoxib did not alter the Ang II response (data not shown).

3.3. Expression of AT_1 and AT_2 receptors in portal veins

To investigate a possible alteration in angiotensin receptor expression between SHR and Wistar rats, we quantified the levels of AT_1R and AT_2R mRNA in samples from portal veins. The results are shown in Fig. 3.

While no differences were detected in AT_1R expression, AT_2R mRNA levels in the portal vein samples were significantly reduced in SHR [0.34 ± 0.13 arbitrary units (a.u.); $n = 7$; $P < 0.05$] compared to Wistar rats (1.05 ± 0.19 a.u.; $n = 4$) (Fig. 3).

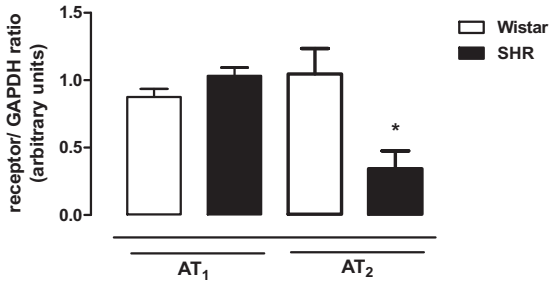

Fig. 3. mRNA expression of angiotensin receptors. Bar graph showing mRNA expression of AT_1R and AT_2R in portal veins from Wistar rats and SHR. Results from real-time PCR represent mean ± SEM, and were normalized to GAPDH mRNA expression; $n = 5$–7. *$P < 0.05$ *versus* Wistar rats.

Immunohistochemical assays revealed similar results. Fig. 4 contains representative images of immunohistochemical staining for AT_1R and AT_2R in SHR and Wistar rats. AT_1R and AT_2R were present in the endothelium, vascular smooth muscle cells, and adventitial layer. There was no difference in AT_1R expression in SHR and Wistar rats, while AT_2R expression was reduced in the portal veins of SHR (6.85 ± 0.50 a.u.; $n = 5$; $P < 0.05$) compared to Wistar rats (9.08 ± 0.25 a.u.; $n = 5$) (Fig. 5).

4. Discussion

Ang II evoked a consistent constriction in mesenteric venules and portal vein from SHR. In both vascular preparations, losartan reduced or nearly abolished the Ang II-mediated constriction, while PD123319 did not modify this response. Ang II-induced venoconstriction was markedly increased by indomethacin, while celecoxib was effective only in mesenteric venules. Whereas vascular responses to Ang II were augmented by HOE-140, L-NAME had no effect. By analyzing our results, we found that Ang II-induced

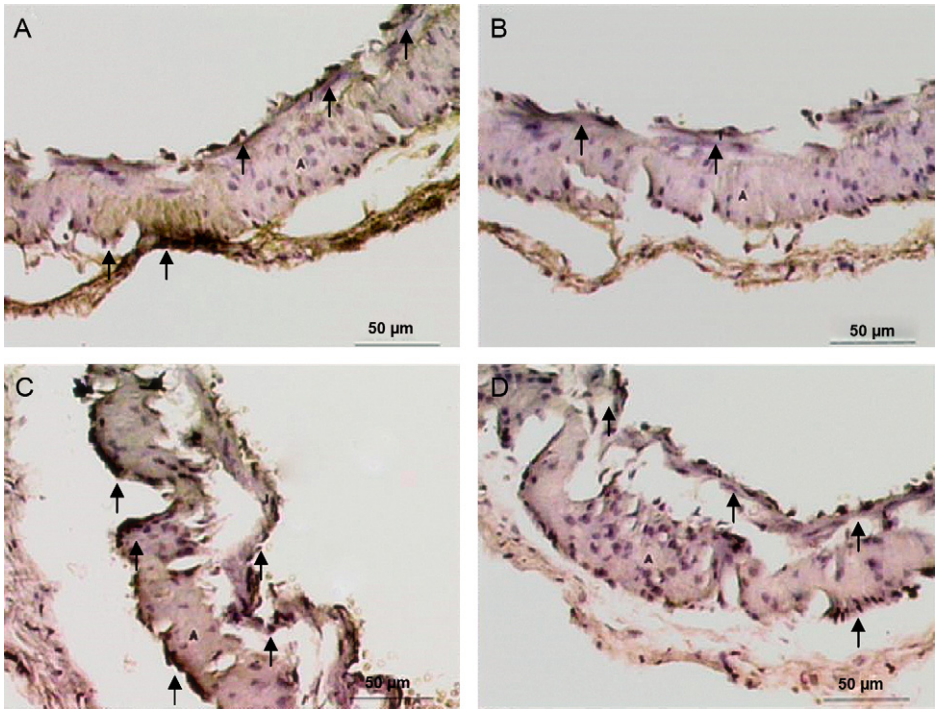

Fig. 4. Representative image of immunohistochemical staining of AT_1R and AT_2R 400× magnification. AT_1R staining in portal vein from Wistar rat (A) and SHR (B). AT_2R staining in portal vein from Wistar rat (C) and SHR (D). [I] indicates intimal layer; [A] indicates adventitial layer and [arrows] indicates antibody staining.

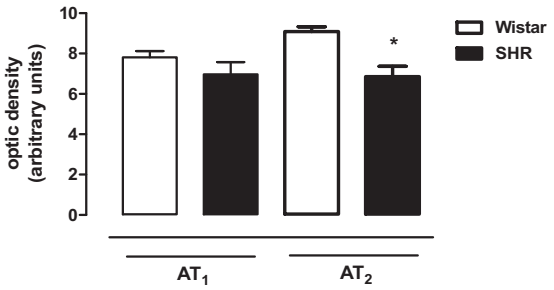

Fig. 5. Protein expression of angiotensin receptors. Bar graphs showing quantification of protein expression of AT_1R and AT_2R in portal veins of Wistar rats and SHR, expressed as arbitrary units. Results represent mean ± SEM, $n = 5$ for each group. $^*P < 0.05$ *versus* Wistar rats.

constriction in mesenteric venules and portal vein from SHR is dependent of AT_1R activation and counterbalanced by COX metabolites and kinin B_2R.

Several aspects of our results may point to important differences between the venous system of normotensive and hypertensive rats. For instance, Ang II-induced constriction was significantly attenuated in portal vein rings from SHR. Besides, Ang II-induced venoconstriction was mediated by both AT_1R and AT_2R in normotensive rats [8]. Considering these findings, we hypothesized that differences between strains could be related to changes in angiotensin receptors expression. In fact, when AT_1R and AT_2R were evaluated, the AT_2R expression was significantly reduced in portal vein from SHR. Although several studies have investigated the influence of AT_2R in the vascular system, the functional role of this subtype is not completely elucidated. Authors have demonstrated that AT_2R activation can induce both vasodilation [39] and vasoconstriction [34,40]. In this regard, a consistent vasoconstrictor effect of Ang II mediated by AT_2R in mesenteric arterioles of SHR has been demonstrated [34,40]. Moreover, AT_2R also participates of contractile effect of Ang II in portal vein preparations from normotensive rats [8,23]. Probably, reduction of AT_2R levels in portal vein from SHR can be responsible for the decreased response observed by us. This result can indicate that AT_2R plays a distinct role in the vasculature of normotensive and hypertensive rats.

The basic hemodynamic disturbance in established hypertension is an elevation of total peripheral resistance, which is determined mainly by resistance vessels from arterial system. In fact, it is well established that hypertensive patients have similar values of cardiac output in comparison with normotensive controls and the elevated blood pressure is maintained by increase in total peripheral resistance [16,26]. Similarly, it was demonstrated that cardiac output is not altered in SHR, a generally accepted model for human essential hypertension [31,36]. From this point of view, reduced Ang II response observed in venous from SHR would not be influencing cardiac output control. However, considering that Ang II-induced constriction is augmented in arteries from hypertensive animals [6,11,13], reduction of Ang II responses in the venous system could be related to a compensatory mechanism, avoiding exaggerated increase in venous return and alterations in cardiac output.

The kalikrein–kinin system plays an important role in the maintenance of cardiovascular homeostasis. In this regard, the kinin B_2R null mice present high sensitivity to hypertensive stimuli [1,5], impairment of endothelium-dependent vasodilation and decrease in NO bioavailability [15]. Moreover, studies have indicated the existence of functional interactions between angiotensin and kinin receptors in vascular cells. In this respect, Seyed et al. [29] demonstrated that Ang II-mediated vasodilation in coronary vessels from dogs is dependent of B_2R. This interaction was also observed in arteries from normotensive [9,19] and hypertensive rats [21]. The present data suggest that Ang II-induced constriction is also counterbalanced by B_2R activation in venules and veins from hypertensive rats. Therefore, the final effects resulted from Ang II, at least on these vascular beds, should be considered as a combination of AT_1R signaling in the presence of a modulating action elicited by B_2R. Further studies will reveal the physiological and pathophysiological consequences of this phenomenon.

Whereas COX metabolites appear to counterbalance the Ang II-induced venoconstriction in SHR, our data do not suggest the participation of NO in this effect. In normotensive rats, Fernandes et al. [8] demonstrated that NO counteracts the Ang II-induced venoconstriction, while COX metabolites were not involved in this response. Similar results were observed in mesenteric arterioles from normotensive rats [19]. It has been suggested that alteration in NO metabolism is implicated in endothelial dysfunction, a common denominator in essential hypertension [7]. In fact, several vascular beds of SHR present impaired endothelium-dependent vasodilation [14,17,33]. In this regard, increased production of superoxide anion in vessels of SHR has been associated to NO inactivation and elevation of the blood pressure [28]. Our data suggest that production of vasodilatory eicosanoids in venous bed from SHR represent an alternative pathway to attenuate the Ang II-induced constriction at low levels of NO. Moreover, COX metabolites probably are involved in impairment of Ang II-induced constriction in portal vein from SHR.

Concluding, in SHR, the attenuation of Ang II-induced venoconstriction by COX metabolites and B_2R may be involved in the local response to conserve the normal cardiac output in established hypertension. Taken together, our data indicate that different mechanisms are involved in the regulation of venous tonus of normotensive and hypertensive rats. These differences probably reflect distinct factors that influence arterial and venous bed in hypertension.

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