BASIC RESEARCH

Renin angiotensin system and cardiac hypertrophy after sinoaortic denervation in rats

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OBJECTIVE: The aim of this study was to evaluate the role of angiotensin I, II and 1–7 on left ventricular hypertrophy of Wistar and spontaneously hypertensive rats submitted to sinoaortic denervation.

METHODS: Ten weeks after sinoaortic denervation, hemodynamic and morphofunctional parameters were analyzed, and the left ventricle was dissected for biochemical analyses.

RESULTS: Hypertensive groups (controls and denervated) showed an increase on mean blood pressure compared with normotensive ones (controls and denervated). Blood pressure variability was higher in denervated groups than in their respective controls. Left ventricular mass and collagen content were increased in the normotensive denervated and in both spontaneously hypertensive groups compared with Wistar controls. Both hypertensive groups presented a higher concentration of angiotensin II than Wistar controls, whereas angiotensin 1–7 concentration was decreased in the hypertensive denervated group in relation to the Wistar groups. There was no difference in angiotensin I concentration among groups.

CONCLUSION: Our results suggest that not only blood pressure variability and reduced baroreflex sensitivity but also elevated levels of angiotensin II and a reduced concentration of angiotensin 1–7 may contribute to the development of left ventricular hypertrophy. These data indicate that baroreflex dysfunction associated with changes in the renin angiotensin system may be predictive factors of left ventricular hypertrophy and cardiac failure.

KEYWORDS: Angiotensins; Left ventricular hypertrophy; SAD; Spontaneously hypertensive rats; Collagen.

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INTRODUCTION

Arterial baroreflex is the main mechanism for the regulation of the cardiovascular system, and sinoaortic denervation (SAD) is a widely used tool to study its function.[1] It is well established that chronic SAD increases blood pressure variability (BPV) without increasing mean blood pressure.[2] Previous studies have demonstrated that BPV,[3] baroreflex sensitivity,[4] and the renin angiotensin system (RAS)[5] were independent determinants in hypertensive end-organ damage in rats.

In addition to the classic circulating RAS, there is growing evidence supporting the existence of a local functioning cardiac RAS.[6] All components of the RAS (renin, angiotensinogen, angiotensin-converting enzyme (ACE), and angiotensin II receptors) are identified in the heart at the mRNA and protein levels,[7] and this system is activated in experimental left ventricular hypertrophy (LVH) induced by hemodynamic overload.[8]

The hypertension-induced LVH initially serves as an adaptive ventricular response to pressure overload; nevertheless, it is a major independent risk factor associated with increased cardiovascular morbidity and mortality.[9]

The aim of this study was to evaluate ventricular hypertrophy parameters induced by baroreflex impairment and the contribution of the local cardiac angiotensins (Ang) I, II and 1–7 in this process in normotensive (Wistar) and hypertensive (SHR) rats.

MATERIALS AND METHODS

Animals

Experiments were performed on male Wistar and SHR rats aged 16 weeks,[10] housed in cages with free access to water and food, and maintained in a room with a constant temperature (23℃) on a 12-hour light–dark cycle. All

surgical procedures and protocols used were in accordance with the Guidelines for Ethical Care of the Experimental Animals and were approved by the Institutional Animals Care and Use Committee (CEP 1016/08). Rats were randomized into four groups: Wistar control (WC), N = 9, Wistar denervated (WD), N = 7, SHR control (SHRC) N = 8, SHR denervated (SHRD) N = 8.

Sinoaortic denervation

SAD was performed under anesthesia with ketamine, 80 mg/kg (Parke-Davis, Brazil) and Xylazine, 12 mg/kg (Bayer, Brazil). A 3-cm midline incision was made, and sternocleidomastoid muscles were reflected laterally, exposing the neurovascular sheath. The common carotid arteries and the vagal trunk were isolated, and the aortic depressor fibers either traveling with the sympathetic nerve or as an isolated aortic nerve were cut. The communicating branch of the aortic fibers was also resected. The third contingent of aortic baroreceptor fibers traveling with the inferior laryngeal nerve was interrupted by resection of the superior laryngeal nerve after the carotid bifurcation was exposed extensively for carotid stripping. To complete the procedure, the sinus nerve, all carotid branches and the carotid body were resected.[11]

The mortality rate was 35% higher in the animals submitted to SAD than in their respective controls.

Hemodynamic measurements

Ten weeks after SAD, animals were anesthetized with ketamine, 80 mg/kg (Parke-Davis, Brazil) and xylazine, 12 mg/Kg (Bayer, Brazil), and femoral artery and vein catheters were inserted for direct measurements of blood pressure (BP), heart rate (HR) and drug administration.

Twenty-four hours after catheterization, the arterial catheter was connected to a transducer (Blood Pressure XDCR, Kent© Scientific, Litchfield, CT), and BP signals were recorded in conscious, freely moving rats, over a 30-min period by a microcomputer equipped with an analog-to-digital converter board (Windaq, 2000 Hz sampling frequency, Dataq Instruments, Inc, Akron, OH). The recorded data were analyzed on a beat-to-beat basis to quantify changes in mean BP and HR. Both BP and HR variability were evaluated using the mean of the standard deviation.

Baroreflex sensitivity evaluation

Increasing doses of phenylephrine (0.25–32.0 μg/kg) and sodium nitroprusside (0.05–1.6 μg/kg) were given as sequential bolus injections (0.1 mL) to produce pressure responses ranging from 5 to 30 mmHg. A 3- to 5-min interval between doses was necessary for BP to return to baseline. Peak increases or decreases in mean BP after vasoactive drugs injection and the corresponding peak reflex changes in HR were recorded for each dose of the drug. Baroreflex sensitivity was evaluated by a mean index that associated changes in HR to the changes in mean BP, thus allowing for a separate analysis of gain for reflex bradycardia and reflex tachycardia.[12] The mean index was expressed as bpm/mmHg.

Left ventricular hypertrophy evaluation

LVH was evaluated in animals at 16 weeks of age by ventricular hypertrophy index (calculated by LV/body mass index (mg/g)), echocardiography and LV collagen volume fraction.

Echocardiography

Echocardiographic variables were obtained according to the recommendations of the American Society of Echocardiography. Transthoracic echocardiography was performed in all the groups, by double-blind observers with the use of a SEQUOIA 512 (ACUSON Corporation, Mountain View, CA), which offers a 10–13 MHz multifrequency linear transducer. Images were obtained with the transducer placed on each animal's shaved chest (lateral recumbence). To obtain a more distinctive image display, a transmission gel was used between the transducer and the animal's chest (General Imaging Gel, ATL, Reedsville, PA). Animals were scanned from below, at a 2-cm depth with focus optimized at 1 cm. All measurements were based on the average of three consecutive cardiac cycles. Rats were anesthetized with a combination of ketamine, 80 mg/kg (Parke-Davis) and xylazine, 12 mg/kg (Bayer) i.p. Wall thickness and left ventricular (LV) dimensions were obtained from a short-axis view at the level of the papillary muscles. LV mass was calculated by using the following formula, assuming a spherical LV geometry and validated in rats: LV mass = $1047 \times [(LVd + PWd + IVSd)3 - LVd3]$, where 1047 is the specific gravity of muscle, LVd is LV end-diastolic diameter, PWd is end-diastolic posterior wall thickness and IVSd is end-diastolic interventricular septum thickness. In addition, another morphological index was evaluated, the relative wall thickness (RWT), which is expressed by $2 \times PWD/LVd$. It represents the relation between the LV cavity in diastole and the LV posterior wall. LV fractional shortening was calculated as $(LVd - LVs)/LVd \times 100$, where LVs is LV end-systolic diameter. Two-dimensional guided pulsed-wave Doppler recordings of LV inflow were obtained from the apical four-chamber view. Maximal early diastolic peak velocity (E) and late peak velocity (A) were derived from mitral inflow. The LV outflow tract velocity was measured just below the aortic valve, from an apical five-chamber view. The velocity of circumferential fiber shortening (VCF) was measured following the formula $(LVd - LVs)/(LVd \times ET)$, where ET is the ejection time. The sample volume was then placed between the mitral valve and LV outflow tract so that the aortic valve closure line and the onset of mitral flow could be clearly identified. The isovolumic relaxation time (IVRT) was measured from aortic valve closure to the onset of mitral flow. Global cardiac function was evaluated by using the myocardial performance index (MPI), which is the ratio of total time spent in isovolumic activity (isovolumic contraction time and isovolumic relaxation time) to the ET. These Doppler time intervals were measured from the mitral inflow and LV outflow time intervals. Interval ''a'', from the cessation to onset of mitral inflow is equal to the sum of the isovolumic contraction time, ET and isovolumic relaxation time. Ejection time ''b'' is derived from the duration of the LV outflow Doppler velocity profile. The MPI was calculated with the formula $(a - b)/b$.

Collagen volume fraction evaluation

At the end of the experimental protocol, a transverse incision below the diaphragm and bilateral thoracotomy incisions were performed in the sodium pentobarbital (40 mg/kg i.p.) anesthetized rats, in order to expose the heart. A needle (40×12) was carefully introduced into the LV apex. The animal's heart was arrested in diastole by perfusion with a NaCl 0.9% plus 14 mM KCl solution

(pressure equal to a 13 cm water column), followed by 4% tamponed formalin for tissue fixation. Hearts were excised, trimmed, weighed and immersed in paraformol 4% in PBS for 24 h. Thereafter, the heart was transected perpendicular to the long axis and processed and embedded in paraffinso that histological sections could be performed. Histomorphometric analyses were performed blinded regarding the identity of the experimental groups. LV sections of 3 μm were stained with picrosirius red for collagen quantification.[13] Computerized image acquisition (Nikon Optiphot) and analysis were used to access collagen volume fraction in LV of all studied animals using the digital image analyzer program Image Pro Plus 6.0 (Media Cybernetics, USA).[14]

Quantification of angiotensins I, II and 1–7 by high-performance liquid chromatography

Tissue was homogenized as described by Oliveira et al.[15] in 100 mmol/L phosphate buffer (PB), pH 7.2, containing 340 mmol/L sucrose, 300 mmol/L NaCl (1 g tissue: 10 mL buffer) and inhibitor PMSF(100 mmol/L). The homogenates were centrifuged at 3.000 rpm, at 40℃ for 10 min and the supernatant was frozen at −70℃ until experimental use.

Angiotensins I, II, and 1–7 were quantified in tissue extracts using reverse-phase high-performance liquid chromatography coupled with ultraviolet (214 nm) detection (HPLC-UV). Peptide separation was accomplished using a 4.6 × 250 mm, 7-μm Aquapore OD 300 column (Applied Biosciences, Foster City, CA), equilibrated with 0.1% phosphoric acid in 5% acetonitrile. The peptides were initially separated by isocratic elution for 5 min, followed by a linear gradient from 5% to 35% acetonitrile in 0.1% phosphoric acid for 20 min at a flow rate of 1.5 mL/min. The left ventriclewas pre-homogenized in 8 mL of 0.1 M sodium phosphate buffer containing 0.34 M sucrose and 0.3 M NaCl (pH 7.2). Angiotensin III (320 ng) was added to each sample as an internal standard. Extraction of angiotensins I, II and 1–7 was achieved using Sep-Pak-C18 column chromatography (Millipore, Milford, MA). The column was activated with the following steps: 5 mL of methanol, 5 mL of tetrahydrofuran, 5 mL of hexane, 5 mL of methanol, and 10 mL of H_2O (MilliQ water). The samples were then loaded onto the column and eluted by the following steps: 10 mL of H_2O, 5 mL of 4% acetic acid, and 5 mL of ethanol/acetic acid/H_2O (9046, v/v). The peptides were eluted in the last phase and were evaporated to dryness in a SpeedVac SC110 sample concentrator (Savant Instruments, Holbrook, NY). Sample extracts were reconstituted with 500 μL of 0.1% phosphoric acid in 5% acetonitrile, after which they were filtered and injected onto the analytical column of the HPLC system. Retention time was used to identify peaks of interest, previously determined by the elution of peptide standards. Calculations were based on peak area, and angiotensins I, II and 1–7 concentrations were expressed as nmol/g of tissue.

RESULTS

Data are presented as mean ± SEM. Two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test was used. Statistical significance was considered when $p < 0.05$.

Hypertensive groups (SHRC and SHRD) showed higher values in mean blood pressure than the normotensive groups (WC and WD) (155 ± 2.2; 162 ± 7.3 vs 100 ± 1.8; 100 ± 2.8 mmHg, respectively).

Baroreflex sensitivity was evaluated to confirm the denervation. Tachycardia and bradycardia indexes in WD group were 0.44 ± 0.08 and −0.63 ± 0.05 bpm/mmHg, respectively; in the SHRD group, the tachycardia index was 0.23 ± 0.03 bpm/mmHg and bradycardia index was −0.67 ± 0.09 bpm/mmHg. These results confirm the baroreflex dysfunction caused by chronic SAD.

The BPV (expressed by standard deviation) was increased in the groups submitted to SAD compared with their respective controls, and the SHRD group presented increased values of BPV compared with the other groups (Fig. 1).

Animals of the WD, SHRC and SHRD groups presented higher ventricular hypertrophy index than WC, and SAD associated with hypertension induced a higher increase in left ventricle mass in the SHRD group (Fig. 2).

The size of left ventricle cavity in diastole (LVDIA) was increased in the animals of the hypertensive groups (SHRC and SHRD). The interventricular septum (IVS) and left ventricle posterior wall (LVPW) were increased in WD and SHR groups. The relative wall thickness (RWT) and left ventricle mass (LVM) were increased in the WD, SHRC and SHRD groups, and the SHR groups presented increased values of LVM compared with WD group.

Isovolumic relation time (IVRT) was increased in the WD, SHRC, SHRD groups. The E/A ratio was lower in the WD group than the WC group; and animals of the SHR groups presented increased values compared with the WD group.

Only the animals of the WD group presented increased values of ejection fraction (EF) and velocity of circumferential shortening (VCF) compared with the WC group (Table 1).

Left ventricular fibrosis, accessed by collagen volume fraction evaluation (Fig. 3A,B), showed that hypertensive rats (SHR and SHRD) presented increased percentage of

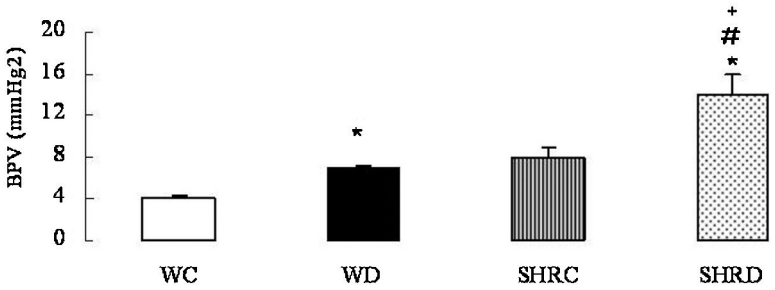

Figure 1 - Blood pressure variability (BPV) of the Wistar control (WC), Wistar denervated (WD), SHR control (SHRC), SHR denervated (SHRD) groups *p<0.05 vs WC; #p<0.05 vs WD; +p<0.05 vs SHRC.

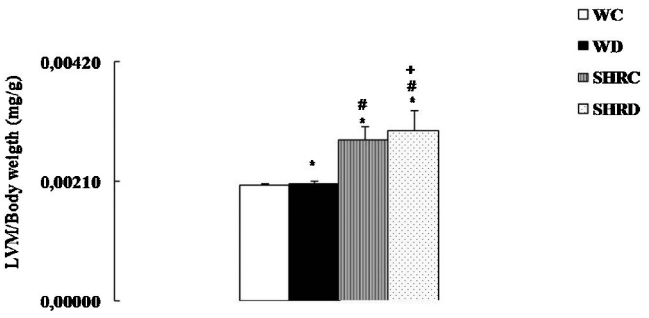

Figure 2 - Hypertrophy ventricular index (LVM/body weight) of the Wistar control (WC), Wistar denervated (WD), SHR control (SHRC), SHR denervated (SHRD) groups *p<0.05 vs WC; #p<0.05 vs WD; +p<0.05 vs SHRC.

collagen compared with normotensive groups. Interestingly, denervated rats (WD and SHRD) had sharply increased collagen content compared with baroreflex intact rats.

Fig. 4 shows the concentration of the Ang I, Ang II and Ang 1–7 in the left ventricle of different groups. Ang II concentration was increased in the SHR groups compared with the WC group, whereas the Ang 1–7 concentration was decreased in the SHRD group compared with the Wistar groups.

DISCUSSION

Chronic SAD (10 weeks) did not increase the BP and HR of normotensive and hypertensive groups. However, BPV was increased in the SHRD group compared with all groups. Additionally, SAD also increased the BPV in Wistar groups, as the variability became the same as the SHR animals without denervation.

The most consistent cardiovascular alteration produced by acute or chronic SAD is the huge increase in BPV. Previous studies have described similar high variability in dogs,[16] cats[17] and rats.[18] Usually, the BPV in SAD animals is characterized by an increase in the BP standard mean, obtained through the moment-to-moment records of BP signals.[18]

The increased BPV may be due to the absence of straight beat-to-beat synchronization between BP and sympathetic nerve activity normally provided by baroreceptors.[2]

The ventricular hypertrophy index was increased in SHRD compared with SHRC, and in the SHRC compared with WC, which suggests that hypertension may play a role in the development of the LVH. This also seems to indicate

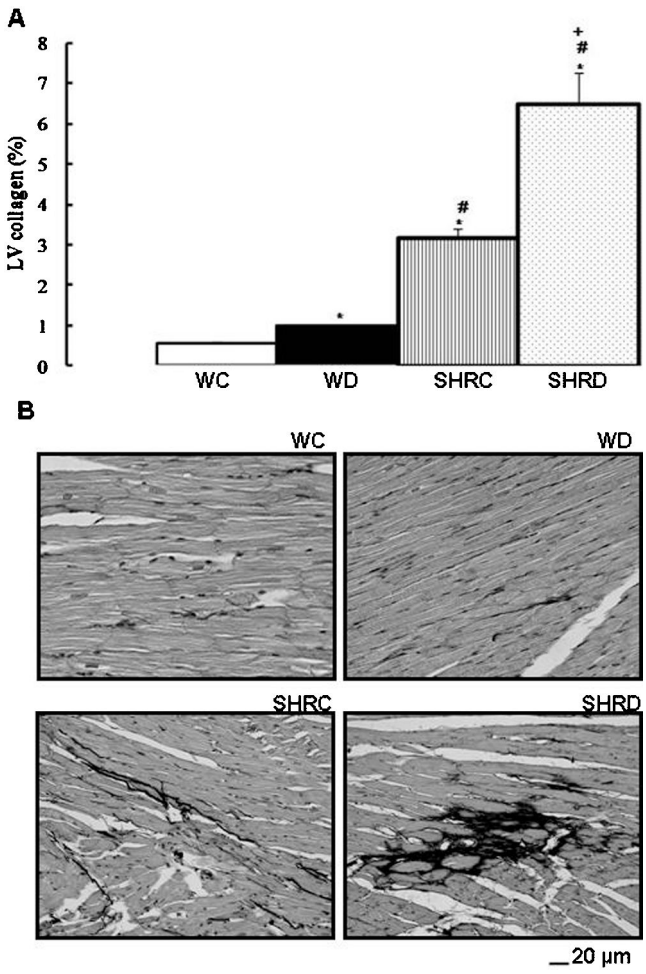

Figure 3 - A, Collagen volume fraction in the left ventricle of Wistar control (WC), Wistar denervated (WD), SHR control (SHRC), and SHR denervated (SHRD) groups. B, Representative photomicrography of collagen fibers in the left ventricle of WC, WD, SHRC, SHRD. *p<0.05 vs WC, #p<0.05 vs WD, + p<0.05 vs SHRC.

that hypertension associated with the BPV and baroreflex sensitivity damage results in a higher LVH.

A similar behavior was observed for the collagen volume fraction, an important marker of pathological LVH. The collagen network may help to maintain cardiac muscle structural organization, may facilitate tensile strength transmission from cardiomyocytes to LV, and may contribute

Table 1 - Echocardiography parameters of the Wistar control (WC), Wistar denervated (WD), SHR control (SHRC), SHR denervated (SHRD) groups.

	WC	WD	SHRC	SHRD
$LVDIA_{CORR}$ (cm/g)	1.85 ± 0.04	1.72 ± 0.06	2.25 ± 0.08*#	2.61 ± 0.12*#
IVS (cm/g)	0.27 ±0.007	0.36 ± 0.01*	0.61 ± 0.01*#	0.56 ± 0.01*#+
LVPW (cm/g)	0.27 ± 0.008	0.37 ± 0.01*	0.61 ± 0.01*#	0.55 ± 0.01*#+
RWT (cm)	0.29 ± 0.008	0.43 ± 0.03*	0.55 ± 0.02*#	0.43 ± 0.02*+
LVM (mg/g)	2.58 ± 0.06	2.88 ± 0.06*	4.02 ± 0.1*#	4.11 ± 0.13*#
IVRT (ms)	23 ± 0.56	26 ± 1.69*	27 ± 0.70*	29 ± 0.73*
E/A ratio	1.92 ± 0.09	1.21 ± 0.66*	1.87 ± 0.08#	1.97 ± 0.21#
EF (%)	72 ± 1	80 ± 2*	77 ± 2	77 ± 3
VCF (circ/s*10^3)	0.004 ± 0,0002	0.0059 ± 0,0004*	0.0053 ± 0,0003	0.0051 ± 0,0003

Mean ± SEM *p<0,05 vs WC, #p <0,05 vs WD,+ p<0,05 vs SHRC.

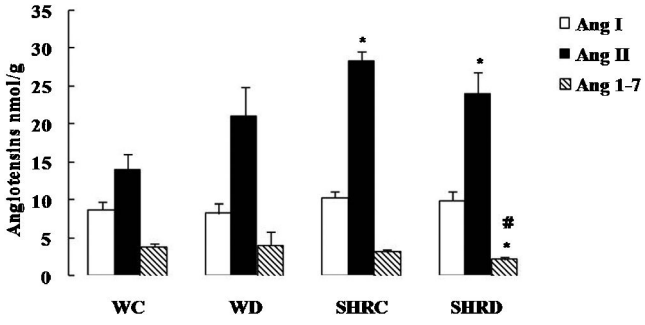

Figure 4 - Angiotensins I, II and 1–7 in left ventricle of Wistar control (WC), Wistar denervated (WD), SHR control (SHRC), SHR denervated (SHRD) groups. *$p<0.05$ vs WC, #$p<0.05$ vs WD.

towards myocardial relaxation. Equally important, the collagen network is responsible for myocardial passive stiffness and for ventricular volume.[13] Despite these beneficial aspects of the collagen network, pressure overload to the heart may cause excessive accumulation of collagen fibers among cardiac cells due to mechanisms yet to be fully understood. This process, known as fibrosis, considerably increases cardiac tissue stiffness, impairing cardiac function.[19] Reparative and reactive fibrosis may occur in different situations. In hypertension, the reactive fibrosis to increased pressure overload seems to be linked to myocyte apoptosis.[20] In addition, since increased BPV is associated with enhanced end-organ damage[4], the amount of collagen in the heart of denervated rats is consequently augmented. In this study, we showed that baroreflex impairment may mediate these stimuli regardless of pressure overload, as denervated rats presented increased collagen volume fraction, and when SAD was associated with hypertension this increase became considerably more evident.

Both hypertension and high BPV are involved in cardiovascular damage. A study carried out by Miao et al.[21] showed that in the SHR (hypertension accompanied by an elevated BPV) the LVH is more severe than in animals with chronic SAD (only elevated BPV, without hypertension), corroborating our findings of a positive correlation between left ventricle mass and BPV ($r = 0.56$). This correlation has also been described by Van Vliet et al.[22] in animals submitted to carotid denervation, SAD and modified SAD (preservation of the carotid chemoreceptors).

Our results for left ventricle angiotensins show that SHR groups presented increased Ang II levels compared with WC. Classically, RAS is a systematic regulator in various biological activities. Ang II is the key effector of RAS and a vasoconstrictor acting in the cardiovascular system.[23] Li et al.[24] have shown that the gene expressions of all components of RAS were elevated in the heart and aorta of the SHR compared with Wistar Kyoto rats. This significant increase lends supports to the hypothesis that activation of local tissue RAS may contribute to cardiac hypertrophy and arterial remodeling.

RAS plays a central role in the development of hypertension and the progression of end-organ damage.[5] Our results showed a positive correlation between the cardiac hypertrophy index and Ang II levels ($r = 0.41$), thus corroborating other data in that this peptide is closely related to the development of the cardiac hypertrophy.[25]

Li et al.[24] have suggested that the LVH in SHR was associated with ACE and ACE2 gene expression cardiac RAS, while aortic hypertrophy was associated with angiotensinogen and ACE2 gene expression of aortic RAS. This seems to support the postulation that, since there is no correlation between the plasma Ang II concentration and organ damage parameters, it is indeed the tissue RAS which may exert a detrimental effect on cardiovascular function[26] rather than the circulating RAS. Miao et al.[27] have shown that plasma and tissue Ang II concentrations remain unaltered after chronic SAD (2, 10 and 16 weeks). Accordingly, our study could not find any significant differences between animals submitted to SAD compared with each control group, although a qualitative tendency may be observed in the WD group compared with the control.

On the other hand, Shan et al.[28] have observed an increase in the Ang II levels in the cardiac and kidney tissues of rats submitted to chronic SAD, and these Ang II levels were more elevated when these animals were submitted to chronic stress, suggesting that elevated BPV and Ang II liberation may be related to end-organ damage development induced by baroreflex dysfunction.

In transgenic animals the overexpression of the angiotensinogen gene in the cardiac myocytes resulted in increased cardiac Ang II concentration and right and left ventricular hypertrophy, unaccompanied by either hypertension or increased circulating levels of Ang II.[29] These data strongly suggest that Ang II may induce cardiac hypertrophy regardless of its hemodynamic effects.

In our data, Ang I levels were not statistically different among groups. This finding corroborates Campbell et al.[30] when comparing SHR to normotensive control animals.

A reduced Ang 1–7 concentration in the SHRD group was observed compared with normotensive groups. Campbell et al.[30] found similar results when comparing SHR (20 weeks) with Donryu rats as a control group.

Previous studies have shown that Ang 1–7 partially prevented the pressoric response elicited by Ang II in SAD rats but not in sham-operated animals.[31] It is well established that Ang 1–7 can act as a physiological antagonist of Ang II by means of the activation of MASreceptors, which in turn blocks the vasoconstrictor effect of Ang II.[32]

Different researchers have shown that opposite effects of Ang II are enhanced in animal models of hypertension.[33] Benter et al.[34] have demonstrated that Ang 1–7 attenuates Ang II vasoconstriction in SHR but not in normotensive animals. Other studies (Höcht et al.[31]) have also demonstrated that Ang 1–7 blocked the pressor response to Ang II in hypertensive SHR and aortic coarcted rats, but not in normotensive control rats. The enhanced effect of Ang 1–7 in situations of Ang II overactivity may be related to an increased sensitivity to Ang 1–7.

In our data, the qualitative and quantitative reduction of the Ang 1–7 levels were accompanied by the significant increase in the Ang II levels. This reduction of Ang 1–7 may be related to the increase in its sensitivity in the attempt to tone down the effects generated by Ang II overactivity.

CONCLUSIONS

Our results suggest that not only BP variability and reduced baroreflex sensitivity may contribute to the development of

left ventricular hypertrophy as previously suggested; our findings indicate that elevated levels of Ang II and reduced concentration of Ang 1–7 may also play a role in this process. These data indicate that baroreflex dysfunction associated with changes of RAS may be predictive factors of LVH and cardiac failure.

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