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Effects of early aldosterone antagonism on cardiac remodeling in rats with aortic stenosis-induced pressure overload

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ABSTRACT

Aldosterone plays a pivotal role in the pathophysiology of systolic heart failure. However, whether early aldosterone antagonism improves cardiac remodeling during persistent pressure overload is unsettled. We evaluated the effects of aldosterone antagonist spironolactone on cardiac remodeling in rats with ascending aortic stenosis (AS).

Methods: Three days after inducing AS, weaning rats were randomized to receive spironolactone (AS-SPR, 20 mg/kg/day) or no drug (AS) for 18 weeks, and compared with sham-operated rats. Myocardial function was studied in isolated left ventricular (LV) papillary muscles. Statistical analyses: ANOVA or Kruskal–Wallis tests.

Results: Echocardiogram showed that LV diastolic (Sham 8.73 ± 0.57; AS 8.30 ± 1.10; AS-SPR 9.19 ± 1.15 mm) and systolic (Sham 4.57 ± 0.67; AS 3.61 ± 1.49; AS-SPR 4.62 ± 1.48 mm) diameters, left atrial diameter (Sham 5.80 ± 0.44; AS 7.15 ± 1.22; AS-SPR 8.02 ± 1.17 mm), and LV mass were higher in AS-SPR than AS. Posterior wall shortening velocity (Sham 38.5 ± 3.8; AS 35.6 ± 5.6; AS-SPR 31.1 ± 3.8 mm/s) was lower in AS-SPR than Sham and AS; E/A ratio was higher in AS-SPR than Sham. Developed tension was lower in AS and AS-SPR than Sham. Time to peak tension was higher in AS-SPR than Sham and AS after post-rest contraction. Right ventricle weight was higher in AS-SPR than AS, suggesting more severe heart failure in AS-SPR than AS. Interstitial collagen fractional area and myocardial hydroxyproline concentration were higher in AS than Sham. Metalloproteinase-2 and -9 activity, evaluated by zymography, did not differ between groups.

Conclusion: Early spironolactone administration causes further hypertrophy in cardiac chambers, and left ventricular dilation and dysfunction in rats with AS-induced chronic pressure overload.

1. Introduction

Aldosterone is a mineralocorticoid hormone involved in renal sodium and potassium homeostasis and blood pressure modulation. Increased systemic and myocardial concentrations of aldosterone are associated with deleterious cardiac effects. Experimental studies have shown that aldosterone directly stimulates myocyte growth and strongly induces myocardial fibrosis [1,2]. The harmful effects of aldosterone on the cardiovascular system also include myocyte apoptosis, myocardial oxidative stress and electrical remodeling, vascular injury, endothelial dysfunction, ventricular arrhythmia, and sudden death [3,4]. After the pioneer studies by Pitt et al. [5–7], aldosterone antagonists started to be evaluated in different cardiac aggression models, which showed that they prevent or attenuate left ventricular (LV) structural, functional, and molecular changes [8–11]. In clinical settings, aldosterone blockers reduced mortality and hospitalizations in patients with systolic heart failure of any cause [5,7], and in patients with systolic dysfunction after myocardial infarction [6]. Therefore, aldosterone blockade is now recommended for symptomatic systolic heart failure patients [12]. In contrast, recent studies have shown that aldosterone blockade failed to reduce cardiovascular morbimortality [13] or improve maximal exercise capacity, symptoms, or quality of life in heart failure patients with preserved ejection fraction [14]. The mechanisms responsible for the lack of response to aldosterone blockers in this situation are not clear.

Chronic pressure overload is a major cause of heart failure with preserved or reduced ejection fraction. During sustained pressure overload, cardiac remodeling is mainly characterized by myocyte hypertrophy and interstitial fibrosis [15,16]. Stable cardiac hypertrophy is usually maintained for a long period and may progress to a decompensated

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state with left ventricular dilation and systolic dysfunction [17,18]. The role of aldosterone antagonism on the development of cardiac hypertrophy during persistent pressure overload is not clear. In spontaneously hypertensive rats with LV hypertrophy and no heart failure features, aldosterone antagonist spironolactone reduced the frequency of heart failure development, improved myocardial function, and attenuated myocardial fibrosis independent of blood pressure levels [8]. In this study we tested the hypothesis that early aldosterone blockade improves cardiac remodeling during chronic pressure overload. Therefore, we evaluated the effects of spironolactone on cardiac structures, ventricular and myocardial function, and myocardial fibrosis in rats when administration was initiated three days after ascending aortic stenosis induction. In this model, 3–4 week-old rats are subjected to a clip placement around the ascending aorta [19]. After clip placement, aorta diameter is preserved; as rats grow, stenosis progressively develops [20,21]. Therefore, spironolactone was started before LV hypertrophy was established.

2. Materials and methods

2.1. Experimental groups

Male Wistar rats weighing 80–100 g were purchased from the Central Animal House at Botucatu Medical School, UNESP. All animals were housed in a temperature controlled room at 23 °C and kept on a 12-hour light/dark cycle. Food and water were supplied ad libitum. All experiments and procedures were approved by the Animal Experimentation Ethics Committee of Botucatu Medical School, UNESP, SP, Brazil.

The animals underwent median thoracotomy under anesthesia with intraperitoneal ketamine hydrochloride (50 mg/kg) and xylidine hydrochloride (10 mg/kg). Aortic constriction was induced by placing a 0.6 mm stainless-steel clip on the ascending aorta via a thoracic incision according to a previously described method [22]. Age-matched sham operated rats were used as controls. Three days after surgery, the rats were randomly assigned into three groups: Sham (n = 22), aortic stenosis (AS, n = 41) and aortic stenosis treated with spironolactone (AS-SPR, n = 36). Treatment was initiated three days after surgery and kept up for 18 weeks. Spironolactone was added to rat chow at a dosage of 20 mg/kg/day. At the end of the experimental period, rats were subjected to transthoracic echocardiogram and euthanized the next day.

2.2. Echocardiographic study

Cardiac structures and left ventricular (LV) function were evaluated by transthoracic echocardiogram using a commercially available echocardiograph (General Electric Medical Systems, Vivid S6, Tirat Carmel, Israel) equipped with a 5–11.5 MHz multifrequency transducer as previously described [23–25]. Rats were anesthetized by intramuscular injection with a mixture of ketamine (50 mg/kg) and xylazine (0.5 mg/kg). A two-dimensional parasternal short-axis view of the left ventricle (LV) was obtained at the level of the papillary muscles. M-mode tracings were obtained from short-axis views of the LV at or just below the tip of the mitral-valve leaflets, and at the level of the aortic valve and left atrium. M-mode images of the LV were printed on a black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 100 mm/s. All LV structures were manually measured by the same observer (KO) according to the leading-edge method of the American Society of Echocardiography [26]. Values obtained were the mean of at least five cardiac cycles on M-mode tracings. The following structural variables were measured: LV diastolic and systolic dimensions (LVDD and LVSD, respectively), LV diastolic and systolic posterior wall thickness (LVDPWT and LVSPWT, respectively), LV diastolic and systolic septal wall thickness (LVDSWT and LVSSWT, respectively), aortic diameter (AO), and left atrium (LA) diameter. Left ventricular mass (LVM) was calculated using the formula $[(LVDD + PWT + SWT)^3 - (LVDD)^3] \times 1.04$. Relative wall thickness (RWT) was calculated as 2 × PWT / LVDD. LV function was assessed by the following parameters: endocardial fractional shortening (EFS), midwall fractional shortening (MWFS), posterior wall shortening velocity (PWSV), early and late diastolic mitral inflow velocities (E and A waves), E/A ratio, E-wave deceleration time (EDT), and isovolumetric relaxation time (IVRT).

2.3. Myocardial functional study

Two days after echocardiographic study, myocardial contractile performance was evaluated in isolated LV papillary muscle preparations as previously described [27,28]. Rats were anesthetized with pentobarbital sodium, 50 mg/kg, intraperitoneally, and decapitated. Hearts were quickly removed and placed in oxygenated Krebs–Henseleit solution at 28 °C. LV anterior or posterior papillary muscle was dissected free, mounted between two spring clips, and placed vertically in a chamber containing Krebs–Henseleit solution at 28 °C and oxygenated with a mixture of 95% O_2 and 5% CO_2 (pH 7.38). The composition of the Krebs–Henseleit solution in mM was as follows: 118.5 NaCl, 4.69 KCl, 1.25 $CaCl_2$, 1.16 $MgSO_4$, 1.18 KH_2PO_4, 5.50 glucose, and 25.88 $NaHCO_3$. The spring clips were attached to a Kyowa model 120T-20B force transducer and a lever system, which allowed for muscle length adjustment. Preparations were stimulated 12 times/min at a voltage 10% above threshold.

After a 60-min period, during which the preparations were permitted to shorten while carrying light loads, muscles were loaded to contract isometrically and stretched to the apices of their length-total tension curves (L_{max}). After a 5-min period, during which preparations performed isotonic contractions, muscles were again placed under isometric conditions, and the apex of the length-total tension curve was determined. A 15 minute period of stable isometric contraction was imposed prior to the experimental period. One isometric contraction was then recorded for later analysis.

The following parameters were measured from isometric contraction: peak of developed tension (DT, g/mm^2), resting tension (RT, g/mm^2), maximum rate of tension development (+dT/dt, g/mm^2/s), maximum rate of tension decline (−dT/dt, g/mm^2/s), and time to peak of tension (TPT). To evaluate contractile reserve, papillary muscle mechanical performance was evaluated at basal conditions and after the following inotropic stimulation: post-rest contraction, extracellular Ca^{2+} concentration increase, and β-adrenergic agonist isoproterenol addition to the nutrient solution [29].

Papillary muscle cross-sectional area (CSA) was calculated from muscle weight and length by assuming cylindrical uniformity and a specific gravity of 1.0. All force data were normalized for the muscle CSA. After dissecting papillary muscle, atria and ventricles were separated and weighed. Atria and left and right ventricular weights were normalized to body weight.

2.4. Morphologic study

Transverse LV sections were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin–eosin and collagen-specific stain picrosirius red (Sirius red F3BA in aqueous saturated picric acid) [30]. In each heart, at least 50 myocyte diameters were measured as the shortest distance between borders drawn across the nucleus. On average, 20 microscopic fields were used to quantify interstitial collagen fractional area. Perivascular collagen was excluded from this analysis. Measurements were performed using a Leica microscope (magnification 40×) attached to a video camera and connected to a computer equipped with image analysis software (Image-Pro Plus 3.0, Media Cybernetics, Silver Spring, MD, USA).

2.5. Myocardial hydroxyproline concentration

Myocardial hydroxyproline (HOP) concentration was assessed to estimate tissue collagen content. HOP was measured in LV tissue as previously described [31,32]. Briefly, the tissue was dried using a Speedvac Concentrator SC 100 attached to a refrigerated condensation trap (TRL 100) and vacuum pump (VP 100, Savant Instruments, Inc., Farmingdale, NY, USA). Dry tissue weight was measured and samples were hydrolyzed overnight at 100 °C with 6 N HCl (1 mL/10 mg dry tissue). A 50 μL aliquot of hydrolysate was transferred to an Eppendorf tube and dried in the Speedvac Concentrator. One milliliter of deionized water was added and the sample transferred to a tube with a Teflon screw cap. One milliliter of potassium borate buffer (pH 8.7) was added to maintain constant pH and the sample was oxidized with 0.3 mL of chloramine T solution at room temperature for 20 min. The addition of 1 mL of 3.6 M sodium thiosulfate and thorough mixing for 10 s stopped the oxidative process. The solution was then saturated with 1.5 g KCl. The tubes were heated in boiling water for 20 min. After cooling to room temperature, the aqueous layer was extracted with 2.5 mL of toluene. One and a half milliliters of toluene extract were transferred to a 12 × 75 mm test tube. Then 0.6 mL of Ehrlich's reagent was added and the color allowed to develop for 30 min. Absorbances were read at 565 nm against a reagent blank. Deionized water and 20 μg/mL HOP were used as the blank and standard, respectively.

2.6. Metalloproteinase activity

Matrix metalloproteinase (MMP)-2 and -9 activity was determined as previously reported [33]. In brief, analysis samples were prepared by dilution in extraction sample buffer with 50 mM Tris, pH 7.4; 0.2 M NaCl; 0.1% Triton X, and 10 mM $CaCl_2$. In sample protein was quantified by the Bradford method. Samples with 20 μg of protein were then diluted in application buffer with 0.5 M Tris, pH 6.8; 100% glycerol, and 0.05% bromophenol blue, and loaded into wells of 8% SDS-polyacrylamide containing 1% gelatin. Electrophoresis was run in a Bio-Rad apparatus at 80 V for 2 h. Gel was removed, washed twice with 2.5% Triton-X-100, and washed with 50 mM Tris, pH 8.4. Gel was then incubated at 37 °C overnight in activation solution with 50 mM Tris, pH 8.4; 5 mM $CaCl_2$, and $ZnCl_2$. Staining was performed for 2 h with 0.5% coomassie blue and destaining in 30% methanol and 10% acetic acid at room temperature until clear bands over a dark background were observed. The gels were photographed and the intensity of gelatinolytic action (clear bands) was analyzed in UVP, UV, White Darkhon image analyzer.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation or median and 25th and 75th percentiles. Comparisons between groups were performed by one way analysis of variance (ANOVA) followed by Tukey test or Kruskal–Wallis followed by Dunn test. Mortality was assessed by log-rank test (Kaplan Meier). Statistical significance was accepted at the level of $p < 0.05$.