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SHORT COMMUNICATION

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Volume-weighted mean nuclear volume and numerical nuclear density in the cardiomyocyte following enalapril and verapamil treatment

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Abstract The estimation of the volume-weighted mean nuclear volume, $\overline{v}_v$, and the numerical nuclear density in the plane, N_A, was used to analyze cardiomyocyte nuclei in the condition of cardiac hypertrophy caused by nitric oxide (NO) synthesis blockade and simultaneous antihypertensive treatment for 40 days (four groups of ten rats each: control, L-NAME, L-NAME+enalapril, L-NAME+verapamil). The blood pressure (BP) increased 71% in the L-NAME group. In the L-NAME+enalapril and L-NAME+verapamil groups, the BP did not show any alteration when compared with the respective controls. In comparison with the control group, $\overline{v}_v$ was 250% greater, and the N_A was 25% smaller in the L-NAME animals, while no difference occurred in the other two groups. With respect to cardiomyocyte nuclear size, the present results suggest a beneficial effect of the angiotensin-converting enzyme inhibitor enalapril and the calcium channel blocker verapamil when NO synthesis is blockaded.

Keywords Nitric oxide · Hypertension · Enalapril · Verapamil · Stereology

Introduction

The process of cellular mass increase in response to growth stimuli is not only an adaptive process of the cardiac myocyte in response to increased workload but also one of the most important clinical complications of cardiovascular disorders [32, 35]. Cardiac hypertrophy, leading to heart failure, is one of the most common causes of debility and death. It is initiated by an increase and/or redistribution of the forces developed and faced by the cardiac myocytes caused by serious diseases of the cardiovascular system, such as arterial hypertension, myocardial infarction, valvular heart disease, or disseminated diseases of the heart muscle [9].

Recent work has demonstrated that hemodynamic overload is only part of a complex interaction between mechanical, neural, hormonal, and genetic factors that culminates in cardiac hypertrophy [13]. Although elevated systemic pressure clearly plays an important role in the pathogenesis of hypertensive cardiac hypertrophy, it is neither essential nor necessarily sufficient for the development of ventricular hypertrophy, and hypertensive magnitude shows no strict correlation with myocardial mass [36]. This dissociation between blood pressure (BP) and the degree of cardiac hypertrophy has been interpreted to indicate that, in addition to hemodynamic stress, other factors, such as the renin-angiotensin-system [30], importantly influence the nature of myocardial remodeling during the pathogenesis of cardiac hypertrophy.

The chronic deficiency of nitric oxide (NO) causes arterial hypertension accompanied by cardiac hypertrophy, loss of cardiomyocytes, and changes in the myocardial microcirculation [19, 25, 26, 27, 28, 29]. However, there is still controversy in relation to the exact interpretation of myocardial alterations caused by this model of arterial hypertension [23].

The class of antihypertensive agents, named angiotensin-converting enzyme (ACE) inhibitors, reduce BP by inhibiting the generation of hemodynamically active octapeptide angiotensin II from inactive decapeptide angiotensin I [10]. The calcium antagonists are also known as calcium channel blockers (CCBs) because they inhibit the movement of calcium ions from plasma into cells through the calcium channels. Then, inhibiting calcium influx across cell membranes leads to vasodilatation and reduction in systemic vascular resistance [13].

As far as events between the plasmatic membrane and the nucleus are concerned, the progress has been enormous in elucidating the intracellular signal transduction pathway for hypertrophic stimuli [7, 16]. However,

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much less is known about the nuclear events and the interaction of transcription factors that determine cardiac hypertrophy [17]. In the stage of dilated cardiomyopathy, the most obvious change in the cardiac myocyte is the occurrence of large nuclei of often-bizarre shape [31]. The present study focuses on the quantitative alteration of the cardiomyocyte nuclei in the condition of cardiac hypertrophy caused by NO synthesis blockade and simultaneous antihypertensive treatment.

Materials and methods

Mature male rats (40 of the Wistar strain) were obtained from colonies maintained at the State University of Rio de Janeiro. In the beginning of the study, the animals had a body mass of 268.6±22.1 g and a tail BP of 116.5±5.5 mmHg (mean±SD). Rats were given food (Nuvilab, Rio de Janeiro, Brazil) and water ad libitum. 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 1985).

The BP was weekly verified using the non-invasive method of the tail-cuff plethysmography (RTBP1007, Kent Scientific Co., Litchfield, Conn.). Four groups of ten rats each were used in the experiments. In the control group, animals were manipulated and sacrificed just as the animals of the experimental groups; they only received water and food ad libitum. In the L-NAME group, animals received L-NAME 50 mg/kg per day, dissolved in drinking water (hydrochloride of *N*ω-nitro-L-arginine-methyl-ester; Sigma Chemical Co., St. Louis, Mo.; lot 67H0876). In the L-NAME± enalapril group, animals received L-NAME (dose and administration as indicated for L-NAME group) and, simultaneously, enalapril maleate 15 mg/kg per day, dissolved in drinking water [(*S*)-*n*-(1-(ethoxycarbonyl)-3-phenylpropyl)-Ala-Pro maleate] (Sigma Chemical Co., St. Louis, Mo.; lot 38H0500). In the L-NAME±verapamil group, animals received L-NAME (dose and administration as indicated for L-NAME group) and, simultaneously, verapamil hydrochloride 15 mg/kg per day dissolved in drinking water ($C_{27}H_{38}N_2O_4$ HCl) (Sigma Chemical Co., St. Louis, MO.; lot 56H0925).

In the morning of the 41st day of experimentation, animals were anaesthetized (inhalation of diethyl ether) and sacrificed (heart injection of 3.0 ml of KCl at 10%). The left ventricular myocardial fragments were obtained by cutting the heart arbitrarily using the procedure denominated "orthrip", which produces isotropic, uniform, and randomly (IUR) oriented sections [21]. The material was embedded in paraplast, sectioned at 3 µm, and stained with hematoxylin and eosin and picro-sirius. Sections were analyzed using video-microscopy with a final on-screen magnification of 2167×, using a 100× objective (numerical aperture 1.25; Leica DMRBE microscope, Kappa CF 15/5 video camera and a Sony Trinitron monitor).

Stereology and statistical analysis

The estimation of the volume-weighted mean nuclear volume, $\bar{v}_v$, was made using the "point-sampled intercepts" method reported by Gundersen and Jensen [14] in IUR tissue. For this procedure, five fields per section, three sections of tissue from each animal, and ten animals per group were analyzed (150 fields per group). A test system consisting of parallel lines associated with test points was superposed on each microscopic field. The direction of the lines on the sample was determined by lottery. For each point inside the unbiased counting frame, which hits a nucleus, the nuclear intercept through the point was measured (Fig. 1).

The measurement of the intercept length was performed using a 32-mm long logarithmic l_0^3-ruler composed of a series of 15 classes, where the width of any class is approximately 17% larger than that of the preceding class [33]. Each individual intercept was cubed, and the mean of all these values was multiplied by $\pi/3$ in every case to give $\bar{v}_v$. The numerical nuclear density in the plane, i.e., the number of nuclear profiles per area (N_A), was also determined using a frame with 5675 µm².

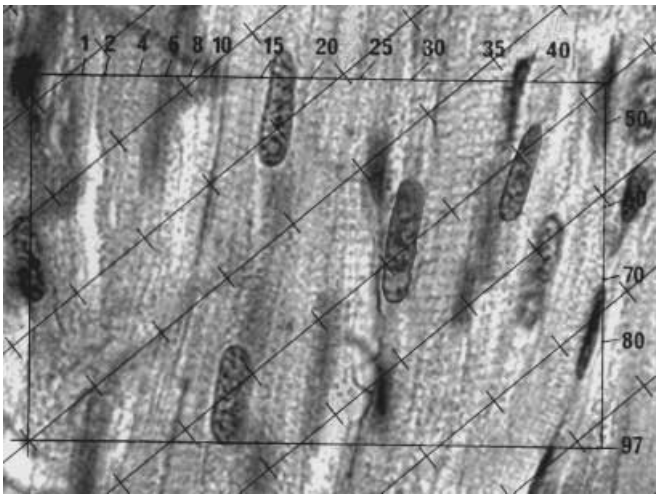

Fig. 1 Photomicrograph of the myocardium (control group). The "point-sampled intercept" method is illustrated using a test system composed for an unbiased counting frame in conjunction with lines associated with test points. The distance between two consecutive test points is 6.5 µm

The differences among the groups were analyzed using analysis of variance and the Tukey multiple comparison test with a significant level of 0.05 after verifying that the four population variances were homogeneous [38].

Results and discussion

The present protocol quantitatively studied the cardiomyocyte nuclei in normotensive rats and in rats with a NO blockade without and with antihypertensive treatment. The cardiomyocyte $\bar{v}_v$ and the nuclear N_A separated these animals into two different groups. The first group had the control rats, the L-NAME+enalapril, and the L-NAME+verapamil rats, while the second group had the L-NAME rats. The L-NAME rats had the $\bar{v}_v$ greater and the nuclear N_A smaller than the other groups.

Table 1 summarizes the results. On the 41st day of the experiment, there were meaningful differences in BP between the control group and the L-NAME group. The BP increased 71% in the L-NAME group. In L-NAME+enalapril and L-NAME+verapamil groups, the BP did not show any alteration when compared with the respective controls. In the animals treated with enalapril and verapamil, the BP values were significantly smaller than those in the L-NAME animals (42% and 44%, respectively). In comparison with the control group, $\bar{v}_v$ was 250% greater and N_A was 25% smaller in the L-NAME animals, while no difference occurred in the other two groups.

The $\bar{v}_v$ is normally used to obtain information on the three-dimensional size of nuclei and to discriminate be-

Table 1 Descriptive statistics of the blood pressure (BP), the volume-weighted mean nuclear volume $\bar{v}_v$ and the numerical cardiomyocyte nuclear density in the plane (N_A). *CE* coefficient of error (SE/mean); *CV* coefficient of variation (SD/mean)

Groups	BP (mmHg)		$\bar{v}_v$ (μm^3)	N_A ($1/mm^2$)
	Day 1	Day 41		
Control				
Mean	116.7	117.6	186.4	436.0
SD	4.3	6.6	136.6	90.0
CE (%)	1.2	1.9	13.3	6.5
CV (%)	3.7	5.6	73.3	20.6
L-NAME				
Mean	119.5	206.8	646.5	323.0
SD	1.6	21.7	569.4	52.0
CE (%)	0.4	3.3	18.0	5.1
CV (%)	1.3	10.5	88.1	16.1
L-NAME+enalapril				
Mean	114.4	115.8	183.8	433.0
SD	5.3	5.7	164.3	60.0
CE (%)	1.6	1.6	15.1	4.4
CV (%)	4.6	4.9	89.4	13.9
L-NAME+verapamil				
Mean	114.0	114.1	219.7	409.0
SD	6.6	4.0	219.6	14.0
CE (%)	1.8	1.1	18.0	1.1
CV (%)	5.8	3.5	100.0	3.4

tween histopathological entities [2]. The estimate of $\bar{v}_v$, has high reproducibility and efficiency and is capable of detecting changes not immediately apparent with microscopic observation [22, 34]. The nuclear weighted volume is better than the morphometric nuclear measurements (the diameter, the area, and the nuclear roundness factor) for staging and gives the prognostic evolution of different tumors [1, 3, 8, 11, 12, 18, 37].

Increased biomechanical stress on cardiomyocytes, either through genetic abnormalities or through excessive stress on the chamber wall due to myocyte loss or severe hemodynamic loading, generates a persistent signal for ventricular growth and hypertrophy [6, 15]. The two-dimensional area of nuclei in age-matched normotensive and hypertensive Goldblatt-operated guinea pig revealed the increase in nuclear area in hypertensive animals [24]. The myocardium of the L-NAME-treated rats has a remarkable increase in nucleic acids content and protein synthesis [20]. Babál and coworkers [4] reported that in L-NAME-treated rats the total RNA content increased by 15%, DNA content increased by 228%, and (^{14}C)leucine incorporation increased by 97%.

In this study, the $\bar{v}_v$ and the nuclear N_A were determined in four different groups. The $\bar{v}_v$ is three-dimensional information relative to size, and the N_A provides information about the density of the myocardial cardiomyocyte nuclei. The high coefficient of variation and coefficient of error, found to be $\bar{v}_v$ in all groups (even the control group), suggests that nuclear polymorphism normally occurs in the rat myocardium. However, these coefficients were low to the nuclear N_A, indicating a homogeneous cardiomyocyte distribution in the rat myocardium.

The ACE inhibitors are cardioprotective drugs suppressing the remodeling of pressure-overloaded myocardium [5]. In relation to the CCB, most of them reduce cardiac mass (as do the ACE inhibitors), although the significance of this effect (particularly in reducing the independent risk of the left ventricular hypertrophy) remains to be shown [10]. The present results, considering the quantitative analysis of the cardiomyocyte nuclei, suggest a beneficial effect of the ACE inhibitor enalapril and the CCB verapamil when NO synthesis is blockaded in rats.

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