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Stereology of the myocardium in hypertensive rats. Differences in relation to the time of inhibition of nitric oxide synthesis

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Abstract Structural changes in the myocardium following inhibition of nitric oxide synthesis were studied quantitatively within two different periods. Four groups of 10 rats were studied: control and L-NAME (N^G-nitro-methyl-ester-L-arginine) groups for 25 and for 40 days. L-NAME was administered at 50 mg/kg per day in the drinking water. On the 26th and 41st days, the hearts were examined. Volume densities of myocytes (Vv[m]), cardiac interstitium (Vv[int], numerical density of myocytes (Nv[m]) and mean cross-sectional area of the myocytes (A[m]) were determined. Comparing the L-NAME animals with their respective controls showed the arterial pressure (AP) and the heart weight (HW) to be increased in the L-NAME animals. At 25 days, and more obviously at 40 days, the myocytes were hypertrophied with increase of myofibrils (A[m], greater in L-NAME rats). There were some areas with ischaemic lesions, inflammatory infiltrates and perivascular and interstitial fibrosis. The intramyocardial arteries had a thick tunica media and tunica intima. At 25 days the myocardium showed no stereological difference between L-NAME and controls, but by 40 days there was decreased Vv[m] and Nv[m] and increased Vv[int] in the exposed group. Inhibition of NO synthesis provoked a time progressive myocardial change, quantified by stereology.

Key words Nitric oxide · Myocardium · Hypertension · Stereology

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Introduction

Myocardial hypertrophy is an adaptive change in response to work overload, but progresses to heart failure when the limits of response are exceeded [16]. In hypertrophied myocardium the microcirculation develops vessels with thick walls and reduced vascular lumina with an increase in collagen biosynthesis. Changes in the structure of the myocardium, including interstitial remodelling with accumulation of fibrillar collagen, cause myocardial rigidity [7, 17, 21]. The action of the renin–angiotensin–aldosterone system also promotes myocardial accumulation of collagen (mainly types I and III), increasing systolic/diastolic ventricular dysfunction. In consequence, the structural remodelling of the cardiac collagen matrix increases the ventricular dysfunction until congestive failure develops [38, 39].

Myofibroblasts have both contractile and metabolic activities and can be phenotypically transformed to fibroblasts expressing alpha actin and responsible for the turnover of collagen in myocardial repair. It is known that de novo synthesis of angiotensina II by myofibroblasts in injured areas produces important autocrine and paracrine effects. Regressive, persistent and progressive myocardial fibrosis are all related to the action of myofibroblasts and the signals that they generate [35].

Inhibition of nitric oxide synthase promotes arterial hypertension in a rapid and permanent way, and is characterized by an increase in the total peripheral resistance of blood vessels [3]. L-NAME (N^G-nitro-methyl-ester-L-arginine), which acts competitively with L-arginine in nitric oxide production, causes L-arginine inhibition with consequent vasoconstriction and elevation of the levels of arterial pressure [12, 29]. Chronic deficiency of nitric oxide causes arterial hypertension accompanied by cardiac hypertrophy [15, 19, 22, 26]. There is still some controversy over the nature of the myocardial alterations caused by this model of arterial hypertension [2, 4, 22], since in animals exposed to L-NAME the myocardial hypertrophy is not proportional to the blood pressure levels and the myocardial alterations in the interstitium are

more intense than those seen in other models of arterial hypertension (Goldblatt II, for example) [21, 27].

This study aimed to quantify the structural changes of the myocardium in rats receiving a nitric oxide synthesis inhibitor.

Materials and methods

We used 40 rats (male adult Wistars) with an initial body weight of 250 g and tail arterial pressure below 120 mmHg. The animals were maintained for 2 weeks under observation, with arterial pressure checked weekly, and were then divided into four groups of 10 rats, maintained in an appropriate environment from the aspects of temperature and light. The groups were: control 25 days (C25), control 40 days (C40), L-NAME 25 days (L25) and L-NAME 40 days (L40).

The animals in the control groups received water and food Purina(r) ad libitum. The animals in the experimental groups (L25 and L40) received L-NAME (N^G-nitro-L-arginine-methyl-ester hydrochlorate; Sigma, St. Louis, Mo., lot 44:0102) for 25 or 40 days, respectively. The average daily intake of water per animal in the conditions of the present study was 20 ml. Thus, each animal received a dose of L-NAME of 50 mg/kg per day (see [29, 33]). This investigation was performed in accordance with 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) and the Animal Subjects Committee of the State University of Rio de Janeiro approved the protocols.

On the 26th and in the 41st days of experimentation the animals in the L-NAME groups and their respective controls were anaesthetized by ether inhalation and killed by cardiac injection of 10% KCl (to stop the heart in diastole). The hearts were weighed according to the method of Scherle [32], suspended by a thread and immersed in physiological solution in a vessel on the plate of the scale. They were then fixed in a buffered solution of 4% formaldehyde (pH 7.2) for 48 h.

The myocardium is an anisotropic structure, but isotropic sections are necessary for a stereological study. The estimation was thus carried out by cutting the organ with the "orthotrip" [20].

Myocardial fragments of the left ventricular wall were obtained by cutting the heart at random; the organ was then placed with the cut edge down and, again at random, the specimen was cut with a section perpendicular to the first plane. The specimen was placed with the new cut surface down and a new random orientation was defined with the organ sectioned perpendicular to the previous plane. The last cut was considered to be uniformly isotropic: that is to say that without reference to the position of the specimen in the first cut the last surface has an orientation that deviates from all possible orientations with a constant probability [18]. The material was embedded in paraffin, sectioned at 5 µm and stained with picro-sirius and van Gieson.

The volume and the numerical densities of the cardiac myocytes were determined. Stereology was accomplished in 15 random microscopic fields using an oil-immersion objective and a video-microscopy system made up of a microscope (Leica model DMRBE) coupled to a video-camera (Kappa CF 15/5) and a Sony triniton monitor. A test system, M42, was put upon the screen of the monitor and calibrated with a Leitz micrometer 1 mm/100.

The volume density was determined by point counting. The numerical density was determined according to the disector method. The optical disector was defined between two parallel planes separated by a known distance controlled automatically with the motorized stage of the microscope moving the *z*-axis. The look-up and look-down planes were defined in the test area of the M42 system test, allowing the construction of the optical disector [9, 14]. Twenty disectors were analysed in each group, with determination of the number of nuclei of myocytes inside the volume of the disector. For efficiency, a nucleus was treated as a myocyte and was only counted in the look-up plane of the disector, since it did not cross the restriction line or its extensions [13, 18].

The volume density of the myocytes (Vv[m]) was calculated as

$$Vv[m] = \frac{Pp[m]}{P_T}\%$$

where Pp[m] is the number of points on the myocytes and P_T is the total number of test points, 42 in the present case.

The numerical density of the myocytes (Nv[m]) was determined as

$$Nv[m] = \frac{Q_A^-}{\text{disector}} / \text{mm}^3$$

where Q_A^- is the number of nuclei counted in the look-up plane and disector is the test volume determined by the product of the test area and the thickness of the disector.

The mean cross-sectional area of the myocytes (A[m]) was determined as

$$A[m] = \frac{A_A}{Q_A}\mu\text{m}^2$$

where A_A is the same as V_V, according to the principle of Delesse.

Myocytes and interstitium make up the myocardium (interstitium is composed of connective tissue, blood vessels and nerves). The volume density of the cardiac interstitium (Vv[int]) was determined as:

$$Vv[\text{int}] = 100 - Vv[m]\%$$

Apart from descriptive statistics, differences comparing control with L-NAME groups at 25 and 40 days were tested with the Mann-Whitney nonparametric test with a significance level of 0.05 [40].

Results

After 25 days of use of L-NAME the arterial pressure was 187.1±2.5 mmHg (mean±SD), 74.5% higher than in control animals of the same age. At 40 days of L-NAME use, the arterial pressure reached 204.8±1.8 mmHg; this is 90.2% higher than in controls. When the hypertensive animals were compared with the corresponding controls of the same age, their heart weight was found to be larger by 50% at 25 days and larger by a further 28.6% at 40 days (Tables 1, 2).

At 25 days, and more intensely after 40 days, of arterial hypertension, the myocytes were hypertrophied, with increased myofibrils. In these animals the myocardium showed some areas with ischaemic lesions and inflammatory infiltrates. This was probably due to myocytic necrosis in these areas, where the thickness of the media and intima of the intramyocardial coronary vessels were both increased, with intense perivascular fibrosis (Fig. 1).

In L-NAME animals the myocardium showed areas of extensive interstitial fibrosis, predominantly in the endomysium and perimysium. When observed by polarized light these areas were characterized by red-orange collagen (probably collagen type I) and, in smaller amounts, green collagen fibres (probably collagen type III). The L-NAME animals showed progressive interstitial and perivascular fibrosis and an increased thickness of the intima of the small intramyocardial arteries.

Significant stereological differences between controls and L-NAME animals are shown in Tables 1 and 2.

Table 1 Cardiac weight and myocardial stereology statistics. Control and L-NAME animals were analyzed in the 25th day (*A* cross sectional area, *CE* coefficient of error, *CV* coefficient of variation, *int* interstitium, *m* myocyte, *Nv* numerical density, *p* probability (Mann Whitney test), *SD* standard deviation, *SE* standard error of the mean, *Vv* volume density)

Group	Weight (g)	Nv [m] (1/mm^3)	Vv [m] (%)	Vv [int] (%)	A [m] (μm^2)
Control					
Mean	0.6	45131.6	55.6	44.4	173.5
Median	0.6	45565.5	56.3	44.7	173.0
SD	0.1	6213.3	4.2	4.5	11.9
SE	0.0	2778.7	1.9	1.4	5.3
CV %	11.4	13.6	7.5	10.1	6.9
CE %	5.1	6.1	3.4	3.2	3.1
L-NAME					
Mean	0.9	44046.6	54.7	45.3	366.8
Median	0.9	42310.8	54.1	46.6	363.3
SD	0.2	5220.5	5.2	5.4	23.8
SE	0.1	1650.9	1.6	1.7	7.5
CV %	20.9	12.3	9.5	11.9	6.5
CE %	6.6	3.9	3.0	3.8	2.1
P	0.002	NS	NS	NS	0.002

Table 2 Cardiac weight and myocardial stereology statistics. Control and L-NAME animals were analyzed in the 40th day. See legend of the Table 1.

Group	Weight (g)	Nv [m] (1/mm^3)	Vv [m] (%)	Vv [int] (%)	A [m] (μm^2)
Control					
Mean	0.7	67480.4	54.0	46.0	350.8
Median	0.6	65093.6	53.0	47.4	358.0
SD	0.1	14696.6	5.3	5.8	58.3
SE	0.0	4647.5	1.7	1.8	18.4
CV %	16.4	22.6	9.95	12.6	16.3
CE %	5.2	7.1	3.1	3.9	5.2
L-NAME					
Mean	0.9	51640.9	47.2	52.8	405.1
Median	0.9	52074.9	46.7	53.2	390.9
SD	0.1	10320.1	5.4	5.6	51.7
SE	0.0	3263.5	1.7	1.8	16.3
CV %	14.1	19.8	11.6	10.6	13.2
CE %	4.5	6.3	3.7	3.4	4.2
P	0.001	0.02	0.01	0.006	0.05

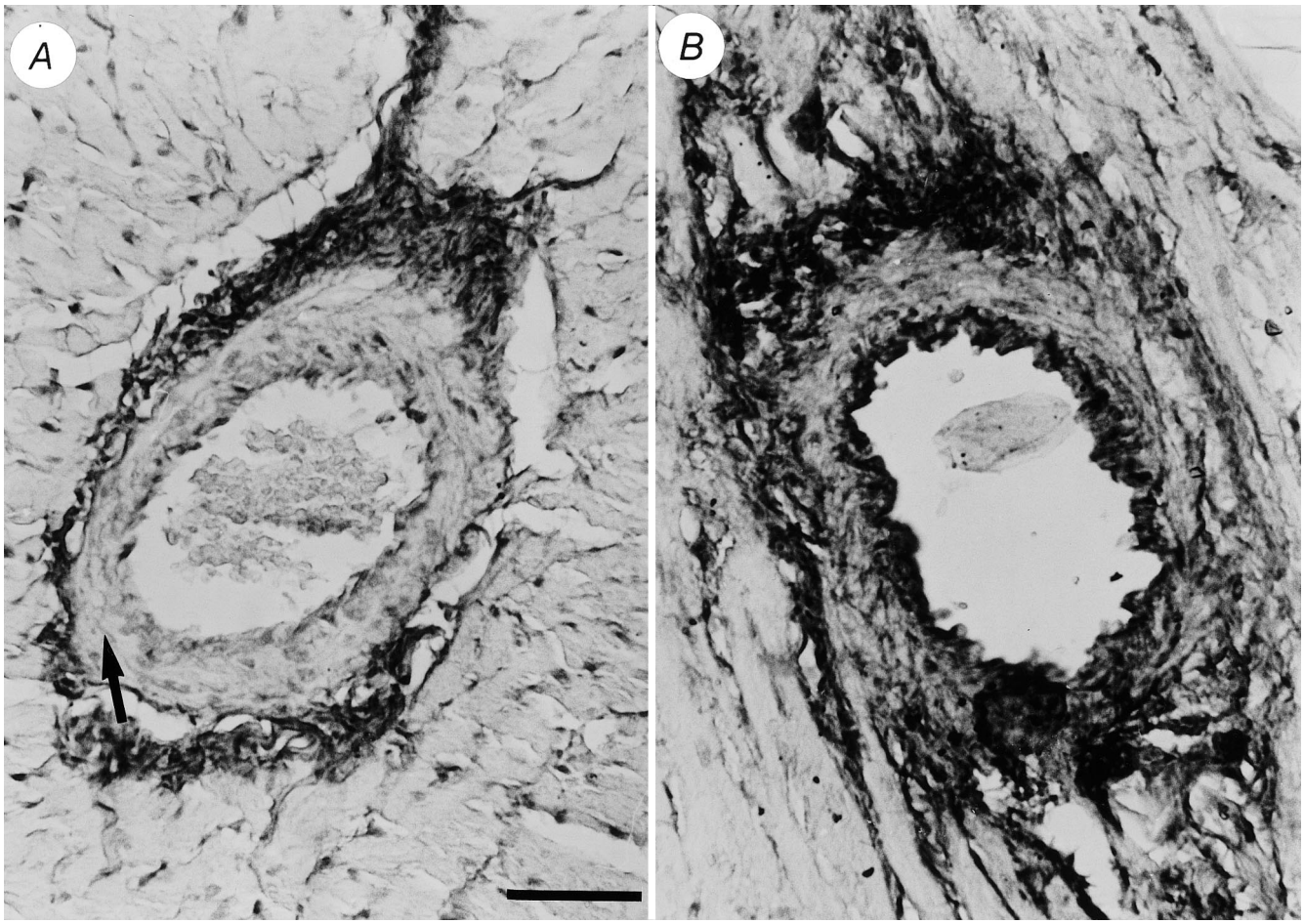

Fig. 1A, B Photomicrographs of the cross-sectioned intramyocardial arteries after 40 days of experimentation. Picro Sirius stain observed without polarized light, *bar* 40 μm **A** Control rat: artery showing a lumen full of erythrocytes. The endothelium cannot be discerned at this magnification. The thick tunica media is composed of numerous layers of circularly disposed smooth muscle cells (*arrow*). The tunica adventitia (*dark*) composed principally of collagenous fibres, is almost as thick as the tunica media. **B** Hypertensive rat: artery showing increased thickness of the tunica intima and tunica media, irregular endothelial surface, increased amount of collagenous fibres in the tunica media and extensive perivascular fibrosis (*dark*)

When the hypertensive animals were compared with the corresponding age-matched controls, the Vv[m] showed no difference at 25 days but had decreased by 12.6% at 40 days. In the same way, the Vv[int] showed no difference at 25 days, but had increased by 14.8% at 40 days. There was no difference in Nv[m] between the hypertensive animals and the controls at 25 days, but it had declined 23.5% in the hypertensive animals at 40 days. The A[m]) was 111.4% larger at 25 days and just 15.5% larger at 40 days.

Discussion

Although we found higher arterial pressures in the L-NAME treated than those found by others using similar protocols [29, 33], the morphological alterations observed resembled those described in other studies of inhibition of NO synthase [19, 21, 24, 26].

The ventricular overload caused by the increase in arterial pressure causes an increase in cardiac size without cellular proliferation [28]. In mammals, the ventricular wall has an extraordinary capability to undergo hypertrophy of the myocytes and hyperplasia of the interstitial cells [1]. The structural remodelling of the myocardium in arterial hypertension is mainly collagenous [35, 36]. It seems that the hypertrophy of the cardiac myocytes contributes to the increase of the myocardial mass and the ventricular wall stiffness. The A[m] may double, with resultant change in the form of the cell [1]. Our results demonstrate a significant increase in A[m] in L-NAME animals. The proliferation of the nonmyocyte cells was not studied but probably occurred in the L-NAME animals, indicating structural remodelling of the cardiac interstitium. This disproportionate increase of the nonmyocytic cells alters the relationship between the muscular and interstitial compartments of the myocardium to what is considered pathologic [36].

In the hypertensive animals at 25 days, the myocardium was not altered in the Vv[int] and Vv[m]. There was a significant increase of the heart weight in this period, probably caused by hypertrophy of the myocytes with a significant increase in A[m]. After 40 days the hypertensive animals had myocytes with a slightly larger A[m] but with a decreased Vv[m] and significantly increased Vv[int]. These results agree with the findings of Contard et al. [8], who confirmed that the increase in heart weight in hypertensive animals is correlated, mainly, with the increase in the content of collagen in the hypertrophied myocardium. It is also in keeping with the findings of Zhang et al. [41], who comment that the size of the heart is primarily determined by the diameter of the cardiac myocytes in the myocardial fibres.

This experimental model causes fibrosis (perivascular and interstitial) and disarray of cardiac muscle which is more intense than that seen in the renovascular model (Goldblatt II) [21]. The L-NAME animals showed remodelling of the coronary microvasculature and myocardial hypertrophy [24], suggesting that the myocardial lesions are not exclusively due to arterial hypertension, but are also associated with chronic inhibition of NO synthase and lesions of the vascular endothelium [19, 21, 24].

Changes in the tunica media of the blood vessels are common in arterial hypertension, while changes in the tunica intima are usually associated with hypercholesterolaemia and atherosclerosis. There is evidence from experimental models of hypertension that the increased blood pressure stimulates the proliferation of cells in the tunica intima [31], possibly as a result of endothelial lesions caused by high intravascular pressure [6]. Coronary vasodilatation in arterial hypertension can be attributed to structural alterations in the resistant coronary vessels. Myocardial hypertrophy may also contribute to extravascular compressive forces that increase the coronary resistance. The administration of L-NAME for 8 weeks was associated with structural changes in the coronary microvessels, increasing the thickness of the tunica media and an increase in perivascular fibrosis, but these alterations did not come about in the larger coronary arteries [24]. Our results showed an increase in perivascular fibrosis with increasing duration of L-NAME administration in rats, which together with the connective tissue in the tunica media, may contribute to a change in vasomotor reactivity and in coronary vasodilator reserve, inducing myocardial ischaemia [10, 11, 23].

Hypertrophied myocytes vary in size in advanced myocardial hypertrophy, with subsequent major variation in the size of the intercapillary space [30]. This, together with perivascular inflammation, also contributes to myocytic ischaemia [25]. Proliferation of myocardial capillaries is insufficient to prevent ischaemic necrosis [34]. The increase in the myocardial metabolic demand in this model is worsened because the microvessels are narrowed and local production of angiotensin II, endothelins and/or related catecholamines may have an important function in myocardial necrosis and subsequent fibrosis [24]. The Nv[m] in the hypertensive animals did not decrease significantly until after 40 days of L-NAME use, while the myocardial ischaemia became accentuated when there were several microscopic fields of recent necrosis and areas of substitution fibrosis.

The inhibition of NO synthase we have produced gives results compatible with the work that characterized this experimental model. The main adaptive normal and pathologic changes that happen in the myocardium were quantified. There is progression in the myocardial lesions, with increasing exposure initially by myocyte hypertrophy, and later with extensive interstitial and perivascular fibrosis. Decrease in the myocyte number was also detected, which was probably associated with necrosis and apoptosis.

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