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Development of cardiomyocyte hypotrophy in rats under prolonged treatment with a low dose of a nitric oxide synthesis inhibitor

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Abstract

Chronic administration of the nitric oxide (NO) synthesis inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) to rats causes hypertension and morphological abnormalities in the heart, consisting mainly of ventricular hypertrophy and foci of necrosis and fibrosis. Since these phenomena have usually been described with high (or moderate) doses of L-NAME, this study was undertaken to evaluate the effects of a low dose of L-NAME on arterial blood pressure, heart weight index, left ventricular weight index, amount of ventricular fibrosis, and cardiomyocyte size. Male Wistar rats received L-NAME (7.5 mg/kg per day) in the drinking water for 2, 4, and 6 months, whereas control animals received tap water alone. At this dose, L-NAME caused 90% inhibition ($P < 0.001$) of brain NO synthase (NOS) activity. The chronic L-NAME treatment caused an approximately 15% reduction in body weight of the animals, and no death was observed. The tail–cuff pressure was markedly ($P < 0.01$) elevated in L-NAME-treated rats. A significant ($P < 0.05$) reduction in both heart weight index (13–20% decrease) and left ventricular weight index (20–34% decrease) at 2, 4, and 6 months of treatment was observed in L-NAME-treated rats. The cardiomyocyte size in subendocardial, subepicardial, and midmyocardial regions of the left ventricles was time-dependently reduced, irrespective of the region studied, as measured at 2 (11% decrease), 4 (28% decrease, $P < 0.05$), and 6 (45% decrease, $P < 0.05$) months of chronic L-NAME treatment. The amount of fibrous tissue was unaltered at 2 and 4 months, but a small (but significant) increase in the amount of fibrous tissue was detected at 6 months (7.1 ± 0.2 %, $P < 0.05$) compared to that of control animals (5.9 ± 0.2%). Our results show that chronic treatment of rats with a low dose of L-NAME for prolonged periods (up to 6 months) causes arterial hypertension accompanied by significant reductions in heart weight, left ventricular weight indexes, and cardiomyocyte size.

Keywords: N^{ω}-nitro-L-arginine methyl ester; Heart lesion; Ventricular hypertrophy; Stereology

1. Introduction

Nitric oxide (NO) plays a major role in modulating regional blood flow and arterial blood pressure in different animal species, including humans (Moncada et al., 1991). Former studies reported that daily administration of NO synthase (NOS) inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) caused marked and sustained arterial hypertension in rats (Baylis et al., 1992; Ribeiro et al., 1992). A number of subsequent studies reported the use of prolonged ingestion of NOS inhibitors to evaluate physiopathological changes mediated by NO in different rat models (see Zatz and Baylis, 1998).

The cardiovascular studies, in which rats ingested L-NAME daily, mainly used relatively high (70–250 mg kg^{-1} day^{-1}) or moderate (40–50 mg kg^{-1} day^{-1}) doses of NOS inhibitors for a period of time varying from 1 to 8 weeks. Using this range of doses, hypertension induced by chronic L-NAME intake may be accompanied by marked pathological changes in both heart (Jover et al., 1993; Rhaleb et al., 1994; Moreno-Jr et al., 1995, 1996) and kidney (Fujihara et al., 1994), as well as in arterial vessels (Delacretaz et al., 1995; Numaguchi et al., 1995; Babal et al., 1997; Chillon et al., 1997; Moreau et al., 1998). In the heart, morphological abnormalities consist mainly of ventricular hypertrophy and foci of necrosis and fibrosis (Numagachi et al., 1995; Moreno-Jr et al., 1996; Babal et al., 1997; Akuzawa et al., 1998; Devlin et al., 1998;

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K-Laflamme et al., 1998; Luvara et al., 1998). However, the mechanism by which these alterations take place in the heart is unclear. Although concomitant ingestion of antihypertensive agents prevents L-NAME-induced hypertension, the resulting hypertrophy and cardiac lesions can either be unaltered (Moreno-Jr et al., 1995) or attenuated by these agents (Numagachi et al., 1995; Oliveira et al., 1999). The purpose of this study was to further understand the relationship between high levels of blood pressure, changes of cardiomyocyte size, and ventricular lesions in response to chronic L-NAME treatment. We therefore treated rats chronically with a low dose of L-NAME (7.5 mg kg^{-1} day^{-1}), and evaluated hypertension, heart weight index, left ventricular weight index, ventricular fibrosis, and cardiomyocyte size after 2, 4, and 6 months of treatment.

2. Material and methods

2.1. Animals

Male Wistar rats (approximately 150 g at the beginning of the study) were provided by the Central Animal House-State University of Campinas (CEMIB-UNICAMP). The animals were maintained under light and temperature-controlled conditions (12 h day/12 h night, 25°C) and were fed with a standard chow (Nuvilab CR-1®, Nuvital Nutrientes, Curitiba, Brazil). All experiments were in accordance with the guidelines of the UNICAMP for animal care.

2.2. Treatment of the animals with L-NAME

The chronic treatment with L-NAME was performed as previously described (Ribeiro et al., 1992). The animals received L-NAME dissolved in the drinking water to give a daily dose of 7.5 mg kg^{-1} day^{-1}. The animals were killed at 2 ($n = 10$), 4 ($n = 10$), and 6 ($n = 15$) months after treatment. Control animals receiving tap water alone were used alongside each experimental group ($n = 10–15$). The concentration of L-NAME in the water would give a total amount of 2 mg rat^{-1} day^{-1}. This was maintained constant; so in the beginning, the rat would have a higher intake than 7.5 mg/kg (first 2 months) and at the end of the experiment (last month), the total dose would be slightly lower than 7.5 mg/kg. The concentration in the water was calculated by our previous experience in the daily intake of water per rat.

2.3. Cardiac weight indices

At the end of the study, the animals were killed with an overdose of sodium pentobarbital (Sagatal®), and the heart was dissected out and washed with saline (0.9%, w/v). The hearts were fixed in a 10% formalin for 24 h. Heart weight was obtained by the removal of both atria, and left ventricular weight was determined by excising the right ventricle and weighing the remaining tissue. Finally, heart weight and left ventricular weight indices were calculated by dividing heart weight and left ventricular weight by body weight measured in the last week of treatment.

2.4. Stereological procedures

Stereological analysis was performed according to the method described by Aherne (1970). For this procedure, formalin-fixed left ventricle and septum were cut into five equidistant rings perpendicular to the long axis of the ventricle. The rings were then embedded in paraffin, and 5-μm sections were stained with Masson's trichrome. Analysis of the slides was performed blindly using a light microscope (Zeiss, Germany), and the relative volume occupied by each element of the ventricle (myocardial fibers and fibrous tissue) was measured with a special ocular containing a 25-point reticulum (five parallel lines with five points each, kpl 8×, Zeiss). For counting, 50 microscopic fields were evaluated and the relative volume (Ppi) occupied by each component was calculated as follows: $Ppi = p/(P - R)$, where p is the number of reticular points hitting each cardiac element, P is the total number of reticular points and R is the number of points hitting artefactual retraction areas. To determine the cardiomyocyte size, the cell diameters were measured by using a light optical system supplied with a graduated eyepiece micrometer and a 40× objective (400 magnification). Fifteen cells, randomly selected from the subepicardial, midmyocardial, and subendocardial regions, were measured for each animal from the different experimental groups.

2.5. Determination of NOS activity in brain

Determination of NOS activity in brains from the both control and L-NAME-treated rats was carried out according to a method previously described, which is based on the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline (Förstermann et al., 1990). For this purpose, the brains from the control and L-NAME-treated rats were rapidly removed, weighed, and individually homogeneized in five volumes of cold incubation buffer (Tris–HCl 50 mM, pH 7.4) containing 1 mM of phenyl methyl sulphonyl fluoride and 1 mM of L-citrulline. The homogenates were incubated at room temperature for 30 min in the presence of 1 mM NADPH, 2 mM $CaCl_2$, and 10 μM L-arginine containing 100,000 dpm of L-[2,3,4,5-^{3}H]arginine monohydrochloride. The determination of NOS activity was also performed in the absence of calcium (omission of $CaCl_2$ and addition of 1 mM EGTA). Protein content of the samples was determined (Peterson, 1977), and brain NOS activity is expressed as picomoles of L-citrulline produced per minute and per milligram of protein.

Table 1
Body weight and tail–cuff pressure in animals treated chronically with L-NAME (7.5 mg/kg per day) and in control animals that received tap water alone
The results represent the means ± S.E.M. for 10–15 rats.
BW, body weight; TCP, tail–cuff pressure.

Months	BW (g)		TCP (mm Hg)	
	Control	L-NAME	Control	L-NAME
2	306 ± 8.4	259 ± 10.5*	122 ± 0.6	151 ± 1.0*
4	341 ± 7.8	291 ± 11.0*	118 ± 1.7	151 ± 1.2*
6	396 ± 7.3	341 ± 11.3*	120 ± 0.7	146 ± 2.0*

* $P < 0.05$ compared to control values.

2.6. Blood pressure measurements

The arterial blood pressure was evaluated weekly. For each animal, mean blood pressure was measured at least in triplicate by a tail–cuff method (Zatz, 1990). Briefly, a small electret microphone, used as a sensor, was connected to the tail by a piece of rubber tubing. This design provides selective attenuation of tail pulsations appearing as the cuff is deflated between systolic and mean arterial pressures. Mean, rather than systolic pressure, appears to be evaluated in the conscious rat with this method.

2.7. Drugs

L-NAME and pentobarbital sodium (Sagatal®) were purchased from Sigma (USA) and May & Baker (UK), respectively. L-[2,3,4,5-^{3}H] arginine (specific activity 60.0 Ci/mmol) was supplied by Amersham (UK). The reagents to measure brain NOS activity were purchased from Sigma.

2.8. Data and statistical analysis

Results are expressed as mean ± S.E.M. Analysis of variance (ANOVA) followed by Bonferroni test was applied in order to assess the differences in body weight and tail–cuff pressure. For stereological procedures, ANOVA was followed by Tukey test. A P-value < 0.05 was considered significant.

3. Results

3.1. Body weight and survival

L-NAME (7.5 mg kg^{-1} day^{-1}) did not significantly affect the body weight of the animals until the fourth week of treatment (249 ± 1.9 and 237 ± 8.1 g, for control and treated, respectively; $n = 10$), after which, a small (but significant) reduction in body weight of approximately 15% was observed in the L-NAME-treated animals ($P < 0.05$; Table 1). All the animals from the control (tap water) and L-NAME groups survived.

3.2. Tail–cuff pressure

L-NAME caused a marked increase ($P < 0.01$) in tail–cuff pressure, reaching submaximal values after 1 month of treatment (142 ± 2.1 mm Hg; $P < 0.01$) compared to that of control animals (120 ± 2.7 mm Hg). In this group of animals, tail–cuff pressure remained significantly ($P < 0.01$) elevated for the whole period of treatment with L-NAME (Table 1). Animals receiving tap water alone had no significant changes in tail–cuff pressure (Table 1).

3.3. Cardiac weight indices

Significant reductions in heart weight index and left ventricular weight index at 2, 4, and 6 months, were observed in the L-NAME-treated animals compared to the control group (Table 2).

3.4. Stereological analysis in subendocardial, subepicardial, or midmyocardial regions: cardiomyocyte size and fibrous tissue

Animals receiving L-NAME showed a marked and progressive decrease in cardiomyocyte size at 4 and 6 months after treatment (Fig. 1). This reduction of cardiomyocyte size was of the same magnitude, irrespective of the region of the left ventricle studied (subendocardial, subepicardial, or midmyocardial) and was clearly detected at 4 (25–30%

Table 2
Heart weight index (HWI), left ventricular weight index (LVWI) and amount (%) of fibrous tissue in animals treated chronically with L-NAME (7.5 mg/kg per day) for 6 months and in control animals that received tap water alone
The results represent the means ± S.E.M. for 10–15 rats for each group.

Months	HWI		LVWI		Fibrous tissue (%)	
	Control	L-NAME	Control	L-NAME	Control	L-NAME
2	2.30 ± 0.07	1.82 ± 0.06*	2.25 ± 0.12	1.80 ± 0.12*	5.7 ± 0.1	5.7 ± 0.2
4	2.90 ± 0.03	1.90 ± 0.10*	1.83 ± 0.03	1.59 ± 0.08*	5.9 ± 0.3	5.7 ± 0.2
6	2.98 ± 0.04	1.95 ± 0.09*	1.87 ± 0.02	1.50 ± 0.08*	5.9 ± 0.2	7.1 ± 0.2*

* $P < 0.05$ compared to control values.

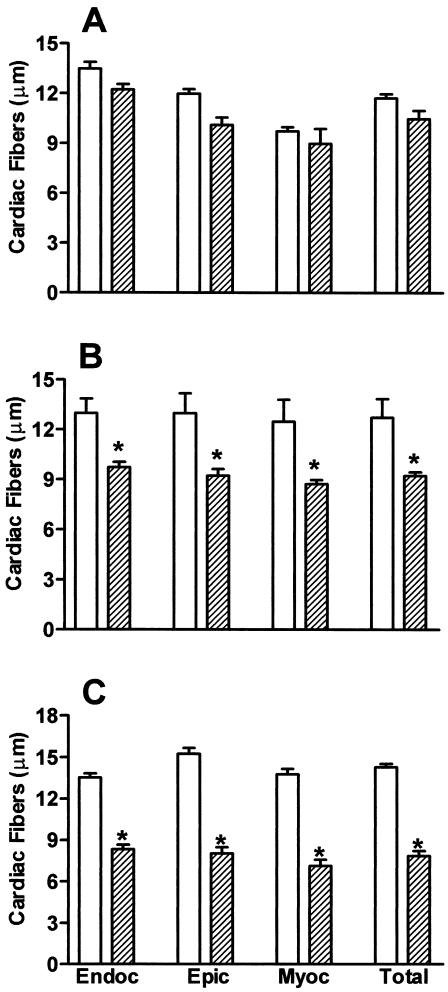

Fig. 1. Cardiomyocyte size (μm) in subendocardial, subepicardial, and midmyocardial regions of the left ventricle from rats treated chronically with L-NAME (7.5 mg/kg per day) for 2 (Panel A), 4 (Panel B), and 6 (Panel C) months. Control animals that received tap water alone are shown by the open columns, whereas L-NAME-treated animals are shown by the hatched columns. $^{*}P < 0.05$ compared to control values. Endoc, subendocardial; Epic, subepicardial; Myoc, midmyocardial.

reduction) and 6 (40–50% reduction) months of chronic L-NAME treatment (Fig. 1).

For measurement of fibrous tissue in the left ventricle, we assumed the amount of fibrous tissue to equal the sum of postnecrotic fibrous scars and interstitial and perivascular fibrosis. With L-NAME treatment, the amount of fibrous tissue was unaltered at 2 and 4 months, but a small (but significant; $P < 0.05$) increase in fibrous tissue was observed at 6 months of treatment (Table 2).

3.5. Brain NOS activity

The brain NOS activity in rats treated chronically with L-NAME for 2 months was markedly reduced (0.59 ± 0.10 pmol citrulline min^{-1} mg^{-1}; $n = 3$) compared to that of control animals (5.04 ± 0.48 pmol min^{-1} mg^{-1}; $n = 3$; $P < 0.01$). The omission of Ca^{2+} and addition of EGTA to the brain homogenates abolished the NOS activity in both the control (0.6 ± 0.1 pmol citrulline min^{-1} mg^{-1}; $n = 3$) and L-NAME-treated animals (0.1 ± 0.06 pmol citrulline min^{-1} mg^{-1}; $n = 3$); thus, indicating that conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was due to constitutive NOS.

4. Discussion

Our results show that chronic treatment of rats with a low dose of L-NAME (7.5 mg kg^{-1} day^{-1}) for prolonged periods (4 and 6 months) causes significant hypertension accompanied by significant reductions in both heart weight and left ventricular weight indexes as well as in cardiomyocyte size. This finding contrasts with the literature, since in other studies (usually using higher doses of L-NAME for shorter periods), ventricular hypertrophy and increase in cardiomyocyte size were reported in association with hypertension (Numagachi et al., 1995; Moreno-Jr et al., 1996; Devlin et al., 1998; Gomes-Pessanha et al., 1999).

The reduction in cardiomyocyte size is not due to the sustained hypertension induced by L-NAME, since it usually leads to ventricular hypertrophy in animals (Dussaule et al., 1986) and humans (Hammond et al., 1988; Levy et al., 1989) in response to the increased afterload (Dominiczak et al., 1997). This discrepancy indicates that cardiac hypotrophy in NO-deficient rats is not due to mechanical overload. Two possible explanations for these results can be proposed: one, based on a systemic deficiency of NO, leading to a decrease in blood supply to the heart muscle; and the other, based on a local deficiency of NO, causing metabolic changes in the cardiomyocyte itself.

The first hypothesis is supported by the findings that ventricular lesions caused by higher doses of L-NAME appear to be mainly the consequence of extensive myocardial ischaemia, which ultimately leads to cardiomyocyte death, necrosis, and subsequent formation of interstitial fibrosis (Moreno-Jr et al., 1996). These phenomena take place independently of arterial hypertension since prolonged treatment of rats with angiotensin-converting enzyme inhibitors (Hropot et al., 1994; Pechanova et al., 1997; Akuzawa et al., 1998; Matsubara et al., 1998) reduces hypertension but fails to affect the accompanying ventricular lesions (Moreno-Jr et al., 1995). Therefore, the reduction of cardiomyocyte size, as evidenced in this study, could represent a prior step before cardiomyocyte death. Thus, a slow (but persistent) reduction in coronary flow in response to a low dose of L-NAME would lead to an inadequate supply of oxygen and impaired myocardial contractility, resulting in cardiomyocyte thinning and, in later stages, in myocyte death and replacement by fibrous tissue. Consistent with this, a significant increase in fibrous tissue was observed 6 months after L-NAME treat-

ment. It is likely that hypotrophy of cardiomyocytes with higher doses of L-NAME was undetectable as intense coronary ischaemia would occur promptly after L-NAME administration, thereby accelerating cardiomyocyte death. Indeed, cardiac infarction, induced by high doses of L-NAME, is observed as soon as 72 h after oral administration (Moreno-Jr et al., 1997).

Mechanical (stretch) and humoral factors (thyroid hormones, catecholamines, and the renin–angiotensin system hormones) are known to regulate the growth of adult hearts (see Hudlicka and Brown, 1996). However, little is known about whether NO is involved in cardiomyocyte growth (Pignatti et al., 1999). Previous studies demonstrated that cardiomyocytes produce NO (Kitakaze et al., 1995) and express both type II (Pinsky et al., 1995; Buchwalow et al., 1997) and type III (Stein et al., 1998) NOS. Since the low dose of L-NAME caused hypotrophy of cardiomyocytes (and inhibited NOS), it is possible that NO has hypertrophic actions in this particular cell type. Indeed, NO has been shown to have proliferative actions in both endothelial cells from coronary postcapillary venules (Ziche et al., 1994) and chick cardiomyocytes in culture (Pignatti et al., 1999).

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