Summary. The rat model of hypertension induced by prolonged treatment with Nω-nitro-L-arginine methyl ester (L-NAME) has been extensively used. However, the effects on cardiac autonomic innervation are unknown. Here, the cardiac sympathetic innervation is analyzed in parallel with myocardial lesions and leukocyte infiltration during L-NAME (40 mg/Kg body weight/day, orally) treatment. The occurrence of cardiomyocyte hypertrophy, a controversial matter, is also addressed. Degenerating cardiomyocytes and focal inflammation occurred one day after treatment. Inflammatory lesions became gradually more frequent until day 7. At day 14 fibroblast-like cells were outstanding. Interstitial and perivascular connective tissue increased from day 28 on. In the left ventricle, cardiomyocyte hypertrophy occurred only around the damaged area during the first 14 days. After 28 days, it became more widespread. In the right ventricle, the hypertrophic cardiomyocytes were restricted to damaged areas. Significant reduction of the noradrenergic nerve terminals occurred from day 3 to 28. The area occupied by ED1+ (hematogenous) macrophages increased until day 7, and dropped to control levels by day 10. ED2+ (resident) macrophages increased from day 3 to 7 and remained higher than control values up to day 77. Animals receiving both L-NAME and aminoguanidine (AG), an inducible nitric oxide synthase (iNOS) inhibitor (65 mg/Kg body weight/day, orally), showed significant decrease in the nitrite serum levels, sympathetic denervation and macrophage infiltration at day 7. No denervation was detectable at day 14 of double treatment, using subcutaneous AG. Our findings favor a role for ED1+ macrophages and iNOS in the hypertension–induced denervation process.

Key words: Nitric oxide, Inducible nitric oxide synthase, Aminoguanidine, Cardiomyocyte hypertrophy, Heart innervation

Introduction

Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) has a pivotal role in the vascular tonus control and, therefore, in blood pressure regulation. Nω-nitro-L-arginine methyl ester (L-NAME) is a non-specific inhibitor of NOS with selectivity for the constitutive NOS isoforms (Darblade et al., 2001). Daily treatment with L-NAME causes sustained hypertension and marked morphological alterations in the heart such as multifocal necrotic and inflammatory/fibrotic lesions (Numaguchi et al., 1995; Moreno et al., 1996; Babál et al., 1997; Devlin et al., 1998). The L-NAME model of hypertension has been considered to simulate the clinical condition of essential hypertension associated with endothelial dysfunction. In this animal model, involvement of the cardiac autonomic innervation remains to be studied and the occurrence of cardiomyocyte hypertrophy is still controversial. Regarding the autonomic innervation, contemporary researches on heart failure have highlighted the relevance of sympathetic dysfunction for prognostic purposes. Indeed, the rate of cardiac noradrenaline spillover correlates with the severity of heart failure. Increased sympathetic efferent neuronal activity induces tachycardia and enhanced the arterial resistance (Esler et al., 1997; Rundqvist et al., 1997). Despite the higher levels of circulating plasma noradrenaline there is a significant reduction of its cardiac content (Chidsey et al., 1965; Eisenhofer et al., 1996). This fact has been taken as indicative of sympathetic denervation of failing hearts (Daly and Sole, 1990). A previous finding from our laboratory (Machado et al., 2000) has demonstrated significant reduction of the cardiac postganglionic autonomic innervation at the end-stage of heart failure in
patients with chronic Chagas’ disease (American trypanosomiasis) or other dilated cardiomyopathy (hypertension, peripartum, idiopathic, rheumatic fever). In a rat model of Chagas’ disease, cardiac autonomic nerve terminals are damaged in parallel with the acute myocarditis (Machado and Ribeiro, 1989; Machado et al., 1994 and references therein) and probably macrophages are involved (Melo and Machado, 1998; Guerra et al., 2001).

The main purpose of the current investigation was to study the cardiac sympathetic efferent innervation in L-NAME-induced hypertension in parallel to the histopathological changes and serum levels of the NO metabolites. In order to test the participation of the inducible nitric oxide synthase (iNOS) on the L-NAME-induced cardiac changes, blockage of this enzyme was achieved by concomitant treatment with amino-guanidine, an iNOS selective inhibitor. Interestingly, administration of L-NAME can result in a compensatory increase of iNOS expression (Miller et al., 1996). Indeed, L-nitro-arginine, the in vivo circulating metabolite of L-NAME, is a weak and rapidly reversible inhibitor of iNOS (Darblade et al., 2001).

Materials and methods

Animal groups and treatments

Male Holtzman rats aged 12 weeks were randomized to groups receiving L-NAME (Sigma Chemical Co) daily in drinking water (40 mg/kg body weight/day) ad libitum or the vehicle (untreated). They were killed at days 1, 3, 7, 10, 14, 28, 63 and 77 of treatment, with 6 treated and 4 untreated rats at each time-point. The animals were housed individually under 12-hour light/12-hour dark cycle. The treatment starts one week after this kind of housing. The systolic blood pressure was measured by tail-cuff plethysmography in conscious restrained rats, before L-NAME treatment and once a week during the treatment. The body weight was also estimated weekly.

For selective inhibition of the iNOS, additional groups were treated with aminoguanidine bicarbonate (AG, Sigma Chemical Co). Two protocols were used: (a) oral administration using AG (65 mg/kg body weight) together with L-NAME in drinking water for 7 days (6 AG+ L-NAME-treated and 4 AG-treated rats); (b) AG subcutaneous administration at 12-hour-interval (30 mg/kg body weight) during 14 days of L-NAME treatment (5 double-treated and 4 untreated).

Care and anesthesia obeyed the guidelines for Laboratory Animals established by The National Institute of Health (Bethesda, MD, USA), as recommended by the Institute of Biological Sciences at the Federal University of Minas Gerais, Belo Horizonte, Brazil. Blood samples were obtained from axillary plexus under 2,2,2-tribromoethanol anesthesia (Aldrich, 25 mg/100 g body weight) immediately before organ dissection. Hearts were rapidly blotted and weighed.

Histological and histoquantitative methods

Transverse slices of both ventricle bases as well as the superior cervical and stellate ganglia were fixed in 4% phosphate-buffered paraformaldehyde and processed for resin embedding (JB-4™ Polysciences, St. Louis, MO). Two-µm-thick sections at 40-µm-intervals were stained with an ice-cold mixture of Giemsa (6.25 ml) and May-Grunwald (10.75 ml) solutions in methanol (33ml) diluted 1:4 in distilled water. A morphometric estimative of cardiomyocyte size was performed in the left ventricle using a computer-assisted morphometric program (KS 400, Kontron Elektronik Imaging System, Germany) coupled to a Zeiss Axiosplan 2 microscope. The transversal axis was measured (µm) in 150 longitudinally cut cardiomyocytes per animal, at a final magnification of 1,000. We used longitudinally sectioned fibers to guarantee enough cells in damaged and non-damaged areas in a same section. All measured cells had clear cellular limits, visible nuclei and no signs of cellular degeneration. Cardiomyocyte sizes in areas with normal morphology were compared with those close to inflammatory foci and fibrosis. In the right ventricle, morphometric analysis was done only at day 77 of L-NAME treatment.

Histochemical demonstration of the sympathetic cardiac innervation

The noradrenergic innervation was identified in 30-µm-thick cryostat sections obtained from left ventricles through a glyoxylic acid-induced fluorescence method. This histochemical technique for catecholaminergic neurotransmitters has high specificity and sensitivity (Cottle et al., 1985) and it is routinely used in our laboratory (Machado et al., 2000, Martinelli et al., 2002). The density of sympathetic innervation was morphometrically estimated with the aid of the KS 400 system. The mean percentage area occupied by the fluorescent nerve terminals was measured in 60 microscopic fields per rat at magnification of 300. Each microscopic field measured 0.38 mm². The values were distributed in classes from the less (class 1) to the more (class 8) innervated myocardium as already described (Machado et al., 2000). The relative frequency of each class in L-NAME -treated and age-matched untreated rats assessed the occurrence of focal denervation.

Immunohistochemical characterization of leukocytes

Left ventricle tissues embedded in Tissue Tek (OCT compound, Miles USA) were immediately frozen in liquid nitrogen and stored at -70°C. Cryostat-obtained sections (7-µm-thick) were mounted on silane precoated slides, and then fixed in acetone, washed in PBS and incubated in 0.1 M sodium azide/0.1% H₂O₂, for endogenous peroxidase activity inhibition. Incubation with mouse antibodies (Serotec, Kidlington, England) at previously tested dilutions (ED1, 1:400; ED2, 1:400;
CD8, 1:100; CD4, 1:100; CD45RA, 1:200 and NKR/CD161, 1:200) was followed by washing and incubation with peroxidase-conjugated goat anti-mouse antibody (Pharmineng, San Diego, CA) diluted 1:50. The peroxidase activity was revealed by 3,3'-diaminobenzidine (Sigma, St. Louis, MO) in PBS buffer with 0.1% H2O2 for 10 minutes. The mean area (µm²) occupied by each immunolabeled cell was estimated by using KS 400 system at a final magnification of 300. The values were expressed as µm²/mm². The identification of ED1⁺, ED2⁺, NKR⁺, CD8⁺, CD4⁺ and CD3⁺ cells in the lymph nodes or spleens from control rats, as well as the suppression of the primary antibody assured the specificity of the immunohistochemical reactions.

Spectrophotometric assay of nitrite/nitrate serum levels

After enzymatic reduction to nitrite, as previously described (Schmidt et al., 1989), the total amount of NO₃⁺ NO₂⁻ (NOx) was obtained by the Griess reaction (Grén et al., 1981). Absorbance at 500 nm was read in Spectra Max 250-Molecular Device, Sunnyvale, CA). Standard curve was obtained by incubating sodium nitrate (1 to 200 mM) with the reductase buffer.

Statistical analyses

SigmaStat Software, version 1.0 (Sigma, St. Lois, MO, USA) was used for all analyses. Student’s t test or Mann-Whitney Rank Sum test, all with significance at p<0.05, were used for comparison of blood pressure, body weight, heart weight, heart/body weight ratio, NOx serum levels and macrophages distribution. The comparisons between the dichotomous variables (innervation) were performed using the chi-square (χ²) test.

Results

Blood pressure, body weight and heart/body weight ratio

All L-NAME-treated rats exhibited higher systolic blood pressure in comparison with untreated animals.
throughout the experimental time (Fig. 1A). Significant reduction of body weight gain occurred during the first 10 days of L-NAME treatment, and at days 70 and 77 there was significant loss of the body weight in the treated rats (Fig. 1B). No significant difference occurred in the absolute heart weight (Fig. 1C). The heart/body weight ratio was significantly higher only at day 7 of treatment (Fig. 1D). For all these parameters, the values for AG + L-NAME- and L-NAME-treated rats were statistically similar.

**Myocardial histopathology**

In both ventricles, scattered areas exhibiting cardiac fiber disruption, and degenerating cardiomyocytes with cytoplasmic vacuoles and myofibril disorganization occurred at day 1 of the L-NAME treatment. All these histopathological findings were absent from untreated rats (Fig. 2A). By this time, despite the presence of infiltrating mononuclear cells and rare neutrophils, some degenerating cardiomyocytes occurred in the absence of close apposition to these infiltrating leucocytes, even when serial sections were carefully examined (Fig. 2B). From day 3 to 7, all heart sections from L-NAME-treated rats exhibited focal mononuclear cell infiltration, usually closely associated with the degenerating myocytes or cardiac fiber disruption (Fig. 2C, D and E). Some fibroblast-like cells and collagen fibers were seen in damaged areas mainly at day 7. At day 14, the myocardial damaged areas showed fewer inflammatory cells and abundant capillary vessels and fibroblast-like cells (Fig. 2F). From day 28 on, collagen deposition increased gradually. At days 70-77, the myocardial alterations consisted mainly of focal interstitial and replacement fibrosis (Fig. 2G). The myocardial necrosis and inflammatory cells could still be seen. None of these abnormalities was observed in the hearts from untreated rats. In AG + L-NAME-treated rats for 7 or 14 days, the myocardial focal lesions were clearly smaller and less frequent in comparison with those of the L-NAME-treated rats.

In the left ventricle, cardiomyocyte hypertrophy...
occurred at all tested time-points (Fig. 3). In the first two weeks, the hypertrophy was restricted to the cardiomyocytes surrounding inflammatory foci. However at days 28 and 77, the hypertrophic cardiomyocytes were found around damaged as well as in undamaged myocardium. In contrast, the right ventricle at day 77 showed hypertrophic cardiomyocytes (p< 0.05) only around damaged areas (23.2±2.86 µm) in comparison with undamaged areas (17.5±1.41 µm) and untreated rat values (17.1±1.58 µm).

**Leukocyte phenotypes**

In the left ventricle NKR+, CD8+, CD4+ and CD3+ cells were rare or absent in both untreated and L-NAME-treated rats. In untreated rats, ED1+ macrophages were sparsely distributed (Fig. 4A), but ED2+ macrophages were numerous (Fig. 4C). In L-NAME-treated rats, ED1+ macrophages occurred mainly in the inflammatory foci (Fig. 4B). In contrast, ED2+ macrophages were rare inside the inflammatory foci, 

**Fig. 3.** Left ventricle cardiomyocyte hypertrophy (mean ± SD) in control and L-NAME-treated rats. During the first 2 weeks, cardiomyocyte hypertrophy occurred only in damaged myocardium (DM). At days 28 and 77, both normal (NM) and damaged myocardium exhibited hypertrophied cardiomyocytes. Unpaired t-test: *P < 0.05, **P < 0.01 and ***P < 0.001 treated vs. control.

**Fig. 4.** Peroxidase immunostained rat macrophages in the left ventricle. 
A. Rare ED1+ macrophages are seen in untreated rats. B. At day 7 of L-NAME treatment numerous ED1+ macrophages are inside the inflammatory foci. C. Diffuse distribution of ED2+ macrophages in untreated rats. D. ED2+ macrophages were mainly outside the same inflammatory focus shown in B. Bar: 50 µm.
remaining dispersed throughout the myocardium (Fig. 4D). The amount of ED1+ macrophages increased significantly from day 1 to 7. Thereafter, no significant difference was detectable (Fig. 5A). The amount of ED2+ cells increased significantly from day 3 to 7 and remained elevated until day 77 (Fig. 5B). In AG + L-NAME-treated rats for 7 days, there was significant reduction of both ED1+ and ED2+ populations (Fig. 5A,B). Double treatment, using subcutaneous AG for 14 days, brought down the ED1+ population to untreated rat values (Fig. 5A).

**NOx serum levels**

In L-NAME-treated rats, the NOx levels were similar to those of the untreated animals in all time-points excepting day 10, when a significant drop occurred (Fig. 5C). The AG administration to L-NAME-receiving rats for either 7 or 14 days caused a significant reduction of NOx levels (Fig. 5C).

**Sympathetic innervation**

The pattern of ventricular sympathetic innervation was similar in untreated (Fig. 6A) and L-NAME-treated rats at days 1 and 77. In all other time-points, the majority of treated animals showed a variable degree of cardiac focal sympathetic denervation (Fig. 6B,C). H&E-staining after UV examination showed that the areas with marked reduction of nerve terminals exhibited inflammatory foci and degenerating cardiomyocytes (Fig. 6D). Areas with light to moderate denervation could correspond to normal myocardium (Fig. 6C).

Morphometric data showed greater frequency of microscopic fields with reduced innervation at days 3 (P<0.02), 7 (P<0.0001), 14 (P<0.0001) and 28 (P<0.001) of L-NAME treatment. This reduction was higher at days 7 and 14. The relative frequency of classes representing the less (classes 1+2) and the more innervated (classes 4-8) myocardium at each time-point is shown in Fig. 7.

In AG + L-NAME-treated rats for 7 days, complete blockage of the cardiac denervation occurred in about 70% of the rats. Morphometric analysis confirmed the significant reduction of the denervated areas (Fig. 8). The frequency of classes 1 and 2 were 34% and 6.7% for L-NAME-treated and AG + L-NAME-treated rats, respectively. After 14 days of double treatment, all animals presented normal innervation (not quantified).

Despite the cardiac sympathetic denervation, qualitative analysis of superior cervical and stellate ganglia showed no detectable histopathological alteration, indicating that the reversible cardiac denervation was a local phenomenon.

**Discussion**

Although our main objective has been the study of the cardiac sympathetic efferent innervation in relation
to macrophage infiltration, some interesting new information regarding the cardiomyocyte hypertrophy and kinetics of leukocytes infiltration were obtained and they will be discussed first.

Controversial reports have been published on the development of heart hypertrophy in L-NAME model of hypertension (Arnal et al., 1993; Delacretaz et al., 1994; Rhaleb et al., 1994; Sládek et al., 1996; Banting et al., 1997; Wickman et al., 1997). Our findings in the left ventricle showed that hypertrophic cardiomyocytes appeared first around inflammatory lesions and later around fibrotic lesions and in undamaged myocardium. However, in the right ventricle, hypertrophic cardiomyocytes remained restricted to damaged areas.

Cardiomyocyte hypertrophy around myocardial lesions indicates a compensatory mechanism for degenerating or lost cardiomyocytes. Despite the widespread cardiomyocyte hypertrophy in the left ventricle from day 28 on, no appreciable difference in the absolute or relative heart weight occurred. Cardiomyocyte loss and/or fibrotic lesions could compensate the hypertrophy. Also, right ventricle hypotrophy could mask the effects of the left ventricle hypertrophy. According to Arnal et al. (1993), the right ventricle weight decreases after 8 weeks of L-NAME treatment, and left ventricle hypertrophy correlates with higher hypertension levels. In our hypertensive rats, widespread hypertrophy occurred only when the mean arterial

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**Fig. 6.** Glyoxilic acid-induced fluorescence technique for catecholamines in the left ventricle. **A.** Normal density of sympathetic nerve terminals in untreated rat. **B.** Well-innervated area at day 7 of L-NAME treatment. **C.** Another area in the same section showed marked reduction of the sympathetic varicose nerve terminals. **D.** The same region showed in B after H&E staining, reveals that myocardium surrounding the damaged area corresponds to the poorly innervated myocardium showed in C. The * marks similar regions in C and D. Bar: 100 µm.
pressure was around or above 140 mm Hg. Interestingly, molecular alterations on left ventricular actin and myosin have been described in this hypertension model (Arnal et al., 1993; Takaori et al., 1997).

Our immunohistochemical findings showed a numeric increase of two macrophage populations, ED1+ and ED2+, with different kinetics. ED1 antibody recognizes phagolysosomes in rat monocytes, hematogenous infiltrating macrophages and some interdigitating cells of lymphoid organs (van der Berg et al., 2001). Therefore, ED1 expression depends on phagocytic activity. The ED1+ cell population increased from day 1 to attain maximal values at day 7, the period of maximal cardiomyocyte damage. Afterwards, in the remodeling phase, the values became similar to that of untreated rats. Increase in ED1+ macrophages was already reported (Tomita et al., 1998) at days 3, 7 and 28 of L-NAME treatment, the highest value occurring at day 3, as happened with expression of the monocyte chemoattractant protein-1 (MCP-1). Our results in AG + L-NAME-treated rats favor a role for iNOS in tissue lesions induced by L-NAME. Indeed, the double treatment diminished both the myocardial lesions and amount of macrophages. Fibroblast-like cells were frequently observed in our L-NAME-treated rats from day 3 on, becoming outstanding at day 14. Interestingly, an increase in fibroblast number has been associated with factors released by ED1+ macrophages in isoproterenol-induced myocardial injury (Nakatsuji et al., 1997).

A new finding was the increase in the amount of ED2+ macrophages from day 3 to 7, the values remaining elevated until the end of the L-NAME treatment. ED2 antibody exclusively labels resident macrophages (van der Berg et al., 2001) which comprise different subpopulations although their functional roles are not clearly understood. There is evidence that one of them may proliferate in inflamed tissues (Nakatsuji et al., 1997; Mueller et al., 2001, 2003). Besides, there are data supporting a physiological turnover of resident macrophages by blood-derived cells (Mueller et al., 2003). This cell type has been related to the regenerative phenomena in skeletal muscle (Massimino et al., 1997) and scar tissue formation in experimentally induced hepatic fibrosis (Ide et al., 2003).

In the current paper, reduction of sympathetic nerve terminals was first observed at day 3 and maximum denervation occurred at days 7 and 14 of L-NAME treatment. Most denervated areas were associated with focal ED1+ macrophage infiltration. In the acute myocarditis provoked by T. cruzi infection in rats, a diffuse and severe autonomic denervation paralleled the acute myocarditis characterized by mononuclear cells predominance (Machado and Ribeiro, 1989). After resolution of this T. cruzi-induced inflammation there is a gradual recovery of nerve terminals assessed by histochemical, biochemical and ultrastructural methods as reviewed (Melo and Machado, 1998). The axonal regrowth is possible because no appreciable lesion is found in the sympathetic ganglia responsible for the cardiac innervation (Camargos et al., 1996). Similarly, no appreciable damage occurred in the sympathetic ganglia of the L-NAME-treated rats. This finding and the focal character of the denervation explain the fast recovery of the sympathetic innervation soon after the drop of ED1+ population to control values. The

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Fig. 7. Percentage area occupied by fluorescent nerve terminals in the left ventricle of control and L-NAME-treated rats. Relative frequency of classes 1–2 and classes 4–8 representing the less and the more innervated myocardium respectively. Chi-square test showed higher relative frequency of less innervated classes at days 3 (P<0.02), 7 (P<0.0001), 14 (P<0.0001) and 28 (P<0.001) days of L-NAME-treatment.

Fig. 8. Relative frequency of classes representing the mean areas occupied by the fluorescent nerve terminals at day 7 of L-NAME, AG and AG + L-NAME treatments. Chi-square test showed less denervation in the AG + L-NAME-treated rats.
possibility that decreased density of sympathetic nerve fibers around myocardial lesions could simply reflect cardiomyocyte hypertrophy (Gerová et al., 1996) is not supported by our results. Indeed hypertrophic cardiomyocytes became widespread from day 28 on, when the recovery of normal innervation pattern was observed.

AG treatment blocked the denervation and significantly reduced the enhancement of ED1+ population. Importantly, the double treatment caused a significant drop on nitrite/nitrate serum levels at day 7 (oral) and 14 (subcutaneous). These findings agree with the notion that endothelial iNOS expression and NO production occur after prolonged treatment with L-NAME (Miller et al., 1996; Darblade et al., 2001). As iNOS is expressed by inflammatory macrophages (Peng et al., 1998) and sympathetic denervation is morphologically associated with the ED1+ macrophage infiltration (present results), our results indicate that these macrophages are involved in the damage of the delicate sympathetic nerve endings. In acute T. cruzi-induced myocarditis, nerve ending damage occurs at the variocities (Machado et al., 1994), sites partially or completely devoid of Schwann cell sheath.

In conclusion, two different macrophage populations increased during L-NAME-induced hypertrophy in rats. Because of its kinetics and distribution, ED1+ cells could be involved with the damage of nerve endings. This assumption is reinforced by the reduction of the focal inflammation, sympathetic denervation and serum NOx levels in AG + L-NAME-treated animals. The latter effect confirms that iNOS is not blocked by L-NAME treatment. Our results also suggest that NO could aggravate the myocardial lesions. Further research is necessary to address, among others, the role for distinct macrophages in the heart remodeling during hypertension.

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References


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