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ORIGINAL ARTICLE

Structural alterations in rat myocardium induced by chronic L-arginine and L-NAME supplementation

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Abstract Structural changes affecting cardiomyocyte function may contribute to the pathophysiological remodeling underlying cardiac function impairment. Recent reports have shown that endogenous nitric oxide (NO) plays an important role in this process. In order to examine the role of NO in cardiomyocyte remodeling, male rats were acclimated to room temperature (22 ± 1 °C) or cold (4 ± 1 °C) and treated with 2.25% L-arginine·HCl or 0.01% L-NAME (N^{ω}-nitro-L-arginine methyl ester)·HCl for 45 days. Untreated groups served as controls. Right heart ventricles were routinely prepared for light microscopic examination. Stereological estimations of volume densities of cardiomyocytes, surrounding blood vessels and connective tissue, as well as the morphometric measurements of cardiomyocyte diameters were performed. Tissue sections were also analyzed for structural alterations. We observed that both L-arginine and L-NAME supplementation induced cardiomyocyte hypertrophy, regardless of ambient temperature. However, cardiomyocyte hypertrophy was associated with fibrosis and extra collagen deposition only in the L-NAME treated group. Taken together, our results suggest that NO has a modulatory role in right heart ventricle remodeling by coordinating hypertrophy of cardiomyocytes and fibrous tissue preventing cardiac fibrosis.

1. Introduction

L-arginine is a precursor in the synthesis of numerous biologically important molecules whose signaling is limited to a great extent by their availability, as well as by the expression and activity of multiple enzymes involved in their metabolism (Wu and Morris, 1998; Jobgen et al., 2006). One of the most important biomolecules originating from L-arginine is NO

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which functions as a cell signaling molecule in mammalian cells, controlling vital functions such as neurotransmission, blood vessel tone, host defense and immunity (Moncada et al., 1991). The enzyme that catalyzes NO production from the guanidine group of L-arginine in an NADPH-dependent reaction is nitric oxide synthase (NOS), which is present in at least three distinct isoforms: neuronal NOS (nNOS, or NOS1), inducible NOS (iNOS, or NOS2), and endothelial NOS (eNOS, or NOS3). The recognition that all three isoforms of NOS are expressed in cardiomyocytes has raised several intriguing questions regarding the signaling role of NO in the heart. NO is produced from virtually all cell types composing the myocardium and regulates cardiac function through both vascular-dependent and -independent effects. NO acts as a negative inotrope in cardiac muscle (Brady et al., 1993), and exerts a hypotensive effect *via* peripheral artery dilatation, thus decreasing afterload, and it reduces the preload by the dilatation of the venous system (Simko and Simko, 2000). Besides its hemodynamic actions, however, the direct antiproliferative effects of NO may play an important role (Kolpakov et al., 1995; Rossi and Colombini-Netto, 2001), since NO seems to be an important factor in modulating hypertrophic heart growth (Matsuoka et al., 1996; Simko and Simko, 2000; Pereira and Mandarim-de-Lacerda, 2001; Simko et al., 2004).

Cardiac hypertrophy refers to the process of cardiac thickening and remodeling and may be due to cardiac pathology, long-term exercise training (Pelliccia et al., 2002; Maron et al., 2003) or chronic cold exposure (Cheng and Hauton, 2008). In pathological cardiac hypertrophy, myocardial structure is affected by two key pathological processes: cardiomyocyte hypertrophy and a progressive accumulation of connective tissue within the myocardial interstitium-myocardial fibrosis (Diez et al., 2001; Rossi, 2001; Gonzalez et al., 2004). The main etiological factor of pathological cardiac hypertrophy is hypertension-induced chronic pressure overload (Perrino et al., 2006). In contrast, during conditions of increased hemodynamic requirements, such are during sustained exercise training or during prolonged cold exposure, so-called physiologic cardiac hypertrophy occurs (a condition known as "athlete heart" as a result of cardiac adaptation to long-term training). Physiologic cardiac hypertrophy is characterized by preserved myocardial structure, with a normal pattern of gene expression and collagen metabolism, and does not progress to ventricular dysfunction (Rajiv et al., 2004).

Since NO is the endothelium-derived relaxing factor essential for regulating vascular tone and hemodynamics (Ignarro et al., 1999), there has been a growing interest in L-arginine supplementation to prevent and treat cardiovascular disorders. Endothelial dysfunction due to impaired NO bioavailability in the vasculature plays an important role in the development of cardiovascular disease. Patients with chronic heart failure display signs of disturbed endothelial function in coronary arteries (Richartz et al., 2001) and in the peripheral circulation (Kubo et al., 1991). Since L-arginine availability is a rate-limiting step for NOS (Baydoun et al., 1990; Mitchell et al., 1990; Sessa et al., 1990) and its decreased availability results in a decreased NO production (Xia et al., 1996), this amino acid is widely available and publicized as having benefits in the prevention and treatment of cardiovascular disorders (Maxwell and Cooke, 1998; Boger and Ron, 2005) and atherosclerosis risk factors, all of which are conditions that are associated with reduced NO biosynthesis (Creager et al., 1992; Clarkson et al., 1996; Pieper et al., 1996; Adams et al., 1997; Lerman et al., 1998). Dietary supplements containing L-arginine are also claimed to have effects in healthy subjects by promoting vasodilatation which improves perfusion and delivery of nutrients and oxygen to various organs. However, less is known about how L-arginine supplementation affects the structure and function of pathologically unaltered hearts.

In contrast, chronic inhibition of NOS by N^{ω}-nitro-L-arginine methyl ester (L-NAME) is a well-established model of experimental hypertension and cardiac hypertrophy (Pechanova et al., 1999). The vascular effects of L-NAME have been demonstrated to involve the removal of the tonic vasodilator action of NO by eNOS inhibition (Griscavage et al., 1995; Cohen et al., 1996; Vaziri and Wang, 1999) and stimulation of the sympathetic nervous system *via* nNOS inhibition (Chowdhary and Townend, 1999).

One of the important factors in increasing heart workload is cold exposure. The ability of homeotherms to make the necessary physiological and metabolic adaptations when subjected to cold is important for their survival. These include increased heat production that is fueled by increased food intake and/or mobilization of body reserves which often results in a decreased body weight gain, nitrogen balance, and skeletal muscle accretion (Samuels et al., 1996; McAllister et al., 2000). There is also an attendant increase in the size of the heart (Heroux and Gridgeman, 1958; Hale et al., 1959; Samuels et al., 1996) and its protein mass (McAllister et al., 2000), which likely reflects its higher workload in support of the increased thermogenesis. As we have already demonstrated L-arginine-induced enhancement of thermogenic activity in brown adipose tissue (Petrovic et al., 2005) and L-arginine-induced intensification of antioxidative defense processes in the skeletal muscle of cold-acclimated rats (Petrovic et al., 2008), it is expected that L-arginine will further affect myocardial load in rats acclimated to cold.

The aim of this study was to determine whether chronic oral administration of L-arginine and L-NAME to adult male rats (maintained at room temperature or acclimated to cold) affected myocardial structure. Structural changes were characterized by microscopic, stereological and morphometric examinations.

2. Materials and methods

2.1. Experimental design

The experiment was approved by the Ethical Committee for the Treatment of Experimental Animals of the Institute of Biological Research "Sinisa Stankovic", University of Belgrade, and the animals were cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals*. Male Mill Hill hybrid-hooded, 2-month-old rats were used. They were divided into three main groups. The first group received L-arginine·HCl (2.25%), and the second group received L-NAME·HCl, (0.01%), in drinking water for 45 days. The third, untreated group served as a control, and received tap water. The animals in all three groups were further divided into two groups – one group was kept in a cold room at 4 ± 1 °C and the other group was kept at room temperature (22 ± 1 °C). Each group consisted of six animals. The

animals were kept in individual cages, with food and drinking liquids *ad libitum*. At the end of the experimental period the animals were sacrificed; hearts were isolated immediately and prepared for microscopic analyses.

2.2. Microscopic analyses

In order to obtain isotropic and uniform random sections for stereological and morphometric studies of right ventricle, the unbiased design-based approach to the quantitative study of anisotropic structures – the orientator method (Mattfeldt et al., 1990) was performed. The right ventricles were cut at random; then placed on cut surface and again cut at random, perpendicular to the first plane. The specimens were again placed on the new cut surface and a new random orientation is defined by cutting the organ in a perpendicular section to the plane. The last cut is considered uniformly isotropic, meaning that without reference to the position of the specimen in the first cut the last surface has an orientation that varies from all possible ones. One half of the randomly selected heart right ventricle samples from each animal was fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in Araldite (Fluka, Germany). Semi-fine serial sections were cut using an ultramicrotome (Leica, Germany) and stained with toluidine blue. Micrographs (magnification 100 × 10) of each section were obtained for stereological and morphometric analyses (Axio Imager M1, Carl Zeiss, Germany). The other half of the randomly selected heart right ventricle samples was fixed in buffered 10% formalin and embedded in paraffin. Five-μm thick paraffin sections were cut using a microtome (Reichert, Austria), stained with Azan trichrome and analyzed microscopically (Axio Imager M1). Micrographs (magnification 100 × 10) were obtained for histological analysis and morphometric analysis of cardiomyocyte diameters.

2.3. Morphometric and stereological analyses

2.3.1. Estimations of volume densities of cardiomyocytes, blood vessels and connective tissue in the myocardium

The volume densities of the major myocardial components (cardiomyocytes, blood vessels and connective tissue) were obtained using a standard point-counting technique (Weibel, 1981) and the ImageJ image analysis system (NIH, USA). This technique allows the determination of the volume fraction of each compartment as a ratio of the number of points falling on the compartment relative to the total number of points falling on the myocardial tissue. An orthogonal grid with 8 × 10 intersections was superimposed on light micrographs at a final magnification of ×1000 (15 micrographs per animal). Volume densities were expressed as percentage fractions of cardiac muscle tissue.

2.3.2. Measurement of cardiomyocyte diameters

Morphometric evaluation of cardiomyocyte diameters was performed using the ImageJ image analysis system. Measurements were performed exclusively on longitudinal azan trichrome-stained sections of myocardium. 150–260 cardiomyocyte diameters per animal were analyzed in the central region of the cell (nuclear level). Results are presented as mean value ±SEM. In order to follow the trend in cardiomyocyte diameter change, a histogram of cardiomyocyte size distribution was constructed with a class interval of 5 μm.

2.3.3. Statistical analysis

To test data for normality, the Kolmogorov–Smirnov test was used. Analysis of variance (ANOVA) was used for within-group comparisons of the data. If the F test showed an overall difference, Student's t-test was used to evaluate the significance of the differences in cases of parametric distribution, or Mann–Whitney test in cases of non-parametric distribution. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Volume densities of cardiomyocytes, blood vessels and connective tissue in the myocardium

The results of stereological analyses of volume densities of cardiomyocytes, blood vessels and connective tissue are shown in Table 1. Compared with the control group acclimated to room temperature, cold acclimation did not significantly change volume density of myocardial tissue components (cardiomyocytes, blood vessels, connective tissue), although a slight decrease in blood vessel volume density was observed.

Chronic treatment with L-arginine at room temperature did not lead to statistically significant changes in volume densities of myocardial tissue components, although blood vessel and connective tissue volume densities have tended to increase. Chronic treatment with L-arginine in cold-acclimated animals also showed no significant changes in volume densities of tissue components, when compared with the appropriate control. However, when compared with L-arginine treatment at room temperature, cardiomyocyte volume density increased, while volume density of blood vessels decreased (Table 1).

Both chronic treatments with L-NAME, irrespective of ambient temperature acclimation, led to increased connective tissue volume density associated with a decrease in cardiomyocyte volume density (when compared with appropriate controls). L-NAME treatment in cold-acclimated animals also decreased blood vessel volume density, when compared with the identical treatment in room-acclimated animals.

3.2. Structural alterations in myocardium

The control and L-arginine-treated groups acclimated to room temperature and cold, respectively, showed correct arrangement of myofibrils inside cardiomyocytes with maintenance of myocardial striation (Fig. 1A, B, D and E). A small quantity of connective tissue with scarce collagen fibers was found around the cardiomyocytes.

In contrast, both the room temperature- and cold-acclimated L-NAME-treated groups (Fig. 1C and F) showed myofibril distortion inside the cardiomyocytes with consequent interruption of the correct myocardial striation pattern. Deposition of collagen fibers around the cardiomyocytes was also increased, leading to connective tissue growth in the myocardium of L-NAME-treated rats.

With regard to the capillary supply in the myocardium, a reduction was evident in all cold-acclimated groups (Fig. 1D–F).

Table 1 Volume densities of cardiomyocytes (Vv_{cm}), blood vessels (Vv_{bv}) and connective tissue (Vv_{ct}) in the right ventricle myocardium of experimental groups. Compared with L-arginine treatment in room temperature-acclimated animals, L-arginine treatment in cold-acclimated animals increases cardiomyocyte volume density and decreases blood vessel volume density. L-NAME treatment increases connective tissue volume density and decreases cardiomyocyte volume density. Values are presented as mean ± SEM. Statistically significant differences *vs.* referent control: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$; statistically significant differences *vs.* identical treatment at room temperature: (##) $p < 0.01$, (###) $p < 0.001$.

	Room temperature			Cold		
	Control	L-arginine	L-NAME	Control	L-arginine	L-NAME
Vv_{cm} (%)	82.25 ± 1.16	79.49 ± 0.86	77.82 ± 1.15 (**)	83.81 ± 1.23	83.06 ± 1.58 (##)	78.88 ± 1.27 (**)
Vv_{bv} (%)	10.03 ± 0.77	11.24 ± 0.71	11.13 ± 0.67	8.97 ± 0.77	7.53 ± 0.62 (###)	7.94 ± 0.68 (##)
Vv_{ct} (%)	7.73 ± 0.84	9.10 ± 0.59	11.06 ± 1.15 (*)	7.66 ± 0.79	9.82 ± 1.69	12.49 ± 0.99 (***)

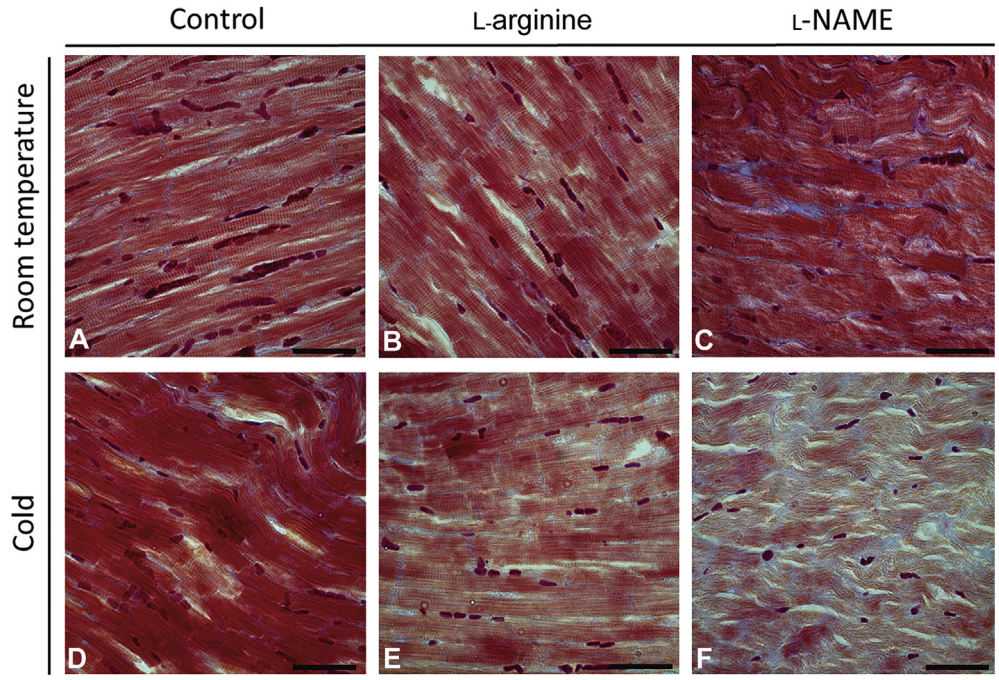

Figure 1 Representative micrographs of longitudinal sections of the right ventricles myocardia from: control groups, L-arginine- and L-NAME-treated groups acclimated to room temperature (22 ± 1 °C) and cold (4 ± 1 °C), respectively. No signs of pathological alterations were observed in (A and D) control and (B and E) L-arginine-treated groups. In (C and F) both L-NAME-treated groups, distortion of correct myofibril arrangement in cardiomyocytes is visible. Also, collagen (blue) content in the interstitium is increased in these groups. Azan trichrome staining. Scale bars – 50 μm.

3.3. Cardiomyocyte diameter

Compared with the control group acclimated to room temperature, increased cardiomyocyte diameter occurred in all experimental groups, however, the diameters were statistically different ($p < 0.01$) only after L-arginine and L-NAME treatment in room temperature-acclimated animals (Table 2).

The distribution histogram of cardiomyocyte diameters confirmed these results, and showed a shift to greater diameters in all treated and cold-acclimated groups when compared to the room temperature-acclimated control group (Fig. 2).

4. Discussion

In the present work, adult male rats were treated with L-arginine and L-NAME for 45 days, respectively, in order to examine the possible effects of NO on myocardial structure during room temperature- or cold-acclimation. Our results demonstrated that cold acclimation *per se* does not significantly alter the volume densities of myocardial tissue components, but leads to a trend in cardiomyocyte hypertrophy followed by a downward trend in capillarity.

L-Arginine treatment in room temperature-acclimated rats led to cardiomyocyte hypertrophy which was followed by a simultaneous increase in capillarity and interstitial connective tissue in the myocardium, maintaining the relative ratio of tissue components unaltered. Regardless of the increase in cardiomyocyte size, no statistically significant changes of their volume density or in blood vessels and connective tissue occurred in the myocardium. L-Arginine treatment following cold acclimation acts synergistically with cold by increasing cardiomyocyte size and decreasing myocardial capillarity. Compared to the room temperature-acclimated group treated

Table 2 Hypertrophy of cardiomyocytes after chronic L-arginine and L-NAME treatment in both room temperature-acclimated and cold-acclimated animals. Cardiomyocyte diameters were measured in longitudinal sections at the nuclear level. Values are presented as mean ± SEM. (**) $p < 0.01$ – statistically significant differences *vs.* referent control.

	Room temperature			Cold		
	Control	L-arginine	L-NAME	Control	L-arginine	L-NAME
Cardiomyocyte diameter (μm)	16.08 ± 0.33	17.25 ± 0.30 (**)	17.29 ± 0.28 (**)	16.98 ± 0.37	17.44 ± 0.37	17.27 ± 0.25

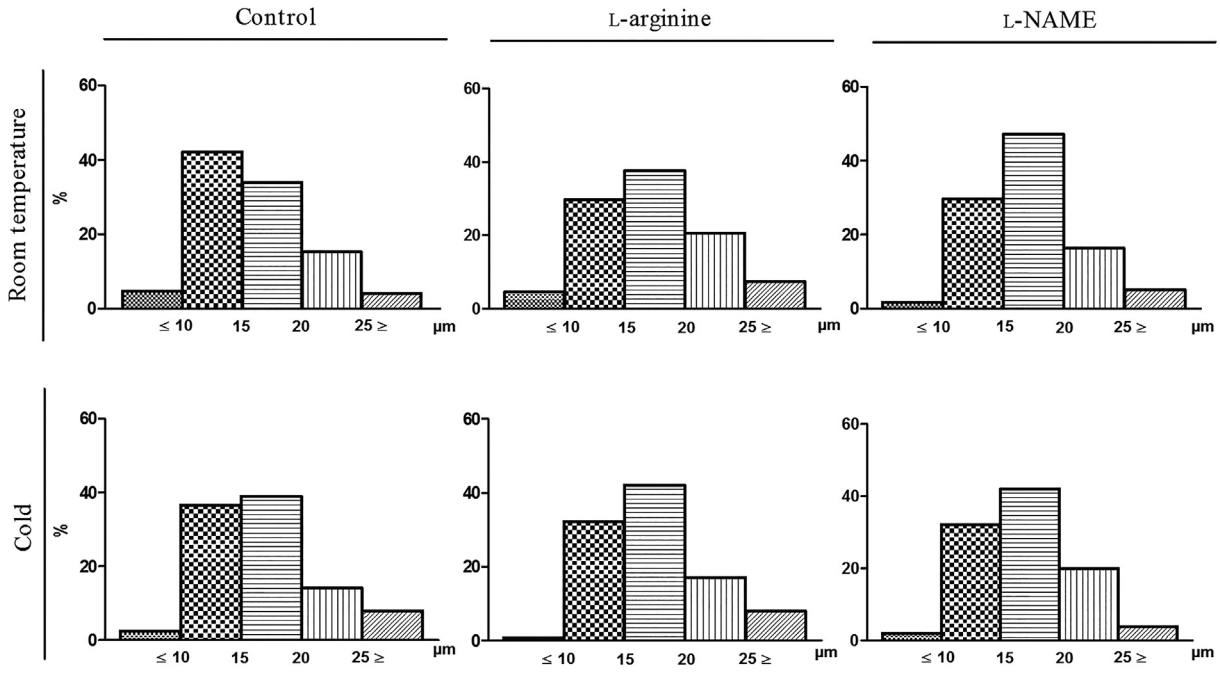

Figure 2 Distribution of cardiomyocyte diameters according to their size. Compared with the room temperature-acclimated group, the percentage of cardiomyocytes with diameters over 15 μm increased in all treated and cold-acclimated groups.

with L-arginine, the volume density was additionally increased and was followed by a decrease in blood vessel volume density. This imbalance between cardiomyocyte enlargement and capillary supply could be considered to be the effect of cold, since a declining trend in myocardial capillarity was observed in all cold-acclimated groups.

Cardiomyocyte hypertrophy (enlargement) occurs *via* intracellular signaling pathways within these cells (Heineke and Molkentin, 2006) and is commonly connected with cardiac hypertrophy, which refers to cardiac thickening and remodeling. This process may be due to cardiac pathology, to long-term exercise training (Pelliccia et al., 2002; Maron et al., 2003) or chronic cold exposure (Cheng and Hauton, 2008). Our results demonstrated that the structural alterations observed corresponded to physiologic cardiac hypertrophy since we were unable to identify any pathological changes in the myocardial structure (no signs of increased collagen deposition or myofibril distortion) after chronic L-arginine treatment in male rats. The observed hypertrophic effect of chronic L-arginine supplementation on cardiomyocytes is inconsistent with previous findings demonstrating the anti-proliferative effects of NO in the heart (Kolpakov et al., 1995). It should be noted that the anti-proliferative effect of NO and therefore of L-arginine, was observed during L-arginine treatment of existing pathological myocardial hypertrophy, a condition that is known to be associated with reduced NO biosynthesis (Pechanova et al., 1999). Our previous studies demonstrated L-arginine-stimulated thermogenic activation in brown adipose tissue and acceleration of cold-induced antioxidative defense in the skeletal muscle of these animals (Petrovic et al., 2005, 2008), suggesting that the observed cardiomyocyte hypertrophy in L-arginine-treated animals is an indirect consequence of increased peripheral circulatory demands. It remains to be elucidated whether L-arginine-induced hypertrophy of cardiomyocytes also includes direct NO-dependent or NO-independent mechanisms.

The most prominent effect of the NOS inhibitor, L-NAME, on rat myocardia observed in this study was fibrosis which was demonstrated as an increase in interstitial connective tissue volume density accompanied by increased collagen abundance in the interstitium. This effect was followed by cardiomyocyte hypertrophy despite a decrease in their volume density. These alterations were observed in both the room temperature- and the cold-acclimated L-NAME-treated groups, compared with the appropriate controls. When compared to the room temperature-acclimated group treated with L-NAME, identical treatment in cold-acclimated animals additionally enhanced interstitial fibrosis.

Pathological cardiac hypertrophy is characterized not only by the growth of myocardial fibers, but also by changes in cardiac architecture and cellular metabolism and, finally, by myocardial dysfunction with an increased morbidity and mortality. Specific genetic expression profiles, different from the adaptive profiles involved in physiological hypertrophy, are activated (McMullen et al., 2003). Pressure or volume overload causes initial hypertrophy, which represents a compensatory mechanism for maintaining cardiac function. If these stimuli persist, structural and functional cardiac anomalies develop. Thus, cardiac sarcomeres become bigger with abnormal proteins, resulting in a bioenergetics deficit that affects their function. The cardiac muscle fibers are disorganized, and separated by an excessive interstitial connective tissue since collagen metabolism is changed, resulting in a decreased degradation and an increased deposition of collagen in the extracellular matrix (Diez et al., 1995; Rossi, 2001). In these initial phases, collagen composition is normal with an enlarged ventricle (Creemers and Pinto, 2011). However, with long-term myocardial remodeling, there is a buildup of fibroblasts and extracellular matrix proteins, causing abnormal structure and function in the heart, resulting in the condition known as myocardial fibrosis (Brown et al., 2005; Krenning et al., 2010) which is closely related to the amount of ventricular hypertrophy (Creemers and Pinto, 2011). Consequently, myocardial fibrosis causes regional myocardial dysfunction (Rajiv et al., 2004).

The mechanisms responsible for myocardial fibrosis in hypertrophy are not fully understood. However, growth factors such as FGF-2, TGFβ, and platelet-derived growth factor, and plasma hormones such as AngII, endothelin-1 and catecholamine, have been found to contribute to the regulation of this process (Creemers and Pinto, 2011). With regard to the effects of chronic L-NAME administration, inhibited NOS activity in the myocardium, brain and kidney, and induced hypertension, hypertrophy and fibrotic remodeling of the left ventricle have been demonstrated, suggesting that chronic L-NAME treatment could be used in a model of pathological cardiac hypertrophy (Simko et al., 2005). Moreover, in various conditions (the absence of coenzyme tetrahydrobiopterin, BH4; L-arginine deficiency; enhanced levels of free radicals, and the presence of different NOS inhibitors, L-NAME) NOS reduces molecular oxygen rather than L-arginine, resulting in the production of superoxide rather than NO, a phenomenon known as "NOS uncoupling" (Münzel et al., 2008). In a positive feedback manner, the activation of superoxide source (uncoupled eNOS) may provoke the formation of superoxide and/or ONOO–, which, in turn, oxidizes BH4 to BH3 radical, and leads to further ROS/RNS formation (Kuzkaya et al., 2003). Consequently, scavenging of endogenous NO by superoxide anions may contribute to the diminished role of NO in the regulation of cardiac hypertrophy.

Myocardial structural alterations such are fibrosis and an increase in collagenous proteins were described after 4 weeks of L-NAME administration (Pechanova et al., 1997, 1999), while extensive areas of fibrosis and myocardial necrosis were observed after 8 weeks of L-NAME administration (Moreno et al., 1995). It should be mentioned that the dose of L-NAME used in our study (approximately 10 mg/kg/day) was four times lower than that in the above-mentioned experiments (40 mg/kg/day), and is considered a low dose which was demonstrated to have no toxic effects (Saha et al., 1996).

In conclusion, chronic L-arginine supplementation induces a low level right ventricular hypertrophy in room temperature-acclimated rats and significantly augments ventricular hypertrophy in cold-acclimated animals. This L-arginine-induced ventricular hypertrophy could be considered physiological, since no signs of myocardial fibrosis were observed. In contrast, chronic treatment with L-NAME, an inhibitor of NOSs, caused pathological right ventricular hypertrophy with signs of myocardial fibrosis, demonstrating the importance of NO depletion in the development of cardiovascular disease.

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