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## L-NAME Treatment Enhances Exercise-induced Content of Myocardial Heat Shock Protein 72 (Hsp72) in Rats

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### Key Words

Hsp72 • Exercise • Nitric oxide synthase • Nitric oxide

### Abstract

**Background/Aim:** Nitric oxide (NO) modulates the expression of the chaperone Hsp72 in the heart, and exercise stimulates both NO production and myocardial Hsp72 expression. The main purpose of the study was to investigate whether NO interferes with an exercise-induced myocardial Hsp72 expression. **Methods:** Male Wistar rats (70-100 days) were divided into control (C, n=12), L-NAME-treated (L, n=12), exercise (E, n=13) and exercise plus L-NAME-treated (EL, n=20) groups. L-NAME was given in drinking water (700 mg·L<sup>-1</sup>) and the exercise was performed on a treadmill (15-25 m·min<sup>-1</sup>, 40-60 min·day<sup>-1</sup>) for seven days. Left ventricle (LV) protein Hsp content, NOS and phosphorylated-NOS (p-NOS) isoforms were measured using Western blotting. The activity of NOS was assayed in LV homogenates by the conversion of [<sup>3</sup>H]L-arginine to [<sup>3</sup>H]L-citrulline. **Results:** Hsp72 content was increased significantly (223%; p<0.05) in the E group compared to the C group, but exercise alone did not alter the NOS content, p-NOS isoforms or NOS activity. Contrary to our expectation, L-NAME

enhanced (p<0.05) the exercise-induced Hsp72 content (EL vs. C, L and E groups = 1019%, 548% and 457%, respectively). Although the EL group had increased stimulatory p-eNOS<sup>Ser1177</sup> (over 200%) and decreased inhibitory p-nNOS<sup>Ser852</sup> (~50%) compared to both the E and L groups (p<0.05), NOS activity was similar in all groups. **Conclusions:** Our results suggest that exercise-induced cardiac Hsp72 expression does not depend on NO. Conversely, the *in vivo* L-NAME treatment enhances exercise-induced Hsp72 production. This effect may be due to an increase in cardiac stress.

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### Introduction

It has been clearly established that exercise training reduces the risk of ischemic cardiac diseases. This cardioprotection seems to depend at least in part on the increased expression of several proteins known as stress proteins which maintain cellular homeostasis under stressful conditions [1, 2]. Overexpression of the heat shock protein-72 (Hsp72), a stress protein, seems to play a critical role for the exercise-induced cardioprotection [1, 2]. In-

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creased body temperature [3], mechanical stress [4], free radicals [5], intracellular  $\text{Ca}^{2+}$  [6] and adrenergic stimulation [7] are hypothesized as putative mechanisms that increase Hsp72 expression during exercise. However, the molecular mechanisms linking exercise to the increased cardiac Hsp72 expression remain unknown.

Malyshev et al. reported that the nitric oxide (NO) is involved in the cardiac Hsp72 expression cascade because its expression was attenuated in presence of NO synthase (NOS) inhibition in rats submitted to heating [8]. Xu et al. also observed that Hsp72 expression is NO-dependent in cultured vascular smooth muscle cells [9]. Similar findings have been observed in intestinal epithelial T84 cells [6]. Direct and indirect effects of NO on the heat shock factor-1 (HSF1) have been speculated to be the causal mechanism of the NO-induced Hsp72 overexpression [6, 8, 9].

NO is a free radical with a half-life of few seconds. It is produced in almost all cardiac cells. This small and highly diffusible molecule acts as an important intra- and intercellular messenger by modulating the activation of protein kinases A and C [10, 11], which are also involved in Hsp72 expression [12-14]. Aside from increasing body temperature, exercise elevates myocardial NO production by increasing NOS expression [15, 16] and shear stress-induced NO production in the coronary vessels [17]. Since exercise represents a stress condition to the heart, it is conceivable that the increased NO production may also contribute to stimulate Hsp72 production during exercise.

In a previous study [18], however, NOS inhibition with  $\text{N}^{\text{o}}$ -nitro-L-arginine methyl ester (L-NAME) did not prevent the exercise-induced Hsp72 expression in the cardiac muscle of rats. However, in this study, Hsp72 expression was measured in rats submitted to the *in vitro* ischemia-reperfusion which can also influence *per se* Hsp72 expression. Moreover, NOS activity and expression were not evaluated in this study. Because *in vivo* L-NAME treatment may induce compensatory NOS-derived NO in the rodent heart [19-21], it is essential to measure either cardiac NOS expression as activity to an adequate evaluation of the potential participation of NO in exercise-induced cardiac Hsp72 expression.

Therefore, our aim was to investigate the possible participation of NO for the exercise-induced myocardial Hsp72 content in the rat. Contrary to our expectation, our results showed that, although the L-NAME treatment does not alter left ventricle (LV) NOS activity, it enhanced the exercise-induced Hsp72 content in the rat LV.

## Materials and Methods

### *Ethical approval*

All animal experiments were approved by the Institutional Ethics Committee at the Federal University of Espírito Santo, Brazil (protocol 003/2009).

### *Groups and treatment*

Male Wistar rats (70 to 100 days old) were used in this study. The animals were housed in collective cages ( $n = 4-5$  per cage) to prevent the well-known stressful situation induced by socially isolated rats [22]. The animals were maintained in a temperature-controlled room (22-25°C) under a 12:12-h light:dark cycle with free access to water and rat chow. One week before experimentation, all animals were subjected to a treadmill exercise test (15  $\text{m}\cdot\text{min}^{-1}$ , 10 min, 0% grade) to evaluate running ability. The rats that completed this preliminary exercise session were randomly divided into four experimental groups: control (C;  $n = 12$ ), L-NAME (L;  $n = 12$ ), exercise (E;  $n = 13$ ) and exercise plus L-NAME (EL;  $n = 20$ ). This selection strategy is necessary because rats with different intrinsic aerobic exercise capacity show unequal cardiomyocyte properties [23]. The L and EL groups received L-NAME continuously (Sigma Aldrich, St. Louis, MO, USA) dissolved in drinking water (700  $\text{mg}\cdot\text{L}^{-1}$ ) for seven days. This L-NAME concentration was similar to that used in other studies [24, 25]. Water intake was measured daily by dividing the total consumption by the number of rats in each cage. Animals from the E and EL groups were simultaneously subjected to a short-term treadmill running protocol for seven days. During the first four days these animals ran daily for 60, 60, 40 and 60  $\text{min}\cdot\text{set}^{-1}$  at treadmill speeds of 15, 18, 20 and 20  $\text{m}\cdot\text{min}^{-1}$ , respectively, 0% grade, to adapt to the running protocol. For the following three days, animals ran daily for 40, 60 and 40  $\text{min}\cdot\text{set}^{-1}$  at speeds of 22.5, 22.5 and 25  $\text{m}\cdot\text{min}^{-1}$ , respectively, 0% grade. The C and L groups remained sedentary during the experimental period.

### *Haemodynamic measurements*

Seven days after L-NAME treatment and 24 h after the final exercise session, the animals were anesthetized with ketamine plus xylazine (90  $\text{mg}\cdot\text{kg}^{-1}$  and 10  $\text{mg}\cdot\text{kg}^{-1}$ , respectively, i.p.), and a fluid-filled polyethylene catheter (PE-50, 10-15 cm) was introduced in the right carotid artery to assess arterial pressure during spontaneous breathing. After a stabilization period, the catheter was inserted into the LV cavity to record the LV systolic and end-diastolic pressures and their first positive and negative maximal time derivatives ( $+\text{dP}/\text{dt}$  and  $-\text{dP}/\text{dt}$ ). Arterial and ventricular pressures were recorded continuously for at least 20 min using a pressure transducer (TRA021, LSI, Letica Scientific Instruments, Spain) connected to a data acquisition system (Powerlab 4sp, sp-4922, ADInstruments, Australia). Haemodynamic parameters from each rat were calculated as mean values from each 20-min record under stable conditions (Chart 5 PRO software, ADInstruments, Australia). The animals were then euthanized and the heart and right tibia were removed. The LV and right ventricle (RV) were weighed and its fragments were stored at -80°C. The length of the right tibia

(mm) was measured with a paquimeter and later it was used to obtain ventricles weight index (ventricle weight/right tibia length).

#### *Western blot procedures to assess total NOS, phosphorylated-NOS (p-NOS), Hsp72 and Hsp73 content*

Western blots for eNOS, nNOS, iNOS and p-NOS (p-eNOS<sup>Thr495</sup> and p-eNOS<sup>Ser1177</sup>; p-nNOS<sup>Ser852</sup>) were performed as previously described [26]. Briefly, LV fragments were lysed at 4°C with lysis buffer solution (in mmol/L: 150 NaCl, 50 Tris-HCl, 5 EDTA.2Na, and 1 MgCl<sub>2</sub>) containing 1% Triton X-100, 0.5% SDS plus a cocktail of protease inhibitors (SigmaFAST). Phosphatase inhibitors were used (NaF 20 mM; Na<sub>3</sub>VO<sub>4</sub> 0.1 mM) in the determination of phosphorylated proteins. The lysate was centrifuged, and the protein concentration in the supernatant was determined spectrophotometrically. Thirty µg were separated using a denaturing SDS polyacrylamide gel (7.5 or 15%) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Blots were blocked with 5% non-fat dry milk in PBS and 0.1% Tween 20 (PBS-T) at room temperature. The following antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used: rabbit polyclonal anti-eNOS (1:1,000), rabbit polyclonal anti-nNOS (1:1,000), rabbit polyclonal anti-iNOS (1:1,000), goat polyclonal anti-p-eNOS<sup>Ser1177</sup> (1:1,000), goat polyclonal anti-p-eNOS<sup>Thr495</sup> (1:1,000), goat polyclonal anti-p-nNOS<sup>Ser852</sup> (1:1,000) and mouse monoclonal anti-β-actin (1:3,000). Immunocomplexes were detected using a chemiluminescent reaction (ECL kit; Amersham, Les Ulis, France) followed by densitometric analyses with the ImageQuant software package. β-Actin content was used to normalize total NOS protein content.

For Hsp72 and Hsp73 western blotting, LV samples were pulverized in liquid nitrogen using a mortar (Fisher Scientific, USA) and then homogenized in ice-cold buffer (600 mM NaCl, 15 mM Tris-HCl, 1 mM PMSF, pH 7.5) as previously described [27]. After 5 min on ice, homogenized tissues were centrifuged (14,000 g, 20 min, 4°C), and the protein concentration in the supernatant was determined using Bradford reagent (Bio-Rad, USA). Protein extracts (75 µg) were electrophoretically separated by 7.5% SDS-PAGE for ~20 h at 40 V in running buffer (25 mM Tris, 190 mM glycine, 0.05% SDS). Proteins were then transferred to PVDF membranes (Amersham, USA) using a Trans-Blot cell system (Bio-Rad, USA) in buffer (25 mM Tris, 190 mM glycine, 20% methanol, 0.05% SDS). Membranes were blocked with 5% non-fat dry milk and then incubated in monoclonal anti-HSP 70 (1:1,000, Sigma-Aldrich), which recognizes both Hsp72 and Hsp73, for ~12 h at 4°C. After washing (10 mM Tris, 100 mM NaCl, 0.1% Tween 20), the membranes were incubated in peroxidase-conjugated goat anti-mouse IgG (1:2,000, Bio-Rad, USA). Immunocomplexes were detected using an enhanced horseradish peroxidase-luminol chemiluminescence system (ECLPlus, Amersham) and subjected to autoradiography (Hyperfilm ECL, Amersham). Signals on immunoblots were quantified using Scion Image software. Because stress events only produce a slight increase in Hsp73 expression that quickly (~4 h) returns to baseline levels [4, 5], total Hsp73 content was used as an internal control for normalizing Hsp72 content, as previously published [4]. A positive control

for cardiac Hsp72 was obtained using heart tissue from an unstressed adult rat that was anesthetized with ketamine plus xylazine (90 mg·kg<sup>-1</sup> and 10 mg·kg<sup>-1</sup>, i.p.) and warmed to 42 ± 0.4°C (15 min), as previously described [2].

#### *NOS activity*

NOS activity was measured using the [<sup>3</sup>H]L-citrulline assay method [28] with slight modifications. LV tissues were suspended in hydroxyethylpiperazine ethanesulphonic acid (HEPES) buffer (in mM: HEPES 20, sucrose 320, DTT 1, EDTA 1, PMSF 1) and lysed with a sonifier (Thorton). The homogenates were centrifuged at 500 g for 10 min at 4°C. Aliquots from the supernatant were incubated in HEPES buffer (30 mM, pH 7.4) containing 1 mM EDTA, 1.25 mM CaCl<sub>2</sub>, 4 µM FAD, 4 µM FMN, 25 µM 5,6,7,8-tetrahydrobiopterin, 1 mM NADPH, 10 µg·ml<sup>-1</sup> calmodulin, 120 nM L-arginine and 0.5 µCi [<sup>3</sup>H]L-arginine for 60 min at 37°C in a total volume of 200 µl. The reactions were stopped by adding 1 ml of a solution containing 20 mM HEPES, pH 5.5. These samples were passed through a 0.3-ml DOWEX 50WX8-400 (Na<sup>+</sup> form) column, and [<sup>3</sup>H]L-citrulline was eluted with 1 ml HEPES buffer (20 mM, pH 5.5). Citrulline, ionically neutral at pH 5.5, passes through the column completely. The radioactivity corresponding to [<sup>3</sup>H]L-citrulline in the eluate was measured by liquid scintillation counting. Protein concentration was determined by the Bio-Rad protein assay.

#### *Statistical analysis*

Data are presented as means ± SEM. Two-way ANOVA and Tukey's *post hoc* test were used to assess the interaction between exercise and L-NAME intervention. The Pearson correlation coefficient (r) was used to determine the association between Hsp72 content and the L-NAME-induced increase in blood pressure. Statistical significance was set at 5%.

## **Results**

Water intake was higher ( $p < 0.05$ ) in the C and E groups (C = 44±1 vs. E = 43±1 mL/day;  $p > 0.05$ ) than in the L-NAME-treated groups (L = 34±1 vs. EL = 34±1 mL/day). The L-NAME dose was similar ( $p > 0.05$ ) in the two treated groups (around 24 mg/day). L-NAME treatment reduced the animals' ability to follow the running protocol used in our study. Five animals (25%) were removed from the EL group because they were unable to complete the running protocol. Another five rats in the EL group died during the experimental period. Death occurred at night, between the fourth and sixth days of the running protocol. As 20 rats were included in the EL group, the results refer to the 10 rats that completed the exercise protocol. Two animals (15%) were also removed from the E group because they did not tolerate the progressive exercise protocol. No dropping out occurred in the C and L groups.

Group	Control (C; n: 12)	L-NAME (L; n: 12)	Exercise (E; n: 11)	Exercise plus L-NAME (EL; n: 10)	Interaction exercise vs. L-NAME ‡
Initial BW (g)	338±8	348±8	338±6	338±9	<i>p</i> = 0.77
Final BW (g)	369±11	359±11	357±11	346±12	<i>p</i> = 0.94
TL (cm)	3.56±0.03	3.52±0.03	3.53±0.03	3.53±0.03	<i>p</i> = 0.37
LVW (g)	0.74±0.02	0.76±0.02	0.75±0.03	0.76±0.02	<i>p</i> = 0.42
RVW (g)	0.20±0.01	0.22±0.01	0.22±0.01	0.21±0.01	<i>p</i> = 0.06
LVW/TL (g.cm <sup>-1</sup> )	0.211±0.007	0.216±0.006	0.216±0.006	0.209±0.006	<i>p</i> = 0.35
RVW/TL (g.cm <sup>-1</sup> )	0.059±0.003	0.063±0.003	0.063±0.003	0.057±0.003	<i>p</i> = 0.06

**Table 1.** Body weights, visceral weights and tibia lengths in the four experimental groups. BW=body weight, LVW=left ventricle weight; RVW=right ventricle weight; TL=right tibia length. Data are shown as mean±SEM, and only animals that completed the experimental protocol were included. ‡, two-way ANOVA.

Group	Control (C; n: 12)	L-NAME (L; n: 12)	Exercise (E; n: 11)	Exercise plus L-NAME (EL; n: 10)	Interaction exercise vs. L-NAME ‡
SAP (mmHg)	131±5	157±4*	130±5	122±6	<i>p</i> = 0.01
DAP (mmHg)	101±4	124±4*	98±4	94±4	<i>p</i> = 0.01
MAP (mmHg)	112±4	135±5*	109±4	103±4	<i>p</i> = 0.01
HR (bpm)	247±10	237±4	215±10	236±11	<i>p</i> = 0.16
LVSP (mmHg)	133±5	150±5*	135±5	131±5	<i>p</i> = 0.04
EDP (mmHg)	9±1	10±1	10±1	7±1	<i>p</i> = 0.08
+dP/dt (mmHg/s)	5406±285	5482±301	4876±272	5305±301	<i>p</i> = 0.55
-dP/dt (mmHg/s)	4248±265	4240±280	3770±253	4129±278	<i>p</i> = 0.50

**Table 2.** Haemodynamic data for the experimental groups. SAP=systolic arterial pressure; DAP=diastolic arterial pressure; MAP = mean arterial pressure; HR=heart rate; LVSP=left ventricular peak systolic pressure; EDP=end-diastolic pressure; and dP/dt=1st derivative of left ventricular pressure. Data are presented as means ± SEM; \* *p* < 0.05 vs. C, E and EL groups. ‡, two-way ANOVA followed by Tukey's post hoc test.

#### Body and ventricular weights

Table 1 shows body and ventricular weights and ventricle weights normalized to tibia length. No significant differences were found in these parameters.

#### Blood pressure and LV function

Table 2 shows the blood pressure and LV function data. The L group exhibited a higher blood pressure and higher LV peak systolic pressure (LVSP) compared to the C and E groups. In the EL group, we observed that exercise prevented the elevation in blood pressure and LVSP induced by L-NAME. End-diastolic pressure, heart rate, +dP/dt, and -dP/dt were similar in all four groups.

#### NOS and p-NOS content by Western blot

Fig. 1 and 2 show the content of NOS isoforms and p-NOS isoforms, respectively. Neither exercise nor L-NAME alone changed total eNOS or iNOS content. The L group exhibited increased total nNOS content. The eNOS phosphorylated at the Thr495 inhibition site was not changed in any group, while the eNOS phosphorylated at the Ser1177 stimulatory site was increased in the EL group. The nNOS phosphorylated at the Ser852 inhibition site was increased in the L group and decreased in the EL group.

#### Total NOS activity

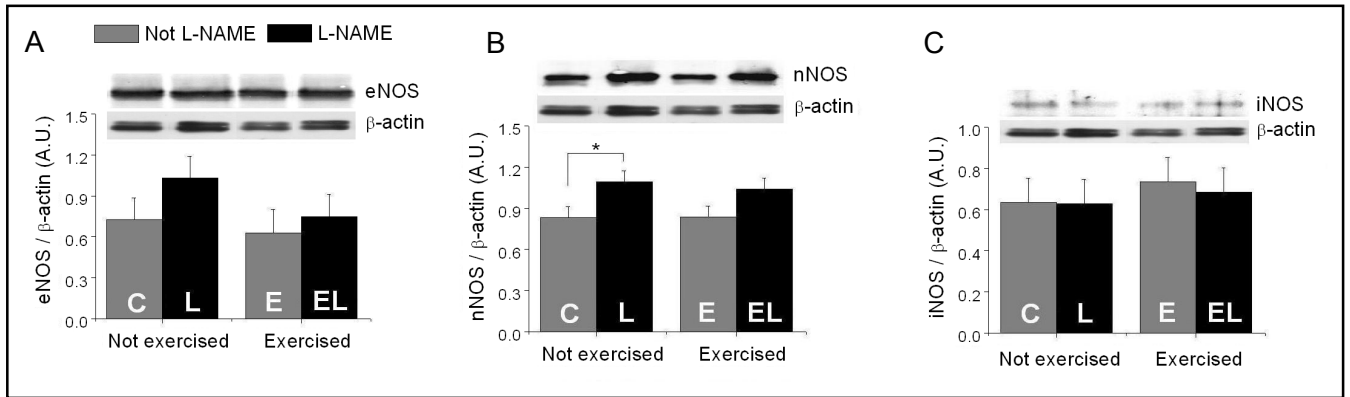
Total NOS activity obtained by measuring the conversion of [<sup>3</sup>H]L-arginine to [<sup>3</sup>H]L-citrulline was similar in the four groups (Fig. 3).

#### Heat shock proteins content

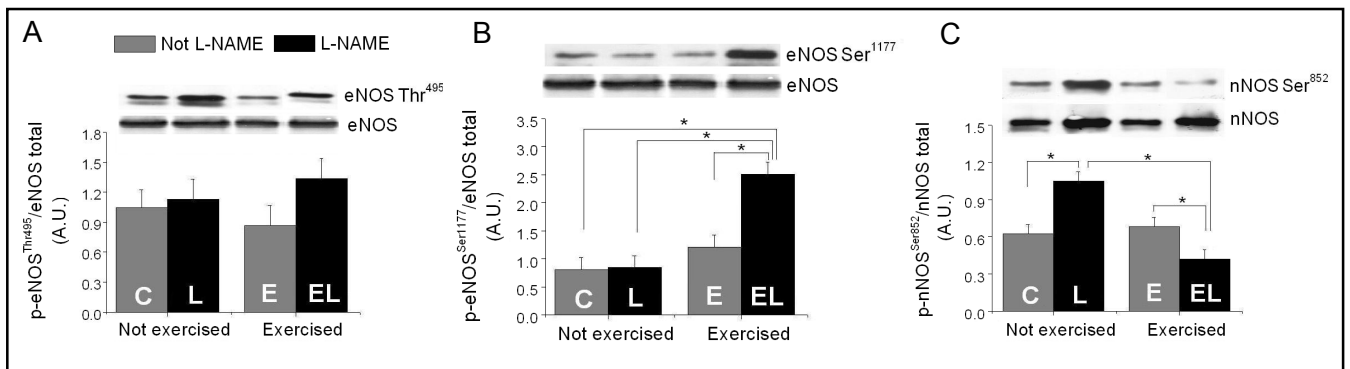
Fig. 4 depicts Hsp72 content and the Hsp72/Hsp73 content ratio. As expected, the constitutive content of cardiac Hsp73 (in arbitrary units – AU) was similar in all groups (C = 9,838±776, L = 9,635±455, E = 9,13±449 and EL = 9,765±413). As expected, cardiac Hsp72 and the Hsp72/Hsp73 content ratio were higher in E group than in the C group. An interaction was observed in the exercised group subjected to L-NAME treatment (EL group). Namely, Hsp72 content in the EL group was increased almost fourfold over its respective control group (E group). No correlation between Hsp72 content and systolic pressure (*r* = -0.13; *p* = 0.6), diastolic pressure (*r* = -0.05; *p* = 0.8) or LVSP (*r* = -0.02; *p* = 0.9) was found.

## Discussion

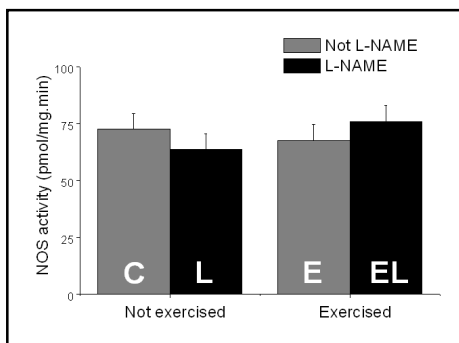
The hypothesis tested in this study was that NO influences the exercise-induced increase in the content of myocardial Hsp72. Contrary to our initial expectations,



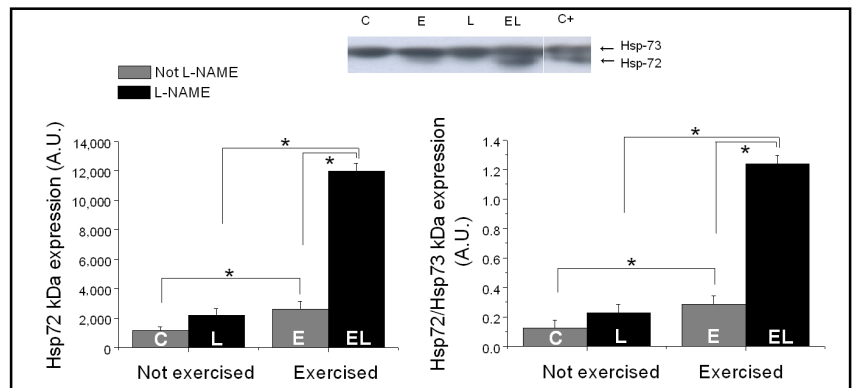
**Fig. 1.** Nitric oxide synthase (NOS) content in the left ventricle of rats in the control group (C=not exercised and not treated with L-NAME), L-NAME-treated group (L group), untreated exercised group (E group) and L-NAME-treated exercised (EL group). A.U.=arbitrary units. \*,  $p < 0.05$  between groups depicted by a horizontal line. Data are presented as mean $\pm$ SEM; two-way ANOVA followed by Tukey's post hoc test. The number of samples studied was 5 or 6 per group.



**Fig. 2.** Content of phosphorylated nitric-oxide synthase (p-NOS) in the left ventricle of rats in the control group (C=not exercised and not treated with L-NAME), L-NAME-treated group (L group), and untreated exercised group (E group) and L-NAME-treated exercised (EL group). A.U.=arbitrary units. \*,  $p < 0.05$  between groups depicted by a horizontal line. Only statistically relevant differences to the study purpose are depicted in panels. Data are presented as mean $\pm$ SEM; two-way ANOVA followed by Tukey's post hoc test. The number of samples studied was 5 to 6 per group.



**Fig. 3.** Conversion of [ $^3$ H]L-arginine to [ $^3$ H]L-citrulline labelled in the left ventricles of rats. Groups are the same as in Fig. 1. Data are presented as mean $\pm$ SEM.  $p > 0.05$ , two-way ANOVA. The number of samples studied was 6 per group.



**Fig. 4.** Content of Hsp72 and Hsp72/Hsp73 in the left ventricles of rats. Groups are the same as Fig. 1. A.U. = arbitrary units; C+ = positive control. \*,  $p < 0.05$  between groups discriminated by a horizontal line, two-way ANOVA followed by Tukey's post hoc test. The number of samples studied was 6 per group.

the use of NOS inhibitor in exercised rats for seven days produced a substantial increase in cardiac Hsp72 content. The rat model was used in our experiments because

the exercise effects on Hsp72 induction in well known in this rodent model [8, 14, 16, 18].

The rationale for our original hypothesis came from

a previous study showing that NO enhances heat shock-induced expression of Hsp72 in the heart [8]. In vitro studies confirmed the importance of the NO in heat shock-induced expression of Hsp72 [6, 9]. Exercise may also induce cardiac Hsp72 expression by increasing body temperature [3]. However, other pathways may influence induction of this stress protein during exercise. Since exercise stimulates cardiac NOS bioavailability [17] and expression [15, 16] the participation of NO on the Hsp70 induction during exercise has been proposed [12, 13].

In the present study, total and phosphorylated NOS and in vitro NOS activity were measured to investigate the direct participation of NO in the exercise-induced expression of Hsp72 in the rat heart. Exercise alone did not alter cardiac NOS or p-NOS content, possibly because we used a short-term exercise protocol, whereas long-term exercise (4 to 10 weeks) increases NOS and p-NOS expression in the rat myocardium [15, 16]. Regardless, our results have shown that exercise-induced content of cardiac Hsp72 increased in rats under inhibition of NOS. Therefore, expression of this heat shock protein due to exercise seems not to depend on NO.

The in situ regulation of NOS expression and activity seems to be complex. For example, cardiac or vascular eNOS activity may involve multi-site phosphorylation of serine/threonine residues [29, 30]. The induction of stimulatory p-NOS may be counterbalanced by increasing inhibitory p-NOS [31]. These results suggest that the regulation of NOS-derived NO is complex because increased NOS expression does not necessarily mean increased NOS activity, what may explain some of our findings. For example, the inhibitory phosphorylation site Ser852 was increased in the L group. However, total nNOS content was also increased in this group, possibly to compensate for the increased inhibition of nNOS activity. Indeed, the L group had a NOS activity that was similar to that in the other groups.

It is evident that acute L-NAME administration inhibits cardiac NOS activity [8, 32, 33], and chronic L-NAME treatment increases blood pressure [24]. However, several studies have shown that rodents treated with different L-NAME doses (from 1 to 50 mg·kg<sup>-1</sup>·day<sup>-1</sup>) and durations (2 to 60 days) may exhibit compensatory eNOS [19], nNOS [20], iNOS [20, 25] and total NOS [34] expressions and activities in different tissues [20, 25], including the myocardium [19-21]. Together, these results suggest that L-NAME treatment for days or weeks may increase NOS expression and activity as a compensatory mechanism.

However, in the EL group, inhibitory p-nNOS<sup>Ser852</sup> was decreased, while stimulatory phosphorylation site Ser1177 on eNOS was increased. These results suggest that NO production would be increased in the EL group. However, cardiac NOS activity, as measured through a more sensitive and specific method that converts radiolabeled L-arginine to radiolabeled L-citrulline [35], was similar in all the groups. Thus, it is possible that other serine/threonine phosphorylation sites that were not assessed in the present study are involved in steady-state NO production. We could not find studies investigating the effects of exercise and L-NAME on cardiac p-eNOS<sup>Ser1177</sup> or p-nNOS<sup>Ser852</sup>. Thus, further investigation is necessary to explain this interaction.

Because cardiac NO production in the four groups was apparently similar, the direct participation of NO in exercise-induced cardiac Hsp72 content is unlikely. Why L-NAME treatment enhances exercise-dependent cardiac Hsp72 content is now a new and emerging question.

Increased cardiac afterload secondary to L-NAME treatment did not appear to contribute to Hsp72 content, because we did not find any correlation between Hsp72 content and arterial or ventricular pressure. Furthermore, the EL group exhibited lower blood and ventricular pressures than the L-NAME group. It has been suggested that exercise prevents L-NAME-induced hypertension [36]; however, the mechanisms responsible for this response of the exercise need further study.

Given our results, myocardial Hsp72 elevated content induced by exercise and L-NAME cannot be explained by the same mechanism as myocardial Hsp72 content induced by exercise alone. First, Hsp72 content was much higher in the EL group. Second, cardiac NO production was similar in all groups. Third, the frequency of rats that did not complete the exercise protocol was higher in the EL group, suggesting more physical effort and, consequently, earlier exhaustion. Fourth, animal deaths only occurred in the EL group, suggesting the existence of pathological stress.

Thus, it is likely that cardiac Hsp72 elevated content in rats simultaneously exercised and treated with L-NAME is a response to pathological myocardial stress. At least two mechanisms can be postulated to explain this phenomenon: 1) ischemia-induced cardiac injury and 2) increased production of free radicals.

L-NAME treatment may induce a reduction in coronary blood flow [18, 33] and subendocardial ischemia, resulting in focal fibrosis and necrosis [19, 37]. Hypoxia induces cardiac Hsp72 expression [38] as a mechanism for preventing apoptosis [39]. It is possible that increased

exercise-induced myocardial oxygen demand [17] exacerbates the L-NAME-dependent ischemic effect. Indeed, rats exercised for 8 weeks and treated with L-NAME during the last 7 days of exercise days exhibited higher cardiac fibrosis compared to rats subjected to L-NAME treatment only [40].

Regarding free radicals, it is known that free radicals induce Hsp72 [38, 41], and exercise may increase free radicals in the myocardium [42]. It is also now known that NOS produces superoxide anions ( $O_2^-$ ) [43]. This phenomenon, known as “NOS uncoupling”, increases the levels of the potent oxidant peroxynitrite (ONOO<sup>-</sup>), which induces protein nitration, DNA damage and cell death [42]. In fact, it has been suggested that NO-induced protein damage is the cause of heat shock factor activation [9]. Exercise-induced oxidative stress could generate ONOO<sup>-</sup> through a direct reaction between mitochondrial  $O_2^-$  and NO. Recently, Waard et al. showed that exercise and elevated cardiac eNOS expression separately attenuate LV dysfunction after myocardial infarction in mice [44]. However, the combination of exercise and eNOS overexpression abolished the beneficial effects of each individual treatment, and this detrimental effect was due to increased oxidative stress secondary to eNOS uncoupling.

Although our results do not confirm the direct participation of NO in cardiac Hsp72 expression, an indirect contribution through ONOO<sup>-</sup> cannot be discounted. Accordingly, increased production of ONOO<sup>-</sup> could explain the suggested NO-dependent Hsp72 overexpression observed in other studies [6, 8, 9]. However, this hypothesis needs to be addressed in further studies.

Ischemia-induced cardiac injury and/or increased production of free radicals could also be responsible for the high mortality of the rats subjected to exercise and simultaneously treated with L-NAME. Such a phenomenon

would characterize an important aspect of exercise intolerance.

Finally, we have to emphasize two study limitations. The first is related to the L-NAME dose, since the animals were maintained in collective cages to reduce psychological stress [22], the L-NAME dose was estimated according to daily water consumption. However, the estimated L-NAME dose was close to values used in other studies [19, 20, 24]. A second limitation was the mortality in animals subjected to exercise under L-NAME treatment, showing the importance of NO to acute exercise adaptations. Mortality occurred out of the exercise training session. The mechanisms leading animals to death deserves more detailed studies because our results may represent only the effects in rats surviving to the combination of aerobic exercise under L-NAME effect.

In summary, our results showed that short-term L-NAME treatment alone seems not to affect the myocardial Hsp72 expression in rats. When L-NAME is used concomitantly with aerobic exercise, it enhances the myocardial content of this stress protein probably because the exercise in presence of reduced NO production may represent a greater stress to myocardium, including development of pathological stress. Because exercise alone increased cardiac Hsp72 content and did not change cardiac NOS content or cardiac NO production, the exercise-induced Hsp72 expression in the heart seems to be unrelated to the direct participation of NO.

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