

Allopurinol attenuates L-NAME induced cardiomyopathy comparable to blockade of angiotensin receptor

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Summary. It is widely recognized that L-NAME exposed rats develop myocardial fibrosis and hypertrophy. The aim of this study was to evaluate the contribution of xanthine oxidase (XO) to these phenomena using allopurinol, isolated or associated with olmesartan. Thirty adult male Wistar rats were divided into 5 groups (n=6) and studied for 5 weeks: L group (L-NAME, 40mg/kg/day); L+A group (L-NAME and allopurinol, 40 mg/kg/day); L+O group (L-NAME and olmesartan, 15mg/kg/day); L+A+O group (L-NAME, allopurinol, and olmesartan); and control group. L-NAME caused arterial hypertension and cardiomyocyte hypertrophy. Hypertension was prevented by olmesartan, but not by allopurinol. There was an increase of left ventricular mass index in the L-NAME group that was prevented by allopurinol, olmesartan and by the combination of both. The increase in mean cardiomyocyte transversal area caused by L-NAME was prevented by the allopurinol and olmesartan combination, or by olmesartan used as monotherapy, but not by allopurinol alone. There was a reduction in the myocardial vascularization index caused by L-NAME which was abolished by allopurinol or by olmesartan, but not by the association. L-NAME caused a reduction in the total number of cardiomyocyte nuclei. This was prevented by olmesartan alone or associated with allopurinol, but not by allopurinol alone. We conclude that XO has an important contribution to adverse cardiac remodeling in L-NAME exposed animals. Moreover, allopurinol acts without interfering with L-NAME induced hypertension. The protective action of this drug is comparable to the results obtained with olmesartan. Antioxidative mechanisms are proposed to account for

the pressure independent effects of allopurinol.

Key words: L-NAME, Allopurinol, Olmesartan, Arterial hypertension, Cardiac remodeling

Introduction

Nitric oxide (NO) plays a role in a broad range of physiological processes and participates in the pathophysiology of cardiovascular diseases, modulating vascular tonus and reactivity (Moncada and Higgs, 1993). The inhibition of the NO synthase enzyme by N ω -nitro-L-arginine methyl ester (L-NAME) is associated with *in situ* activation of renin-angiotensin-aldosterone system (RAAS) (Usui et al., 2000) and with the activation of enzymatic systems related to the production of reactive oxygen species (ROS) in the cardiovascular tissues (Niu et al., 1994).

Anti-hypertensive drugs have been associated with heart tissue preservation in L-NAME experimental model. Our group (Pereira et al., 2004) and others (Sanada et al., 2001) previously described the results obtained with drugs that act on RAAS. Olmesartan medoxomil is the latest angiotensin II (ATI) receptor blocker approved for clinical use (Brousil and Burke, 2003). This drug ameliorates adverse cardiovascular remodeling, as verified in the kidney and the heart of genetic models of hypertension (Izuhara et al., 2005; Yokoyama et al., 2005).

Allopurinol is a xanthine oxidase (XO) inhibitor, largely employed for management of gout patients, based on its long described uric acid reducing properties (Neumann, 2003). However, recent studies prompted new interest in this drug, due to reports of antioxidant and cardiovascular protective effects (Mellin et al., 2005; George et al., 2006).

Considering that L-NAME determines metabolic

and histological alterations in myocardial tissue (Pereira and Mandarim-de-Lacerda, 1999), the objective of this study was to evaluate the effect of allopurinol on L-NAME induced cardiomyopathy. Accordingly, we determined the degree of structural modification occurring when both XO and NOS are inhibited. We were able to identify the magnitude of the effects obtained with allopurinol and olmesartan, either isolated or in association. We used light microscopy and stereological techniques in order to quantify multiple heart structural parameters of hypertensive rats.

Materials and methods

We studied male Wistar rats aged 16 weeks obtained from colonies maintained in the State University of Rio de Janeiro. The animals were individually housed in a temperature ($21 \pm 2^\circ\text{C}$) and humidity-controlled ($60 \pm 10^\circ\text{C}$) room, submitted to 12-h dark/light cycle (artificial lights, 7:00 p.m. to 7:00 a.m.) and air exhaustion cycle (15 min/h). This investigation was approved by the Ethics Committee for Animal Experimentation at the State University of Rio de Janeiro in conformity with the "Guide for the care and Use of Laboratory Animals", published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

The rats were randomly divided into five groups ($n=6$ each group) and studied for five weeks. All drugs were dissolved in drinking water and the control rats (C group) were given only food (Nuvilab, Nuvital, PR, Brazil) and water *ad libitum*. L group received L-NAME 40 mg/kg/day (hydrochloride of N ω -nitro-L-arginine methyl ester – Sigma-Aldrich Co., St. Louis, USA), L+A group received L-NAME plus allopurinol 40 mg/kg/day (Sigma-Aldrich Co., St. Louis, USA), L+O group received L-NAME plus olmesartan medoxomil 15 mg/kg/day (Sankyo Pharma, SP, Brasil) and L+A+O group received L-NAME plus allopurinol and olmesartan.

Animals were weekly weighed and the systolic blood pressure (BP) was verified by the non-invasive method of tail-cuff plethysmography (Letica LE 5100, Panlab, Barcelona, Spain) in conscious rats. At the end of the experiment, animals were deeply anaesthetized (intraperitoneal sodium pentobarbital), the thorax was opened and blood samples were taken from the right atrium for biochemical analysis. Sodium and potassium were measured by means of a selective electrode (AVL; Roche Diagnostics, Basel, Switzerland), creatinine was evaluated by the Jaffe method of kinetic determination and uric acid was assayed by the enzymatic method (Weiner Lab, Rosario, Argentine).

The left ventricle (LV, including the interventricular septum) was separated from the entire heart and measured (liquid displacement in which the volume was recorded by weight, W) (Scherle, 1970). As the isotonic saline specific gravity (σ) is of 1.0048, the respective volume was obtained by $V[\text{LV}] = W[\text{LV}] / \sigma$ or simply $V[\text{LV}] (\text{cm}^3) = W[\text{LV}] (\text{g})$ (Weibel, 1979). LV mass

index was determined as LV mass/body mass (mg/g).

Light microscopy and stereology

LV fragments were immersed in fixative solution (1.27 mol/l formaldehyde in 0.1 M phosphate buffer, pH 7.2) for 48h, embedded in Paraplast plus (Sigma-Aldrich Co., St. Louis, USA), and sectioned at 2 and 5 μm thick. Slices were stained with hematoxylin and eosin and Masson trichrome.

The myocardium was stereologically analyzed considering the cardiomyocytes (cmy), the connective tissue (ct) and the intramyocardial vessels (ve). Briefly, the cmy mean cross-sectional area was estimated as $A[\text{cmy}] = Vv[\text{cmy}] / 2 \cdot Q_A[\text{cmy}] (\mu\text{m}^2)$, where $Q_A[\text{cmy}] = N[\text{cmy}] / A_T (1/\text{mm}^2)$, N is the number of cmy profiles counted in the test-area, the frame AT. The volume densities were estimated as $Vv[\text{structure}] = Pp[\text{structure}] / PT$, where Pp is the number of points that hit the structure and PT is the total number of test-points inside the test-system (Mandarim-de-Lacerda, 2003).

To estimate the number of cardiomyocyte nuclei (cmyn), we used the disector method, a three-dimensional probe that samples structures proportional to their number regardless of their size or shape. Briefly, two surface planes of a section are used to create a sampling volume with an upper reference plan view containing a test frame (optical *disector*) (Sterio, 1984). For each frame, the thickness of the section was measured by focusing on the upper and lower section planes, verified objectively by employing an auto-focus device and read-out using a microscope equipped with a z-axis motorized focus controller microcator with a resolution of 0.1 μm . Light microscopy was performed using a Leica DMRBE microscope (Wetzlar, Germany with 100x planachromatic immersion oil objective, NA = 1.25), a Kappa videocamera (Gleichen, Germany) and a Sony Trinitron monitor (Pencoed, UK).

The numerical density (Nv) of cmyn (number of cardiomyocyte nuclei per mm^3) was determined by analyzing ten random disector pairs for each rat. This sampling design was based on a pilot experiment to determine inter-animal variability. Estimates of relative variance ($= \text{variance} / \text{mean}^2 = [\text{coefficient of variation}]^2$) of around 10% was considered acceptable (Gundersen and Osterby, 1981).

$$Nv_{[\text{cmyn}]} = Q_{\text{cmyn}}^- / t \cdot A_T$$

The total number of cardiomyocyte nuclei ($N[\text{cmyn}]$) was then estimated as the product of $Nv[\text{cmyn}]$ and LV volume. In all analyses, the observer was blinded to the group studied.

Data analysis

The data showing normal distribution and equal variances (Barlett's test) were, therefore, tested with one-way ANOVA and the post-hoc test of Newman-Keuls, otherwise with nonparametric Kruskal-Wallis

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ANOVA and the post-hoc test of Dunn (GraphPad Prism version 5, San Diego, USA). A P-value of 0.05 was accepted for statistical significance.

Results

Blood pressure, biochemistry and biometry

BP increased significantly in animals of L and L+A groups, since allopurinol showed no effect on hypertension. Olmesartan, both used as monotherapy and in association with allopurinol, was able to avoid the increase of BP caused by L-NAME (Fig. 1).

The results of blood biochemistry are shown on Table 1. The UA plasma level decreased in groups treated with allopurinol (L+A and L+A+O). Neither L-NAME nor olmesartan interfered in UA levels. The creatinine, sodium and potassium plasma levels were not different among the groups.

The L group showed greater LV mass index than the

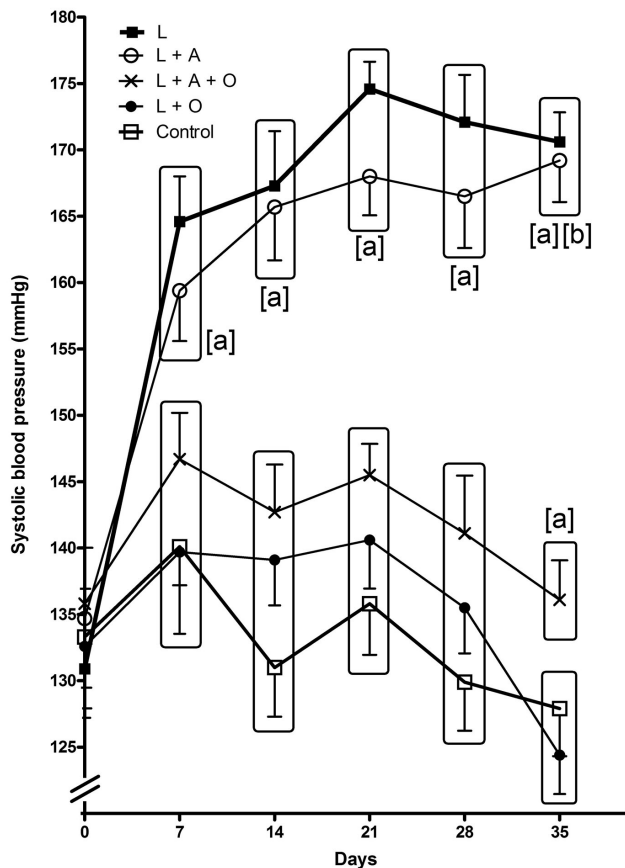


Fig. 1. Systolic blood pressure (mean and SEM). Differences were tested by one-way ANOVA and post-hoc test of Tukey. The groups were put together when they were not different. $P < 0.05$ when: [a] different from the group containing the control group, [b] different from L+A+O group.

C group. This effect of L-NAME was prevented by the administration of both allopurinol and olmesartan, as monotherapy or in combination. The LV mass index in the L+O group was significantly smaller than in the C group (Table 2 and Fig. 2).

Myocardial structure

The normal pattern of myocardial fiber organization, collagen deposition, and vessel dimensions (Fig. 3A,B) was profoundly distorted by NO inhibition (Fig. 3C,D). Allopurinol attenuated these changes, as demonstrated by histological intermediate features between L and C

Table 1. Plasma biochemistry (mean and SEM).

Group	Uric Acid (mg/dl)	Na ⁺	K ⁺	Creatinine
Control	1.0 (0.1)	142.0 (6.4)	5.2 (0.4)	0.36 (0.01)
L	0.9 (0.08)	140.7 (9.1)	4.8 (0.2)	0.34 (0.01)
L+A	0.35 (0.04) ^{[a][b]}	141.2 (2.3)	5.0 (0.3)	0.37 (0.01)
L+O	1.0 (0.2) ^[c]	141.3 (2.8)	4.8 (0.5)	0.38 (0.03)
L+A+O	0.37 (0.06) ^{[a][b][d]}	140.5 (5.8)	5.0 (0.4)	0.34 (0.02)

Differences verified by one-way analysis of variance and post hoc test of Tukey. $P < 0.05$ when: [a] different from the control group, [b] different from the L group, [c] different from the L+A group, [d] different from the L+O group.

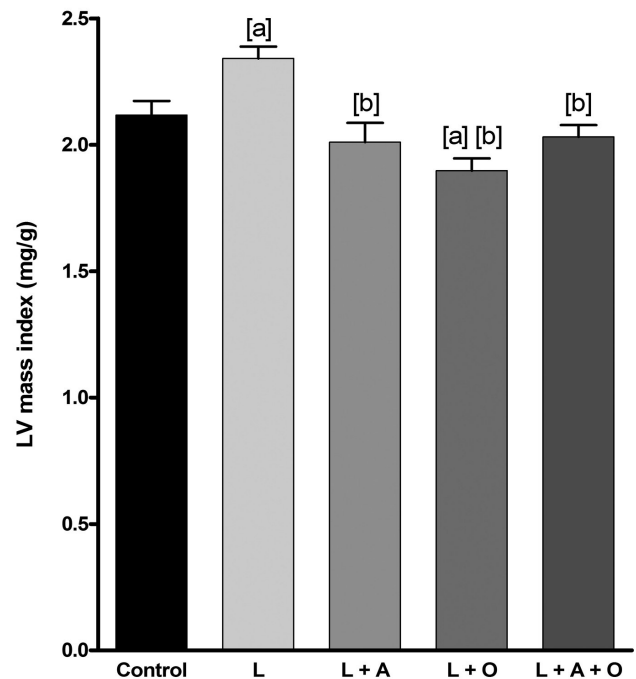


Fig. 2. Left ventricle mass index (mean and SEM). Differences were tested by one-way ANOVA and post-hoc test of Tukey. $P < 0.05$ when: [a] different from the control group, [b] different from the L group.

groups (Fig. 3E,F), while the groups treated with olmesartan (L+O group, Fig. 3G, and L+A+O group, Fig. 3H) showed tissue architecture similar to C group. These changes were quantified by the stereological methods employed, summarized in Table 3.

Stereology

The $Vv[ct]$ was more than 300% greater in the L group than in the C group. The L+A and L+O groups showed a reduction of 15 and 50% of $Vv[ct]$ when compared to the L group. The combination of allopurinol and olmesartan (in the L+A+O group) showed an intermediate effect between the C group and L group. Neither $Vv[cmj]$ nor $Vv[ve]$ showed differences among the groups (Fig. 4).

The $Vv[ve]/Vv[cmj]$ ratio was 30% smaller in the L

group than in the C group. Both allopurinol and olmesartan attenuated this L-NAME induced alteration. The L+A+O group showed no statistical difference from

Table 2. Biometric heart parameters (mean and SEM).

Group	Heart Mass (mg)	LV Mass (mg)	LV Mass Index (mg/g)
Control	1100 (43)	736 (48)	2.1 (0.07)
L-NAME	1028 (56)	765 (41)	2.3 (0.05) ^[a]
L+A	1081 (75)	690 (49)	2.0 (0.01) ^[b]
L+O	951 (47)	639 (55)	1.9 (0.06) ^{[a] [b]}
L+A+O	957 (48)	699 (37)	2.0 (0.06) ^[b]

Differences verified by one-way analysis of variance and post hoc test of Tukey. $P < 0.05$ when: [a] different from the control group, [b] different from the L group.

Table 3. Cardiac stereological parameters (mean and SEM).

Group	$Vv[cmj]$ %	$Vv[ve]$ %	$Vv[ct]$ %	$A[cmj]$ μm^2	$Vv[ve]/Vv[cmj]$ %
Control	92.9 (2.5)	5.3 (2.1)	1.8 (0.2)	125.2 (11.0)	5.7 (0.3)
L	88.1 (3.2)	3.9 (1.5)	8.0 (0.5) ^[a]	239.5 (15.4) ^[a]	4.4 (0.2) ^[a]
L+A	88.5 (3.5)	4.7 (1.8)	6.9 (0.5) ^{[a] [b]}	239.6 (15.7) ^[a]	5.3 (0.3) ^[b]
L+O	91.6 (2.7)	5.0 (1.9)	3.4 (0.2) ^{[a] [b] [c]}	177.6 (12.7) ^{[a] [b] [c]}	5.5 (0.3) ^[b]
L+A+O	90.1 (2.6)	4.4 (2.0)	5.4 (0.3) ^{[a] [b] [c] [d]}	196.6 (14.4) ^{[a] [b] [c]}	4.9 (0.2)

Differences verified by one-way analysis of variance and post hoc test of Tukey. $P < 0.05$ when: [a] different from the control group, [b] different from the L group, [c] different from the L+A group, [d] different from the L+O group.

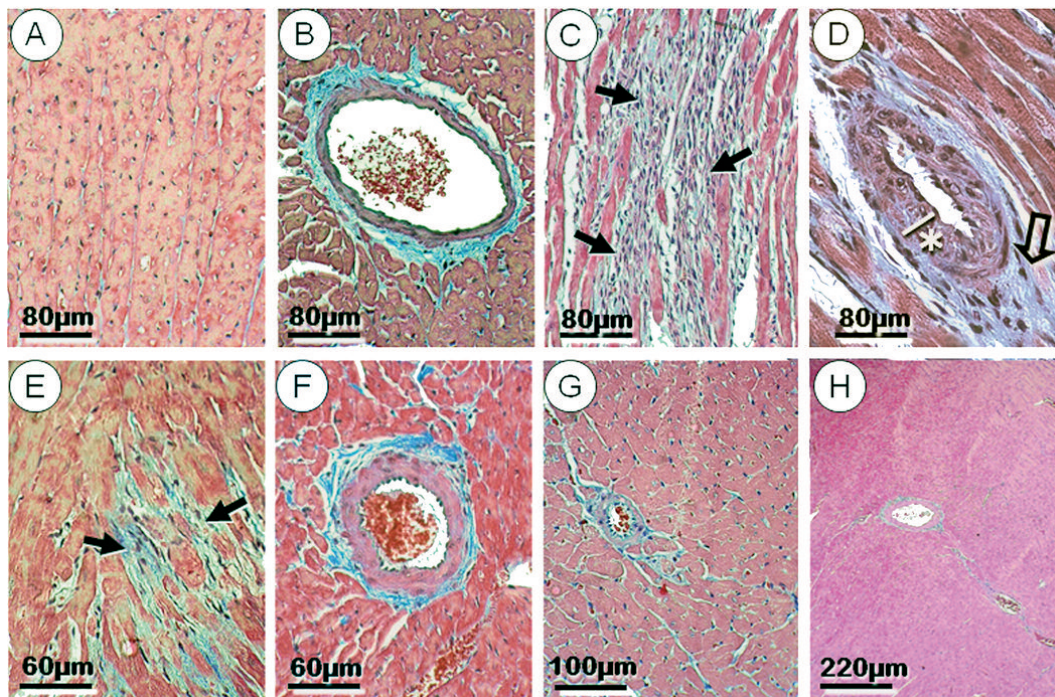


Fig 3. Photomicrographs of the myocardium in the different groups (Masson's trichrome stain). **A and B.** Control animal showing the usual pattern of muscular fiber organization and transversal section of intramyocardial vessel with perivascular collagen deposition. **C and D.** Animal from L group exhibiting inflammatory infiltrate (**C**, arrows) and transversal section of intramyocardial vessel (**D**) with media hypertrophy (*) and abundant perivascular collagen deposition (open arrow). **E and F.** L+A animal showing area of interstitial fibrosis (**E**, arrows) and intramyocardial vessel (**F**) with near normal appearance. **G and H.** Panoramic views of L+O and L+A+O animals, respectively. The structure of heart tissue closely resembles the control group.

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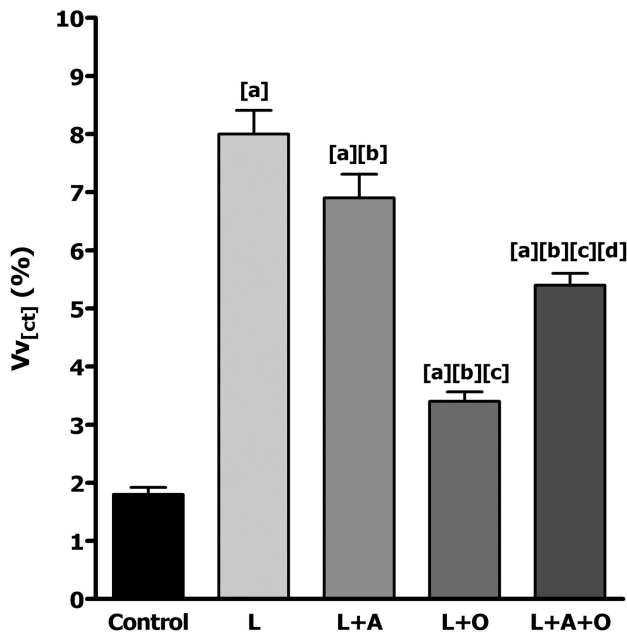


Fig 4. Volume density of the myocardial connective tissue (mean and SEM). Differences were tested by one-way ANOVA and post-hoc test of Tukey: P<0.05 when: [a] different from the control group, [b] different from the L group, [c] different from the L+A group, and [d] different from the L+O group.

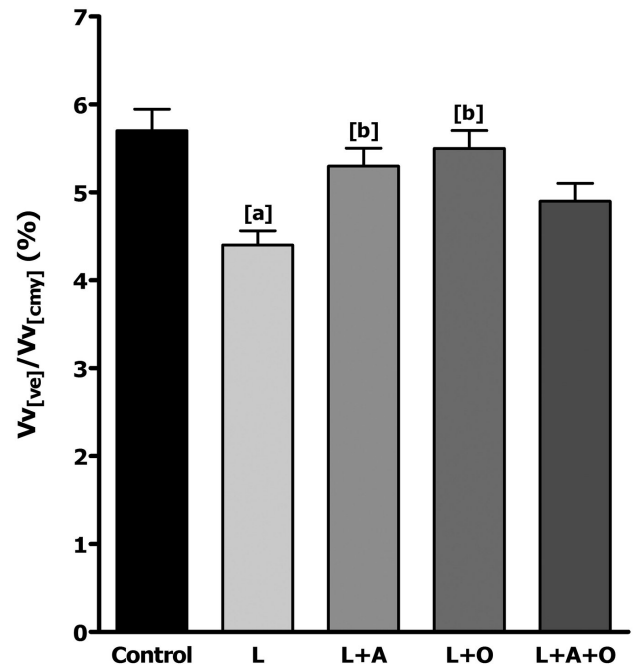


Fig 5. Ratio between the volume densities of the intramyocardial vessels to cardiomyocytes (mean and SEM). Differences were tested by one-way ANOVA and post-hoc test of Tukey. P<0.05 when: [a] different from the control group, [b] different from the L group.

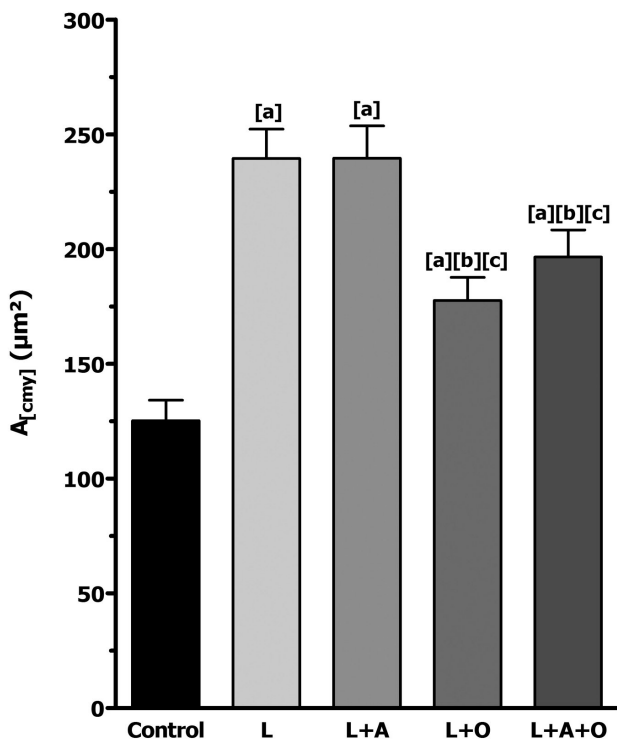


Fig 6. Cardiomyocyte mean cross-sectional area (mean and SEM). Differences were tested by one-way ANOVA and post-hoc test of Tukey. P<0.05 when: [a] different from the control group, [b] different from the L group, [c] different from the L+A group.

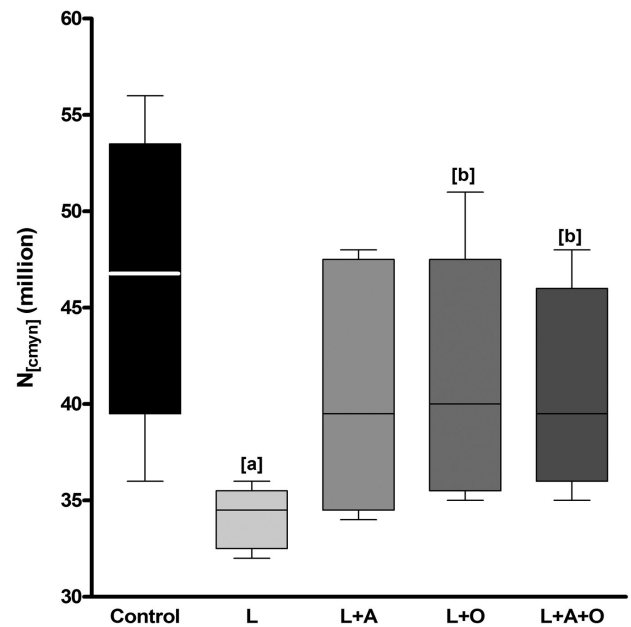


Fig 7. Number of cardiomyocyte nuclei (median and interquartile interval). Differences verified by nonparametric Kruskal-Wallis ANOVA and post-hoc test of Dunn. P<0.05 when: [a] different from the control group, [b] different from the L group.

the L group (Fig. 5).

The cardiomyocyte hypertrophy was measured by the A[cm], which was 90% greater in the L group than in C group. Olmesartan was effective in treating the cardiomyocyte hypertrophy in the L+O and L+A+O groups. Allopurinol used as monotherapy showed no effect on this parameter (Fig. 6).

In the L group the N[cm] was 30% smaller than in the C group ($P=0.004$). Olmesartan preserved the number of cardiomyocyte nuclei, which was 20% greater in the L+O and L+A+O groups when compared to the L group (Fig. 7).

Discussion

In this study, olmesartan reduced the BP in NO deficient animals to control levels which resembled the effect of losartan administration on L-NAME exposed rats (Sanada et al., 2001). In contrast, allopurinol had no effect on the hypertension caused by L-NAME, as seen in a previous study restricted to aorta analysis (Usui et al., 1999). The lack of antihypertensive effect of allopurinol was verified in different experimental models of hypertension (Zhang et al., 2005). However, allopurinol lowered the BP in the oxonic acid induced hyperuricemia model (Mazzali et al., 2001) and in the dexamethazone induced hypertension model (Wallwork et al., 2003). These data suggest that the hemodynamic repercussion of allopurinol varies with the experimental model chosen, probably depending on the amount of XO activity contribution. Allopurinol reduced the plasma UA concentrations in the animals studied. This effect of XO inhibition is in agreement with the results from other experimental models using rats (Mellin et al., 2005; Zhang et al., 2005).

Confirming the results obtained by the use of losartan in another study (Sanada et al., 2001), the LV mass index in the olmesartan treated group was inferior to the control group. Allopurinol attenuated the rise in LV mass index in a similar way to olmesartan treatment. Left ventricular hypertrophy (LVH) can be induced by L-NAME through several mechanisms, including pressure overload (Sladek et al., 1996) and RAAS activation (Kato et al., 1998). These pathogenic features are attenuated by olmesartan, which prevented LVH in the present study. The effects of allopurinol on L-NAME induced hypertrophy are not yet clear. In this study, allopurinol did not prevent LVH caused by NO inhibition. In contrast, a direct effect of allopurinol reducing cardiomyocyte hypertrophy has been demonstrated after myocardial infarction in mice (Engberding et al., 2004).

NO inhibition enhanced myocardial fibrosis, as measured by the Vv[ct], which is supported by previous experimental findings (Ono et al., 1999). This adverse remodeling effect was efficiently treated by olmesartan. The influence of RAAS antagonism in preventing myocardial fibrosis in the L-NAME model was described by others using olmesartan (Takemoto et al.,

1997). Interestingly, in the present study, allopurinol produced a similar effect, without lowering BP, which agrees with a report on experimental heart infarction, when allopurinol was able to reduce interstitial fibrosis (Engberding et al., 2004).

We observed a mismatch between vascular supply and cardiomyocyte volume in L-NAME treated animals. Considering that L-NAME inhibits vascular cell proliferation (El Mabrouk et al., 2000), the net result may favor vascular cell loss during L-NAME administration. Olmesartan and allopurinol prevented the decrease in the vessel/myocyte relationship in our study. Vascular cell loss was prevented by olmesartan in cell culture studies (Akishita et al., 2005).

The advantages of olmesartan in L-NAME induced cardiomyopathy find explanation in its pharmacological properties. It is known that L-NAME stimulates ACE activation (Linardi et al., 2004) and both AT1 and AT2 receptor expression in rat cardiac tissue (Kato et al., 1998). While blocking AT1 receptor, olmesartan leaves AT2 receptor action unopposed (Levy, 2004). The anti-hypertrophy effects associated with AT2 may account for some of the cardiac structural effects observed in our study.

The reduced number of cardiomyocyte nuclei in L-NAME exposed rats confirmed previous studies from our group (Pessanha and Mandarim-de-Lacerda, 2000). In the present study, the cardiomyocyte loss was prevented by olmesartan, in accordance with different reports that have linked RAAS interference with cardiomyocyte preservation (Fiordaliso et al., 2000; Mandarim-de-Lacerda and Pereira, 2001). Allopurinol did not cause the same effect, even though there was a clear trend. Cardiomyocyte loss could occur as result of ischemic necrosis or apoptosis, and continuous myocardial ischemia may provoke early cardiomyocyte apoptosis followed by necrosis (Pessanha and Mandarim-de-Lacerda, 2000).

The pressure-independent effect of allopurinol in preserving the cardiac structure of hypertensive animals probably reflects its action over tissue metabolism. The XO enzyme produces superoxide and hydrogen peroxide during the formation of uric acid (Berry and Hare, 2004). Several lines of evidence, based on both animal and clinical studies, associate ROS production by XO with cardiovascular damage (Cappola et al., 2001; Saavedra et al., 2002).

Accordingly, the beneficial effects of allopurinol in the present study suggest an important contribution of xanthine oxidase (XO) to myocardium redox state. Additionally, these findings support a possible role of XO in the histological derangements associated with L-NAME. Indeed, L-NAME induces wide areas of hypoxia and infarction (Moreno et al., 1996). Hypoxia is a stimulus for the transcription of XO by endothelial cells (Terada et al., 1997). Furthermore, an excess of XO activity has been documented in tissues of L-NAME exposed animals (Laakso et al., 1999). This imbalance may be counteracted by allopurinol.

Improved myocardial remodeling may be the basis for the beneficial effects of allopurinol observed in heart failure patients (Struthers et al., 2002), another condition associated with relative chronic hypoxemia. Interestingly, L-NAME exposure mimics heart failure in isolated heart preparations and causes hemodynamic disturbances that are reversed by allopurinol (Saavedra et al., 2002). Another relevant issue is to establish whether urate reduction per se or the antioxidant action of allopurinol is responsible for the effects described. Additional studies using other urate reducing drugs, mainly probenecid, may be performed to address this topic. The recent study of George and colleagues, however, suggests that urate reduction is not the mechanism underlying allopurinol benefits (George et al., 2006).

One limitation of the present study is the absence of a direct measurement of myocardial ROS levels. However, the systemic effects of allopurinol administration were demonstrated by the reduction of UA plasma concentrations in animals receiving the drug. The decrease in UA plasma concentrations caused by allopurinol is associated to reductions in ROS levels at the myocardium (Mellin et al., 2005). Future studies directly addressing oxidative stress in myocardium of L-NAME exposed animals and the effect of allopurinol treatment may elucidate this issue.

In conclusion, we demonstrated that allopurinol reduces cardiac structural lesion in L-NAME exposed rats. These effects are significant, albeit smaller than the results obtained with olmesartan, a well-established antihypertensive agent. This principle must be explored by additional research, clarifying the interplay between NO, XO and other ROS producing enzymatic systems occurring at the myocardium.

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