

Chronic Low-dose L-NAME Treatment Increases Nitric Oxide Production and Vasorelaxation in Normotensive Rats

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Running title: Chronic Low-dose L-NAME Treatment and NO Synthesis

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Summary

N^G-Nitro-L-arginine methyl ester (L-NAME) is a non-specific nitric oxide (NO) synthase inhibitor, commonly used for the development of NO-deficient hypertension. The aim of this study was to investigate the effect of chronic low-dose administration of L-NAME on NO production, vascular function and structure of the heart and selected arteries of rats. Adult male Wistar rats were treated with L-NAME in the dose of approximately 1.5 mg/kg/day in drinking water for 8 weeks. Basal blood pressure (BP) of rats (determined by tail-cuff) was 112 ± 3 mm Hg. The low-dose administration of L-NAME significantly elevated BP measured on the third and sixth week of treatment vs. controls by approximately 9% and 12%, respectively. After this period, BP of L-NAME-treated rats returned to the control values. The relative left ventricular mass, heart fibrosis and collagen III/collagen I ratio were not affected by L-NAME. Similarly, there were no alterations in the cross sectional area and wall thickness/diameter ratio of the aorta and the femoral artery of L-NAME-treated rats. NO synthase activity (determined by conversion of [³H]-L-arginine to [³H]-L-citrulline) was not altered in the hypothalamus of L-NAME-treated rats. Interestingly, chronic low-dose L-NAME treatment significantly elevated NO synthase activity in the left ventricle and aorta, increased endothelium-dependent acetylcholine-induced vasorelaxation and reduced serotonin-induced vasoconstriction of the femoral artery. The data suggest that chronic low-dose L-NAME treatment can increase NO production and vasorelaxation in normotensive rats without negative structural changes in the cardiovascular system.

Key words: hypertension, blood pressure, cardiac and vascular structure, negative feedback regulation, acetylcholine, serotonin

Introduction

Nitric oxide is a widespread biological mediator produced in various tissues by one of four isoforms of nitric oxide (NO) synthase (Guix *et al.* 2005, Šimko 2007). Besides its role in neurotransmission and hemodynamic control, NO participates in the regulation of cell proliferation and growth (Pechářová and Šimko, 2007). It has been shown that chronic pharmacological reduction of NO synthesis with the NO synthase (NOS) inhibitor N^G-Nitro-L-arginine methyl ester (L-NAME) resulted in metabolic alterations, hypertension and myocardial hypertrophy (Bernátová *et al.* 1999b, Šimko and Šimko, 2000, Kuneš *et al.* 2004), reduced vasorelaxation (Bernátová *et al.* 2002, Gerová *et al.* 2004, Török and Kristek 2002, Paulis *et al.* 2006) and vascular wall thickening (Bernátová *et al.* 1999a, Gerová and Kristek 2001, Šimko *et al.* 2007). Additionally, the reduction of NO synthesis led to remodelling of myocyte junctions (Fialová *et al.* 2007), angiogenesis, mitochondrial damage (Okruhlicová *et al.* 2000, Tribulová *et al.* 2000) and myocardial fibrosis (Babál *et al.* 1997).

Although the model of L-NAME-induced hypertension has been one of the most frequently used models of experimental hypertension since 1990, there is little information on the effects of chronic low-dose L-NAME treatment (less than 2 mg/kg/day) on the cardiovascular system. This issue may be particularly important because several studies have provided evidence for the negative-feedback regulatory role of NO on endothelial NOS, and therefore on vascular function (Cohen *et al.* 1996, Griscavage *et al.* 1995, Vaziri and Wang 1999). Additionally, negative feedback regulation by NO was observed also for nNOS and iNOS (Park *et al.* 1997, Vickroy and Malphurs 1995). However, the above mentioned studies investigated the effects of

exogenous NO donors on NO production *in vitro* or in acute experiments. Thus the question arose whether chronic reduction of NO bioavailability *in vivo* may activate NO production.

The purpose of this study was to investigate the effect of long-term low-dose L-NAME treatment on NO production, vascular function and cardiovascular structure of normotensive rats.

Methods

Animals and treatment

Male 12-week-old Wistar rats were randomly divided into a control (n = 8) and an L-NAME-treated group (n = 7). L-NAME was administered orally in tap water for eight weeks. All rats were housed at 22-24°C on a 12:12-h dark-light cycle (06.00-18.00h lights on) and had food and water (or L-NAME solution) *ad libitum*. The concentration of L-NAME in tap water was calculated on the basis of body mass and drinking volume of rats to reach approximately the daily dose of 1.5 mg/kg/day L-NAME. The calculated average daily dose of L-NAME was 1.49 ± 0.053 mg/kg/day. All procedures used in this study were approved by the State Veterinary and Food Administration of the Slovak Republic.

One week before experimentation, the rats were handled and accustomed to the tail-cuff procedure of blood pressure recording. Blood pressure (BP) was determined before experiment (basal) and on the 1st, 3rd, 6th and 8th week of experiment. After 8 weeks of experiment, the rats were killed by decapitation following a brief diethyl ether

anaesthesia. Body mass (BM) as well as the wet mass of the left (LV) and the right ventricle (RV) were determined for calculation of their relative masses (LV/BM, RV/BM).

NO synthase activity

NO synthase activity was measured in the homogenates of the hypothalamus, thoracic aorta and the left ventricle by determination of [^3H]-L-citrulline (L-Cit) formation from [^3H]-L-arginine (MP Biomedicals, USA), as previously described (Bredt and Snyder 1990), with minor modifications. Briefly, crude homogenates of the aorta and LV containing 200 mg of wet tissue in 1 ml of homogenization solution containing 50 mmol/l Tris-HCl, pH 7.4 and 1% Protease Inhibitor Cocktail (Sigma, Germany) were centrifuged at 10 000 g for 15 min at 4°C. After centrifugation, 50 μl of supernatant was incubated in the presence of 10 $\mu\text{mol/l}$ [^3H]-L-arginine (specific activity 5 GBq/mmol, about 100 000 DPM), 5 $\mu\text{g/ml}$ calmodulin, 0.5 mmol/l β -NADPH, 250 $\mu\text{mol/l}$ tetrahydrobiopterin, 4 $\mu\text{mol/l}$ FAD, 4 $\mu\text{mol/l}$ FMN, 1 mmol/l Ca^{2+} , 1 mmol/l Mg^{2+} , in the total volume of 100 μl . After 20-min incubation at 37°C, the reaction was stopped by 1 ml of ice-cold stop solution containing 20 mmol/l HEPES, pH 5.5, 2 mmol/l EDTA, 2 mmol/l EGTA and 1mmol/l L-Cit and applied to 50WX-8 Dowex columns (Na^+ form). [^3H]-L-citrulline was eluted by 1 ml of water and determined by liquid scintillation counting. NO synthase activity was expressed as pmol/min/mg of proteins.

Vascular responses

Femoral arteries were carefully excised, cleaned of adipose and connective tissue, cut into segments (approximately 1 mm long) and mounted as ring-shaped preparations in a Mulvany – Halpern's small vessel myograph chamber (Dual Wire Myograph System 410A, DMT A/S, Aarhus, Denmark) to determine the vascular reactivity during isometric conditions in the arteries with intact endothelium, as described elsewhere (Púzserová *et al.* 2006a). Endothelium-dependent NO-mediated vasorelaxation was determined after pre-constriction of the segments with phenylephrine (10^{-4} mol/l). Acetylcholine was applied in cumulative manner (10^{-9} - 10^{-5} mol/l) when the contractile response to phenylephrine reached a plateau, and the extent of relaxation was expressed as the percentage of pre-contraction. Other segments of the arteries were used to test contractile responses of the arteries induced by serotonin (5-hydroxytryptamine). Serotonin was applied after a 40-min stabilization period in cumulative manner (10^{-9} - 10^{-5} mol/l) and the extent of vasoconstriction was expressed as mN/mm.

Morphological analysis

Samples of the heart, aorta and femoral artery were fixed for one day in 4% formaldehyde (Sigma Chemie, Germany). Fixed tissue samples were embedded in paraffin, cut in 5µm slices and stained routinely with hematoxylin and eosin. Modified picrosirius red staining technique (Dolber and Spach 1993) was used to determine the level of fibrosis. Briefly, after deparaffinization in xylene and rehydration in distilled water, the slides were incubated for 5 minutes in 0.2% aqueous solution of phosphomolybdic acid (Sigma Chemie, Germany) and stained with 0.1% sirius red in saturated solution of picric acid (Sigma Chemie, Germany) for 90 minutes. Finally the

slides were washed for two minutes in 0.01 mol/l hydrochloric acid. Total fibrosis of each tissue sample was measured by histomorphometry in 10 microscopic fields at 20x magnification. Results are expressed as per mill of collagen positivity of the total analyzed area. The area rich for collagen type I (red color in polarized light) and collagen type III (green color in polarized light) was analyzed by the digital color subtraction, and the ratio of collagen type I to collagen type III was determined.

The standard morphometric analysis of the aorta and femoral artery was realized on hematoxylin and eosin-stained slides. The cross-sectional area (CSA) expressed in μm^2 and the wall thickness to internal diameter ratio of vessels were measured using the ImageJ morphometric software v.1.33 (National Institutes of Health, USA). The picrosirius red stained sections were evaluated by light microscopy and in polarized light at 40x magnification. Results were calculated as ratio of collagen type I to collagen type III presence expressed in pixels.

Statistical analysis

Blood pressure and vascular responses were analyzed using two-way ANOVA. Significant differences were identified by Duncan's post-hoc test. All other data were analyzed by Student's t-test. Values were considered to differ significantly when $p < 0.05$. All results are presented as mean \pm SEM.

Results

Blood pressure and morphological parameters

Basal blood pressure of all rats before experiment was 112 ± 3 mm Hg (Fig. 1). Low-dose L-NAME administration resulted in a transient elevation of BP at the 3rd and 6th week by approximately 9% and 12% vs. control value ($p < 0.001$). However, normalization of BP was observed after eight weeks of L-NAME treatment.

The relative masses of the LV and RV, heart fibrosis and collagen III/collagen I ratio in the heart of control and L-NAME treated rats did not differ significantly. Similarly, there were no alterations in the cross-sectional area and wall thickness/diameter ratio of the aorta and femoral artery of L-NAME-treated rats when compared to control (Table 1).

NO synthase activity

Basal NO synthase activity in the aorta, LV and hypothalamus were 5.6 ± 0.3 , 3.8 ± 0.4 and 38.2 ± 5.9 pmol/min/mg, respectively (Fig. 2 A, B and C). Eight weeks lasting low-dose L-NAME treatment led to a significant elevation of NO synthase activity in the aorta and LV by approximately 43% and 45% ($p < 0.007$) vs. control values while there were no significant changes in hypothalamic NO synthase activity.

Vascular function

Maximal acetylcholine-induced vasodilatation of the femoral artery (Fig. 3A) observed in control rats was $88 \pm 3\%$, which was significantly lower than in the L-NAME-treated rats ($105 \pm 3\%$, $p < 0.05$). Maximal serotonin-induced vasoconstriction of the femoral artery in

control rats (Fig. 3B) was 19.8 ± 2.7 mN/mm and L-NAME reduced it to 13.9 ± 1.8 mN/mm ($P < 0.03$).

Discussion

The most important finding of this study was that the NO synthase inhibitor L-NAME was paradoxically able to increase NO production in the left ventricle and aorta of normotensive rats *in vivo* when administered in a low dose and for a long time. Chronic low-dose L-NAME-treatment resulted also in augmentation of the endothelium-dependent vasorelaxation and reduced vasoconstriction of the femoral artery. Additionally, no negative effects of L-NAME on the morphological parameters of the heart, aorta and femoral artery were observed.

Several studies addressed the role of NO in the regulation of cardiac and vascular functions using pharmacological inhibition of NO production in normotensive rats. Administration of high doses of L-NAME (50 -100 mg/kg/day) for 6-8 weeks increased BP by about 40-80 % (Arnal *et al.* 1992, Ribeiro *et al.* 1992, Kristek *et al.* 1996, Mandarim-de-Lacerda and Pereira 2001). Similar elevation of BP was observed using lower doses of L-NAME (10-20 mg/kg/day) for 4-8 weeks (Arnal *et al.* 1992, Delacretaz *et al.* 1994, Ferreira-Melo *et al.* 2006). In our previous study, approximately 27% elevation of BP was observed after the first day of L-NAME administration at the dose of 40 mg/kg/day (Pecháňová *et al.* 1997). However, low doses of L-NAME (1 mg/kg/day for 25 days and 2 mg/kg/day for 7 days) had no significant effect on BP of normotensive rats (Ralay Ranaivo *et al.* 2004, Arnal *et al.* 1992). A mechanism responsible for the increase of BP during L-NAME-treatment is associated with NO deficiency and

alterations in various blood pressure regulating systems. Several authors observed elevation of vasoconstriction and attenuation of vasorelaxation in different parts of the vascular tree, increased sympathetic activity and alterations in renin-angiotensin system in L-NAME treated rats (Jover *et al.* 2001, Bernátová *et al.* 2002, Kuneš *et al.* 2004, Rossoni *et al.* 2007). These metabolic and hemodynamic changes were associated with the development of left ventricular hypertrophy, heart fibrosis, necrosis and protein remodelling, as well as with vascular wall hypertrophy (Babál *et al.* 1997, Ferreira-Melo *et al.* 2006, Pereira and Mandarim-de-Lacerda, 1999, Bernátová *et al.* 2000, Šimko *et al.* 2007).

In this study, administration of low doses of L-NAME (~1.5 mg/kg/day) resulted in a transient mild elevation of PB after three and six weeks of treatment and an extension of treatment to eight weeks resulted in normalization of BP. This was associated with elevation of NO synthase activity in the left ventricle and aorta. Although elevated NOS activity in the aorta does not necessarily mean improvement of NO bioavailability in the femoral artery, the augmentation of endothelium-dependent NO-mediated vasorelaxation along with the decrease of vasoconstriction are indicative of elevated NO availability also in this artery (Dabire *et al.* 1990). The increase of NO production may result from the negative feedback regulation between NO and its own production. In addition to the studies which used exogenous NO donors, Grumbach *et al.* (2005) have recently observed that eNOS promoter activity and mRNA transcription in bovine aortic endothelial cells were increased in the presence of the NOS inhibitor L-NAME. However, to our knowledge, our study is the first to show that long-term administration of low doses of NO synthase inhibitor may induce improvement of NO

production in the rat heart and aorta *in vivo* and simultaneously influence vascular function. Based on the elevation of blood pressure after the third and sixth week of L-NAME treatment, we assume that the low doses of L-NAME led to a transient mild NO deficiency, which in turn activated NO production. Thus, our data suggest that chronic low-dose L-NAME administration could provide a useful tool for activation of NO production *in vivo*. On the other hand, an important limitation of using L-NAME may result from the fact that L-NAME can induce cardiac fibrosis and vascular wall hypertrophy independently of blood pressure and thus impair vascular function and accelerate the development of hypertension (Bernátová *et al.* 1999). Nevertheless, as observed in this study, low dose of L-NAME had no negative effect on the structure of the heart and vasculature. These findings suggest that long-term administration of low doses of L-NAME could be used to increase NO synthesis in contrast to supplementation with exogenous NO donors, which may inhibit endogenous NO production.

In conclusion, the results showed that chronic administration of the low doses of L-NAME activated NO synthesis in the cardiovascular system without modifying central NO synthesis. In addition, low doses of L-NAME enhanced endothelium-dependent vasorelaxation and reduced vasoconstriction of the femoral artery. The results provide evidence for negative feedback regulation of NO synthesis by NO in normotensive rats *in vivo*. However, more experiments are needed to elucidate whether this regulatory mechanism is operating only in physiological conditions or if it may be used to increase endogenous NO production (directly or after some modification in the dosage and/or duration of treatment) in pathological conditions, such as hypertension or ischemic heart disease.

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Table 1 Biometric and morphological parameters of Control and L-NAME-treated rats.

		Control	L-NAME
		n = 8	n = 7
Final BM (g)		374±5	382±6
LV/BM (mg/g)		1.29±0.03	1.31±0.04
RV/BM (mg/g)		0.48±0.02	0.50±0.02
Heart	Fibrosis (‰)	12.53±1.74	10.20±1.98
	Col III/Col I	49.33±2.05	55.38±2.96
Aorta	WT/ID	65.50±2.50	71.00±2.45
	CSA (µm ²)	534±30	518±40
Femoral a.	WT/ID	94.05±10.25	108.11±17.04
	CSA (µm ²)	63.8±11	46.5±10

Results are presented as Mean±SEM. Abbreviations: BM- body mass, LV- left ventricle, RV- right ventricle, Col- collagen, WT- wall thickness, ID- internal diameter, CSA- cross sectional area

Figure captions

Figure 1 Effect of chronic low-dose L-NAME treatment on blood pressure of normotensive rats. Results are presented as Mean \pm SEM. *p<0.001 vs. control. Abbreviations: B-basal value

Figure 2 Effect of chronic low-dose L-NAME treatment on nitric oxide synthase activity in the aorta (A), left ventricle (B), and hypothalamus (C) of rats. Results are presented as Mean \pm SEM. *p<0.007 vs. control. Abbreviations: C- Control

Figure 3 Effect of chronic low-dose L-NAME treatment on acetylcholine-induced vasorelaxation (A) and serotonin-induced vasoconstriction (B) of the femoral artery. Results are presented as Mean \pm SEM. *p<0.05 vs. control, ⁺p<0.03 vs. control.

Figure 1

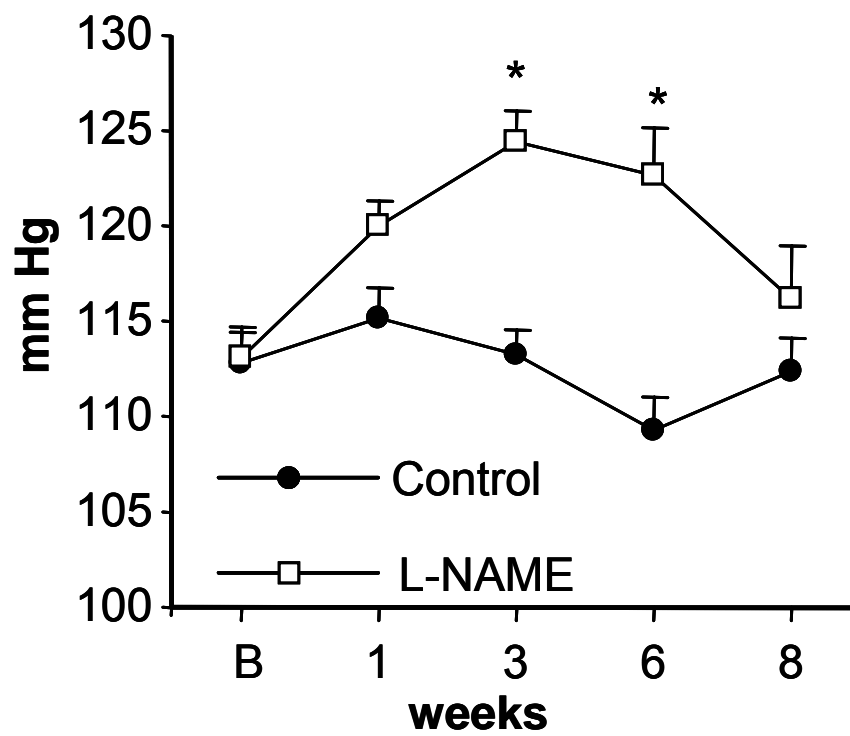


Figure 2

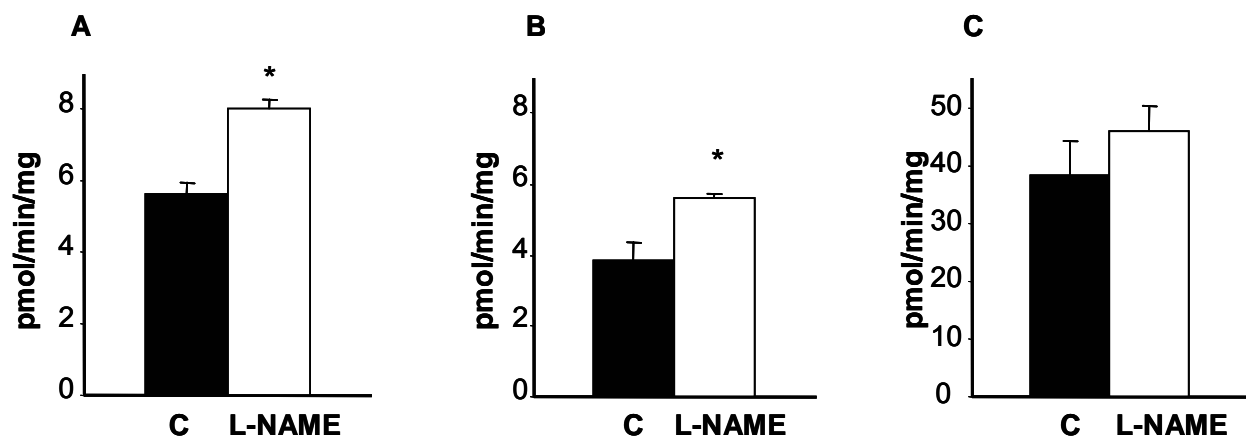


Figure 3

