

# Chronic low-dose L-NAME treatment effect on cardiovascular system of borderline hypertensive rats: Feedback regulation?

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## Abstract

**OBJECTIVES:** The effect of 8-week-lasting low-dose treatment of N<sup>G</sup>-Nitro-L-arginine methyl ester (L-NAME), a nitric oxide (NO) synthase inhibitor, was investigated in borderline hypertensive rats (BHR) to examine, whether dose of 1.5 mg/kg/day affects feedback regulation of NO synthesis.

**METHODS:** Blood pressure (BP) of 12 weeks old Wistar and BHR rats was determined non-invasively by tail-cuff. NO synthase (NOS) activity was determined by conversion of [<sup>3</sup>H]-L-arginine to [<sup>3</sup>H]-L-citrulline in the aorta, left ventricle (LV) and hypothalamus. Vascular function of the femoral artery was determined using Mulvany's myograph in isometric conditions.

**RESULTS:** Chronic low-dose L-NAME treatment of BHR induced sustained blood pressure elevation and left ventricular hypertrophy associated with the decrease in NOS activity in left ventricle and unaltered NOS activity in the aorta. By contrast, the improvement of LV and aortic NOS activity was found in Wistar rats. In hypothalamus, no changes in NOS activity were found in both BHR and Wistar. In Wistar, acetylcholine-induced relaxation of the femoral artery was increased and serotonin-induced and noradrenalin-induced constriction were reduced in L-NAME treated group. These effects, however, were not seen in BHR.

**CONCLUSION:** The results indicate that NOS/NO feedback regulation works differently under conditions of normotension and prehypertension. Low-dose L-NAME treatment accentuated NO production in normotensive rats, but it failed to improve NOS activity in BHR.

## Abbreviations

L-NAME	- N <sup>G</sup> -Nitro-L-arginine methyl ester	RV	- right ventricle
NO	- nitric oxide	BW	- body weight
NOS	- nitric oxide synthase	LVW	- left ventricle weight
BHR	- borderline hypertensive rats	RVW	- right ventricle weight
BP	- blood pressure	L-NMA	- N <sup>G</sup> -monomethyl-L-arginine
LV	- left ventricle	NA	- noradrenalin
		B	- basal value,
		WKY	- Wistar-Kyoto rats

## INTRODUCTION

Both local and central blood pressure regulation requires presence of appropriate amounts of nitric oxide (NO). This small gas molecule is produced from L-arginine by one of four isoforms of nitric oxide synthase (NOS) which have been distinguished until recently by either constitutive or inducible expression of enzyme (Forstermann *et al.*, 1998; Guix *et al.*, 2005; Nakata *et al.*, 2005; Müller & Kerschbaum, 2006; Maes *et al.*, 2007).

L-arginine analogues are widely used to block enzymatic activity of NOS (Rees *et al.*, 1990) and thus to induce so called NO-deficient hypertension. The administration of NOS inhibitor N<sup>G</sup>-Nitro-L-arginine methyl ester (L-NAME) to normotensive rats in doses about 10 mg/kg/day and higher leads to the development of sustained hypertension (Arnal *et al.*, 1992; Bernátová *et al.*, 1999b), left ventricular hypertrophy and heart fibrosis (Pechánová *et al.*, 1997; Bernátová *et al.*, 2000; Babál *et al.*, 1997) and attenuation of vasorelaxation (Bernátová *et al.*, 2002). On the other hand, exogenous NO donors failed to fully compensate these pathologic alterations (Gerová & Kristek, 2001).

Several studies on exogenous NO donors brought also evidence for the negative-feedback regulatory role of NO on endothelial, neuronal and inducible NOS in cell cultures, *in vitro* or in acute *in vivo* experiments (Buga *et al.*, 1993; Rogers & Ignarro, 1992; Vaziri & Wang, 1999; Griscavage *et al.*, 1995; Park *et al.*, 1997). Considering that administration of exogenous NO leads to decrease of endogenous production, the question arose whether reduced bioavailability of NO induced by partial inhibition of NOS may invoke prolonged activation of L-Arginine/NO pathway based on negative feedback *in vivo*. Consequently, Grumbach and

colleagues (2005) observed enhanced eNOS expression in bovine aortic endothelial cells after incubation with NOS inhibitor L-NAME. Additionally, we observed the improvement of NO synthesis in cardiovascular system of normotensive rats after chronic low-dose L-NAME treatment (Bernátová *et al.*, 2007). However, the question arose, if the same negative feedback can modify NO production also in prehypertensive period.

## METHODS

### *Animals and treatment*

Male 12-week-old Wistar rats and borderline hypertensive rats (BHR, offspring of spontaneously hypertensive dams and Wistar sires) were randomly divided into a control (n=8) and an L-NAME-treated group (n=7), each phenotype separately. All rats were housed at 22–24°C on a 12:12-h dark-light cycle (06.00–18.00h lights on) and maintained on standard pellet diet and tap water (or L-NAME solution) *ad libitum*. L-NAME was administered orally in tap water for eight weeks and its concentration in tap water was calculated on the basis of body mass and drinking volume of rats for purpose of reaching approximate daily dose of 1.5 mg/kg/day L-NAME. The calculated average daily intake of L-NAME was 1.49±0.053 mg/kg/day for Wistar and 1.52±0.12 mg/kg/day for BHR. The daily drinking volume was not affected by presence or absence of drug in the water (see Table 1). 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 (BP) recording. Blood pressure was determined before experiment (basal) and on the 1st,

**Table 1.** Basic biometric parameters and average responses of the femoral artery of Wistar and BHR control and L-NAME-treated rats. Results are presented as Mean±SEM.

	Wistar control n=8	Wistar L-NAME n=7	BHR control n=8	BHR L-NAME n=7
<b>Final BW</b> (g)	374±5	382±6	369±9	388±5
<b>Daily drinking volume</b> (ml/100g of BW)	9.3±0.4	9.9±0.5	12.1±0.6†	12.5±0.9
<b>LVW/BW</b> (mg/g)	1.29±0.03	1.31±0.04	1.50±0.03†	1.64±0.06*
<b>RVW/BW</b> (mg/g)	0.48±0.02	0.50±0.02	0.52±0.01	0.47±0.01
<b>Average vasorelaxation</b> (%)	57±4	64±7*	68±5†	69±4
<b>Average vasoconstriction to serotonin</b> (mN/mm)	10.4±1.7	6.9±0.9*	5.5±1.2†	6.4±1.1
<b>Average vasoconstriction to NA</b> (mN/mm)	1.3±0.2	0.6±0.2*	0.5±0.1†	0.5±0.2

Abbreviations: BW – body weight, LVW – left ventricle weight, RVW – right ventricle weight, NA – noradrenalin, \**p*<0.05 vs. control, †*p*<0.05 vs. Wistar

3rd, 6th and 8th week of experiment. At the end of experiment, the rats were killed by decapitation after brief diethyl ether anesthesia. Body weight (BW) as well as the weights of wet mass of the left (LVW) and the right ventricle (RVW) were determined for calculation of their relative weights (LVW/BW, RVW/BW).

#### NO synthase activity

NO synthase activity was assessed by measuring the conversion of [<sup>3</sup>H]-L-arginine (Amersham, UK) to [<sup>3</sup>H]-L-citrulline in homogenates of the hypothalamus, the aorta and the left ventricle (LV) as previously described (Púzszerová *et al.*, 2006). NO synthase activity was expressed as pmol/min/mg of proteins.

#### Vascular responses

Femoral arteries were carefully excised, cleaned of connective and adipose tissue and cut into segments (approximately 1 mm long). These arterial rings were mounted 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 (Púzszerová *et al.*, 2006). To evaluate relaxation responses, the vessels were first precontracted with a 10<sup>-4</sup> mol/l dose of phenylephrine. Acetylcholine was applied in cumulative manner (10<sup>-9</sup>–10<sup>-5</sup> mol/l) when the contractile response to phenylephrine reached a plateau and the extent of relaxation was expressed as the percentage of precontraction. Other segments of the arteries were used for testing the contractile responses induced by serotonin (5-hydroxytryptamine) or noradrenalin. Both serotonin and noradrenalin were applied after a 40-min stabilization period in cumulative manner (10<sup>-9</sup>–10<sup>-5</sup> mol/l) and the extent of vasoconstriction was expressed as active wall tension (mN/mm). The average values of relaxation and constriction (effect of

strain/L-NAME interaction) were calculated from the individual dose-response curves.

#### Statistical analysis

Blood pressure and vascular responses were analyzed using three-way ANOVA. Significant differences were identified by Duncan’s post-hoc test. All other data were analyzed by two-way ANOVA. Values were considered to differ significantly when *p*<0.05. All results are presented as Mean ± SEM.

## RESULTS

#### Blood pressure and basic biometric parameters

Basal blood pressure of Wistar and BHR before experiment was 112±3 mm Hg and 136±2 mm Hg, respectively (Figure 1). Low-dose L-NAME administration in both L-NAME-treated groups resulted in a transient elevation of BP at the 3rd and the 6th week by approximately 11% in Wistar and 14% in BHR (*p*<0.05 vs. control value). However, after eight weeks of L-NAME treatment the normotensive rats normalized their BP back to control values, while BHR were not able to cope with the low dose L-NAME-treatment and their blood pressure remained elevated until the end of study (*p*<0.05 vs. control value).

We observed increased relative LVW of the left ventricle (Table 1) in BHR compared to Wistar (*p*<0.05) and L-NAME treatment induced additional hypertrophy in prehypertensive rats (*p*<0.05 vs. control values). No differences in relative RVW were found (Table 1).

#### NO synthase activity

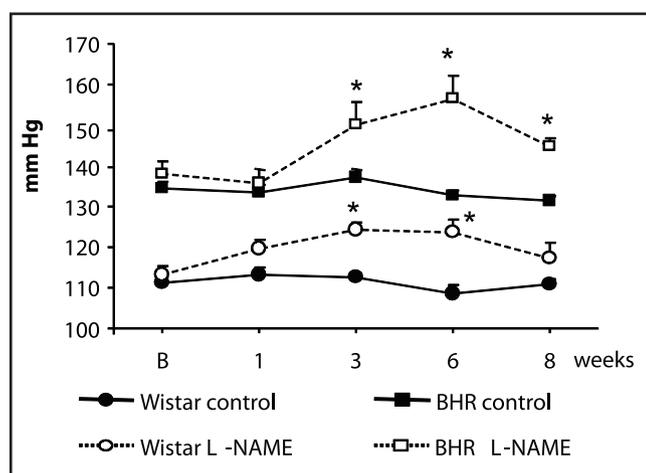
In Wistar, control NO synthase activity in the aorta and LV were 5.6±0.3 and 3.8±0.4 pmol/min/mg, respectively (Figure 2 A, B) and as mentioned, eight weeks lasting low-dose L-NAME treatment led to a significant elevation both in the aorta and LV by 43% and 47%, respectively (*p*<0.05 vs. control values). However, under conditions of borderline hypertension L-NAME treatment failed to enhance activity of NOS. Instead, the NOS activity in LV was decreased by 57% vs. control values while NOS activity in aorta was not significantly changed.

Hypothalamic activity was not affected by L-NAME neither in Wistar nor in BHR (Figure 2 C).

#### Vascular function

The average acetylcholine-induced vasodilatation of the femoral artery (Table 1) of control Wistar was 57±4% and L-NAME treatment improved it significantly. However, no significant change of relaxation ability was observed in BHR. The dose responses curves of both BHR did not differ in any concentration. L-NAME treatment enhanced the relaxation response to the maximal concentration of acetylcholine (10<sup>-5</sup> mol/l) in Wistar (Figure 3).

L-NAME treatment also attenuated the average serotonin-induced and noradrenalin-induced vasoconstriction



**Figure 1.** Effect of chronic low-dose L-NAME treatment on blood pressure of Wistar and BHR. Results are presented as Mean±SEM. \**p*<0.05 vs. control. Abbreviations: B – basal value.

tion of the femoral artery in Wistar without affecting the constrictor response in BHR (Table 1).

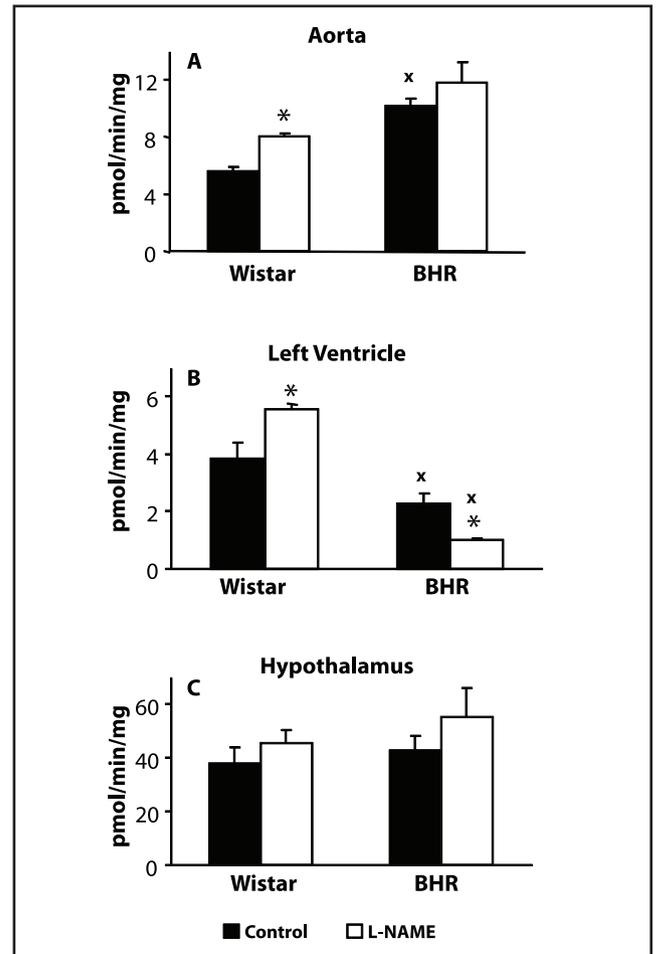
## DISCUSSION

We have shown that chronic treatment of BHR with a low dose (1.5 mg/kg/day) of NOS inhibitor L-NAME induces sustained blood pressure elevation and left ventricular hypertrophy associated with the decrease in NOS activity in the left ventricle. Additionally, no changes of NOS activity and vascular wall function of the femoral artery were observed in BHR. These findings were different from those observed in Wistar, where elevated NOS activity was found in the aorta and LV together with improvement of vascular function.

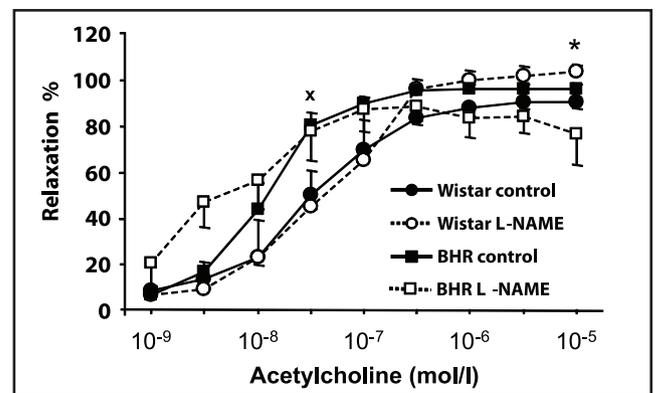
Several studies opened the question of negative feedback effect of NO to its synthesizing enzyme. The cases investigated dealt with the impact of exogenous NO donors on either NOS enzymatic activity (Buga *et al.*, 1993; Assreuy *et al.*, 1993; Rogers & Ignarro, 1992; Griscavage *et al.*, 1994, 1995) or expression of individual isoforms (Park *et al.*, 1997; Vaziri & Wang, 1999). Additionally, it was found that pretreatment of pulmonary arterial rings with exogenous NO donor caused a marked decrease in relaxation response to all three electrical field stimulation, acetylcholine and bradykinin without a significant change in the response to NO donor itself (Buga *et al.*, 1993).

Afterwards, the attention turned to NOS expression and several observations were made to clarify the involvement of NO to these biological processes. NO was considered to affect NOS expression in feedback regulation (Sheffler *et al.*, 1995) perhaps via a cGMP-mediated process (Vaziri & Wang, 1999) and/or influencing NF $\kappa$ B (Colasanti *et al.*, 1995; Park *et al.*, 1997; Peng *et al.*, 1998; Aktan, 2004). It became clear, that NOS expression is limited rather by levels of the enzyme product i.e. NO, than by the NOS protein itself.

Logically, the question arose whether, if elevated levels of NO serve as NOS activity and/or expression inhibiting agent, a mild NOS inhibition and thus lowering the feedback signal would be able to set up new equilibrium maintained on higher level of NO. Luss *et al.*, (1994) described 1.5-fold increased expression of iNOS mRNA and protein *in vivo* in a model of chronic liver inflammation after 7-day-long treatment by N<sup>G</sup>-monomethyl-L-arginine (L-NMA) suggesting the loss of feedback mechanism on iNOS expression. Recently, Kojšová *et al.*, (2007) observed elevated NOS expression and activity in the heart of Wistar rats treated with L-NAME at the dose of 40 mg/kg/day for 7 weeks, which, however, were associated with elevated blood pressure and reduced central NO production. The results suggest that the effects of L-NAME may be time-, dose- and tissue-dependent. However, expression of NOS mRNA or protein may not reflect the functional state of enzyme, and similarly, elevated activity may not reflect NO bioavailability, especially in hypertension (Adler & Huang, 2004), due to



**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 Wistar and BHR. Results are presented as Mean $\pm$ SEM. \* $p$ <0.05 vs. control., <sup>x</sup> $p$ <0.05 vs. Wistar.



**Figure 3.** Effect of chronic low-dose L-NAME treatment on acetylcholine-induced vasorelaxation of the femoral artery of Wistar and BHR. Results are presented as Mean $\pm$ SEM. \* $p$ <0.05 Wistar L-NAME vs. Wistar control, <sup>x</sup> $p$ <0.05 BHR control vs. Wistar control.

uncoupling of the NOS dimer and the elevated reactive oxygen species production (Münzel *et al.*, 2005; Lassegue & Griendling, 2004, Pečivová *et al.*, 2006, Zúrová-Nedelčevová *et al.*, 2006).

As supposed, in our experiment the treatment with the given low dose of L-NAME enhanced the activity of NOS in homogenates of LV and aorta in normotensive strain perhaps due to adjustment of negative feedback balance to higher levels.

However, different situation occurred under the conditions of borderline hypertension, as no changes of NOS activity in aorta and attenuation of NOS activity in left ventricle were observed. These data suggest the different sensitivity of NOS/NO pathway to NOS inhibitors in hypertension (Sventek *et al.*, 1996) eliciting the different response of NO system to the same treatment. This is in accordance with the study by Sylvester (1997), who subjected Wistar-Kyoto (WKY) to L-NAME treatment of approximately 16.9 mg/kg/day (L-NAME solution of concentration 150 mg/l) and BHR rats to the same concentration for three weeks. Such L-NAME concentration induced significant elevation of BP in WKY which returned back to control values by two weeks after L-NAME withdrawal, while no decrease of blood pressure was observed in BHR.

Our observation of elevated NOS activity in the aorta of Wistar rats was accompanied with the augmentation of endothelium-dependent vasorelaxation and attenuation of serotonin- and noradrenalin-induced vasoconstriction of the femoral artery indicating enhanced NO bioavailability. This provides evidence that the functional regulation of NO system via partial reduction of negative feedback signal *in vivo* is possible – at least under physiological conditions. In BHR, no changes in NOS activity in the aorta and acetylcholine-induced relaxation responses of the femoral artery were observed after low-dose L-NAME treatment, suggesting that NO/cGMP signaling was not disturbed in the large arteries. Furthermore, the acetylcholine-induced relaxation was elevated in control BHR compared to control Wistar, arguing that the etiology of borderline hypertension is not due to impaired NO bioavailability in large arteries (Púzszerová *et al.*, 2007).

However, elevated basal BP in BHR was associated with the left ventricle hypertrophy and this was even increased after the L-NAME treatment, while no LV hypertrophy was observed in Wistar rats. The question whether accentuation of hypertrophy is the consequence of more heightened BP or of NO deficiency itself is hard to answer. In our previous experiment using the high dose of L-NAME (40 mg/kg/day) in normotensive rats, we found LV and aortic hypertrophy which were associated rather with NO deficiency than with elevated BP (Pecháňová *et al.*, 1997; Bernátová *et al.*, 1999a, b). On the other hand, some authors did not found hypertrophy of the left ventricle even when administering 50–100 mg/kg/day L-NAME dose (Matsubara *et al.*, 1998) if plasmatic renin activity was not elevated (Arnal *et al.*, 1993). The LV hypertrophy may be a beneficial compensatory mechanism to chronic pressure overload (Brancaccio *et al.*, 2003). On the other hand, the decreased NOS activity in the LV

is indicative of reduced levels of NO. It is well known that angiotensin II is one of the most important growth factors affecting cardiac myocytes (Sadoshima & Izumo, 1993). NO blockade could result in increased sensitivity to angiotensin II and so cause hypertrophy (Kato *et al.*, 1998). Moreover, NO possesses antiproliferative properties and these may antagonize the trophic action of angiotensin II (Devlin *et al.*, 1998). The absence of L-NAME-induced LV hypertrophy and enhanced NOS activity in normotensive rats in this experiment bear the evidence that the mechanisms may operate in this way.

Higher sensitivity to NOS inhibitors in hypertension was also examined in the work of Tucker *et al.*, (2000) who investigated the effect of 3-week-lasting inhibition of NOS with the doses 2, 5, 10 and 20 mg/kg/day in the brain of Wistar and genetically hypertensive rats and found several dissimilarities. While the dose of 2 mg/kg/day reduced NOS activity in one of five cerebral regions investigated in normotensive strain, in hypertensive rats, the same dose caused more than 50% decline of NOS but in distinct three regions. However, the authors did not observed any improvement of cerebral NOS activity at the abovementioned dose of L-NAME similarly to our experiment. The final absence of L-NAME effect on hypothalamic NO synthase in both Wistar and BHR in our experiment may be due to different feedback settings in central NO system or due to restoration of new balance on the same level after transient elevation of blood pressure in the third and the sixth week of treatment.

In conclusion, this study brought the evidence of different NO regulation and/or sensitivity to NO synthase inhibition in rats with positive family history of hypertension compared with normotensive rats. The question still remains, whether sustained elevation of blood pressure during the low-dose L-NAME treatment of BHR is the result of different recoverability of central BP regulation and would regenerate in a longer time period or whether it is simply a consequence of fact, that the dose of 1.5 mg/kg/day is still too high to rearrange the feedback relations. These dependences are unclear and need to be elucidated in further studies.

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