ORIGINAL ARTICLE

Chronic Stress Deteriorated Nitric Oxide Production in Wistar Rats Exposed to a Low Dose of L-NAME

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Abstract

The aim of this study was to investigate the effect of chronic crowding stress in condition of disturbed nitric oxide (NO) production by a low dose of NO synthase inhibitor N^G-Nitro-L-arginine methyl ester (L-NAME).

Male, 12 weeks old, Wistar rats were exposed to crowding stress (200 cm^2 per rat, 5 rats per cage), L-NAME treatment (1.5 mg/kg/day in drinking water) or their combination for 8 weeks. Control and L-NAME treated rats were kept 4 rats per cage (480 cm^2 per rat). Blood pressure (BP) was determined by tail-cuff method. Nitric oxide synthase activity was determined by conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline. Vascular function was investigated using Mulvany's myograph in isometric conditions.

Stress and L-NAME alone failed to affect BP at the end of experiment. However, combined L-NAME + stress exposure resulted in significant elevation of BP and left ventricular (LV) hypertrophy. Chronic stress failed to affect NOS activity in the hypothalamus, hypophysis, LV and aorta. Low-dose L-NAME-treatment paradoxically significantly elevated NO synthase activity in the aorta and LV, had no effect in the hypothalamus and reduced NO production in the hypophysis. Combined L-NAME + stress exposure reduced NO production in all tissues investigated. Acetylcholine-induced relaxation of the femoral artery was elevated in stressed and L-NAME-treated rats but significantly reduced in the L-NAME + stress group.

Results suggest that chronic stress can markedly deteriorate NO production and vascular function in conditions when NO production is disturbed by a low dose of L-NAME in normotensive rats.

Abbreviations:
N^G-nitro-L-arginine methyl ester (L-NAME); blood pressure (BP); left ventricle (LV); nitric oxide (NO); cardiovascular system (CVS); hypothalamic-hypophyseal-adrenal axis (HHA); body mass (BM)

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). Besides its role in hemodynamic control (Torok 2008), NO participates in regulation of cell proliferation and growth. In our previous experiments, chronic pharmacological reduction of NO synthesis with NO synthase inhibitor N^G-Nitro-L-arginine methyl ester (L-NAME) resulted in decreased locomotor activity (Halcak *et al* 2000), in metabolic alterations and hypertension (Bernatova

et al 1999b), reduced vasorelaxation (Bernatova *et al* 2002b) and elevated aortic wall thickness (Bernatova *et al* 1999a). Additionally, the reduction of NO synthesis led also to the remodeling of myocyte junctions (Tribulova *et al* 2002), angiogenesis, mitochondrial damage (Okruhlicova *et al* 2000; Tribulova *et al* 2000) and myocardial fibrosis (Babal *et al* 1997). Although the model of NO-deficient hypertension (or L-NAME-induced hypertension) is one of the most frequently used models of experimental hypertension in the last decade, there is little information on the effects of more than 4 week-lasting low-dose L-NAME-treatment (less than 2 mg/kg/day) in the cardiovascular system of rats. Recently, we have shown that a low dose of L-NAME can increase NO production in the left ventricle and aorta in normotensive rats but not in borderline hypertensive rats (Kopincova *et al* 2008).

Additionally, there is still conflicting data on the effect of chronic stress on cardiovascular system (CVS) and results of the individual studies differ depending on the stress model, duration and intensity of stressor, animal strain, gender, age etc (McDougall *et al* 2000; Grippo *et al* 2002; Andrews *et al* 2003). To investigate the effect of chronic stress on NO production, we used social stress produced by crowding because it evokes social-stress reactions with prominent psychosocial components mimicking emotional state alterations (Bugajski 1999). Although crowding is a relatively mild stressor, it affects signal transduction in hypothalamic-hypophyseal-adrenal axis (HHA) (Bugajski *et al* 2006; Gadek-Michalska *et al* 2005).

The finding that acute stress may enhance NO production in the neuroendocrine system of normotensive rats (Kawa *et al* 2002; Sanchez *et al* 1999; Leza *et al* 1998) suggests that NO may represent one of the stress-limiting systems. Recently, we have shown elevated NO synthase activity and nitrate/nitrite levels in the heart and aorta of Wistar-Kyoto rats exposed also to chronic stress (Bernatova *et al* 2007c). However, the influence of chronic social stress on NO production in CVS and HHA in conditions of impaired NO synthesis is unknown. The reduction of NO in the CVS, CNS and/or in the HHA could participate in development of hypertension while an increase of NO could represent a protective mechanism against stress-induced hypertension.

Thus, the purpose of this study was to investigate the effect of chronic stress produced by crowding on blood pressure, NO production and endothelial function in conditions when NO production was affected by a low dose of NO synthase inhibitor L-NAME in normotensive Wistar rats.

Material and Methods

Males, 12 weeks old Wistar rats, were randomly divided into control (C), stressed group (S), L-NAME (1.5 mg/kg/day in tap water) and the L-NAME + stress (LNS) group for eight weeks. Controls and L-NAME-treated rats were kept in groups of 4 rats/cage (35/55/20 cm, 480 cm^2/rat). Rats exposed to crowding stress or to L-NAME + stress were kept in groups of 5 rats/cage (25/40/15 cm), where their living-space was reduced to 200 cm^2/rat (Bernatova *et al* 2007a) for eight weeks. All rats were housed at 22–24 °C on a 12:12-h dark-light cycle (07.00–19.00h lights on) and had food and water (or L-NAME solution) *ad libitum*. All procedures used in this study were approved by the State veterinary and food committee 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) and heart rate (HR) were determined before experiment (basal) and after the 1st, 3rd, 6th and 8th week of experiment. After eight weeks of experiment, the rats were killed by decapitation and body mass (BM) as well as the wet mass of the left ventricle (LV) were determined for calculation of its relative mass (LV/BM).

NO synthase activity was measured in the 20% tissue homogenates of the aorta, left ventricle, hypothalamus and hypophysis by determination of [^{3}H]-L-citrulline formation from [^{3}H]-L-arginine (Amersham, UK), as described previously (Bernatova *et al* 2007b). NO synthase activity was expressed as pmol/min/mg of proteins.

Vascular function was investigated in the femoral arteries, which 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 (Puzserova *et al* 2006). Endothelium-dependent 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. Average relaxation was calculated on the basis of individual dose-response curves. The extent of relaxation was expressed as the percentage of pre-contraction.

Statistical analysis

Data were analyzed using two-way ANOVA followed by Duncan's post-hoc test. Values were considered to differ significantly when $p<0.05$. All results are presented as mean ± SEM.

Results

Basal BP of all rats before experiment was 111±3 mm Hg. Crowding alone failed to affect BP (***Figure 1A***). Low dose of L-NAME resulted in a transient elevation of BP vs. basal value after the 3rd and 6th week of treatment by approximately 11% *vs.* control value ($p<0.05$). However, normalization of BP was observed at the end of

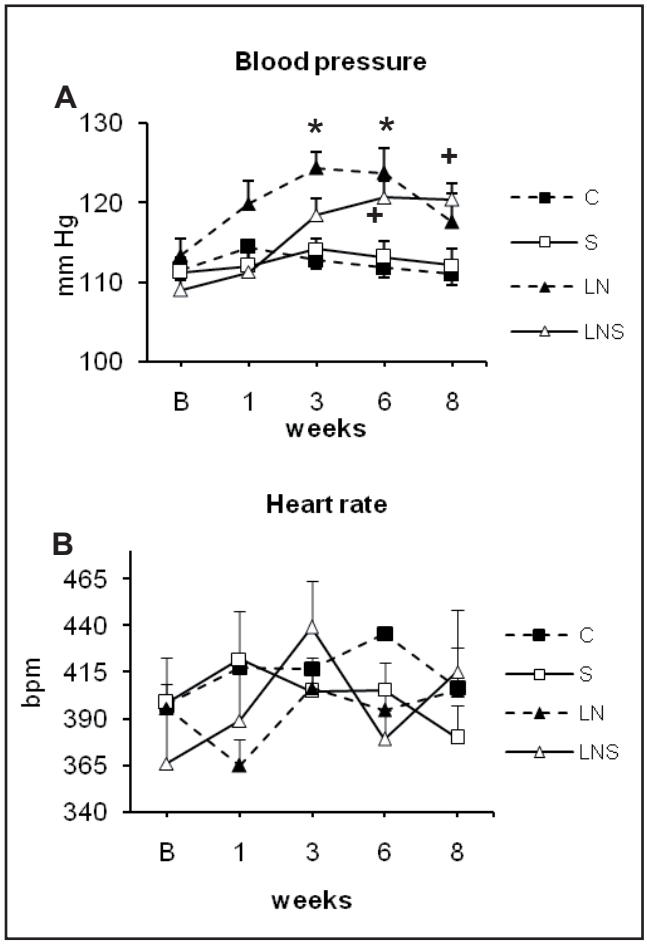

Fig. 1. The effect of stress, L-NAME (1.5 mg/kg/day) and their combination on blood pressure (A) and heart rate (B) of Wistar rats. Abbreviations: controls (C), crowding stress (S), L-NAME (LN), combination of L-NAME + stress (LNS), basal measurement (B). Results are mean ± SEM, *p<0.05 LN *vs.* control, ^{+}p<0.05 LNS *vs.* control.

experiment. Simultaneous exposure of rats to crowding and a low dose of L-NAME resulted in the significant elevation of BP after the 6th and 8th week of exposure.

Basal HR of all rats before experiment was 403±4 bpm and it was not changed significantly in course of experiment in any group investigated (***Figure 1B***)

Relative mass of the LV of control (1.29±0.03 mg/g), stressed (1.29±0.05 mg/g) and L-NAME treated (1.32±0.04 mg/g) rats did not differ significantly. However, simultaneous exposure to L-NAME + stress led to significant elevation of LV/BM ratio *vs.* the control group (1.43±0.04 mg/g, p<0.05)

Basal NO synthase activity in the hypothalamus, hypophysis, aorta and LV were 38.2±5.8, 45.3±7.6, 5.6±0.3 and 3.8±0.5 pmol/min/mg (***Figures 2A–D***). Stress itself failed to affect NO synthase activity in all tissues investigated. Chronic low-dose L-NAME-treatment led to the significant elevation of NO synthase activity in the aorta and LV by approximately 43% and 45% *vs.* control value (p<0.05). No alterations were seen in the hypothalamus but reduced NO production was seen in the hypophysis by approximately 66% *vs.* control (p<0.05). Interestingly, simultaneous exposure to stress and L-NAME resulted in significant reduction of NO synthase activity in all tissues investigated as compared to the control values.

Acetylcholine-induced relaxation of the femoral artery (***Figure 3A***) observed in the L-NAME-treated was significantly higher than that in control rats. Stress alone also improved vasorelaxation vs. control. However, combined exposure to the L-NAME + stress reduced acetylcholine-induced relaxation *vs.* control. Accordingly, the average relaxation, which was significantly elevated in both L-NAME and stress groups, was reduced significantly in the L-NAME + stress group *vs.* control (Figure 3B).

Discussion

This study brought several interesting results. The most interesting finding of this study was that NO synthase inhibitor L-NAME can paradoxically activate NO production in the heart and aorta of normotensive rats when it is administered in a low dose and for a long time. This, however, was not observed in the hypothalamus and hypophysis. Additionally, the NO production in the heart, aorta, hypothalamus and hypophysis was significantly inhibited by simultaneous crowding stress exposure. Reduced NO production in rats co-exposed to stress and L-NAME resulted in the elevation of BP, LV hypertrophy and endothelial dysfunction.

Several studies addressed the role of NO in regulation of cardiac and vascular functions using pharmacological inhibition of NO production. Using of high dose of L-NAME (50–100 mg /kg/day) for 6–8 weeks increased BP by about 40% at the end of treatment (Arnal *et al* 1992; Ribeiro *et al* 1992; Kristek *et al* 1996). Similar effect of L-NAME on BP was observed also in our experiments using 40 mg/kg/day already after the 1st day of treatment (Pechanova *et al* 1997). Lower dose of L-NAME (10 mg/kg/day) for 6–8 weeks elevated BP by about 25–36% (Arnal *et al* 1992; Delacretaz *et al* 1994). Dose of L-NAME 2 mg/kg/day had no significant effect on BP of normotensive rats during 7 days of treatment (Ralay *et al* 2004). Mechanisms responsible for the increase of BP in L-NAME-induced hypertension are associated with the alterations in several blood pressure-regulating systems. Several authors observed elevation of vasocontractility and attenuation of vasorelaxation in the different parts of the vascular tree, effect of circulating angiotensin II and increased sympathetic activity in L-NAME treated rats (Bernatova *et al* 1996; Holecyova *et al* 1996; Zicha *et al* 2001; Jover *et al* 2001; Kunes *et al* 2004).

In this study, using of a low dose of L-NAME (1.5 mg/kg/day) resulted in a transient mild elevation of BP after 3 weeks of treatment while an extension of treatment on 8 weeks resulted in normalization of BP. This was associated with an unexpected activation of

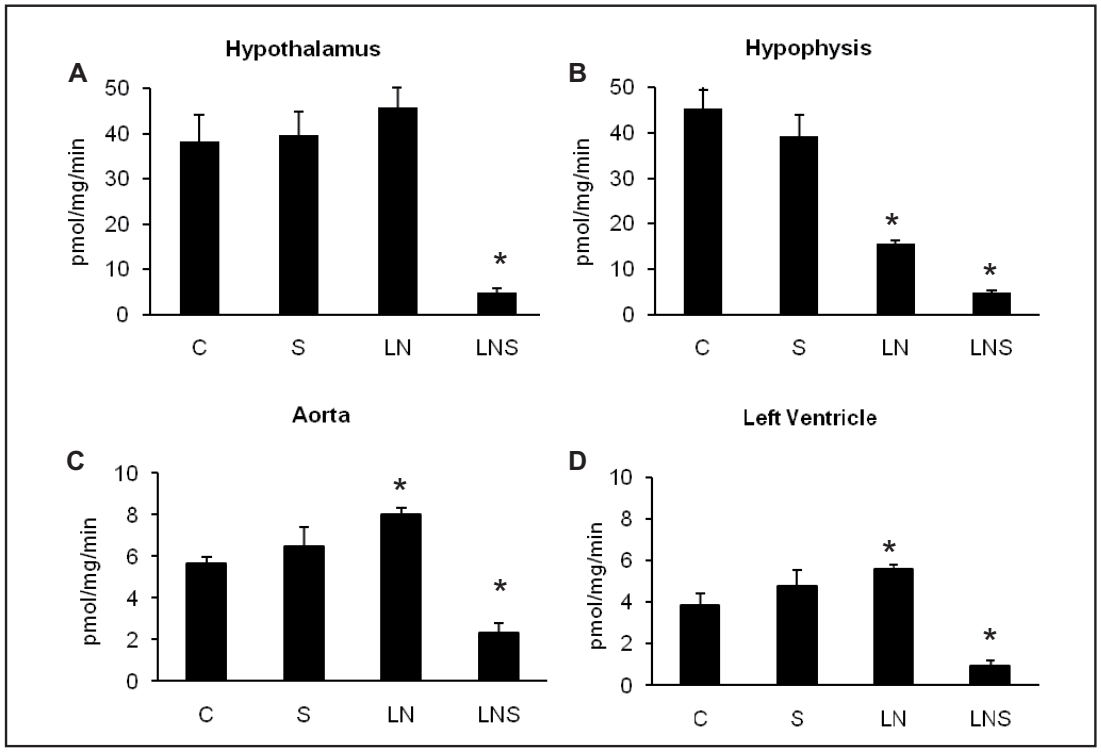

Fig. 2. The effect of stress, L-NAME (1.5 mg/kg/day) and their combination on nitric oxide synthase activity. Abbreviations: controls (C), crowding stress (S), L-NAME (LN), combination of L-NAME + stress (LNS). Results are mean±SEM, *p<0.05 *vs.* control.

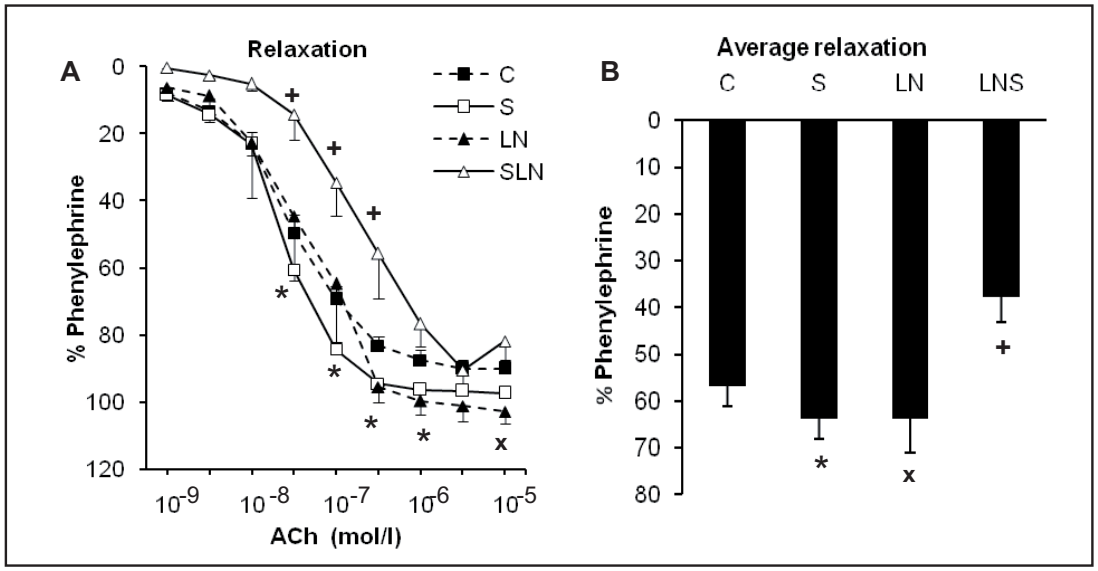

Fig. 3. The effect of stress, L-NAME (1.5 mg/kg/day) and their combination on dose-dependent responses (A) and average values (B) of acetylcholine-induced relaxation in the femoral artery. Abbreviations: controls (C), crowding stress (S), L-NAME (LN), combination L-NAME + stress (LNS). Results are mean±SEM, *p<0.05 stress *vs.* control, ^{x}p<0.05 LN *vs.* control, ^{+}p<0.05 LNS *vs.* control.

NO synthase in the heart and aorta. Moreover, in agreement with these findings we observed an improvement of endothelium-dependent vasorelaxation of the femoral artery. All these results led us to the hypothesis that adequately long-term administration of a low dose of L-NAME could provide a useful tool to activate NO production, which could be used to prevent development of social stress-induced hypertension. Mechanism for activation of NO production by a low dose of L-NAME may be associated with a transient mild decrease of NO levels in the tissue, which may in turn lead to activation of NO synthase activity and/or expression. Such negative feedback between NO and NO synthase expression was observed for inducible

(Park *et al* 1997), endothelial (Grumbach *et al* 2005) and also for neuronal NO synthase (Vickroy & Malphurs 1995). However, in the hypothalamus no change in NO production was observed and even reduced NO synthase activity was seen in the hypophysis after chronic low-dose L-NAME-treatment. This suggests differences in negative feedback regulation of neuronal NO synthase supposedly due to the higher affinity of this isoform to L-NAME (Boer *et al* 2000).

Regarding stress, there are still conflicting data as to the nature of cardiovascular changes induced by stress (Andrews *et al* 2003; Bernatova *et al* 2002a; Williams *et al* 1993; Lemaire & Mormede 1995). Although crowding stress alone had no effect on BP and NO synthesis of normotensive rats in this study, it induced the development of hypertension in rats with a positive family history of hypertension (Bernatova *et al* 2007a). The effect of various stressors to central and peripheral NO production is also unclear. As mentioned above acute stress can increase NO production in the selected parts of HHA and thus L-Arg/NO system can act as antistress system (Stefano *et al* 2006). In this study however, we did not observe elevated NO production in crowded rats in any tissue investigated. Similarly, chronic crowding failed to affect NO production in the cortex, hippocampus, striatum and cerebellum of Wistar rats (Moiseeva *et al* 2009) suggesting no effect of crowding on the nitrergic system of the brain.

Interestingly, the combination of chronic stress with low dose of L-NAME led to massive decrease of NO production. This suggests that although L-NAME alone improved NO synthesis in normotensive rats at least in the CVS, its combination with crowding stress had negative influence in CVS as well as in the selected parts of HHA axis. Both of them can participate on the elevation of BP. On the other hand, the elevation of BP without changes in HR suggests that peripheral vascular changes (i.e. elevated vascular resistance) rather than central mechanisms were responsible for elevation of BP. This is in agreement with the finding of endothelial dysfunction only in rats co-exposed to L-NAME + stress.

Surprisingly, the elevation of BP in the L-NAME + stress group was relatively mild as compared to the model of NO-deficient hypertension, inspite of comparable degree of NO synthase inhibition (Bernatova *et al* 1999b). Results suggest that other pressoric systems, for example the renin-angiotensin system, could be affected by a high dose of L-NAME (Bernatova *et al* 1999b) leading to a rapid and more pronounced elevation of BP in association with structural alterations in the heart and blood vessels (Babal *et al* 1997), which was not observed after low-dose L-NAME-treatment (Bernatova *et al* 2007b).

On balance then, data showed that chronic administration of the low dose of L-NAME can activate NO synthesis in the heart and aorta of normotensive rats but not in the hypothalamus and hypophysis. However, simultaneous exposure to social stress in conditions, when NO production is disturbed by a low dose of L-NAME, resulted in massive reduction of NO synthesis associated with the elevation of BP, LV hypertrophy and endothelial dysfunction. Thus, the results suggest that chronic stress can markedly impair NO production and vascular function in conditions when NO production is slightly disturbed by a low dose of NO synthase inhibitor in normotensive rats.

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