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 NG-Nitro-L-arginine methyl ester (L-NAME). Male, 12 weeks old, Wistar rats were exposed to crowding stress (200 cm² 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 cm² per rat). Blood pressure (BP) was determined by tail-cuff method. Nitric oxide synthase activity was determined by conversion of [3H]-L-arginine to [3H]-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: N⁶-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 N⁶-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 on the stress model, duration and intensity of stressor, and results of the individual studies differ depending on the effect of chronic stress on CVS) (Kawa et al 2002). Motivational rats but not in borderline hypertensive rats (Kopincova et al 2008). 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 [3H]-L-citrulline formation from [3H]-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 (10⁻⁸–10⁻⁵ 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.

**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 cm²/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 cm²/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).

**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...
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
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
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 anti-stress 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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