Original Article

Chronic *N*-Acetylcysteine Administration Prevents Development of Hypertension in *N*^ω-Nitro-L-Arginine Methyl Ester-Treated Rats: The Role of Reactive Oxygen Species

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The aim of this study was to evaluate the production of superoxide anions as well as their role in the induction and/or maintenance of high blood pressure in rats with *N*^ω-nitro-L-arginine methyl ester (L-NAME)-induced hypertension. In the preventive study, we compared adult Wistar rats treated with L-NAME for 4 weeks with L-NAME-treated rats that were simultaneously given *N*-acetylcysteine (NAC) in their drinking water. Basal blood pressure, superoxide production, conjugated dienes concentration and NO synthase (NOS) activity were measured at the end of the experiment. Chronic NOS inhibition by L-NAME treatment increased blood pressure, enhanced superoxide production in the aorta and elevated the concentration of conjugated dienes in the heart and kidney. All these changes were prevented by simultaneous NAC administration, which augmented NOS activity in L-NAME-treated rats. In the therapeutic study, the effects of chronic NAC treatment were studied in rats with established hypertension which developed during 4 weeks of L-NAME administration. The blood pressure effects of chronic NAC treatment in established L-NAME hypertension were only moderate, although this treatment also restored NOS activity and lowered conjugated dienes in the heart and kidney. Since chronic NAC treatment had better preventive than therapeutic effects, it seems that reactive oxygen species play a more important role in the induction than in the maintenance of L-NAME hypertension. (*Hypertens Res* 2005; 28: 475–482)

Key Words: *N*^ω-nitro-L-arginine methyl ester, nitric oxide synthase, superoxide anions, conjugated dienes, *N*-acetylcysteine

Introduction

It is well known that oxidative stress plays an important role in the pathogenesis of hypertension (for review see Berry *et al.* (1) and Wilcox (2). Endothelial dysfunction due to

increased oxidative stress is also characteristic of human hypertension (3). Most of the experimental evidence has been obtained in spontaneously hypertensive rats (SHR), in which enhanced production of reactive oxygen species (ROS) has repeatedly been reported (4–6). The acute administration of various ROS scavengers such as tempol or melatonin lowers

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Fig. 1. Preventive study. Mean arterial pressure (MAP) as well as NO synthase (NOS) activity in the left ventricle and kidney of controls (C), L-NAME-treated rats (L) and rats treated simultaneously with L-NAME and N-acetylcysteine (L+N). Data are the means $\pm$ SEM. *Indicate significant differences ($p < 0.05$) of L-NAME-treated rats from control rats, whereas full dots represent significant differences ($p < 0.05$) between L and L+N animals.

Table 1. BW and Relative Organ Weight in Control Rats, L-NAME-Treated Rats and Rats Treated Simultaneously with L-NAME and NAC Which Were Involved in Either “Preventive Study” or “Therapeutic Study”

	Controls	L-NAME rats	NAC-treated L-NAME rats
Preventive study	<i>n</i> =8	<i>n</i> =8	<i>n</i> =8
BW (g)	333 $\pm$ 10	331 $\pm$ 6	304 $\pm$ 9
Heart weight (mg/100 g BW)	222 $\pm$ 6	233 $\pm$ 3	233 $\pm$ 4
Kidney weight (mg/100 g BW)	338 $\pm$ 5	339 $\pm$ 8	446 $\pm$ 14*
Therapeutic study	<i>n</i> =7	<i>n</i> =7	<i>n</i> =7
BW (g)	364 $\pm$ 13	368 $\pm$ 20	371 $\pm$ 8
Heart weight (mg/100 g BW)	215 $\pm$ 4	221 $\pm$ 11	212 $\pm$ 6
Kidney weight (mg/100 g BW)	305 $\pm$ 7	311 $\pm$ 13	326 $\pm$ 9

Data are means $\pm$ SEM. *Significantly different ($p < 0.01$) from all other groups. L-NAME, *N*^ω-nitro-L-arginine methyl ester; NAC, N-acetylcysteine; BW, body weight.

blood pressure of SHR (7, 8). Moreover, chronic therapy with various antioxidants—including vitamin E (9, 10), vitamin C (11), tempol (12, 13), melatonin (14, 15), and N-acetylcysteine (NAC) (16, 17)—has been shown to attenuate the development of spontaneous hypertension, decreasing blood pressure by about 15–20%. On the other hand, high blood pressure *per se* also seems to be a cause of endothelial dysfunction (18–20).

There is only scant information on the role of ROS in hypertension elicited by chronic NOS inhibition with *N*^ω-nitro-L-arginine methyl ester (L-NAME), although increased oxidative stress has been reported in L-NAME hypertensive rats (21–24). Surprisingly, only a few attempts have been made to influence the development of this type of hypertension by various chronic antioxidant treatments (24, 25). However, such treatment failed to lower blood pressure even when the administration of antioxidants lowered superoxide production. Increased superoxide production in the kidney of L-NAME hypertensive rats is mediated by NADPH oxidase (24) and is attenuated by chronic administration of vitamin E

or angiotensin II type 1 receptor (AT₁R) blocker, although only the latter drug lowers the blood pressure of these animals (26). It should also be noted that the elevated superoxide production in NO-deficient animals might be prevented by an AT₁R blocker given in low, subantihypertensive doses (23). Usui *et al.* (22) demonstrated enhanced superoxide generation in the aorta of L-NAME hypertensive rats, and this enhanced production could be decreased by simultaneous treatment of animals with L-arginine, allopurinol, ebselen or NAC for 1 week, although none of these co-treatments lowered the high blood pressure of NO-deficient rats. Thus the relationship between ROS and blood pressure has not been fully clarified. Moreover, the endothelium may not be the only source of superoxide production; other sources, *e.g.*, vascular smooth muscle cells or monocytes infiltrating the vascular wall, must also be considered (25–27).

The aims of our study were to evaluate 1) whether the augmentation of antioxidant capacity by chronic administration of NAC would affect superoxide production, formation of conjugated dienes (CD) and hypertension development in rats

Fig. 2. Preventive study. Reactive oxygen species (ROS) measured by lucigenin chemiluminescence in the abdominal aorta of controls (C), L-NAME-treated rats (L) and rats treated simultaneously with L-NAME and N-acetylcysteine (L+N). Symbols are as described in Fig. 1.

treated with L-NAME for 4 weeks; 2) whether superoxide anions play any significant role in the maintenance of elevated blood pressure in rats with established L-NAME hypertension; and 3) whether chronic NAC administration exerts therapeutic effects in rats with already developed L-NAME hypertension.

Methods

Animals

Adult male rats aged 12 weeks (Institute of Physiology AS CR, Prague, Czech Republic) were housed under standard laboratory conditions (temperature $23 \pm 1^\circ\text{C}$, 12-h light-dark cycle), drank tap water *ad libitum* and were fed a standard ST1 diet (containing 1% NaCl). All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Physiology AS CR, and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use.

Preventive NAC Study

Wistar rats were divided into three experimental groups ($n=8$ each): 1) intact controls; 2) rats treated with L-NAME (60 mg/kg/day); and 3) rats treated simultaneously with L-NAME and NAC (1.5 g/kg/day, 20 g/l drinking fluid). After 4 weeks of the experiment, blood pressure was measured by direct carotid puncture under light ether anesthesia (28), and the aorta, heart and kidney were dissected for biochemical measurements (see below).

Acute Tempol Study

An additional group of adult Wistar rats was treated with L-NAME (60 mg/kg/day) in the drinking water for 4 weeks.

Fig. 3. Preventive study. Conjugated dienes (CD) concentrations in the left ventricle and kidney of controls (C), L-NAME-treated rats (L) and rats treated simultaneously with L-NAME and N-acetylcysteine (L+N). Symbols are as described in Fig. 1.

One day before blood pressure measurement, polyethylene catheters were inserted into the left carotid artery and jugular vein and exteriorized in the interscapular region. Blood pressure was recorded in conscious animals after 24-h recovery using a Statham pressure transducer and multichannel Hewlett-Packard recorder. The blood pressure response to acute tempol administration (25 mg/kg i.v.) was determined in 9 rats with established L-NAME hypertension and in 6 intact normotensive controls.

Therapeutic NAC Study

The effects of chronic NAC treatment (20 g/l drinking water for 4 weeks) were examined in Wistar rats that were made hypertensive by prior L-NAME treatment for 4 weeks. Untreated animals served as controls ($n=7$ in each group). At the end of the experiment, blood pressure, superoxide production, NO synthase (NOS) activity and CD were measured as in the "Preventive Study."

Superoxide Production in the Aorta

The aortas were dissected and carefully cleaned of periadventitial tissue. Molecular O_2^- was measured in aortic segments by means of lucigenin chemiluminescence using a liquid scintillation counter (Tricarb; Hewlett Packard, Andover, USA) as described earlier (29, 30).

Total NOS Activity

The kidney and left ventricle were excised, weighed and homogenized for determination of NOS activity. Total NOS activity was determined in crude homogenates by measuring the formation of L-[^3H]citrulline (L-Cit) from L-[^3H]arginine (Amersham Biosciences, Buckinghamshire, UK) as previ-

Fig. 4. Preventive study. The relationships of aortic reactive oxygen species (ROS) production to systolic blood pressure (SBP) (left panel) or conjugated dienes level (CD) (right panel) in rats of this study ($n = 23$).

ously described by Bredt and Snyder (31) with minor modifications (32).

Conjugated Dienes Concentration

The concentration of CD was determined in lipid extracts of the heart (left ventricle) and kidney homogenates according to Kogure *et al.* (33). Briefly, after chloroform evaporation under an inert atmosphere and addition of cyclohexane, the CD concentration was determined using a GBC 911A spectrophotometer ($\lambda = 233$ nm; Bio-Rad, Hercules, USA).

Statistical Analysis

Results were expressed as the means $\pm$ SEM. The statistical differences were evaluated by one-way analysis of variance followed by the least significant difference test. Values of $p < 0.05$ were considered to indicate statistical significance. The relationships between particular parameters were evaluated by correlation analysis.

Results

Preventive Study

Chronic treatment with NAC completely prevented the development of L-NAME-induced hypertension (Fig. 1). NOS activity was reduced by 25% in the left ventricle and by 33% in the kidney of L-NAME-treated rats. This reduction was abolished by simultaneous NAC treatment (Fig. 1). There were no significant differences in body weight or relative heart weight (HW/BW ratio) among the study groups (Table 1). Kidney weight was considerably increased by NAC treatment (probably due to metabolic acidosis caused by chronic NAC administration); the same effect was observed in NAC-treated normotensive rats (data not shown).

Superoxide production in the abdominal aorta was enhanced in L-NAME-treated animals, and this rise was prevented by NAC treatment (Fig. 2). Similarly, CD were elevated in the left ventricle and kidney of L-NAME hyperten-

sive rats, and this elevation was completely prevented by the chronic NAC treatment (Fig. 3). There were positive correlations between superoxide production and CD level or systolic blood pressure (SBP) (Fig. 4). The same was true for the relationship of SBP to CD in the left ventricle ($r = 0.755$, $p < 0.001$) and kidney ($r = 0.888$, $p < 0.001$).

Acute Tempol Study

In this study, the mean arterial pressure of L-NAME hypertensive rats reached 162 ± 4 mmHg, whereas it was 121 ± 3 mmHg in the normotensive controls. The acute administration of tempol (a superoxide dismutase mimetic) significantly lowered blood pressure in L-NAME-treated but not in control animals (Δ MAP: -14 ± 3 vs. -5 ± 2 mmHg; $p < 0.05$), indicating that a moderate contribution of superoxides to the blood pressure maintenance of L-NAME hypertensive rats.

Therapeutic Study

The administration of L-NAME for 8 weeks induced hypertension, increased the aortic production of superoxides (Fig. 5), reduced NOS activity in the kidney and heart, and elevated CD in the organs (Fig. 6). There were no significant changes in body weight or organ weight (Table 1). These effects were similar to those elicited by 4-week L-NAME administration in the "Preventive Study." Nevertheless, chronic NAC treatment performed during the last 4 weeks of L-NAME administration, when hypertension was already established, had only a non-significant effect on blood pressure and aortic superoxide production (Fig. 5), although it normalized NOS activity and CD in both the kidney and heart (Fig. 6). Thus the therapeutic effects of chronic NAC treatment are inferior to its preventive effects.

Discussion

The present study demonstrated that 1) the production of ROS was enhanced in rats with L-NAME hypertension; 2) these superoxides significantly contributed to the actual mainte-

Fig. 5. Therapeutic study. Mean arterial pressure (MAP, left panel) and reactive oxygen species (ROS) measured by lucigenin chemiluminescence in the abdominal aorta (right panel) of control rats (C), rats treated with L-NAME for 8 weeks (L) and rats treated with L-NAME for 8 weeks and with N-acetylcysteine for the last 4 weeks (L+N). *Indicate significant differences ($p < 0.05$, $p < 0.01$) from control rats.

nance of elevated blood pressure in rats with established L-NAME hypertension; 3) the development of L-NAME hypertension was completely prevented by chronic NAC treatment; and 4) the therapeutic effects of NAC in rats with established L-NAME hypertension were less pronounced than the preventive effects of NAC on the development of L-NAME hypertension.

Our data confirmed previous reports (22–24) that superoxide production is increased in blood vessels of L-NAME hypertensive rats. Increased ROS production causes subsequent augmentation of lipoperoxidation, as evidenced by the elevated levels of CD in the heart and kidney of our L-NAME hypertensive animals. The lowering of superoxide levels by NAC also decreased the levels of CD. The relationship between overall superoxide production and CD formation was documented by a positive correlation between aortic lucigenin chemiluminescence and the levels of CD in the heart or kidney.

Our parallel experiments (34) indicated that chronic NAC administration exerted a more pronounced antioxidant effect and a more efficient antihypertensive action than chronic tempol treatment. It should be noted that both NAC and tempol enhanced the activity of NOS, probably by protecting its essential cofactor, tetrahydrobiopterin, from oxidation by the excess superoxide. This is in agreement with previous reports that antioxidant treatment with NAC or melatonin augmented NO-dependent vasodilation in SHR (14, 35). There are several possible explanations for the finding that NAC had a more pronounced antihypertensive effect than tempol. The former drug augments the levels of reduced glutathione, which is a part of the natural antioxidant defense of the organism. Vaziri *et al.* (36) reported the rapid induction of pronounced hypertension in normal rats in which glutathione

Fig. 6. Therapeutic study. NO synthase activity (NOS, upper panels) and conjugated dienes concentrations (CD, lower panels) in the kidney (left panels) and left ventricle (LV) (right panels) of control rats (C), rats treated with L-NAME for 8 weeks (L) and rats treated with L-NAME for 8 weeks and with N-acetylcysteine for the last 4 weeks (L+N). *Indicate significant differences ($p < 0.05$, $p < 0.01$) from control animals, whereas full dots represent the differences ($p < 0.05$, $p < 0.01$) between L and L+N rats.

synthase was chronically inhibited. In another study, acute inhibition of glutathione synthase induced a greater decrease of glutathione concentration in L-NAME-treated rats than in controls (37). Moreover, NAC has been shown to protect the sulfhydryl groups of NOS from destruction by free radicals and thus to maintain its activity (38). It has also been hypothesized that NO reacts with an –SH group of sulfhydryl donors to form S-nitrosothiols, which directly activate guanylate cyclase (39). In this regard, it has been reported that NAC augments flow-induced endothelium-dependent relaxation *in vitro* (40) and enhances the hypotensive effect of acetylcholine *in vivo* (41). On the other hand, tempol, a mimetic of superoxide dismutase, converts superoxide to hydrogen peroxide, the detoxification of which would require increased catalase activity. Lower antihypertensive effects of tempol compared to NAC were also observed when we studied the role of ROS in the development of spontaneous hypertension (42).

However, the peripheral interaction of ROS with NO-

dependent vasodilation is not the only blood pressure-regulating mechanism that can be targeted by antioxidant therapy. As we mentioned in our previous paper (43), enhanced sympathetic vasoconstriction in rats with L-NAME hypertension results from the inhibition of NOS in the rostral ventrolateral medulla (44). It should be noted that the inhibition NOS in L-NAME-treated rats is more pronounced in the central nervous system than in the cardiovascular apparatus (Pechánová *et al.*, under submission). It is evident that the enhanced NO bioavailability in the rostral ventrolateral medulla will lower high blood pressure by the attenuation of sympathetic outflow. This can be achieved by the augmentation of NO production, *e.g.*, by means of eNOS gene transfer (45), or by the reduction of elevated superoxide levels, *e.g.*, by means of SOD gene transfer (46). The same central effects might be exerted by chronic treatment with certain antioxidants. Indeed, we have demonstrated that chronic NAC treatment completely prevented the development of salt hypertension in Dahl rats by a strong attenuation of the sympathetic blood pressure component (47).

The present study also demonstrated that chronic NAC administration had only a borderline therapeutic effect in rats with established L-NAME hypertension, although the same treatment completely prevented the development of L-NAME hypertension when NAC treatment was applied simultaneously with L-NAME administration from the very beginning of this hypertensive regimen. This cannot be ascribed to the different degree of hypertension, because maximal blood pressure elevation is achieved within 1–2 weeks of L-NAME treatment (32, 43). Thus it seems that ROS participate in the development rather than in the maintenance of this form of experimental hypertension. The remodeling of resistance vessels due to extensive fibrosis (48–51) seems to be the most probable mechanism maintaining the elevated blood pressure in chronic stages of L-NAME hypertension. It is difficult to imagine that lowering of superoxide levels and/or augmented NO bioavailability could efficiently counteract the structurally based increase of peripheral resistance, *i.e.*, the already developed morphological changes of resistance vessels. Our conclusions on the mechanisms of the early and late phases of L-NAME hypertension are strongly supported by our findings in SHR, in which chronic NAC treatment considerably attenuated the development of spontaneous hypertension in weanling prehypertensive animals, but had no significant effect on blood pressure of adult SHR with established hypertension (42).

It can be concluded that chronic NAC administration prevented the development of L-NAME-induced hypertension, indicating the important role of ROS in its pathogenesis. However, reactive oxygen species seem to play a less important role in the maintenance of this form of experimental hypertension.

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