INTRODUCTION

Although pathophysiological mechanisms of genetic hypertension are still not completely understood, it is evident that sympathetic hyperactivity and/or endothelial dysfunction are responsible for a major part of blood pressure (BP) elevation seen in SHR (1-3). Despite numerous attempts to use antioxidants for prevention or therapy of genetic hypertension (for reviews see 4, 5), only moderate BP lowering effect was usually achieved.

Chronic administration of N-acetylcysteine (NAC), which is a free radical scavenger increasing NO bioavailability, prevented the development of high BP in young SHR (6) but had only negligible therapeutic effects on established hypertension in this rat strain (7). Chronic preventive NAC treatment also attenuated hypertension development in rats subjected to long-term inhibition of NO synthase (NOS) by L-NAME administration, whereas it had no major therapeutic effects on the established form of NO-deficient hypertension (8). Chronic NAC treatment of SHR increased total NOS activity by 32% in the brainstem and by 67% in the cerebellum. After the incubation of brainstem and cerebellum with SMTC there were no significant differences in NOS activity of NAC-treated rats compared to strain-matched controls. Taken together, NOS seems to be responsible for the increase of total NOS activity in the brain of SHR. SMTC inhibited 86% and 70% of NAC-induced increase of total NOS activity in the brainstem and cerebellum, respectively. Thus, nNOS is responsible not only for strain differences but also for NAC-induced increase of total NOS activity in the brain.

Key words: N-acetylcysteine, S-methyl-L-thiocitrulline, neuronal NOS, spontaneous hypertension, brainstem, cerebellum

CONTRIBUTION OF NEURONAL NITRIC OXIDE (NO) SYNTHASE TO N-ACETYLCYSTEINE-INDUCED INCREASE OF NO SYNTHASE ACTIVITY IN THE BRAIN OF NORMOTENSIVE AND HYPERTENSIVE RATS

The goal of our study was to determine a contribution of nNOS to the increase of brain NO synthase activity induced by chronic N-acetylcysteine (NAC) treatment. Young 4-week-old male Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were subjected to treatment with NAC (1.5 g/kg/day) for 8 weeks. At the end of experiment total NOS activity was determined in the brainstem and cerebellum with and without specific nNOS inhibitor S-methyl-L-thiocitrulline (SMTC, 10^-6 mol/l) by measuring the formation of L-[3H] citrulline from L-[3H] arginine. Chronic NAC treatment had no effect on blood pressure (BP) of WKY, while it attenuated BP increase in young SHR. Total NOS activity was increased in the brainstem of SHR compared to WKY, but this strain difference was abolished by SMTC. Chronic NAC treatment of SHR increased total NOS activity by 32% in the brainstem and by 67% in the cerebellum. After the incubation of brainstem and cerebellum with SMTC there were no significant differences in NOS activity of NAC-treated rats compared to strain-matched controls. Taken together, nNOS seems to be responsible for the increase of total NOS activity in the brain of SHR. SMTC inhibited 86% and 70% of NAC-induced increase of total NOS activity in the brainstem and cerebellum, respectively. Thus, nNOS is responsible not only for strain differences but also for NAC-induced increase of total NOS activity in the brain.
when administered in vivo (21), this nNOS inhibitor was chosen for the estimation of nNOS contribution in our in vitro assay of NOS activity in brain homogenates.

The aims of the present study were 1) to evaluate the effect of chronic NAC treatment on NOS activity during the development of high blood pressure in young SHR, and 2) to estimate the contribution of neuronal NO synthase (nNOS) to possible strain differences as well as to NAC-induced changes in the total NOS activity in brainstem and cerebellum of these animals. To achieve the second goal of our study we determined NOS activity also in the presence of a specific nNOS inhibitor S-methyl-thiocitrulline (SMTC).

**METHODS**

**Animals and treatment**

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. All the chemicals used were purchased from Sigma Chemicals Co. (Germany) except of [3H]-L-arginine (Amersham, UK).

Young 4-week-old male WKY (n=12) and SHR (n=15) were randomly divided into water drinking control groups and groups receiving N-acetylcysteine (NAC, 1.5 g/kg/day, 20 g/l) in tap water for 8 weeks. All animals were housed in the room with a stable temperature of 23±1°C and fed a regular pellet diet ad libitum. At the end of treatment, the blood pressure was determined by direct puncture of carotid artery under a light ether anesthesia. Thereafter the animals were sacrificed and body weight (BW) and heart weight (HW) were determined. Brainstem and cerebellum were dissected (17) and used for determination of total NOS activity.

**Total NO synthase activity**

Total NO synthase activity was determined in crude homogenates (Potter, teflon homogenizer) of the cerebellum and brainstem by measuring the formation of [3H]-L-citrulline from [3H]-L-arginine as previously described by Bredt and Snyder (22) with minor modifications (23). Briefly, 50 µl of crude homogenate of the brain part (7.5 mg of wet tissue) was incubated in the presence of 50 mmol/l Tris/HCl, pH 7.4, containing 1 µmol/l [3H]-L-arginine (specific activity 5 GBq/mmol, approx. 100000 d.p.m.), 0.5 mg/ml calmodulin, 0.5 mmol/l β-NADPH, 250 µmol/l tetrahydrobiopterin, 4 µmol/l FAD, 4 µmol/l flavin mononucleotide and 1 mmol/l Ca²⁺, in a total volume of 100 µl. Aliquots of the homogenate were also incubated in the presence of SMTC (10⁻⁶ mol/l). Fig. 1 shows dose-dependent inhibition of cerebellar NOS activity by increasing SMTC concentrations obtained in our preliminary experiments carried out in Wistar rats. The above concentration of SMTC has been chosen to demonstrate its predominant effect on nNOS because 10⁻⁵ mol/l SMTC seem to inhibit also the activity of other NOS isoforms (20). After a 30 min incubation at 37°C, the reaction was stopped (by adding 0.02 M Hepes containing 2 mM EDTA, 2 mM EGTA and 1 mM L-citrulline), the samples were centrifuged, and supernatants were applied to 1 ml Dowex 50WX-8 columns (Na⁺ form). L-citrulline was eluted with 2 ml of water and [3H]-L-citrulline radioactivity was determined by liquid scintillation counting. Total NO synthase activity was expressed as pkat/g of proteins.

**Statistical analysis**

Results were expressed as means ± SEM. The data were evaluated by means of one-way analysis of variance with Fisher least significant difference (LSD) post-hoc test. P<0.05 value was taken as a criterion of significant relationship.

**RESULTS**

SHR were characterized by increased blood pressure and higher relative heart weight compared to WKY controls (Table 1). Total NOS activity was increased in brainstem of SHR compared to WKY (4.4±0.4 vs. 3.3±0.1 pkat/g proteins, p<0.05), but this difference was not significant in the cerebellum (5.8±0.6 vs. 5.0±0.7 pkat/g proteins). These strain differences were due to the changes in SMTC-sensitive NOS activity, whereas no strain

![Fig. 1. Dose-dependent inhibition of NO synthase (NOS) activity by S-methyl-L-thiocitrulline (SMTC) in the homogenate of cerebellum from Wistar rats. Data are means±S.E.M. (n=4).](image-url)

**Table 1.** Blood pressure, body and heart weight in untreated and NAC-treated WKY and SHR rats

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>WKY-NAC</th>
<th>SHR</th>
<th>SHR-NAC</th>
</tr>
</thead>
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<tr>
<td>Number of animals</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>294±16</td>
<td>230±12*</td>
<td>297±5</td>
<td>272±4*</td>
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<tr>
<td>Systolic BP (mm Hg)</td>
<td>128±4</td>
<td>123±3</td>
<td>194±5*</td>
<td>165±4*</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>107±4</td>
<td>95±5</td>
<td>157±5*</td>
<td>134±5*</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>90±4</td>
<td>77±5</td>
<td>126±6*</td>
<td>105±6*</td>
</tr>
<tr>
<td>Heart weight (mg/100 g bw)</td>
<td>255±7</td>
<td>247±9</td>
<td>336±6*</td>
<td>318±5*</td>
</tr>
</tbody>
</table>

Data are means ± S.E.M. MAP - mean arterial pressure. Significantly different (p<0.05): * from WKY, # effect of NAC treatment.
Nitric oxide synthase (NOS) activity in the brainstem of untreated and NAC-treated Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). Total NOS activity (hatched column), SMTC-sensitive NOS activity (black column), and SMTC-insensitive NOS activity (white column). Data are means±S.E.M. SMTC was used in 10⁻⁶ M concentration. Significantly different (p<0.05): *- from WKY, #- effect of NAC treatment.

Nitric oxide synthase (NOS) activity in the cerebellum of untreated and NAC-treated Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). Total NOS activity (hatched column), SMTC-sensitive NOS activity (black column), and SMTC-insensitive NOS activity (white column). Data are means±S.E.M. SMTC was used in 10⁻⁶ M concentration. Significantly different (p<0.05): *- from WKY, #- effect of NAC treatment.

Chronic NAC treatment reduced blood pressure and heart weight in SHR only (Table 1). Chronic NAC treatment increased total NOS activity by 45% in the brainstem and by 38% in the cerebellum of Wistar-Kyoto rats. Similarly, NAC treatment of SHR increased total NOS activity by 32% in the brainstem and by 67% in the cerebellum. This effect was due to the rise of SMTC-sensitive activity. In contrast, no significant change in SMTC-insensitive NOS activity was found in brainstem or cerebellum of both strains (Figs. 2 and 3). Thus, in SHR SMTC inhibited 86% and 70% of NAC-induced increase of total NOS activity in the brainstem and cerebellum, respectively. Taken together, nNOS is responsible for increased total NOS activity in the brain of SHR as well as for NAC-induced changes of NOS activity.

**DISCUSSION**

Our study demonstrated higher SMTC-sensitive NOS activity in the brainstem of 12-week-old SHR compared to age-matched WKY rats. This difference in neuronal NOS activity was further augmented by chronic N-acetylcysteine treatment, which lowered blood pressure in SHR but not in WKY rats. Chronic NAC treatment augmented SMTC-sensitive NOS activity not only in the brainstem but also in the cerebellum. This effect was also present in WKY brain but to a smaller extent compared to the changes seen in SHR brain. Neuronal NOS plays not only an important role in the central control of sympathetic tone (see below) but also in the modulation of the response of hypothalamo-pituitary-adrenal axis to α-adrenergic or β-adrenergic stimulation (24).

The mechanisms of N-acetylcysteine-induced increase of NO bioavailability in the cardiovascular tissues and in the brain are not completely elucidated yet. NAC has been reported to posses potent antioxidant effects by both direct neutralization of reactive oxygen species (25) and increased GSH production (26). Using TEAC assay, the antioxidant activity of NAC was found to be comparable with the activity of Trolox (7). Furthermore, NAC treatment reduced NF-κB protein expression in SHR (6, 7) and aldosterone-salt hypertensive rats (27). Regarding GSH production, chronic NAC treatment increased GSH concentration in SHR rats without a parallel decrease in GSSG concentration, suggesting an elevated level of L-cysteine which is the rate-limiting component in GSH synthesis (26). Reduced level of reactive oxygen species may further lead to a protection of NO synthase from its uncoupling resulting in preserved or even increased NOS activity. Moreover, NAC was found to elevate eNOS protein expression in the heart and kidney (6). In isolated SHR femoral artery NAC elicited dose-dependent relaxation which was completely abolished in the presence of NO synthase inhibitor L-NAME (7). Being a thiol, NAC may also protect NO by forming S-nitrosothiols and thus prolonging the NO lifetime (24). The above mentioned antioxidant mechanisms were also considered in NAC-induced attenuation of hypoxia-induced downregulation of chemically and mechanically-induced cough (29).

These beneficial effects of NAC were described rather in the peripheral tissues. Nevertheless, antioxidant effects of NAC accompanied by improvement of NO pathway may operate within the brain as well. Recently, Arakawa and Ito (30) and Jayalakshmi et al (31) described neuroprotective effect of NAC which includes redox interactions, protection against oxidative stress-induced damage and against hypoxia. Moreover, using the cerebrovascular arteries Sury et al. (32) demonstrated that NAC was able to inhibit endothelin-1 upregulation induced by TNF-α. Mitogen and stress-activated protein kinase was involved in this inhibitory effect. Improvement of NO pathway within the central nervous system may further modulate sympathetic stimulation of peripheral arteries. Macarthur et al. (33) documented that chronic NAC administration reduced nerve-stimulated norepinephrine overflow but increased neuropeptide Y overflow in the mesenteric arterial bed from the SHR. This property of NAC was prevented by inhibiting NO synthase with L-NAME demonstrating that the effect of NAC was due to the preservation of NO pathway.
Our present findings on NAC-induced augmentation of SMTC-sensitive NOS activity in the brain of SHR seem to be compatible with the suggested inhibitory role of brain NO in the central control of sympathetic tone (10, 34). It was demonstrated at the level of rostral ventrolateral medulla that NO counterbalances angiotensin II-mediated rise of sympathetic outflow (35-37). Chronic NAC treatment of young SHR attenuated the development of hypertension (6) and this effect was ascribed to antioxidant and NOS-stimulating effects of this treatment. Girouard et al. (38) reported that NAC improves nitric oxide vasodilatation and attenuates alpha-adrenergic vasoconstriction in mesenteric beds of spontaneously hypertensive rats. Our study in salt hypertensive Dahl rats (39) indicated that blood pressure lowering effect of chronic NAC treatment was almost exclusively due to a profound reduction of sympathetic vasoconstriction which is greatly enhanced in this hypertensive model. It seems that the antioxidant effects of chronic NAC treatment might be even more important for blood pressure reduction than NAC-induced stimulation of NOS activity because chronic administration of NO donor - pentaerythrityl tetranitrate - did not attenuate hypertension development in young SHR (40).

On the basis of the above findings, we can suggest that NAC-induced elevation of neuronal NOS activity in the brainstem of SHR might significantly contribute to the observed blood pressure decrease by the reduction of sympathetic tone in SHR. This is further combined with the improvement of endothelial dysfunction and NO-dependent vasodilatation in peripheral tissues which were also reported to be impaired in SHR (41).

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