Effect of chronic N-acetylcysteine treatment on the development of spontaneous hypertension

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ABSTRACT

The imbalance between NO (nitric oxide) and ROS (reactive oxygen species) is an important factor in the development of hypertension. The aim of the present study was to determine the preventive and therapeutic effects of NAC (N-acetylcysteine) in SHRs (spontaneously hypertensive rats). Young and adult SHRs and WKY (Wistar–Kyoto) rats were treated with NAC (20 g/l in the drinking water). After 8 weeks of treatment, BP (blood pressure) and NOS (NO synthase) activity, conjugated dienes and GSH (reduced glutathione) in the kidney and left ventricle were determined. Protein expression of eNOS (endothelial NOS), inducible NOS and NF-κB (nuclear factor κB) were also determined in the left ventricle and kidney. Chronic NAC treatment partially attenuated the rise in BP in young SHRs (179 ± 6 compared with 210 ± 8 mmHg in untreated animals), but it had no significant effect on BP in adult SHRs. The antioxidant action of NAC, measured as a decrease of the concentration of conjugated dienes or inhibition of NF-κB expression, was greater in young than in adult SHRs. Similarly, eNOS protein expression was attenuated more in young than in adult SHRs, although NAC treatment increased NOS activity to a similar extent in both young and adult rats. In conclusion, both decreased ROS production and increased NOS activity appear to participate in the BP changes after NAC treatment in young SHRs. In adult SHRs with established hypertension, however, the secondary alterations (such as pronounced structural remodelling of resistance vessels) might attenuate the therapeutic effect of NAC.

INTRODUCTION

NO (nitric oxide) plays an important role in the maintenance of vascular tone, which affects the level of BP (blood pressure) [1–3]. In various models of hypertension, impaired endothelium-dependent relaxation has been described, implying endothelial dysfunction and an apparent decrease in the production of bioactive NO [4]. The increased release of endothelial vasoconstricting factors, such as thromboxane A2, endothelins and endoperoxides, as well as the production of O2− (superoxide anion), may contribute to this endothelial dysfunction in hypertension. Indeed, enhanced formation of endothelial O2− has been described in vessels of rats with genetic [4], NO-deficient [5], salt-sensitive [6] or angiotensin II-induced [7] hypertension. Such an increase in O2− production probably accelerates the inactivation of NO and accounts for the apparent decrease in bioactive NO.

Although it is generally accepted that such an effect may explain the blunted vasodilator responses observed in various forms of hypertension, controversy exists

Key words: N-acetylcysteine, genetic hypertension, nuclear factor κB (NF-κB), nitric oxide synthase, reactive oxygen species (ROS).

Abbreviations: BP, blood pressure; BW, body weight; CD, conjugated diene; DBP, diastolic BP; HW, heart weight; t-NAME, N^G-nitro-L-arginine methyl ester; LV, left ventricle; MAP, mean arterial pressure; NAC, N-acetylcysteine; NF-κB, nuclear factor κB; NO, nitric oxide; NOS, NO synthase; eNOS, constitutive NOS; iNOS, inducible NOS; O2−, superoxide anion; ROS, reactive oxygen species; SBP, systolic BP; SHR, spontaneously hypertensive rat; WKY, Wistar–Kyoto.

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regarding the impact of hypertension on eNOS [endothelial NOS (NO synthase)] activity and expression [8]. It has been demonstrated that pharmacologically induced BP elevation up-regulates NO production [9]. The release of NO by endothelial cells, as well as mRNA and protein expression of eNOS, can be altered by changes in shear stress, pulsatile stretch and other mechanical forces [10]. Indeed, in spontaneous hypertension, the production of NO in the vessels and heart is increased; however, the level of cGMP, a marker of NO efficiency, is similar to normotensive controls or even decreased.

Thus conflicting data exist regarding basal NO production in hypertension. Briones et al. [11] demonstrated similar basal cNOS (constitutive NOS) as well as iNOS (inducible NOS) activity and expression in arteries from both normotensive rats and SHRs (spontaneously hypertensive rats). Radaelli et al. [12] suggested that NO-dependent vasodilation is preserved (if not enhanced) during the developmental phase of spontaneous hypertension, namely in the prehypertensive and early established hypertensive stages, such that a putative impairment of this function provides no significant pathogenetic contribution to the onset of genetic hypertension. Vaziri et al. [13] also supposed that the development of hypertension is not due to a primary impairment of NO production in SHRs because they observed an increased NO production in young SHRs both before and after the onset of hypertension. Nava et al. [9] concluded that the NO pathway is up-regulated in the resistance vessels and heart of SHRs by a mechanism involving the induction of the cNOS, leading to an overproduction of NO. Despite this fact, endogenously produced NO is not available in sufficient amounts to stimulate the formation of cGMP and to maintain an adequate NO-dependent vasodilatory tone in SHRs. This discrepancy between high NO production and low NO bioavailability is probably due to the increased production of ROS (reactive oxygen species) or due to a physical barrier, such as fibrotic intimal layer of hypertensive vessels [4].

The imbalance between NO formation and ROS production appears to be a common feature of experimental [14–16] and human [17,18] hypertension. In addition, NO and superoxide react to form the powerful oxidant peroxynitrite, which can form hydroxyl radicals and nitrate protein tyrosine residues, resulting in an impairment of cellular signalling [19]. It has been reported that NO could be protected from free radical destruction by forming S-nitrosothiol compounds. Furthermore, sulphydryl donors such as NAC (N-acetylcysteine) or thiosalicylic acid enhance NO bioavailability by decreasing the level of ROS [20]. Chronic administration of NAC had a protective effect against vascular dysfunction in spontaneous hypertension [21]. Giroud et al. [22] also documented the hypotensive effect of NAC in this form of hypertension, which was probably mediated by the increase in NO-mediated vasodilation.

The aim of the present study was to determine the preventive effect of NAC on the development of spontaneous hypertension and to analyse the mechanisms responsible, particularly the effects of chronic NAC administration on the levels of ROS and GSH (reduced glutathione), as well as on NO synthase activity and expression in young SHRs in which hypertension was just developing. In the second experiment, the therapeutic effects of chronic NAC treatment were studied in adult SHRs with established hypertension.

METHODS

Animals and treatments

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 of the chemicals used were purchased from Sigma, except for l-[3H]arginine (Amersham Biosciences).

In experiment 1, young (5-week-old) male WKY (Wistar–Kyoto) rats and SHRs were divided randomly into two groups: (i) a control group drinking just tap water; and (ii) a group receiving NAC (1.5 g·day$^{-1}$·kg$^{-1}$ of body weight; 20 g/l) in tap water for 8 weeks ($n = 7$ in each group).

In experiment 2, male adult SHRs aged 12 weeks received NAC at the same dose and for the same period of time as in experiment 1.

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, BP was determined by direct puncture of carotid artery under a light ether anaesthesia. Thereafter the animals were killed and BW (body weight) and HW (heart weight) were determined. Samples of the LV (left ventricle) and kidney were used for the determination of NOS activity and CD (conjugated diene) and GSH concentrations, as well as for Western blot analysis. The GSH concentration was also determined in the liver.

Determination of CD and GSH concentrations

The concentration of CDs was measured in lipid extracts of LV and kidney homogenates as described by Kogure et al. [23]. Briefly, after chloroform evaporation under the inert atmosphere and addition of cyclohexane, CD concentration was determined spectrophotometrically ($\lambda = 233$ nm; GBC 911A; Bio-Rad Laboratories).

GSH was determined by the method of Ellman [24]. Briefly, the samples of LV, kidney and liver were homogenized in 0.5 vol. of ice-cold 5% sulphosalicylic acid and, after centrifugation at 12 000 g for 15 min, the concentration of GSH was analysed spectrophotometrically in the acid-soluble fractions ($\lambda = 412$ nm; GBC 911A; Bio-Rad Laboratories).
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Table 1  Effect of NAC treatment on BW, relative HW (HW/BW), BP and GSH in young WKY rats and SHRs

Values are means ± S.E.M. *P < 0.05 compared with WKY controls (− NAC); †P < 0.05 compared with SHR controls (− NAC).

<table>
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<tr>
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<th>Young WKY rats</th>
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<th>Young SHRs</th>
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<td>− NAC</td>
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<td>n</td>
<td>7</td>
<td>7</td>
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<tr>
<td>BW (g)</td>
<td>291.7 ± 10.7</td>
<td>257.4 ± 8.1*</td>
<td>292.0 ± 2.6</td>
<td>285.4 ± 4.1</td>
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<td>HW/BW (mg/g)</td>
<td>2.65 ± 0.03</td>
<td>2.66 ± 0.10</td>
<td>3.35 ± 0.04*</td>
<td>3.22 ± 0.02†</td>
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<td>SBP (mmHg)</td>
<td>134.7 ± 4.4</td>
<td>131.3 ± 4.0</td>
<td>210.2 ± 7.5*</td>
<td>178.6 ± 6.6†</td>
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<td>MAP (mmHg)</td>
<td>110.3 ± 3.3</td>
<td>110.1 ± 3.0</td>
<td>160.0 ± 6.8*</td>
<td>143.0 ± 6.6†</td>
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<td>DBP (mmHg)</td>
<td>83.3 ± 5.6</td>
<td>91.1 ± 2.8</td>
<td>126.3 ± 6.0*</td>
<td>111.3 ± 6.9†</td>
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<td>GSH (µmol/g of tissue)</td>
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<tr>
<td>Kidney</td>
<td>6.34 ± 0.30</td>
<td>6.87 ± 0.29</td>
<td>6.33 ± 0.17</td>
<td>6.78 ± 0.17</td>
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<td>Liver</td>
<td>10.64 ± 0.11</td>
<td>10.65 ± 0.17</td>
<td>12.02 ± 0.21†</td>
<td>12.20 ± 0.21†</td>
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Total NOS activity

Total NOS activity was determined in crude homogenates of LV and kidney by measuring the formation of L-[^3]H]citrulline from L-[^3]H]arginine, as described previously by Bredt and Snyder [25] with minor modifications [26].

Western blot analysis of eNOS and iNOS

Samples of LV and kidney (50 mg of wet tissue) were homogenized in 25 mmol/l Tris/HCl (pH 7.4) containing 5 mmol/l EDTA, 50 mmol/l NaCl, 1 µmol/l leupeptin, 0.3 µmol/l aprotinin, 0.1 mmol/l PMSF, 1 mmol/l pepstatin and 1 % (w/v) SDS. After the centrifugation (15 000 g, 20 min, twice), supernatants were subjected to SDS/PAGE using 10 % (w/v) acrylamide gels. Following electrophoresis, proteins were transferred on to nitrocellulose membranes and were probed with polyclonal rabbit anti-eNOS and anti-iNOS antibodies (Alexis Biochemicals) and a polyclonal rabbit anti-NF-κB (nuclear factor-κB) antibody (Santa Cruz Biotechnology). Bound antibodies were detected using a secondary peroxidase-conjugated anti-rabbit antibody (Alexis Biochemicals). The bands were visualized using the ECL® (Amersham Biosciences) and were analysed densitometrically using Photo-Capt V.99 software.

Statistical analysis

Results are expressed as means ± S.E.M. One-way ANOVA and Bonferroni test were used for statistical analysis. Values were considered to differ significantly if the P value was < 0.05. Linear regression analysis was used for evaluation of relationships between studied parameters.

RESULTS

Effect of chronic NAC treatment on young SHRs (experiment 1)

In young SHRs, chronic NAC treatment attenuated the rise in BP occurring during the development of spontaneous hypertension, whereas it had no effect on the BP of WKY rats (Table 1). This treatment also reduced cardiac hypertrophy in young SHRs, but it did not affect the HW/BW ratio in WKY rats.

Figure 1 shows that the CD levels were always higher in SHRs compared with WKY rats. NAC treatment did not affect CD levels in WKY rats, but lowered them considerably in both LV and kidney of SHRs. Nevertheless, CD levels remained significantly elevated in NAC-treated SHRs compared with NAC-treated WKY rats. GSH levels were similar in kidneys of WKY rats and SHRs, irrespective of the treatment, whereas GSH levels in liver were increased in SHRs compared with WKY rats (in both controls and NAC-treated animals; Table 1). NF-κB protein expression in LV of WKY rats and SHRs was attenuated after chronic NAC treatment (Figure 2).

Figure 3 shows the positive correlation of SBP (systolic BP) with CD concentration in LV or kidney. A similar relationship was also observed for MAP (mean arterial pressure; r = 0.668 and r = 0.809 in LV and kidney respectively) and DBP (diastolic BP; r = 0.571 and r = 0.717...
Figure 2 Western blot analysis demonstrating the effect of NAC treatment on NF-κB protein expression in the LV
Representative blots are shown with densitometric readings expressed as a percentage of WKY or adult SHR controls. Expression of β-actin was used as a loading control. \(^* P < 0.05\) compared with untreated rats of the same genotype; \(^+ P < 0.05\) compared with the corresponding WKY rat group.

Figure 3 Relationship between SBP and CD concentrations in the LV and kidney in SHRs and normotensive controls

in LV and kidney respectively; \(n = 28; P < 0.001\) for all).

Figure 4 shows that NOS activity was significantly higher in LV and kidney of SHRs compared with WKY rats. Chronic NAC treatment augmented NOS activity in both tissues of the two strains. Expression of eNOS was significantly greater in the LV of SHRs than WKY rats. NAC treatment activated further eNOS protein expression in both WKY rats and SHRs (Figure 5). On the other hand, NAC treatment had no effect on the protein expression of iNOS in the LV of the two strains (results not shown).

Effect of chronic NAC treatment on adult SHRs (experiment 2)
In contrast with that found in young rats, chronic administration of NAC to adult SHRs with developed
hypertension caused no significant reduction in BP and cardiac hypertrophy (Table 2). NAC treatment lowered CD concentrations (Figure 6, upper panels) in both LV and kidney of adult SHRs, although this decrease was less pronounced than in young animals. Chronic NAC administration also inhibited NF-κB protein expression in the LV of adult SHRs (Figure 2). GSH levels were not different in kidney or liver of adult control and NAC-treated SHRs (Table 2).

Table 2 Effect of NAC treatment on BW, relative HW (HW/BW), BP and GSH in adult SHRs

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<th>− NAC</th>
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<tr>
<td>n</td>
<td>8</td>
<td>8</td>
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<tr>
<td>BW (g)</td>
<td>294.0 ± 6.2</td>
<td>270.5 ± 5.7</td>
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<tr>
<td>HW/BW(mg/g)</td>
<td>3.41 ± 0.04</td>
<td>3.34 ± 0.06</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>191.4 ± 4.6</td>
<td>181.1 ± 6.4</td>
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<tr>
<td>MAP (mmHg)</td>
<td>149.6 ± 3.3</td>
<td>138.0 ± 7.7</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>118.9 ± 3.0</td>
<td>105.4 ± 7.5</td>
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<tr>
<td>GSH (µmol/g of tissue)</td>
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<tr>
<td>Kidney</td>
<td>6.20 ± 0.25</td>
<td>6.16 ± 0.35</td>
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<tr>
<td>Liver</td>
<td>12.21 ± 0.47</td>
<td>12.14 ± 0.54</td>
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Values are means ± S.E.M. *P < 0.05 compared with SHR controls (− NAC).

NOS activity was elevated (Figure 6, lower panels) in both LV and kidney of SHRs treated with NAC in adulthood. NAC treatment enhanced eNOS protein expression (Figure 5), whereas it had no effect on iNOS protein expression (results not shown).

**DISCUSSION**

This is the first report documenting that the chronic effect of NAC administration on BP in SHRs is dependent on the stage of development of hypertension. It is evident that chronic administration of NAC partially attenuated the increase in BP occurring in young SHRs. On the contrary, in adult SHRs with fully developed hypertension, the effect of NAC was negligible. The same was true for the age-dependent effect of chronic NAC administration on cardiac hypertrophy, the effect being significant only in young, but not in adult, SHRs. The mechanisms responsible for the reduction in BP appear to be related to both the decrease in the level of ROS and the increase of NO production (indicated by the elevation of NOS activity and eNOS protein expression).

Several authors have documented an increase in NO production in young SHRs both before and after the onset of hypertension [12,13], but increased ROS generation appears to account for decreased NO bioavailability in young, as well as in adult, SHRs. Indeed, increased activity and/or enhanced expression of xanthine oxidase and NADPH oxidase, the enzymes responsible for ROS generation, were found in different tissues of SHRs [15,16]. Since the increased expression of NADPH oxidase in kidney preceded the development of hypertension, it could be one of the causes, rather than a consequence, of the hypertension [16]. Therefore antioxidant treatment should be more efficient in young SHRs before the development of secondary hypertensive changes. Our results support this hypothesis reasonably well as antioxidant treatment with NAC attenuated hypertension development in young SHRs (experiment 1), but had no significant effect on BP in adult SHRs (experiment 2). The failure in the present study to lower BP in adult SHRs by chronic NAC treatment is in good agreement with the original study of Cabassi et al. [27], who reported no changes of SBP or HW in 16-week-old SHRs consuming very high doses of NAC (4 g · day −1 · kg −1 of body weight) for 2 weeks. However, Cabassi et al. [21] subsequently demonstrated that such NAC treatment was capable of significantly reducing SBP but not DBP in adult SHRs aged 13 weeks, suggesting that NAC-induced BP change in adult SHRs need not be due to a reduced tone of resistant vessels.

There are several possible mechanisms responsible for the reduction in the increase in BP in young SHRs treated with NAC. First, NAC treatment was able to inhibit NF-κB protein expression, indicating a decreased...
production of ROS during NAC treatment. This inhibition was more pronounced in young than in adult SHRs. This is in good agreement with our further findings that antioxidant action of NAC, measured as a decrease in CD concentration, was greater in young than in adult SHRs (by 20% compared with 17% in LV respectively, and 24% compared with 12% in kidney). The age-dependent antioxidant action of NAC might be a possible explanation for the different action of NAC on BP in young and adult SHRs. In fact, Cabassi et al. [21] also observed that NAC exerted its antioxidant effect by reducing aortic lipid peroxidation, as indicated by a lower concentration of malondialdehyde. It should be mentioned that NAC treatment also reduces NF-κB concentration in aldosterone-salt hypertensive rats [28]. Furthermore, NAC as a thiol may protect NO from oxidation by scavenging of oxygen free radicals [20] and forming nitrosothiols [29]; both effects could prolong NO half-life and potentiate its effect. Alternatively, this protective effect of NAC could be due to the augmentation of the activity of NOS and cGMP cyclase, which are thiol dependent [30]. We documented that NAC treatment may increase NOS activity further by the enhancement of eNOS protein expression, which was more pronounced in young than in adult SHRs. A similar NAC-induced activation of eNOS protein expression was observed in cultured bovine aortic endothelial cells [31].

Although the mechanism by which antioxidant treatment improves vasorelaxation and decreases BP is not clear, it is generally accepted that antioxidants scavenge superoxides and thus increase the bioavailability of NO. Kinetic constants, however, suggest that the rate of reactions between common antioxidants and superoxide is much slower than the rate of the reaction between superoxide and NO [32–34]. Thus other mechanisms, including enhancement of eNOS expression, may also be responsible for increased NO availability induced by antioxidant treatment. With the exception of NAC-induced activation of eNOS protein expression, the same effect on eNOS expression was observed for vitamins C and E, nordihydroguaiaretic acid, catechol and glutaryl prolubocil and other antioxidants [31,35,36].

NAC has also been reported to increase GSH concentration in SHR aorta [21] without a parallel decrease in GSSG concentration, suggesting an elevated level of l-cysteine which is the rate-limiting component in GSH synthesis [37]. Unfortunately, our results did not confirm such an effect of NAC treatment on GSH level. Interestingly, in our present study, GSH concentration in liver was higher in both young and adult SHRs compared with the normotensive WKY rat controls. This could represent part of the adaptive mechanisms counteracting the increased oxidative stress in SHRs. Although decreased GSH concentration and altered turnover rate of the GSH redox cycle with the increased intracellular content of GSSG in different tissues has been described in SHRs [38], this adaptive mechanism was probably not sufficient.

Recently, we have demonstrated [39] that chronic NAC treatment (1.5 g·day⁻¹·kg⁻¹ of body weight for 4 weeks) prevented the development of l-NAME (NG- nitro-l-arginine methyl ester)-induced hypertension in adult WKY rats. This effect was associated with increased NOS activity and decreased level of oxygen free radicals in l-NAME-treated rats, as was observed in young SHRs in the present study. Thus chronic NAC administration might interfere with the developing hypertension, rather than lowering BP in animals with already established hypertension. Another study from our group [40] has shown that chronic NAC treatment also completely abolished the development of salt hypertension in young salt-sensitive Dahl rats. In this case, the antihypertensive effect was mainly mediated by a reduction in sympathetic vasoconstriction, although there was also a certain reduction in vasodilator deficit. This finding would be compatible with the observations of Xu et al. [41,42], who reported the NO-independent effects of tempol on BP and sympathetic nervous activity. Finally, we must also consider the possibility that enhanced ROS formation in the central nervous system might interfere with NO synthesis by nNOS (neuronal NOS). Lowering of central NO bioavailability appears to enhance sympathetic tone generated by central mechanisms involving brain angiotensin II [43,44].

In conclusion, both the increase in NOS activity and the decrease in production of ROS might be responsible for the preventive effect of NAC on the development of hypertension in young SHR, but do not exclude other possible mechanisms involved in hypertension development. In adult SHRs with developed hypertension, however, the secondary alterations (such as pronounced remodelling of resistance vessels) might attenuate the beneficial effect of chronic NAC treatment on the BP level.

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