Angiotensin-Converting Enzyme Inhibitors and AT1-Receptor Antagonist Restore Nitric Oxide Synthase (NOS) Activity and Neuronal NOS Expression in the Adrenal Glands of Spontaneously Hypertensive Rats

Fatimunnisa Qadri*, Thomas Arens, Eike C. Schwartz, Walter Häuser and Peter Dominiak

Institute for Experimental and Clinical Pharmacology and Toxicology, Medical University of Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany

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ABSTRACT—During development of hypertension in spontaneously hypertensive (SHR) rats, the activity of adrenal nitric oxide synthase (NOS) was investigated. SHR and Wistar-Kyoto (WKY) rats were studied at different ages: 3–4, 7–8 and 12–13 weeks after birth. Basal NOS activity was measured by the ability of homogenate to convert [3H]-L-arginine to [3H]-L-citrulline. At all ages, SHR rats exhibited 50–60% reduction in NOS activity when compared to age-matched WKY rats. In a following study, SHR rats (12–13 weeks) were treated chronically with the angiotensin I-converting enzyme inhibitors (ACE-I) captopril or enalapril, or the AT1-receptor antagonist losartan (2/8G2b 25, 10 and 60 mg/kg per day for 10 days, respectively). The total NOS activity and protein expression of NOS isoenzymes from adrenals were determined. The basal NOS activity and protein expression of neuronal NOS (nNOS) was significantly increased in treated SHR rats when compared to control rats. The isoforms endothelial NOS and inducible NOS were undetectable. We conclude that impaired NO synthesis in the adrenal glands of SHR rats may contribute to the onset and maintenance of hypertension. The upregulation of nNOS protein in the adrenal glands may be one of the mechanisms by which ACE inhibitors and AT1-receptor antagonists by restoring the NO synthesis, mediate their antihypertensive effects.

Keywords: Nitric oxide synthase, Spontaneously hypertensive rat, Adrenal gland, Angiotensin-converting enzyme inhibitor, Losartan

*Corresponding author. FAX: +49-451-500-3327
E-mail: qadri@medinf.mu-luebeck.de

NO within the adrenal medulla may modulate the release of catecholamines into the circulation, thereby playing an important role in the maintenance of blood pressure (BP) at physiological levels. We therefore hypothesized that an impaired synthesis of NO within the adrenal medulla in spontaneously hypertensive (SHR) rats may be one of the crucial factors contributing to the development and maintenance of hypertension. Furthermore, the reduction in BP following treatment with angiotensin-converting enzyme inhibitors (ACE-I) or AT1-receptor antagonists may affect the NO production within the adrenal gland. In the present study, we investigated the activities of NOS with respect to different stages in the development of hypertension in SHR and compared them with those of the age-matched normotensive Wistar-Kyoto (WKY) rats. Furthermore, the effect of chronic treatment with ACE-I and an AT1-receptor antagonist was tested to evaluate the influence on the protein expression and activity of different isoenzymes of NOS from the adrenal glands of adult SHR rats.
MATERIALS AND METHODS

Animals
Male SHR rats were studied at three different ages: 3–4 (pre-hypertensive phase), 7–8 (onset of hypertension) and 12–13 (maintenance phase) weeks after birth (74±2, 148±2 and 265±3 g body weight, respectively). Age-matched male WKY rats served as controls (74±1, 220±2 and 334±2 g body weight, respectively). All animals were purchased from Charles River Wiga (Sulzfeld, Germany), and they were housed under a 12-h light/dark cycle with food and water given ad libitum. Animals were cared for in accordance with the Declaration of Helsinki and with the guide for the care and use of laboratory animals as adopted by the “Ministerium für Natur und Umwelt des Landes Schleswig-Holstein, Germany (animal protocol No. 9/v/97)”.

Determination of basal NOS activity in the adrenal glands of age-matched SHR and WKY rats
The basal NOS activity was determined in the adrenal glands of SHR and WKY rats at three different ages; i.e., 3–4, 7–8 and 12–13 weeks after birth. Animals were sacrificed, adrenals were removed and immediately frozen in liquid nitrogen and stored at −80°C until assayed for NOS activity as described below.

Effects of chronic treatment with ACE-I and AT₁-receptor antagonist on NOS activity and expression in adrenal glands of adult SHR rats
Adult 12–13-week-old SHR rats were divided into four groups. The antihypertensive drugs were delivered orally by gavage daily for 10 days. Group 1 was treated with captopril (2×25 mg/kg per day), group 2 with enalapril (10 mg/kg per day), group 3 with losartan (60 mg/kg per day), and group 4 with tap water, which served as a control. Group 5 consisted of age-matched normotensive WKY rats that were also treated with tap water and served as a control.

The doses of drugs applied were equi-effective in lower- ing BP in SHR rats (preliminary data from our laboratory). Systolic blood pressure (SBP) and heart rate (HR) were measured by tail plethysmographically 2 days prior and 8 days after the beginning of treatment. On the tenth day of treatment, rats were sacrificed, adrenal glands were removed and immediately frozen in liquid nitrogen and stored at −80°C until assayed for NOS activity and protein content as described below. Different animal groups were taken for each experimental protocol.

NOS activity assay
NOS activity was determined by measuring the conversion of [³H]-l-arginine to [³H]-l-citrulline in the presence of tissue homogenate and NADPH as described previously with slight modifications (6). In brief, adrenal glands were homogenized in ice-cold buffer. A 200-μg sample of protein (approximately 50 μl homogenate) were incubated in HEPES buffer (10 mM, pH 7.5) containing non-radioactive l-arginine (10 μM) and [³H]-l-arginine (2000 Bq/tube), l-valine (60 mM), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (3 μM) and calcium chloride (2 mM) for 20 min at 37°C. The reaction was stopped by adding 1 ml of ice-cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Arginine was removed from the reaction mixture by adsorption to Dowex 50W (Na⁺ form). The amount of [³H]-l-citrulline was measured in a Beckman scintillation counter, and the activity of NOS was expressed as the formation of citrulline in pmol·mg protein⁻¹·min⁻¹.

Western blot analysis of NOS protein
A 30-μg sample of protein were separated on a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane using PharmSystem Model TE77 (Pharma- cia Biotech, San Francisco, CA, USA). The membrane was blocked with 1% BSA in phosphate-buffer solution (PBS) (pH 7.5) containing 0.1% Tween 20 for 1.5 h at room temperature. Thereafter, the membrane was incubated overnight at 4°C with rabbit polyclonal anti-nNOS (neuronal NOS) (1:3000), anti-eNOS (endothelial NOS) (1:2000) or anti-iNOS (inducible NOS) (1:5000) antibodies (Transduction Laboratories, Lexington, KY, USA) in PBS containing 0.1% Tween 20. The membrane was then washed with washing buffer (PBS-0.1% Tween 20) and finally incubated with a 1:5000 dilution of anti-rabbit IgG conjugated to horseradish peroxidase for 2 h at room temperature. After successive washes with washing buffer, the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence reaction (ECL RPN2106; Amersham Pharmacia Biotech, Little Chalfont, UK) and exposed to a x-ray film (Kodak, Germany) for 2–5 min. The optical density of respective bands was quantified by densitometric analysis of the western blots using Scion Image-software (NIH Image, Bethesda, MD, USA).

Statistical analyses
Data are expressed as the mean ± S.E.M. Differences between and within groups were evaluated by 2-way ANOVA followed by the Bonferroni procedure. A value of P<0.05 was considered significant.

RESULTS

Basal NOS activity in adrenal glands of age-matched SHR and WKY rats
The basal NOS activity in the adrenal glands of SHR
Adrenal NOS Activity/Expression in SHR

rats was greatly reduced compared to age-matched WKY rats. No age-related difference in NOS activity was observed among SHR and WKY rats (Fig. 1).

Effects of chronic treatment with ACE-I and AT1-receptor antagonist on SBP and NOS activity in adrenal glands of adult SHR rats

Chronic treatment of adult SHR rats significantly reduced SBP by 25–30 mmHg compared to their initial SBP values. There was no significant change in HR and body weight before and after the drug treatment except for captopril, which reduced HR significantly (Table 1). The NOS activity was significantly higher in SHR rats treated with ACE-I- or AT1-receptor antagonist compared to control SHR rats. There was no significant difference in NOS activity between treated SHR and normotensive WKY controls (Fig. 2).

NOS protein expression in adrenal glands of adult SHR rats after chronic treatment with ACE-I and AT1-receptor antagonist

The basal protein expression of nNOS in adrenal glands of adult SHR rats was significantly lower than in age-matched WKY rats. The nNOS protein expression, however, was significantly increased in SHR rats following chronic blockade of ACE-I and AT1 receptor when compared to control SHR or WKY rats (Fig. 3). An expression of eNOS and iNOS protein remained undetectable either under the basal condition or after antihypertensive treatment (data not shown).

DISCUSSION

The main findings of this study are that SHR rats in comparison to WKY rats showed a reduced NOS activity in adrenal glands at all stages of development of hypertension, and blockade of ACE or AT1 receptors in adult SHR rats increases both expression and activity of nNOS.

Endogenously produced NO has been shown to reduce the basal catecholamine release from peripheral sympathetic nerve terminals and from adrenal glands (5, 7–10). On the other hand, L-NAME significantly decreases tyrosine hydroxylase (TH) activity as well as TH mRNA levels in the adrenal medulla of WKY rats (11). This suggests...
that spontaneous release of catecholamines from adrenal glands, as described previously by many authors (12–14), is tonically controlled by the NO-cGMP system. Recently, Schwarz et al. (4) have demonstrated that chromaffin cells exhibit a basal NOS activity that could be stimulated by acetylcholine and inhibited by L-NAME. These findings provide strong evidence that chromaffin cells possess an autocrine NO/cGMP pathway that tonically controls catecholamine release. Furthermore, NO was also able to inhibit the biological activity of catecholamines (15). Taken all into account, the data described above suggest that the control of adrenal sympathetic function by NO contributes to the physiological regulation of BP and possibly to its pathological derangement.

In the present study, the reduced NOS activity at all stages during the development and maintenance of hypertension implies impaired synthesis of NO within the adrenal glands due to a reduced nNOS protein expression. It could be speculated that this may result in an increased release of catecholamines and, as a result, BP will increase. Donohue et al. (16) have demonstrated in detail the changes in catecholamine content with age (2 days to 17-week-old) in a variety of tissues from SHR and WKY rats. Adrenaline levels in adrenal glands were similar in SHR and WKY rats at all ages. However, NA levels in adrenal glands of SHR rats increased in parallel to the development of hypertension. In the present study, the reduced synthesis of NO in the adrenal glands from young age in SHR rats may correspond to a disinhibition of sympathetic outflow which along with some other factors initiate and, in adulthood, maintain high BP. However, the effect of reduced NOS activity at the pre-hypertensive phase (3–4 weeks postnatal) on synthesis/release of catecholamines could not be explained, since at this age, there was no difference in plasma or tissue content of catecholamines between age-matched SHR and WKY rats. On the other hand, it has been demonstrated that at this age, the sympathetic nervous system (SNS) plays an important role in the development of spontaneous hypertension in rats, since sympathectomy at this age prevented the development of hypertension in adulthood (17, 18). Based on our data, we suggest that reduced NO synthesis in the adrenals of SHR is not an epiphenomenon, but rather one of the causes in the development of spontaneous hypertension in rats.

There is quite a number of observations suggesting an inverse relationship between the expression/activity of ACE and eNOS from blood vessels via feed-back regulation (19, 20). ACE expression or activity is increased when eNOS expression or activity is decreased (21, 22). There are, however, very few data available regarding the effect of AT1 blockers (1) and to our knowledge, no data exist regarding ACE inhibitors on the expression/activity of nNOS. In the present study, the reduced NOS activity in the adrenal glands of SHR rats was due to a reduced expression of nNOS protein, and chronic treatment with blockers of the renin-angiotensin system (RAS) significantly increased the nNOS protein expression and, as a result, an increase in the levels of its activity. Our present results concerning the upregulation of nNOS are consistent with the data shown by Iwai et al. (1) who demonstrated that an increase in nNOS mRNA levels in adrenal glands of adult SHR rats following chronic treatment with antihypertensive drugs. In our study, we did not use antihypertensive drugs other than RAS-blockers, since it was demonstrated that hydralazin increased the nNOS gene expression (1). It seems that reduction in BP in adult SHR rats induced by inhibitors of the RAS or by vasodilators triggers the augmentation of nNOS mRNA expression. The mechanism(s) behind the upregulation of nNOS following blockade of RAS or other vasodilators is not fully understood. However, based on our data we speculate that decrease in BP following either ACE inhibition or AT1-receptor blockade induced an increase in nNOS protein expression and, as a consequence, an increase in NO synthesis which in turn inhibits the sympathetic-adrenal system, since it has been previously shown that
ACE inhibition and AT1 blockade could affect the release of catecholamines (23 – 25). From our data, we conclude first that since SHR rats possess a reduced NOS activity beginning from birth, reduction in NO synthesis is not a epiphenomenon, but rather a cause that may along with some other factors play a role in the development of spontaneous hypertension; second, in adult SHR rats, restoration of NO synthesis due to an upregulation of nNOS expression in adrenal glands may contribute as a sympato-inhibitory mechanism to the BP lowering effect of RAS-blockers such as ACE inhibitors and AT1 antagonists.

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