INTRODUCTION

Nitric oxide (NO), an important modulator of vascular tone and blood pressure is synthesized by the enzyme NO-synthase (NOS), which is constitutively expressed in endothelial (eNOS) and neuronal (nNOS) isoforms. The treatment of normotensive as well as spontaneously hypertensive rats (SHR) by non-specific inhibitors of NOS, such as NG-nitro-L-arginine methylester (L-NAME), evoked an unequivocal cardiovascular response: increases in systolic blood pressure (SBP) and contractility associated with arterial wall hypertrophy and impaired endothelial function (1-7). Essential hypertension is a multifactorial disease and NO system might be directly or secondary (via entering to other regulatory systems) engaged in its etiopathogenesis. Nevertheless, neither the role of NO generally nor the participation of nNOS specifically is still not clearly dedicated in genetic hypertension.

Several studies showed that the different NO production by nNOS in the brain of SHR may act as important factor for the onset and the development of high blood pressure (8, 9). On the other hand, the enhanced expression of nNOS reported in different brain areas as well as outside of central nervous system seems to be a compensatory mechanism against increased vascular tone rather than pathogenic mechanism (10-12). 7-nitroindazole (7-NI), a selective inhibitor of nNOS, has been widely used to study the role of the neuronal NO pathway in the cardiovascular system. Most reports documenting 7-NI treatment in normotensive rats have failed to demonstrate a long-term induction of increase in SBP, and ambiguous effects of 7-NI on vessel wall in vitro and in vivo have been reported (13-15). Our previous experiments demonstrated that nNOS inhibition by 7-NI for six weeks in adult Wistar rats induced: no changes in SBP at the end of the treatment, only mild inhibition of endothelium-dependent relaxation of thoracic aorta, no inhibition of relaxation of mesenteric artery in vitro, and no changes in hypotensive response to acetylcholine in vivo (16, 17).

Based on these findings, which are in distinct opposition to eNOS inhibition effects, we wondered whether the treatment with 7-NI in SHR could exert similar effects. Moreover, data related to the effect of chronic inhibition of nNOS on the vascular function and structure in SHR are unavailable. Therefore, the aim of this study was to investigate the effect of long-term administration of the nNOS inhibitor 7-NI on the cardiovascular system in SHR, with a specific focus on vasoactivity and geometry of conduit arteries.

EFFECT OF CHRONIC NEURONAL NITRIC OXIDE-SYNTHASE INHIBITION ON ARTERIAL FUNCTION AND STRUCTURE IN SPONTANEOUSLY HYPERTENSIVE RATS

While the effect of chronic non-specific NO-synthase inhibition in the cardiovascular system has been recognized under normotensive and hypertensive conditions, there are no data relating the long-term inhibition of neuronal NO-synthase (nNOS) in essential hypertension. The aim of this study was to investigate the long-term effect of nNOS inhibitor 7-nitroindazole (7-NI) administration on arterial function and structure in spontaneously hypertensive rats (SHR). Ten weeks old SHR were divided in two groups: control group and group administered 7-NI (10 mg/kg/day) for six weeks in drinking water. Systolic blood pressure (SBP) was measured using the plethysmographic method. The vasoactivity of isolated thoracic aorta (TA) and mesenteric artery (MA) was recorded via changes in isometric tension, and the geometry of both arteries was measured using light microscopy. Chronic treatment with 7-NI did not affect either SBP or heart/body weight ratio. Acetylcholine-induced relaxation of both arteries was unchanged after 7-NI. 7-NI administration did not affect the sensitivity and contraction to exogenous noradrenaline in TA, whereas both parameters were augmented in MA. The contractile response of MA induced by transmural nerve stimulation (endogenous noradrenaline) was unaffected after 7-NI. The mass of TA wall was unchanged, whereas hypertrophy was observed in MA after 7-NI. In summary, although SBP and endothelial function were not changed after chronic nNOS inhibition, the contractile and structural properties of TA and MA were affected differently. The data suggest that nNOS triggers original and tissue-specific regulatory pathways in essential hypertension.

Key words: 7-nitroindazole, arterial structure, endothelial function, hypertension, neuronal nitric oxide-synthase, spontaneously hypertensive rat, vasomotor activity
MATERIAL AND METHODS

Guide for the Use and Care of Laboratory Animals

Procedures were performed in accordance with institutional guidelines and were approved by the State Veterinary and Food Administration of the Slovak Republic and by an Ethical committee according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Directive 2010/63/EU of the European Parliament. All rats were housed under a 12 h light-12 h darkness cycle, at constant humidity (45-65%) and temperature (20-22°C), with free access to standard laboratory rat chow and drinking water. The Institute of Normal and Pathological Physiology provided veterinary care.

Experimental animals and treatments

Ten-weeks-old male SHR were used in the present study. The animals were divided randomly into two groups of twenty animals each. Animals in each group had the similar weight at the beginning of the experiment and we used offspring of different mothers in each group. The first group received 7-NI (10 mg/kg/day) dissolved in drinking water for six weeks. This dose represented the most commonly used dose of 7-NI that was dissolved in drinking water and has been found to inhibit nNOS isoform in vivo (18). 7-NI uptake was monitored daily, according to the respective drinking intake. The second group of animals represented the control group. For both groups, the SBP was measured noninvasively by tail-cuff plethysmographic method once a week. During the week before treatment, the rats were trained to be accustomed to SBP measurements. At the end of the experiment, ten animals from each group were used for functional studies, and ten animals from each group were used for morphological investigation.

Functional study

Rats were killed by decapitation after a brief CO2 anaesthesia and the thoracic aorta and mesenteric artery were isolated. Arteries were cleaned of connective tissue, and cut into rings (3-4 mm in length). The rings were vertically fixed between two stainless steel wires in 20 ml incubation organ bath with Krebs solution (NaCl 118 mM, KCl 5 mM, NaHCO3 25 mM, MgSO4 1.2 mM, KH2PO4 1.2 mM, CaCl2 2.5 mM, glucose 11 mM, ascorbic acid 1.1 mM, CaNa2EDTA 0.032 mM). The solution was oxygenated with 95% O2 and 5% CO2 and kept at 37°C. The upper wire triangles were connected to electromechanical transducers (Experimetria) and the changes in isometric tension were registered using AD converter and Dewetron software. The resting tension was adjusted to 1 g and applied to each ring. Subsequently, the preparations were allowed to equilibrate for 60-90 minutes until stress relaxation no longer occurred. The relaxant responses were followed on rings precontracted with a sub-maximal dose of phenylephrine (10-6 mol/l) to produce a stable plateau of contraction. The rings were then exposed to cumulative doses of acetylcholine (10-6-3×10-4 mol/l). The extent of relaxation of the arterial rings was expressed as a percentage of the phenylephrine-induced contraction. Contractile responses were induced by increasing concentrations of noradrenaline (10-8-3×10-2 mol/l) in a cumulative manner and expressed as developed tension/cross-sectional area of tissue (mN×mm-2) and as percentages of the maximum tissue response to noradrenaline (demonstrating the sensitivity to noradrenaline). The concentrations producing the half-maximum response (EC50) were calculated from individual dose-response curves and expressed as the negative logarithm of the noradrenaline molar concentration. Neurogenic contraction of isolated mesenteric artery was induced by the electric stimulation of intramural nerves (square pulses of supramaximal intensity 35 V, 0.2 ms duration, 8 Hz frequency, for a period of 20 s).

Morphological study

The animals were sacrificed by a dose of anaesthesia - mixture of xylazine (1 g/kg b.w.) and ketamine (2.5 g/kg b.w.) administered intraperitoneally, the chests of the rats were opened and, via a cannula inserted in the left ventricle, the cardiovascular system was perfused with a fixative (300 mmol/l glutaraldehyde in 100 mmol/l phosphate buffer) under 120 mm Hg pressure. Thoracic aorta and mesenteric artery was excised, cleaned, divided into four segments about 1 mm long, fixed with the same fixative, postfixed with 40% alcohol, and embedded in Durcupan ACM. Three randomly selected blocks of each artery were cut perpendicularly to the longitudinal axis. The inner circumference and arterial wall thickness (tunica intima and tunica media) were measured using light microscopy. The arterial wall thickness (tunica intima and tunica media) was measured at points in about 45° intervals around the circumference of the artery. The inner diameter and cross sectional area (tunica intima and tunica media) were calculated.

Drugs

The following drugs were used: phenylephrine (Sigma-Aldrich, St Louis, Missouri, USA), acetylcholine (Sigma-Aldrich, St Louis, Missouri, USA), 7-nitroindazazole (Sigma-Aldrich, St Louis, Missouri, USA), noradrenaline (Sigma-Aldrich, St Louis, Missouri, USA), xylazine (Spofa, Prague, Czech Republic), and ketamine (Spofa, Prague, Czech Republic), 7-nitroindazazole was dissolved in drinking water that had been heated to 80°C.

Statistical analysis

The data were expressed as means ±S.E.M. For the statistical evaluation of differences between groups un-paired t-test was used. The differences between means were considered significant at p<0.05.

RESULTS

Basic parameters

The time-course of SBP changes in untreated SHR (n=20) and SHR treated with 7-NI (n=20) for six weeks is shown in Fig. 1. The treatment with 7-NI did not affect the SBP over the course of treatment, and there were no differences between treated and untreated group. There were no differences in body weight at the end of the treatment between untreated SHR (336.2±7.1 g) and 7-NI-treated SHR (334.6±5.2 g). Similarly, the treatment with 7-NI did not affect heart weight (1.21±0.022 mg in untreated and 1.23±0.027 mg in treated group) and heart weight/body weight ratio (3.6±0.043 mg/g in untreated and 3.7±0.052 mg/g in treated group).

Functional study

The application of acetylcholine induced concentration-dependent relaxation of the thoracic aorta pre-contracted by phenylephrine (10-6 mol/l) in untreated SHR (n=10). Chronic treatment with 7-NI did not affect the relaxation to acetylcholine (n=10, Fig. 2a). The application of exogenous noradrenaline to the incubation bath induced a dose-dependent contraction of the
Fig. 1. Time course of tail SBP in control SHR (control, n=20) and in SHR treated with 7-nitroindazole (7-NI, n=20) for six weeks. Values are mean ±S.E.M.

Fig. 2. Reactivity of thoracic aorta. Acetylcholine concentration-response curves (a), noradrenaline concentration-response curves (b), and curves expressed as per cent values of the maximum tissue response to noradrenaline (c) in thoracic aorta isolated from control SHR (control, n=10) and SHR after six weeks of treatment with 7-nitroindazole (7-NI, n=10). Values are mean ±S.E.M.

Fig. 3. Reactivity of mesenteric artery. Acetylcholine concentration-response curves (a), noradrenaline concentration-response curves (b), and curves expressed as per cent values of the maximum tissue response to noradrenaline (c) in mesenteric artery isolated from control SHR (control, n=10) and SHR after six weeks of treatment with 7-nitroindazole (7-NI, n=10). Values are mean ±S.E.M. * p<0.05 with respect to the value of control group.
thoracic aorta in untreated SHR (n=10), and chronic treatment with 7-NI did not affect this contraction (n=10, Fig. 2b).

Expressing the data as a percentage of the maximal noradrenaline-induced contraction also revealed no differences between the untreated and treated rats (Fig. 2c).

Concerning mesenteric artery, we observed no significant difference in the acetylcholine induced relaxation between untreated (n=10) and 7-NI treated SHR (n=10, Fig. 3a). However, chronic treatment with 7-NI shifted the dose-dependent response of mesenteric artery to exogenous noradrenaline to the left, and contraction induced by low doses (10^-9–10^-7 mol/l) was significantly augmented in treated (n=10) compared to untreated rats (n=10, p<0.05, Fig. 3b). Expressing the data as a percentage of the maximal contraction also revealed that 7-NI treatment shifted the dose-response curve to the left, and there was a significant difference between untreated and treated groups in terms of contractile response induced by 3×10^-1-3×10^-7 mol/l noradrenaline (p<0.05, Fig. 3c).

The –logEC50 value for the thoracic aorta was not affected by 7-NI treatment (7.58±0.15, n=10, in untreated group, 7.74±0.07, n=10, in treated group), however, this value for mesenteric artery was significantly higher in treated (7.34±0.13, n=10, p<0.05) than in untreated rats (6.66±0.25, n=10). These data indicate that the sensitivity of mesenteric artery to exogenous noradrenaline in SHR was significantly increased after the treatment with 7-NI.

Transmural nerve stimulation (8 Hz) induced contraction of mesenteric artery in untreated SHR (n=10). The contraction was not affected after the treatment with 7-NI (n=10, Fig. 4).

**Morphological study**

Geometry of the thoracic aorta revealed that wall thickness (WT) (tunica intima+media) and cross sectional area (CSA) (tunica intima+media) in SHR treated with 7-NI (n=10) did not differ from those in untreated SHR (n=10) (Fig. 5a). Similarly, inner diameter (ID), and WT/ID of thoracic aorta in SHR treated with 7-NI did not differ from those in untreated SHR (Fig. 5b). Significant (p<0.01) increase of WT (by 25%), CSA (by 20%) (Fig. 5a), and WT/ID (by 32%) (Fig. 5b) was observed in mesenteric artery of SHR after 7-NI administration. The inner diameter of the mesenteric artery of SHR receiving 7-NI did not differ from that in control SHR (Fig. 5b).

**DISCUSSION**

The results of our study revealed that the long-term inhibition of nNOS in SHR by 7-NI did not affect SBP, heart weight or endothelial function. However, we observed the following effects on contractile properties and the geometry of conduit arteries: no changes in thoracic aorta and increased vasoconstriction and sensitivity to noradrenaline associated with wall hypertrophy in mesenteric artery. These results distinguish from that observed in Wistar rats (16, 17), where we found out arterial hypotrophy in both thoracic aorta and mesenteric artery. The intrinsic modification of the arterial wall structure might promote the alterations of the mechanical and functional properties of the conduit arteries. The treatment with 7-NI in Wistar rats significantly decreased the absolute contraction represented as a change in active tension (the dose-response curves to exogenous noradrenaline were shifted to the right) and did not affect the sensitivity to noradrenaline. So, we suggested that the decrease in
contractile force was probably related to the decrease of smooth muscle mass as we found in 7-NI group (17). Results from SHR confirmed the idea that the functional changes seen in large vessels are concomitant with the morphological changes: no changes in wall trophy after 7-NI treatment observed in thoracic aorta were accompanied by no changes in contractile response to noradrenaline and hypertension of mesenteric artery was associated with increased vasoconstriction (the dose-response curve was shifted to the left). Nevertheless, since in the mesenteric artery the increased sensitivity to noradrenaline was also observed, the increased contraction might be associated with alterations in contractile apparatus sensitivity and/or receptor affinity too. Moreover, we recently demonstrated that chronic treatment of SHR with 7-NI could be associated with a higher Ca2+ transport from the intracellular stores (19) and several authors have confirmed in different arteries that differences in Ca2+ handling were associated with sensitization of contractile elements to Ca2+ and enhanced vasoconstriction (20-22).

NO synthesized by nNOS was identified as a neurotransmitter that decreases vascular smooth muscle tone in the peripheral autonomic system. Our previous results, along with observations of other authors, confirmed that NO released from perivascular nerves blunted the sympathetic response by inhibition of neurogenic noradrenaline release in mesenteric artery of normotensive rats (17, 23, 24). However, the study of Rabelo et al. (24) demonstrated that the neuronal NOS activity is most likely suppressed in perivascular nerves of mesenteric artery in SHR. This is in a good agreement with our results, which show that the contraction induced by perivascular nerve stimulation (8 Hz) remained unchanged in the mesenteric artery after 7-NI. Several other studies have shown that transmural nerve stimulation was able to induce the release of NO from perivascular nitrergic nerves in mesenteric circulation of SHR, however, Ferrer et al. (25) demonstrated a simultaneous increased production of superoxide anions, which blunted the effect of peripherally released neuronal NO on sympathetic-mediated vasoconstriction. In summary, the disturbances/alterations in the release of noradrenaline (a strong proliferative agent and vasoconstrictor) from nerve endings were most likely not responsible for increased contractility and hypertrophy of mesenteric artery after chronic 7-NI treatment.

The nNOS enzyme isoform has also been identified in non-neural cells, including endothelial and vascular smooth muscle cells. In SHR, several studies have confirmed an increased expression and/or activity of nNOS as a counter-regulatory response to increased vascular tone. Boulanger et al. (10) observed an increased expression of nNOS in carotid artery smooth muscle cells from SHR and the generation of NO from this isoform was stimulated only in arteries from SHR (not in normotensive rats). Briones et al. (26) demonstrated that the expression of basal nNOS was approximately 2-times higher in the vessel wall of mesenteric artery from SHR than in normotensive rats. This action of nNOS appears to be specific to organ and animal strain. Piech et al. (11) demonstrated that nNOS protein abundance in SHR is not uniform but is differentially regulated according to the tissue specificity. Our results fit this paradigm well; we observed that treatment with 7-NI increased contractility and hypertrophy only in mesenteric artery and not in the thoracic aorta or heart. Consequently, increased contraction and hypertrophy of mesenteric artery could be (beside alterations in contractile apparatus sensitivity) the result of inhibited overproduction of NO derived from nNOS specifically in the mesenteric arterial wall. Moreover, our study showed that treatment with 7-NI did not affect the SBP, which aligns with the finding of Berg (27), who confirmed that the peripheral nNOS system was up-regulated in SHR to counteract the rise in total peripheral vascular resistance but without any positive effect on SBP.

NO, independently of the source, has an antiproliferative and SBP decreasing effect, so its inhibition should lead to the positive trophic and SBP increasing effect. In this study, in SHR, the inhibition of nNOS did not affect SBP and only partially induced the arterial hypertrophy. We found an increase of CSA, WT, and WT/ID only in mesenteric artery. No effect in this respect was observed in thoracic aorta. The data suggest that the positive trophic effect of NO deficiency after 7-NI administration is more pronounced in muscular than elastic type of artery. In our previous study, in Wistar rats, we observed no effect on SBP, and moreover, arterial and heart hypertrophy associated with decreased contractility (16, 17). To explain this discrepancy requires further functional and biochemical investigation.

To the best of our knowledge, there are no other studies available on chronic inhibition of the nNOS enzyme isoform in SHR for direct comparison with our findings. However, studies using L-NAME, an inhibitor with predominant activity towards the eNOS isoform are particularly noteworthy. SHR treated with L-NAME (2-4 weeks) developed sustained malignant hypertension accompanied by reduced body weight gain (2, 28), whereas the six week time course of 7-NI treatment did not increase SBP, reduce body weight or result in a high mortality rate. Administration of L-NAME in SHR led to the general accentuation of vascular hypertrophy (3, 4, 7), an enhanced contractility in response to vasoconstrictors and inhibited endothelium-dependent vasorelaxation in different conduit arteries (1-3, 29). This is in contrast to our results, which showed no changes in SBP and endothelial function and non-uniform arterial wall hypertrophy after 7-NI treatment. We suggest, that similarly as in Wistar rats (16, 17), the specific inhibition of the nNOS isoform triggers different metabolic pathways compared to eNOS in SHR.

Understanding of courses leading to hypertension enable to reveal new preventive and therapeutic decisions. However, for understanding of individual pathological processes is necessary as a first step to reveal basic mechanisms and inter-relationship between structure and function of the heart-vascular system. Our results suggest several implications. Firstly, 7-NI treatment did not affect SBP as well as endothelial function and differently influenced the contractility and trophicity of conduit arteries in SHR. Secondly, because inhibition of nNOS did not lead to either a blood pressure increase and impaired vasorelaxation or a uniform increase of vasoconstriction and general hypertrophy of cardiovascular system, we can conclude that nNOS influences regulation of the SHR cardiovascular system by a different mechanism than eNOS. The data suggest that nNOS triggers individual and tissue-specific regulatory pathways in essential hypertension.

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