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CHANGES IN THE VASOACTIVE EFFECTS OF NITRIC OXIDE, HYDROGEN SULFIDE AND THE STRUCTURE OF THE RAT THORACIC AORTA: THE ROLE OF AGE AND ESSENTIAL HYPERTENSION

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Several studies have already confirmed the specific vasomotor effect of hydrogen sulfide (H_2S) and its interaction with the nitric oxide (NO) system in normotensive rats, but results in spontaneously hypertensive rats (SHRs) are limited. The aim of this study was to describe the age- and blood pressure-dependent effects of endogenous NO and exogenous Na_2S and their interaction in vasomotor responses of the thoracic aorta (TA) in normotensive Wistar rats and SHRs. The systolic blood pressure (sBP), vasoactivity, NO-synthase (NOS) expression and activity, cystathionine gamma-lyase (CSE) expression, and geometry of the isolated TA were evaluated at 4 and 16 weeks of age. Although hypertrophy of the heart was observed in young and adult SHRs, the sBP was increased only in adulthood. The contractile responses were decreased in young as in adult SHRs with the key participation of the endogenous NO system. However, the hypotrophy in the young and the hypertrophy (mainly at the expense of extracellular matrix) in the adult SHRs were found in the TA. While unchanged in young SHRs, in adult SHRs, partially impaired endothelial function was confirmed. Nevertheless, the NO-dependent component of acetylcholine-induced relaxation was higher in both young and adult SHRs. Consistently, even though there was an age-dependent decrease in NOS activity in both strains, NOS activity was higher in both young and adult SHRs compared to age-matched normotensive rats. Application of exogenous Na_2S evoked a concentration-dependent dual vasoactive effect of TAs in both strains, regardless of age. Increased sensitivity in favor of vasorelaxant responses of Na_2S in prehypertensive SHRs, and an enhanced maximal vasorelaxation in adult SHR was observed. The acute NO inhibition generally increased the relaxant phase of Na_2S responses; nevertheless, the development of hypertension potentiated this effect. The TA of the SHRs is endowed with a unique inherent predisposition of vasoactive mechanisms, which serve as compensatory processes during the developed stage of hypertension: the NO component and H_2S signaling pathways are implicated. The decreased contractility seems to be a deleterious effect. The increased participation of the H_2S system on vasorelaxation after acute NO inhibition could be considered a reserved mechanism in case of endogenous NO deficiency.

Key words: *nitric oxide, hydrogen sulfide, prehypertension, hypertension, thoracic aorta, spontaneously hypertensive rats, noradrenaline, nitric oxide synthase, cystathionine gamma-lyase*

INTRODUCTION

Hypertension and aging are considered important cardiovascular factors evoking functional and structural changes at the level of the endothelium, the smooth muscle cells and the extracellular matrix of blood vessels. Age- and blood pressure-dependent alterations can be manifested as damage in the equilibrium between endothelial vasorelaxant and vasoconstrictor agents as well as by an abnormal cardiovascular remodeling (1-3). The possible mechanisms engaged in the regulation of these processes represent a huge group of interactions among many systems.

In conduit arteries of normotensive rats, such as the thoracic aorta (TA), endothelium-dependent vasorelaxation is primarily mediated by gaseous transmitter - nitric oxide (NO) - that is released predominantly by endothelial cells. The involvement of NO in the pathological processes of aging and hypertension has been investigated by numerous studies. Briones *et al*. (4) showed an age-dependent upregulation of endothelial NO-synthase (eNOS) in the mesenteric artery bed; in contrast, Yoon *et al*. (5) observed that aging decreased the production of NO and the activity and expression of eNOS in endothelial cells of human umbilical vein. In spontaneously hypertensive rats (SHRs), an animal model of essential hypertension, contradictory results were observed related to endothelial NO regulation; in adult SHRs, the inhibition and potentiation of NO-dependent relaxation associated with an increased NO production by vessel wall were shown (6, 7). We have previously confirmed that the TA of young SHRs disposed with strengthened NO-dependent vasorelaxant mechanisms and decreased adrenergic contractility is associated with hypotrophy of the arterial wall compared with age-matched normotensive rats. Since the study was performed in the juvenile ontogenic stage of the SHR, when an acceleration of systolic blood pressure was not recorded (8), we could assume that the TA was endowed with a unique inherent predisposition, which serves as an adaptive mechanism later during the

developed phase of hypertension. One of the goals of this study was to test this suggestion.

Another gaseous transmitter - hydrogen sulfide (H_2S) - through its vasoactive properties has been identified as protective compound with anti-inflammatory effects (9). Moreover, experiments using adult SHRs, in which H_2S was administered, proved that H_2S could partially prevent hypertension and the remodeling of the aorta (10). At the same time, several authors confirmed 'crosstalk' between H_2S and NO, at the molecular level and at the level of their endogenous production. Ondrias *et al*. (11) used adult normotensive rats to prove the H_2S-NO interaction at the molecular level. The authors demonstrated that low doses of H_2S donor potentiated NO release from exogenous donor S-nitrosoglutathione (GSNO) and increased its vasorelaxant effect. Geng *et al*. (12) demonstrated that H_2S inhibited NO synthesis at the level of eNOS expression and decreased eNOS activity in normotensive conditions. Our previous study showed that H_2S evoked a dose-dependent dual vasoactive effect in precontracted TA in both young Wistar rats and SHRs; however, the H_2S donor (Na_2S) regulated the arterial tone in favor of vasorelaxant responses with a higher sensitivity in SHRs than in normotensive rats and more effectively during acute NO deficiency (8). We suggest that the TA of the prehypertensive SHR is endowed with strengthened endothelium-regulated vasorelaxant mechanisms involving an NO and H_2S interaction, which can serve as an adaptive mechanism later. This study also sought to test this hypothesis.

Taken together, the position of NO in the etiopathogenesis of essential hypertension is still not clear. Moreover, investigations of the direct vasoactive effects of H_2S and the engagement of endogenous NO in these responses with respect to the impact of age and increased blood pressure have not yet been described. Therefore, in the present study, we aimed to characterize and compare the participation of the NO and H_2S systems and their interaction in vasomotor responses of the TA in young and adult Wistar rats and SHRs. We evaluated the endothelium-dependent vasorelaxation, NOS expression and activity, CSF expression, dual vasoactive effect of exogenous H_2S, and adrenergic vasocontraction and geometry of the TA.

MATERIALS AND METHODS

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 used in the study were born in an accredited breeding establishment of the Institute of Normal and Pathological Physiology, Slovak Academy of Sciences and were housed in groups of 3 animals, each strain separately, under a 12 h light/12 h dark cycle, at a 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.

Functional study

Briefly, 4-week-old and 16-week-old male Wistar rats and SHRs (n = 18 in each group) were used in this study. Systolic blood pressure was measured in prewarmed rats by the non-invasive tail-cuff method before the beginning of the *in vitro* study (except handling). The body weight (BW) of each rat was determined before decapitation after a brief anesthetization with CO_2. The weight of the heart (HW) was recorded for calculation of heart weight/body weight ratio to evaluate the degree of cardiac hypertrophy. Although the thoracic aorta (TA) is a large conduit artery to avoid the impairment of the endothelium TA was carefully cleaned of connective tissue and cut into 5 mm length rings using the binocular microscope. The rings were vertically fixed between 2 stainless wire triangles and immersed in 20 mL incubation organ bath with Krebs solution (NaCl 118 mmol/L, KCl 5 mmol/L, $NaHCO_3$ 25 mmol/L, $MgSO_4.7H_2O$ 1.2 mmol/L; KH_2PO_4 1.2 mmol/L, $CaCl_2$ 2.5 mmol/L, glucose 11 mmol/L, $CaNa_2EDTA$ 0.032 mmol/L). This solution was oxygenated with 95% O_2 and 5% CO_2 mixture and kept at 37°C. The upper triangles were connected to sensors of the isometric tension (FSG-01, MDE, Budapest, Hungary), and the changes in tension were registered by an AD converter NI USB-6221 (National Instruments, Austin, USA; MDE, Budapest, Hungary) and registered by DEWEsoft (Dewetron, Prague, Czech Republic) and SPEL Advanced Kymograph (MDE, Budapest, Hungary) software. The resting tension of 1 g was applied to each ring and maintained throughout a 45 – 60 min of equilibration period until stress relaxation no longer occurred. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (10^{-5} mol/L) to induce at least 50% relaxation of phenylephrine (10^{-6} mol/L) pre-contracted arteries.

Increasing concentrations of exogenous noradrenaline (NA, $10^{-10} – 3 \times 10^{-5}$ mol/L) were used to demonstrate the adrenergic receptor-dependent contractile abilities of smooth muscle cells. The extent of the contractile responses was expressed as developed changes in isometric tension (g) and as a percentage of the maximum tissue response to the agonist (demonstrating the sensitivity of contractile apparatus). The concentrations of NA producing the half-maximum response (EC_{50}) were calculated from individual dose-response curves and expressed as the negative logarithm of NA molar concentration.

The relaxant responses were followed on rings precontracted with phenylephrine (PE; 10^{-6} mol/L) after the achievement of a stabile plateau of the contraction. The isolated TA rings were then exposed to cumulative doses of acetylcholine (Ach; $10^{-10} – 3 \times 10^{-5}$ mol/L). The rate of relaxation was expressed as a percentage of the PE induced contraction. The concentrations producing 50% inhibition of contraction to PE (IC_{50}) were calculated from individual dose-response curves and expressed as the negative logarithm of Ach molar concentration.

Na_2S was used as a H_2S donor that dissociates in water solution to Na^+ and S^{2-}, which reacts with H^+ to yield HS^- and H_2S. We use the term Na_2S to encompass the total mixture of H_2S, HS^- and S^{2-}. Direct vasoactive effects of Na_2S were observed on NA-pre-contracted (10^{-6} mol/L) rings by administration of increasing doses of Na_2S (20, 40, 80, 100, 200, 400 μmol/L).

The participation of endogenous NO system in the vasomotor responses of TA was followed before and 20 minutes after pretreatment with a non-specific inhibitor of NOS, N^G-nitro-L-arginine methyl ester (L-NAME; 10^{-6} mol/L) to block the basal and the receptor-induced endogenous NO production.

Morphological study

Four groups (n = 10 in each group) of Wistar rats and age-matched SHRs aged 3 – 4 weeks (4 w) and 16 weeks (16 w) were used for the study. The geometry of the TA as well as the structure of the TA was assessed. The animals were sacrificed by an overdose of anesthesia (Rometar and Zoletile, 1 and 6 mg/100 g, respectively) administered i.p. The chest was opened, a cannula was placed into the left ventricle, and the cardiovascular system was perfused at a constant pressure of 90 mm Hg (4 w) and 120

mm Hg (16 w) for 10 minutes with a fixative solution (300 mol/L glutaraldehyde in 100 mmol/L phosphate buffer). The middle part of the TA was excised and immersed in the same fixative overnight, post-fixed with 40 mmol/L OsO4 in 100 mmol/L phosphate buffer, stained en block with 1% uranyl acetate, dehydrated by a graded alcohol series and propylene oxide, and embedded in Durcupan ACM. Semi-thin sections were cut perpendicularly to the long axis. Both the wall thickness (tunica intima + tunica media) and inner circumference were measured by light microscopy. The wall thickness was measured at 45° intervals around the circumference of the artery. The inner diameter, cross-sectional area (tunica intima + tunica media), and wall thickness/inner diameter ratio were evaluated from these data. Sections of approximately 70 nm thickness were cut on an ultramicrotome (Reichert Nova, Charleston, South Carolina, USA), stained with alkaline lead citrate and examined in a transmission electron microscope (Tesla BS 500, Brno, Czech Republic). The volume densities (proportional representation) of endothelial cells (ECs), smooth muscle cells (SMCs), and the extracellular matrix (ECM) in the tunica intima and media were determined with the Weibel *et al*. (13) point counting method by electron microscopy, and their cross-sectional areas were counted from the volume densities.

Nitric oxide synthase activity

Total NOS activity was determined in crude homogenates of aorta by measuring the formation of [^{3}H] - L-citrulline from [^{3}H] - L-arginine (ARC, Montana, USA), as previously described and slightly modified by Pechanova *et al*. (14). [^{3}H] - L-citrulline was measured with the Quanta Smart TriCarb Liquid Scintillation Analyzer (Packard Instrument Company, Meriden, CT, USA). NOS activity was expressed as pkat/min per gram of protein.

Endtohelial NO-synthase (eNOS) and cystathionine gamma-lyase (CSE) expressions

Protein expressions of eNOS and CSE were determined in the aorta by Western blot analysis. Sample of the aortas were homogenized in lysis buffer 0.05 mmol/L Tris containing protease inhibitor cocktail (Calbiochem, Germany). After centrifugation (15,000 rpm at 4°C for 20 min), protein concentrations were determined according to Lowry assay. Proteins were subjected to 12% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were blocked with 5% non-fat milk in Tris-buffer solution (TBS; pH 7.6) containing 0.1% Tween-20 (TBS-T) for 1 hour at room temperature and then incubated in primary antibodies overnight at 4°C with polyclonal rabbit anti-endothelial NOS (Abcam, Cambridge, UK) and mouse monoclonal anti-gamma cysthationase antibodies (Proteintech, Menchester, UK). Membranes were washed and finally incubated with secondary antibodies for 2 hour at room temperature using a secondary peroxidase-conjugated anti-rabbit and anti-mouse antibody, respectively (Abcam, Cambridge, UK). The intensity of bands was visualized using chemiluminiscence system (ECL, Amersham, UK), quantified by using ChemiDocTM Touch Imagine System (Image LabTM Touch software, Bio-Rad), and normalized to GAPDH bands (Abcam, Cambridge, UK).

Statistical analysis

The data are expressed as the mean ± S.E.M. For the statistical evaluation of differences between groups, one-way and two-way analysis of variance (ANOVA) with the Bonferroni post hoc test and paired t-test were used. The differences between means were considered significant at $P < 0.05$.

DRUGS

The following drugs were used: phenylephrine (Sigma-Aldrich, St. Louis, Missouri, USA), noradrenaline (Zentiva, Czech Republic), acetylcholine (Sigma-Aldrich), sodium sulphide nonahydrate (Sigma-Aldrich), N^G-nitro-L-arginine methyl ester (Sigma-Aldrich), durcupan ACM (Fluka), and glutaraldehyde (Sigma).

RESULTS

Basic cardiovascular parameters

The heart weight/body weight ratio and the values of the systolic blood pressure (sBP) are shown in *Fig. 1*. Although in young rats (n = 16), there was no difference between the SHR

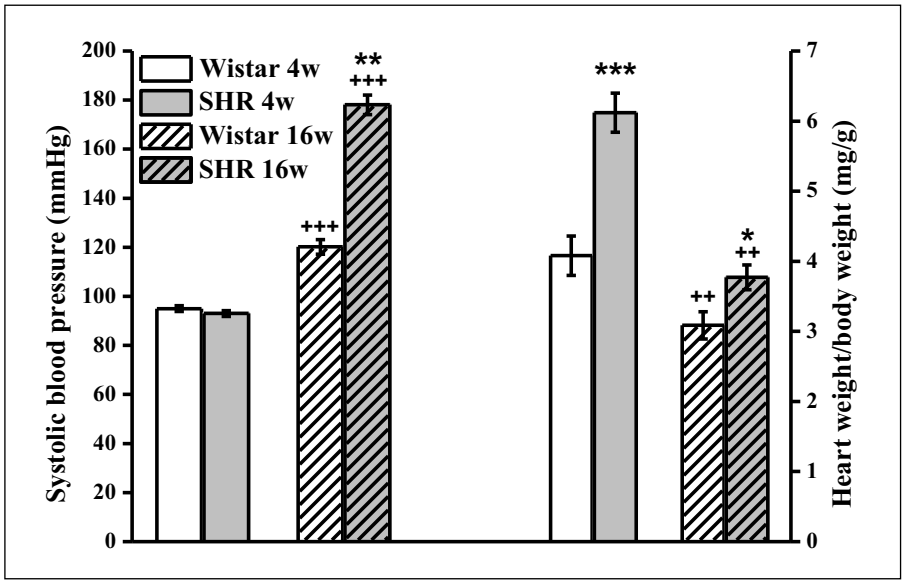

Fig. 1. Comparison of the systolic blood pressure and heart weight body weight ratio between young and adult normotensive Wistar rats and spontaneously hypertensive rats (SHR). White columns represent young, 4-weeks-old Wistar rats (Wistar 4w), gray columns represent young, 4-weeks-old SHR (SHR 4w), hatched white columns represent adult, 16-weeks-old Wistar rats (Wistar 16w), hatched grey columns represent adult, 16-weeks-old SHR (SHR 6w). Values are mean ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 versus Wistar of the same age; $^{++}$P < 0.01, $^{+++}$P < 0.001 versus 4-week-old rats within the strain.

and normotensive group (n = 16) in terms of the values of sBP, an age-dependent increase in this parameter was observed in both groups ($P < 0.001$); moreover, in adult SHRs, the increase was accentuated compared to the age-matched normotensive group ($P < 0.01$). SHRs had a reduced body weight compared with Wistar rats: 61.75 ± 3.33 g for young SHRs versus 107.00 ± 4.55 g for age-matched Wistar rats ($P < 0.001$); 299.17 ± 18.52 g for adult SHRs versus 366.2 ± 13.89 g for age-matched Wistar rats ($P < 0.05$). We did not observed any differences in heart weight between the experimental groups (young Wistar rats:

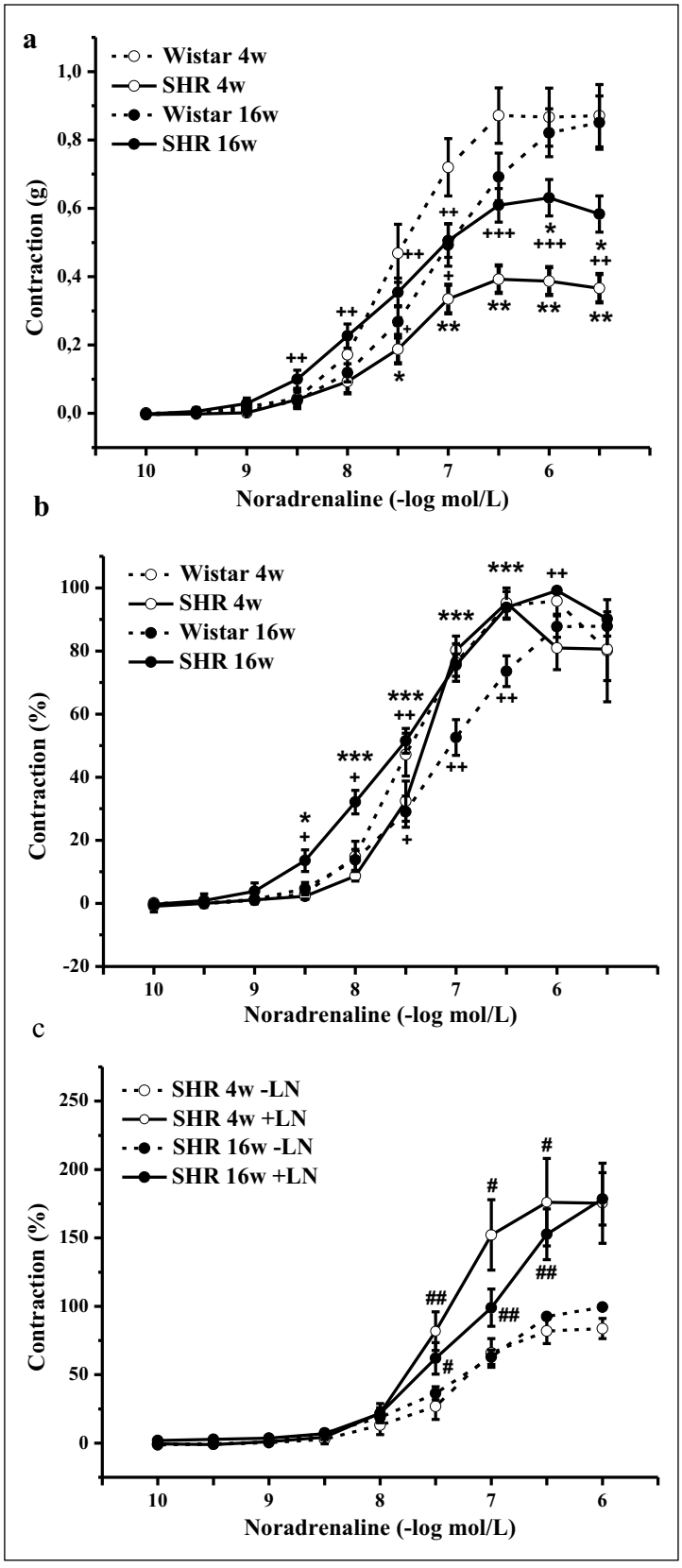

Fig. 2. Noradrenaline-induced concentration-dependent responses of thoracic aorta. The curves are expressed: a) in grams; b) in per cent values of the maximum tissue response to noradrenaline; c) before (–LN) and after (+LN) acute pre-treatment with NO-synthase inhibitor L-NAME (10^{-6} mol/L) in 4-week-old normotensive rats (Wistar 4w), spontaneously hypertensive rats (SHR 4w), and in 16-week-old normotensive rats (Wistar 16w), spontaneously hypertensive rats (SHR 16w). Values are mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Wistar of the same age; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ versus other age group within the strain; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus -LN of the same age.

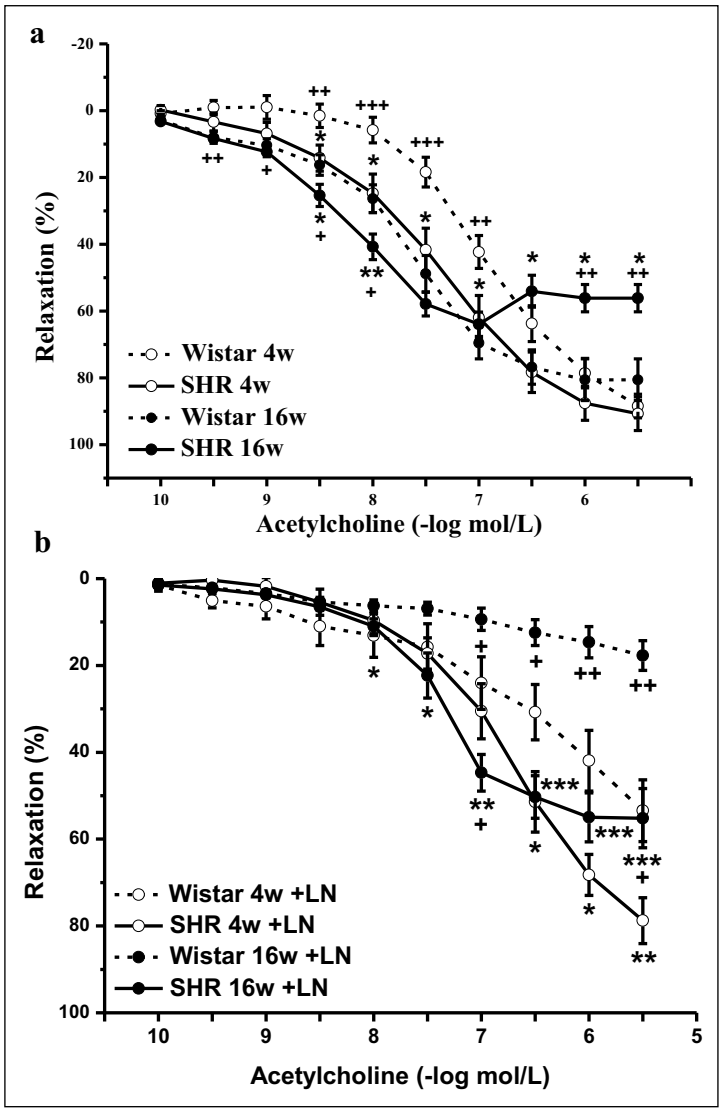

Fig. 3. Endothelium-dependent relaxation of thoracic aorta. Acetylcholine-induced concentration-dependent responses in 4-week-old normotensive rats (Wistar 4w), spontaneously hypertensive rats (SHR 4w), and in 16-week-old normotensive rats (Wistar 16w), spontaneously hypertensive rats (SHR 16w) before (a) and after (b) the treatment with NO-synthase inhibitor L-NAME (LN) (10^{-6} mol/L). Values are mean ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 versus Wistar of the same age; $^{+}$P < 0.05, $^{++}$P < 0.01, $^{+++}$P < 0.001 versus other age group within the strain.

437.70 ± 35.09 mg, young SHRs: 378.90 ± 27.39 mg, adult Wistar rats: 1123.52 ± 55.4 mg, adult SHRs: 1109.26 ± 27.51 mg). A significantly increased (P < 0.001; P < 0.05) heart weight to body weight ratio was demonstrated in both SHR groups compared with the age-matched Wistar rats. However, with age, this ratio was reduced in both strains (P < 0.01).

Vasocontractile responses

Cumulative application of NA (10^{-10} – 3 × 10^{-6} mol/L) induced vasoconstriction in a concentration-dependent manner in both strains, regardless of age (n = 8 rats in each group). Although the absolute force of contractile responses was significantly reduced in SHRs compared to age-matched Wistar rats (*Fig. 2a*; P < 0.05; P < 0.01), an age-related increase in the contractile force was observed in SHRs. Expressing the data as a percentage of the maximal NA-induced contraction revealed no differences between the two young groups (Wistar: 7.8 2 ± 0.16; SHR: 7.26 ± 0.25). On the other hand, in adult SHRs, we demonstrated an increased sensitivity to NA compared to adult Wistar rats (*Fig. 2b*). Aging modified the sensitivity of smooth muscle cells to NA in both strains, but in the opposite manner: it was reduced in Wistar rats (7.06 ± 0.14) and enhanced in SHRs (7.75 ± 0.1; P < 0.05; P < 0.01). An acute inhibition of endogenous NO production by L-NAME (10^{-6} mol/L) significantly increased the dose-dependent contractile responses to NA similarly in young and adult SHRs; endogenous NO participated in adrenergic vasocontraction to the same extent in both age groups (*Fig. 2c*).

Endothelium-dependent vasorelaxation

The application of Ach (10^{-10} – 3 × 10^{-6} mol/L) relaxed the rings of TA in both strains and both age groups (n = 8 rats in each group) in a concentration-dependent manner (*Fig. 3*). The maximum of the endothelium-dependent vasorelaxation (Ach, 3 × 10^{-6} mol/L) was comparable between young Wistar rats and SHRs; on the other hand, in adult SHRs, the achieved relaxation at this concentration was lower in the age-matched Wistar rats and young SHR (P < 0.05; P < 0.01) than that in the age-matched Wistar rats. According to the sensitivity of smooth muscle cells to Ach, similar trends were observed: 1) SHRs had an increased sensitivity to Ach compared with age-matched Wistar rats (young Wistar: 6.9 ± 0.13 versus SHR: 7.59 ± 0.2; P < 0.05; adult Wistar: 7.73 ± 0.07 versus SHR: 8.13 ± 0.09; P < 0.01), and 2) there was an age-dependent increase in sensitivity in both strains (Wistar: young 6.9 ± 0.13 versus adult 7.73 ± 0.07; SHR: 7.59 ± 0.2 versus 8.13 ± 0.09; P < 0.01). After the treatment with L-NAME the

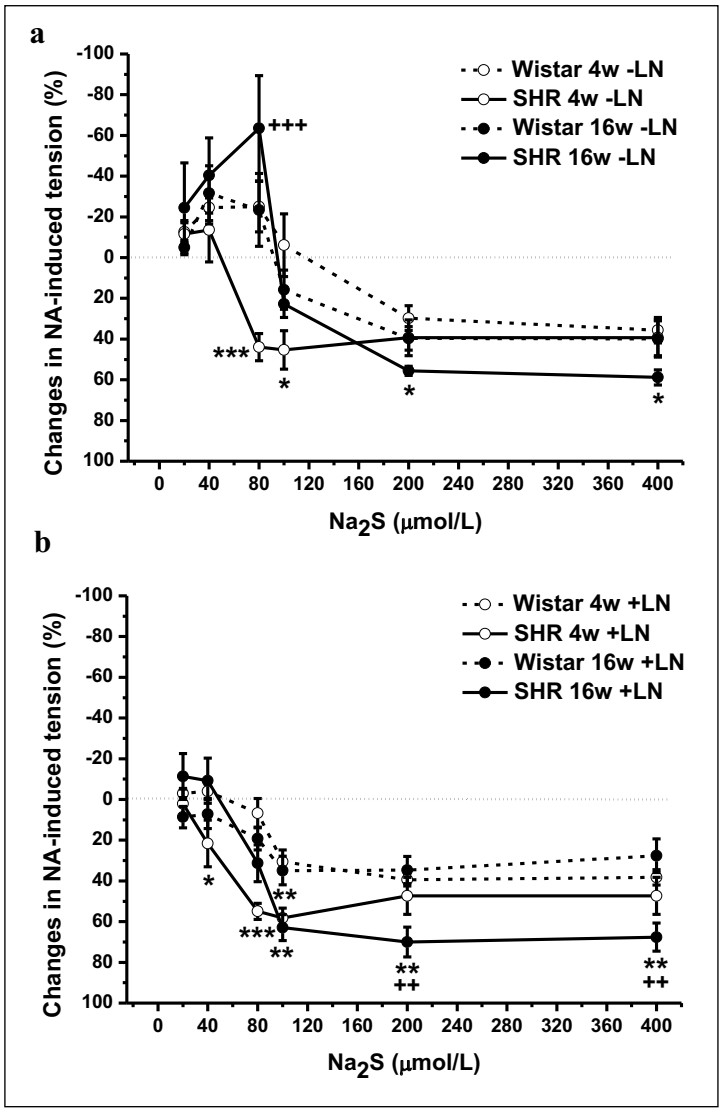

Fig. 4. Dual vasoactive effects of exogenous H_2S donor (Na_2S) in thoracic aorta of Wistar rats and SHR before (a) and after (b) acute NO-synthase inhibition with L-NAME (LN). Changes in noradrenaline (NA, 10^{-6} mol/L) - increased tension induced by application of cummulative concentrations of Na_2S in 4-week-old normotensive rats (Wistar 4w), spontaneously hypertensive rats (SHR 4w), and in 16-week-old normotensive rats (Wistar 16w), spontaneously hypertensive rats (SHR 16w). Values are mean ± S.E.M. *P < 0.05, **P < 0.01, ***P < 0.001 versus Wistar of the same age; $^{+}$P < 0.05, $^{++}$P < 0.01, $^{+++}$P < 0.001 versus other age group within the strain.

endothelium-dependent relaxation in response to Ach was significantly smaller in young Wistar rats (n = 6) compared to age-matched SHR (n = 6) (P < 0.05; P < 0.01; *Fig. 3b*) demonstrating the higher participation of NO in the vasorelaxation in young SHRs than that in young Wistar rats. The difference between the strains was preserved also in adult rats. After the L-NAME treatment the acetylcholine-induced vasorelaxation was significantly smaller in adult Wistar rats (n = 6) compared to adult SHR (n = 6) (P < 0.05; P < 0.01; P < 0.001; *Fig. 3b*). In both strains, an age-dependent significant decrease observed in maximal vasorelaxant response to acetylcholine was observed decreased by age (P < 0.05; P < 0.01 in Wistar rats; P < 0.05 in SHR).

Vasoactive effect of hydrogen sulfide donor

The cumulative application of the H_2S donor (Na_2S; 20, 40, 80, 10, 200, and 400 μmol/L) on NA-pre-contracted TA rings induced a dual effect in all experimental groups (n = 8 rats in each group): lower concentrations of Na_2S induced contraction, whereas the higher concentrations evoked vasorelaxation of the arterial wall (*Fig. 4a*). A drift in vasomotor responses in favor of vasorelaxation was observed only in young SHRs compared to young Wistar rats but not in adult rats. The vasorelaxant response was triggered in young SHRs by a lower concentration of Na_2S (80 μmol/L) than in Wistar rats (100 μmol/L) (P < 0.001). The vasorelaxant effects of higher Na_2S doses (200, 400 μmol/L) were significantly enhanced in adult SHRs compared to the age-matched Wistar rats (P < 0.05). Unlike in Wistar rats, an age-dependent drift in favor of vasorelaxation (80 μmol/L) was evident in SHRs. Acute inhibition by L-NAME generally diminished the contractile part of the Na_2S-induced vasomotor responses in all groups (*Fig. 4b*). Similarly as before L-NAME treatment, a drift in vasomotor responses in favor of vasorelaxation was observed in young SHRs compared to young Wistar rats (P < 0.05; P < 0.01; P < 0.001) and the vasorelaxant effect induced by higher Na_2S doses was significantly enhanced in adult SHRs compared to the age-matched Wistar rats. In Wistar rats there were no age-dependent differences in H_2S donor effects (*Fig. 4b*). However, in the SHRs, we observed that the increase in maximal vasorelaxation induced by Na_2S positively correlated with age (P < 0.01).

Nitric oxide synthase activity

The total NO synthase activity (NOS) of TA in 4-week-old SHRs was increased by 126% in comparison to the age-matched Wistar rats (136.57 ± 10.2 pkat/g, n = 7; versus 60.39 ± 4.3

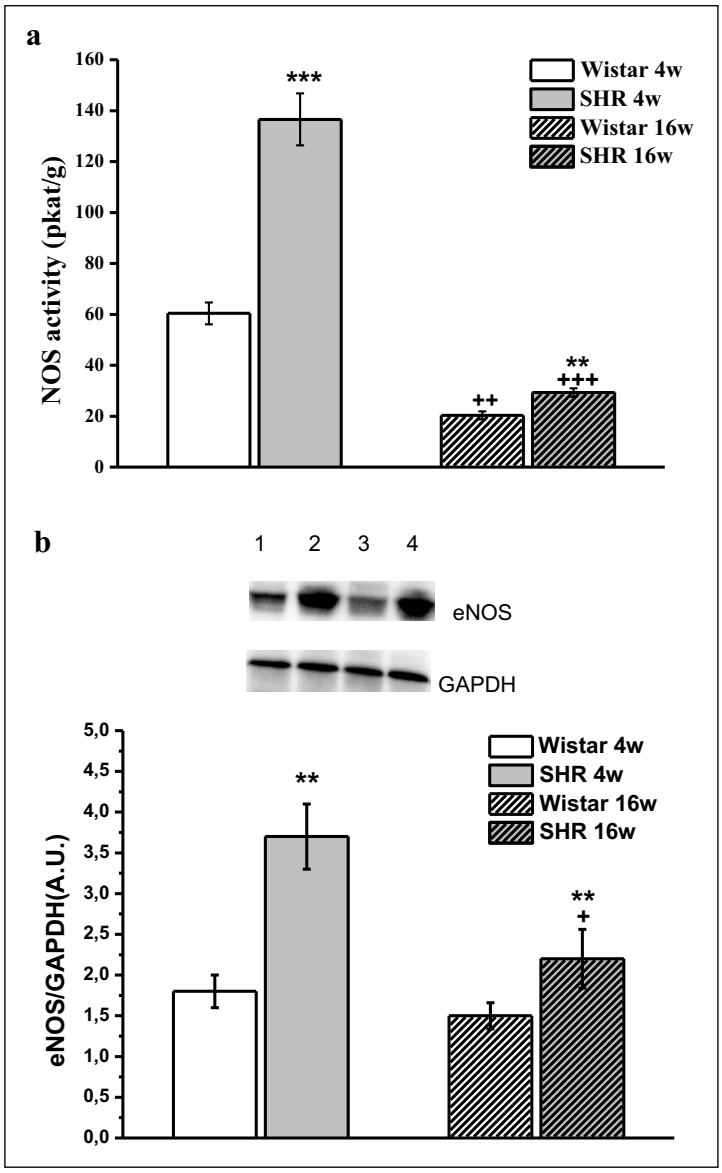

Fig. 5. NO-synthase in the thoracic aorta. The total NOS activity (a) and the endothelial eNOS expression (b) in 4-week-old normotensive rats (Wistar 4w), spontaneously hypertensive rats (SHR 4w), and in 16-week-old normotensive rats (Wistar 16w), spontaneously hypertensive rats (SHR 16w). Lanes: 1 = Wistar 16w; 2 = SHR 16w; 3 = Wistar 4w; 4 = SHR 4w. Values are mean ± S.E.M. **P < 0.01, ***P < 0.001 versus Wistar of the same age; $^{+}$P < 0.05, $^{++}$P < 0.01, $^{+++}$P < 0.001 versus other age group within the strain.

pkat/g, n = 8; respectively; P < 0.001; *Fig. 5a*). At the age of 16 weeks, NOS activity was still significantly higher by 44% in the SHR group than in the Wistar rat group (20.3 ± 1.58 pkat/g, n = 8; versus 29.28 ± 1.68 pkat/g, n = 8; respectively; P < 0.01). Moreover, we also confirmed age-dependent decrease in NOS activity in both strains (P < 0.01 in Wistar rats; P < 0.001 in SHR; *Fig. 5a*).

Endothelial nitric oxide synthase expression and cystathionine gamma-lyase (CSE) expression

Endothelial NOS protein expression in the aorta of 4-week-old SHR (n = 6) was increased significantly in comparison to the age-matched Wistar rats (n = 6) (P < 0.01). The same effect was found in 16-week-old animals (n = 6; P < 0.01; *Fig. 5b*). Moreover, a significant decrease in endothelial NOS expression in adult compared young SHR was demonstrated (P < 0.05; *Fig. 5b*).

On the other hand, protein expression of CSE in the aorta did not show any significant differences between Wistar and SHR animals in both examined ages. Similarly, there were no age-dependent significant changes in protein expression of CSE in both strains (*Fig. 6*).

Geometry and composition of the thoracic aorta

The thickness of the thoracic wall in 4-week-old SHRs (n = 8) compared to age-matched Wistar rats (n = 8) was significantly decreased by 36%, as was the cross-sectional area (tunica intima + tunica media), which was decreased by 53%. The wall thickness/inner diameter ratio was significantly lowered by 21%, despite no significant changes in the inner diameter (–12%) of the TA (*Fig. 7*). In the TA of the 16-week-old SHRs, compared to normotensive Wistar rats of the same age, the wall thickness was already significantly increased by 12% compared to normotensive rats. The cross-sectional area of the TA wall was increased by 15%. The inner diameter was not changed (4%), and the wall thickness/inner diameter ratio was slightly (though not significantly) decreased (–10%) (*Fig. 7*). We also confirmed age-dependent changes in trophicity of TA wall in both strains. The thickness of TA was increased in 16-week-old SHR (n = 8) compared to 4-week-old SHR (P < 0.01). The cross-sectional area (tunica intima + tunica media) of TA was increased in both 16-week-old Wistar rats and SHR compared to young Wistar rats (P < 0.01) and SHR (P < 0.01) (*Fig. 7*).

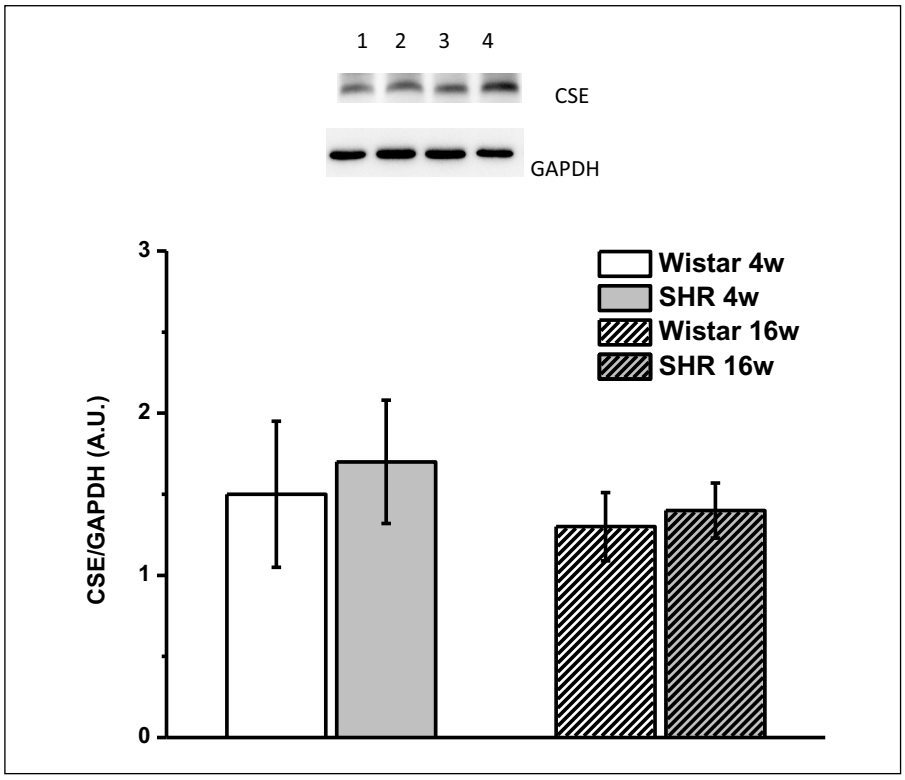

Fig. 6. Cystathionine gamma-lyase (CSE) evaluation in the thoracic aorta. CSE expression in the thoracic aorta in 4-week-old normotensive rats (Wistar 4w), spontaneously hypertensive rats (SHR 4w), and 16-week-old normotensive rats (Wistar 16w), spontaneously hypertensive rats (SHR 16w). Lanes: 1 = Wistar 16w; 2 = SHR 16w; 3 = Wistar 4w; 4 = SHR 4w. Values are mean ± S.E.M.

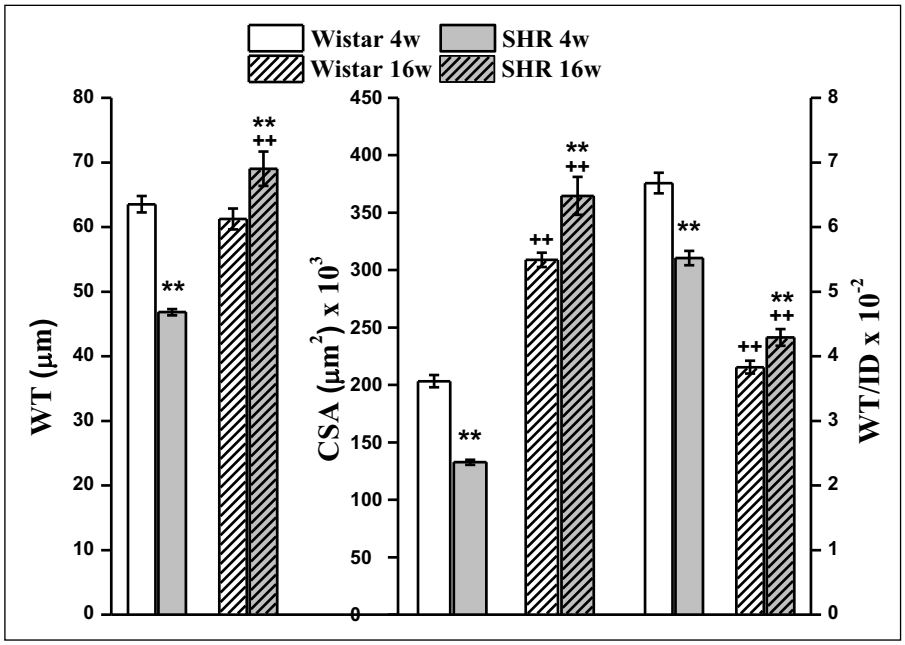

Fig. 7. Geometry of the thoracic aorta. Wall thickness (WT), cross sectional area (CSA) of the wall (tunica intima + media), wall thickness-inner diameter ratio (WT/ID) in 4-week-old normotensive rats (Wistar 4w), spontaneously hypertensive rats (SHR 4w), and in 16-week-old normotensive rats (Wistar 16w), spontaneously hypertensive rats (SHR 16w). Values are mean ± S.E.M. **P < 0.01 versus Wistar of the same age; $^{+}$P < 0.05, $^{++}$P < 0.01 versus 4-week-old rats within the strain.

Analysis of the composition of the arterial wall of the TA in 4-week-old SHRs (n = 8) compared to age-matched Wistar rats (n = 8) revealed that all components of the wall were hypotrophied (*Fig. 8*). The cross-sectional areas of smooth muscle cells, endothelial cells, and the extracellular matrix were decreased by 38%, 33%, and 32%, respectively. This finding means that the level of participation of all components is approximately the same in the hypotrophy of the TA wall at this stage of ontogenic development of the SHR. The analysis of the TA wall in 16-week-old animals proved that all components in both strains were hypertrophied (*Fig. 8*), but in SHRs, hypertrophy was more intensive than in normotensive TA. The most increased component participating in the hypertrophy of the TA wall was the extracellular matrix, which participated at a level of 33%, and less smooth muscle cells, at a level of 7% (still significantly increased in comparison with Wistar rats). No significant difference was observed in the cross-sectional area of the endothelial cells (+12%). As related to the age-dependent changes within the strain we observed in 16-week-old animals an increase in cross-sectional areas of smooth muscle cells ($P < 0.01$ in Wistar rats; $P < 0.001$ in SHR), extracellular matrix ($P < 0.01$ in Wistar rats; $P < 0.001$ in SHR) and endothelial cells ($P < 0.05$ in Wistar rats; $P < 0.05$ in SHR) compared to young 4-week-old animals (*Fig. 8*).

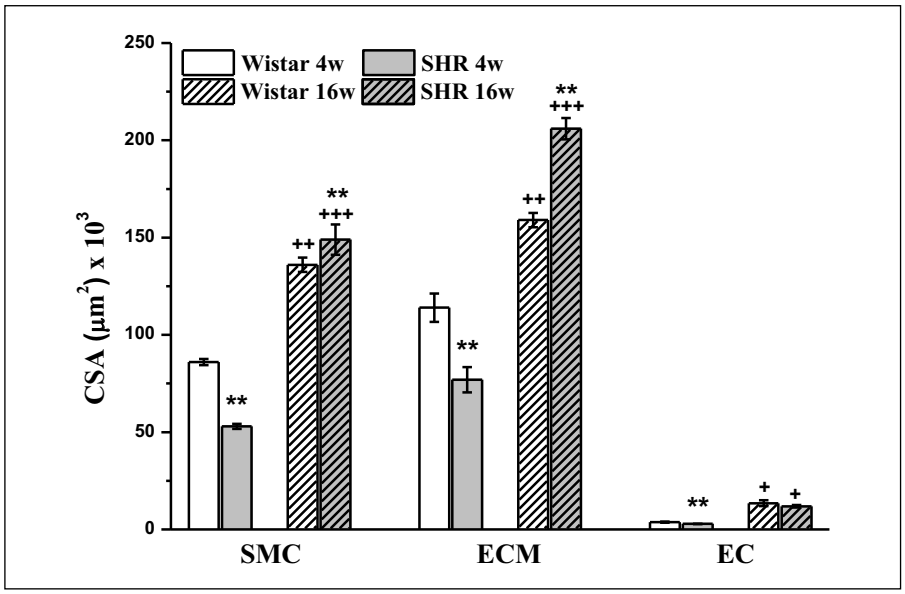

Fig. 8. Composition of the thoracic aorta wall. Cross sectional area (CSA) of smooth muscle cells (SMC), extracellular matrix (ECM) and endothelial cells (EC) in thoracic wall in 4-week-old normotensive rats (Wistar 4w), spontaneously hypertensive rats (SHR 4w), and in 16-week-old normotensive rats (Wistar 16w), spontaneously hypertensive rats (SHR 16w). Values are mean ± S.E.M. **P < 0.01 versus Wistar of the same age; $^{+}$P < 0.05, $^{++}$P < 0.01, $^{+++}$P < 0.001 versus 4-week-old rats within the strain.

DISCUSSION

Our study focused on the comparison of vasoactive and structural properties of the thoracic aorta (TA) in young and adult normotensive rats and SHRs. We confirmed i) reduced contractility in young and adult SHR; ii) preserved NO participation in vasorelaxation in adult Wistar rats and SHRs; and iii) that age- and blood pressure-dependent enhanced vasorelaxant effects of H_2S were potentiated by endogenous acute NO deficiency. For the first time, we noted the age-and blood pressure-dependency of crosstalk between NO and H_2S.

The recorded values of sBP were in good agreement with data from the literature, including the original description of the SHR strain. At the age of 4 weeks, there was no significant difference in sBP between SHRs and normotensive rats. An age-dependent increase in sBP was observed in both Wistar rats and SHRs; however, sBP increased faster in SHRs than in Wistar rats (*Fig. 1*). Similar results were reported by several other studies, which found that blood pressure accelerated from 6 weeks of age, and the continual increasing of sBP was stopped after approximately 36 weeks of the age (15). There is a relative consensus that the main alterations in SHRs, such as changes in the heart and arterial wall trophicity, start from approximately the 5th week of life (3). We determined that the heart-body weight ratio was significantly higher in both young and adult SHRs than in Wistar rats (*Fig. 1*). This could be attributed to the lower body weight registered in SHRs, which was approximately 42% lower in young rats and approximately 18% lower in adult SHRs. Since the SHR mothers had sustained hypertension, the blood supply for the fetus might be assumed to have been compromised, causing a reduced body weight during ontogenesis, especially in the early periods. Similar results were declared by Kristek and Gerova (16) who observed a lower body weight in 4-week-old rats from NO-deficient hypertensive mothers. Moreover, Cebova and Kristek (15) showed that the heart-body weight ratio was significantly higher in SHRs than in normotensive rats in all periods of ontogeny and already lower in 3-week-old rats. Although several studies reported that increasing blood pressure stimulated hypertrophy of the heart (17, 18), our present data demonstrated that changes in heart trophicity could not be fully blood pressure-dependent.

Our morphological analysis revealed the hypotrophy of the TA wall in 4-week-old SHRs compared to Wistar rats. On the other hand, in adulthood, together with a rapid blood pressure increase, the TA was hypertrophied compared to normotensive rats (*Fig. 8*). In our recent paper, we showed that in the prehypertensive period of SHRs (3 weeks of age) normotrophy of the carotid artery and hypertrophy of the iliac artery were observed (3). Nevertheless, similar to in the TA, the arterial wall of all arteries was hypertrophied along with the blood pressure elevation. These data indicate that the most important changes in the structure of conduit arteries occur during the development of hypertension. Moreover, our results revealed that the changes in arterial wall trophicity can also be age dependent. Although, the WT of the TA was the same in 4- and 16-week-old Wistar rats, the WT/ID ratio was decreased, and the CSA was increased in adulthood. ID, WT and BP determine the circumferential stress (BP × ID/WT) that participates in the remodeling of the arterial wall. A pronounced decrease in the WT/ID ratio in the prehypertensive period of SHRs (compared to young Wistar rats) and in adult Wistar rats (compared to young Wistar rats) can result in an increase in the circumferential stress on the arterial wall. The increased circumferential stress together with disorder in the wall structure can affect arterial wall stiffening, pulse wave, and cushioning function.

Several studies showed that in SHRs, the large conduit arteries had increased sympathetic innervation and adrenergic contractile responses compared to normotensive rats (19). Moreover, Oliver *et al*. (20) declared that in SHRs, age-dependent enlargement of the presence and sensitivity of α-adrenergic receptors resulted in an enhanced sympathetic tone and adrenergic contraction. Our results showed that adrenergic contractile responses of the TA were significantly decreased in young and adult SHRs compared to Wistar rats; however, this reduction was more extended in prehypertensive SHRs than in adults (*Fig. 2a*). Changes in the trophicity of the arterial wall can, but do not have to, go hand-in-hand with functional changes in the contractility of the vessels. In young SHRs, the decreased absolute adrenergic contractility corresponded to hypotrophy of the arterial wall, where the smooth muscle cells and extracellular matrix participated at the same level. In adult SHRs, despite the arterial hypertrophy and increased α-adrenergic receptor sensitivity (*Fig. 2b*), as good predictors of increased contractile responses, we demonstrated an attenuated contractility of TA. However, in our study, we also confirmed

that the most increased component participating in hypertrophy of the TA in adult SHRs was the extracellular matrix. In pathological conditions, the development of hypertension in particular could be closely related to increased contents of collagen fibers, elastin filaments and extracellular matrix and be the reason for the decreased contractile efficiency of the TA (21). On the other hand, Gendron *et al*. (22, 23) suggested an early remodeling of TA already in young prehypertensive SHRs, and Arribas *et al*. (23) demonstrated the abnormal organization of elastin, which could diminish the mechanical properties of the arterial wall, in carotid arteries of one-week-old SHRs and in the TA of one-month-old SHRs. At any rate, the compromised contractile function of smooth muscle cells in SHRs may be related to changes in the arrangements of arterial wall components (collagen fibers, elastin filaments, and extracellular matrix). Despite the observed decreased mass of endothelial cells in the TA of young SHRs (compared to young Wistar rats), SHRs are probably not NO deficient. Our results confirmed no significant difference in the cross-sectional area of endothelial cells in adult SHRs, and the acute NOS inhibition significantly enhanced the adrenergic contraction to the same extent in young and adult SHRs (*Fig. 2c*). Key participation of the endogenous NO/NOS system in the attenuation of adrenergic contractile responses could serve as an effective and compensatory tool against the increased adrenergic sensitivity. On the other hand, the continued presentation of the reduced absolute contractile force of hypertrophied arterial wall could represent a deleterious effect. Consequently, a question arose regarding whether the decreased contractility of the TA in the early ontogeny period of SHRs could be an inherent adaptive mechanism or already related to negative structural remodeling.

Information related to NO signaling is limited in young SHRs and controversial in adult SHRs. Balis *et al*. (24) observed endothelial dysfunction in the femoral artery of 9-week-old SHRs; however, this dysfunction was due to the release of endothelium-derived contractile factor(s) and/or reduced hyperpolarization. Nevertheless, Torok *et al*. (25) proved an absolutely maintained endothelium-derived vasorelaxation of the TA in 4-week-old SHRs. In this study, we confirmed, similarly to our previous study (8), that endothelium-dependent vasorelaxation of the TA was increased, and the dose-response curve to Ach was shifted to the left in young prehypertensive rats compared to normotensive rats. These results indicated that the endothelial dysfunction was absent in young rats before the development of hypertension and that the sensitivity to endogenous NO was increased. Moreover, the expression as well as the activity of NOS was significantly higher in young SHR than in Wistar rats (*Fig. 5b*), which correlated with the functional finding that acute pretreatment with L-NAME inhibited acetylcholine-induced dose-response curve to a lower extent in young SHRs than in normotensive rats (*Fig. 3b*). The application of L-NAME was not able to fully inhibit the production of NO and in young SHR there was still enough amount of available NO. These data proved the prevalent participation of the NO-dependent component in vasoactive control of prehypertensive SHRs. We also recorded age-related changes in responses to Ach: maximal relaxation was reduced in SHRs and unchanged in Wistar rats; however, the dose-response curve was shifted to the left in both strains, though the hypertension potentiated this shift. We suggest that the reason for this increase in the sensitivity of smooth muscle cells could be a feedback reaction to attenuated NOS system , as confirmed in adulthood for both strains. It is in agreement with the results of several studies that have shown that the decreased endothelial NO efficiency of isolated arteries might be compensated by the increased sensitivity to NO (26). Hussain *et al*. (27) also confirmed that up-regulation of sGC ± cyclic GMP signalling could occur rapidly in response to a reduction in NO levels. On the other hand, our findings proved that even if the expression of endothelial NOS and NOS activity were decreased in adult SHRs compared to young SHR, there was still significantly higher NOS activity than in aged-matched Wistar rats. Tang *et al*. (28) reported that in adult SHRs, during the developed phase of hypertension, the effectivity of NO can be decreased but through the declared overproduction of superoxides, which could deteriorate the endothelium-dependent vasorelaxation. Previously it has been also proved that the impaired endothelial functions could not be primarily associated with reduced NO synthesis but rather with the prevalence of vasoconstrictors produced by cyclooxygenase (COX) (29). Matz *et al*. (2), Versari *et al*. (30) found that with age, graduated production of COX-derived vasocontractile agents in normotensive and hypertensive conditions was evident. Additionally, Puzserova *et al*. (1) also showed an important role for contractile prostanoids in hypertension-related endothelial dysfunction of the femoral artery in SHRs. It seems that our finding of reduced maximal relaxation of TA in adult SHRs could be associated rather with other mechanisms than with an inhibited NO signaling pathway.

Different possible alternative sources of vasoactive NO have been reported in SHRs. Zhao *et al*. (31) demonstrated that in adult SHRs, the production of endothelium-derived, NOS-independent NO as the inherent alternative production of NO from nitrites and nitrates was evident. Boulanger *et al*. (32) demonstrated that in the carotid artery of SHRs, neuronal NOS isoform-derived NO compensated for a weakened endothelial response. We suggest that NO can preserve a compensatory effort against the pathological effects of hypertension. We assume that age-related pathological changes could be well compensated in normotensive conditions, at least until early adulthood; however, during the development of hypertension, the prevalence of other negative impacts (reactive oxygen species, COX) eliminates these compensatory sources of the cardiovascular system.

In our previous study, we confirmed that the TA of prehypertensive SHRs is endowed by strengthened endothelium-regulated vasorelaxant mechanisms, which also involve another gaseous transmitter, namely, H_2S. In this study, we confirmed the dual vasoactive effect of H_2S and the existence of crosstalk between NO and H_2S in both rat strains and in both ontogeny stages. Even if Yan *et al*. (10) found the decreased gene expression and activity of CSE in arterial wall of SHR, we did not confirm the differences in CSE expression in thoracic aorta between Wistar rats and SHR regardless of age. Nevertheless, there were differences as related to the vasoactive responses induced by exogenously applied H_2S donor. We showed that in young and adult Wistar rats and in adult SHRs, the concentrations of 20 – 80 μmol/L Na_2S induced vasoconstriction, and concentrations of 100 – 400 μmol/L led to vasorelaxation of the noradrenaline precontracted aortic rings. In young SHRs, Na_2S induced vasorelaxation at 80 μmol/L. While in Wistar rats we did not document changes in vasoactive responses to Na_2S with age; in SHRs, the maximal Na_2S-induced relaxation was enhanced in the adult SHRs compared with age-matched Wistar rats (*Fig. 4a*). Several other studies have already confirmed a concentration-dependent dual effect of Na_2S in the endothelium-denuded and intact normotensive rat and human arteries and veins (33, 34). However, the results regarding Na_2S-induced vasomotor responses in SHR are limited. Zhao *et al*. (35) demonstrated that the vasorelaxant part of Na_2S effects was mediated predominantly by hyperpolarization of smooth muscle cells due to the activation of K_{ATP}. Our previous study also confirmed these findings, inasmuch as pretreatment with glybenclamide (specific K_{ATP} inhibitor) inhibited the Na_2S-induced relaxation but did not affect the contractile responses *in vivo* (36). Previous *in vitro*

experiments (8) and the present data also suggested that NO-independent pathways are responsible for the vasorelaxant effects of H_2S. The pretreatment with L-NAME diminished the contractile part but did not inhibit the relaxant part of the vasoactive Na_2S effects in young and adult normotensive rats as well as it did in SHR (*Fig. 4b*). We supposed that the vasoconstrictor effects of H_2S were probably related to its inhibitory effect on the endogenous NO/NOS system since acute L-NAME treatment disabled the inhibitory effect of Na_2S on NO production, thus masking the contractile effect of H_2S. Other authors also confirmed that NaHS, another H_2S donor, directly inhibited the activity of recombinant endothelial NOS, possibly *via* the interaction of H_2S with co-factors for NOS, such as NADPH or tetrahydrobiopterin (37). Moreover, Geng *et al*. (12) confirmed that NaHS inhibited NO generation in cultured aortic tissue and that low doses of NaHS could downregulate the L-arginine/NO pathway through the inhibition of endothelial NOS expression and L-arginine transporters and/or a decrease in NOS activity. On the other hand, the acute NO inhibition enhanced the vasorelaxant part of Na_2S-induced vasoactive effects, as shown in young Wistar and SHRs (8) and confirmed in adult SHR by the present study. We showed that acute NOS inhibition with L-NAME changed the contractile response to relaxation in both strains, and this switch-over was shifted to a lower Na_2S concentration in SHRs than in Wistar rats. Additionally, we documented age-dependent changes in the participation of NOS-derived NO in maximum Na_2S-evoked relaxation. Interestingly, in SHRs, after acute NOS inhibition, a significant age-dependent increase of the maximal vasorelaxation induced by Na_2S was observed (*Fig. 5b*). Taken together, in SHRs, H_2S regulated the arterial tone towards the vasorelaxant phase of responses, which was 'doubled' by inhibition of endogenous NO. Our findings confirmed that H_2S could take part in compensatory mechanisms of SHRs. This role of H_2S is triggered in the early ontogeny period and is enhanced in adulthood, so the development of hypertension can potentiate its balancing properties.

The present study provides original data about age- and blood pressure-dependent changes in functional and structural properties of the rat thoracic aorta. We confirmed our previous hypothesis that the thoracic aorta of the SHR is endowed with a unique inherent predisposition for vasoactive mechanisms, which serve as compensatory processes during the developed stage of hypertension; whereas the NO component and H_2S signaling pathways are implicated, the decreased contractility seems to be excluded. Moreover, the increased participation of the H_2S system on vasorelaxation after acute NO inhibition could be considered a reserved mechanism in cases of endogenous NO deficiency.

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