

Endothelial Dysfunction in Femoral Artery of the Hypertensive Rats Is Nitric Oxide Independent

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Summary

This study examined nitric oxide (NO) production, oxidative load and endothelium-dependent relaxation (NO-dependent and NO-independent) in adult male borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats as compared to normotensive Wistar-Kyoto (WKY) rats. Systolic blood pressure (BP) was determined by tail-cuff. NO production was determined by conversion of [³H]-L-arginine. Conjugated dienes (CD) and concentrations of thiobarbituric acid-reactive substances (TBARS) were measured for assessment of oxidative load. Vascular function was investigated in rings of the femoral artery (FA) using a wire myograph. BP of WKY, BHR and SHR was 106±2, 143±3 and 191±3 mm Hg, respectively ($p < 0.01$ for each). Significant left ventricle (LV) hypertrophy and elevated levels of CD and TBARS in the LV were present in BHR and SHR as compared to WKY. NO production was elevated significantly in the aorta of BHR and SHR vs. WKY as well as in the LV of SHR vs. WKY. Acetylcholine (ACh)-induced relaxation of the FA was reduced significantly in both BHR and SHR vs. WKY. The NO-dependent component of ACh-induced relaxation had increasing tendency in hypertensive groups and it correlated positively with BP. The NO-independent component of vasorelaxation was reduced significantly in BHR and SHR vs. WKY and it correlated negatively with BP. In conclusion, the results showed that endothelial dysfunction in the experimental model of borderline hypertensive and hypertensive rats is NO-independent. The results suggest that borderline hypertension represents a risk of other cardiovascular disorders which is qualitatively similar to that of fully developed hypertension.

Key words

Acetylcholine • Borderline hypertension • Endothelium • SHR • Vascular reactivity

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Introduction

Chronic hypertension is generally considered to be a risk factor of several cardiovascular diseases. However, prehypertension is not a disease category (Chobanian *et al.* 2003) and relatively little attention has been paid to prevention of cardiovascular risk resulting from marginal elevation of blood pressure (BP) in adult individuals. Although the vascular mechanisms involved in prehypertension and hypertension are not well-defined, there are studies suggesting the involvement of endothelial dysfunction (ED) in conduit and resistance arteries in association with altered nitric oxide (NO) production and oxidative stress, which may significantly contribute to the pathogenesis of hypertension by increasing peripheral vascular resistance.

Observations in spontaneously hypertensive rats (SHR), a widely used experimental model of human essential hypertension (Yamori and Okamoto 1973, Pintérová *et al.* 2011, Kuneš *et al.* 2012), showed considerable differences related to ED (Vapaatalo *et al.*

2000, Török 2008, Gündüz *et al.* 2011). As shown in the literature, ED in SHR depends on several factors including age, artery size and methodological approaches (Bernatova *et al.* 2009). Since hypertension in SHR occurs already at juvenile age (about 5-8 weeks), it is impossible to examine metabolic and vascular alterations in the prehypertensive period in adult (12+ week-old) SHR. For this reason, borderline hypertensive rats (BHR) are more suitable for investigating the early stage of hypertension development in adulthood, which is obvious in the human population, and needs to be better understood.

Borderline hypertensive rats, an experimental model of human prehypertension and early stage hypertension, can be produced by mating spontaneously hypertensive dams with normotensive sires (Lawler *et al.* 1980, Fuchs *et al.* 1998, Bernatova *et al.* 2007, Pechánová *et al.* 2009). Resting systolic BP of adult offspring in the first-generation is in the range 130-150 mm Hg (Sanders and Lawler 1992, Mansi and Drolet 1997, Bernatova *et al.* 2007), which allows investigating the vascular function in adult animals with mild elevation of blood pressure.

In general, ED is characterized by alterations in the production and/or bioavailability of endothelium-derived relaxing factors (EDRFs) and endothelium-derived constricting factors (EDCFs) (Török 2008, Vanhoutte 2009). The functional status of the endothelium is usually tested by the acetylcholine (ACh) test in isolated pre-constricted arteries. ACh-induced relaxation results from stimulated release of at least three different vasodilating agents from the endothelial cells – nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived hyperpolarizing factor(s) (EDHFs) (Fujimoto and Fujimoto 2001, Stankevicius *et al.* 2003, Gündüz *et al.* 2011). NO seems to be the most powerful vasorelaxing factor, however the extent of NO-dependent relaxation depends on the size of the artery. The greatest NO-dependency of vasorelaxation was observed in the aorta, while the role of NO is much smaller in the regulation of endothelial function in the small resistant arteries where EDHFs seem to be more important (Shimokawa *et al.* 1996, Török 2008).

In this study we investigated vascular function (contractile responses and vasorelaxation including its NO-dependent and NO-independent components) in the femoral artery as well as the oxidative load and NO production in selected tissues of normotensive, borderline hypertensive and spontaneously hypertensive rats in order

to determine the role of NO and the seriousness of vascular changes in conditions of borderline hypertension in adulthood.

Methods

Animals

All rats used in the present study were born in our certified animal facility (Institute of Normal and Pathological Physiology SAS) in order to maintain the same environmental background of all animals. Adult, 22-week old males of Wistar-Kyoto (WKY, negative control), BHR and SHR (positive control) were used in this study (n=10 each). BHRs were the first filial offspring of SHR dams and normotensive WKY sires. Rats were housed in groups of 5 rats per cage (35/55/20 cm), each genotype separately, in an air-conditioned room at constant temperature (22-24 °C) and humidity (45-60 %) at a 12:12-h light/dark cycle (06:00-18:00 h lights on) and maintained on a standard pellet diet and tap water *ad libitum*. Drinking water consumption was measured in each group from the age of 12 weeks (beginning of adulthood) to 22 weeks of age (end of experiment). All procedures used were in accordance with the EU Directive 2010/63/EU and they were approved by the State Veterinary and Food Administration of the Slovak Republic.

Experimental design

Twenty-two-week-old Wistar-Kyoto, borderline hypertensive and spontaneously hypertensive rats were killed by decapitation after a brief CO₂ anesthesia between 7:30 and 9:30 AM. Wet mass of the left heart ventricle (LVM) and body mass (BM) were determined for calculation of the relative left ventricular mass (LVM/BM) to evaluate the degree of cardiac hypertrophy.

Blood pressure and heart rate

Systolic blood pressure (BP) and heart rate (HR) were determined non-invasively in conscious rats by tail-cuff plethysmography (using the Statham Pressure Transducer P23XL, Hugo Sachs, Germany). One week before experimentation, the rats were handled and accustomed to the tail-cuff procedure of blood pressure recording in three independent sessions (Durante *et al.* 2010). Blood pressure and heart rate were determined between 9:00-12:00 h and were calculated as the average value of 4-5 successive measurements (Púžserová *et al.* 2013).

Assessment of vascular reactivity by wire myograph

Femoral arteries (FA) were carefully dissected, immediately immersed in modified cold physiological salt solution (PSS) and cleaned of adipose or connective tissue. Arteries were then cut into segments (1.33 ± 0.04 mm long) and mounted as ring-shaped preparations in the Mulvany-Halpern style small vessel wire myograph chamber (Dual Wire Myograph System 410A, DMT A/S, Aarhus, Denmark) to determine the vascular reactivity during isometric conditions as described elsewhere (Mulvany and Halpern 1977). After mounting in oxygenated (5% CO₂, 95% O₂ mixture) PSS (composition in mmol/l: NaCl 118.99, KCl 4.69, NaHCO₃ 25, MgSO₄·7H₂O 1.17, KH₂PO₄ 1.18, CaCl₂·2H₂O 2.5, Na₂EDTA 0.03, glucose 5.5), pH 7.4, at 37 °C, a standardized computer-assisted normalization procedure was performed to set the pre-tension of the arteries. The arteries were set to a circumference equivalent to 90% of that which they would have at a transmural pressure of 100 mm Hg (90% of the normalized inner circumference). The change in wall tension (active wall tension) was calculated as measured force divided by the double segment length and expressed in mN/mm. Resting wall tension (which arises from the properties of the passive elements in the vascular wall) was also determined after the normalization procedure (Mulvany and Halpern 1977).

Femoral artery reactivity

Before the start of the normalization procedure, the vessels were allowed to stabilize in PSS for 30 min. The experimental protocol consisted of the following steps: 45 min after normalization, PSS was changed to KPSS in which NaCl was exchanged for an equimolar concentration of KCl (in whole KPSS 125 mmol/l for 2 min) followed by wash-out with PSS to baseline (15 min). After noradrenaline (NA) addition (10 µmol/l, maximal stimulation, waiting to plateau) and wash-out (PSS, 20 min), pre-contraction was achieved by serotonin (5-hydroxytryptamine, 1 µmol/l, waiting to plateau). When the contraction of the femoral artery to serotonin reached a steady state, increasing concentrations of the vasodilator acetylcholine (ACh, 0.001 to 10 µmol/l) were added in cumulative manner to perform endothelium-dependent concentration-response curves (Fujimoto and Fujimoto 2001). When the concentration-relaxation curve was completed, the drugs were washed-out (PSS, 20 min) and the same experiment was repeated in presence of N^G-nitro-L-arginine methyl

ester (L-NAME, nitric oxide synthase inhibitor, 300 µmol/l) after a 25-min pre-incubation period. In the presence of L-NAME, we could preclude ACh-induced NO release, the curve thus represented L-NAME-resistant (i.e. NO-independent) component of ACh-induced relaxation. When concentration-response curve for ACh was completed, drugs were wash-out (PSS, 30 min), and the nitric oxide donor sodium nitroprusside (SNP, 0.001 to 10 µmol/l) was added by cumulative manner to the pre-constricted arteries (serotonin, 1 µmol/l).

After the following wash-out (PSS, 20 min), the femoral artery rings were stimulated with high concentration of K⁺ (125 mmol/l) in depolarizing solution to induce maximal contraction (PSS was changed to KPSS) and then left to achieve plateau. The maximal tension achieved with depolarizing solution was set as 100% to express the active tension generated by different vasoconstrictors (Puzserova and Bernatova 2010).

The NO-dependent (i.e. L-NAME-sensitive) component of endothelium-dependent relaxation was calculated as the difference between ACh-induced relaxation before and after acute L-NAME pre-treatment (Paulis *et al.* 2008, Puzserova and Bernatova 2010) and expressed as area under the curve (AUC, in arbitrary units – a.u.) based on the individual concentration-response curves (Puzserova and Bernatova 2010). The extent of vasorelaxation was expressed in relative values as the percentage of the initial contraction induced by serotonin as well as in absolute values as agonist-induced wall tension decrease (mN/mm) (Webb *et al.* 1987, Hansen and Nedergaard 1999, Lišková *et al.* 2011). Vasoconstrictions were determined as the maximal tension and they were expressed as active wall tension in mN/mm. Responses to each drug concentration were always allowed to stabilize before addition of a subsequent dose of the same drug or of another drug (Asano *et al.* 1982, Webb *et al.* 1987).

All chemicals used were purchased from Sigma-Aldrich (Germany), except noradrenaline hydrogenotartras (Zentiva, Czech Republic). NA was prepared daily. All drugs were dissolved in distilled water and concentrations were expressed as final concentration in the myograph chamber.

Oxidative stress markers

For assessment of oxidative load, conjugated dienes (CD) and concentrations of thiobarbituric acid-reactive substances (TBARS) were measured in the left ventricle (LV), as described previously (Hu *et al.* 1989),

with some modifications.

To determine TBARS, 1 ml of tissue homogenate of the left ventricle (10 % w:v, in 1.15 % KCl in 0.01 mol/l phosphate buffer, pH 7.4) was added to 2 ml of 7.5 % trichloroacetic acid and mixed. After centrifugation at 1 000g for 10 min, 1 ml of the supernatant was added to 0.5 ml of 0.7 % 2-thiobarbituric acid and incubated in a water bath at 100 °C for 10 min. After cooling, TBARS were measured at 532 nm (GBC UV/VIS 911 A). An extinction coefficient of 156 000 mol⁻¹.l.cm⁻¹ was used for calculation of the results.

CD were determined from the same tissue homogenates as TBARS. Homogenate (0.4 ml) was mixed with 1.5 ml chloroform:methanol (1:2) for 1 min, and 0.5 ml of chloroform was added and mixed for 30 s. Afterwards, 0.5 ml of deionized water was added and mixed for additional 30 s. After centrifugation for 10 min at 1 000g, 0.5 ml of the chloroform (lower) layer was removed and dried under nitrogen. The lipid was dissolved in 2 ml of cyclohexane, and conjugated dienes were measured at 233 nm using an extinction coefficient of 27 000 mol⁻¹.l.cm⁻¹.

All chemicals used were purchased from Sigma-Aldrich (Germany) and Merck Chemicals (Germany).

Nitric oxide synthase activity

Total NO synthase (NOS) activity was measured in tissue homogenates of the aorta and left ventricle (200 mg/ml) by determination of [³H]-L-citrulline formation from [³H]-L-arginine, as described previously (Púzserová *et al.* 2006, Puzserova *et al.* 2013) and expressed as pmol/min/mg of tissue proteins as determined by the Lowry method (Lowry *et al.* 1951). Briefly, crude homogenates of the aorta and LV containing 200 mg of wet tissue per 1 ml of homogenization solution containing 50 mmol/l Tris-HCl, pH 7.4 and 1 % Protease Inhibitor Cocktail (Sigma, Germany) were centrifuged at 10 000 g for 10 min at 4 °C. After centrifugation, 50 µl of supernatant was incubated in the presence of 10 µmol/l [³H]-L-arginine (MP Biomedicals, USA, 50 Ci/mmol), 5 µg/ml calmodulin, 0.5 mmol/l β-NADPH, 250 µmol/l tetrahydrobiopterin, 4 µmol/l FAD, 4 µmol/l FMN, 1 mmol/l Ca²⁺, 1 mmol/l Mg²⁺ in the total volume of 100 µl. After 20-min incubation at 37 °C, the reaction was stopped by 1 ml of ice-cold stop solution containing 20 mmol/l HEPES, pH 5.5, 2 mmol/l EDTA, 2 mmol/l EGTA and 1 mmol/l L-citrulline and applied to 50WX-8

Dowex columns (Na⁺ form). [³H]-L-citrulline was eluted by 1 ml of distilled water and determined by liquid scintillation counting (Perkin Elmer Liquid Scintillation Analyzer, TriCarb 2910TR).

Statistical analysis

Data are presented as group mean values ± SEM of the number (n) of observations. Results were analyzed by analysis of variance (ANOVA). One-way ANOVA was used to compare basic biometric and cardiovascular parameters, basal tension and inner diameter, vascular constrictions, nitric oxide synthase activity, CD and TBARS concentrations. In case of significant results pairwise comparison with Bonferroni adjustment was employed. Concentration response curves were compared using two-way ANOVA, followed by vertical contrast with Bonferroni adjustment. Homogeneity of variances and normality of distribution were tested by Levene's test and by Shapiro-Wilk's test, respectively. To assess depression present at high concentration of ACh-cumulative concentration response curves, the maximal response and the response at higher ACh concentration at a particular response curve was compared with Dunnet's test (see Fig. 2). Means were considered to differ significantly when p<0.05.

Results

Basic biometric and cardiovascular parameters, NO production and oxidative stress markers

Systolic blood pressure, heart rate, body mass, water intake and LVM/BM ratio of WKY, BHR and SHR rats are shown in Table 1. Normalized inner diameter was decreased by 10 % in SHR and increased by 5 % in BHR compared to WKY (Table 1). There was a significant positive correlation between normalized inner diameter at 13.3 kPa and BM (r=0.58, p=0.006, n=21). However, there were no significant differences in the resting FA wall tension of borderline hypertensive and hypertensive rats vs. WKY rats (Table 1). There were also no significant differences in the FA segment length after mounting among the groups (Table 1).

The NOS activity in the aorta was enhanced by 81 % in BHR and by 106 % in SHR vs. WKY (p<0.01; Fig. 1). In the left ventricle, NOS activity was increased only in SHR, as compared to both BHR and WKY (p<0.01; Fig. 1). Additionally, there were significant increases in TBARS and CD concentrations in the left ventricle of BHR and SHR (Table 1).

Table 1. Basic biometric and cardiovascular parameters, oxidative stress markers, basal tension and inner diameter of adult Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats.

	WKY	BHR	SHR
Water intake (ml/100 g/day)	8.1±0.2	8.9±0.2**	9.9±0.2***
BM (g)	425.7±9.9	481.7±5.9**	385.3±10.1***
BP (mm Hg)	106±2	143±3**	191±3***
HR (bpm)	385±18	405±13	545±14***
LVM/BM (mg/g)	1.45±0.02	1.63±0.05*	2.18±0.05***
TBARS-LV (nmol/g)	8.25±0.48	13.99±1.64**	15.55±0.75**
CD-LV (nmol/g)	1054±26	1238±78**	1813±40***
FA length (mm)	1.33±0.06	1.32±0.07	1.36±0.08
ND (µm)	769±12	806±11*	690±13***
WT (mN/mm)	0.91±0.07	0.92±0.05	0.76±0.06

Values represent mean±SEM of 7-10 rats. BM – body mass, BP – blood pressure, CD – conjugated dienes, FA – femoral artery, HR – heart rate, LV – left heart ventricle, LVM/BM – left ventricular mass-to-body mass, ND – normalized inner diameter of the femoral artery at 100 mm Hg, TBARS – thiobarbituric acid-reactive substances, WT – resting wall tension of the femoral artery. ** p<0.01, * p<0.05, compared to WKY rats; ++ p<0.01, + p<0.05, compared to BHR rats

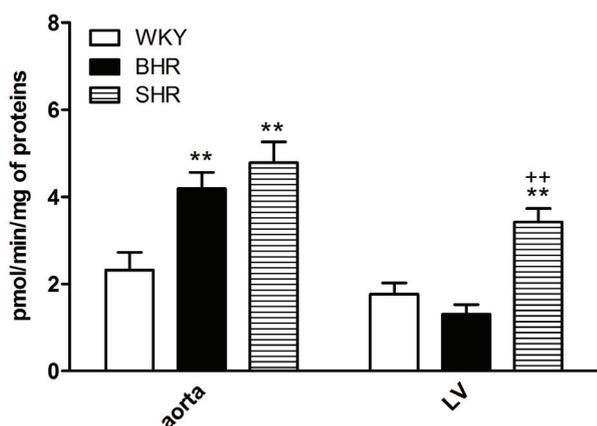


Fig. 1. Nitric oxide synthase activity of adult Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats. LV – left heart ventricle. Values represent mean±SEM of 6-8 rats. ** p<0.01, compared to WKY rats; ++ p<0.01, compared to BHR rats

Vascular responsiveness to vasoconstrictors

Both noradrenaline (10 µmol/l) and serotonin (1 µmol/l) induced contractile responses in the femoral arteries with intact endothelium; the maximum contractions are shown in Table 2. NA-induced responses were biphasic: a transient contraction (early response, phasic contraction), which occurred within the first 10-15 s and returned nearly to baseline was followed by sustained contraction (delayed response, tonic contraction), which reached steady maximum levels at 5 to 20 min (Fig. 2D). Maximum phasic and tonic contraction induced by NA was significantly greater in SHR than in WKY, but unchanged in BHR vs. WKY

(Table 2). However, only tonic contraction induced by NA determined in relative values (calculated as percentage of maximal response induced by KPSS) was significantly greater in SHR than in WKY (Table 2).

In the arteries pre-treated with the NOS inhibitor, the response to serotonin was augmented as compared to responses before L-NAME administration (Table 2). There were no significant changes in serotonin responses after L-NAME administration in BHR and SHR rats as compared to WKY rats. However, the difference between serotonin-induced contraction after and before L-NAME (to reveal basal, non-stimulated NO synthesis) was significantly increased in BHR rats compared to WKY and SHR, but comparable between the WKY and SHR groups (Table 2).

The absolute values for the maximum responses to KPSS were similar among the groups (Table 2).

Endothelium-dependent and -independent vasorelaxation

ACh (1 nmol/l - 10 µmol/l) and SNP (1 nmol/l - 10 µmol/l) relaxed the femoral artery in a concentration-dependent manner (Figs 2, 3A, 3C, 4A, 4C). Since serotonin (1 µmol/l) induced a reduced response in SHR vessels, resulting in a smaller pre-relaxation active tension level as compared to WKY (Table 2), the results were quantitatively expressed as mN/mm. ACh in serotonin-contracted femoral arteries could not induce a sustained relaxation and femoral arteries started to constrict again after a transient relaxation response (Fig. 2). WKY vessels relaxed significantly more in

Table 2. Vascular constrictions induced by noradrenaline, high-potassium solution, pre-relaxation active tension responses induced by serotonin and maximal vasorelaxations based on the individual concentration-response curves of the femoral artery of Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats.

	Vasoconstriction (mN/mm)		
	WKY	BHR	SHR
<i>NA – phasic</i>	1.07±0.17	1.39±0.16	1.68±0.29*
<i>NA – tonic</i>	1.13±0.19	1.20±0.22	2.30±0.22***++
<i>Ser before L-NAME</i>	7.41±0.15	7.07±0.42	6.05±0.50*
<i>Ser after L-NAME</i>	8.94±0.31 ^{xx}	9.90±0.40 ^{xx}	7.80±0.47 ^{++xx}
Δ <i>Ser after and before L-NAME</i>	1.53±0.30	2.83±0.42*	1.76±0.22 ⁺
<i>KPSS</i>	9.17±0.90	8.99±0.58	9.78±0.48
	Relative vasoconstriction (%)		
	WKY	BHR	SHR
<i>NA – phasic</i>	11.72±1.76	15.60±1.59	16.79±2.36
<i>NA – tonic</i>	13.46±3.19	11.41±1.34	23.35±1.68***++
<i>Ser before L-NAME</i>	85.57±8.41	77.04±6.65	62.09±4.69*
<i>Ser after L-NAME</i>	102.04±8.13	112.76±7.28 ^{xx}	79.82±3.15 ^{***++xx}
	Maximal vasorelaxation (mN/mm)		
	WKY	BHR	SHR
<i>ACh before L-NAME</i>	4.80±0.15	3.96±0.12**	2.83±0.19***++
<i>ACh after L-NAME</i>	4.54±0.36	3.68±0.28	1.99±0.40***++xx

Values represent mean±SEM of 7-10 rats. Δ – difference, ACh – acetylcholine, KPSS – high-potassium physiological salt solution (identical with PSS, except with NaCl replaced by KCl on an equimolar basis), NA – noradrenaline (10 μ mol/l), L-NAME – pre-treatment with N^G-nitro-L-arginine methyl ester (300 μ mol/l, 25 min), Ser – serotonin (1 μ mol/l). ** p<0.01, * p<0.05, compared to WKY rats; ++ p<0.01, + p<0.05, compared to BHR rats; xx p<0.01, x p<0.05, compared to the respective value without L-NAME. Receptor-independent, maximum contraction response to KPSS (125 mmol/l K⁺) is expressed as 100 % for calculation of the relative vasoconstrictions.

response to high, yet not to low acetylcholine concentrations than did BHR and SHR vessels (Figs 3A, 4A). In BHR and SHR, relaxing responses produced by ACh at concentrations greater than 0.3 μ mol/l were smaller than maximal relaxations of the given genotype (Figs 2, 3A, 3B, 4A, 4B). Results revealed a greater endothelial dysfunction in SHR as compared to BHR (Figs 3A, 4A). However, there were small but significant differences in the relative values of ACh-induced relaxations between BHR and SHR (Fig. 4A). Maximal absolute relaxations induced by ACh, determined from the individual concentration-response curves, are shown in the Table 2. Relative maximal relaxations induced by ACh (based on the individual concentration-response curves) were significantly smaller in SHR (58.47±2.52 %, p<0.01) and BHR (60.88±2.75 %,

p<0.05) than in WKY (68.88±1.68 %). Inhibition of nitric oxide synthesis with L-NAME in WKY and BHR had no effect on the maximal vasorelaxation, however it reduced significantly in SHR (Table 2). When the arteries were pre-treated with L-NAME, the femoral arteries from BHR and SHR rats responded by smaller relaxation to ACh than did the normotensive WKY arteries (Figs 3B, 4B). The NO-dependent component of ACh-induced relaxation, calculated as area under the individual curves, had increasing tendency in WKY, BHR and SHR (4.15±1.53 a.u., 4.80±1.23 a.u., 5.18±1.04 a.u. calculated from absolute relaxations and 102±17 a.u., 118±17 a.u., 146±16 a.u. calculated from relative relaxations, respectively, n.s.) rats. However, significant positive correlation was found between systolic BP and NO-dependent vasorelaxation (Fig. 6B). The NO-dependent

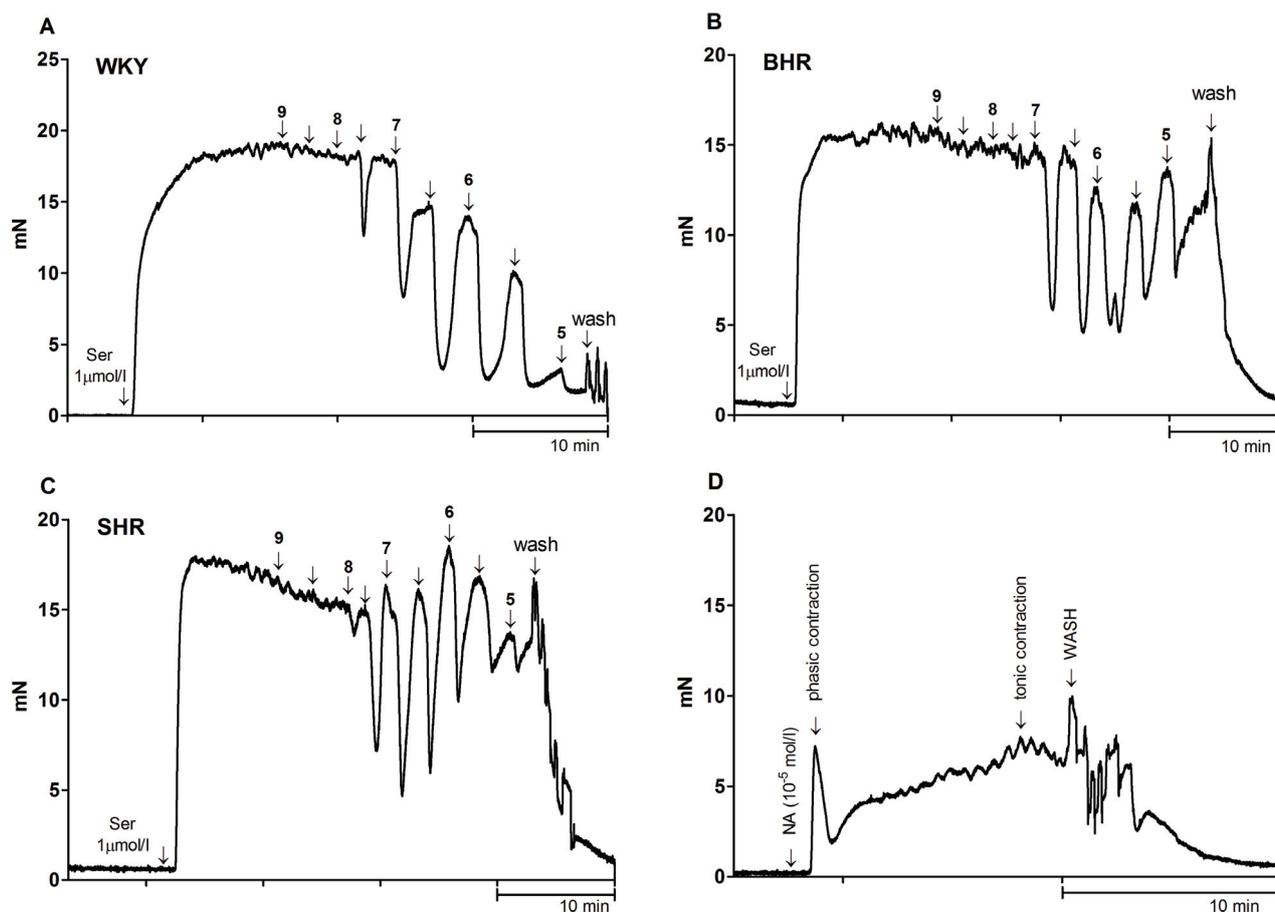


Fig. 2. Representative traces showing the typical relaxation response of serotonin (Ser) pre-contracted femoral artery on acetylcholine (ACh, 1 nmol/l - 10 μ mol/l) in Wistar-Kyoto (WKY, **A**), borderline hypertensive (BHR, **B**) and spontaneously hypertensive (SHR, **C**) rats. Rings were exposed to increasing concentrations of ACh in $-\log$ mol/l units (see charts). The time axis intervals represent 10 minutes. Charts of BHR and SHR femoral artery depicting impaired relaxation responses at higher ACh concentrations as compared to maximal relaxation at lower-concentrations of ACh, indicating release of counterbalancing vasoconstrictive factors in hypertensive animals. **D** shows the typical contraction response to noradrenaline (NA, 10 μ mol/l) in WKY rats.

relaxation calculated as the difference between total relaxation (Figs 3A and 4A) and L-NAME-resistant relaxation (Figs 3B and 4B) are shown in Fig. 5A and Fig. 5B. The NO-independent component of ACh-induced relaxation was decreased in BHR (12.61 ± 1.47 a.u. as compared to 17.49 ± 1.83 a.u. in WKY, $p < 0.05$) and SHR (7.32 ± 1.68 a.u. as compared to WKY and BHR, $p < 0.05$). There was also a significant negative correlation between BP and absolute NO-independent vasorelaxation ($r = -0.656$, $p = 0.0004$, $n = 25$). Moreover, significant negative correlations were found between systolic BP and relative NO-independent vasorelaxation (Fig. 6A).

Cumulative addition of NO donor sodium nitroprusside (SNP) produced similar relaxation responses in the femoral artery from BHR, SHR and WKY rats (Figs 3C, 4C).

Discussion

The presented study was designed to reveal the severity of vascular alterations in adult borderline hypertensive rats as compared to normotensive and hypertensive rats. Special attention was paid to the NO-dependent and independent components of vasorelaxation. The study revealed attenuated acetylcholine-induced relaxation in the serotonin pre-contracted FA of both adult BHR and SHR, which was associated with reduction of the NO-independent component of relaxation. Additionally, there were significant between-strain related alterations in mechanism of relaxation resulting in negative correlation between BP and NO-independent component and positive correlation between BP and NO-dependent component of vasorelaxation. Moreover, biochemical analysis showed elevated NO production as well as oxidative stress in rats with increased BP.

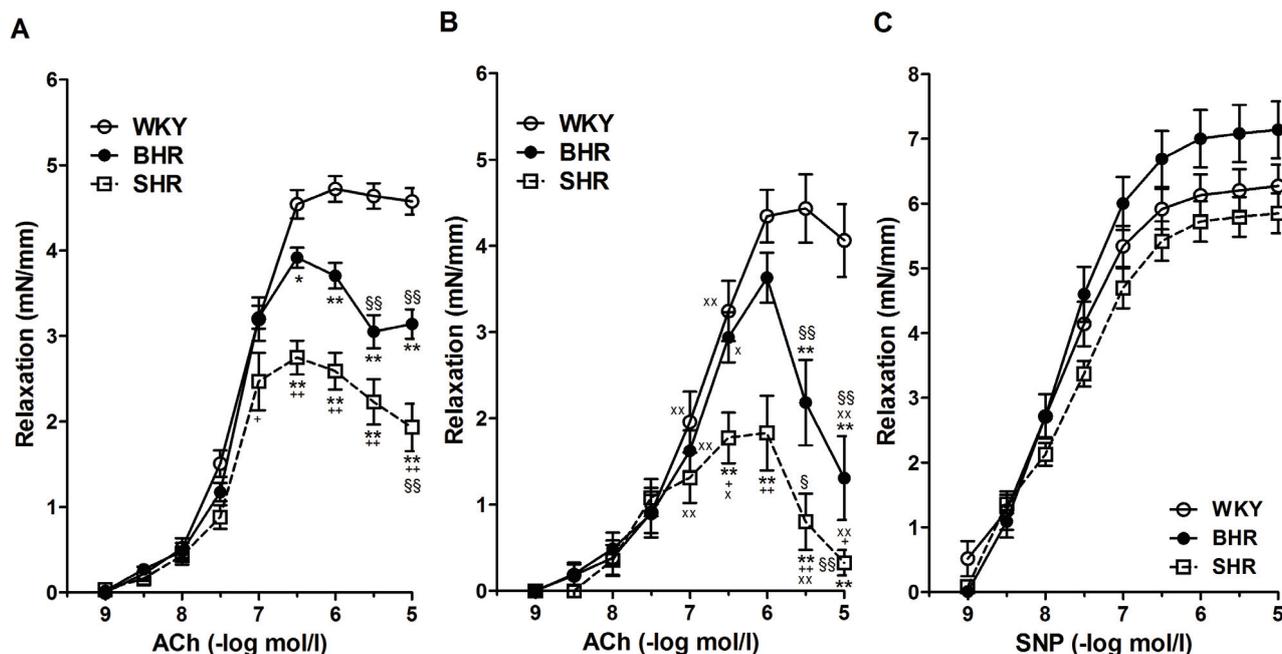


Fig. 3. Vascular responses (absolute changes) to acetylcholine (ACh) in isolated femoral arteries of Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats. In serotonin-contracted rings with endothelium of WKY, BHR and SHR, ACh causes relaxations which at higher concentrations are blunted in the arteries of the hypertensive strain. Endothelium-dependent relaxations before (A) and after (B) incubation with the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, i.e. NO-independent component of ACh-induced relaxation); Sodium nitroprusside (SNP)-induced endothelium-independent relaxation (C). Values represent mean±SEM of 7-10 rats. ***p*<0.01, **p*<0.05, compared to respective value in WKY rats; ++*p*<0.01, +*p*<0.05, compared to respective value in BHR rats; xx*p*<0.01, x*p*<0.05, compared to respective value without L-NAME; ss*p*<0.01, s*p*<0.05, compared to maximal relaxation at ACh concentrations 0.3 μmol/l (A) and 1 μmol/l (B)

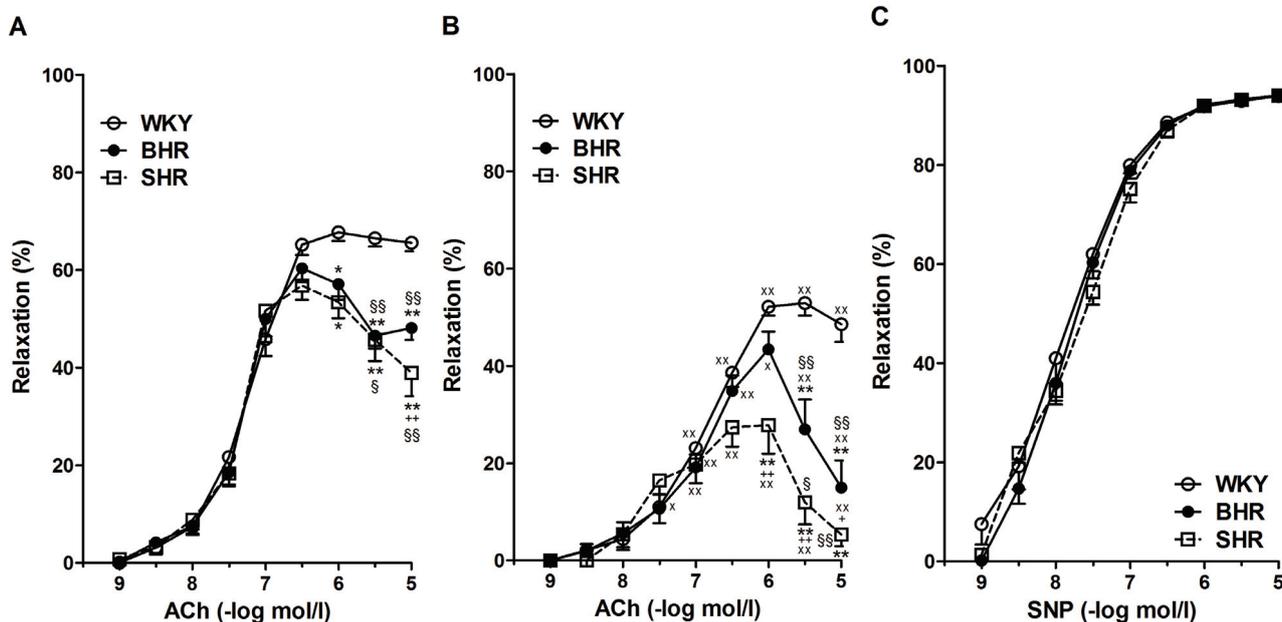


Fig. 4. Vascular responses (relative changes) to acetylcholine (ACh) in the isolated femoral arteries of Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats. Relaxation responses were expressed as the percentage of relaxation respect to the pre-contraction induced by serotonin (1 μmol/l). The pre-contraction plateau value reached after serotonin administration corresponds to 100 %. In serotonin-contracted rings with endothelium of WKY, BHR and SHR, ACh causes relaxations which at higher concentrations are blunted in the arteries of the hypertensive strain. Endothelium-dependent relaxations before (A) and after (B) incubation with the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, i.e. NO-independent component of ACh-induced relaxation); Sodium nitroprusside (SNP)-induced endothelium-independent relaxation (C). Values represent mean±SEM of 7-10 rats. ***p*<0.01, **p*<0.05, compared to respective value in WKY rats; ++*p*<0.01, +*p*<0.05, compared to respective value in BHR rats; xx*p*<0.01, x*p*<0.05, compared to respective value without L-NAME; ss*p*<0.01, s*p*<0.05, compared to maximal relaxation at ACh concentrations 0.3 μmol/l (A) and 1 μmol/l (B)

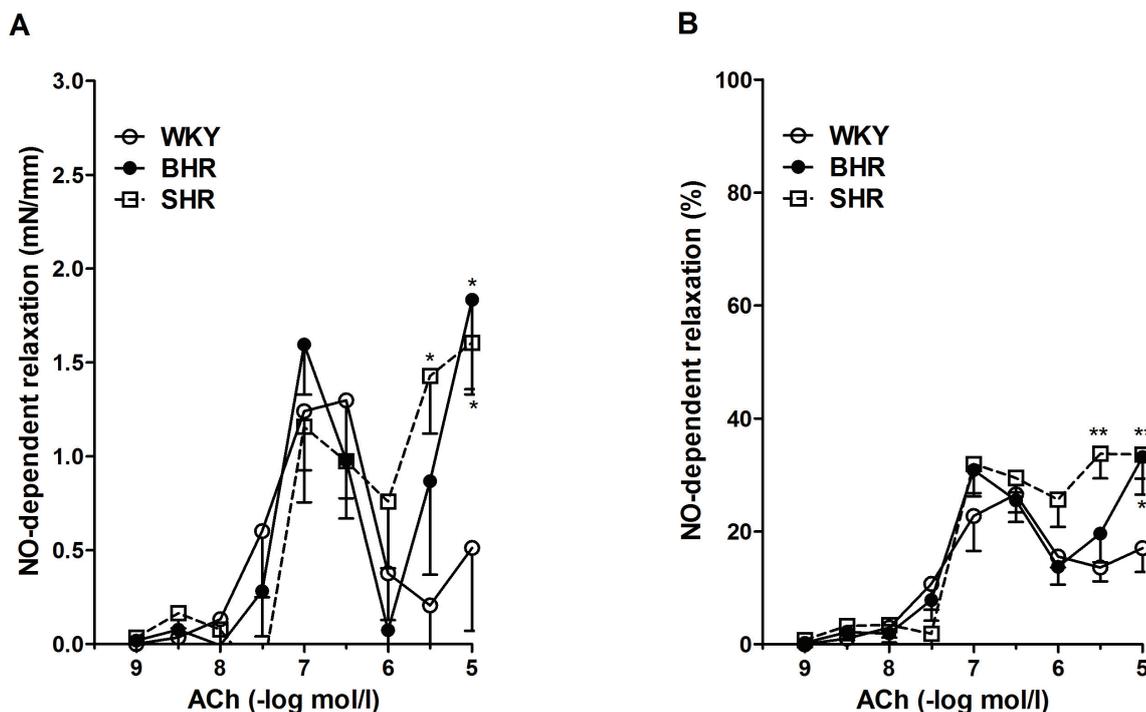


Fig. 5. Nitric oxide (NO)-dependent relaxations (relative and absolute changes) in the isolated femoral arteries of Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats. Nitric oxide (NO)-dependent relaxation (in mN/mm) calculated as the difference between total acetylcholine (ACh)-induced relaxing curves (Fig. 3A) and L-NAME-resistant relaxation (Fig. 3B) (**A**). Nitric oxide (NO)-dependent relaxation (in percentage) calculated as the difference between total acetylcholine (ACh)-induced relaxing curves (Fig. 4A) and L-NAME-resistant relaxation (Fig. 4B) (**B**). Relaxation responses are expressed as the percentage of relaxation to the pre-contraction induced by serotonin (1 μ mol/l). Values represent mean \pm SEM of 7-10 rats. L-NAME – N^G-nitro-L-arginine methyl ester; ** $p < 0.01$, * $p < 0.05$, compared to respective value in WKY rats

Elevated systolic blood pressure in both BHR and SHR was accompanied by an increase of relative left ventricle mass, indicating left ventricular hypertrophy. It is well known that left ventricular hypertrophy results from the interaction of systemic hemodynamic overload and local non-hemodynamic factors, and thus NO may play a significant role in its development. Indeed, in the model of NO-deficient hypertension, local reduction of NO production rather than hemodynamic overload were associated with the degree of LV hypertrophy (Bernátová *et al.* 1999). According to our current data, however, LV hypertrophy occurred without alterations in NO production in the LV of BHR and even in the presence of increased NOS activity in SHR. It is yet to be noted that the oxidative stress markers were increased in the LV of both BHR and SHR. Thus oxidative stress may contribute to the development of hypertrophy induced by multiple mechanisms including G-protein-coupled receptor agonists and mechanical stretch (Grieve *et al.* 2006) as well as reduction of NO bioavailability in the LV (Pechánová *et al.* 2007, 2009). Similarly, vascular wall remodeling is common feature in hypertension (Paulis *et al.* 2007, 2008). We observed reduced internal diameter

in the FA in SHR and increased diameter in BHR without alterations in resting wall tension, which suggest no changes in the passive elements of the vascular wall (Koprdoва *et al.* 2009). In fact we observed that internal diameter in the FA correlated positively with BM.

As mentioned above, despite enormous effort, the mechanisms underlying ED in hypertension are not satisfactorily understood and methodological aspects represent a significant limitation of our current knowledge. In contrast to our previous findings of unchanged ACh-induced relaxation in the phenylephrine (Phe) pre-contracted FA of BHR and even improved relaxation in SHR vs. WKY rats (Púzserová *et al.* 2007) and vs. Wistar rats (Bernátová *et al.* 2007), our current study revealed ED in both BHR and SHR as compared to WKY. Since between-strain differences were detected in the magnitude of pre-contraction induced by serotonin, the role of differences in pre-relaxation tone cannot be rule out (Li and Bukoski 1993). As Hansen and Nedergaard (1999) have previously demonstrated that the ACh-induced relaxation was inversely related to precontractile tension when expressed as a percentage, but independent when expressed in absolute values,

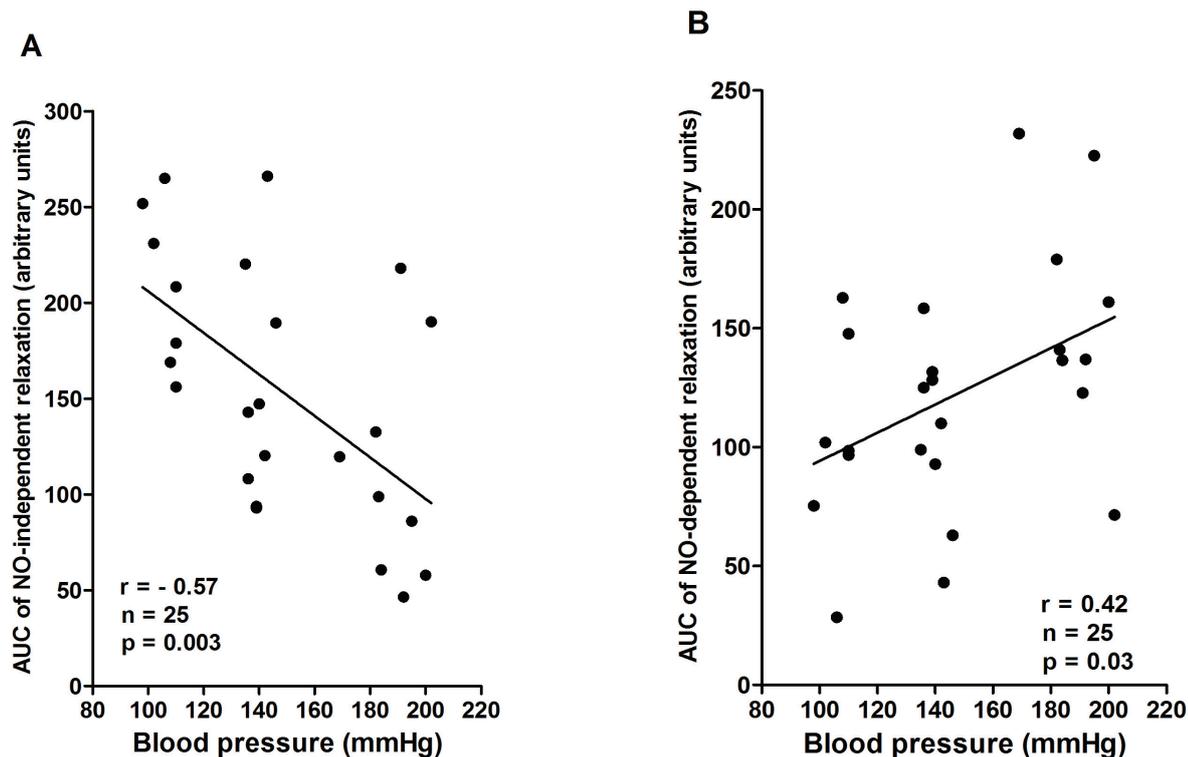


Fig. 6. Vascular responses (relative changes) to acetylcholine (ACh) in the isolated femoral arteries of Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats. Correlation between NO-independent component of vasorelaxation (calculated from relative relaxations) and blood pressure (**A**). Correlation between NO-dependent component of vasorelaxation (calculated from relative relaxations) and blood pressure (**B**). Values represent mean \pm SEM of 7-10 rats. AUC – area under the curve

vasorelaxations in this study were determined also in absolute values. The results calculated on the basis of absolute tension were similar to those obtained in percentages (Fig. 3, Fig. 4), however, determination of relaxation in absolute values revealed a more pronounced ED in SHR vs. BHR, which was not associated with NO deficiency. The present results also suggest that the responsiveness of vascular smooth muscles to NO was not impaired since relaxant responses to SNP were unchanged. Differences in our studies were presumably related to methodological aspects. Namely, relaxant responses to ACh were determined in serotonin (Fujimoto and Fujimoto 2001) pre-contracted arteries instead of phenylephrine (Bernatova *et al.* 2007, Púzserová *et al.* 2007) in order to avoid spontaneous relaxation, which can occur in the Phe pre-contracted FA. In this context, it has been reported that endothelium-dependent vasorelaxation of SHR resistance arteries is not impaired under any conditions and appears to be a function of the agonist used to pre-constrict the vessels (Li and Bukoski 1993).

Moreover, addition of antioxidants, e.g. ascorbic acid (AsA), to solution (to PSS or to solution for dilution of some drugs) used in vascular *in vitro* studies may also

be of importance (Bernatova *et al.* 2009). In addition to above mentioned modifications, our previous observations of the absence of ED in BHR and SHR were achieved in the presence of AsA (Bernatova *et al.* 2007, Púzserová *et al.* 2007). Similarly to our studies, absence of ED was shown in the presence of AsA in various arteries of adult SHR (Török and Kristek 2001, Cacányiová and Kristek 2008, Lisková *et al.* 2010). However, AsA might artificially reverse ED by improvement of oxidative status resulting in better NO bioavailability (May 2000, Bernatova *et al.* 2009). On the other hand, preserved ACh-induced relaxation was reported also in the adult SHR aorta in absence of antioxidants (Lorkowska *et al.* 2006). Furthermore even greater relaxation responses were observed in the adult SHR FA compared to their normotensive counterparts (Konishi and Su 1983). On the contrary, a reduced ACh-induced vasorelaxation was reported in the iliac artery in SHR in the presence of AsA (Cacányiová *et al.* 2006).

The investigation of vasorelaxation by cumulative addition of ACh revealed also considerable differences in the course of vascular responses of WKY, BHR and SHR, mainly at higher ACh concentrations. It is well known that higher concentrations of ACh may

elicit production of endothelium-derived vasoconstrictive cyclooxygenase-generated prostaglandins in adult SHR arteries, simultaneously with EDRFs (Lüscher and Vanhoutte 1986, Jameson *et al.* 1993, Vanhoutte 2009). Previously reported EDCFs include endothelin, cyclooxygenase-derived prostanoids or free radicals (Li and Bukoski 1993, Paulis *et al.* 2008, Török 2008, Cacányiová and Kristek 2008, Vanhoutte *et al.* 2005, Vanhoutte 2009). The dysbalance between EDRFs and EDCFs was shown to be progressively increasing not only with advancing age, but also by hypertension development (Lišková *et al.* 2011). Similarly, in this study the EDCFs release was present only in hypertensive animals as determined by stronger depression of relaxation curves at higher concentration (more than 1 $\mu\text{mol/l}$ in this study) of acetylcholine compared to normotensive animals. Moreover, the release of EDCFs was not observed in age-matched WKY rats, similarly as it was found previously (Lišková *et al.* 2011). In contrast, the EDCFs-mediated contractions were not seen in the FA of BHR and SHR pre-contracted with phenylephrine in the presence of AsA (Púzserová *et al.* 2007), which is in agreement with the idea that reactive oxygen species, namely superoxide anion, accelerate endothelium-dependent contractions to ACh in SHR (Jameson *et al.* 1993, Vanhoutte *et al.* 2005, Vanhoutte 2009). Furthermore, the EDCFs-mediated contractile responses in the FA of BHR and SHR were augmented after pre-incubation with L-NAME, suggesting that NO counterbalances the effect of EDCFs.

An interesting result of this study was the observation of reduced NO-independent (i.e. L-NAME-resistant) component of ACh-induced relaxation. It is an important finding as many studies investigating vascular function by ACh test assume that NO deficiency is the major factor of endothelial dysfunction. As the N^G -nitro-L-arginine (an inhibitor of NOS) in the combination with tetraethylammonium or charybdotoxin (inhibitors of EDHFs-mediated responses) and indomethacin (cyclooxygenase inhibitor) completely abolished the relaxant response to ACh in the adult SHR femoral artery (Fujimoto and Fujimoto 2001) we assume that the NO-independent component is related to endothelium-derived hyperpolarizing factors and PGI_2 dependent vasorelaxation in this artery. This study showed that ED (at least in the femoral artery) observed in experimental model of genetic prehypertension and hypertension was related rather to the decreased EDHFs-mediated and/or PGI_2 -mediated vasorelaxation (Li *et al.* 1994, Kähönen *et*

al. 1995, Fujimoto and Fujimoto 2001, Mori *et al.* 2006) than to lack of NO. Indeed, there was positive correlation between NO-dependent component of relaxation and BP, similarly as we observed previously (Púzserová *et al.* 2007).

Also of interest is our observation that maximal ACh-induced relaxation of SHR vessels was inhibited by L-NAME, whereas this blocker was without effect on maximal relaxations of WKY and BHR vessels. Similar observations were reported by Li and Bukoski (1993). This observation suggests that compensatory EDHFs production may occur in normotensive rats after acute NOS inhibition (Bauersachs *et al.* 1996) while this mechanism is impaired in SHR rats (Púzserová *et al.* 2010).

Regarding vasoconstriction, this study demonstrated that phasic and tonic contractile responses to noradrenaline were larger in SHR than in WKY. Similar findings were observed by Asano *et al.* (1982) in the FA of adult female SHR, which was associated with the decrease in β -adrenoceptor relaxing responses and accompanied by an enhanced vasoconstriction induced by NA. Reduced β -adrenoceptor-mediated relaxation was found in the femoral artery in adult SHR males (Konishi and Su 1983) as well as in young SHR males in the prehypertensive period (Fujimoto *et al.* 1987). Thus it is likely that a decrease in β -adrenoceptor activity would result in potentiation of vasoconstriction by unopposed α -adrenoceptor stimulation (Asano *et al.* 1982). Our results also suggest differences in sensitivity and/or distribution of alpha and beta adrenoceptors between adult BHR and SHR since NA-induced tonic vasoconstriction of BHR was similar to that in WKY and significantly lower compared to SHR. Additionally, Lišková *et al.* (2011) suggested the enhanced contribution of EDCFs in NA-induced contraction of the femoral arteries in adult SHR.

Biochemical findings support the idea that the L-arginine/NO system may serve as a compensatory mechanism activated in response to increased blood pressure in SHR, because NO is known to counterbalance the effect of sympathetic stimulation on the peripheral as well as central level (Török 2008). Indeed, several studies, including ours, observed elevated NO production in SHR (Chen and Hu 1997, Radaelli *et al.* 1998, Vaziri *et al.* 2000, Zalba *et al.* 2001, Hojná *et al.* 2007, Pechánová *et al.* 2007, 2009, Púzserová *et al.* 2007, Török 2008). Considering the presence of oxidative stress, elevated NO production may be a consequence of free radical-induced activation of NF- κ B, transcriptional

factor for NOS (Kopincová *et al.* 2011). On the other hand, oxidative load may also reduce NO bioavailability (Pechánová *et al.* 2007), so that NO may be unable to induce greater NO-dependent vasodilatation, despite increased vascular NO synthesis. Moreover, even though NO-dependent vasorelaxation was not reduced in hypertensive rats, we cannot exclude relative NO deficiency as compared to the existing sympathetic overactivity (Kunes *et al.* 2004, Paulis *et al.* 2007, Török 2008, Behuliak *et al.* 2011) as suggested by increased drinking volume (Kraly *et al.* 1985) and elevated HR in SHR as compared to both WKY and BHR.

The observations of elevated CD and TBARS support the idea that oxidative stress may be a factor contributing to LV hypertrophy as well as ED in genetic hypertension. However, persistent elevation of NO and superoxide levels can result in generation of peroxynitrite, a potent oxidant and tissue-damaging agent, resulting in impairment of cellular signaling (Durackova 2010, Kopincová *et al.* 2011). It is thus not clear whether NO would remain a protective compensatory molecule over a prolonged time-course of hypertension. Taking into account all aforementioned results, the reduced endothelium-dependent relaxations to ACh in the femoral arteries of BHR and SHR were not due to a decreased release of NO but rather to other EDRFs and/or to the simultaneous release of EDCFs.

In conclusion, this study showed that LV hypertrophy and ED were present in both adult BHR and SHR. Moreover, the results revealed that the degree of

ED was associated with the level of BP. LV hypertrophy and ED observed in this study did not result from reduced NO synthesis. The most important finding of this study was that the NO-independent component of endothelium-dependent ACh-induced relaxation in serotonin pre-contracted FA correlated negatively with BP. Thus, the results suggest that both in BHR and SHR rats, ED was not associated with reduced NO production. Additionally, the results indicate that borderline hypertension in adulthood represents qualitatively a similar risk of other cardiovascular diseases as does fully developed hypertension. Therefore it is important to study vascular mechanisms in conditions of moderately increased blood pressure in order to prevent pressure-induced organ damage, which can further accelerate the development of serious hypertension.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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