

**Effect of Blood Pressure on L-NAME-sensitive component of relaxation in adult rats**

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## Summary

The aim of this study was to investigate nitric oxide (NO) production and L-NAME-sensitive component of endothelium-dependent relaxation in adult normotensive Wistar-Kyoto rats (WKY), borderline hypertensive rats (BHR) and spontaneously hypertensive rats (SHR). Blood pressure (BP) of WKY, BHR and SHR (determined by tail-cuff) was  $111\pm 3$ ,  $140\pm 4$  and  $184\pm 6$  mm Hg, respectively. NO synthase activity (determined by conversion of [ $^3\text{H}$ ]-L-arginine) was significantly higher in the aorta of BHR and SHR vs. WKY and in the left ventricle of SHR vs. both BHR and WKY. L-NAME-sensitive component of endothelium-dependent relaxation was investigated in the precontracted femoral arteries using the wire myograph during isometric conditions as a difference between acetylcholine-induced relaxation before and after acute N<sup>G</sup>-nitro-L-arginine methyl ester pre-treatment (L-NAME,  $10^{-5}$  mol/l). Acetylcholine-induced relaxation of SHR was significantly greater than that in WKY. L-NAME-sensitive component of relaxation in WKY, BHR and SHR was  $20\pm 3\%$ ,  $29\pm 4\%$  ( $p < 0.05$  vs. WKY) and  $37\pm 3\%$  ( $p < 0.05$  vs. BHR), respectively. There was a significant positive correlation between BP and L-NAME-sensitive component of relaxation of the femoral artery. In conclusion, results suggest the absence of endothelial dysfunction in the femoral artery of adult borderline and spontaneously hypertensive rats and gradual elevation of L-NAME-sensitive component of relaxation with increasing BP.

## Key words

endothelial dysfunction, pre-hypertensive period, borderline hypertension, spontaneously hypertensive rats, nitric oxide

## Introduction

The level of tone in the vascular smooth muscle is a key determinant of local blood flow and peripheral resistance. The endothelium of blood vessels appears to play a central role in the regulation of tone and thus in blood pressure regulation via the synthesis and release of vasoactive substances (Das and Kumar 1995). Endothelial cells regulate the underlying smooth muscle layer by release of endothelium-derived relaxing factors such as nitric oxide (NO), prostacycline (PGI<sub>2</sub>) and endothelium-derived hyperpolarizing factor (EDHF) as well as by liberation of vasoconstricting factors (Stankevičius *et al.* 2003). Injury of the endothelial monolayer can result in the impairment of vascular function and thus in impairment of blood pressure regulation.

NO, one of the most potent vasodilators is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) (Moncada and Higgs 1993, Pecháňová and Šimko, 2007). There is evidence that NO is the main mediator of endothelium-dependent acetylcholine-induced relaxation in the large conduit arteries, whereas hyperpolarizing factor plays an important role in the resistance arteries (Hwa *et al.* 1994, Brandes *et al.* 2000).

The use of inhibitors of NOS showed the important role of NO in the regulation of blood pressure and in processes accompanying the development of cardiovascular disorders. It has been shown that chronic reduction of NO synthesis resulted in hypertension (Zatz and Baylis 1998, Gerová *et al.* 2004), reduced relaxation (Török and Kristek 2001, Bernátová *et al.* 2002, Paulis *et al.* 2006) and myocardial hypertrophy (Šimko *et al.* 2004).

However, observations in the spontaneously hypertensive rats (SHR), which are widely used experimental model of human essential hypertension, showed considerable differences related to NO production and/or endothelial dysfunction (Vapaatalo *et al.* 2000). Thus, the role of NO and endothelial dysfunction in pre-hypertensive period and established hypertension is still conflicting. For such studies, besides of spontaneously hypertensive rats with blood pressure above 180 mm Hg, a model of borderline hypertensive rats (BHR) produced by the matting of spontaneously hypertensive dams with normotensive sires can be used (Lawler *et al.* 1980). Resting mean arterial pressure of adult offspring in the F1 generation is in the range 130 – 150 mm Hg (Sanders and Lawler 1992, Mansi and Drolet 1997, Csizmadiová *et al.* 2006), which allows investigating vascular function in adult objects in pre-hypertensive period.

The purpose of this study was to determine the nitric oxide synthase activity in the aorta and left ventricle in rats with borderline and established hypertension. In addition, we investigated endothelium-dependent relaxation and L-NAME-sensitive component of this relaxation in the femoral artery of BHR and SHR in comparison with age-matched normotensive Wistar-Kyoto rats. Furthermore, we investigated relation between L-NAME-sensitive component of relaxation and blood pressure.

## **Methods**

### *Animals*

All rats used in the study, n = 10 in each group, were born in our animal facility in order to keep the same environmental background of all objects. Three cardiovascular phenotypes of 20 weeks old male rats were used in the study:

normotensive Wistar-Kyoto rats (WKY), spontaneously hypertensive rats (SHR) and borderline hypertensive rats. BHR were F1 offspring of SHR dams and normotensive WKY sires. Rats were housed at 22 - 24°C on a 12:12-h dark-light cycle and maintained on a standard pellet diet and tap water *ad libitum*. All procedures used were in accordance with institutional guidelines and they were approved by the State Veterinary and Food Administration of the Slovak Republic.

One week before experimentation, the rats were handled and accustomed to the tail-cuff procedure of blood pressure recording. Blood pressure (BP) and heart rate (HR) were determined between 9.00 - 12.00 h and were calculated as the average value of 5 - 6 measurements. Rats were killed by decapitation after a brief CO<sub>2</sub> anaesthesia. Body mass (BM) as well as the wet masses of the left ventricle (LV) and right ventricle (RV) were determined for calculation of their relative masses (LV/BM, RV/BM).

#### *NO synthase activity*

NO synthase activity was measured in the homogenates of the aorta and left ventricle by determination of [<sup>3</sup>H]-L-citrulline (L-Cit) formation from [<sup>3</sup>H]-L-arginine (MP Biomedicals, USA), as described previously (Bredt and Snyder 1990), with minor modifications. 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 15 min at 4°C. After centrifugation, 50 µl of supernatant was incubated in the presence of 10 µmol/l [<sup>3</sup>H]-L-arginine (specific activity 5 GBq/mmol, about 100 000 DPM), 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 Ca<sup>2+</sup>, 1 mmol/l Mg<sup>2+</sup> 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-Cit and applied to 50WX-8 Dowex columns (Na<sup>+</sup> form). [<sup>3</sup>H]-L-citrulline was eluted by 1 ml of water and determined by liquid scintillation counting. NO synthase activity was expressed as pmol/min/mg of proteins.

### *Vascular responses*

Femoral arteries were carefully dissected out, immediately immersed in Krebs-Ringer solution and cleaned of adipose or connective tissue. Then arteries were cut into segments (approximately 1 mm long) and mounted as ring-shaped preparations in the Mulvany – Halpern's style small vessel wire myograph (Mulvany and Halpern 1977) chamber (Dual Wire Myograph System 410A, DMT A/S, Aarhus, Denmark) to determine the vascular reactivity during isometric conditions. Relaxation was determined in the arteries (with mean normalized internal diameter 611±20 µm) with intact endothelium, as described elsewhere (Púzserová *et al.* 2006). To assess relaxation, dose-response curves were constructed using endothelium-dependent vasodilator acetylcholine (ACh) after the precontraction of the segments with phenylephrine (10<sup>-4</sup> mol/l). ACh was applied in cumulative manner (10<sup>-9</sup>- 10<sup>-5</sup> mol/l) when the contractile response to phenylephrine reached a plateau. When the dose-dependent relaxing curve was completed, the drugs were washed-out (with 4x10 ml of Krebs-Ringer solution) and the same experiment was repeated after 20-min preincubation with the nitric oxide synthase inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) in the bath medium. The difference between ACh-induced response before and after preincubation with L-NAME (10<sup>-5</sup> mol/l) represented L-NAME-sensitive component of ACh-induced vasodilatation at the given concentration of L-

NAME. The extent of relaxation was expressed as the percentage of precontraction and the average value of relaxation was calculated as a mean value of relaxation reached in the groups based on the individual dose-response curves.

All the chemicals used were purchased from Sigma-Aldrich (Germany). All drugs were dissolved in distilled water and concentrations are expressed as final concentration in the myograph chamber.

### *Statistical analysis*

Data were analyzed using Statistica 6.0 (Statsoft, Inc., Tulsa, OK). All results are presented as mean  $\pm$  SEM. Vascular function was analyzed using two-way ANOVA. All other data were analyzed using one-way ANOVA. Analyses were followed by Duncan's post-hoc test. Values were considered to differ significantly when  $p < 0.05$ .

## **Results**

### *Basic parameters*

Basic biometric and cardiovascular parameters ( $n = 8 - 10$ ) of WKY, BHR and SHR rats are described in the Table 1. BM was significantly reduced in SHR when compared to both WKY and BHR. Blood pressure was significantly higher in both BHR and SHR compared to age-matched WKY rats. BP of BHR was elevated by about 26% vs. WKY. In SHR, BP was elevated vs. WKY and BHR by about 66% and 31%, respectively. There were significant differences in the LV/BM ratio among WKY, BHR and SHR rats. The augmentation of BP was accompanied by increased LV/BM ratio in both BHR and SHR. Moreover, relative mass of left ventricle was elevated in

SHR group compared to BHR group. HR and the RV/BM ratio were similar in WKY and BHR, however both parameters were elevated in SHR vs. WKY.

#### *Nitric oxide synthase activity*

Nitric oxide synthase activity (n = 6 in each group) in the aorta of WKY rats was  $2.63 \pm 0.18$  pmol/min/mg and in the left ventricle was  $2.19 \pm 0.42$  pmol/min/mg. NOS activity in the aorta of BHR and SHR rats was significantly higher than that in WKY rats (Fig. 1). In the left ventricle, nitric oxide synthase activity was elevated in SHR vs. both WKY and BHR.

#### *Endothelium-dependent relaxation*

The average acetylcholine-induced relaxation of the femoral artery in WKY, BHR and SHR (n = 6 in each group) was  $61 \pm 5$  %,  $63 \pm 6$  % (ns),  $79 \pm 4$  % ( $p < 0.05$  vs. WKY), respectively. In SHR, relaxant response to acetylcholine was markedly increased in the range of concentration  $5 \cdot 10^{-9}$  –  $10^{-7}$  mol/l compared to normotensive group ( $p < 0.05$ ) and the dose-response curve to acetylcholine was shifted to the left indicating increased sensitivity to acetylcholine. There was no significant difference in relaxant response to acetylcholine between WKY and BHR. Acute blockade of nitric oxide synthesis by L-NAME ( $10^{-5}$  mol/l) significantly reduced relaxations in all groups investigated. Individual dose-response curves in the absence and presence of the NOS inhibitor L-NAME are presented in the Figure 2. The L-NAME-sensitive component of ACh-induced relaxation was significantly increased in BHR ( $p < 0.05$  vs. WKY) and SHR ( $p < 0.05$  vs. BHR) (Fig. 3A). There was a significant positive correlation between L-NAME-sensitive component of relaxation and BP ( $r = 0.614$ ,  $p < 0.007$ ,  $n = 18$ ; Fig. 3B).

## Discussion

This study investigated vascular NO production and endothelium-dependent relaxation of the femoral artery in adult rats with one or two hypertensive progenitors. The most important finding of this study was that magnitude of L-NAME-sensitive component of ACh-induced relaxation in the femoral artery of adult BHR and SHR positively correlated with BP. Furthermore, vascular NO production in BHR and SHR was greater than that in normotensive rats and ACh-induced relaxation of the femoral artery of SHR rats was greater than that in WKY.

In this study elevated values of blood pressure were accompanied by an increase in relative left ventricle mass in both SHR and BHR indicating left ventricular hypertrophy. The degree of blood pressure magnitude was closely associated with severity of cardiac hypertrophy. Since left ventricular hypertrophy is the result of interaction of hemodynamic overload and local non-hemodynamic factors, NO may play a significant role in its development. Indeed, pharmacological inhibition of NO production can induce hypertrophic myocardial growth (Kristek and Gerová 1996, Šimko and Šimko 2000) as well as to damage myocardial structure and function (Okruhlicová *et al.* 2000, Tribulová *et al.* 2000). However, our data showed occurrence of left ventricular hypertrophy without alterations in NO production in BHR and even simultaneously with increased NOS activity in SHR. This suggests that hemodynamic factors and/or other local growth factors rather than NO modified myocardial growth. However, it is worthy to note that local NO bioavailability in the heart may be either significantly reduced by oxidative stress (Pecháňová *et al.* 2007) or it may be still insufficient in relation to sympathetic activity of hypertensive rats (Kuneš *et al.* 2004, Pecháňová *et al.* 2004).

The question whether endothelial dysfunction is a consequence or a cause of hypertension remains still opened. This is documented by several studies showing impaired, unaltered or improved endothelial function in spontaneously hypertensive rats. There are many studies, which showed that reduced NO production led to attenuation of vasodilatation, elevation of vasoconstriction and to the development of hypertension in normotensive rats (Holéciová *et al.* 1996, Török and Kristek 2001, Šimko *et al.* 2004, Fialová *et al.* 2007). Based on this knowledge, it may be assumed that the development of hypertension in spontaneously hypertensive rats may be associated with NO deficiency and/or endothelial dysfunction. Indeed, several authors observed reduced relaxation of various conduit and resistance arteries such as the aorta, iliac artery, basilar artery and coronary and mesenteric arteries of adult SHR rats (Mayhan 1990, Wuorela *et al.* 1994, Küng and Lüscher 1995, Pourageaud and Freslon 1995, Čačányiová *et al.* 2006). Reduced relaxation of the aorta was also observed by Konishi and Su (1983). However, the same study showed the improvement of vasodilatation in the femoral artery of SHR as it was found in our study. Similarly, greater magnitude of the ACh-induced vasodilatation was observed in the mesenteric arteries of adult SHR (Chang *et al.* 2002). Moreover, Gerová *et al.* (2005) showed enhanced hypotensive response to ACh in adult SHR *in vivo*, which was associated with improvement of relaxation of resistance arteries but attenuated relaxation of the conduit iliac artery *in vitro*. In adult BHR, relaxation of the thoracic aorta was higher than in normotensive controls, while no differences were observed in the mesenteric artery (Stratton *et al.* 1994, Fuchs *et al.* 1998). In addition to above mentioned studies, no differences were observed in the magnitude of ACh-induced relaxation of the carotid artery and aorta of SHR compared with normotensive rats (Török and Kristek 2001). Altogether, these findings suggest that endothelial

dysfunction in hypertension may not be present in all parts of vascular tree and it appears to be the consequence rather than a cause of hypertension in rats.

In this study, simultaneously with elevated relaxation in SHR rats, we observed that reduction of ACh-induced relaxation after acute NO synthase inhibition (at the dose of L-NAME  $10^{-5}$  mol/l) was more pronounced in SHR and BHR than in WKY. This elevated sensitivity to L-NAME suggests that vascular function of rats with positive family history of hypertension was more NO-dependent than in normotensive rats. Elevated sensitivity of relaxation of the femoral artery of SHR to acute NO deficiency was observed also in our previous study when we used lower dose of L-NAME ( $10^{-6}$  mol/l). Although this dose had no effect on ACh-induced relaxation in BHR rats, it significantly attenuated relaxation in SHR (Bernátová *et al.* 2006). Thus, the sensitivity of endothelium-dependent relaxation to acute NO deficiency was the greatest in rats with established hypertension and the lowest in normotensive rats.

Regarding NO production, reduced expression of endothelial NO synthase in coronary arterioles and aorta of SHR was demonstrated (Crabos *et al.* 1997, Chou *et al.* 1998). On the other hand, several studies showed that NO formation and/or release were upregulated in cardiovascular system of SHR (Hayakawa and Raji 1997, Nava *et al.* 1998, Vaziri *et al.* 1998). Physiological stimuli for NO production in blood vessels are shear stress (Rubányi *et al.* 1986), cyclic strain and intraluminal blood pressure, which may increase NO production *in vitro* as well as *in vivo* (Buga *et al.* 1991, Awolesi *et al.* 1994, Hoyer *et al.* 1996). Elevated constitutive NOS activity in SHR was observed in the aorta and left ventricle and there was a striking positive correlation between NO production and blood pressure (Hayakawa and Raji 1997). Because NO is known to counterbalance the effect of sympathetic stimulation on the peripheral as well as central level (Safar *et al.* 2001, Stefano *et al.* 2006), elevated

basal vascular NO synthesis in rats with a positive family history of hypertension may be considered an adapting mechanism, preventing them from excessive BP elevation.

In conclusion, our results showed that adult rats with borderline and established hypertension did not developed endothelial dysfunction and they were able to maintain high levels of vascular NO production. Moreover, we showed positive correlation between BP and magnitude of L-NAME-sensitive component of relaxation of adult rats with elevated blood pressure. This suggests that reduction of cardiovascular NO production and endothelial dysfunction do not participate in the initiation of hypertension in these experimental models.

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**Table 1.** Basic biometric and cardiovascular parameters of Wistar-Kyoto rats (WKY), borderline hypertensive rats (BHR) and spontaneously hypertensive rats (SHR).

	<b>n</b>	<b>WKY</b>	<b>BHR</b>	<b>SHR</b>
<b>BM (g)</b>	10	390±11	400±10	339±5* <sup>+</sup>
<b>BP (mm Hg)</b>	10	111±3	140±4*	184±6* <sup>+</sup>
<b>HR (bpm)</b>	10	403±11	411±9	448±10*
<b>LV/BM (mg/100g)</b>	8	148±3	161±3*	226±5* <sup>+</sup>
<b>RV/BM (mg/100g)</b>	8	58±1	58±2	69±5* <sup>+</sup>

BM- body mass; BP- blood pressure; HR- heart rate; LV- left ventricle; RV- right ventricle; Results are mean ± SEM. \*p<0.05 vs. WKY, <sup>+</sup>p<0.05 vs. BHR.

## Figure captions

**Figure 1** Nitric oxide synthase activity in Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats. Results are mean  $\pm$  SEM. \* $p < 0.05$  vs. WKY;  $^{\dagger}p < 0.05$  vs. BHR.

**Figure 2** Concentration-response curves of acetylcholine (ACh)-induced relaxation of the femoral arteries of Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats in the absence (A) and in the presence (B) of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME,  $10^{-5}$  mol/l). Results are mean  $\pm$  SEM. \* $p < 0.05$  vs. WKY at the same concentration of ACh;  $^{\dagger}p < 0.05$  vs. BHR at the same concentration of ACh.

**Figure 3** Average values of acetylcholine-induced relaxation (entire column) of the femoral artery and L-NAME-sensitive component of relaxation (black column) of Wistar-Kyoto (WKY), borderline hypertensive (BHR) and spontaneously hypertensive (SHR) rats (A) and correlation between L-NAME-sensitive component of relaxation and blood pressure (B). Results are mean  $\pm$  SEM. \* $p < 0.05$  vs. WKY;  $^{\dagger}p < 0.05$  vs. BHR.

Figure 1

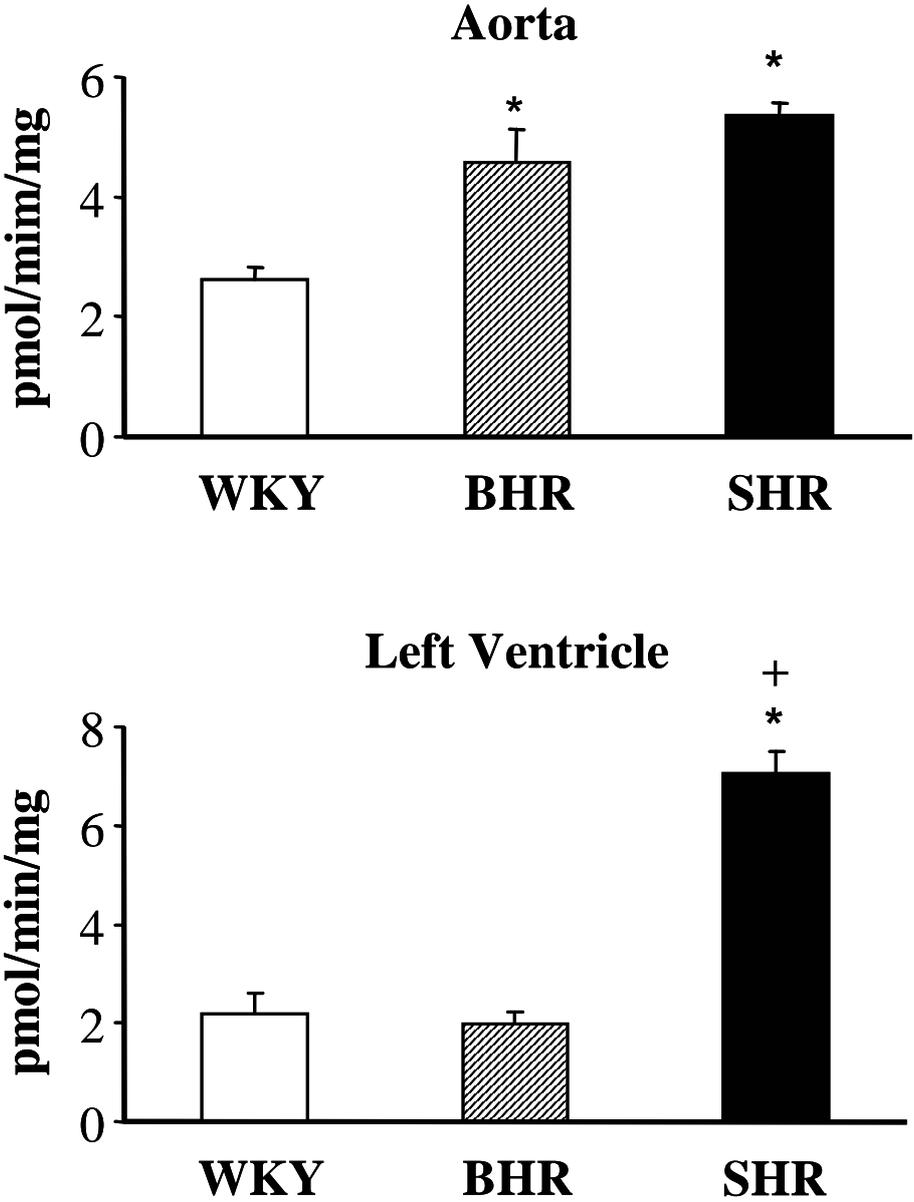


Figure 2

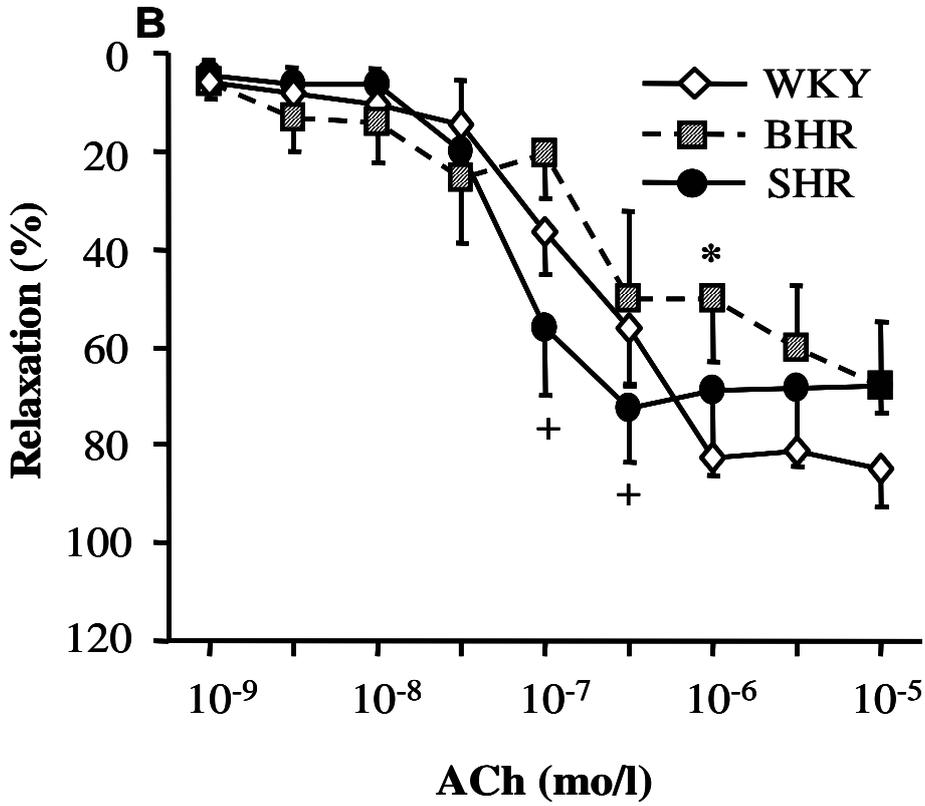
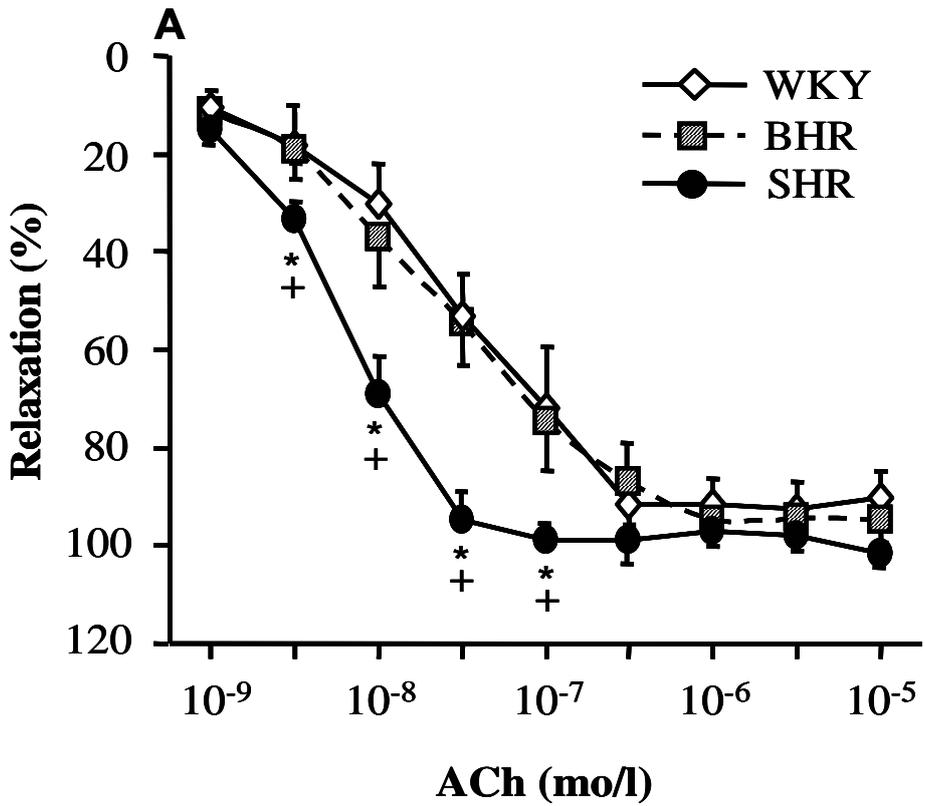


Figure 3

