Comparison of the Effect of Simvastatin, Spironolactone and L-arginine on Endothelial Function of Aorta in Hereditary Hypertriglyceridemic Rats

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Running title: Simvastatin improves endothelial function

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Summary

Hereditary hypertriglyceridemic (hHTG) rats are characterized by enhancement of blood pressure and impairment of endothelium-dependent relaxation of conduit arteries. The aim of this study was to investigate the effect of long-term (4 weeks) treatment of hHTG rats with three drugs which, according to their mechanism of action, may be able to modify the endothelial function: simvastatin (an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase), spironolactone (an antagonist of aldosterone receptors) and L-arginine (a precursor of nitric oxide formation).

At the end of 4th week the systolic blood pressure in the control hHTG group was 148 ± 2 mm Hg and in control normotensive Wistar group 117 ± 3 mmHg. L-arginine failed to reduce blood pressure, but simvastatin (118 ± 1 mmHg) and spironolactone (124 ± 4 mmHg) treatment significantly decreased the systolic blood pressure. In isolated phenylephrine-precontracted aortic rings from hHTG rats endothelium-dependent relaxation was diminished as compared to control Wistar rats. Of the three drugs used, only simvastatin improved acetylcholine-induced relaxation of the aorta.

We conclude that both simvastatin and spironolactone reduced blood pressure but only simvastatin significantly improved endothelial dysfunction of aorta. Prominently increased expression of eNOS in large conduit arteries may be the pathomechanism underlying the protective effect of simvastatin in hHTG rats.

Key words: Thoracic aorta • Blood pressure • Simvastatin • Spironolactone • L-arginine • Relaxation • Contraction

Introduction

Hereditary hypertriglyceridemic rats (hHTG), which were developed from the colony of Wistar rats (Vrána and Kazdová 1990) are characterized by the presence of insulin resistance, hyperinsulinaemia and hypertriglyceridemia (Klimeš *et al*. 1995, 1997), and a number of hemodynamic, structural and functional abnormalities such as arterial hypertension, hypertrophy of conduit arteries and endothelial dysfunction (Štolba *et al*. 1992, Šimko *et al*. 2002, Török *et al*. 2002). It is typical that arterial hypertension in hHTG rats is only of mild degree and the rats are not obese (Štolba *et al*. 1992). Deterioration of endothelial function is characterized by decreased acetylcholine-induced relaxation of the aorta (Katakam *et al*. 1998, Török *et al*. 2002, Bartuš *et al*. 2005) and other conduit arteries (mesenteric, iliac, carotid) and mesenteric resistance arteries as well (Kuesterer *et al*. 1999, Čačanyiová *et al*. 2006, Bartuš *et al*. 2005). Interestingly, weaning (4-week-old) hHTG rats are still normotensive and normoglyceridemic with endothelial function similar to controls, and their blood pressure and triglycerides begin to rise as late as during the second postnatal month (Zicha *et al*. 2006). These changes are associated with deterioration of endothelium dependent relaxation (Török *et al*. 2003, 2006).

We have shown recently that 4- week treatment of hHTG rats with angiotensin converting enzyme inhibitor captopril normalized elevated systolic blood pressure and left ventricle weight, and improved reduced endothelium-dependent relaxation of thoracic aorta (Šimko *et al*. 2002, Török *et al*. 2002).

The aim of this study was to compare the protective effect of simvastatin, spironolactone and L-arginine) on blood pressure and endothelial function in hHTG rats.

Materials and methods

Experimental animals

Experiments were performed on adult male normotensive Wistar rats and Prague hypertriglyceridemic (hHTG) rats. hHTG rats were obtained from Institute of Physiology AS CR. Animals were housed in a temperature - and light - controlled (12:12 dark-light cycle) room and had *ad libitum* access to standard chow and water. The Institutional Ethical Committee approved all experimental procedures, which conform to the European Convention on Animal Protection.

Animals were divided in five groups:

1. Rats with hereditary hypertriglyceridemia (hHTG); 2. hHTG rats treated with L-arginine (precursor of nitric oxide production) 1g/kg/day (in the drinking water, 4 weeks); 3. hHTG rats treated with spironolactone (antagonist of aldosterone receptors) 200mg/kg/day (dissolved in methylcelulose, given by gavage, 4 weeks); 4. hHTG rats treated with simvastatin (inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase) 10mg/kg/day (in drinking water, 4 weeks); 5. Age-matched control normotensive Wistar rats.

Systolic blood pressure was measured weekly by the tail-cuff plethysmographic method. At the end of the experiment, the animals were sacrificed by decapitation, thoracic aorta removed and prepared for pharmacological treatment. Body weight (BW) as well as the wet left ventricle weight (LVW) were determined and the LVW/BW was calculated. Samples of the thoracic aorta were used for the determination of eNOS protein expression.

Vascular responses

Rings of isolated thoracic aorta were mounted in organ baths for measurement of isometric contractile force. The rings were set up for isometric tension recording in 20 ml organ baths containing modified Krebs bicarbonate solution at 37°C and bubbled continuously with a 95% O_2 and 5% CO_2 gas mixture to maintain the pH at 7.3 to 7.4. The modified Krebs bicarbonate solution used had the following composition (in mmol/l): NaCl 118; $NaHCO_3$ 25; KCl 5; $MgSO_4.7H_2O$ 1.2; $CaCl_2.2H_2O$ 2.5; glucose 11; $CaNa_2EDTA$ 0.03 and ascorbic acid 1.1. The rings were mounted on stainless hooks, and one side of the tissue was connected by a thread to a force-displacement transducer (Sanborn FT 10, USA) to measure changes in isometric contraction. A resting tension of 10 mN was applied to the tissue and was readjusted every 15 min during a 60 to 90 min equilibration period.

Arterial endothelium-dependent relaxation was measured after active tension had been elicited with phenylephrine (1 µmol/l), alpha1-adrenergic agonist. When the contractile response had reached a plateau, acetylcholine was added to the organ bath in a cumulative manner. Aortic contractions to cumulative doses of noradrenaline were tested in quiescent preparations. All preparations were pretreated with indomethacin (10 µmol/l) to avoid the possible participation of prostaglandins in endothelium-dependent relaxation.

Endothelial NO synthase expression

Isolated thoracic aorta (50 mg of wet tissue) was homogenized in 50 mmol/l Tris HCl. pH 7.4, containing 1% Triton X-100, leupeptin 1 µmol/l, aprotinin 0.3 µmol/l, PMSF 0.1 mmol/l, and pepstatin 1 mmol/l for Western blot analysis. After centrifugation (15 000 x g, 20 min, twice) supernatants were subjected to SDS-

PAGE using 8% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and probed with polyclonal rabbit anti-endothelial NO synthase antibody (Santa Cruz Biotechnology, USA). Bound antibodies were detected with a secondary peroxidase-conjugated anti-rabbit antibody (Zymed, Germany). The bands were visualized using the enhanced chemiluminescence system (ECL, Amersham, UK).

The chemicals used were purchased from Sigma-Aldrich Co. and Léčiva – Czech Republic (noradrenaline). All drugs were dissolved in distilled water and concentrations are expressed as final concentration in the incubating bath.

Data analysis

Statistical significance was assessed by ANOVA or unpaired Student´s t-test. Data are presented as means ± S.E.M. $P < 0.05$ value was considered as statistically significant.

Results

Cardiovascular parameters

After 4 weeks´ treatment, the body weight of animals in all investigated groups did not differ in comparing with the control group (Table 1). Also, the left ventricle/body weight ratio was not significantly affected in any experimental group.

Mean systolic blood pressure in hHTG rats before treatment was in the range of 145 - 149 mmHg in all groups. At the end of 4th week blood pressure in control untreated hHTG group was 148 ± 2 mmHg and in control normotensive

Wistar group 117 ± 3 mmHg. Long-term treatment of hHTG rats with L-arginine did not significantly influence blood pressure (Fig.1). On the other hand, spironolactone and simvastatin already in second week slightly lowered SBP and at the end of 4th week the blood pressure was reduced to the levels observed in control Wistar rats (spironolactone 124 ± 4 mmHg and simvastatin 118 ± 1 mmHg, respectively; $P < 0,01$).

Relaxant response

In phenylephrine-precontracted aortic rings from hereditary hypertriglyceridemic rats endothelium -dependent relaxation to acetylcholine was diminished (Fig.2). Chronic treatment of hHTG-rats with L-arginine and spironolactone did not influence endothelium-dependent relaxation of the aorta induced by acetylcholine, but in spironolactone-treated group was a tendency for the responses to be similar to relaxant responses observed in control Wistar rats, however this effect failed to attain statistical significance. On the other hand, simvastatin significantly improved the magnitude of relaxation induced by higher concentration of acetylcholine.

Contractile response

Effect of long-lasting treatment of hHTG rats with L-arginine, spironolactone and simvastatin on the contractile responses of thoracic aorta is illustrated in Fig.3. Neither of used drugs had significant effect on dose-response curves to noradrenaline.

Endothelial nitric oxide synthase protein expression

The Western blot analysis revealed a decrease in aortic eNOS protein expression in untreated hHTG rats comparing to control Wistar rats. Long-term treatment of hHTG rats with L-arginine did not significantly changed the expression of eNOS; treatment of hHTG rats with spironolactone and simvastatin clearly increased eNOS protein expression in comparing with hHTG rats without pharmacological treatment (Fig. 4).

Discussion

It was shown that long-lasting treatment of hereditary hypertriglyceridemic rats with

a/ L-arginine influenced neither systolic blood pressure nor endothelium-dependent relaxation of thoracic aorta;

b/ spironolactone decreased elevated systolic blood pressure but did not influence endothelium-dependent relaxation of thoracic aorta;

c/ simvastatin decreased elevated systolic blood pressure and improved endothelium-dependent relaxation of thoracic aorta.

In phenylephrine-precontracted aortic rings from hHTG rats endothelium-dependent relaxation induced by ACh was diminished. Endothelial dysfunction has been linked with vasoconstriction and elevation of blood pressure. The potential mechanism responsible for this endothelial dysfunction with decreased NO-production in hHTG rats (Török *et al*. 2002) may be related to decreased NO synthase activity (Zicha *et al*. 2006), cGMP concentration (Kazdová *et al*. 2001) and increased oxidative stress (Banos *et al*. 2005). Since L-arginine, spironolactone and statins are considered to increase NO-availability and bioactivity, we supposed that all three substances will confer similar benefit in hHTG rats with respect to endothelial dysfunction and blood pressure. Surprisingly, there were substantial differences in the action of these protectives.

L-arginine seems to have discrepant effect on blood pressure of rats with hemodynamic overload during acute and chronic administration. In acute experiments, administration of L-arginine decreased blood pressure (Nakaki *et al*. 1990) and partially improved impaired endothelium-dependent relaxation of aorta in rats with NO-deficient hypertension (Török *et al*. 1998, Šimko 2007a). This acute antihypertensive effect of L-arginine may be related to its direct relaxation effect on vascular smooth muscle (Moritoki *et al*. 1991). On the other hand, chronic L-arginine treatment neither decreased blood pressure nor improved relaxation of aorta in hHTG rats in this experiment. Similarly in L-NAME –induced hypertension (Šimko *et al.* 2005), or in spontaneously hypertensive rats (SHR) (Kristek 1998) and stroke-prone SHR (Stier et al.1991) chronic L-arginine treatment failed to prevent or reverse hypertension and left ventricular hypertrophy (LVH). This failure of L-arginine to exert the expected protection may be explained by the fact that L-arginine stimulates the activity of L-arginase and moreover induces a neurohumoral activation, which may counteract the potential benefit of L-arginine administration (Romero et al. 2006).

Aldosterone, independently of its effect on blood pressure, may impair vascular structure and function (Ikeda *et al*. 1995). On the other hand, aldosterone receptor antagonists are known to improve endothelial dysfunction in variable experimental conditions. In rats with chronic heart failure aldosterone receptor antagonist, eplerenone improved NO-mediated relaxation of the aorta (Schafer *et al*. 2003). Quaschning *et al*. (2001) demonstrated that spironolactone normalized blood pressure, prevented upregulation of vascular endothelin-1 and restored NO-mediated endothelial dysfunction in liquorice hypertension. This favourable effect of aldosterone receptor blockers might be partly related to the improvement of NO-bioactivity through the preservation of thiol and/or S-nitrosothiol groups as it was shown with spironolactone in L-NAME–induced hypertension (Pecháňová *et al*. 2006).

In this experiment on hHTG rats, spironolactone normalized elevated systolic blood pressure but only slightly improved acetylcholine-induced relaxation. It suggests that reduction of blood pressure may be induced also by other spironolactone effects, like reduction of aldosterone-induced volume expansion or deterioration of vascular elasticity.

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase – statins reliably reduce morbidity and mortality in the primary and secondary prevention of coronary heart disease. It has been shown in the recent years that the therapeutic potential of statins may exceed the benefits achieved by their lipid lowering potential. One of the most important “pleiotropic effects” of statins is their protection of the endothelial function (Vaughan *et al*. 1996, Takemoto *et al*. 2001, Liao and Laufs 2005, Kucharská *at al.* 2007, Šimko 2007b).

In our experiments simvastatin completely normalized elevated systolic blood pressure and improved impaired endothelium-dependent relaxation of the aorta. Beneficial effect of simvastatin against endothelial dysfunction in the aorta of hHTG rats may have several plausible explanations:

a/ Statins act beneficially on vascular smooth muscle. Alvarez de Sotomayor *et al*. (2000) have shown that simvastatin improved both endothelium dependent and independent relaxation of the aorta. Perez-Guerrero *et al*. (2005) documented that simvastatin-evoked relaxation of phenylephrine-precontracted arteries from SHRs independently of the presence of endothelium.

b/ Statins may protect the vessels by reducing oxidative stress. Simvastatin attenuated superoxide anion formation in the endothelium along with the increase of NO synthase activity and restoration of endothelial NO-mediated vasorelaxation in large arteries after myocardial infarction (Wagner *et al*. 2000, Bates *et al*. 2002).

c/ Statins may improve endothelial function through enhancing eNOS expression and activity. In this experiment, simvastatin, along with blood pressure reduction, improved acetylcholine-relaxation of the aorta. Simultaneously, simvastatin increased the expression of endothelial NO synthase protein, which could participate on improved NO-production and represent a mechanism underlying the improvement of endothelium-dependent vascular relaxation (Fig.4).

We conclude that both simvastatin and spironolactone reduced blood pressure but only simvastatin significantly improved endothelial dysfunction of aorta. Increased expression of eNOS in large conduit arteries may be the pathomechanism underlying the protective effect of simvastatin.

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Figure captions

Figure 1 Systolic blood pressure (SBP) profile of hereditary hypertrigyceridemic (hHTG) rats treated with L-arginine (ARG), spironolactone (SPIR) and simvastatin (SIMV). Values are means ± SEM. *P<0.05, **P<0.01 compared with untreated hHTG rats.

Figure 2 Endothelium-dependent relaxation of thoracic aorta induced by acetylcholine in control hereditary hypertriglyceridemic (hHTG) rats and in hHTG rats treated with L-arginine (ARG), spironolactone (SPIR) and simvastatin (SIMV). Values are means ± SEM. *P<0.05 compared with control (Wistar) rats.

Figure 3 Effect of L-arginine (ARG), spironolactone (SPIR) and simvastatin (SIMV) treatment on the contractile responses of thoracic aorta from hereditary hypertriglyceridemic (hHTG) rats induced by noradrenaline. Values are means ± SEM.

Figure 4 Western blot analysis with representative blots demonstrating the effect of L-arginine (ARG), spironolactone (SPIR) and simvastatin (SIMV) treatment on endothelial nitric oxide synthase (eNOS) protein expression in the thoracic aorta in hereditary hypertriglyceridemic (hHTG) rats. *P<0.05 compared with untreated hHTG rats.

Table 1. Effect of L-arginine (L-ARG), spironolactone (SPIR) and simvastatin(SIMV) on the body weight (BW), left ventricle weight (LVW) and left ventricle to body weight ratio (LVW/BW) in hHTG rats.

	Control (Wistar)	**hHTG untreated**	**hHTG + L-ARG**	**hHTG + SPIR**	**hHTG + SIMV**
Body weight (BW, g)	**309.23±6.39**	**292,38±7,48**	**301,67±5,46**	**284,38±17,79**	**306,67±9,01**
Left ventricle weight (LVW, mg)	**388.40±12.01**	**377,88±13,11**	**392,00±10,54**	**360,88±21,09**	**371,33±21,4**
LVW/BW (mg/g)	**1.26±0.05**	**1,30±0,04**	**1,30±0,03**	**1,21±0,04**	**1,21±0,06**

Values are means ± SEM

Figure 1

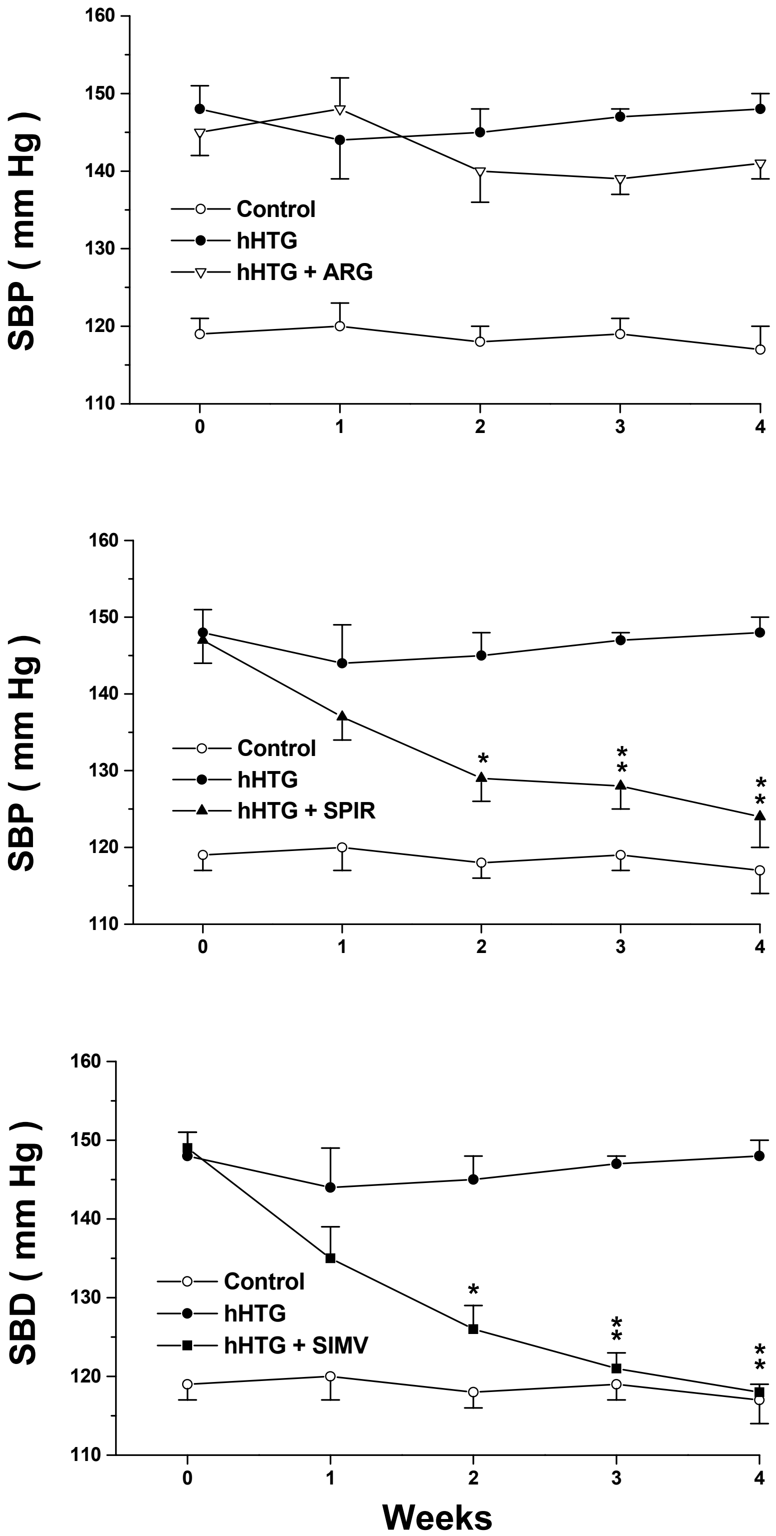

SBP (mm Hg)
Control
hHTG
hHTG + ARG
SBP (mm Hg)
Control
hHTG
hHTG + SPIR
SBD (mm Hg)
Control
hHTG
hHTG + SIMV
Weeks

Figure 2

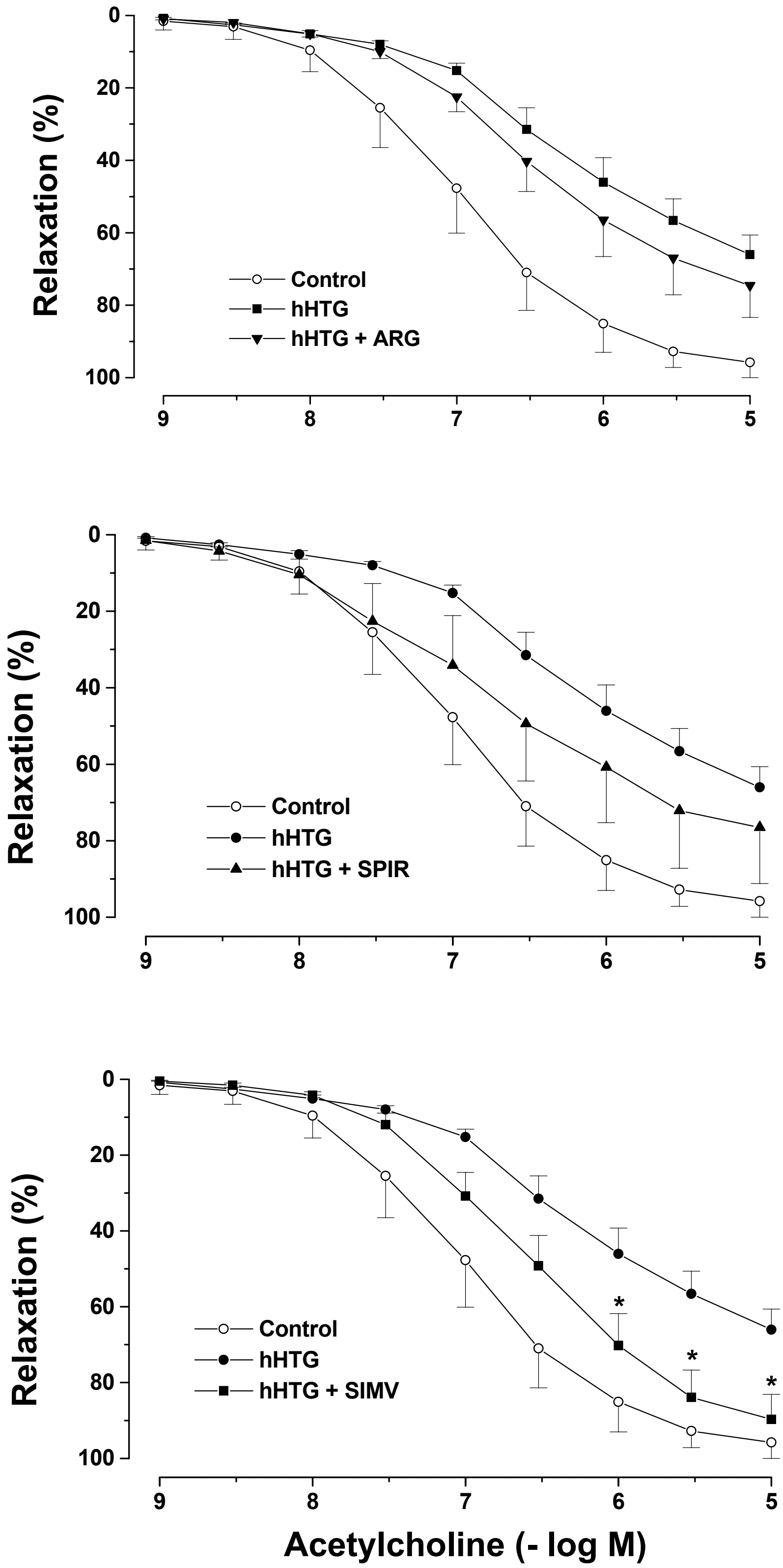
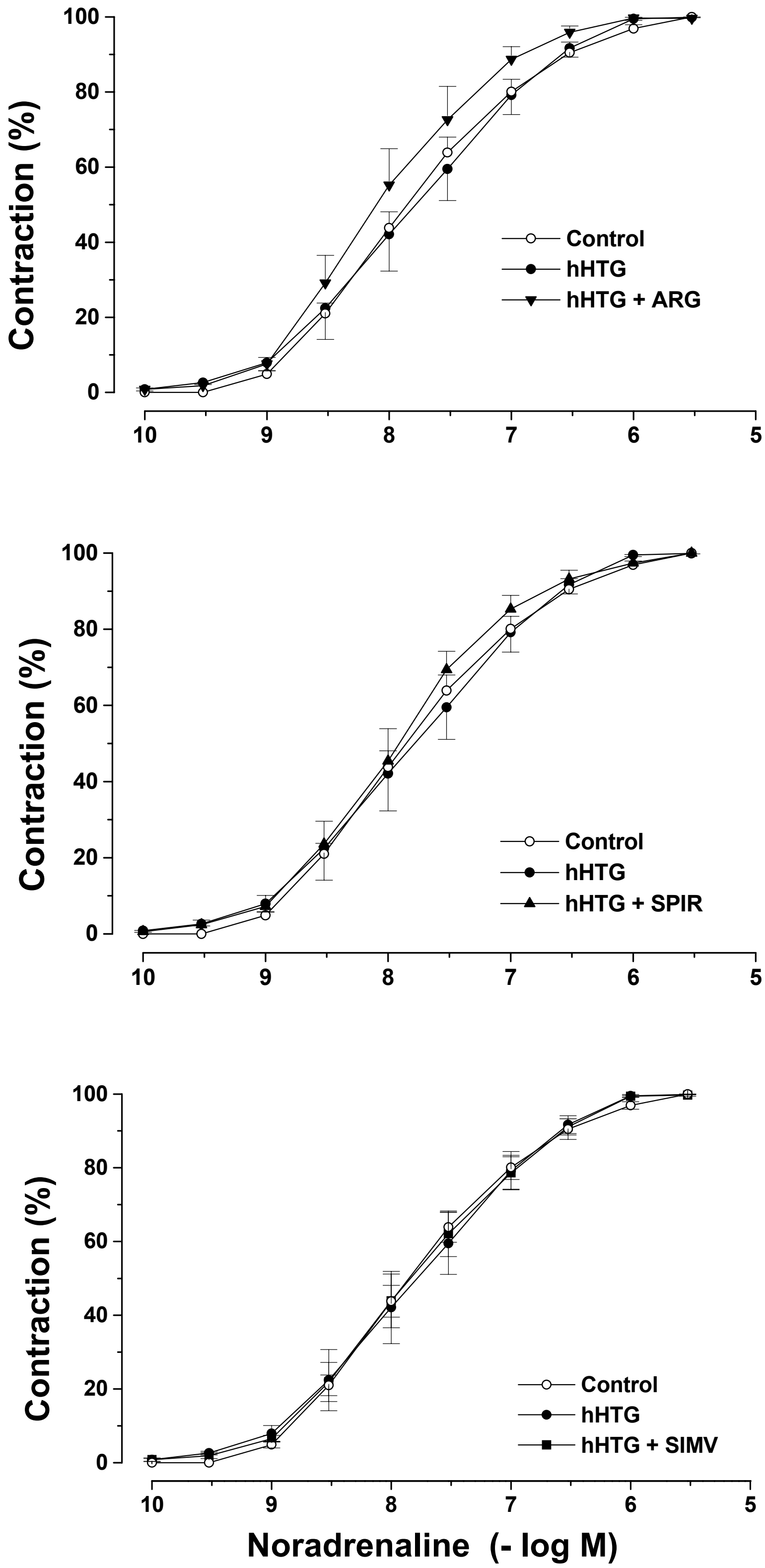
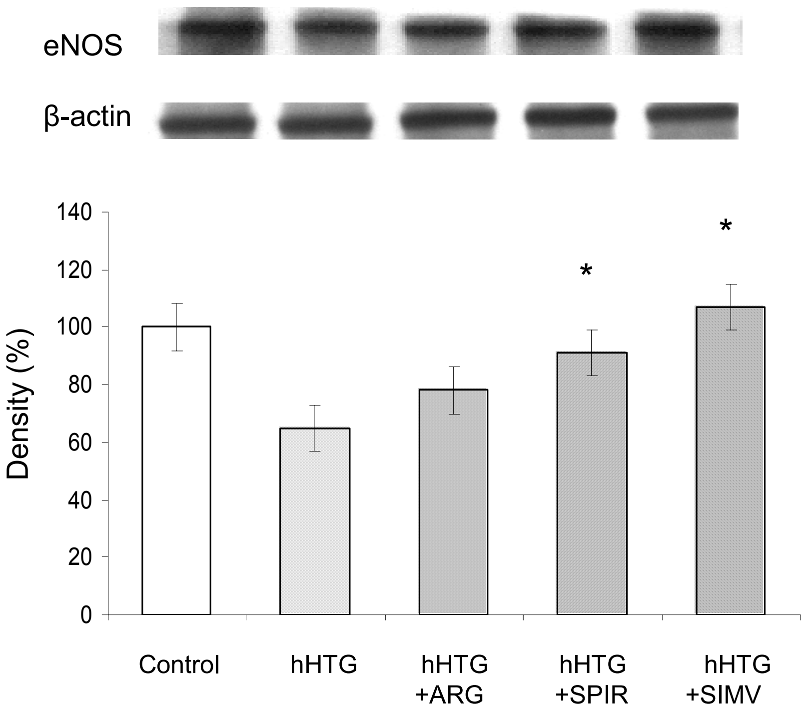

Relaxation (%)
0
20
40
60
80
100
9
8
7
6
5
Control
hHTG
hHTG + ARG
Control
hHTG
hHTG + SPIR
Control
hHTG
hHTG + SIMV
*
*
*
Acetylcholine (- log M)

Figure 3



Contraction (%)
Control
hHTG
hHTG + ARG
hHTG + SPIR
hHTG + SIMV
Noradrenaline (- log M)

Figure 4


eNOS
β-actin
140
120
100
80
60
40
20
0
Density (%)
*
*
Control
hHTG
hHTG
+ARG
hHTG
+SPIR
hHTG
+SIMV