Physiol. Res. 55 (Suppl. 1): S39-S47, 2006

Vascular Effects of Red Wine Polyphenols in Chronic Stress-Exposed Wistar-Kyoto Rats

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Received October 27, 2006
Accepted November 17, 2006
On-line available December 22, 2006

Summary

Present study investigated the effect of red wine polyphenolic compounds (Provinols™) on blood pressure (BP), nitric oxide synthase (NOS) activity and vascular function in Wistar-Kyoto (WKY) rats exposed to chronic social stress produced by crowding. Adult male rats were divided into four groups: control (480 cm^2/rat), Provinols™-treated (20 mg/kg/day, 480 cm^2/rat), crowded (200 cm^2/rat) and crowded treated with Provinols™ (20 mg/kg/day, 200 cm^2/rat) for 8 weeks. No differences in BP were observed among the groups at the end of experiment, however, reduced BP was observed in Provinols™-treated rats after 3 weeks of treatment. NOS activity in the aorta was significantly elevated in crowded rats, while Provinols™ alone had no effect on nitric oxide (NO) production. Acetylcholine-induced relaxation of the femoral artery was significantly improved in stressed and Provinols™-treated rats *vs.* control, without significant changes in their noradrenaline-induced vasoconstriction. Interestingly, Provinols™ blunted the elevation of NO production and vasorelaxation during crowding. Increased endothelium-dependent vasorelaxation and NO synthesis in crowded rats may represent the adaptation mechanisms, resulting in unaltered blood pressure in stress-exposed normotensive rats. This study further demonstrated that elevated release of NO during chronic stress may be prevented by Provinols™. Thus, Provinols™ might maintain equilibrium between endothelium-derived vasoconstrictor and vasodilator factors in stress.

Key words

Crowding • Chronic social stress • Provinols™ • Blood pressure • Nitric oxide

Introduction

In socially organized mammals including man, a significant portion of stress arises from their interaction with social environment rather than from any physical events. Crowding typically evokes social stress reactions with prominent psychosocial components mimicking emotional state alterations (Bugajski 1999). Intensity of the stress reaction is determined by the relationship among the activation of the stress systems in response to

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the stressor and the activation of „stress-limiting systems“ (Meerson 1987, Malyshev and Manukhina 1998, Bernátová *et al.* 2004), which may restrict the excessive activation of the stress systems and, thereby, the detrimental effect of stress. With regard to cardiovascular system, some experimental models of psychosocial stress were able to induce hypertension in normotensive rats (Szilagyi 1991, Andrews *et al.* 2003), while other authors did not observed changes in BP (Harrap *et al.* 1984, Lemaire and Mormede 1995, Mansi and Drolet 1997). Thus, although Selye´s theory of general adaptation syndrome (Selye 1936) presupposed the same response to a variety of stimuli (stressors), there are still conflicting data as to the nature of cardiovascular changes induced by stress, depending on the nature, duration and intensity of the stressor, animal strain, age, gender etc.

Nitric oxide (NO) is one of the most potent vasodilating substances, which participates in modulation of vascular resistance and thus in BP regulation (Rees *et al.* 1989, Török and Gerová 1996, Kuneš *et al.* 2004). The evidence that NO production may be considerable modified in stress and during adaptation to diverse stressors have immediately entailed a hypothesis that NO plays an important role in stress and adaptive responses of the organism (Malyshev and Manukhina 1998). Cardiovascular vulnerability, which may involve endothelial dysfunction and thus reduction of NO synthesis, following chronic social stress has not been adequately studied in normotensive animal models.

Various epidemiological reports indicate that consumption of foods and beverages rich in polyphenols, such as red wine, is associated with lower incidence of cardiovascular diseases (Stoclet 2001, Arts and Hollman 2005). The beneficial effects (vasoprotective, anti-angiogenic, anti-atherogenic, vasorelaxant, anti-hypertensive etc.) of polyphenolic compounds might be explained by their actions on a wide range of biological activities affecting mammalian metabolism, including nitric oxide (Zenebe and Pecháňová 2002, Stoclet *et al.* 2004, Curin and Andriantsitohaina 2005). It has been reported that grape juices, grape skin extracts and different wine extracts can induce endothelium-dependent vasorelaxation (Fitzpatrick *et. al* 1993, 1995). Moreover, the endothelium-dependent vasorelaxant effect of wine polyphenols was probably due to increased synthesis of NO (Andriambeloson *et al.* 1997, 1998, Zenebe *et al.* 2003, Duarte *et al.* 2004). In our previous studies we provided an evidence that oral administration of red wine polyphenolic compounds (Provinols^{TM}) prevented the development of cardiovascular alterations in NO deficient hypertension (Pecháňová *et al.* 2004) as well as it induced a faster and more profound decrease of blood pressure in developed NO deficient hypertension (Bernátová *et al.* 2002).

However, to our best knowledge, there is only one study investigating the influence of natural polyphenols during chronic stress (Henry and Stephens-Larson 1984). Therefore, the present study was designed to determine as yet unknown effect of red wine polyphenolic compounds Provinols^{TM} on blood pressure (BP), nitric oxide synthase (NOS) activity and vascular functions of the femoral artery in normotensive Wistar-Kyoto (WKY) rats exposed to chronic social stress produced by crowding.

Methods

Animals

Normotensive Wistar-Kyoto (WKY) rats used in this study were born in our animal facility. Rats were housed in an air-conditioned room at constant temperature (22-24 ˚C) and humidity (45-60 %) on 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*. All procedures used in this study were approved by the State veterinary and food administration of the Slovak Republic.

After weaning (25th day), male rats were kept in groups of 4 rats per cage (35/55/20 cm, 480 cm^2/rat). Adult males, 12 weeks old, were randomly divided into four groups: control rats (C) kept in groups of 4 rats/cage (35/55/20 cm, 480 cm^2/rat), group treated with Provinols^{TM} (P, 20 mg/kg/day, 480 cm^2/rat), stressed group (S, 200 cm^2/rat) and stressed group treated with Provinols^{TM} (P+S, 20 mg/kg/day, 200 cm^2/rat) simultaneously. Rats exposed to crowding stress were kept in groups of 5 rats/cage (25/40/15 cm), where their living-space was reduced to 200 cm^2/rat (Bernátová and Csizmadiová 2006). Daily water consumption of rats was estimated one week before the experiment and it was controlled daily during all experiment. Calculated amount of Provinols^{TM} was administered in drinking water and Provinols^{TM} concentration in the drinking water was adjusted, if necessary. The Provinols^{TM} is an alcohol-free dry powder extract from red wine (Languedoc-Roussillon regions in the South-East of France). The content of polyphenols in Provinols^{TM} (in mg/g of dry powder) was:

480 proanthocyanidins, 61 total anthocyanins, 19 free anthocyanins, 38 catechin, 18 hydroxycinnamic acids, 14 flavonols, 370 polymeric tanins (100 mg of Provinols™ corresponds to the polyphenol content of 1 glass of red wine).

Rats were killed after eight weeks of experiment by decapitation after a brief CO_2 anesthesia.

Blood pressure

Blood pressure (BP) was determined non-invasively by tail-cuff plethysmography (using the Statham Pressure Transducer P23XL, Hugo Sachs, Germany) before experiment (basal) and after 1st, 3rd, 6th and 8th week of experiment. One week before experimentation, the rats were handled and accustomed to the tail-cuff procedure of blood pressure recording in three independent sessions.

Nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was measured in the homogenates of the thoracic aorta. Homogenates were centrifuged at 10 000 rpm, 15 min at 4ºC and NOS activity was measured by determination of [^{3}H]-L-citrulline formation from [^{3}H]-L-Arginine (Amersham, UK), as described previously (Bredt and Snyder 1990), with minor modifications (Bernátová *et al.* 2002). NOS activity was expressed as pmol/min/mg of proteins.

In vitro assessment of vascular reactivity by wire myograph

Femoral arteries were carefully dissected out, cleaned of adipose and connective tissue, cut into segments (approximately 1 mm long, with mean normalized internal diameter 657±12 μm) and mounted as ring shaped preparations in a Mulvany-Halpern´s small vessel myograph chamber (Dual Wire Myograph System 410A, DMT A/S, Aarhus, Denmark), to determine the vascular reactivity during isometric conditions. Arteries were taken from the right hind limb. The procedures for investigation of small vessels using wire myograph and apparatus has been described in detail elsewhere (Mulvany and Halpern 1977). Briefly, two 40 μm stainless steel wires were passed through the lumen of the vessel with care taken not to damage the endothelium and mounted in the jaws of an wire myograph. After equilibration in oxygenated (5 % CO_2, 95 % O_2 mixture) Krebs-Ringer`s solution (composition in mmol/l: NaCl 118, KCl 5, $NaHCO_3$ 25, $MgSO_4.H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, EDTA 0.03, ascorbic acid 1.1, glucose 11), pH 7.4, at 37 °C, a standardized computer-assisted normalization procedure was performed to set the pretension of the arteries. This defines the lumen diameter (L_{100}) that the artery would have had in vivo, when relaxed and under a transmural pressure of 100 mm Hg. The arteries were then set to the lumen diameter $L_1 = 0.9 \times L_{100}$ where active force development was maximal (Mulvany and Halpern 1977). After normalization procedure and 45-min rest period, the arteries were exposed to 120 mmol/l KCl, to ensure that the arteries were viable. After a wash-out with 4 × 10 ml of Krebs-Ringer solution and 15-min equilibration, the arteries were preconstricted with phenylephrine (PE, final concentration 10^{-4} mol/l). When the contraction of the femoral artery to PE reached a steady state, increasing concentrations of vasodilator acetylcholine (Ach, 10^{-9} to 10^{-5} mol/l) were added in cumulative manner to perform endothelium-dependent dose-response curves. The extent of relaxation was expressed as the percentage of PE-induced contraction. When the concentration-relaxation curve was completed, the drugs were washed-out (with 4×10 ml of Krebs-Ringer solution) and the same experiment was repeated after 20-min preincubation with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-5} mol/l) in the bath medium.

Noradrenaline (NA)-induced responses were examined in the another segments of the femoral arteries after the testing of viability of the segments by 120 mmol/l KCl. The dose-response curve to NA was constructed in a cumulative manner (final concentrations 5.10^{-8} - 10^{-5} mol/l).

The contractile responses of the femoral arteries were reported as active tension (mN/mm). The effect of interaction of Provinols™ and stress was also expressed as the average value of vasorelaxation and vasoconstriction, which was calculated as a mean value of vasorelaxation (or vasoconstriction) reached in the groups based on the individual dose-response curves.

Statistical analysis

All results are presented as mean ± SEM. Blood pressure was analyzed using two-way ANOVA (group × week of experiment). Vasoconstriction to phenylephrine and KCl was analyzed using one-way ANOVA. Vascular reactivity was analyzed using two-way ANOVA (group × concentration of agonist). All analyses were followed by Duncan`s post-hoc test. Values were considered to differ significantly when $p<0.05$.

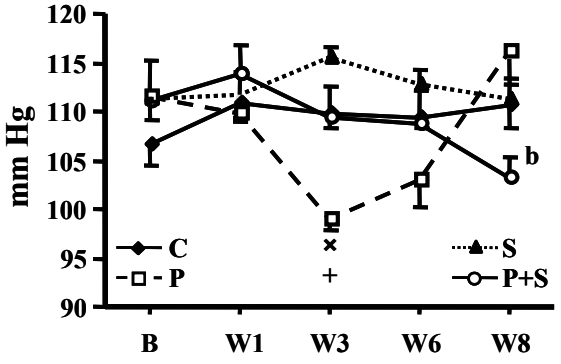

Fig. 1. Effect of Provinols™ and stress on blood pressure after 1st (W1), 3rd (W3), 6th (W6) and 8th (W8) week of experiment (n = 6 in each group). Abbreviations: B – Basal value before experiment; C – control; P – Provinols™; S – stress; P+S – Provinols™+stress; Results are mean ± SEM. $^{\times}p<0.04$ *vs.* control at the same week; $^{+}p<0.02$ *vs.* basal value; $^{b}p<0.04$ *vs.* Provinols™ at the same week.

Results

Blood pressure

Basal blood pressure of all rats before experiment was 110±2 mm Hg and it was not significantly different among the groups (n = 24). In the control group, BP did not change significantly during experiment (Fig. 1). Three weeks of Provinols™ treatment produced a significant decrease of BP compared with control rats ($p<0.04$). Although extension of Provinols™ treatment resulted in a progressive increase of BP, BP of Provinols™-treated rats was not significantly different from the controls at the end of experiment. Similarly, chronic crowding alone as well as in combination with Provinols™ failed to alter BP *vs.* the control group.

NO synthase activity

NOS activity in the thoracic aorta of control rats (n = 6) was 2.63±0.18 pmol/min/mg (Fig. 2A). Chronic crowding increased significantly NOS activity in the aorta *vs.* control by about 80 % ($p<0.04$). Although Provinols™ alone failed to affect aortic NOS activity, it blunted the elevation of NO production in rats simultaneously exposed to stress.

Vascular reactivity

High concentration of KCl (120 mmol/l) induced a sustained contraction of the femoral artery with a maximal tension 7.39±0.75 mN/mm in control group (n = 10). The KCl induced contraction was not affect either by Provinols™ or by stress (Table 1). PE (10^{-4} mol/l) induced a sustained contraction of the femoral artery with a peak tension 4.29±1.57 mN/mm in control rats (n = 5). Similarly to KCl, the maximal responses to PE were not affected significantly by Provinols™ and stress. However, exposure to stress slightly impaired contraction to PE in femoral arteries (Table 1). After acute L-NAME pretreatment (10^{-5} mol/l), the PE contraction was significantly reduced in stressed rats compared with control rats pre-treated with L-NAME (Table 1).

The average acetylcholine-induced vasodilatation of the femoral artery was 64.7±5 % (n = 45) and stress increased it by about 18 % *vs.* the control group ($p<0.0001$) (Fig. 2B). In the Provinols™ – treated group, the endothelium-dependent relaxation to acetylcholine (n = 45) was significantly improved compared with the control group ($p<0.04$). However, Provinols™ blunted the enhanced endothelium-dependent responses of the femoral artery in rats simultaneously exposed to stress. Blockade of endothelial nitric oxide synthesis by L-NAME (10^{-5} mol/l) significantly reduced endothelium-dependent relaxation in all groups. However, the inhibition of NOS activity with L-NAME had a significantly greater effect on Ach-induced vasorelaxation in arteries from stressed rats compared with control WKY rats ($p<0.002$) (Fig. 2B). Individual dose-response curves in the absence or presence of the NOS inhibitor L-NAME are presented in the Figure 3.

The average NA-induced vasoconstriction of the femoral artery was 0.048±0.082 mN/mm in control group (n = 30). Although chronic crowding had no significant effect on NA-induced responses *vs.* control, NA caused rather relaxation of the femoral artery (Fig. 4). However, Provinols™ (alone or in combination with stress) produced significant contractile responses to NA as compared to stress alone.

Discussion

The present study investigated the effect of 8-week administration of red wine polyphenolic compounds Provinols™ on blood pressure, NOS activity and vasoreactivity of femoral arteries of normotensive Wistar-Kyoto rats exposed to chronic crowding stress. We found that chronic crowding failed to affect BP of WKY rats. This was associated with elevation of vascular NO synthesis and improvement of the endothelium-dependent relaxation of the femoral artery. Chronic Provinols™ treatment also improved vasorelaxation of

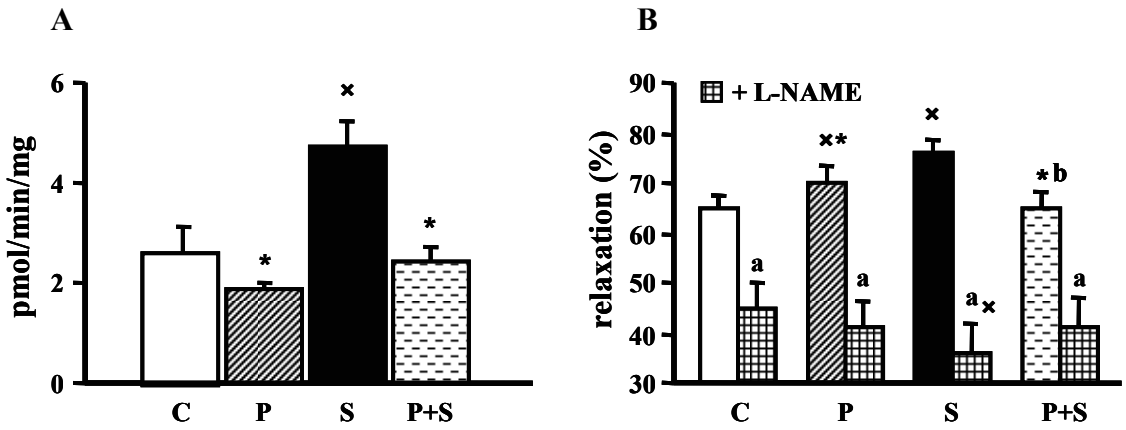

Fig. 2. Effect of Provinols™ and stress on nitric oxide synthase activity in the thoracic aorta (A, n = 6 in each group) and the average values of endothelium-dependent acetylcholine-induced relaxation of the isolated femoral arteries in the absence and presence of L-NAME (10^{-5} mol/l) (B, n = 45 in each group). Abbreviations: C – control; P – Provinols™; S – stress; P+S – Provinols™+stress; Results are mean ± SEM. $^{*}p<0.03$ *vs.* stress; $^{\times}p<0.04$ *vs.* control; $^{a}p<0.0001$ *vs.* control in the same group; $^{b}p<0.04$ *vs.* Provinols™.

Table 1. Effect of Provinols™ and stress on phenylephrine (PE, 10^{-4} mol/l) - (in the absence or presence of L-NAME) and KCl (120 mmol/l)-induced contraction in the rings of the femoral artery.

	C	P	S	P+S
KCl [mN/mm] (10)	7.39 ± 0.75	7.56 ± 0.73	8.71 ± 1.15	9.61 ± 0.92
PE [mN/mm] (5)	4.29 ± 1.57	3.08 ± 1.04	1.16 ± 0.24	4.52 ± 0.89
PE after L-NAME [mN/mm] (5)	7.53 ± 1.72	4.70 ± 1.50	3.33 ± 0.65$^{\times}$	9.52 ± 0.78 ^{a}*

Values are mean ± SEM. Numbers in parentheses denote number of experiments in each group. Abbreviations: C – control; P – Provinols™; S – stress; P+S – Provinols™+stress; $^{\times}p<0.05$ *vs.* control; $^{*}p<0.004$ *vs.* stress; $^{a}p<0.02$ *vs.* P+S in the absence of L-NAME.

the femoral artery, however, this was not associated with significant alterations in NO production. Interestingly, BP, NO production and vasoreactivity of the femoral artery to vasodilators and vasoconstrictors was not changed in rats simultaneously exposed to stress and Provinols™ treatment compared to the controls.

Although chronic stress is considered to be a significant risk factor for the development of cardiovascular disorders, cardiovascular pathology following chronic stress has not been consistently shown in normotensive animal strains. Development of hypertension and significant cardiac pathology were observed by Andrews *et al.* (2003) using territorial stress in young WKY rats. On the other hand, no changes in BP were observed in adult WKY rats exposed to 6-month psychosocial stress (Henry *et al.* 1993). The same author observed chronic psychosocial stress-induced hypertension in CBA mice, which was prevented by chronic decaffeinated green tea treatment (Henry and Stephens-Larson 1984). Similarly, using of a complex population cage produced stress resulting in hypertension in the CBA Agouti mice (Webb *et al.* 1987). The same authors also demonstrated increased responsiveness to endothelium-dependent vasodilators in this model of stress. Additionally, Fuchs *et al.* (1998) demonstrated that endothelium-dependent relaxation to Ach was unaltered quantitatively in the small mesenteric arteries isolated from WKY rats after 10 days of air-jet stress. However, the mechanism mediating vasorelaxation was altered in stressed rats because acute L-NAME-pretreatment reduced vasorelaxation only in stress-exposed rats (Fuchs *et al.* 1998). We observed that identical model of chronic crowding stress as it was used in this study induced hypertension in rats with spontaneously hypertensive mother but not in Wistar rats (Bernátová *et al.* 2006). No changes in BP of crowded WKY rats, may result from elevated vascular NO synthesis and from elevated endothelium-dependent relaxation observed in femoral arteries. Moreover, mild hyporeactivity to PE and rather relaxation to NA in the femoral rings could contribute to this process. Although the exact mechanisms of the slightly impaired contraction to PE and NA cannot be

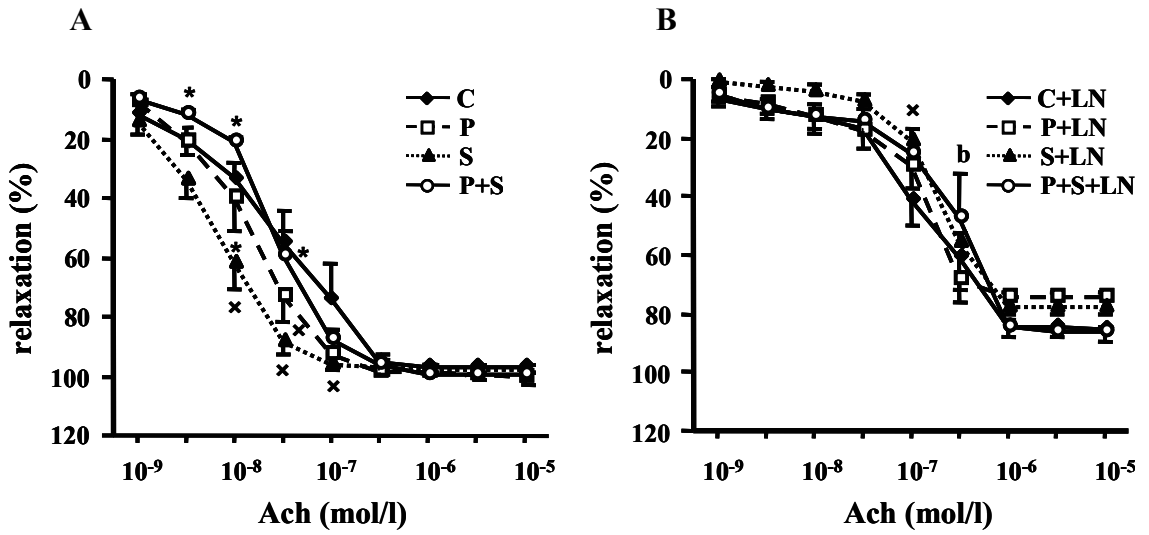

Fig. 3. Effect of Provinols™ and stress on dose-response curves for acetylcholine (Ach) in phenylephrine (10^{-4} mol/l) pre-contracted femoral arteries in the absence (A) and in the presence (B) of L-NAME (10^{-5} mol/l). Abbreviations: C – control; P – Provinols™; S – stress; P+S – Provinols™+stress; LN – L-NAME; Results are mean ± SEM. $^{*}p<0.03$ *vs.* stress; $^{\times}p<0.05$ *vs.* control; $^{b}p<0.04$ *vs.* Provinols™.

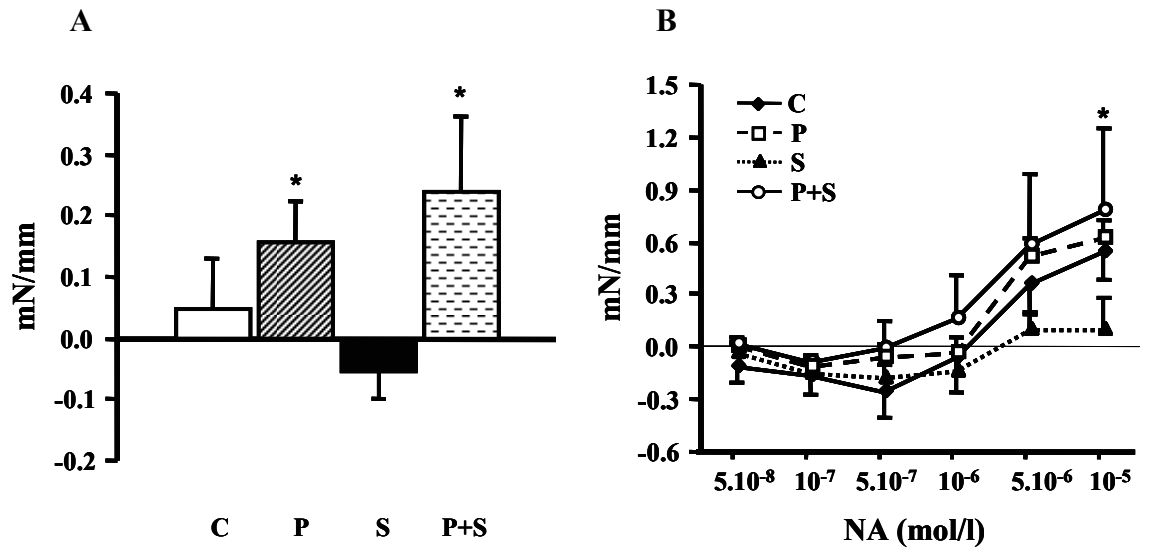

Fig. 4. Average values of reactivity of the femoral artery to noradrenaline (NA) (A) and tension development to noradrenaline in femoral arteries (Fig. 4B). Abbreviations: C – control; P – Provinols™; S – stress; P+S – Provinols™+stress; Results are mean ± SEM. $^{*}p<0.05$ *vs.* stress.

determined from the results of this study, α-adrenoceptors downregulation, reduced sensitivity to NA stimulation and/or altered distribution of adrenergic α and β receptor subtypes may contribute to reduced vasoconstrictor responses of the femoral artery to NA stimulation in crowded rats (Fuchs *et al.* 1998). Moreover, elevated vascular NO production may attenuate NA-induced vasoconstriction in blood vessels (Safar *et al.* 2001). All together, these changes may be considered for adapting mechanisms of normotensive rats to chronic social stress, which may participate in maintenance of normal BP.

Interestingly, the improvement of vasorelaxation of the femoral artery and normal values of BP were not associated with elevated NO production in rats treated chronically with Provinols™ alone. This is in contrast to the several studies investigating the mechanisms of action of natural polyphenolic compounds. An increase of NO production induced by red wine polyphenolic compounds has been reported *in vitro* using cultured endothelial cells (Martin *et al.* 2002), rat aortic rings with functional endothelium (Andriambeloson *et al.* 1999) as well as *in vivo* in rats. Diebolt *et al.* (2001) observed that short-term

(7 days) oral administration of red wine polyphenolic compounds produces a decrease in blood pressure in normotensive rats. This hemodynamic effect was associated with an enhanced endothelium-dependent relaxation and an induction of gene expression of inducible NO synthase and cyclooxygenase-2 (COX-2) within the arterial wall (Diebolt *et al.* 2001). The progressive increase of BP observed in presented study after 6-week Provinols^{TM}-treatment (however still in normal values), may be due to increased expression of COX-2 with subsequent increased release of endothelial thromboxane A_2 (TXA_2) (Diebolt *et al.* 2001). However, our findings indicate that the enhanced Ach-induced endothelium-dependent relaxation of the femoral arteries of rats treated with Provinols^{TM} for 8 weeks may involve other mechanisms than elevation of NO production. It is known that in addition to NO, endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (PGI_2) may be released by the endothelium after Ach stimulation. It is thought that Ach induced elevation in EDHF production may be involved in mechanism of action of Provinols^{TM} when it is administered for a long time. The stimulation of EDHF-mediated vasorelaxation was observed by Duarte *et al.* (2004) in the mesenteric arteries and by Ndiaye *et al.* (2003) in the coronary arteries *in vitro*. However, both these studies used an acute administration of red wine polyphenols to isolated arteries. Interestingly, Provinols^{TM} blunted the enhanced endothelium-dependent responses of the femoral artery in rats simultaneously exposed to stress. This effect may be due to simultaneous stimulation of two different pathways, vasorelaxing L-Arginine/NO and vasoconstrictor arachidonic acid/TXA_2, which compete between each other, in rats simultaneously exposed to stress and Provinols^{TM}. Thus, more studies are needed to elucidate the exact mechanisms of vasorelaxation induced by chronic Provinols^{TM} as well as its effects on vascular function during chronic social stress.

In conclusion, increased endothelium-dependent relaxation and vascular NO synthesis in crowded WKY rats may represent the adaptation mechanisms, resulting in unaltered values of blood pressure. This study further demonstrated that elevated release of NO during chronic psychosocial stress may be prevented by red wine polyphenols. Thus, Provinols^{TM} might maintain equilibrium between endothelium-derived vasoconstrictoric and vasodilating factors in stress-exposed normotensive rats.

Acknowledgments

This study was supported by the grants No. APVT-51-018004 and VEGA 2/4156/26. The Provinols^{TM} was gift from Mr. David Ageron, Societé Française de Distilleries, Vallon Pont d'Arc, France. The authors would like to thank Mrs. Yvonne Hanáčková and Mrs. Jana Peťová for their competent technical assistance. Preliminary results were presented at COST844 Meeting "The Role of Nitric Oxide in Cardiovascular System", April 8-10, 2005, Bratislava, Slovakia (Csizmadiová *et al.* 2005).

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