Physiol. Res. 55 (Suppl. 1): S89-S97, 2006

Acetylcholinesterase Inhibition Affects Cardiovascular Structure in Mice

I. BERNÁTOVÁ[1,2], P. BABÁL[3], R. D. GRUBBS[1], M. MORRIS[1]

[1]Department of Pharmacology and Toxicology, Boonshoft School of Medicine, Wright State University, Dayton, Ohio, USA, [2]Institute of Normal and Pathological Physiology Slovak Academy of Sciences, Bratislava, Slovak Republic, [3]Institute of Pathological Anatomy, Medical Faculty, Comenius University, Bratislava, Slovak Republic

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

Summary

Experiments were performed in C57BL/6J male mice to determine the effects of acetylcholinesterase (AChE) inhibitor pyridostigmine bromide (PB) and stress on cardiovascular function, structure, and apoptosis. Mice were studied for seven days under the following conditions: Controls (osmotic minipump with saline), PB (10 mg/kg/day, minipumps), shaker stress (45 stressors/day, minipump with saline) and PB+Stress combination. AChE activity was significantly reduced in all PB-treated mice. PB caused no changes in 24-h mean arterial pressure (MAP) or heart rate (HR). Stress increased 24-h MAP on day 1 and 24-h HR on day 7 in both Stress and PB+Stress groups. A significant reduction in the aortic wall thickness/diameter ratio ($P < 0.05$ *vs.* control) and slightly reduced relative heart weight were observed in the PB group. These effects were blunted by simultaneous stress exposure. Immunochemistry was used to stain for Bax and Bcl-2 (apoptosis markers). There was a four-fold increase in Bax/Bcl-2 ratio in the heart of PB and PB+Stress treated mice while an attenuation was observed in aortic endothelium. Results suggest that a relatively short-term continuous PB exposure may have adverse effects on the heart and blood vessels, independently of changes in MAP and HR.

Key words

Blood pressure • Heart rate • Pyridostigmine • Stress reactivity • Apoptosis • Vascular structure

Introduction

Pyridostigmine bromide (PB) is a quaternary ammonium compound that inhibits hydrolysis of acetylcholine (ACh) by reversibly binding to acetylcholinesterase (AChE). It is used clinically to treat myasthenia gravis, as a prophylactic against organophosphate poisoning (Keeler *et al.* 1991) and as a putative treatment for heart disease (Breyer-Pfaff *et al.* 1985, Nobrega *et al.* 2001, Sueta 2003). With regard to its cardiovascular actions, there is evidence that PB reduced heart rate, increased left ventricular diastolic function and reduced QTc dispersion in healthy subjects (Castro *et al.* 2002, Nobrega *et al.* 2001, Sant'anna et *et al.* 2003, Raj *et al.* 2005). Although PB does not readily enter the brain, there is data indicating that there are interactions between PB and stress in animals and humans (Beck *et al.* 2003, Grabe-Guimaraes *et al.* 1999). We reported that the combined exposure of mice to PB and stress resulted in marked increases in heart rate

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

variability and baroreflex function, while PB by itself caused no changes (Joaquim *et al.* 2004).

Since AChE inhibitors such as PB enhance cholinergic transmission, it is likely that cardiovascular effects of PB are mediated by changes in ACh. ACh, in addition to its role in neurotransmission, is one of the most potent vasorelaxant factors in the cardiovascular system, affecting vascular tone and blood pressure. ACh signal transduction is associated with enhancement of nitric oxide (NO) production in endothelial cells and cardiomyocytes (Kelly *et al.* 1996). Pharmacological attenuation of NO production resulted in elevation of blood pressure and in pressure-independent structural alterations in the heart and blood vessels (Pecháňová *et al.*1999, Bernátová *et al.* 1999, Török and Kristek, 2002, Kuneš *et al.* 2004). Additionally, AChE has been shown to be associated with tissue remodelling during development and differentiation (Coleman and Taylor 1996, Chan *et al.* 1998). It has been detected in various cell types including endothelial and smooth muscle cells and pharmacological inhibitors of AChE as well as antisense AChE prevented apoptosis in *in vitro* experiments (Zhang *et al.* 2002). However, there is little information either on the influence of continual PB infusion (alone or combined with stress) on cardiovascular function and structure.

In this study we investigated the effect of continual subcutaneous infusion of PB, shaker stress and their interaction on blood pressure, heart rate and stress-induced cardiovascular reactivity. Additionally we determined the effects of PB and stress on structure and apoptosis in the heart and aorta.

Methods

Animals

Male C57BL/6j mice (Harlan Sprague-Dawley, Indianapolis, IN), 10-12 weeks of age, were used. Mice were housed at 22 °C with a 12:12-h dark-light cycle (05:00-17:00 h lights on). Animals were housed individually in plastic cages with a bedding of wooden shavings. They were maintained on *ad libitum* access to standard pellet diet (Harlan Teklad, 0.4 % sodium by weight) and tap water. Mice were randomly assigned to experimental groups as follows: Control, Stress, PB and PB+Stress. The drug and stress treatments were given chronically over a 7 day period. The Laboratory Animal Care and Use Committee of Wright State University approved all experiments.

Pyridostigmine bromide treatment

PB (Sigma Chemical Co., St. Louis, MO) was infused s.c. at 10 mg/kg/day using Alzet^{TM} minipumps (model 1007D, volume 0.5 µl/h, DURECT Corporation, Cupertino, CA). Osmotic pumps were implanted on the back of the mice under ketamine-xylazine anesthesia (6:1 mg/kg, i.m.). Control and Stress groups had minipumps filled with isotonic saline.

Shaker stress

Shaker stress was delivered as previously reported (Bernátová *et al.* 2002). Briefly, cages were attached to a shaking platform (Model 5901, Eberbach Inc., Ann Arbor, MI) that was programmed to produce intermittent shaking (2 min shaking, 150 cycles/min, 2.8 cm stroke). The stress periods were followed by variable rest periods from 13 to 45 min to induce an unpredictability to the stress. Mice were exposed to 45 shaking sessions/24h for 7 days.

Blood cholinesterase activity

Total blood cholinesterase (ChE), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were determined at the end of treatment by a modified version of the Ellman's colorimetric method (Bernátová *et al.* 2003).

Plasma corticosterone

Corticosterone concentration was determined in all experimental groups after 7 days of treatment. Blood samples were collected from trunk blood between 09.00 h and 11.00 h, 30 min after the last shaking session (in the stress and PB+stress groups). Samples were taken into heparinized test-tubes, centrifuged, and stored at –30°C. Plasma corticosterone was measured using the ImmuChem^{TM} double antibody corticosterone ^{125}I RIA kit (ICN Biochemicals, Inc. Costa Mesa, CA).

Cardiovascular measurements

For cardiovascular studies, mice were prepared with chronic carotid arterial catheters as previously described (Li *et al.* 1999, Bernátová *et al.* 2002). After surgery, a heparinized isotonic saline solution (100 U/ml) was continuously infused into the catheter at 25 µl/h using a syringe pump (Model 220, Kd Scientific, Boston, MA). The mice were allowed to recover from surgery for at least 6 days, by which time water and food intake had returned to normal levels.

Blood pressure (BP) and heart rate (HR) were

recorded continuously (24 h) before minipump implantation (basal values) and on days 1 and 7 of treatment. The recording on day 1 began 12 h after implantation of the minipump. Systolic and diastolic BP were recorded directly at a sampling rate of 100 Hz using the Biopac system MP150 (BIOPAC Systems, Santa Barbara, CA). HR was derived from BP data. The data were converted from digital to numeric form using acquisition software. Data were processed by calculation of 10-min means of the respective variable and averaged for calculation of 24-h mean arterial pressure (MAP) and HR.

Cardiovascular reactivity

The effect of PB on cardiovascular stress reactivity was investigated by comparison of immediate MAP and HR responses during individual shaking sessions in mice exposed to Stress and Stress+PB. Responses were evaluated before minipump implantation (basal, no treatment) and on stress days 1 and 7 during the light and dark periods (11:00 h and 23:00 h, respectively). Responses were calculated as percentage change as compared to pre-stress levels (2 min before shaking).

Histology and morphometry

Separate groups of mice (without catheter surgery) were used for evaluation of structural and apoptotic changes in heart and aorta. The groups were the same as those described previously. Mice were decapitated and tissues (heart and descending aorta) were collected and fixed in 10 % buffered formalin (pH 7.4). The hearts were bisected between the apex and the top of the ventricle. The tissues were embedded in paraffin and sectioned (5 μm). Sections were stained with hematoxylin and eosin for basic histological evaluation.

The perpendicular cross sections of the aorta (5 μm) were stained with phosphotungstic acid hematoxylin, converted to digital form. Vascular wall thickness (tunica intima and tunica media) was measured by Impor software (KVANT, Slovak Republic) at four different points and the average was calculated. The inner diameter was calculated as the average of the smallest and the biggest diameters perpendicular one to the other. The wall thickness/diameter ratio was calculated for each sample.

Apoptosis

The pro-apoptotic marker Bax and anti-apoptotic marker Bcl-2 expressions were evaluated using immunochemical staining in sections of the aorta and the heart. Primary mouse monoclonal anti-Bax and anti-Bcl-2 antibodies (Novocastra, Laboratories, Newcastle upon Tyne, UK) were applied in a histochemical staining technique as described previously (Danihel *et al.* 2002) Negative controls employed mouse non-immune IgG instead of specific primary monoclonal antibodies. The apoptosic markers in the aortic endothelium were evaluated using a semiquantitative score, considering the intensity and the area of expression. The categories were as follows: 0.5 – weak intensity in the area less than 10 %, 1 – weak to moderate intensity on the area up to 30 %, 2 – weak to moderate intensity on the area over 30 %, 3 – strong intensity on over 30 % of the evaluated area.

Statistical analysis

All results are presented as mean ± SEM. One-way ANOVA and Duncan's test were used for evaluation of body weight, heart-to-body weight ratio, cholinesterase activities and corticosterone. For evaluation of 24-h MAP and HR, 2-way ANOVA (treatment x day of experiment) and Duncan's test were used. In order to evaluate cardiovascular reactivity, 4-way ANOVA (treatment x day of experiment x day phase x time course) and Duncan's test were used. One-way ANOVA and Newman-Keuls's test were used for evaluation of morphological and immunohistochemical data. Values were considered to differ significantly if $p < 0.05$.

Results

Basic parameters

There were no differences in the initial body weight (BW) of mice (n=9/group, Table 1). Final body weight was decreased in mice exposed to stress ($p<0.04$ in Stress and PB+Stress *vs.* Control). In agreement with the BW data, there was a reduction in the absolute heart weight in mice exposed to stress ($p<0.04$) and PB+Stress ($p<0.05$). Heart weight was also reduced in the PB group ($p<0.05$ *vs*. control), although no changes in BW were observed in PB-treated mice.

Blood cholinesterase

Total ChE, AChE and BChE activities in the Control group (n=6) were 1.1±0.04, 0.7±0.04 and 0.5±0.02 μmol/ml/min, respectively (Fig. 1). PB treatment reduced ChE, AChE and BChE activities in

Table 1. The effect of pyridostigmine bromide (PB) and stress on body weight, plasma corticosterone and apoptotic markers (Bax and Bcl-2) in the heart and endothelium (Endo) of mice.

		Control	Stress	PB	PB+Stress
Initial body weight	[g]	25.14±0.49	24.81±0.53	24.54±0.29	24.92±0.22
Final body weight	[g]	26.58±0.48	25.35±0.31*	26.57±0.35	25.40±0.25*
Heart weight	[mg]	137.5±3.3	126.2±3.4*	127.5±2.4*	128.1±4.1*
Corticosterone	[ng/ml]	22.1±4.3	87.2±6.7*	25.0±4.9	48.2±8.1*x
Heart					
Bax	[IOD]	4.27±1.46	6.35±0.98	9.68±2.57	8.36±1.96
Bcl-2	[IOD]	39.6±13.9	27.02±11.94	23.45±5.76	20.15±2.34
Bax/Bcl-2		0.11	0.23	0.41	0.41
Endo					
Bax	[score]	1.8±0.11	1.1±0.07*	0.5±0.11*	0.5±0.27*
Bcl-2	[score]	2.0±0.19	2.7±0.14*	1.1±0.11*	1.7±0.27
Bax/Bcl-2		0.9	0.4	0.5	0.3

Bax and Bcl-2 were determined using integrated optical density (IOD) or using semi-quantitative score as described in Material and Methods. Results are Mean ±SEM. * p< 0.05 *vs.* control, x p< 0.001 *vs.* stress.

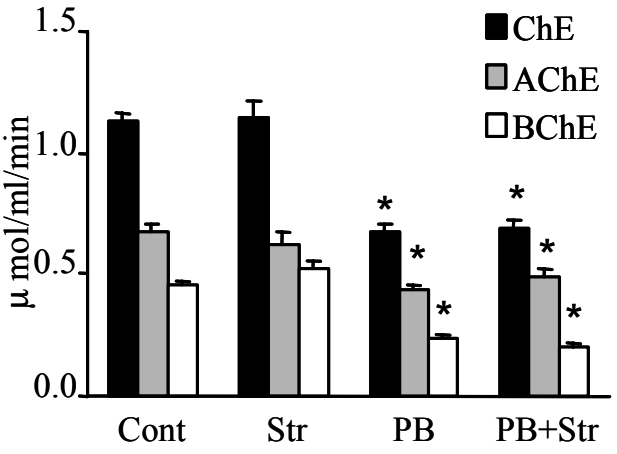

Fig. 1. The effect of pyridostigmine bromide (PB) and stress (Str) on the total cholinesterase (ChE), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities. Results are mean ±SEM. *P < 0.001 *vs.* control (Cont).

both, PB and PB+stress groups (p<0.0001, n= 6-7 in each group).

Plasma corticosterone:

Stress (n=11) increased significantly plasma corticosterone concentration by 295 % (p<0.001) *vs.* the control group (n=15, Table 1). No differences were observed in the PB-treated mice (n=15). In mice co-exposed to PB+stress (n=11), corticosterone level was increased as compared to the control group (by 118 %, p<0.005) but reduced as compared with the stress group (by 45 %, p<0.0001).

Blood pressure and heart rate

The average basal 24-h MAP and HR in all mice investigated was 107±1 mm Hg and 550±8 bpm, respectively (n = 27, Fig. 2A and 2B). On day 1, 24-h MAP was increased significantly in the Stress (p<0.007, n=6) and PB+Stress groups (p<0.003, n=6) *vs*. Control on day 1 (n=8) without significant changes in HR. On day 7, 24-h HR was significantly increased in the Stress (p<0.001) and PB+Stress groups (p<0.02) *vs*. Control on day 7, without changes in MAP. There were no significant differences in MAP and HR of PB-treated mice (n=7) *vs*. Control.

Cardiovascular reactivity

In order to evaluate cardiovascular reactivity, we examined the pressor and heart rate responses to stress in the light and dark periods on days 1 and 7 of treatment. Stress delivered during the light period produced greater MAP and HR responses than stress delivered in the dark period ($F(1,162) = 8.4$, $p<0.005$, main effect of circadian factor for MAP, $F(1,162) = 8.5$, $p<0.004$, main effect of

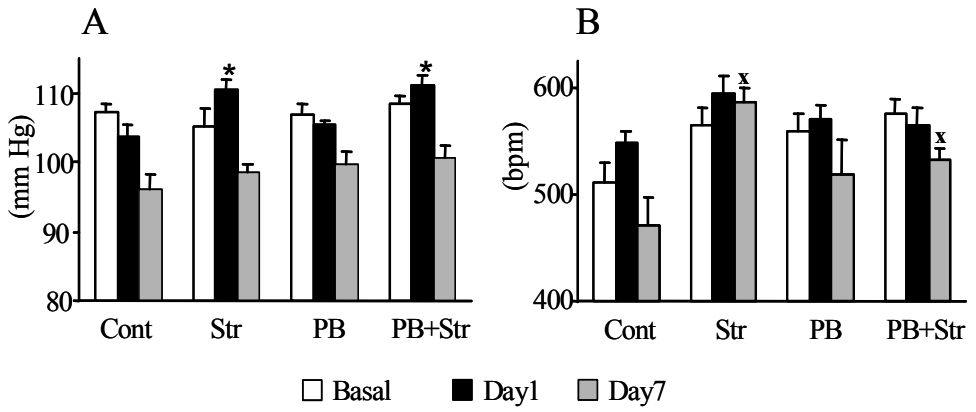

Fig. 2. The effect of pyridostigmine bromide (PB) and stress (Str) on 24-hour average of mean arterial pressure (A) and heart rate (B). Results are mean ±SEM. *P < 0.007 *vs.* day 1 of control, ^{x}P < 0.02 *vs.* day 7 of control (Cont).

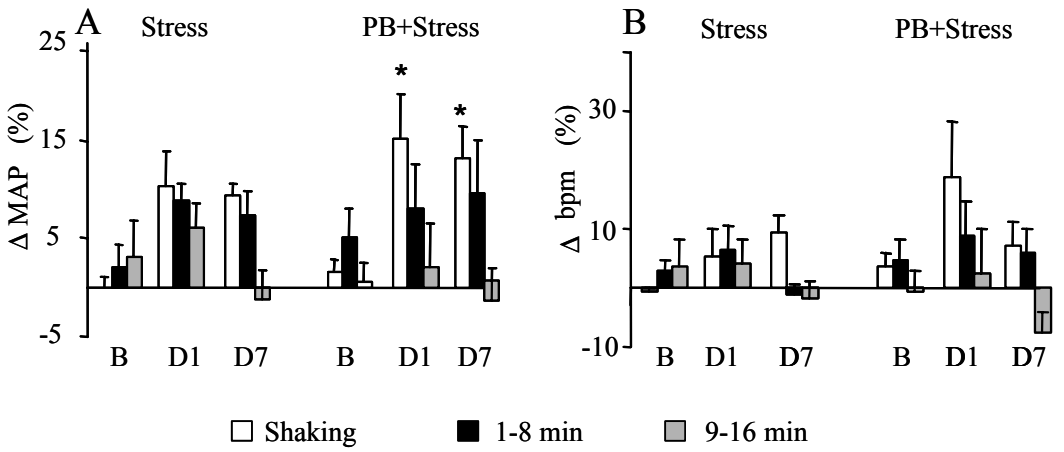

Fig. 3. Pressor (A) and heart rate (B) reactivity in mice exposed to stress and PB+Stress on basal day (B, before treatment) and on day 1 and 7 of treatment (D1, D7). Mean arterial pressure and heart rate were determined during shaking and post-shaking (1-8 min and 9-16 min) in the dark period. Results are mean ±SEM.*P <0.03 *vs.* shaking on basal day in the PB+Stress group.

circadian factor for HR). In the dark period, the immediate shaking-induced pressor responses were increased significantly in the PB+Stress group on days 1 and 7, an effect that was not observed in mice exposed to stress only (Fig. 3A). The immediate shaking-induced increases in HR were similar in the Stress and PB+Stress groups (Fig. 3B). In the light period, no differences in the stress-induced MAP and HR reactivity were observed between the Stress and PB+stress groups (data not presented).

Histology and morphometry

No differences in heart weight-to-body weight ratio were observed in the Stress and PB+Stress groups, while a marginally significant reduction (p=0.08 *vs*. Control) was seen after PB alone (Fig. 4A). The aortic wall thickness/diameter ratio was reduced significantly only in PB-treated mice (p<0.005, Fig. 4B).

Apoptosis

In general, the signal of the anti-apoptotic marker Bcl-2 was more intense than the signal of the pro-apoptotic Bax protein in both the heart and aorta (Table 1). In the heart, there were no significant differences in Bax and Bcl-2 expression among the groups. However, apoptotic index calculated as Bax/Bcl-2 ratio (from the mean values of Bax and Bcl-2) was four-times higher in PB and PB+Stress treated mice as compared to controls (Table 1). The expression of Bax and Bcl-2 in the heart was heterogeneous, with areas of stronger as well as relatively weaker expression of Bax and/or Bcl-2. Both markers were observed in the cardiomyocytes rather than in other cell types, predominantly in the subendocardial myocardium.

In the aorta, there was weak or no expression of Bax and Bcl-2 in smooth muscle cells. On the other hand, relatively strong signal of both markers was observed in

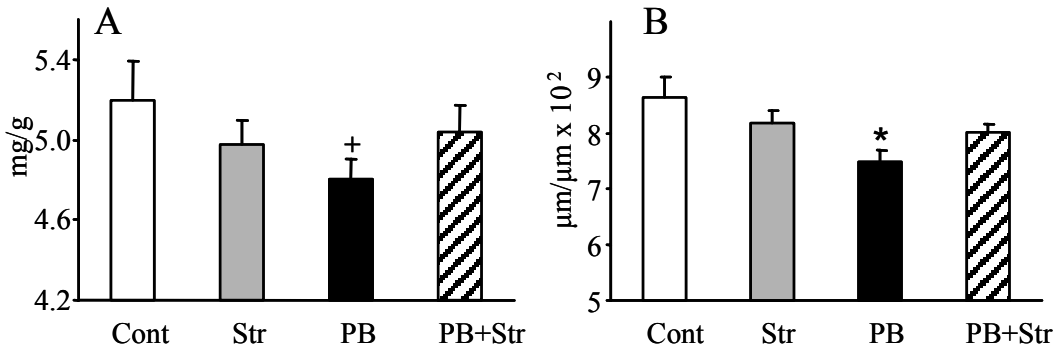

Fig. 4. Effect of pyridostigmine bromide (PB) and stress (Str) on relative heart weight (A) and aortic thickness/diameter ratio (B). Results are mean ±SEM. $^{+}p=0.08$ *vs.* control (Cont), *P <0.05 *vs.* control.

the aortic endothelium. Bax/Bcl-2 ratio in the endothelial cells decreased in all groups investigated as compared to controls, with the lowest values observed in mice co-exposed to PB+Stress (Table 1).

Discussion

The main finding of this study was that a relatively short-term continual subcutaneous infusion of PB affected cardiac and vascular structure and apoptotic processes in mice. This was seen as a reduction in aortic wall thickness/diameter ratio, partial reduction in relative heart weight and alterations in apoptotic index in the heart and aortic endothelium. Reduction in wall thickness/diameter ratio and relative heart weight of PB-treated mice occurred without changes in MAP and HR and it was blunted by simultaneous stress exposure.

There has been much research into the physiological effects of AChE inhibitors since the 1990's when deployed military were treated with PB as a prophylactic drug and possibly exposed to AChE-affecting chemical warfare agents. In fact, one of the theories of the etiology of the "Gulf War Syndrome" was related to interactions between AChE inhibitors and environmental and psychological stressors (Sinton *et al.* 2000, Das *et al.* 2000). However, there is little information on the interactions between PB (AChE inhibitor) and stress in the regulation of cardiovascular function. In the present study, chronic shaker stress increased 24-h MAP on the first day of exposure. A previous study showed that the same stress paradigm produced similar changes in BP with predominant changes occurring in the light (sleeping) period (Bernátová *et al.* 2002). Prolongation of the stress exposure, however, resulted in adaptation of the BP response (Bernátová *et al.* 2002, Farah *et al.* 2004). However, when arterial pressure data were subjected to autoregressive spectral analysis, a pattern of changes emerged. This was seen as increased HR variability in both low and high frequency domains in animals co-exposed to PB+Stress or PB alone (Joaquim *et al.* 2004, Soares *et al.* 2004). Likewise, a study of persons with the "Gulf War Syndrome" showed alterations in autonomic balance (HR variability) as compared to controls (Haley *et al.* 2004). When the effect of mental stress was tested in healthy young people pretreated with PB, there was a reduction in stress-induced tachycardia and pressor response (Sant'anna *et al.* 2003). The authors suggested that effects of PB on cardiac dynamics may be related to activation of the endothelial L-Arg/NO system. An association between NO and structural effects produced by AChE inhibition was seen in rats (Jeyarasasingam *et al.* 2000). The results showed that inhibition of NO synthase reduced paraoxon-induced muscle degeneration (paraoxon is an AChE inhibitor and frequently used insecticide), suggesting that increased NO production contributed to tissue damage.

In this study, a decrease in the heart/body weight and aortic wall thickness/diameter ratios in mice exposed to PB alone suggest that chronic PB exposure may have adverse effects also on cardiovascular system. We hypothesize that direct metabolic effects of elevated NO levels on proliferation and growth (Garg and Hassid, 1993, Kolpakov *et al.* 1995) may play a role in this process because structural alterations in the aorta occurred without changes in MAP and HR. Adult cardiomyocytes however do not proliferate under normal conditions, rather, cardiac growth is mediated by hypertrophy. For hypertrophy, however, sufficient levels of ATP are needed. Both PB and NO have been shown to

inhibit mitochondrial respiration and produce ATP depletion (Kato *et al.* 1989, Sarti *et al.* 2003). Thus, the decrease in energy stores may inhibit normal trophic process in cardiomyocyte and results in relative hypotrophy of the heart and aorta in PB-treated mice.

On the other hand, stress activates the sympathetic and HPA axes. This was seen as an elevation of 24-h HR (day 7) and corticosterone in all mice exposed to stress (alone or with PB). We also observed reduced body mass gain in stressed mice that was associated with reduced heart weight. However, results showed that the heart/body weight and wall thickness/diameter ratios in the Stress and PB+Stress groups were not different from controls. Thus, in mice co-exposed to PB and stress, stress blunted negative structural effects of PB, supposedly due to increased sympathetic activation that counterbalanced increased parasympathetic action and/or elevated peripheral NO synthesis. The interaction between PB and stress was also seen in modulation of neuroendocrine activation when PB blunted stress-induced corticosterone secretion. With regard to cardiovascular stress responsiveness, shaking-induced pressor reactivity was accentuated in mice treated with PB+stress suggesting a dissociation of pressor and endocrine activation in mice simultaneously exposed to PB and stress. Since similar dissociation was observed in oxytocin-knockout mice (Bernátová *et al.* 2004), it is assumed that PB may alter synthesis and/or release of antistress hormone oxytocin (Ježová *et al.* 1995, Morris *et al.* 1995).

To investigate the possible effect of PB on apoptosis, pro-apoptotic marker Bax and anti-apoptotic marker Bcl-2 were measured. Since both markers may be affected by PB, the Bax/Bcl-2 ratio was calculated to see whether there was an enhancement or attenuation of apoptosis. In the hearts of PB and PB+Stress treated mice, a 4-fold increase of the Bax/Bcl-2 ratio was observed. This suggests an elevation of apoptosis in both PB-treated groups, which may be again associated with a PB-mediated increase of NO (Arstall *et al.* 1999, Ing *et al.* 1999, Uchiyama *et al.* 2002).

In contrast to the heart, decreased Bax/Bcl-2 ratio was observed in the aortic endothelium of PB+Stress and PB-treated mice. This finding is in agreement with previous studies showing an opposite effect of NO on apoptosis in the endothelial cells as compared to the other cell types, including cardiomyocytes (Ho *et al.* 1999, Choy *et al.* 2001). Moreover, elevation of NO synthesis due to shaking-induced elevation of shear stress may be responsible for attenuated apoptosis in the Stress and PB+Stress groups (Dimmeler *et al.* 1996).

On the other hand, our results suggest adverse influence of relatively short-term PB treatment on cardiac growth and vascular structure. Observed effects of PB in the heart and aorta were not associated with changes in BP and HR and they were blunted by simultaneous stress exposure. Although the investigation of the exact molecular mechanism of PB action was out of the scope of this study, data suggest that the cardiovascular effects of PB may result from the elevation of ACh-induced NO levels in the heart and vasculature.

Acknowledgments

We express thanks to members of the Chemical Toxicology group at WSU, including the special contributions of Dr. Sara J. Paton, Dr. James Lucot and Mr. William Price. Study was supported by the US Department of Defense contract No. DAMD17-00-C-0020 and by Air Force Research Labs/Dayton Area Graduate Studies Institute grant No. HE-WSU-00-15. IB was partially supported by VEGA grant No. 2/4156/26. Preliminary results were presented at COST844 Meeting "The Role of Nitric Oxide in Cardiovascular System", April 8-10, 2005, Bratislava, Slovakia (Bernátová *et al.* 2005).

References

ARSTALL MA, SAWYER DB, FUKAZAWA R, KELLY RA: Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. *Circ Res* **85**: 829-840, 1999.

BECK KD, BRENNAN FX, MOLDOW RL, OTTENWELLER JE, ZHU G, SERVATIUS RJ: Stress interacts with peripheral cholinesterase inhibitors to cause central nervous system effects. *Life Sci* **73**: 41-51, 2003.

BERNÁTOVÁ I, PECHÁŇOVÁ O, KRISTEK F: Mechanism of structural remodelling of the rat aorta during long-term NG-nitro-L-arginine methyl ester treatment. *Jpn J Pharmacol* **81**: 99-106, 1999.

BERNÁTOVÁ I, KEY MP, LUCOT JB, MORRIS M: Circadian differences in stress-induced pressor reactivity in mice. *Hypertension* **40**: 768-773, 2002.

BERNÁTOVÁ I, DUBOVICKY M, PRICE WA, GRUBBS RD, LUCOT JB, MORRIS M: Effect of chronic pyridostigmine bromide treatment on cardiovascular and behavioral parameters in mice. *Pharmacol Biochem Behav* **74**: 901-907, 2003.

BERNÁTOVÁ I, RIGATTO KV, KEY MP, MORRIS M: Stress-induced pressor and corticosterone responses in oxytocin-deficient mice. *Exp Physiol* **89**: 549-557, 2004.

BERNÁTOVÁ I, BABÁL P, GRUBBS RD, MORRIS M: Acetylcholinesterase inhibition affects cardiovascular structure in mice. *Physiol Res* **54**: 52P, 2005.

BREYER-PFAFF U, MAIER U, BRINKMANN AM, SCHUMM F: Pyridostigmine kinetics in healthy subjects and patients with myasthenia gravis. *Clin Pharmacol Ther* **37**: 495-501, 1985.

CASTRO RR, PORPHIRIO G, SERRA SM, NOBREGA AC: Cholinergic stimulation with pyridostigmine reduces the QTc interval in coronary artery disease. *Braz J Med Biol Res* **35**: 685-689, 2002.

CHAN RY, ADATIA FA, KRUPA AM, JASMIN BJ: Increased expression of acetylcholinesterase T and R transcripts during hematopoietic differentiation is accompanied by parallel elevations in the levels of their respective molecular forms. *J Biol Chem* **273**: 9727-9733, 1998.

CHOY JC, GRANVILLE DJ, HUNT DW, MCMANUS BM: Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol.* **33**: 1673-1690, 2001.

COLEMAN BA, TAYLOR P: Regulation of acetylcholinesterase expression during neuronal differentiation. *J Biol Chem* **271**: 4410-4416, 1996.

DANIHEL L, GOMOLČÁK P, KORBEL M, PRUŽINEC J, VOJTASSÁK J, JANÍK P, BABÁL P: Expression of proliferation and apoptotic markers in human placenta during pregnancy. *Acta Histochem* **104**: 335-338, 2002.

DAS A, KAPOOR K, SAYEEPRIYADARSHINI AT, DIKSHIT M, PALIT G, NATH C: Immobilization stress-induced changes in brain acetylcholinesterase activity and cognitive function in mice. *Pharmacol Res* **42**: 213-217, 2000.

DIMMELER S, HAENDELER J, RIPPMANN V, NEHLS M, ZEIHER AM: Shear stress inhibits apoptosis of human endothelial cells. *FEBS Lett* **399**: 71-74, 1996.

FARAH VM, JOAQUIM LF, BERNÁTOVÁ I, MORRIS M: Acute and chronic stress influence blood pressure variability in mice. *Physiol Behav* **83**: 135-142, 2004.

GARG UC, HASSID A: Mechanisms of nitrosothiol-induced antimitogenesis in aortic smooth muscle cells. *Eur J Pharmacol* **237**: 243-249, 1993.

GRABE-GUIMARAES A, ALVES LM, TIBIRICA E, NOBREGA AC: Pyridostigmine blunts the increases in myocardial oxygen demand elicited by the stimulation of the central nervous system in anesthetized rats. *Clin Auton Res* **9**: 83-89, 1999.

HALEY RW, VONGPATANASIN W, WOLFE GI, BRYAN WW, ARMITAGE R, HOFFMANN RF, PETTY F, CALLAHAN TS, CHARUVASTRA E, SHELL WE, MARSHALL WW, VICTOR RG: Blunted circadian variation in autonomic regulation of sinus node function in veterans with Gulf War syndrome. *Am J Med* **117**: 469-478, 2004.

HO FM, LIU SH, LIAU CS, HUANG PJ, SHIAH SG, LIN-SHIAU SY: Nitric oxide prevents apoptosis of human endothelial cells from high glucose exposure during early stage. *J Cell Biochem* **75**: 258-263, 1999.

ING DJ, ZANG J, DZAU VJ, WEBSTER KA, BISHOPRIC NH: Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. *Circ Res* **84**: 21-33, 1999.

JEYARASASINGAM G, YELUASHVILI M, QUIK M: Nitric oxide is involved in acetylcholinesterase inhibitor-induced myopathy in rats. *J Pharmacol Exp Ther* **295**: 314-320, 2000.

JEŽOVÁ D, ŠKULTETYOVÁ I, TOKAREV DI, BAKOŠ P, VIGAŠ M: Vasopressin and oxytocin in stress. *Ann NY Acad Sci* **771**: 192-203, 1995.

JOAQUIM LF, FARAH VM, BERNATOVA I, FAZAN R JR., GRUBBS R, MORRIS M: Enhanced heart rate variability and baroreflex index after stress and cholinesterase inhibition in mice. *Am J Physiol* **287**: H251-H257, 2004.

KATO T, SUGIYAMA S, HANAKI Y, FUKUSHIMA A, AKIYAMA N, ITO T, OZAWA T: Role of acetylcholine in pyridostigmine-induced myocardial injury: possible involvement of parasympathetic nervous system in the genesis of cardiomyopathy. *Arch Toxicol* **63**: 137-143, 1989.

KEELER JR, HURST CG, DUNN MA: Pyridostigmine used as a nerve agent pretreatment under wartime conditions. *JAMA* **266**: 693-695, 1991.

KELLY RA, BALLIGAND JL, SMITH TW: Nitric oxide and cardiac function. *Circ Res* **79**: 363-380, 1996.

KOLPAKOV V, GORDON D, KULIK TJ: Nitric oxide-generating compounds inhibit total protein and collagen synthesis in cultured vascular smooth muscle cells. *Circ Res* **76**: 305-309, 1995.

KUNEŠ J, HOJNÁ S, KADLECOVÁ M, DOBEŠOVÁ Z, RAUCHOVÁ H, VOKURKOVÁ M, LOUKOTOVÁ J, PECHÁŇOVÁ O, ZICHA J: Altered balance of vasoactive systems in experimental hypertension: the role of relative NO deficiency. *Physiol Res* **53** (Suppl 1): S23-S34, 2004.

LI P, SUR SH, MISTLBERGER RE, MORRIS M: Circadian blood pressure and heart rate rhythms in mice. *Am J Physiol* **276**: R500-R504, 1999.

MORRIS M, LI P, BARRETT C, CALLAHAN MF: Oxytocin antisense reduces salt intake in the baroreceptor-denervated rat. *Regul Pept* **59**: 261-266, 1995.

NOBREGA AC, DOS REIS AF, MORAES RS, BASTOS BG, FERLIN EL, RIBEIRO JP: Enhancement of heart rate variability by cholinergic stimulation with pyridostigmine in healthy subjects. *Clin Auton Res* **11**: 11-17, 2001.

PECHÁŇOVÁ O, BERNÁTOVÁ I, PELOUCH V, BABÁL P: L-NAME-induced protein remodeling and fibrosis in the rat heart. *Physiol Res* **48**: 353-362, 1999.

RAJ SR, BLACK BK, BIAGGIONI I, HARRIS PA, ROBERTSON D: Acetylcholinesterase inhibition improves tachycardia in postural tachycardia syndrome. *Circulation* **111**: 2734-2740, 2005.

SANT'ANNA ID, DE SOUSA EB, DE MORAES AV, LOURES DL, MESQUITA ET, DA NOBREGA AC: Cardiac function during mental stress: cholinergic modulation with pyridostigmine in healthy subjects. *Clin Sci* **105**: 161-165, 2003.

SARTI P, ARESE M, GIUFFRE A: The molecular mechanisms by which nitric oxide controls mitochondrial complex IV. *Ital J Biochem* **52**: 37-42, 2003.

SINTON CM, FITCH TE, PETTY F, HALEY RW: Stressful manipulations that elevate corticosterone reduce blood-brain barrier permeability to pyridostigmine in the Rat. *Toxicol Appl Pharmacol* **165**: 99-105, 2000.

SOARES PP, DA NOBREGA AC, USHIZIMA MR, IRIGOYEN MC: Cholinergic stimulation with pyridostigmine increases heart rate variability and baroreflex sensitivity in rats. *Auton Neurosci* **113**: 24-31, 2004.

SUETA CA: Heart rate variability in chronic heart failure: target for therapy? *Am Heart J* **146**: 385-387, 2003.

TÖRÖK J, KRISTEK F: Beneficial effect of pentaerythrityl tetranitrate on functional and morphological changes in the rat thoracic aorta evoked by long-term nitric oxide synthase inhibition. *Vascul Pharmacol* **38**: 177-182, 2002.

UCHIYAMA T, OTANI H, OKADA T, NINOMIYA H, KIDO M, IMAMURA H, NOGI S, KOBAYASHI Y: Nitric oxide induces caspase-dependent apoptosis and necrosis in neonatal rat cardiomyocytes. *J Mol Cell Cardiol* **34**: 1049-1061, 2002.

ZHANG XJ, YANG L, ZHAO Q, CAEN JP, HE HY, JIN QH, GUO LH, ALEMANY M, ZHANG LY, SHI YF: Induction of acetylcholinesterase expression during apoptosis in various cell types. *Cell Death Differ* **9**: 790-800, 2002.

Reprint requests

I. Bernátová, Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Sienkiewiczova 1, 813 71 Bratislava, Slovak Republic. Fax: +421-2-52968516. E-mail: Iveta.Bernatova@savba.sk