

Bcl-2 AND Bax EXPRESSION IN RAT MYOCARDIUM AFTER ACUTE EXERCISE AND ENDURANCE TRAINING

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ABSTRACT. Some factors, as reactive oxygen species (ROS), glucocorticoids, catecholamines, and others can induce apoptosis. In contrast, it was shown that some free radicals attenuate apoptosis. Strenuous exercise modulates several factors, which may alter apoptosis. We studied the capability of two different types of exercise – acute and chronic, to influence anti- and pro- apoptotic proteins (Bcl-2, Bax) and corresponding Bcl-2/Bax-ratio in myocardium. Male Wistar rats were divided into 3 groups: One group was trained on the treadmill for 8 weeks, the other group of untrained rats was subjected to incremental running test at the end of the experiment, and the last group was sedentary. For the immunohistochemically detection of apoptosis-related proteins Bcl-2 and Bax, polyclonal rabbit antibodies were used. The highest Bcl-2/Bax-ratio was determined in endurance trained group in comparison with sedentary ($P<0.001$) and acute exercised rats ($P<0.01$). The reaction for Bcl-2 was strongest in the smooth muscle cells of vessels in the endurance trained (ET) group. Such alterations in these anti- and pro-apoptotic proteins possibly suppress apoptosis in heart and prevent cardiomyocytes loss and damage of myocardial vessel wall in case of oxidative stress.

KEY WORDS. myocardium, rat, exercise, Bcl-2, Bax, immunohistochemistry

INTRODUCTION

Apoptotic cell death differs morphologically from necrotic cell death, and both appear to occur after exercise. Exercise-induced apoptosis is a normal regulatory process that serves to remove certain damaged cells (1). Some factors, as reactive

oxygen species (ROS), glucocorticoids, catecholamines, a rise in intracellular Ca^{2+} levels, and others can induce apoptosis (2, 3). In contrast, it was shown that some free radicals can attenuate apoptosis (1, 3).

Oxygen radicals cause mitochondrial deterioration, nuclear and mitochondrial DNA damage, and eventually influence apoptosis in myocardium, as a typical postmitotic tissue (2, 4, 5). In postmitotic cells mechanisms preventing apoptosis must be available, since postmitotic cell replacement does not occur.

Alterations of Bcl-2 family proteins (especially Bcl-2/Bax-ratio) during periods of acute or chronic oxidative stress on myocardium may be critical in determining consecutive outcomes – repair or apoptosis.

The aim of this study is to determine the capability of two different types of exercise – acute and chronic, to influence anti- and pro- apoptotic proteins (Bcl-2, Bax) and corresponding Bcl-2/Bax-ratio in myocardium.

MATERIAL AND METHODS

Male Wistar rats (initial body weight 200-220g) were used in the experiment. As running on a treadmill is a skilled activity for rats, before the experiments rats were exercised on the treadmill (Columbus Instruments, Columbus, USA) for 5 $\text{min}\cdot\text{d}^{-1}$, 3 $\text{d}\cdot\text{wk}^{-1}$ for two weeks. Such work load induces no training adaptations but familiarises the rats with treadmill running and allows selection of rats that run spontaneously.

In our study, all rats were divided into 3 groups ($n=6$). One group was trained on the treadmill at the belt speed of 27 $\text{m}\cdot\text{min}^{-1}$, 5° elevation (about 70 -75% $\text{VO}_{2\text{max}}$), 5 $\text{d}\cdot\text{wk}^{-1}$ for 8 weeks (**ET**). The duration of the exercise was increased by 5 min every day. By the end of the second week it reached 40 $\text{min}\cdot\text{d}^{-1}$ and remained so till the end of experiment. The other group of untrained rats (**UT**) was exercised on the treadmill 3 $\text{d}\cdot\text{wk}^{-1}$ for 5 min at the same speed and elevation as the training group to ensure familiarization with treadmill running. The last group of rats was sedentary (**S**) and served as a control.

Submaximal running endurance and incremental running tests

At the end of the experiment the rats of ET and UT groups were subjected to submaximal running endurance test. It was determined in rats by having them run at 27 $\text{m}\cdot\text{min}^{-1}$, and 5° elevation of the belt until they could no longer sustain their position on the treadmill belt.

After a one-day recovery period the same rats were subjected to incremental running test according to Bedford et al., 1979 (6). Each step of exercise was 3 min long. Rats were removed from the test until they could no longer maintain their position on the treadmill belt.

Twenty-four hours after the last test of the UT and ET groups all animals were decapitated under thiopental narcosis (10 $\text{mg}\cdot\text{kg}^{-1}$) and small pieces from the left heart ventricle were taken immediately. The material was fixed in Bouin's fixative for 24 hours at room temperature and embedded in paraffin. Seven μm thick paraffin

sections were mounted onto silane-coated slides. For the antigen detection the avidin-biotin peroxidase complex (ABC) method was applied using Vectastain ABC kit (Vector Lab, USA). The sections were incubated for 24 hours at 4° C in humid chamber with specific primary polyclonal rabbit anti-Bcl-2 and anti-Bax (Santa Cruz Biotechnology, Inc., USA) in dilution 1:200. The peroxidase activity was then developed by means of the peroxidase substrate kit (DAB) (Vector Lab, USA). The sections were counterstained with hematoxylin. In the negative controls the primary antibody was replaced by phosphate-buffered saline (PBS) and only the peroxidase activity was visualized.

The color saturation and intensity of Bcl-2 and Bax expression in cardiomyocytes of the three experimental groups were measured by means of “DP-Soft” software package (Olympus, Japan) on ‘Microphot’ microscope (Nikon, Japan) completed with Camedia-5050Z digital camera (Olympus, Japan). Approximately 15 000 pixels were analyzed on random microscopic fields of different slices for each animal of group and average data for each animal were counted. Sections without counterstaining were used. In the quantitative analysis Bcl-2 and Bax in the blood vessels walls were not considered.

Results are expressed as means ± SEM. Data were evaluated for statistically significant differences by one-way ANOVA followed by Tukey’s post hoc test. Significance was accepted at $P < 0.05$.

RESULTS

Immunohistochemical assessment of apoptosis-related proteins revealed positive staining in the hearts of all experimental groups. Bcl-2 and Bax were localized in the cytoplasm of cardiomyocytes and in the vessel wall (Fig1; Fig. 2). In the negative controls (without corresponding primary antibody) immunoreactivity was absent.

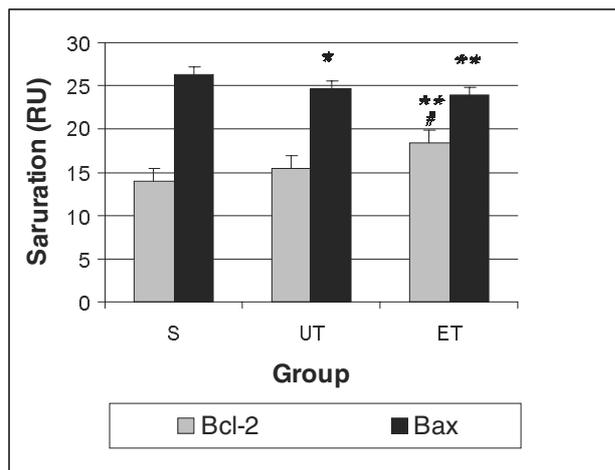


Fig. 3. *Bcl-2 and Bax expression in cardiomyocytes of the experimental groups presented by saturation (in relative units). * $P < 0.05$ vs. S-group; ** $P = 0.001$ vs. S-group; # $P < 0.05$ vs. UT-group.*

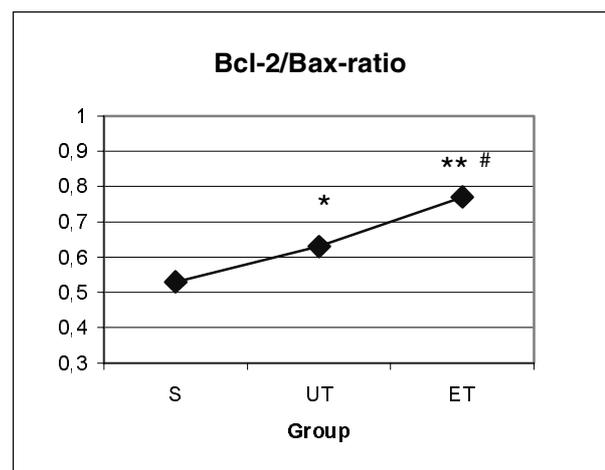


Fig. 4. *Bcl-2/Bax-ratio in experimental groups. * $P < 0.05$ vs. S-group; ** $P < 0.001$ vs. S-group; # $P < 0.01$ vs. UT-group.*

Bcl-2

Myocardial immunoreactivity of Bcl-2, analyzed by its saturation, is shown graphically in Fig. 3. Significant differences were observed between Bcl-2 expression in endurance trained group (ET) and sedentary (S) group (18.4 ± 0.75 vs. 13.9 ± 0.62 ; $P=0.001$) and between endurance trained (ET) and acute exercised (UT) groups (18.4 ± 0.75 vs. 15.5 ± 0.72 ; $P<0.05$). Data of intensity parameters were in similar pattern, but in reciprocal model – in gray scale ($0 \div 256$; 0 = black) (not shown).

Positive Bcl-2 immunohistochemical staining was also seen in the myocardial vessels of exercised groups. The reaction was strongest in the smooth muscle cells of vessels in the endurance trained (ET) group.

Bax

Expression of Bax immunoreactivity is shown in Fig. 3. Cardiomyocytes of sedentary group (S) demonstrated higher Bax immunoreactivity than acute exercised group (UT) (26.3 ± 0.29 vs. 24.6 ± 0.33 ; $P<0.05$) and endurance trained rats (ET) (26.3 ± 0.29 vs. 23.9 ± 0.40 ; $P=0.001$). The decrease of Bax levels in acute exercised and endurance trained group was significant in comparison with the sedentary. The differences in measured intensities were similar, but they didn't reach significance ($P>0.05$).

Positive immunoreactivity for Bax was almost absent in the myocardial vessels of all experimental groups.

Bcl-2/ Bax-ratio

We found significant differences in the saturation ratio of Bcl-2 and Bax between the three experimental groups (Fig. 4). The highest Bcl-2/Bax-ratio was determined in endurance trained group in comparison with sedentary ($P<0.001$) and acute exercised rats ($P<0.01$). Intensity ratio showed similar differences, but only these between endurance trained group (ET) and sedentary group (S) reached statistical significance ($P<0.05$).

DISCUSSION

The results of the present study demonstrated positive immunoreactivity of Bcl-2 and Bax in cardiomyocytes and vessel wall of the experimental groups. Image analysis revealed that system exercise training was associated with increased cytoplasmic expression of the anti-apoptotic protein Bcl-2 and corresponding higher levels of Bcl-2 relative to Bax. Acute exercise with pre-existing accommodation of animals to treadmill running also showed tendency to increase Bcl-2 levels, but without statistical significance. In this group Bcl-2/Bax-ratio was also higher than in sedentary animals.

Bcl-2 is antiapoptotic protein, which has been reported to reside in mitochondrial, endoplasmic reticulum, and nuclear membranes, and for each of these subcellular localizations, a different protective mechanism has been proposed (7, 8). During exercise, muscle metabolism is increased, that leads to an increased production of reactive oxygen species. DNA damage, caused of oxidants can directly induce apoptosis. It has been suggested that Bcl-2 inhibits cell death by reducing the generation of reactive oxidants, thus preventing critical intracellular oxidations that trigger the apoptotic program (3, 9). Mitochondria play a key role in this process (1, 2). An increase in mitochondrial membrane permeability involves release of apoptogenic factors (cytochrome c, apoptosis-inducing factor) through the outer membrane and dissipate the gradient of the inner membrane (10). Opening of a large pore in the inner mitochondrial membrane, which causes uncoupling of the respiratory chain, matrix swelling and efflux of Ca^{2+} appears to be critical event in the induction of apoptosis (11; 12). In previous investigation on myocardium of the same experimental animals by electron microscopy we found preserved mitochondrial structure in the cardiomyocytes of endurance trained rats (13). This substantiate paradigm of Bcl-2 function as a potential anti-apoptotic factor, related to chronic exercise training.

Bax, a member of the Bcl-2 family, forms heterodimers with Bcl-2 and accelerates apoptotic death. Bax overexpression could be induced by chronic cellular response against various stresses, such as chronic ischemia, mechanical overloading of cardiomyocytes near old stage of infarction (14). Obviously, factors which trigger Bax overexpression are not related to treadmill running with submaximal intensity, as used in our study. Bax immunoreactivity decreased without significance in acute exercised and endurance trained groups, as presented by intensity comparison, and showed significance, as presented by saturation aspect. However, Bcl-2/Bax-ratio revealed well define trend to increase in endurance-trained animals. Such alterations in these anti- and pro-apoptotic proteins possibly suppress apoptosis in heart and prevent cardiomyocytes loss and damage of myocardial vessel wall in case of oxidative stress.

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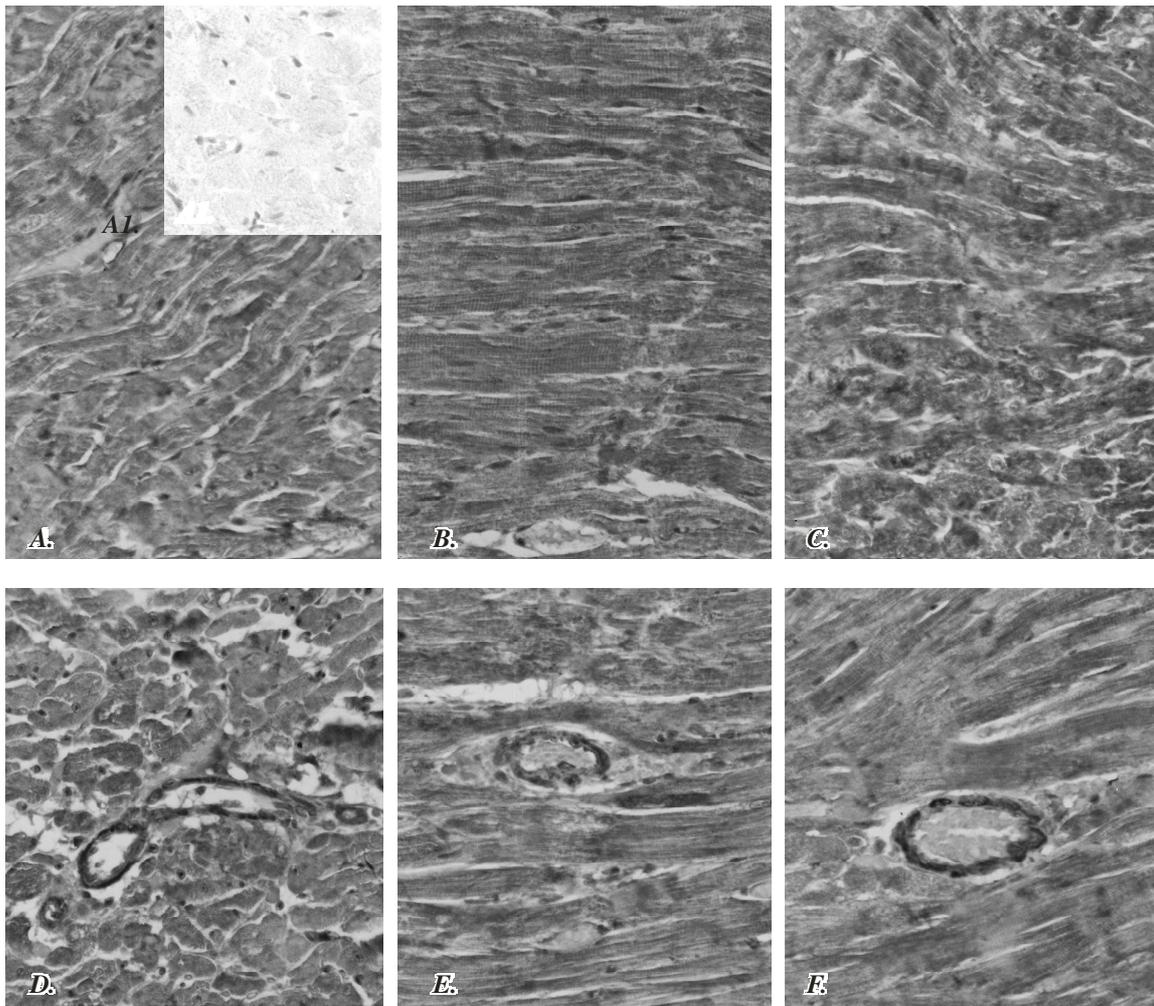


Fig. 1. Immunohistochemical reaction for *Bcl-2* in the experimental groups. *Bcl-2* expression in cardiomyocytes of: A. rat of sedentary group (S); B. rat of acute exercised group (UT); C. rat of endurance trained group (ET). A1 - negative control (x200)
Bcl-2 expression in vessel wall of: D. rat of sedentary group (S); E. rat of acute exercised group (UT); F. rat of endurance trained group (ET). (x200)

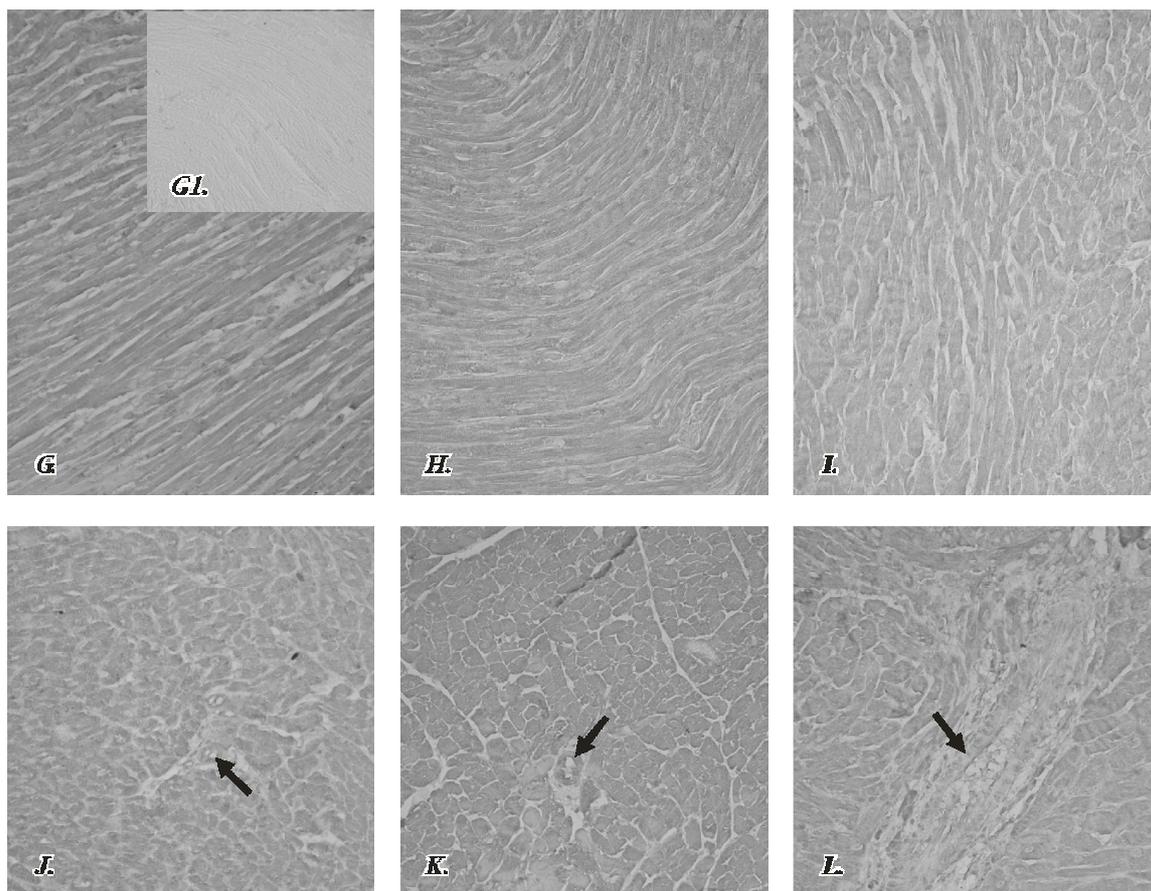


Fig. 2. Immunohistochemical reaction for Bcl-2 in the experimental groups. Bax expression in cardiomyocytes of: G. rat of sedentary group (S); H. rat of acute exercised group (UT); I. rat of endurance trained group (ET). G1 - negative control (x200)
Bax expression in vessel wall of: J. rat of sedentary group (S); K. rat of acute exercised group (UT); L. rat of endurance trained group (ET). (x200)