Original article (short paper)

Aerobic exercise training induces an anti-apoptotic milieu in myocardial tissue

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Abstract—This study evaluated modulators of apoptosis in the myocardium of rats subjected to exercise training. Rats were assigned to non-trained and exercise-trained groups, respectively. The animals ran for 1 h per day, 6 times per week and, for a total of 13 weeks. The left ventricle was processed for analysis of gene and protein anti- (Bcl-2, c-IAP1, c-IAP2, Survivin, ILK, Akt and pAkt) and pro- (Bad) apoptotic expression by real-time PCR (except for Akt and pAkt) and Western blot, respectively. The Bad mRNA (p<0.05), but not the protein expression (p = 0.19), was significantly lower after training. The exercise training significantly increased the gene and protein expression for all anti-apoptotic factors. However, a significant change in the c-IAP2 was seen only for gene expression (p<0.05). The present findings indicate that exercise can create a favorable milieu for the survival of cardiomyocytes when apoptosis is increased.

Keywords: apoptosis, exercise training, myocardial

Resumo—"Treinamento físico aeróbico induz ambiente anti-apoptótico em tecido miocárdico." Este estudo analisou moduladores de apoptose no miocárdio de ratos submetidos a treinamento físico. Os ratos foram distribuídos nos seguintes grupos, respectivamente: não treinados; treinados. Os animais realizaram exercício em esteira (60 min./dia; 6 x semana) por 13 semanas. O ventrículo esquerdo foi processado para análise da expressão gênica e protéica de fatores anti-apoptóticos (Bcl-2, c-IAP1, c-IAP2, Survivina, ILK, Akt e pAkt) e pro-apoptóticos (Bad) por PCR em tempo real (exceto Akt e pAkt) e Western blot, respectivamente. O teor de RNAm da Bad (p<0,05) foi significativamente reduzido após treinamento. Porém, a expressão protéica da Bad não foi diferente entre os grupos. A expressão gênica e proteica de todos os fatores anti-apoptóticos foi significativamente aumentada com o treinamento. A exceção foi para c-IAP2, que aumentou somente em nível transcripcional (p<0,05). Os achados deste estudo indicam que o exercício cria um ambiente favorável para sobrevivência dos cardiomiócitos a apoptose.

Palavras-chave: apoptose, treinamento físico, miocárdio

Resumen—"La práctica de ejercicio aeróbico induce un ambiente anti-apoptótico en tejido miocárdico." El estudio analizó moduladores de la apoptosis en el miocardio de ratas entrenadas físicamente. Las ratas se dividieron en no entrenado y entrenadas. Los animales se ha ejecutado (60 por día x 6 semanas) a las 13 semanas. El ventrículo izquierdo se procesan para el análisis de la expresión de sus genes y proteínas que inhiben (Bcl-2, c-IAP1, c-IAP2, survivina, ILK, Akt y pAkt) y causa (Bad) de la apoptosis por PCR en tiempo real (excepto Akt y pAkt) y Western blot. El nivel de ARNm de Bad (p<0,05) se redujo después de la entrenamiento, pero no era diferente de proteína. La expresión de los inhibidores de la apoptosis fue significativamente mayor con la entrenamiento. La excepción fue para c-IAP2, que aumentó sólo en el nivel transcripcional (p<0,05). Los resultados de este estudio indican que el ejercicio crea un entorno buen para la supervivencia de la apoptosis de los cardiomiocitos.

Palabras clave: apoptosis, el entrenamiento físico, miocardio

Introduction

Physically active lifestyle is recommended to maintain health and improve quality of life. In respect to the type of physical activity, aerobic exercise training (AET) is always recommended, especially when the goal is to improve cardiovascular fitness (Garber et al., 2011). Thus, several lines of evidence have shown that a regular AET program can reduce the risk for cardiovascular diseases (Bocalini, Santos, & Serra, 2008). Moreover, AET has been shown to be cardioprotective to different insults. In this issue, our group has shown cardioprotection to injury by ischemia and/or sustained sympathetic hyperactivity in rats submitted AET in pool and treadmill, respectively (Serra et al., 2008; Veiga et al., 2013).

An issue of interest is to identify the molecular mechanisms for exercise-induced cardioprotective profile. Several potential candidates have been previously evaluated in the myocardium, as follows: antioxidant defense (Pinho et al., 2012); increased angiogenesis (Leosco et al., 2012); favorable inflammatory status (Beavers, Brinkley, & Nicklas, 2010). There is a growing body of evidence showing that AET is associated with an beneficial modulation of programmed cell death (apoptosis) of the cardiomyocytes (Huang et al., 2012; Siu, Bryner, Martyn, & Alway, 2004). The apoptosis is a type of cellular death that allows the elimination of nonfunctional, abnormal, or damaged as well as harmful cells (Cohen, 1997; Thompson, 1995). However, when excessive (e.g. on pathological condition), apoptosis can lead to abnormal change in the structure and myocardial function.

The execution of an apoptotic program is principally controlled under influence of both endogenous pro- and antiapoptotic factors. Thus, in this study, we examined the effects of a regular AET program on genes and proteins modulating apoptosis in the myocardial of rats. We tested the hypothesis that apoptotic suppressors (Bcl-2, c-IAP1, c-IAP2, Survivin, ILK and Akt) are upregulated after AET. We also tested the hypothesis that an apoptotic trigger (Bad) is downregulated post-exercise.

Methods

Animals and exercise training protocol

The research conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The protocol was approved by the Institutional Research Ethics Committee of the Federal University of São Paulo, Brazil (Nº process: 1121/03). Twenty Wistar male rats, weighing 150-180 g, were assigned to one of two groups (n = 10 per group): non-trained rats (NT); exercise-trained rats (EXT). The animals were kept in plastic cages on an environment with temperature and light/dark cycle controlled.

We take into account an aerobic exercise training protocol has been shown to be effective in improving myocardial performance and induce cardioprotection (Serra et al., 2008; Serra et al., 2010).The rats were subjected to run on a motor-driven treadmill for 1 h per day, 6 times per week, and for a total of 13 weeks. The animals were submitted to a pre-training familiarization protocol of 12 days before the start of the formal exercise protocol. In this stage, running velocity was progressively increased by 3 m/min every 2 days until the final velocity of 18 m/min. The sessions initially lasted for 5 min and were increased by 5 min each day to reach 60 min on day 12. On main protocol, the speed race was 18 m/min for 30 min and 22 m/min for the remaining 30 min for each session.

Biological tissue preparation

After 24 hours of the last exercise session, the animals received a urethane overdose (4.8 g kg^{-1} i.p.) and hearts were quickly removed and placed in 5% saline solution to remove excess blood. The left ventricle was removed, stored in cryogenic tube and kept frozen in liquid nitrogen for later analysis of gene expression and protein.

Gene expression analysis

RNA Extraction

The left ventricle tissue was mixed with 1ml of TRIzol Reagent (Gibco BRL, Gaithersburg, USA) for isolation of the total RNA according to the manufacturer's directions. The chloroform (200 µl) was placed to the homogenate and the mixture was stirred for 15s. Then, mixture was kept at room temperature for 5 min and centrifuged for 15min (12,000 x g at 4° C). The aqueous layer of samples was transferred to a 1.5 ml Eppendorf sterile tube and 500 µl of isopropanol were added. After 10 min at room temperature, the samples were again centrifuged (12 000 x g / 4° C / 10 min) and supernatants were removed. The RNA pellets were washed with 1ml of 75% ethanol (prepared with water treated with diethylpyrocarbonate, DEPC, 0.01%) and samples were once again centrifuged (12.000xg/4°C/5 min) and the supernatants were discarded. The pellets were dried in the open air, and then resuspended with 50 µl of DEPC water. The RNAs were measured in spectrophotometer (Eppendorf) on a filter of 260 nm. The integrity of the RNAs was determined on gel electrophoresis in 1% agarose stained with ethidium bromide. The RNA samples with integrity of the subunits 18S and 28S of the RNA ribosomal were used in the experiments.

Total RNA

To eliminate the contamination by genomic DNA, 1 µg of total RNA was incubated with 1 unit of DNAse I / RNAse free (Invitrogen, USA), 0.5 unit of RNase OUT (Invitrogen, USA), 0.5 µl of MgCl2 (50 mM) and DEPC water, in a reaction of 13.5 µl. The samples were incubated at 37°C for 15 min, and 95°C per 5 min to inactivate the DNAse I.

Reverse transcription (RT)

For RT for cDNA synthesis, the treated RNA 2,0 µl of incubation buffer (50 mM KCl, Tris-HCl pH 8.4, 20 mM and 2.5 mM MgCl2) was added, 1.0 unit of MuLV-RT reverse transcriptase

(Invitrogen, USA), 0.5 unity of RNase-OUT (Invitrogen, USA), 1 µl of dideoxinucleotideos (dATP, dCTP, dGTP, dTTP (dNTPs, 10 mM, Amersham Biosciences, USA), 50 ng of a mixture of primers (Random Primer Hexamer, RH, Amersham Biosciences, USA) and 1 µl of dithiothreitol (10 mM LGC Biotechnology, Brazil) in a reaction of final volume of 200 µl. Then, samples were incubated: 20°C per 10 min, 42°C per 45 min, 95°C per 5 min. The complementary DNA (cDNA) samples were kept at -20° C. To ensure the efficacy of the treatment with DNAseI, RT reactions were performed without the reverse transcriptase enzyme, as negative control, RNAs treated as described above.

Polymerase Chain Reaction in real time (RT-PCR)

The RT-PCR was done with a ABI 7500 (Applied Biosystems, USA) using a SYBRGreen core reaction kit (Applied Biosystems, USA). The RT- PCR reaction was performed in the following manner: 0.5 µl of sense primer (10 uM), 0.5 ul of antisense primer (10 µM), 7.5 µl of buffer 2 x Master Mix (Applied Biosystems, USA) and enough water to 15 µl of reaction were added to 1 µl of cDNA. The samples were incubated at 95°C for 10 min, and underwent 40 thermal cycles at 95°C for 15s, 60°C for 30s and 72°C for 90s. All experiments were performed in triplicate. Two parameters were taken into account for control of specificity: presence of only one peak in the fluorescence dissociation protocol; absence of amplification in the reactions with negative control for RT. Table 1 illustrates all primers set used.

Quantification of mRNA expression

The quantification of mRNA was performed as a value relative to an internal reference for Glyceraldehyde phosphate dehydrogenase (GAPDH). The mRNA values were obtained from an arbitrary threshold of fluorescence, analyzed in the exponential phase of the amplification curve. Thus, the cycle threshold (Ct) was calculated for each sample, reaction and gene. The average Ct was calculated and relative expression was done by subtracting this average Ct gene from the average Ct gene for GAPDH, obtaining the ΔCt. Since it is unusual to use the relative expression of a given gene in ΔCt values due to their logarithmic characteristics, the $2^{-\Delta\Delta Ct}$ parameter was used in order to analyze the relative expression of mRNA.

Western blot analysis

The frozen myocardial tissue was homogenized as previously described (Serra et al., 2010). Samples containing 30 µg of the homogenate were subjected to SDS-PAGE (10% polyacrylamide gels) and separated proteins were transferred onto Hydrophobic Polyvinylidene membranes (Hybond-P, Amersham Biosciences; Piscataway, NJ, USA). The membrane was soaked in a blocking buffer (5% non-fat dry milk, 10mM Tris–HCl, pH7.6, 150mM NaCl and 0.1% Tween 20) for 2 h at room temperature and then incubated overnight at 4◦C with Rabbit anti-Bad (1:200 dilution; Santa Cruz Biotechnology, INC.), Mouse anti-Bcl-2 (1:200 dilution; Santa Cruz Biotechnology, INC), Rabbit anti c-IAP1, (1:200 dilution; Santa Cruz Biotechnology, INC), Rabbit anti c-IAP2 (1:200 dilution; Santa Cruz Biotechnology, INC), Mouse anti-Survivin (1:200 dilution; Santa Cruz Biotechnology, INC), Rabbit anti-ILK (1:200 dilution; Santa Cruz Biotechnology, INC), Rabbit anti-Akt (1:200 dilution; Santa Cruz Biotechnology, INC), Rabbit anti-phospho -Akt (Ser 473) (1:200 dilution; Santa Cruz Biotechnology, INC). After overnight incubation, membranes were washed three times and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution; Zymed, San Franscisco, CA, USA). Detection was performed with enhanced chemiluminescence reagents (Amersham Biosciences). The GAPDH expression was used to normalize the results.

Statistical analysis

The Kolmogorov-Smirnov test was used to verify normal statistic distributions. The Student or Mann-Whitney tests were applied when appropriate. The results are shown as mean ± standard error and significance level was set at $p \leq 0.05$.

Table 1. Primers set used for gene expression.

Gene	GenBank	Forward
Bad	NM_022698	F: 5′-CAGTGATCTGCTCCACATTC-3′ (sense) R: 5′-ATATT TCCAGCTAGGATGATAGGAC-3′ (antisense)
Bcl-2	NM_016993	F: 5′-GCTACGAGTGGGATACTGG-3′ (sense) R: 5′-GTGTGCAGATGCCGGTTCA-3′ (antisense)
c-IAP1	AF183430	F: 5’ -CTCCAGCCTTTCTCCAAACCC-3’ (sense) R: 5’-CCAGTTACTGAGCTTCCCACCAC-3’ (antisense)
c-IAP2	AF183431	F: 5’-TCCATCAAATCCTGTAAACTCC-3’ (sense) R: 5’-AGCAAGCCACTCTGTCTCC-3’ (antisense)
Survivin	AF276775	F: 5’-GACCACCGCATCTCTACATTCAAG-3’ (sense) R: 5’-AAGGAAAGCGCAACCGGAC-3’ (antisense)
ILK	NM_133409	F: 5’- ACCCAACCCTCATCACACACT-3’ (sense) R: 5’- GCCTCTTGCCATGTCCAAA-3’ (antisense)
GAPDH	NM_017008	F: 5’- TGCACCACCAACTGCTTAGC-3’ (sense) R: 5’-GCCCCACGGCCATCA-3’(antisense)

F, forward primer; R, reverse primer.

Table 2. Gene expression by real-time RT-PCR in myocardium of non-trained (NT) and exercise-trained (EXT) rats.

Gene	NT	EXT	p value
Bad (a.u.)	2.12±0.18	1.46±0.3	0.001
Bcl-2 (a.u.)	0.12±0.02	0.35±0.11	0.0006
c-IAP1 (a.u.)	0.23±0.04	0.43±0.08	0.0005
c-IAP2 (a.u.)	0.25±0.12	0.45±0.09	0.04
Survivin (a.u.)	0.27±0.05	0.39±0.05	0.007
ILK (a.u.)	1.36±0.14	2.31±0.36	0.003

Results

The AET protocol was well tolerated, thereby all animals completed the study without presenting any complication. Thus, no animal of the non-trained or exercise-trained group was discharged.

The transcriptional results for modulators of apoptosis are summarized in Table 2. As shown by quantitative RT-PCR analysis, the AET over a 13 week period resulted in significant reduction in the pro-apoptotic Bad mRNA content. More importantly, this exercise training protocol was effective to induce a significant increase in all modulators well known to have anti-apoptotic actions in myocardial tissue.

In respect to protein analysis conducted with Western blot approach, the results were wispy different to the findings in transcriptional level. As illustrated in Figure 1, the pro-apoptotic Bad expression was not significantly different between NT and EXT groups. This condition was also observed for the anti-apoptotic c-IAP2 protein, in which there was no significant trend of increase caused by exercise (p= 0.06). On the other hand, several proteins that restrict myocardial apoptosis were affected by training. Therefore, out to Bad, all anti-apoptotic proteins were significantly increased in myocardial as in gene expression analysis. To expand our investigation (Figure 1), we also analyzed the expression of another molecule well known to promote cell survival, the Akt, in which the AET shown to induce a significant increase. To date, the active Akt form was also affected by training. Thus, the phosphorylated Akt expression was significantly higher in EXT group compared with NT group.

Discussion

The present study provides interesting evidence to a beneficial exercise training role on apoptosis in postmitotic myocytes. We showed that rats subjected to chronic AET for 13 wk exhibited a significant increase in several anti-apoptotic factors when measurements are taken 24 h after the last exercise bout. It is important to note that the several anti-apoptotic factors were increased in transcriptional and translational level.

In respect to Bad, a well-known trigger for apoptosis, we saw a distinct expression pattern. The AET decreased the Bad

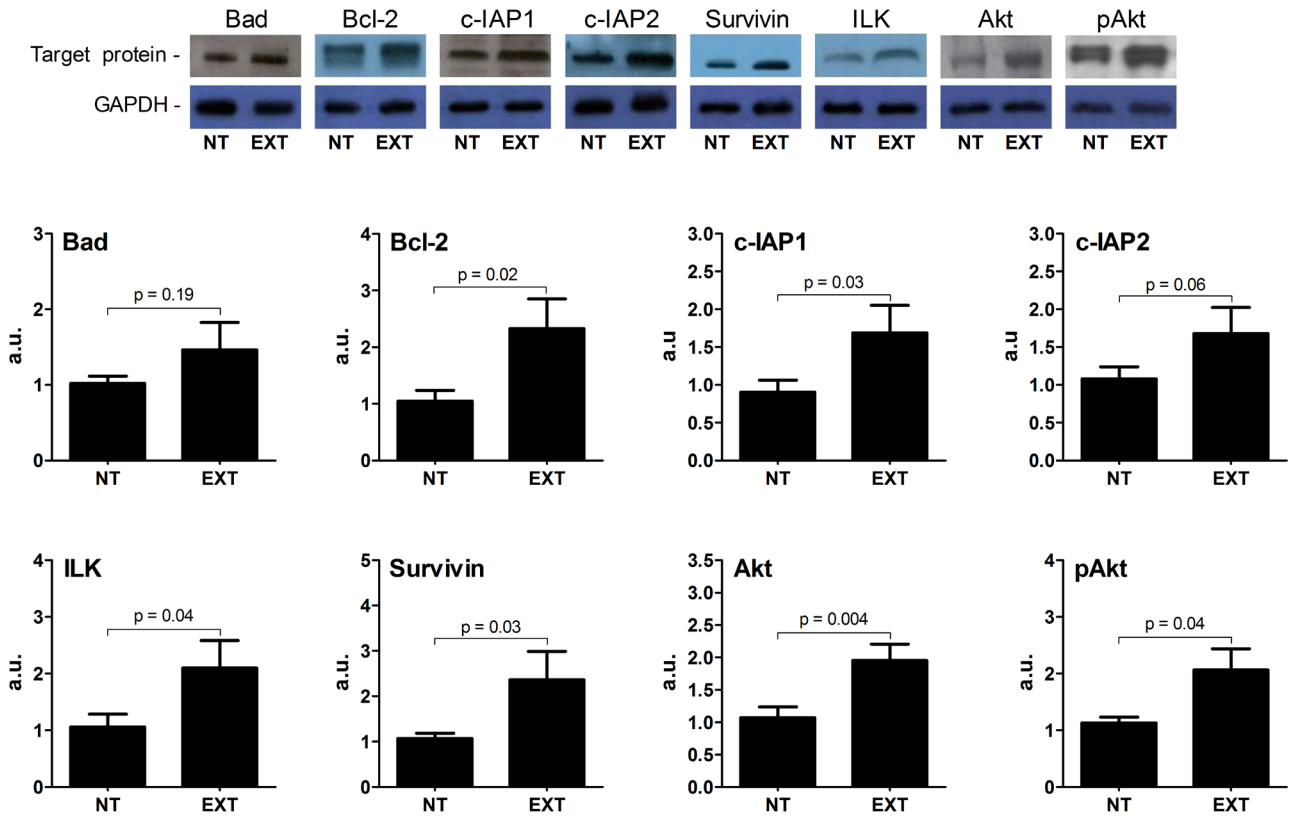

Figure 1. The protein expression for pro- (Bad) and anti (Bcl-2, c-IAP1, c-IAP2, ILK, Survivin, Akt and pAkt) - apoptotic factors by Western blot in myocardium. The upper panel is a representative Western blot for target proteins. All values were normalized for levels of GAPDH. The specific data for each protein are shown in lower panel.

expression in transcript level, but the protein Bad content was similar to that observed in untrained rats. These findings suggest that may occur post-transcriptional changes to protein Bad expression also not be increased as in transcript level. Our findings for a similar protein Bad level are consistent with the idea that myocardial adaptation to AET is not associated with cardiac apoptosis. A study on the cardiac repercussions of treadmill exercise for 13 wk demonstrated that there was no positive cardiomyocytes for staining with digioxigenin-dUTP terminal dexytransferase - a method to indicate apoptosis. Siu, Bryner, Martyn, and Always (2004) investigated the influence of treadmill exercise for 13 wk on apoptosis in myocardial. They found that the proteases activity of caspase-3 was not altered for similar exercise intensity as used in our study.

In respect to analysis for factors inhibiting the execution of apoptosis in myocardial, we have confirmed results of previous studies in which the Bcl-2 was up-regulated after AET (Delchev, Georgieya, Koeya, & Atanassova, 2006; Siu, Bryner, Martyn, & Always, 2004). In our line of investigation, the effects of exercise training were wider and we demonstrate for the first time a relationship between regular AET and increased transcriptional and translational levels for other members of anti-apoptotic IAP family, which included c-IAP1 and Survivin (Pei et al., 2011; Seki et al., 2009). Moreover, we also observed that the c-IAP2 mRNA expression was significantly increased and there was a pronounced tendency to increase the protein content in trained animals (p=0.06). The extent for AET effects on this family of factors suppressing apoptosis was previously reported by Siu, Bryner, Murlasits, and Always (2005). The authors showed a 14% increase in the myocardial XIAP content for animals that were trained by running 5 days weekly for 8 weeks.

This study was also designed to investigate the AET effects on myocardial ILK and Akt signaling expression. The ILK is a multifunctional kinase linking the extracellular matrix to intracellular signaling pathways, whose activation in the heart gives rise to a number of functional results (Gu et al., 2012). Of our interest are the cardioprotective effects for apoptosis associated with ILK. This issue was evident in a recent study conducted by Gu et al. (2012). In a experimental model of doxorubicin-induced cardiomyopathy, the authors observed that the treatment with adeno-ILK was associated with a reduction in apoptosis of cardiomyocytes. These findings are particularly important when considering that we showed a myocardial ILK increase with exercise. We have found that Akt content and its active form were also increased with AET. These findings are important because the Akt is involved in diverse cellular processes, including the promotion of cell survival and inhibition of apoptosis (DeBosch, Sambandam, Weinheimer, Courtois, & Muslin, 2006; Fujio, Nguyen, Wencker, Kitsis, & Walsh, 2000; Zhang, Xia, La Cour, 7 Ren, 2011). Since ILK overexpression can promote activation of Akt (White et al., 2006), we can assume that ILK exerts these beneficial effects through increasing the phosphorylation and hence activation of Akt. In fact, this issue may be acceptable since we have found a similar increase in ILK and Akt in the present study.

In conclusion, our data support the hypothesis that exercise training is able to induce a favorable milieu in the myocardium by modulation of several anti-apoptotic suppressors.

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