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Exercise reverses chronic stress-induced Bax oligomer formation in the cerebral cortex

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Abstract

Chronic stress may lead to neuronal atrophy and functional impairments within the CNS, and increasing evidence indicates that exercise can protect the brain from these changes. Bax is a key protein of the B-cell lymphoma (Bcl) family that complexes within the mitochondrial membrane and forms pores to initiate cellular apoptosis. Herein, we measured cortical Bax levels following chronic and acute stress via immunoblotting. We reveal that chronic, but not acute, stress increases cortical levels of Bax oligomer 270, a complex revealed in previous studies to be associated with apoptosis. Several recent studies have revealed that physical exercise can protect rodents from neurochemical and/or behavioral changes occurring with stress. Previous studies have also revealed that voluntary exercise enhances the expression and activation of cellular proteins associated with enhanced neuronal survival. Herein, we reveal that three weeks of daily restraint led to increased oligomerization of Bax within the cerebral cortex, and that chronic corticosterone administration had a similar effect. Voluntary wheel running, concurrent with chronic restraint, prevented an increase in Bax oligomer 270. Analysis of subcellular fractions also revealed that the combination of exercise with chronic stress reduced the percent of total Bax localized to the mitochondria. Ours is the first study to investigate dynamic molecule complexes associated with the initiation of apoptosis with stress, and the influence of exercise upon the levels of these complexes, suggesting that exercise is an effective preventative measure that can promote neuronal survival and protect the brain against the damaging effects of chronic stress.

Keywords

neuroprotection; apoptosis; mitochondria

Introduction

Chronic stress is known to lead to atrophy and functional impairments in several key brain areas, including the cerebral cortex [21], and has been linked to the development of major depression [8]. Evidence is emerging that voluntary physical activity can protect the brain from stress-induced changes in neuronal function. Changes in rodent behavior due to chronic stress, such as decreased sucrose consumption, decreased motor activity and impaired learning have been prevented when animals were allowed voluntary access to a running wheel during or immediately following the period of stress [15,33]. Also, neurochemical changes due to chronic

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stress, such as altered glucocorticoid sensitivity, were prevented with concurrent wheel running [14]. Neurodegeneration occurs with chronic stress; coupled to these changes is enhanced activation of apoptosis and decreased neuronal survival [21,25]. Voluntary exercise, on the other hand, robustly increases growth factor and growth-associated molecule expression in the cerebral cortex and hippocampus [24,35], and has recently been shown to enhance the activation of intracellular signal transduction pathways promoting neuronal survival [10,34].

In this study, we investigated the effects of chronic restraint stress on the level of activation, reflected in the quaternary structure, of cortical B-cell associated X protein (Bax), a key protein of the B-cell lymphoma (Bcl) family that promotes apoptosis. Endogenous Bax is cytoplasmic in growing cells, and is drawn to the mitochondrial membrane following an apoptotic stimulus. Bax integrates into mitochondrial membranes as a multispinning membrane protein monomer that is subsequently polymerized into an oligomer in order to form pores in the membrane [3]. Previous studies have demonstrated the formation of high molecular weight oligomers in the mitochondrial membranes of apoptotic cells, with a core structure of several Bax subunits and approximate molecular weights of 260,000 and 96,000 Dal [4]. Using native (non-denaturing) Western analysis, we studied the size and concentration of Bax oligomers within cortical neurons of rats subjected to 3 weeks of restraint stress with and without access to a running wheel throughout the time of stress exposure. Total Bax localized to the mitochondrial cellular compartment vs. the cytosol was also measured using differential centrifugation and standard Western analysis. In addition to chronic restraint stress, we investigated the effects of acute immobilization stress and chronic corticosterone exposure on Bax oligomer formation in the cerebral cortex. These alternate stress paradigms have led to significant neurochemical changes in previous studies [1,9], and the effects of acute immobilization have been prevented with voluntary exercise [1].

Materials and Methods

Experimental Subjects and Handling

Male Sprague-Dawley rats (3 months of age, 250–300 g) were obtained from Charles River (Wilmington, MA). Rats were housed singly with wood chips as bedding and fed standard rodent chow and water *ad libitum*. All handling procedures were in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (1996) and our university's Institutional Animal Care and Use Committee.

Chronic Restraint, Voluntary Exercise

Rats in the chronic restraint study were assigned randomly to four groups, at $n = 7$: 1) Sedentary/No Stress, 2) Sedentary/Stress, 3) Activity/No Stress and 4) Activity/Stress. Animals assigned to restraint stress were restrained for 21 consecutive days, for 6 hours/day (0900 to 1500) in polyethylene tubes (diameter = 9 cm). This is a model of chronic stress utilized in several previous studies [16,19,28]. Animals assigned to voluntary exercise were given free access to running wheels within their home cages (34.5 cm in diameter; Nalgene OR.) throughout the experiment. The distance traveled per 24-hr period was recorded by computer using Ratrun Software (C. Hage Associates, CA). Sedentary animals remained in polyethylene cages without running wheels. Plasma corticosterone levels were measured weekly via a corticosterone ELISA kit (Assay Designs, Ann Arbor, MI). Animals were sacrificed at 0600h following the last restraint period. Left motor cortices were extracted and stored at -80°C until analysis.

Acute Restraint

During the acute stress study, rats ($n = 7$) were restrained with tapered plastic film tubes (DecapiCones, Braintree Scientific, Braintree, MA; average diameter = 8 cm) for 4 hours per

day $\times$ 3 days, as utilized in previous studies [1,22]. Control animals were not restrained and remained in their home cages during the experiment.

Experimentally Elevated Serum Corticosterone Concentration

Animals assigned to chronic corticosterone treatment had three pellets (200 mg; Innovative Research of America, FL) surgically implanted subcutaneously at back of the neck one week prior to experimentation. These pellets remained for the entire 3 weeks of experimentation. The animals assigned to the placebo group had three placebo pellets surgically implanted in the same location as the corticosterone pellets, also for the same time course. Cage control animals did not undergo surgery and remained in their cages throughout the experiment. Plasma corticosterone levels were measured weekly via a corticosterone ELISA kit (Assay Designs, Ann Arbor, MI). Plasma corticosterone levels in animals receiving active pellets was 34.36 ± 1.3 ug/dl at the time of the experiment, compared to 17.17 ± 2.8 ug/dl in the placebo group ($p < 0.01$). These levels model those demonstrated during chronic mild stress [7], and are similar to corticosterone levels obtained during previous chronic hormone administration studies [9].

Differential Centrifugation for the Isolation of Specific Subcellular Fractions

Left motor cortices were thawed over ice and homogenized using a motor-driven teflon pestle in ice-cold buffer consisting of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, and 1 mM EGTA, containing 1 protease inhibitor tablet (*Complete*®, Roche Diagnostics, Mannheim, Germany) per 10 ml, pH 7.4. Samples were centrifuged at $1,200 \times g$ at 4°C for 20 minutes and the pellet was discarded. The supernatant was then centrifuged at $10,000 \times g$ at 4°C for 30 minutes resulting in a mitochondrial pellet and a cytosolic supernatant fraction. The mitochondrial pellet was then resuspended in sucrose buffer consisting of 395 mM sucrose, 0.4 mM EGTA, and 40 mM HEPES, pH 7.4. Protein concentrations were determined using the Lowry method [18]. Samples were stored at -80°C for Western blotting (below).

SDS-PAGE and Western Blotting

Equal amounts of protein (30 μg) were applied to each lane of an SDS-PAGE, the proteins electro-transferred to nitrocellulose (Amersham-Pharmacia Biotech, Piscataway, NJ). Western blotting was performed according to the antibody manufacturer's instructions for Bax (Cell Signaling, Beverly, MA) and immunoreactivity visualized using enhanced chemiluminescence (ECL, Amersham-Pharmacia Biotech, Piscataway, NJ). To control for inadvertent differences in protein loading, each blot was then stripped (100 mM 2-mercapto-ethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, 55°C , 10 min, with agitation) and re-probed with anti-GAPDH in accordance with the manufacturer's instructions (Advanced Immunochemicals, Long Beach, CA), followed by ECL.

Quantification from film was implemented using computer-assisted densitometry (MCID Image Processing System, St. Catherine's, Ontario, Canada). Bax optical density levels were divided by corresponding GAPDH optical density levels. Data were then statistically normalized with respect to values obtained with controls (Sedentary/No stress). A one-way ANOVA and Fisher's Least Significant Difference post hoc Test (PLSD) were used to test for differences ($p < .05$) among the 4 treatment groups at $n = 7$ rats per treatment.

Native PAGE and Western Blotting

Native gel electrophoresis and subsequent Western blotting were identical to conventional SDS-PAGE and Western blotting, except that all denaturing conditions (e.g., boiling, SDS, 2-

mercaptoethanol) were omitted, thereby allowing the proteins to maintain their native structure [13]. Thus, the level of Bax present as monomer, dimer and oligomer species were assessed.

Four proteins of known molecular weights were used as markers to calibrate a standard curve of electrophoretic mobility of native proteins: bovine serum albumin (132 kDa, dimer; 66 kDa, monomer), chicken egg albumin (45 kDa), urease, 545 kDa, hexamer; 272 kDa, trimer), and carbonic anhydrase (29 kDa) (Sigma, St. Louis, MO) were electrophoresed on non-denaturing gels. These markers were electrophoresed at the polyacrylamide concentrations of 6%, 8%, 10% and 12%. Gels were then silver-stained to determine banding pattern obtained by the separation of the protein markers. The distance traveled by the protein marker, including all isomeric forms of each protein marker, were measured relative to the tracking dye front, given by $R_f = \text{distance of protein migration} / \text{distance of tracking dye migration}$, where R_f is the electrophoretic mobility of the particular the protein marker. The R_f values were then used to calculate $(x=100 \times (\log(R_f \times 100)))$, and plotted against the percent polyacrylamide concentration to produce a linear graph for each protein marker. The slope obtained for this linear graph yields a retardation coefficient (K_R), whose logarithm of the negative slope for each protein marker is plotted against the logarithm of the known molecular weights of the markers, generating a linear plot, and thereby enabling the determination of the molecular weight of the unknown. (For this study: $R^2 = .7228$, $p = .0018$). Once gel electrophoresis was complete as indicated by the separated molecular weight markers, the gels were silver-stained using conventional procedures [23]. Refer to figure 1 for a sample native blot, indicating the different Bax complexes measured via densitometry.

Results

Non-denaturing Western blot of cortical homogenates revealed increased Bax oligomer (270 kDa) levels with chronic (but not acute) restraint stress

This result was duplicated when animals received corticosterone treatment via slow-release subcutaneous pellets in place of restraint. Increases in Bax oligomer 270 due to chronic restraint were prevented when animals engaged in voluntary wheel running during the period of stress. There was no difference in the level of activity between stressed (4,840 $\pm$ 412 meters/day) and non-stressed (4,015 $\pm$ 385 meters/day) animals. Plasma corticosterone levels were not significantly changed due to chronic restraint or exercise at any point during the experiment (data not shown).

Native Western analysis of cortical homogenates revealed no significant differences in Bax monomer (26.6 kDa), dimer (48.4 kDa), or oligomer 2 (720 kDa) levels between any of the treatment groups in our experiments (data not shown). In contrast, there was a 2-fold increase in Bax oligomer (270 kDa) formation in cortical homogenates of animals that underwent chronic (21 days) restraint stress (Figure 2; $F_{(3,18)} = 3.29$, $p = 0.044$). This result was not evident with the combination of exercise and stress. In contrast, no changes in Bax levels were evident following acute (4 days) restraint stress, either with or without prior exercise (data not shown).

Chronic corticosterone administration via subcutaneous pellet was followed by a 2-fold increase in Bax oligomer (270 kDa) formation in cortical homogenates as compared to control animals (Figure 3; $F_{(2,19)} = 2.16$, $p = 0.05$). As noted in the chronic restraint study, no changes were noted in the levels of Bax monomer (26.6 kDa), dimer (48.4 kDa), or oligomer 2 (720 kDa) within the cerebral cortices of animals receiving chronic corticosterone (data not shown). There was no significant correlation between individual plasma corticosterone levels and levels of Bax oligomer 270.

Subcellular fractionation of cortical homogenates revealed that the combined treatment of activity and stress significantly reduced Bax localization to the mitochondria

Subcellular fractionation followed by (standard denaturing) Western immunoblotting indicated that the combined treatment of both stress and activity significantly reduced the percent of total Bax (28 kD band) localized to the mitochondria as compared to control ($p = 0.041$) and animals that underwent exercise alone (Figure 4; $F_{(3,15)} = 2.32$, $p = 0.034$).

Discussion

A wealth of evidence from recent years indicates that regular, repeated voluntary exercise promotes neuronal survival and growth. In animal studies, voluntary wheel running rapidly enhances the expression of the neurotrophin brain-derived neurotrophic factor (BDNF) in the cerebral cortex and hippocampus [26,29] and enhances the activation state of a number of molecules along survival-promoting intracellular pathways [10]. Evidence also indicates that prolonged stress may inhibit growth and survival mechanisms at the cellular level, leading to neuronal atrophy and apoptosis [20,21]. The results of our study indicate that chronic, but not acute, stress activates a key molecular complex essential for mitochondrial apoptosis, and that regular exercise during the period of stress prevents this activation.

Both human clinical and animal studies indicate that chronic stress or chronic depressive disorders may lead to neuronal atrophy within the brain. Chronic stress has been shown to induce features of neuropathology similar to those observed in major depression, such as neurite beading, dendritic branch retraction and reduced tissue volume [20,30]. Stress-induced cellular atrophy and changes in neurotrophin expression have been observed in the frontal cortex [5,6]. A recent study has shown that with the *in vitro* application of stress hormones, there is an observed up-regulation of pro-apoptotic Bax, making cells more prone to apoptosis [2].

Exercise has shown much promise as a therapeutic and/or protective intervention for stress disorders and depression. As noted earlier, exercise elevates BDNF expression and activates survival-promoting intracellular signaling cascades, such as MAPK and PI-3K [10,31]. The activation of these pathways inhibits several pro-apoptotic molecules, thereby promoting cellular survival [12]. Exercise also reduces oxidative damage induced by stress [27]. In both rats and humans, chronic exercise improves cognitive performance and coping capabilities [11,32].

Our experimental evidence indicates that regular voluntary exercise can prevent molecular changes that set the stage for cellular apoptosis in the cerebral cortex during chronic stress. Chronic restraint stress increased the concentration of Bax oligomer 270, which is associated with mitochondrial pore-forming activity and the activation of apoptosis [4]. This phenomenon of increased oligomer formation was also evident with chronic corticosterone administration, supporting the possibility that chronic restraint-induced Bax oligomerization may be associated with long-term secretion of corticosteroids. Nevertheless, chronic restraint did not lead to measurable changes in Cort levels, so other important factors may be involved. Also, increased oligomer formation was not evident following acute restraint, suggesting that a more long-term process is required to produce the observed cellular change. Nevertheless, it should be noted that the acute restraint procedure used in this study also varied from chronic restraint in the number of hours per day and the device used, and this could account for the difference in results. Also, only left frontal cortices were studied, and possible changes on the right side of the brain may have been missed.

Voluntary exercise concurrent with chronic stress prevented the increased concentration of Bax oligomer 270. Importantly, it was also observed that the combination of exercise with

stress reduced the concentration of total Bax localized to the mitochondria, suggesting that exercise suppressed the translocation of Bax to the cellular compartment where oligomerization and pro-apoptotic activity would occur when stress was present [28]. Previous data from our laboratory indicates that exercise strongly activates the PI-3 kinase pathway [10], which, as noted above, promotes survival by influencing the activity of Bcl family molecules. Activation of the PI-3K pathway stimulates the phosphorylation of Akt (protein kinase B), which promotes Bcl-2 expression and inactivates GSK-3 β , a pro-apoptotic protein that stimulates Bax oligomerization [17]. Therefore, it is very likely that one mechanism of exercise-induced neuroprotection is the intracellular activation of the PI-3 kinase pathway, reduced translocation of Bax to the mitochondria and reduced oligomer formation. Our results suggest that exercise is an effective preventative measure that can promote neuronal survival and protect the brain against the damaging effects of chronic stress.

Acknowledgements

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Oligomer 2 (389 kDal) →
Oligomer 1 (270 kDal) →
Dimer (48 kDal) →
Monomer (24 kDal) →

Figure 1.

Sample native Western blot for Bax, indicating bands corresponding to the various complexes of Bax assessed in this study: monomer (24 kDa), dimer (48 kDa), oligomer 1 (270 kDa) and oligomer 2 (389 kDa).

Figure 2.

The presence of the Bax oligomer (270 kDa) was increased approximately twofold following chronic (3 weeks) restraint stress. This change was not evident when animals engaged in voluntary exercise concurrent with the period of stress. Statistical analyses entailed the use of one-way ANOVA followed by Fisher's PLSD multiple comparisons test. The asterisk indicates a statistically significant difference from control values; brackets indicate groups that are significantly different from each other ($p < 0.05$).

Figure 3.

Chronic corticosterone administration via subcutaneous pellet was followed by a 2-fold increase in Bax oligomer (270 kDa) formation in cortical homogenates as compared to control animals. Statistical analyses were performed via one-way ANOVA followed by Fisher's PLSD multiple comparisons test, and the asterisk indicates the significant increase compared to control ($p < 0.05$).

Figure 4. Subcellular fractionation followed by (standard denaturing) Western immunoblotting revealed that the combined treatment of both stress and activity significantly reduced the percent of total Bax localized to the mitochondria as compared to controls and animals that underwent exercise alone. Statistical analyses entailed the use of one-way ANOVA followed by Fisher's PLSD multiple comparisons test. The asterisk indicates statistically significant difference from the control value ($p < 0.05$); brackets denote significant difference between other groups ($p < 0.05$).