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Intense Exercise Induces Mitochondrial Dysfunction in Mice Brain

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Abstract There are conflicts between the effects of free radical over-production induced by exercise on neurotrophins and brain oxidative metabolism. The objective of this study was to investigate the effects of intense physical training on brain-derived neurotrophic factor (BDNF) levels, COX activity, and lipoperoxidation levels in mice brain cortex. Twenty-seven adult male CF1 mice were assigned to three groups: control untrained, intermittent treadmill exercise (3×15 min/day) and continuous treadmill exercise (45 min/day). Training significantly ($P < 0.05$) increased citrate synthase activity when compared to untrained control. Blood lactate levels classified the exercise as high intensity. The intermittent training significantly ($P < 0.05$) reduced in 6.5% the brain cortex COX activity when compared to the control group. BDNF levels significantly ($P < 0.05$) decreased in both exercise groups. Besides, continuous and intermittent exercise groups significantly ($P < 0.05$) increased thiobarbituric acid reactive species levels in the brain cortex. In summary, intense exercise promoted brain mitochondrial dysfunction due to decreased BDNF levels in the frontal cortex of mice.

Keywords Cytochrome c oxidase · Citrate synthase · Oxidative stress · BDNF · Physical exercise · Brain

Abbreviations

BDNF	Brain-derived neurotrophic factor
COX	Cytochrome c oxidase
CREB	Cyclic AMP response element-binding protein
CS	Citrate synthase
ETC	Electron transport chain
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NMDA	N-methyl-D-aspartate receptor
TBARS	Thiobarbituric acid reactive species
ROS	Reactive oxygen species

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Introduction

Neurological functions and plasticity are well influenced by experiences that intrinsically affect the brain bioenergetics status, such as learning [3], dietary restriction [43] and enriched environment [35]. The physical activity affects the synaptic and cognitive plasticity with changes in brain-derived neurotrophic factor (BDNF) protein and mRNA levels [72]. BDNF signaling at synapses enhances long-term potentiation, a process of synaptic strengthening

associated with learning and memory [70]. BDNF is also involved in controlling energy metabolism with increases in mitochondrial activity [69]. Physical activity decreased the aging-associated development of oxidative stress by preventing the decrease in mitochondrial cytochrome c oxidase (complex IV) activity observed in old mice brains [46].

Most of these studies utilized the running wheel model of physical activity [9, 11], with unknown exercise intensity markers as VO_2max or lactate threshold. The intensity of exercise should be considered when we design programs to optimize physical performance [6, 13] or health benefits because their effects are dose-dependent [48]. So, these experimental procedures should determine the relative work load applied to the biologic system [14]. The running wheel group probably performed exercise of low-level intensity and the effect of high-level intensity exercise remains unknown. Intense physical exercise may cause deleterious biologic adjustments and adaptations like exhaustion and over-training, respectively. Exhaustion and lack of training [67] showed high free radical formation during training and competition. Exercise produces high reactive oxygen species levels only when it is exhaustive [57]. High ROS levels induce oxidative damage near the radical production sites, mainly in tissues with high mitochondrial energy metabolism and poor antioxidant defenses, like the brain.

Mitochondrial oxidative phosphorylation generates most of the ROS in the neuron increased by inhibition of the electron transport chain (ETC). Additionally, the oxidative phosphorylation system itself is vulnerable to damage by ROS [54]. Impaired ETC, in turn, leads to decreased ATP production, increased formation of toxic oxygen species, and altered calcium homeostasis, leading to neuron degeneration and death [30]. These situations are associated with low-brain function. An inhibition of 75% on ETC complexes II–IV and 25% on ETC complex I induces oxidative stress by decreasing BDNF levels [44]. The importance of BDNF in impacting energy metabolism is seen in disorders of energy balance, evidenced by mitochondrial involvement in aging and neurodegenerative diseases [19]. Also, cytochrome c oxidase is used as marker of neurological function in aged mice [46].

Free access to running wheels is a common model of exercise [53]. However, humans mostly engage in regimental physical training instead and the neurological mechanisms of adaptations to intense exercise remain unclear. On the treadmill, performance model and aerobic capacity in mice are generally assessed by total exercise time and incremental speed [17, 37]. In the present study, we employ a forced treadmill running regimen with intensity control. The aim was to analyze the lipid peroxidation and BDNF levels, and cytochrome c oxidase activity on the brain cortex after exposure to intense exercise.

Experimental procedure

Animals

A total of 27 male CF1 mice (weighing 30–35 g, 6 weeks old) were utilized in the experiments. Nuvilab CR1 food (Nuvital Nutrientes S/A, Curitiba/PR, Brazil) and water were available ad libitum. The room was kept at 70% humidity/20 ± 2°C on a 12 h light/dark cycle with lights on at 06.00 h. Each animal was weighed upon inclusion into the study and checked for weight loss.

All procedures were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and were approved by the Ethics Committee of the Universidade do Extremo Sul Catarinense, Brazil. The number of animals and their suffering were minimized in all experimental conditions. Mice were randomly assigned to three groups designated ($n = 9$ each group): control untrained, intermittent exercise and continuous exercise.

Exercise protocol and sacrifice

All groups were habituated on a nine-channel motor-drive treadmill with the speed of 8 m/min for 10 min/day during 1 week to reduce their stress to the new environment. The mice did not receive any stimuli to run. The intermittent and continuous exercise groups performed an incremental running program to obtain progressive levels of intensity during 8 weeks for 5 days/week and for a total period of 40 days (Table 1). The intermittent exercise group performed the exercise three times/day of 15 min (07.00, 14.00, and 19.00 h) and the continuous group exercised once, for 45 min, at 19.00 h. The untrained control animals were put on the switched-off treadmill during the same 8 weeks as the exercise-trained groups.

The exercise training protocol was stopped 48 h before sacrifice. The mice were anesthetized with CO_2 and

Table 1 The treadmill running program performed by CF1 mice resulted in intense exercise and was performed above the anaerobic threshold

Week	Belt speed (m/min)	Daily duration (min)	Inclination (degrees)
1	13.5	45	0
2	13.5	45	0
3	13.5	45	0
4	13.5	45	0
5	16.5	45	0
6	16.5	45	0
7	16.5	45	0
8	16.5	45	0

sacrificed by cervical dislocation. The soleus muscle and brain were immediately excised and placed on ice, while the frontal cortex was removed, weighed, stored and frozen at –80°C until analysis.

Physical exercise intensity

Blood lactate level was defined after the last session of exercise from 50 µl of tail capillary blood, using a commercial kit according to the manufacturer's instructions (Roche, Penzberg, Germany). The blood sample was put onto a glass fiber fleece where the erythrocytes were retained. Lactate was determined by reflectance photometry at a wave length of 657 nm via colorimetric lactate-oxidase mediator reaction.

Muscle oxidative capacity

Due to collaborative tissue requirements and dissection time limitations, citrate synthase (CS) analysis [2] was performed only on the soleus. The tissue was weighed and homogenized with a glass homogenizer on ice in 100 mM Tris–HCl at a constant weight-to-volume ratio. Sample homogenate was then added to a reaction mix of 100 mM Tris–HCl, 1.0 mM dithio-bis (2-nitrobenzoic acid), and 3.9 mM acetyl coenzyme A. After addition of 1.0 mM oxaloacetate, absorbance at 412 nm was recorded for a 2-min period. Mean absorbance change per minute was recorded for each sample, and CS activity in millimole per minute per gram was then calculated by using an extinction coefficient of 13,600.

Lipid peroxidation assay

The 2-thiobarbituric acid reactive species (TBARS) levels were measured [18] and expressed like malondialdehyde (MDA) equivalent. Briefly, the samples were mixed with 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid; subsequently, they were heated in a boiling water bath for 15 min. TBARS were determined by 535 nm absorbance and results are given as nmol MDA/mg protein.

Cytochrome c oxidase (COX) activity

Brain cortex was homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 U/ml heparin). The homogenates were centrifuged at $800 \times g$ for 10 min and the supernatants kept at –70°C until use for enzyme activity determination. The maximal period between homogenate preparation and enzyme analysis was always less than 5 days.

The cytochrome c oxidase (COX, complex IV) was measured [56] by following the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1$ mM$^{-1} \times$ cm^{-1}). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM n-dodecyl-d-maltoside, 2–4 µg homogenate protein and the reaction was initiated with addition of 0.7 µg reduced cytochrome c. COX activity was measured at 25°C for 10 min.

Brain-derived neurotrophic factor (BDNF)

Brain-derived neurotrophic factor protein was quantified using an enzyme-linked immunosorbent assay (ELISA) and standard protocols (ChemiKine™ Brain Derived Neurotrophic Factor, Sandwich ELISA, Chemicon, Temecula, CA, USA). Briefly, Nunc MaxiSorp 96 well plates were coated with 0.1 ml of a monoclonal antibody against BDNF in a buffer containing 0.025 M sodium bicarbonate and 0.025 M sodium carbonate (pH 9.7) for 16 h at 4°C. After being washed in TBST [(20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20)], wells were incubated with 0.2 ml of a blocking buffer at room temperature for 1 h and then washed in TBST again. Samples, six serial dilutions of a BDNF standard (500 pg/ml), and a blank (no BDNF) were added in triplicate into separate wells. Plates were incubated for 2 h at room temperature and washed five times in TBST. A polyclonal antibody against BDNF (1:500 dilution) was added to each well and plates were incubated for 2 h at room temperature. After five washes in TBST, 0.1 ml of a secondary anti-IgY antibody with a horseradish peroxidase conjugate was added to each well and plates were incubated for 1 h at room temperature. Wells were washed five times with TBST. A hydrogen peroxidase solution with a peroxidase substrate was added and incubated for 10 min at room temperature. Reactions were stopped with 1 M phosphoric acid and absorbance at 450 nm was measured using an automated microplate reader. Standard curves were plotted for each plate. Triplicates were averaged and values were corrected for total amount of protein in the sample.

Measurement of protein

Protein concentration was estimated with bovine serum albumin as standard [39].

Statistical analysis

Comparison between means was performed by Student t test and ANOVA followed by the Tukey post hoc test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a compatible computer. A $P < 0.05$ was considered significant.

Results

Animals performed an intense exercise program

The anaerobic threshold is a term that refers to the oxygen consumption during exercise above which the rate of lactate production exceeds the rate of lactate removal, thus causing increase in tissues blood lactate levels [71]. In the final stage of the last day of exercise, blood lactate levels were 7.3 ± 0.8 mmol/l in the untrained control group and 4.3 ± 0.4 mmol/l in the continuous exercise group, respectively. These data indicate a significantly ($P < 0.05$) higher blood lactate content in untrained animals than in those of the continuous exercise group. The aerobically trained mice remove more lactate from anaerobic metabolism in exercise than the untrained animals [14]. The mice of our study performed the exercise above the anaerobic threshold interval [41]. For rats, this occurs at a blood lactate concentration of ~4.2 mmol/l [50].

The soleus muscle showed adaptations to exercise

Several studies have been carried out to determine the influence of exercise training in the mitochondrial enzyme adaptation in skeletal muscle of rats [51, 61]. The soleus muscle CS activity in continuous exercise groups (0.589 ± 0.050 U CS/mg protein) were significantly ($P < 0.05$) higher than in untrained control groups (0.327 ± 0.042 U CS/mg protein). These results indicated that the treadmill-training program used was sufficient to increase the oxidative metabolism in the skeletal muscle of mice.

Intense exercise increases lipid peroxidation levels in the frontal cortex

The mitochondrial work constantly supplies energy for neuronal processes through oxidative phosphorylation. A normal by-product of mitochondrial respiration is free radical formation. When an imbalance occurs between the production of free radicals and the ability of cells to guard against them, it is commonly referred to as oxidative stress [20, 60].

We wanted to examine the possibility that intense exercise can limit the amount of oxidative stress occurring in the brain frontal cortex by employing mitochondrial metabolism. Therefore, we assessed lipid peroxidation levels as markers of oxidative stress [73] following exercise.

We showed that intense continuous (0.27 ± 0.05 µg MDA/mg protein) and intermittent (0.24 ± 0.04 µg MDA/mg protein) physical exercise increased significantly ($P < 0.05$) the level of frontal cortex lipid peroxidation in relation to untrained groups (0.11 ± 0.02 µg MDA/mg protein) (Fig. 1).
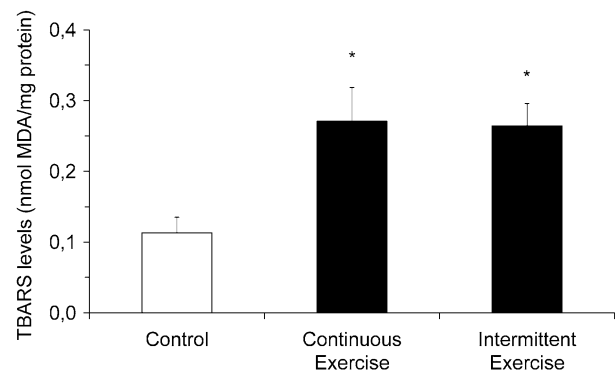

Fig. 1 Lipid peroxidation in frontal cortex was measured by TBARS levels. Forced intense exercise resulted in significantly increased lipid peroxidation levels in the frontal cortex of trained mice compared to untrained control. Treadmill training and TBARS assay are described in Experimental Procedures. Values are mean ± SEM. for nine animals per group. *Asterisk* $P < 0.05$ vs. control, ANOVA, Tukey post hoc test

Intense exercise decreases COX activity in the frontal cortex

Inhibition of the ETC may initially cause oxidative stress rather than ATP depletion, and this may subsequently induce irreversible changes in ETC function providing the basis for a cycle of damage [27].

We investigated partial inhibition of the COX in the frontal cortex of mice and our results showed that intermittent exercise (122.9 ± 3.9 nmol/min mg protein) significantly ($P < 0.05$) decreased COX activity above untrained control (139.5 ± 6.4131.4 ± 3.8 nmol/min mg protein), while continuous exercise (131.4 ± 3.8 nmol/min mg protein) did not show significant ($P < 0.05$) changes in COX activity of control frontal cortex (Fig. 2).
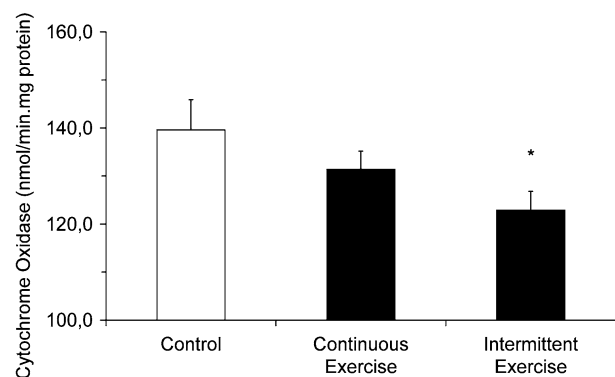

Fig. 2 COX activity in the frontal cortex of mice. COX activity in the intermittent exercise group showed partial inhibition when compared to untrained control values. Treadmill training and enzyme assay are described in Experimental Procedures. Values are mean ± SEM. for nine animals per group. *Asterisk* $P < 0.05$ vs. control, ANOVA, Tukey post hoc test

Frontal cortex BDNF levels decreased in the final stage of exercise program

Based on animal findings, Hellweg et al. [24] proposed that early after initiation of an acute or chronic illness neurotrophins are reduced, followed by a compensatory up-regulation with a second decrease in the end-stages.

The exercise modulates elements central to energy metabolism to subsequently affect synaptic plasticity, specifically by changes in key molecular components underlying learning and memory, i.e., BDNF, CREB, synapsin I, and p-CAMKII [69].

To determine the possibility that energy metabolism may modulate BDNF protein levels, we measured BDNF levels in the frontal cortex of mice. The continuous (152.7 ± 16.0 ρg BDNF/μg protein) and intermittent (189.2 ± 21.0 ρg BDNF/μg protein) running treadmill program significantly ($P < 0.05$) reduced frontal cortex BDNF levels when compared to untrained animals (264.5 ± 14.4 ρg BDNF/μg protein) (Fig. 3).

Discussion

Evidences in humans suggest that intense exercise is associated with accelerated oxygen radical generation that results in acute [1, 63] and chronic [25, 68] blood oxidative stress. In relation to the brain, there are conflicting results in the literature as to whether brain oxidative stress occurs after physical exercise of different intensities. The brain has a large potential oxidative capacity and high-oxygen consumption [8]. There are evidences indicating that capillary growth occurs in motor areas of the cerebral cortex as a robust adaptation to prolonged physical training and
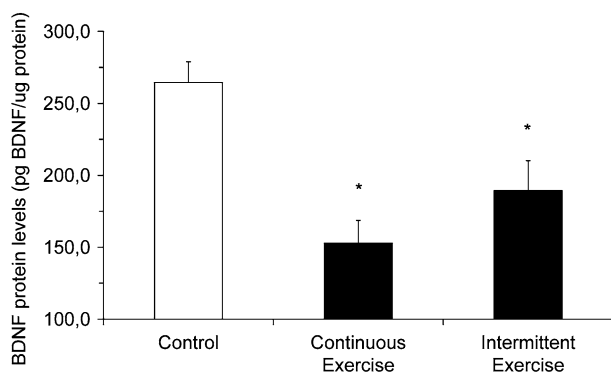

Fig. 3 The BDNF protein levels in frontal cortex were measured by ELISA method. Quantitative BDNF protein levels revealed that intense exercise significantly decreased them in the continuous and intermittent groups. Treadmill training and enzyme assay are described in Experimental Procedures. Values are mean ± SEM. for nine animals per group. *Asterisk* $P < 0.05$ vs. control, ANOVA, Tukey post hoc test

heightened blood flow under conditions of neuronal activation [66]. During exercise, there is an increase in regional cerebral blood flow and blood speed in the major cerebral arteries, as well as an increase in blood flow in the internal carotid artery, suggesting an increase in blood flow to a large part of the brain [26]. While the regional cerebral uptake of oxygen increases during exercise, the global value is regarded as being constant [59].

The brain antioxidant capacity is limited by a high content of easily oxidizable fatty acids [50, 62] and free iron [5, 21, 33], and low levels of antioxidants enzymes and substrates, respectively, catalase and superoxide dismutase [10, 22] and reduced glutathione (GSH) [8, 58]. Some studies have identified significant decreases in free radicals content in the nervous system of rodents, such as decreased lipid and protein oxidation levels in the cerebral cortex, hippocampus and cerebellum of adult rats that swam 40 days, 30 min/day, with a load of 3% body weight [28]. The oxidized glutathione (GSSG) levels decreased and the GSH levels remained unaltered in the cerebral cortex and striatum of adult rats that ran 7.5 weeks on a treadmill with a load of 52% VO$_2$max [62]. Young rats with free access to the running wheel during 14 or 28 days decreased lipoperoxidation of the brain [65].

Other authors did not find brain exercise-induced oxidative stress, as for example Radák et al. [52] who also found improved memory when aged rats were subjected to 8 weeks of swimming training, 5 days/week, with the first 4 weeks for periods of 60 min/day and the last 4 weeks, 120 min/day. Ogonovszky et al. [49] did not find oxidative damage to DNA and lipids in the brain of old rats that performed strenuous training (the swimming duration increased by 30 min each week until it reached 4.5 h in the last week) and over-training (1 h swimming/day, five times/week, for 6 weeks, when the duration was abruptly increased to 4.5 h for the remaining 2 weeks). Also, Ogonovszky et al. [49] showed improved memory and decreased brain protein oxidation.

Our results are really conflicting with these data. We found that the frontal cortex TBARS levels increased in the brains of exercised mice when compared to the untrained control group (Fig. 1). Coskun et al. [10] also found similar TBARS levels in the brain of adult diabetic rats that ran 6.5 weeks on constant speed of 27 m/min and 15% inclination. Özkaya et al. [50] trained diabetic rats with treadmill running during 8 weeks, 5 days/week, with lactate blood levels of 6 mmol/l and showed high brain TBARS levels via increased Xanthine Oxidase (XO) activity and decreased Xanthine Dehydrogenase (XDH) activity.

Exercise intrinsically influenced brain energy metabolism by involving mitochondrial oxidative phosphorylation [15]. Vaynman et al. [69] showed that 3 days of free access to wheel running increased activity of COX-II and reduced

oxidized proteins in the hippocampus of adult rats. Ten weeks of voluntary exercise on a running wheel increased the COX activity of the rats' spinocerebellum [23]. We found that the exercise-induced lipid peroxidation was accomplished by a reduced COX activity in the frontal brain cortex of the intense intermittent exercise group (Fig. 2). Mitochondria in the described condition are called dysfunctional mitochondria [7, 29]. There are evidences correlating oxidative stress to respiratory complex deficiencies [8, 42, 47]. Jacobson et al. [27] suggest that in the context of the whole cell even a limited inhibition of complex IV, although not compromising ATP production, may give rise to increased free radical production, which in an autocatalytic cycle could cause further inhibition of mitochondrial electronic transport chain, more reactive oxygen species production and so on.

Oxidative stress induces energy depletion [36] and can result in impairments to the NMDA channel function [40], related to decreased BDNF levels in aging [55]. We observed decreased BDNF levels in the frontal cortex of mice after 8 weeks of intense exercise (Fig. 3), associated with mitochondrial dysfunction. In turn, brain oxidative stress induced by a diet high in saturated acid interrelated with decreased BDNF protein levels [73]. Hellweg et al. [24] proposed that early after initiation of an acute or chronic illness neurotrophins are reduced, followed by a compensatory up-regulation with a second decrease in the end-stages. Short periods of low-intensity physical activity such as those carried out by Vaynman et al. [69] showed that 3 days of free access to voluntary wheel running increased BDNF protein and mRNA levels in the hippocampus of adult rats. Twelve days of 20 m/min treadmill exercise during 30 min/day increased BDNF and TrκB protein levels in the contralateral hemisphere of adult rats [31]. Ding et al. [16] also showed increased BDNF levels in cortical neurons and striatal glia after 3 weeks of treadmill running at a speed of 15 m/min for 30 min/day followed by induction of stroke in adult rats.

It is well known that different forms of exercise result in different levels of tissues stress [4]. Treadmill running is usually chosen over swimming because swimming causes other forms of stress and aerobic responses are highly variable [38]. Jolitha et al. [28], Radák et al. [52] and Ogonovszky et al. [49] utilized a swimming model of exercise that imposes less mechanical stress due to water pressure, recruitment of different muscles and reduced effects of gravity, according to Jolitha et al. [28]. Suzuki et al. [64] used the running wheel, an intermittent physical activity, voluntary and free access model of exercise [11, 45] with low-intensity levels of running activity [34, 64]. A substantial evolutionary increase in daily movement distances can be achieved by increasing running speed in the running wheel, without remarkable increases in total energy expenditure [32]. The treadmill forces the animal to run according to the exercise demands: time, duration and intensity [4]. Somani et al. [62] imposed 7.5 weeks of moderate intensity treadmill running to rats with daily exercise duration of 30 min.

In summary, we chose 8 weeks of high intensity treadmill running for adult mice with a daily exercise duration of 45 min. This intense physical training decreased COX activity and increased TBARS levels in the brain cortex of mice, similarly to the findings of those induced by mitochondrial dysfunction, followed by low cortical BDNF levels. But incomplete corresponding exercise designs, especially concerning exercise intensity, as well as the exercise responses of trained and untrained controls, may be responsible for the inconsistent results found in literature.

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