Available online at www.sciencedirect.com
ScienceDirect
Neuroscience Letters 426 (2007) 171–174
Neuroscience Letters
www.elsevier.com/locate/neulet

Mitochondrial IV complex and brain neurothrophic derived factor responses of mice brain cortex after downhill training

Aderbal S. Aguiar Jr.[a], Talita Tuon[a], Cléber A. Pinho[a], Luciano A. Silva[a], Ana C. Andreazza[b], Flávio Kapczinski[c], João Quevedo[d], Emílio L. Streck[e], Ricardo A. Pinho[a,*]

[a] *Laboratório de Fisiologia e Bioquímica do Exercício, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, 88806-000 Criciúma, SC, Brazil*
[b] *Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, 90035-003 Porto Alegre, Rio Grande do Sul, Brazil*
[c] *Bipolar Disorders Program, Centro de Pesquisas, Hospital das Clínicas de Porto Alegre, 90035-003 Porto Alegre, RS, Brazil*
[d] *Laboratório de Neurociências, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, 88806-000 Criciúma, SC, Brazil*
[e] *Laboratório de Fisiopatologia Experimental, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, 88806-000 Criciúma, SC, Brazil*

Received 5 February 2007; received in revised form 28 July 2007; accepted 31 August 2007

Abstract

Twenty-four adult male CF1 mice were assigned to three groups: non-runners control, level running exercise (0° incline) and downhill running exercise (16° decline). Exercise groups were given running treadmill training for 5 days/week over 8 weeks. Blood lactate analysis was performed in the first and last exercise session. Mice were sacrificed 48 h after the last exercise session and their solei (citrate synthase activity) and brain cortices (BDNF levels and cytochrome *c* oxidase activity) were surgically removed and immediately stored at −80 °C for later analyses. Training significantly increased ($P < 0.05$) citrate synthase activity when compared to untrained control. Blood lactate levels classified the exercise intensity as moderate to high. The downhill exercise training significantly reduced ($P < 0.05$) brain cortex cytochrome *c* oxidase activity when compared to untrained control and level running exercise groups. BDNF levels significantly decreased ($P < 0.05$) in both exercise groups.

Keywords: BDNF; Citrate synthase; Cytochrome *c* oxidase; Physical exercise

Learning, memory and synaptic plasticity are well influenced by physical activity, a behavior that intrinsically affects the cerebral bioenergetics metabolism [19]. When performed at low intensity, there are improvements in brain-derived neurotrophic factor (BDNF) protein and mRNA levels, mainly in the hippocampus and cerebral cortex [8]. In addition, BDNF is also involved in controlling brain energy metabolism with changes in mitochondrial activity [20]. Moderate exercise ameliorated the activities of enzymes that are critical for brain mitochondrial function such as cytochrome oxidase (COX) [13].

We verified precisely the opposite: a high-intensity level continuous and intermittent training schedule induced brain mitochondrial dysfunction and decreased BDNF levels [1]. This chronic stress behavior may negatively affect multiple neural systems and promote BDNF down-regulation [12]. Exercise that includes a large eccentric component such downhill training is characterized by muscle damage and inflammation [5]. In the brain, downhill exercise increased brain IL-1β levels in association with intensified muscle fatigue [4].

The effect of downhill exercise on neurochemical data has been fairly well studied because the actual investigations of eccentric-biased exercise include mainly measuring muscle damage [7]. Therefore, we focused our investigation on the frontal cortex – the major efferent motor structure – where extensive angiogenesis had been found after exercise [18]. Our objective was to investigate the effects of a forced 8-week

* Corresponding author at: Laboratório de Fisiologia do Exercício, PPGCS, Universidade do Extremo Sul Catarinense, 88806-000 Criciúma, SC, Brazil. Fax: +55 48 3431 2750.
E-mail address: rap@unesc.net (R.A. Pinho).

0304-3940/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2007.08.058

downhill treadmill-running regimen on BDNF protein levels and cytochrome *c* oxidase (COX) activity on the frontal brain cortices.

A total of 24 male CF1 mice (20–27 g, 6 weeks old) were used. 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 24 November 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 ($n = 8$ each group) designated: non-running controls, level runners and downhill runners.

All groups were habituated to a nine-channel motor-driven treadmill at a speed of 8 m/min for 10 min/day during 1 week. Afterwards, the downhill (16° decline) and level (0° incline) trained groups performed an incremental running program to obtain progressive levels of intensity during 8 weeks for 5 days/week, performing a total period of 40 days. Non-runner control animals were put on the switched off treadmill during the same 8 weeks of the trained groups and performed only the last session of exercise to blood lactate data.

The exercise training protocol was discontinued 48 h before sacrifice. The mice were anesthetized with CO_2 and sacrificed by cervical dislocation. The soleus muscle and the brain were immediately excised and placed on ice, while both frontal cortices were removed, weighed, stored and frozen at −80 °C until analysis.

Blood lactate level was acutely determined after the last session of exercise from 15 to 50 μl of tail capillary blood, using a commercial kit according to the manufacturer's instructions (Roche, Germany). Lactate was determined by reflectance photometry at a wavelength of 657 ηm via colorimetric lactate-oxidase mediator reaction.

Due to collaborative tissue requirements and dissection time limitations, citrate synthase (CS) analysis was performed only on the soleus [2]. 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 dithiobis(2-nitrobenzoic acid), and 3.9 mM acetyl coenzyme A. After addition of 1.0 mM oxaloacetate, absorbance at 412 ηm 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 13.6.

Right and left brain cortices were homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin). The homogenates were centrifuged at 800 × *g* for 10 min and the supernatants kept at −70 °C until used for determining enzyme activity. The maximum period between homogenate preparation and enzyme analysis was always less than 5 days.

Cytochrome *c* oxidase (COX, complex IV) was measured by decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 ηm with 580 ηm as reference wavelength ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$) [15]. 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*. The activity of COX was measured at 25 °C for 10 min.

BDNF protein was quantified using an enzyme-linked immunosorbent assay (ELISA) and standard protocols (ChemiKine™ Brain Derived Neurotrophic Factor, Sandwich ELISA, Chemicon, 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 washing 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 ρg/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 into 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 ηm 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.

Folin-phenol reagent was added to binds with present protein and colorimetric changes were read at 700 ηm on a spectrophotometer [10]. Protein concentration was estimated with bovine serum albumin (BSA) as standard.

Results were expressed as mean ± standard error of the mean (S.E.M.). Comparison between means was performed by analysis of variance (ANOVA) followed by Tukey's post hoc test. Correlations were calculated using Pearson's correlation analysis. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a compatible computer. $P < 0.05$ was considered significant.

At the end of the training, there was no significant difference in body weight among the three groups. Our results show that in the end stage of the last day of exercise blood lactate levels were 7.4 ± 0.6 mmol/l in the non-runners control group, 4.4 ± 0.3 mmol/l in the level and 4.8 ± 0.8 mmol/l in the downhill exercise group. These data indicated a significantly ($P < 0.05$) lower blood lactate content in trained animals than in untrained animals after a session of exercise meaning improvement of the oxidative metabolism in the runners. The mice of our study performed the exercise above the anaerobic threshold

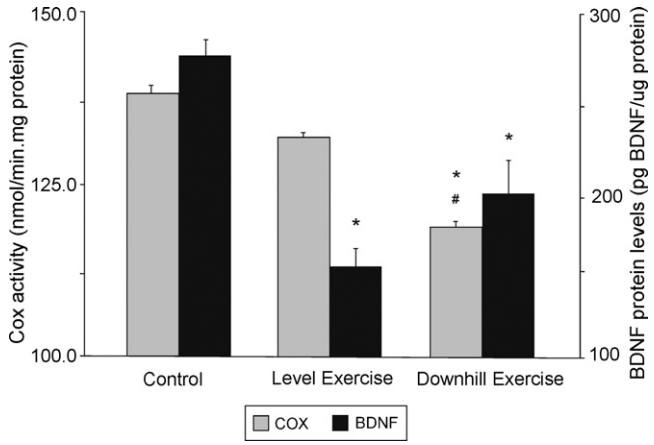

Fig. 1. (Left) COX activity of cortical brain in eccentric exercise group showed significantly low activity when compared to level trained and untrained control values. (Right) Quantitative BDNF protein levels revealed that downhill and level exercise significantly decreased BDNF protein levels when compared to sedentary control. Treadmill training and biochemistry assay are described in the text. Values are mean ± S.E.M. ($n = 8$ per group). *$P < 0.05$ vs. control, #$P < 0.05$ vs. level training, ANOVA, Tukey's post hoc.

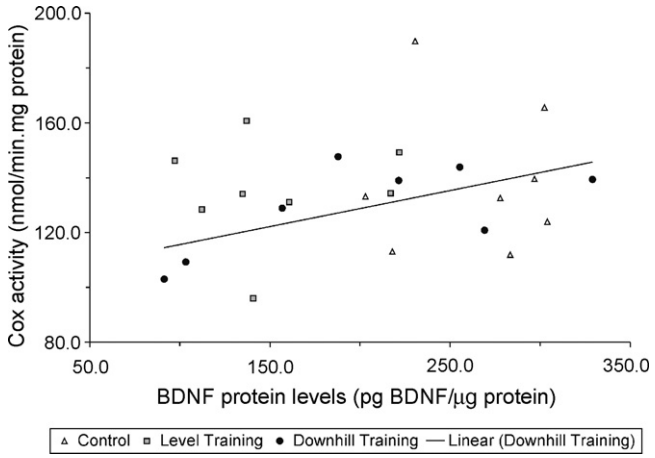

Fig. 2. Weak positive correlation ($P < 0.05$) between COX activity and BDNF protein levels in frontal cortex after 8 weeks of downhill training. Treadmill training and biochemistry assay are described in the text. (△) control; (◯) level training; (●) downhill training. Pearson correlation ($n = 8$ per group).

interval of approximately 4.2 mmol/l for rats [11] meaning high-intensity physical training.

The soleus muscle CS activity in the level (0.578 ± 0.049 U CS/mg protein) and in the downhill (0.601 ± 0.085 U CS/mg protein) exercise groups was significantly ($P < 0.05$) higher than in the non-runners control group (0.316 ± 0.031 U CS/mg protein). These results indicate that the treadmill-training program used was sufficient to increase the oxidative metabolism in skeletal muscle of mice.

We investigated the partial inhibition of COX in the frontal cortex of mice and our results showed that downhill exercise (118.7 ± 4.8 nmol/min.mg protein) significantly ($P < 0.05$) decreased COX activity compared to non-runners control (139.5 ± 6.4 nmol/min mg protein) and level exercise (131.4 ± 3.8 nmol/min mg protein) (Fig. 1).

To determine the possibility that energy metabolism may modulate BDNF protein levels, we measured BDNF levels in the frontal cortex of mice. The level (152.7 ± 16.0 ρg BDNF/μg protein) and downhill (201.8 ± 29.3 ρg BDNF/μg protein) treadmill-running program significantly ($P < 0.05$) reduced frontal cortex BDNF levels when compared to untrained animals (264.5 ± 14.4 ρg BDNF/μg protein) (Fig. 1).

Therefore, in order to examine the relationship between COX activity and BDNF protein levels further, the data of animals from both exercise groups were combined and checked through Pearson's product-moment correlation analysis. There was a significantly weak positive correlation between COX activity and BDNF protein levels for the downhill training group [$r = 0.65$ ($P < 0.05$)] (Fig. 2). In addition, the untrained control [$r = 0.04$ ($P < 0.05$)] and the level trained groups [$r = 0.10$ ($P < 0.05$)] failed to correlate COX activity and BDNF protein levels (Fig. 2).

Exercise seems to activate the neural circuitry involved in learning and memory by its action on BDNF [19]. Low-moderate intensity treadmill exercise for short duration increased hippocampal [14] and cortical BDNF [9]. At moderate-intensity running, which elevated blood lactate and corticosterone levels, induction of BDNF mRNA, but not its protein, was even depressed [17] and a high-intensity treadmill exercise decreased BDNF levels in the frontal cortex of mice [1]. We found that high-intensity downhill and level training decreased BDNF concentrations in the frontal cortex of mice (Fig. 1).

The intensity and mode of running exercise induce different effects on important modulators of synaptic plasticity [14]. Treadmill running may be considered a stressor to rodents [16]. In this case, the downhill mode of exercise generally is a stressor model of exercise, associated with greater fiber damage, soreness, inflammation, fatigue, and other functional deficits [5]. The excessive repetition of the training stimulus the local inflammation can generate a systemic inflammatory response [3]. The main actors in these processes are the cytokines – polypeptides that modulate the hypothalamic-pituitary – adrenal axis function inside and outside the brain at nearly every level of activity [3]. These results are consistent with previous studies reporting a decrease in BDNF mRNA expression in the hippocampus induced by stress and glucocorticoids, as well as immobilization and exhaustive exercise [8].

BDNF is intimately connected with brain energy metabolism [20] and has been shown to impact mitochondrial activity [6]. Low-intensity running wheel increased BDNF mRNA and protein and COX-II levels in the hippocampus [20]. We found that the low BDNF level had a weak correlation (Fig. 2) with low mitochondrial IV complex activity in the frontal brain cortex (Fig. 1) in the downhill training. We had already observed this after high-intensity continuous and intermittent running exercise [1].

We suggest that in the brain mitochondria, the activities of enzymes critical for mitochondrial function (COX) decrease during high-intensity exercise of continuous or intermittent frequency and performed downhill. However, the mechanisms involved in these events have remained obscure. In summary, our results show that high-intensity downhill exercise down regulated BDNF protein and decreased brain IV mitochondrial complex in the frontal cortex of mice.

Acknowledgments

This research was supported by grants from CNPq/MCT (Brazil), CAPES/MEC (Brazil), UNESC (Brazil) and FAPESC (Brazil).

References

[1] A.S. Aguiar, T. Tuon, C.A. Pinho, L.A. Silva, A.C. Andreazza, F. Kapczinski, J. Quevedo, E.L. Streck, R.A. Pinho, Intense exercise induces mitochondrial dysfunction in mice brain, Neurochem. Res., in press.

[2] P.R. Alp, E.A. Newsholme, V.A. Zammit, Activities of citrate synthase and NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenase in muscle from vertebrates and invertebrates, Biochem. J. 154 (1976) 689–700.

[3] A. Angeli, M. Minetto, A. Dovio, P. Paccotti, The overtraining syndrome in athletes: a stress-related disorder, J. Endocrinol. Invest. 6 (2004) 603–612.

[4] M.D. Carmichael, J.M. Davis, E.A. Murphy, A.S. Brown, J.A. Carson, E.P. Mayer, A. Ghaffar, Role of brain IL-1beta on fatigue after exercise-induced muscle damage, Am. J. Physiol. Regul. Integr. Comp. Physiol. 291 (2006) 1344–1348.

[5] D. Chapman, M. Newton, P. Sacco, K. Nosaka, Greater muscle damage induced by fast versus slow velocity eccentric exercise, Int. J. Sports Med. 27 (2006) 591–598.

[6] A. El Idrissi, E. Trenker, Growth factors and taurine protect against excitotoxicity by stabilizing calcium homeostasis and energy metabolism, J. Neurosci. 19 (1999) 9459–9468.

[7] R.G. Eston, S. Finney, S. Baker, V. Baltzopoulos, Muscle tenderness and peak torque changes after downhill running following a prior bout of isokinetic eccentric exercise, J. Sports Sci. 14 (1996) 291–299.

[8] A.M. Huang, C.J. Jen, H.F. Chen, L. Yu, Y.M. Kuo, H.I. Chen, Compulsive exercise acutely upregulates rat hippocampal brain-derived neurotrophic factor, J. Neural. Transm. 7 (2006) 803–811.

[9] M. Kim, M. Bang, T. Han, Y. Ko, B. Yoon, J. Kim, L. Kang, K. Lee, M. Kim, Exercise increased BDNF and trkB in the contralateral hemisphere of the ischemic rat brain, Brain Res. 1052 (2005) 16–21.

[10] O.H. Lowry, N.G. Rosebough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[11] A. Mader, H. Heck, A theory of metabolic origin of the anaerobic threshold, Int. J. Sports Med. 7 (1986) 45–65.

[12] S. Murakami, I. Hiroki, M. Yoshihiro, K. Chiharu, S. Emiko, Chronic stress, as well as acute stress, reduces BDNF mRNA expression in the rat hippocampus but less robustly, Neurosci. Res. 53 (2005) 129–139.

[13] A. Navarro, A. Boveris, Brain mitochondrial dysfunction in aging: conditions that improve survival, neurological performance and mitochondrial function, Front. Biosci. 112 (2007) 1154–1163.

[14] M. Ploughman, S. Granter-Button, G. Chernenko, B.A. Tucker, K.M. Mearow, D. Corbett, Endurance exercise regimens induce differential effects on brain-derived neurotrophic factor, synapsin-I and insulin-like growth factor I after focal ischemia, Neuroscience (2005) 991–1001.

[15] P. Rustin, D. Chretien, T. Bourgeron, B. Gerard, A. Rotig, J.M. Saudubray, A. Munnich, Biochemical and molecular investigations in respiratory chain deficiencies, Clin. Chim. Acta 228 (1994) 35–51.

[16] M.A. Smith, S. Makino, R. Kvetnansky, R.M. Post, Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotro-phin-3 mRNAs in the hippocampus, J. Neurosci. 15 (1995) 1768–1777.

[17] H. Soya, T. Nakamura, C.C. Deocaris, A. Kimpara, M. Iimura, T. Fujikawa, H. Chang, B.S. McEwen, T. Nishijima, BDNF induction with mild exercise in the rat hippocampus, Biochem. Biophys. Res. Commun. 358 (2007) 961–967.

[18] R.A. Swain, A.B. Harris, E.C. Wiener, M.V. Dutka, H.D. Morris, B.E. Theien, S. Konda, K. Engberg, P.C. Lauterbur, W.T. Greenough, Prolonged exercise induces angiogenesis and increases cerebral blood volume in primary motor cortex of the rat, Neuroscience 117 (2003) 1037–1046.

[19] S. Vaynman, F. Gomez-Pinilla, Revenge of the "sit": how lifestyle impacts neuronal and cognitive health through molecular systems that interface energy metabolism with neuronal plasticity, J. Neurosci. Res. 84 (2006) 699–715.

[20] S. Vaynman, Z. Ying, A. Wu, F. Gomez-Pinilla, Coupling energy metabolism with a mechanism to support brain-derived neurotrophic factor-mediated synaptic plasticity, Neuroscience 4 (2006) 1221–1234.