Spinal Cord (2009) 47, 453–457

www.nature.com/sc

ORIGINAL ARTICLE

The effect of exercise and oxidant–antioxidant intervention on the levels of neurotrophins and free radicals in spinal cord of rats

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Study design: This study was designed to investigate the effects of oxidant and antioxidant treatment, as well as regular exercise, on neurotrophin levels in the spinal cord of rats.
Objectives: Reactive oxygen species (ROS) play a role in neurodegenerative diseases, but ROS at moderate levels could stimulate biochemical processes through redox-sensitive transcription.
Methods: Exercised or sedentary animals were injected subcutaneously with hydrogen peroxide (H_2O_2), *N*-tert butyl-α-phenyl nitrone (PBN) or saline for the last 2 weeks of a 10-week experimental period to challenge redox balance. Free radical (FR) concentration was evaluated in the spinal cord by electron spin resonance, protein carbonyls, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) levels and the mRNA expression of BDNF receptor and tyrosine kinase receptor B (TrKB).
Setting: Research Institute of Sport Science, Semmelweis University, Budapest, Hungary.
Results: Exercise or PBN decreased the concentration of FR, whereas the carbonyl content did not change. BDNF was significantly decreased in exercised sham and sedentary PBN-treated groups, and its content correlated with the level of FR. GDNF was significantly increased in sedentary H_2O_2-treated groups. No differences were observed in TrkB mRNA expression among groups.
Conclusions: Results suggest that regular exercise alone and PBN in sedentary animals can successfully decrease FR levels in the spinal cord. Redox alteration seems to affect the levels of GDNF and BDNF, which might have clinical consequences, as neurotrophins play an important role in cellular resistance and regeneration.
Spinal Cord (2009) **47,** 453–457; doi:10.1038/sc.2008.125; published online 21 October 2008

Keywords: reactive oxygen species; spinal cord; hormesis; antioxidants; exercise

Introduction

Frequent, intermittent exposure to low doses of oxidative stress has proven to be beneficial to the human organism.[1] Regular exercise has been shown to be one of the best precipitators of this type of intermittent reactive oxygen species (ROS) exposure, which is known to result in a wide range of adaptive responses to oxidants.[1] Evidence suggests that exercise-induced adaptation, or exposure to low levels of oxidizing agents, could decrease ROS concentration in the cerebellum,[2] as well as reactive carbonyl derivatives in rat brain.[3] This phenomenon coincides with the hormesis theory, which was developed after observing that low doses of toxins and/or radiation can exert beneficial effects on organisms.[1]

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Received 21 April 2008; revised 31 July 2008; accepted 29 August 2008; published online 21 October 2008

Neurotrophins, especially brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), are important functional regulators of neurons and cell survival. Evidence suggests that, in general, their concentration can be modified by exercise and an altered redox homeostasis.[2,4] As most of these observations are tissue dependent, and the response of the spinal cord to regular exercise is poorly understood, we decided to study the response of spinal cord neurotrophins, as the resistance to oxidative stress could be vitally important in the prevention of oxidative stress-associated spinal cord alterations. According to the hormesis theory, low levels of oxidants, such as in exercise, have a beneficial effect, while high levels of oxidants and/or antioxidant treatment would have an unfavorable effect on BDNF and GDNF. Moreover, adaptation to moderate levels of ROS treatment, which involves regular exercise, would stimulate neurotrophins, which could be an important means to cope with oxidative

challenge. It is known that ROS, but especially H_2O_2, have a concentration-dependent diverse effect on the spinal cord and at high concentrations they can induce apoptosis and serious cell damage,[5] although recent findings suggest that low levels of endogenous H_2O_2 is required for wound healing in different tissues.[6] In addition, oxidative challenge, such as ischemic preconditioning, has been shown to attenuate oxidative stress in the spinal cord.[7] Preconditioning involves the induction of GDNF, which appears to promote axonal growth of injury-primed sensory neurons in a concentration-dependent manner.[8] Therefore, it cannot be excluded that treatment with low levels of H_2O_2 or physical exercise could have beneficial effects on preconditioning of the spinal cord, against oxidative challenge.

Materials and methods

Animals

Thirty-six five-month old male Wistar rats were used in the study and were cared for according to the guiding principles for the Care and Use of Animals on the basis of the Helsinki Declaration. The study was approved by the local Animal Welfare Committee of the university. Animals were housed in standard polyethylene cages with food and water available *ad libitum*.

Six rats were randomly assigned to each of six groups: non-exercised control, injected with saline (NEC); non-exercised injected with H_2O_2 (NEH); non-exercised injected with *N*-tert-butyl-α-phenylnitrone (PBN) purchased from Sigma-Aldrich, (B7263) served as (NEP), exercised injected with saline (EC); exercised injected with H_2O_2 (EH); and exercised injected with PBN (EP). Non-exercised groups remained sedentary for the 10 weeks of the study. After a 1-week adaptation period, which consisted of 1 h of running per day, with the intensity starting at 17 m min^{-1} and reaching 27 m min^{-1} on the last day of the adaptation period, exercised groups were subjected to forced treadmill running for 9 additional weeks, 5 days per week, 1 h per day, at 27 m min^{-1} intensity. During weeks 9 and 10, all animals were injected subcutaneously, every other day, 1 h before exercise with saline (NEC and EC), or 0.5 mM H_2O_2 (NEH and EH). To establish the H_2O_2 dose, a pilot study was designed (data not shown) in which rats were injected with 1 or 0.5 mM of H_2O_2 and the later one was found to be applicable for this study based on physiological and biochemical outcomes. The remaining groups (NEP and EP) were injected in a similar manner during weeks 9 and 10, every other day and 1 h before exercise, with 13 mg per 100 g of body weight of PBN diluted in saline.[9] One day after the last training session, animals were killed by decapitation, the spinal cord was extracted and the cervical region was separated. The cervical section was chosen because this is a frequently injured part of the spinal cord, and injury is known to be associated with ROS production.[10] The sections were frozen in liquid nitrogen and stored at −80 °C until analyses.

Biochemical assays and equipment

BDNF and GDNF determination. Spinal cord samples were homogenized in eight volumes of lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (10 mg ml^{-1}), leupeptin (1 μg ml^{-1}) and 0.5 mM sodium vanadate.

The concentrations of BDNF and GDNF were determined, from the spinal cord using the E-MAX ImmunoAssay System according to the manufacturer's protocol (Promega, Madison, WI, USA). The absorbance was read at A450 (Molecular Devices ThermoMax microplate reader, with SOFTmax PRO v3.1 software, Sunnyvale, CA, USA).

Determination of reactive carbonyl derivatives. Determination of reactive carbonyl derivatives was performed by western blot as described earlier.[3] Exposure to hyperfilm (CL-XPosure no. 34089) was carried out using enhanced chemiluminescence (Pierce, no. 34080).

RNA isolation. RNA isolation was carried out by the FastRNA Pro Green Kit (6045-050 QBioGene) using the FastPrep Instrument (6001-120, QBioGene) according to the manufacturer's protocol.

Reverse transcription reaction-cDNA synthesis. Two micrograms of RNA from each sample were reverse-transcribed in a total volume of 40 μl. The reverse transcription reaction mixture, consisting of 8 μl of 5 × reverse transcription buffer, 8 μl of $MgCl_2$, 4 μl of dNTP, 1 μl of random primers, 1 μl of RNase inhibitors and 1 μl of reverse transcriptase, was incubated at 45 °C for 50 min, and heated at 90 °C for 10 min to discontinue the reaction. The solution was quick-chilled at −80 °C for 10–20 min. cDNA samples were stored at −20 °C for subsequent RT-PCR analysis.

Real-time quantitative RT-PCR. The ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used to quantify mRNA transcription for each gene. The real-time RT-PCR reaction mixture consisted of 2 μl of cDNA, 1.3 μl of primer, 12.5 μl of TaqMan universal PCR master mix and 9.2 μl of RNase-free water for a total volume of 25 μl. The amplification profile involved an initial step at 50 °C for 2 min, a second step at 95 °C for 10 min, which was followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. All reactions were set up in duplicate and repeated once.

Analyses of the real-time quantitative PCR data were performed using the comparative threshold cycle (C_t) method as suggested by Applied Biosystems (user bulletin no. 2). The sequence of probe, forward and reverse primer for tyrosine kinase receptor B (TrkB), was designed by Applied Biosystems: TrkB: (5′-CCAGGGCAGAGTCCTTCAG-3′); forward: (5′-TTCCCGCTGCCAGCAT-3′); reverse: (5′-CATCAGC TCGTACACCTC-3′). As an internal standard housekeeping gene, the expression of β-actin (AppliedBiosystems, Rn00560868_m1) was utilized.

The electron paramagnetic resonance (EPR) measurements were carried out as described by Stadler *et al*.,[11] with an

X-Band computer-controlled spectrometer, constructed by Magnetech GmbH (Berlin, Germany).

Statistical analyses

To test differences among normally distributed variables, one-way ANOVA followed by Tukey's HSD *post hoc* test were used. The nonparametric test Kruskal–Wallis ANOVA by rank was utilized to test differences among the non-normally distributed TrkB mRNA variable. Pearson's correlation was utilized to test relationships among groups. The significance level was set at $P<0.05$.

Results

The obtained biomarkers were measured when the animals were in a rested state. The levels of free radical (FR) concentration obtained from EPR measurements in cervical spinal cord of EC and NEP groups decreased by 27 and 21% ($P<0.05$), respectively, compared with control (NEC) (Figure 1). In addition, NEP group decreased to 74% of NEH group ($P<0.05$; Figure 1). A strong tendency was observed in the cervical spinal cord of EH in which FRs decreased to 81% of NEH group ($P=0.06$; Figures 1 and 2). Oxidative damage of proteins, as indicated by western blot assay of carbonylated proteins, showed no differences among the pooled samples of the groups, indicating that the accumulation of FRs was tolerated by the cells, and that the increase could still be in the physiological range of redox homeostasis (data not shown). BDNF protein level in EC and NEP groups decreased to 49 and 48% ($P<0.05$), respectively, of control (NEC) (Figure 3). Interestingly when FR concentration and BDNF protein concentration are correlated, a significant positive correlation is acquired ($r=0.50$, $P<0.05$), which suggests either an associative or a causative relationship (Figure 4). The mRNA level of BDNF receptor, TrkB, was expressed in all groups but no significant differences were observed (data not shown). The level of GDNF protein in the cervical spinal cord of the NEH group increased beyond 153% of control (NEC) and 140% of NEP ($P<0.05$), respectively, suggesting a stimulating role of H_2O_2 injection on GDNF levels (Figure 5).

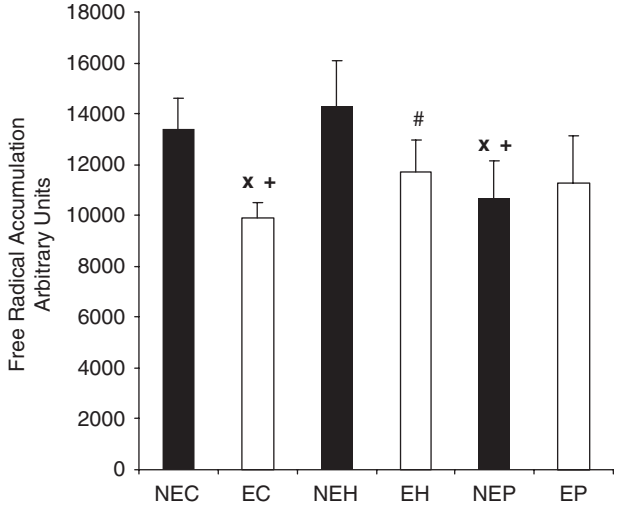

Figure 1 Free radical (FR) accumulation in the spinal cord: bars show the FR accumulation in the cervical spinal cord region, obtained by EPR measurement. Values are means ± s.d. Exercise reduced significantly the FR concentration in the EC group ($^{x}P<0.05$ vs NEC and $^{+}P<0.05$ vs NEH) and showed a strong tendency to reduce FR concentration in EH group ($^{\#}P=0.06$ vs NEH). *N*-tert butyl-α-phenyl nitrone (PBN) administration reduced significantly the FR concentration in non-exercised animals ($^{x}P<0.05$ vs NEC and $^{+}P<0.05$ NEH). Group identification: NEC, non-exercised control; EC, exercised control; NEH, non-exercised H_2O_2-injected; EH, exercised H_2O_2-injected; NEP, non-exercised PBN-injected; EP, exercised PBN-injected.

Discussion

The spinal cord is the main pathway for information transmission between brain, the peripheral nervous system and muscle. Its ability to cope with oxidative stress-related challenges could be of vital importance. Regular exercise results in systemic adaptation of the body,[12] although the oxidative stress-associated adaptation of the spinal cord is poorly investigated. The results of the present investigation

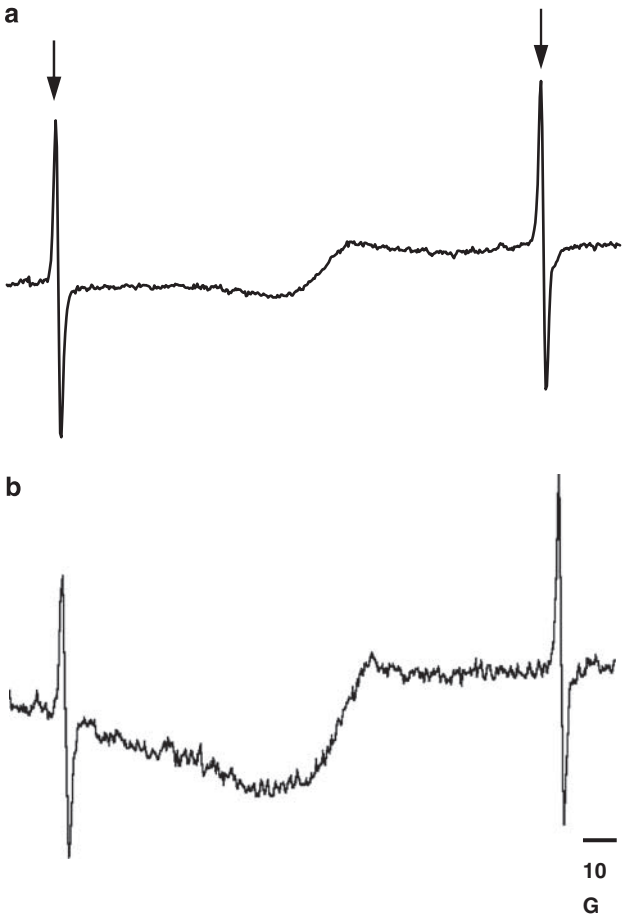

Figure 2 EPR spectra: EPR spectra taken *ex vivo* at 77 K showing steady-state native free radical concentrations in the cervical spinal cord of (**a**) exercised and (**b**) non-exercised rats treated with H_2O_2. Spectra containing two Mn/MnO signals as internal standards are indicated by arrows. The area observed between the two signals of standards was double-integrated for evaluation.

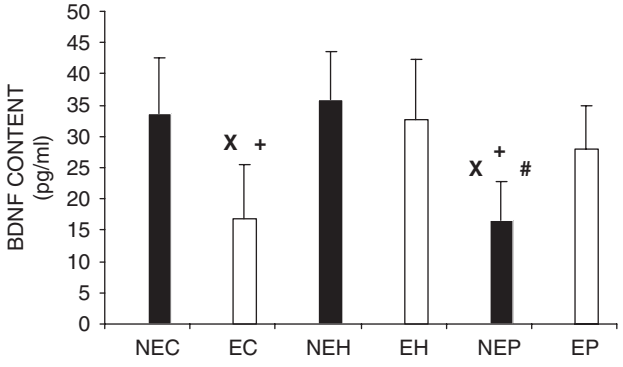

Figure 3 Brain-derived neurotrophic factor (BDNF) protein concentration in spinal cord: BDNF was determined using the E-Max ImmunoAssay System. Samples were obtained from cervical spinal cord homogenates. Values are means ± s.d. for six animals. Exercise alone significantly reduced BDNF content in the cervical spinal cord region ($^{x}P<0.05$ vs NEC and $^{+}P<0.05$ vs NEH). Similarly, PBN administration in non-exercised animals significantly decreased BDNF content as compared with non-exercised control and to both non-exercised and exercised H_2O_2-treated animals ($^{x}P<0.05$ vs NEC, $^{+}P<0.05$ vs NEH, $^{\#}P<0.05$ vs EH). Group identification: NEC, non-exercised control; EC, exercised control; NEH, non-exercised H_2O_2-injected; EH, exercised H_2O_2-injected; NEP, non-exercised PBN-injected; EP, exercised PBN-injected.

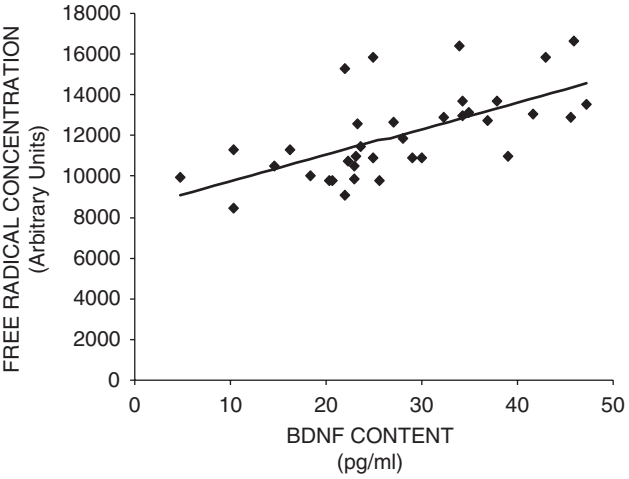

Figure 4 BDNF protein content and free radical (FR) level positive correlation: The plot shows the positive correlation between FR concentration and BDNF content in the cervical spinal cord region ($r=0.50$, $P<0.05$). Animals with higher reactive oxygen species concentration had higher BDNF content and vice versa.

revealed that chronic exercise training decreases the level of FRs in the cervical section of the spinal cord. We have previously shown that chronic swimming training decreases FR concentrations in the cerebellum.[2] This decrease in FR concentration in the spinal cord of the exercised group could be related to an upregulated antioxidant enzyme capacity as a result of chronic training[12,13] or the result of more tightly controlled ROS generating systems.

On the other hand, our hypothesis that a higher level of oxidative challenge through exercise and oxidant administration could further diminish FR concentration was not supported. Even though oxidant addition did not result in a

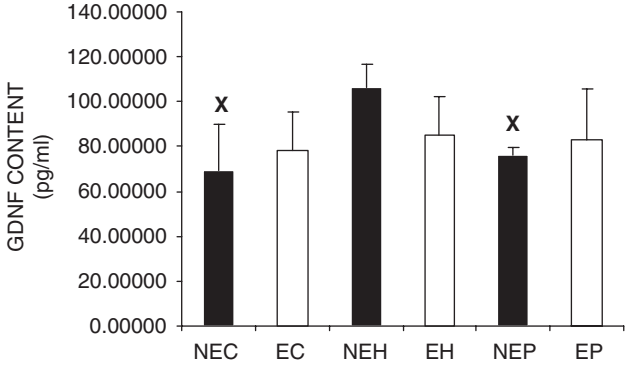

Figure 5 Glial cell line-derived neurotrophic factor (GDNF) protein content in spinal cord: GDNF content was determined using the E-Max ImmunoAssay System. Values are means ± s.d. for six animals per group. H_2O_2 increased significantly GDNF content in the cervical spinal cord region of non-exercised animals, whereas PBN in non-exercised animals maintained GDNF production at control levels ($^{x}P<0.05$ vs NEH). Group identification: NEC, non-exercised control; EC, exercised control; NEH, non-exercised H_2O_2-injected; EH, exercised H_2O_2-injected; NEP, non-exercised PBN-injected; EP, exercised PBN-injected.

further lowering of FR concentrations in the exercised groups, a strong tendency to do so was observed in EH group. The magnitude of the oxidative stress that can be tolerated by the spinal cord is vital, as spinal cord injuries easily cause severe oxidative stress resulting in apoptosis and necrosis, which can be attenuated by preconditioning.[14] Mildly, oxidant-challenged cells increase their viability through complex adaptations, that result in maintained physiological functions that induce adaptations in normal cell populations.[15] The strong tendency of lower FR concentrations suggests a preconditioning effect of exercise vs H_2O_2.[1]

N-tert butyl-α-phenyl nitrone is reported to have multiple pharmacological activities to reduce ROS, including the ability to trap alkoxyl radicals, superoxide radicals and hydroxyl radicals and to decrease inducible cytochrome (cyclooxygenase-2), as well as to inhibit nuclear factor-κB transduction.[16] As suspected, PBN intervention in the sedentary group was shown to be effective in decreasing FR concentrations as compared with the non-exercised control and non-exercised H_2O_2 groups. This finding is supported by other studies, which have reported a neuroprotective effect of PBN that was derived by successfully attenuating hydroxyl radical in rat striatum.[16]

The combined effects of exercise and PBN did not result in a decreased FR level, possibly due to an antioxidant interaction in the exercise-induced adaptation process. Although data on spinal cord are not available, it has been shown in skeletal muscle that allopurinol, a potent inhibitor of xanthine oxidase, attenuates the exercise-induced adaptation to ROS.[17]

The level of oxidative protein damage, assessed by reactive carbonyl derivatives, was not changed as a result of regular exercise and oxidant and antioxidant treatment. Toldy *et al*.[18] have similarly reported no effect of regular exercise on brain reactive carbonyl derivatives.

Evidence suggests that BDNF and its cognate receptor are upregulated by physical activity in both brain and spinal cord[4] and linked to ameliorative function. We have also observed improved spatial memory with regular exercise (data will be published elsewhere), but it appears that it is not associated with a higher concentration of BDNF protein or mRNA levels of TrkB in the spinal cord, emphasizing the importance of the local environment. In this study, BDNF decreased significantly as a result of exercise (EC vs NEC). Similar findings have been reported recently by Engesser-Cesar *et al*.,[19] who noted a decrease in the levels of BDNF in the thoracic region of spinal cord of mice, with exercise. It appears that exercise of low intensity increases, but high intensity decreases, the BDNF content in the frontal cortex of the brain.[20] These findings suggest that exercise of moderate intensity could be more beneficial to brain in terms of BDNF levels. It cannot be excluded that a similar phenomenon could occur in the spinal cord. The sensitivity of BDNF on ROS could be due to the fact that the cyclic adenosine monophosphate-responsive element-binding protein, which is one of the transcription factors of BDNF, is redox sensitive.[21] Indeed, groups with the lowest BDNF levels have been shown to have the lowest ROS concentrations.

Glial cell line-derived neurotrophic factor protein was produced to a greater extent in the sedentary group injected with H_2O_2 than in the other sedentary groups. GDNF has been shown previously to rescue neuro-cells exposed to H_2O_2 (Onyango *et al*.[22]) and FR-mediated cell damage. The neuro-protective effect of GDNF has been linked to increased antioxidant enzyme activities.[3] Exercise, on the other hand, induced no alteration in spinal cord GDNF content. Widenfalk *et al*.[23] similarly reported insensitivity of this neurotrophin to exercise stimulus in the hippocampus.

The results of this study suggest that regular exercise and PBN administration in sedentary animals are effective means of decreasing FR concentrations in the cervical region of the spinal cord. This is of primary clinical importance, as exercise can provide an effective preconditioning stimulus over oxidative stress-associated diseases or oxidative stress associated with traumatic spinal cord injury. PBN may, as well, serve as a direct means of attenuating ROS.

Apparently, at least in this investigation, the levels of ROS play a role in determining BDNF protein content in the cervical spinal cord. GDNF release seems to be H_2O_2 sensitive and its production may be associated with survival-promoting effects. The data obtained in this study support the hormesis theory.

Acknowledgements

This work was supported by grants OTKA 42629 and TéT JAP 13/02 to ZR.

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