The effects of exercise, oxidants and antioxidants on neurotrophins and oxidative damage on spinal cord of rats

Outlined Booklet of the PhD Thesis

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I. INTRODUCTION

Frequent, intermittent exposure to low doses of oxidative stress has been proven to be beneficial. Regular exercise could be one of the best examples for this kind of intermittent ROS exposure, which is known to result in a wide range of adaptive responses to oxidants. Evidence suggests that exercise-induced adaptation, or exposure to low levels of oxidizing agents, could decrease ROS concentration in the cerebellum, as well as reactive carbonyl derivatives in rat brain and rat myocardium. This phenomenon coincides with the hormesis theory, (dose response phenomenon characterized by a low dose of stimulation, high dose of inhibition, resulting in either a J-shaped or an inverted U-shaped dose response, which is a non-monotonic response), which was developed after observing that low doses of toxins and/or radiation can exert beneficial effects on lower organisms. Increasing evidence suggests that hormesis may operate in higher animals, as well.

Neurotrophins especially, brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), are important functional regulators of neurons and cell survival and evidence suggests that, in general, their concentration can be modified by exercise and an altered redox homeostasis. In addition, Extracellular signal kinase (ERK) is a signalling molecule whose phosphorelation has been associated to neurotrophins stimulation and its activation has been linked to exercise and altered redox state. Since, most of these observations are tissue dependent, and according to our knowledge, the response of spinal cord to regular exercise, oxidative challenge is poorly understood, we aimed to study the response of neurotrophins at spinal cord to asses the response of these important components. Since the resistance against oxidative stress, could be vitally important at the prevention of oxidative stress-associated spinal cord alterations. We suggested that low level of oxidant, like exercise has a beneficial, while high level of oxidant and/or antioxidant treatment would have unfavourable effect on

BDNF and GDNF according to the hormesis theory. Moreover, adaptation to moderate level of ROS treatments, which involves regular exercise, would stimulate neurotrophins, which could be an important mean to cope with oxidative challenge.

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

II. OBJECTIVES

Most of these observations described briefly above are tissue dependent, and according to our knowledge, the response of spinal cord to regular exercise, oxidative challenge is poorly understood:

- Therefore, we aimed to study the response of neurotrophins and free radical concentration at spinal cord to asses the response of these important components.

Since the resistance against oxidative stress, could be vitally important at the prevention of oxidative stress-associated spinal cord alterations:

- We suggested that low level of oxidant, like exercise has a beneficial, while high level of oxidant and/or antioxidant treatment would have unfavourable effect on BDNF and GDNF according to the hormesis theory. Moreover, adaptation to moderate level of ROS treatments, which involves regular exercise,

would stimulate neurotrophins, which could be an important mean to cope with oxidative challenge. Therefore, it cannot be excluded that treatment with low level of H2O2 or physical exercise could have beneficial effects on preconditioning of the spinal cord against oxidative challenge.

III. METHODS

III. 1. Subjects

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 based upon the Helsinki Declaration. The study was approved by the local Animal Welfare Committee of the university.

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 ten weeks of the study. After a one week adaptation period, which consisted of one hour of running/day with the intensity starting at 17m/min and reaching 27m/min on the last day of the adaptation period, exercised

groups were subjected to forced treadmill running for nine additional weeks, five days/week, one hour/day at 27 m/min intensity.

During the 9^{th} and 10^{th} weeks all animals were injected subcutaneously, every other day, one hour prior to exercise with saline (NEC & EC), or 0.5 Mm H_2O_2 (NEH & EH). The remaining groups (NEP & EP) were injected in a similar manner during the 9^{th} and 10^{th} week, every other day and one hour prior to exercise, with 13mg/100gr of body weight of PBN diluted in saline. One day after the last training session, animals were sacrificed by decapitation, the spinal cord was extracted and the cervical region separated. The sections were frozen in liquid nitrogen and stored at -80 ^{o} C until analyses.

III. 2. Biochemical Analyses

BDNF and GDNF: 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). The absorbance was read at A450 (Molecular Devices ThermoMax microplate reader, with SOFTmax PRO v3.1 software, Sunnyvale, CA).

Determination of reactive carbonyl derivatives (RCD): was performed by Western blot as described earlier by Radak et al.[17, 18]. Exposure to hyper-film (CL-XPosure^{TM} #34089) was carried out using enhanced chemiluminescence (PIERCE #34080). Exposure time was set at 1 min.

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 5X reverse transcription buffer, 8µl of $MgCL_2$, 4µl of dNTP, 1µl of Random Primers, 1µl of RNase-inhibitors, and 1µl of Reverse Transcriptase, was incubated at 45C° for 50 minutes,

and heated at 90C˚ for 10 minutes in order to discontinue the reaction. The solution was quick-chilled at -80C˚ for 10-20 minutes. cDNA samples were stored at -20C˚ for subsequent RT-PCR analysis.

Real time quantitative RT-PCR: The ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used to quantify mRNA transcription for each gene. The real time RT-PCR reaction mixture consisted of 2µl of cDNA, 1.3µl primer, 12.5 µl of TaqMan universal PCR master mix, and 9.2µl RNase-free water for a total volume of 25µl. The amplification profile involved an initial step at 50 ˚C for two minutes, a second step at 95 ˚C for ten minutes, which was followed by 40 cycles at 95 ˚C for 15 seconds and at 60 ˚C for one minute. 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 [Ct] method as suggested by Applied Biosystems (User Bulletin #2). The sequence

of probe, forward and reverse primer for TrkB was designed by Applied Biosystems: TrkB: (5'- CCAGGGCAGAGTCCTTCAG-3'); forward: (5'- TTCCCGCTGCCAGCAT-3'); reverse: (5'- CATCAGCTCGTACACCTC-3'). As an internal standard housekeeping gene the expression of b-actin (AppliedBiosystems, Rn00560868_m1) was utilized.

The EPR measurements: were carried out as described by Stadler et al.[19]. The measurements were carried out with an X-Band computer-controlled spectrometer, constructed by Magnetech GmbH (Berlin, Germany).

Determination of extracellular signal kinase (ERK1/2): homogenates of spinal cord samples were set equal among groups utilizing the Bio-Rad protein assay for cytocolic proteins according to the manufacturer's protocol. Groups were then pooled and subsequent protein determinations utilizing Bio-Rad protein assay (3 protein assays were performed and the mean value was calculated) were once more performed in order to set equal protein concentration

among the pooled groups. Once protein content set equal an end volume of 20ul was removed from each pooled sample and 2ul of sample buffer containing 1% bromphenol blue was added. The samples were then vortex and incubated at 95^{o}C for 5 min. Followed, samples were centrifuged for 5 min on 16 000 g at 22^{o}C and loaded to an 8% gel along with 20ul of molecular weight marker indicator (BenchMark^{TM}, Pre-Stained Protein Ladder). Electrophoresis was then carried out and subsequent separation of proteins was performed. Once electrophoresis was over the protein transferred to a PVDF membrane. Then the membrane was incubated for 1 h in blocking buffer containing TBST + 5% skimmed milk and then washed 3 times by TBST. Membrane was then treated with primary phosphor-ERK1/2 antibody, mouse monoclonal (Cell Signaling, #9106S) and incubated overnight at 4oC. Primary antibody concentration was 1:500. The next day membrane was washed 3 times for 10 min in TBST and incubated for 1 h in mouse secondary-

HRP antibody (1:5000). The membrane was once again washed 3 times for 10 min in TBST.

Exposure to hyper-film (CL-XPosure^{TM} #34089) was carried out using enhanced chemiluminescence (PIERCE #34080). Exposure time was set to 20 min. For the detection of unphosphorelated-ERK1/2 the same membrane was used. First the membrane was treated with 50 ml stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS and 100 mM b-mercapto ethanol added only before use) at 40Oc shacking for 30 min. Then the membrane was washed 3 times with TBST for 10 min or until the smell of b-mercapto ethanol wash away. After this step the immunostaining procedure was as described for the detection of phosphor-ERK. The primary antibody used was a rabbit polyclonal (Cell Signaling, #9102) with concentration 1:500. The secondary antibody was an anti-rabbit-HRP (1:5000). Exposure to hyper-film (CL-XPosure^{TM} #34089) was carried out using enhanced chemiluminescence (PIERCE #34080). Exposure time was set to 30 seconds.

III. 3. Statistical analyses

Statistical tests were performed using STATISTICA 7, software. Initially, all dependent variables underwent a Normality test (Shapiro-Wilk's W test). Non-normally distributed variables include: free radical concentration and TrkB mRNA. To test differences among normally distributed variables we use one way ANOVA followed by Tukey HSD post-hoc test. Non-parametric test Kruskal-Wallis ANOVA by ranks was utilized to test differences among the non-normally distributed followed by Mann-Whitney U test when significant. Non-parametric Kendall Tau correlation test (non-parametric alternative to parametric Pearson's correlation) was utilized to test relationships among groups since the variables of interest were not normally distributed. The significance level was set at $p<0.05$.

IV. RESULTS

The obtained biomarkers were measured when the animals were in a rested state. The level of free radical concentration obtained from EPR measurements in cervical spinal cord of EC and NEP groups decreased by 27% and 21% ($p<0.05$) compared to control (NEC) respectively. In addition, NEP group decreased to 74% of NEH group ($p<0.05$) (Fig.1). A strong tendency was observed in the cervical spinal cord of EH in which free radicals decreased to 81% of NEH group ($p=0.06$).

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 free radicals was tolerated by the cells, and that the increase could be still in the physiological range of redox homeostasis.

No expression of BDNF mRNA could be detected in any of the groups.

BDNF protein level in EC and NEP groups decreased to 49% and 48% ($p<0.05$) of control (NEC). Interestingly when free radical concentration and BDNF protein concentration are plotted together in a correlation plot, a significant positive correlation is acquired ($r = 0.50$, $p<0.05$), suggesting either an associative or a causative relationship.

The mRNA level of BDNF receptor, TrkB, was expressed in all groups but no significant differences were observed.

The level of GDNF protein in the cervical spinal cord of the NEH group increased beyond 153% of control (NEC) and 140% of NEP group ($p<0.05$) respectively suggesting a stimulating role of H_2O_2 injection on GDNF level.

Immunoblots demonstrated that ERK1 was mainly activated in groups EH and EC suggesting a possible exercise-associated ERK1 phosphorelation. On the other hand, ERK2 remained unaffected by the treatments utilized.

V. CONCLUSIONS

Following our results and the limitations of our study we conclude that:

1. Chronic exercise decreased the free radical concentration in the cervical spinal cord of exercised control rats. PBN was effective in decreasing free radical concentration only in non-exercised animals while PBN in exercised animals seems to attenuate the ROS-induced adaptive responses to exercise. Thus, chronic exercise alone, and PBN administration in non-exercised animals are effective means in decreasing free radicals in cervical spinal cord of young male Wistar rats.
2. Interestingly, ROS concentration and BDNF protein concentration are significantly and positively correlated. That is, animals with higher ROS concentration had higher BDNF protein concentration and vice versa. Apparently, at least in the present study the level of ROS concentration in the

cervical spinal cord of young male Wistar rats seems to determine the level of BDNF protein concentration.

3. No differences were observed in oxidative protein damage among group's pooled samples indicating that the accumulation of free radicals were tolerated by the spinal cord cells and that the increase was still in the physiological range of the redox homeostasis. This suggests that the level of ROS is not the only determinant for oxidative damage to occur.
4. According to the findings of the present study, activation of ERK is not the only pathway responsible for BDNF protein induction under the present experimental conditions.
5. In the present investigation H_2O_2 seems to be the major determinant of GDNF protein concentration in cervical spinal cord. Exercise showed to have no effect in inducing GDNF release.

6. Lack of BDNF mRNA expression in cervical region suggests that the detected BDNF protein in the area was transported there either retrogradely form lumbar region or BDNF was possibly expressed elsewhere in CNS and transported there through the blood.

OWN PUBLICATION LIST

1. Radak Z., Toldy A., Szabo Z., **Siamilis S**., Nyakas C., Silye G., Jakus J., Goto S. (2006). Neurochemistry International, volume 49, page 387- 392.
2. **Siamilis S**., Jakus J., Nyakas C., Costa A., Mihalik B., Falus A., Radak Z. (2008). Spinal Cord.