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Basal plasma levels and reactivity of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) to standardized acute exercise in multiple sclerosis and controls

**Basal serum levels and reactivity of nerve growth factor (NGF)
and brain-derived neurotrophic factor (BDNF) to standardized
acute exercise in multiple sclerosis and controls.**

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Abstract

Neurotrophins like brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are thought to play an important role in neuronal repair and plasticity. Recent experimental evidence suggests neuroprotective effects of these proteins in multiple sclerosis (MS). We investigated the response of serum NGF and BDNF concentrations to a standardized acute exercise in MS patients and controls. Basal NGF levels were significantly elevated in MS. Thirty minutes of moderate exercise significantly induced BDNF production in MS patients and controls, but no differential effects were seen. We conclude that moderate exercise can be used to induce neurotrophin production in humans. This may mediate beneficial effects of physical exercise in MS reported recently.

Key words: BDNF - NGF - Multiple Sclerosis - Exercise - Neuroprotection

Introduction

Neurotrophic factors are a family of proteins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and others, that are thought to play a role in preventing neural death and in favoring the recovery process, neural regeneration, and remyelination (cf. Ebadi et al., 1997). Multiple sclerosis (MS) is an inflammatory and degenerative disease, characterized by different patterns of demyelination and axonal loss (Lucchinetti et al., 2000). Many findings indicate an immune-mediated pathogenesis, but the triggers of the initial attack have not yet been elucidated (Keegan & Noseworthy, 2002). While myelin basic protein (MBP) specific T cells are generally thought to play a major role in disease-onset and progression, recent reports have shown – seemingly paradoxical - neuroprotective (side)-effects of these cells in MS (Moalem et al., 1999; Schwartz et al., 1999). The underlying mechanisms of this phenomenon are still unknown. However, Hohlfeld et al. (2000) have suggested that the ability of immune cells to produce neurotrophic factors (Torcia et al., 1996; Besser & Wank, 1999; Kerschensteiner et al., 1999, Skaper et al., 2001) may promote neuroprotection. There is furthermore evidence that intracerebroventricular administration of NGF can delay onset of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Villoslada et al., 2000). Linker et al. (2002) demonstrated that EAE was more severe in ciliary neurotrophic factor (CNTF) knock-out mice and this effect could be counterbalanced by treatment with antiserum against tumor necrosis factor (TNF), thus suggesting that neurotrophic factors exert their protective effects via immunomodulation.

Increased levels of neurotrophic factors have been reported in lesions and cerebrospinal fluid of MS patients (Bracci-Laudiero et al., 1992; Kerschensteiner et al., 1999; Valdo et al., 2002; Stadelmann et al., 2002). Sarichelli et al. (2002) showed that BDNF production by peripheral blood mononuclear cells (PBMCs) was significantly increased during relapse and in the recovery phase compared with values detected in the stable phase of the disease. They also

found decreased levels in secondary progressive (SPMS) patients, and postulated that reduced BDNF production by peripheral cells may contribute to progression and axonal loss. There might be a direct association between neural degeneration or recovery in the central nervous system and peripheral production of BDNF and NGF. Despite their size, these proteins can cross the blood-brain barrier (BBB), at least under experimental conditions (Kastin et al., 1999; Pan et al., 1998; Poduslo et al., 1996).

There is accumulating evidence that physical exercise can promote brain health and function. Animal research suggests that exercise increases neuronal survival and resistance to brain insult, promotes brain vascularization, stimulates neurogenesis, enhances learning, and contributes to maintenance of cognitive function. A series of studies has demonstrated that voluntary wheel-running for several days enhances BDNF production in the hippocampus and other CNS areas of rats, suggesting a mediating role of exercise-induced neurotrophin release (cf. Cotman & Berchtold, 2002 for review).

Acute stress transiently enhances serum levels of NGF in animals and humans (cf. Alleva & Santucci, 2001). Preliminary evidence suggests that chronic stress of caregiving is associated with increased basal NGF levels in elderly women (Hadjiconstantinou et al., 2001). It is, however, not known how serum levels of neurotrophic factors are affected by acute physical stress.

In this study, we investigated the acute effects of a standardized physical exercise stressor (bicycle ergometry) on BDNF and NGF serum concentrations in healthy controls and patients with MS. Based on the literature, we expected to find increased baseline levels of neurotrophic factors in MS patients compared to controls. We hypothesized that moderate acute exercise would increase BDNF and NGF levels. Furthermore, potential alterations of exercise effects on neurotrophic factors in MS were tested.

Methods

Subjects

Effects of acute exercise on neurotrophic factor concentrations were investigated using a quasi-experimental control group design. Forty-eight patients (16 female, 9 male; mean age 39.2 ± 1.8 years) with definite MS according to Poser criteria (Poser, 1983) and 20 age-matched healthy volunteers (14 female, 6 male; mean age 40.4 ± 2.0 years) were recruited for this study. Eighteen patients had to be excluded because they were not able to complete the exercise paradigm as described below. Twenty-five patients had complete data in the requisite physiological data (lactate, cardiovascular, BDNF, NGF) and were thus included in the analyses. Before entering the study, participants underwent standardized clinical scoring (see below).

Disease course was classified according to Lublin et al. (1996). Patients were included if they had an Expanded Disability Status Scale (EDSS, Kurtzke, 1983) <5.0 , and no steroid therapy or immunosuppressive therapy with mitoxantrone within the past 4 weeks. They were not eligible for participation if they had received interferon / copaxone or intravenous immunoglobulins (IVIG) the day prior to the session of the 30 minutes endurance test (see below). Patients were also excluded if their diagnosis was not clearly established, they were suffering from an acute relapse or severe cognitive deficiencies, or had signs of any psychiatric disease. They were further not enrolled in the study when they had experienced worsening of their symptoms under physical exercise. The protocol was approved by the ethics committee, and written informed consent was obtained prior to entering the study.

Neurological scores

EDSS scores were rated by experienced neurologists in our out-patient clinic. Furthermore, objective clinical tests were performed: Time to walk eight meters (T8), and the Symbol Digit Modalities Test (SDMT). The SDMT has been suggested as an easy to handle, sensitive

cognitive screening test in MS measuring information processing, which is a neuropsychological key deficit in this disease (Rudick et al., 1997). Results are given as the standard deviations (SD) from an age and education matched healthy control group. A value below -2.5 SD is regarded as a significant cognitive impairment. Disease impact was furthermore classified according to the Cambridge Multiple Sclerosis Basic Score (CAMBS; Mumford and Compston, 1983) for the dimensions disability (CAMBS-D; graded 0-5 = fully independent to totally dependent), relapse (CAMBS-R; graded 0-5 = stable – relapse which requires hospitalisation), progression (CAMBS-P; graded 0-5 = stable – marked malignant progression) and handicap (CAMBS-H; grade 0-5 = 1 = no effect on role in life to 5 = incapable of any useful role).

Acute physical exercise stress paradigm

The physical stress paradigm consisted of two sessions in order to control for potential interindividual differences in fitness levels, especially between the patient and the control group. In session 1, the individual fitness level was examined in a standardized “step-by-step” bicycle ergometry test. The maximal O₂ intake measured during this session was then used to determine the intensity level in the 30 minutes endurance test, which was considered a stress test standardized to the individual level of fitness.

Visit 1: Subjects presented to the ergometry lab of the Institute for Sports and Exercise Medicine in the afternoon (3:00 to 5:00 p.m.). After 5 minutes of rest (sitting on the ergometer), they were asked to start cycling, beginning at 25 watts. Every 2 minutes, the resistance was increased by 25 watts. Subjects were asked to keep cycling until they were exhausted. VO₂ max was recorded continuously. We further recorded lactate concentrations and heart rate for every step.

Visit 2: One week after the initial step-by-step test, the participants returned to the lab and performed an endurance test. This time, they were asked to cycle for 30 minutes at 60% of

VO₂ max based on the previous step-by-step test. Lactate concentrations were recorded before and after the 30 minutes of exercise. Maximal heart rate was recorded and compared to basal heart rate assessed under resting conditions. Blood samples for determination of BDNF and NGF were drawn at baseline, directly after the 30 minutes test and 30 minutes after the test (60 minutes post baseline). Blood was collected in 5 ml heparinized tubes for analysis of serum BDNF and NGF levels. Serum was frozen and stored at -80 °C until assayed.

Biochemical assays

Chemicals of analytical grade were purchased from Merck (Darmstadt, Germany). Each 140 µl of plasma was diluted with sample buffer and endogenous NGF was quantified by a highly sensitive and specific two-site enzyme immunoassay as described in detail previously (Hellweg et al. 1989). Briefly, black 96-well flat bottom immunoplates (Dynatech Laboratories, Inc., Virginia) were coated with 50µl per well of 1.0 µg/ml monoclonal anti-mouse-β-NGF antibody 27/21 (Chemicon, Hofheim, Germany). Parallel wells were coated with mouse IgG₁ (MOPC 21, Sigma Chemie, Deisenhofen, Germany) for evaluation of non-specific signals. After 2h at room temperature (20°C) the plates were washed three times with 200µl per well of washing buffer. Unlike the previous protocol (Hellweg et al. 1989), this assay did not contain gelatin and the samples were incubated in the coated wells (50µl each) overnight at 4°C (Hellweg et al. 2001). After an additional three washes the immobilized antigen was incubated with 0.75 mU per well of monoclonal antibody 27/21 conjugated with β-D-galactosidase (Chemicon, Hofheim, Germany) for 1.5h at room temperature. The plates were again washed with washing buffer for 1 h, and then finally washed twice with substrate buffer. To start the enzyme reaction, 50µl substrate buffer containing 0.2 mM 4-methylumbelliferyl-β-D-galactoside (Sigma Chemie, Deisenhofen, Germany) was added to each microwell. After overnight incubation at 4°C, the enzyme reaction was stopped with 200 µl per well of stopping buffer. The fluorescent reaction product, 4-methylumbelliferone, was

measured in a microplate fluorometer (Labsystems Flouroskan II, Germany). NGF concentrations were determined from the regression line for the NGF standard (ranging from 0.25 to 1000 pg/ml purified mouse 2.5 S NGF) incubated under similar conditions in each assay. The measured levels for NGF were corrected for mean recoveries of added mouse NGF (125 pg/ml), which were determined in each assay. Determinations of recovery, specific and unspecific NGF binding, each involving quadruplicate fluorescence determinations, were run for each serum sample. Similarly, BDNF serum concentrations were quantified by a modified ELISA (Promega Co., Madison, WI, USA). A detailed description can be found in Hellweg et al. (in press). The assay has a detection limit of 1 pg/ml and no cross-reactivity with other neurotrophins.

Statistical analysis

Group differences between MS patients and healthy controls were investigated using one-way analysis of variance (ANOVA) for age, height, weight, and body mass index (BMI). Sex distribution differences were tested using Pearson's chi-square statistics.

The effectiveness of the physical stress paradigm was tested examining the lactate and heart rate response to the 30-minute endurance test in MS patients and controls. Pre vs. post levels in these two parameters were used as the within-subject factor in a repeated measures ANOVA. A significant main effect for Time in both Lactate and heart rate was considered to support the effectiveness of the stressor. Based on the literature (Beneke, 1995), a mean increase of >1 mmol/l lactate was taken as an indicator of having surpassed the anaerobic threshold. This was examined using a one-sample t-test (test value 1, i.e. it was tested whether the mean change was significantly larger than 1 mmol/l).

NGF and BDNF concentrations showed considerable interindividual variation. Thus, outliers with levels more than three standard deviations from the mean were excluded. To examine whether MS patients and healthy controls differed with respect to their basal levels of

neurotrophic factors NGF and BDNF, we used one-way ANOVAs with group as the between-subjects factor. Differential effects of acute physical exercise in MS patients and healthy controls were tested by employing repeated measures ANOVAs. Group (MS vs. controls) was entered as the between-subjects factor with Time entered as the within-subjects factor.

Results are given in means \pm standard error of mean (SEM). Analyses were computed using statistical software (SPSS 11.0, English version). A p value of $p < .05$ was considered significant while $p < .10$ was accepted in order to detect trends.

Results

Demographic variables

There were no statistical differences in age (MS: 39.2 ± 1.8 years; Controls: 40.4 ± 2.0 years; ANOVA $F(1, 43)=0.2$; $p=.67$), weight (MS: 69.5 ± 2.3 kg; Controls: 70.0 ± 2.3 kg; ANOVA $F(1, 43)=0.1$; $p=.88$), and height (MS: 172.7 ± 1.6 cm; Controls: 171.6 ± 1.7 cm; ANOVA $F(1, 43)=0.2$; $p=.65$). There were further no significant differences in the distribution of sex (MS: 16 females, 9 males; Controls: 14 females, 6 males; chi-square= 0.18 ; $p=.67$) between the groups. Thus, none of these variables was statistically controlled for in the analyses.

Within the patient sample, 20 subjects had been diagnosed with relapsing-remitting disease (RRMS), 4 with secondary progressive disease course (SPMS) and 1 with primary progressive MS (PPMS). Mean EDSS score was 2.3 ± 0.2 (median 2.5) and the disease had been diagnosed 10.5 ± 1.5 years ago on average. Mean scores on the CAMBS were 1.7 ± 0.1 (CAMBS-D), 1.1 ± 0.1 (CAMBS-R), 1.3 ± 0.1 (CAMBS-P) and 2.2 ± 0.2 (CAMBS-H). The patients' average on the T8 test was 4.5 ± 0.3 seconds. Mean SDMT scores were -0.3 ± 0.3 (given in standard deviations below the mean).

Effectiveness of the exercise paradigm

MS patients as well as controls showed strong increases in heart rate and lactate under the 30 minute endurance test. There was a highly significant main effect for Time in heart rate ($F(1, 40)=1018.9$; $p<.0001$) as well as lactate response ($F(1, 40)=70.7$; $p<.0001$).

Mean lactate delta scores (post – pre endurance test) were 2.4 ± 0.3 in our sample. One-sample t-test revealed that this was significantly above the cut-off of 1 mmol/l ($t(41)=4.8$; $p<.0001$).

Basal levels of NGF and BDNF

MS patients exhibited markedly higher baseline concentrations of serum NGF (MS: $44.0 + 14.3$ pg/ml; Controls: $10.8 + 1.0$ pg/ml). The differences was statistically significant ($F(1, 43)=4.3$; $p=.04$). However, no significant baseline differences were seen in BDNF levels (MS: 4435.1 ± 533.4 pg/ml; Controls: 4717.2 ± 491.8 pg/ml; $F(1, 43)=0.1$; $p=.71$).

Differential effects of acute exercise on serum NGF and BDNF

Figure 1 shows the response patterns and recovery of neurotrophic factors NGF and BDNF to 30 minutes of an acute and standardized exercise at 60% VO_2 max. Because of the significant baseline differences reported above, the following analyses were conducted using change from baseline as the between-subjects factor. It should be noted that the same p values for interaction terms were found when using raw scores.

For BDNF levels, there was a significant main effect for Time ($F(2, 42)=3.9$; $p=.03$). No significant effects were found for Group ($F(1, 43)=0.1$; $p=.85$) or Time x Group interaction ($F(2, 42)=0.2$; $p=.83$).

Statistical evaluation of NGF responses to the standardized physical exercise paradigm showed a trend towards main effect for Time ($F(2, 42)=2.6$; $p=.09$). The main effect for Group was not significant ($F(1, 43)=0.8$; $p=.37$) and the Time x Group interaction also failed to reach significant levels ($F(2, 42)=2.1$; $p=.14$).

Discussion

Consistent with the literature showing elevated NGF in MS lesions and CSF (Valdo et al., 2002; Stadelmann et al., 2002; Kerschensteiner et al., 1999; Bracci-Laudiero et al., 1992) and expanding these findings, we found baseline serum concentrations of NGF increased in MS patients compared to controls. In contrast to a recent study by Sarchielli et al. (2002), we did not detect differences in basal BDNF. This might be due to the fact that we measured unstimulated concentrations while Sarchielli's group employed in vitro stimulation. However, it should be noted that also in their study, unstimulated BDNF levels of MS patients in a stable clinical phase did not differ from healthy controls, while concentrations in patients with an ongoing relapse were significantly elevated. This is in line with our findings, since acute relapse was an exclusion criterion in our study.

To the best of our knowledge, this is the first study providing evidence that acute moderate exercise can induce increased BDNF serum concentrations in humans. We also found a marginally significant increase of NGF levels. Because there was strong interindividual variability of NGF levels, subgroups of MS patients who may exhibit strongly elevated NGF concentrations should be examined in future research. However, the findings of neurotrophin induction by acute exercise are in line with preliminary evidence that acute stress can enhance NGF levels in animals and humans (see Alleva et al., 2001). In one of the human studies (Aloe et al., 1994), a parachute jump was used as an acute stressor. Another report found significantly induced NGF levels after experimental acute stress (Lang et al., 2002). This latter study employed a mixed physiologic and psychologic stressor of acute experimental nicotine withdrawal in habitual smokers, making the interpretation of results difficult. Hoffmann-Goetz and Pedersen (1994) argue that acute exercise may be a good alternative as a stress paradigm, since it is easier to control and standardize.

We have recently reported that neuroendocrine and neuroimmune dysregulation is present in MS (Heesen et al., 2002a, 2002b). Here, we could not find differential effects of exercise on neurotrophic factor induction, i.e. there was an increase in healthy controls as well as MS patients. NGF levels were significantly higher at baseline and increased after the stressor in patients while there was little variance over time in controls (see figure 1). Although no statistically significant interaction was detected with the conservative two-group ANOVA approach, the data are suggestive of an enhanced NGF response in MS. This clearly needs further investigation.

The findings of transient neurotrophic factor induction (esp. BDNF) by acute exercise may be important with respect to recent findings of beneficial effects of exercise on brain health and recovery (Cortman & Brechtold, 2002). Given the neurodegeneration and cognitive decline observed in MS, this might be particularly important, since via the pathway of BDNF induction, moderate exercise might help to promote neural recovery and plasticity.

The function of neurotrophic factors is poorly understood. As is the case with IGF-1, NGF might exert its effects via immunomodulation. This is supported by Villoslada's study (2000) which showed a prominent effect on inflammation and demyelination. The authors reported a decrease in IFN γ -producing cells and an increase in IL-10 production of astrocytes as well as markedly reduced demyelination in NGF treated animals. However, remyelination could not be assessed in this study due to the low level of neurodegeneration in the treatment group.

NGF has been shown to be elevated in the serum of several inflammatory and autoimmune diseases (e.g., SLE, chronic juvenile arthritis, asthma, for review see Hellweg et al., 1998). In these studies, elevated NGF levels seem to correlate with disease severity. It is unclear, however, whether this represents a causal or counter-regulatory mechanism. Based on animal findings, Hellweg et al. (1998) proposed that early after initiation of an acute or chronic illness NGF is reduced, followed by a compensatory upregulation with a second decrease in end-

stages. Alternatively, in chronic degenerative as well as in inflammatory diseases there might be a persistent overactivity which may then have detrimental effects. The different neurotrophins bind similarly with low affinity to the p75 neurotrophin receptor, which belongs to the TNF receptor super family. Neurotrophins also bind to their respective high affinity Trk receptor tyrosine kinases, namely NGF to trkA and BDNF to trkB (see Dechant & Barde, 2002 for review). Binding of neurotrophins to Trk receptor tyrosine kinases initiate signaling cascades that promote cell survival and differentiation. In contrast, p75 neurotrophin receptor has been shown to modulate the susceptibility to death of selective cellular populations (Casaccia-Bonofil et al., 1999). This further underlines that increased levels of NGF should be interpreted with caution since their effect seems to depend on the respective receptors and could be neuroprotective or detrimental in nature.

As we have shown in this study, neurotrophic factors can be induced in MS patients to the same extent as in healthy controls. It remains to be elucidated whether longer-lasting increases of BDNF and other neurotrophic factors can be achieved by moderate exercise training over an extended period of time. To date, this hypothesis is only supported by animal data (e.g. Berchtold et al., 2001).

Aerobic training has been found to enhance quality of life, ameliorate depression, and increase physical fitness in MS (Petajan et al., 1996; Mostert & Kesselring, 2002). The same studies reported no adverse side effects of exercise. MS patients have long been advised to refrain from physical exercise since some symptoms tend to transiently worsen when body temperature increases (Utthoff's phenomenon). Based on the cited studies, this view has recently been abandoned. In fact, moderate exercise is now recommended (Petajan & White, 1999). Induction of neurotrophic factors under acute exercise observed in our study in healthy controls as well as MS patients may potentially be involved in beneficial effects of aerobic training. While it has been shown that neurotrophic factors from the blood stream can cross

the BBB under experimental conditions (e.g. Pan et al., 1998), it is premature to conclude that an upregulation of these factors in the blood will have beneficial effects in the brain. Whether such alterations are associated with increased neural plasticity and improved cognitive function remains to be elucidated in human studies. To address this question, we recently started a study on the effects of a eight-week exercise training on endocrine and neurotrophic factors as well as immune and physical functions.

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