Archives Italiennes de Biologie, 145: 99-110, 2007.

BDNF EXPRESSION IN RAT SKELETAL MUSCLE AFTER ACUTE OR REPEATED EXERCISE

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INTRODUCTION

The expression of several growth factor genes in skeletal muscle is regulated by muscle activity (2, 11, 12, 13, 19, 24, 27, 29). The activity-induced expression of neurotrophins is of particular interest because of their possible trophic action on motoneurons (9, 15).

Brain-derived neurotrophic factor (BDNF) is a neurotrophin expressed and released by skeletal muscle (8, 22) and plays a trophic role on motoneurons. It is generally accepted that muscle activity regulates BDNF expression, but the kind of regulation is controversial. In fact, BDNF expression is elevated in muscle during the perinatal period until one week after birth, when rats start their explorative activity (9), and rapidly decreases during the two subsequent weeks. It rises again in adulthood after muscle denervation (6, 8, 9). Consistent with these findings, electrical stimulation of sciatic nerve depresses muscular BDNF expression (9). On the other hand, repetitive muscle activity increases BDNF expression, but a single session of running does not change it until 6 hours after the cessation of that activity (14). Moreover, BTX-paralyzed muscle expresses low levels of BDNF mRNA (15).

In order to shed light on the effects of muscle activity on BDNF expression, we studied the time course of BDNF mRNA and protein expression in rats following one or five consecutive days of exercise on a treadmill. The study was protracted until 72 hours after a single exercise and 24 hours after the last session of repetitive exercise. *Soleus* muscle, a preeminently slow muscle activated during running (30), was used.

Timmusk *et al.* (36) have shown that BDNF gene has a complex structure in rat: one 3' exon (V) which contains the region encoding the BDNF protein and four 5' untranslated exons (I, II, III, IV) linked to different promoters (17). Alternative splicing gives rise to four different mRNAs (I-V, II-V, III-V and IV-V) encoding the same protein. Knowledge of the expression of different exons is important for under-

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standing factors and mechanisms regulating BDNF induction. Hence, the exons expressed in *soleus* muscle after acute and repetitive exercise were investigated separately.

METHODS

1. Animals and exercise.

The experimental procedures described below were conducted in accordance with the American Physiological *Society's Guiding Principles in the Care and Use of Animals* and with italian law regarding animal experimentation.

Adult Sprague-Dawley male rats (3 months of age) were randomly assigned to control (n = 13) or exercise (n = 39) groups. All animals were kept under standard breeding conditions: 12:12 h of light-dark cycle and food and water *ad libitum*. During the two days preceding the exercise sessions, rats of the exercise group were acclimated to the treadmill, exposing them once a day to the apparatus moving at low speed (5 m/minute). They were then exercised on a motor-driven treadmill for 30 minutes, only once in the acute exercise group and over five consecutive days (once a day) in the repetive exercise group. Each exercise session of 30 minutes consisted of alternate 5 minutes-periods of running and rest; each period of 5 minutes of running consisted of 4 minutes at constant acceleration (0-27 m/minute) and 1 minute at maximum speed of 27 m/minute. Exercise sessions were always carried out at the same time. The animals that refused to run were discarded to avoid confusing effects induced by stress. At the end of the last session, the rats were anesthetized with sodium-pentobarbital (45 mg/kg of body weight, *via* i.p.) and *soleus* muscle of both sides was dissected and quickly immersed in RNAlater (Qiagen) for RT-PCR analysis or in liquid N_2 for Western-Blot analysis; the rats were subsequently killed with an intracardiac injection of the same anaesthetic. In the acute exercise group, *soleus* muscles were removed immediately (0 h) or 6, 24, 48 and 72 h after the single exercise session, while in the animals of the repetitive exercise group, *soleus* muscles were removed 2, 6 and 24 h after the last exercise session.

2. Isolation of mRNA and qualitative RT-PCR.

RNA was extracted from muscles using Rneasy kit and proteinase K (Qiagen). Two micrograms of Dnase I (Ambion)-treated total RNA were reverse transcribed using Omniscript reverse transcriptase (Qiagen) according to the manufacturer's instructions.

An analysis of the expression of different BDNF transcripts in *soleus* was performed by RT-PCR as described by Nemoto *et al.* (26). PCR was performed adding to 1 µl of reverse transcriptase reaction the selected primers 0.4 µM and the HotStarTaq Master Mix (Qiagen) to a final volume of 25 µl. After an initial activation step of 15 minutes at 95 °C, 30, 35 and 40 amplification cycles were performed (denaturation at 94 °C, 45 sec; annealing at 60 °C, 45 sec; extension at 72 °C, 45 sec) followed by an elongation at 72 °C, 10 minutes. Total RNA from rat hippocampus was reverse transcribed and used as a positive control in PCR, showing that reported conditions allow efficient amplification of all the different BDNF splice variants.

In order to check the quality of the cDNAs, PCR was also performed with primers for β-actin (Forward: 5'-GCACCAGGGTGTGATGGTGGG-3'; and Reverse: 5'-GGTGGTGAAGCTGTAGCCACG-3') under the same conditions described for BDNF for 30 cycles. All the reactions were performed in a PTC-200 Peltier Thermal Cycler from MJ Research (M-Medical srl, GENENCO – Life Science). PCR products were analysed by 1.6% agarose gel electrophoresis using a molecular weight marker (GeneRuler™ 100bp DNA Ladder Plus from M-Medical srl, GENENCO-Life Science). Sequencing of amplicons was performed using an ABI 377 DNA sequencer (Perkin-Elmer, Applied Biosystems Div.).

3. Real-time quantitative RT-PCR.

Real-time quantitative RT-PCR was used to determine the relative levels of both total BDNF and exons III and IV. Amplification of GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)

mRNA was used as an internal control. Specific primers for quantitative PCR were the following: BDNF exon V (Forward: 5'-GGACATATCCATGACCAGAAAGAAA-3'; Reverse: 5'-GCAACAAACCACAACATTATCGAG-3') and GAPDH (Forward: 5'-CAAGGTCATCCATGACAACTTTG-3'; Reverse: 5'-GGGCCATCCACAGTCTTCTG-3'). The primers used for exons III and IV were those described by Altieri *et al.* (1). PCR was performed in a Bio-Rad icycler iQ Multi-Color real-time PCR Detection System using 2X Quantitect SYBR Green PCR kit (Qiagen). The quantitative PCR reaction consisted of: 95 °C for 10 minutes to activate HotStart DNA polimerase followed by 50 cycles of the two steps at 95 °C for 30 minutes and at 60 °C for 30 minutes. The specificity of the amplification products obtained was confirmed by examining thermal denaturation plots and by sample separation in a 3% DNA agarose gel. The amount of the target transcript was related to that of the reference gene using the method described by Pfaffl (28). Each sample was tested in triplicate by quantitative PCR, and samples obtained from at least four muscles were used to calculate the means and standard errors.

4. Electrophoresis and Western blotting.

The muscles were homogenized with 0.5% Collagenase (Sigma St. Louis, MO) in 1 ml of ice-cold lysis buffer [50 mM Tris–HCl, pH 7.8, 0.25 M sucrose, and 1% (w/v) SDS (sodium dodecyl sulfate), 1 µg/ml pepstatin, 10 µg/ml leupeptin, 2 mM sodium orthovanadate, 10 mM NaF, 5 mM EDTA, 5 mM NEM (Nethylmaleimide), 40 µg/ml PMSF (phenylmethylsulfonyl fluoride), and 0.1% Nonidet-P40] and sonicated for 60 sec at 100 W. Samples were boiled for 4 minutes and then centrifuged for 10 minutes at 14,000 g to remove insoluble debris. Supernatants were mixed 1:1 (v:v) with sample buffer (0.5 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 4% of 2-mercaptoethanol, and 0.05% bromophenol blue) and 50 µg of sample proteins were loaded onto 15% SDS – polyacrylamide slab gels and subjected to electrophoresis (21). Pre-stained molecular weight markers (Bio-Rad, Milan, Italy) were run on adjacent lanes. The gels were electroblotted and stained with Coomassie blue (38). Blots were probed with the specific anti-BDNF (Santa Cruz Biotech, USA) as the primary antibody. Horseradish-peroxidase-conjugated goat anti-rabbit IgG (1/3000) (Bio-Rad, Milan, Italy) was used as a secondary antibody. After antibody probing, nitrocellulose membranes were stripped for 30 minutes at 50 °C with a stripping buffer (62.5 mM Tris-HCl, pH 6.7, containing 10 mM β-mercaptoethanol and 2% SDS) and reprobed with anti-actin (1:200) (Sigma St. Louis, MO). Immune complexes were visualized using an enhanced chemioluminescence Western blot analysis system (Amersham – Pharmacia, Milan, Italy), following the manufacturer's specifications. Blot images were digitized (Chemidoc, Bio-Rad, Milan, Italy) and the area of bands was quantified using the computerized imaging system (Quantity One, Bio-Rad).

5. Statistical analyses.

Statistical analyses were performed using the one-way ANOVA test followed by post-hoc comparison using Tukey's test. The differences were considered significant when $p < 0.05$.

RESULTS

1. BDNF gene expression by soleus muscle.

Qualitative PCR performed on different transcripts showed that splice variants I-V and II-V were not detectable at 40 cycles of amplification. Splice variants III-V and IV-V were already detectable at 30 cycles, revealing a discrete level of expression in all the samples examined. The reported results were obtained at 40 cycles for exon III and IV in control rats, as well as in animal subjected to acute and repetitive exercise. Only three samples are reported (Fig. 1) because no significant differences in the level of expression were detectable at any time by RT-PCR, a qualitative technique that cannot discern between changes in relative expression if they are not very

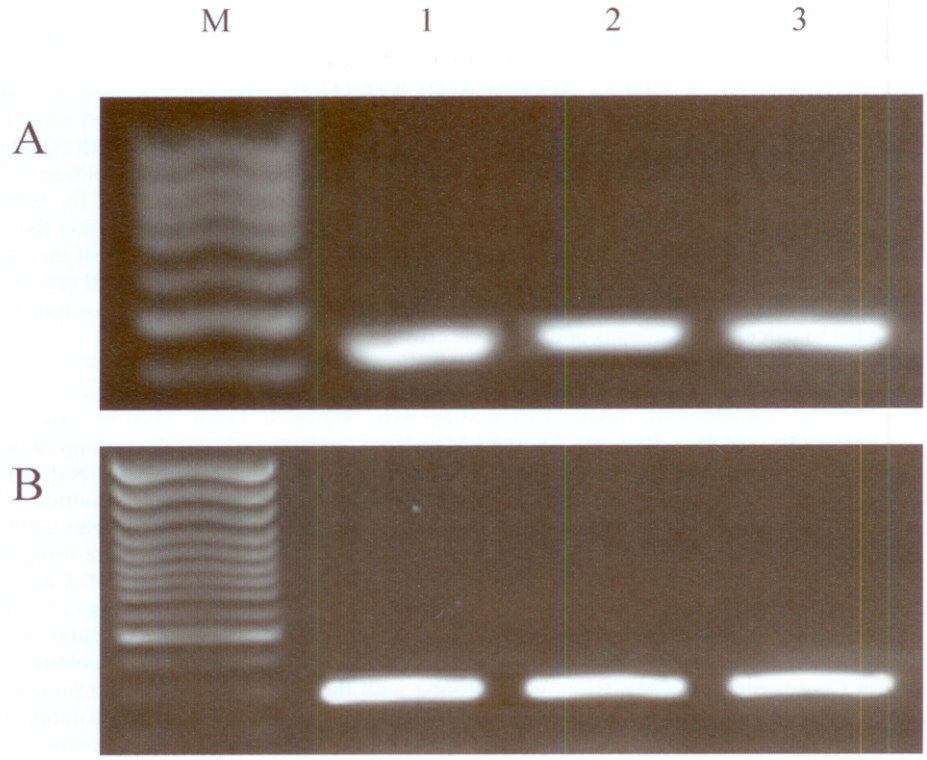

Fig. 1. – *Qualitative analysis of BDNF gene transcripts in soleus muscle.*

In A exon III product, in B exon IV product detected in controls (1), acutely exercised rats (2) and repetitively exercised rats (3). In M molecular marker.

important. Hence, we made a further investigation of the expression of total BDNF and transcripts III and IV by real-time RT-PCR, which allowed us to detect subtle changes of relative expression.

2. Total BDNF mRNA expression following acute or repetitive exercise.

Due to the organization of BDNF gene, real-time RT-PCR performed on exon V may be used to obtain information on the total expression of BDNF.

Immediately after acute exercise, the BDNF mRNA level did not change in comparison to controls, and the same level was found 6 hours after the end of the exercise session (Fig. 2A). The BDNF mRNA level was found to increase 24 hours after exercise and a similar level was maintained after 48 hours. Seventy-two hours after exercise mRNA returned to the control level.

A different time course of BDNF mRNA level was found after repetitive exercise. It was enhanced about 2 hours after the last session of repetitive exercise (Fig. 2B). This increase completely disappeared at 6 hours, but the mRNA level increased again compared to controls at 24 hours.

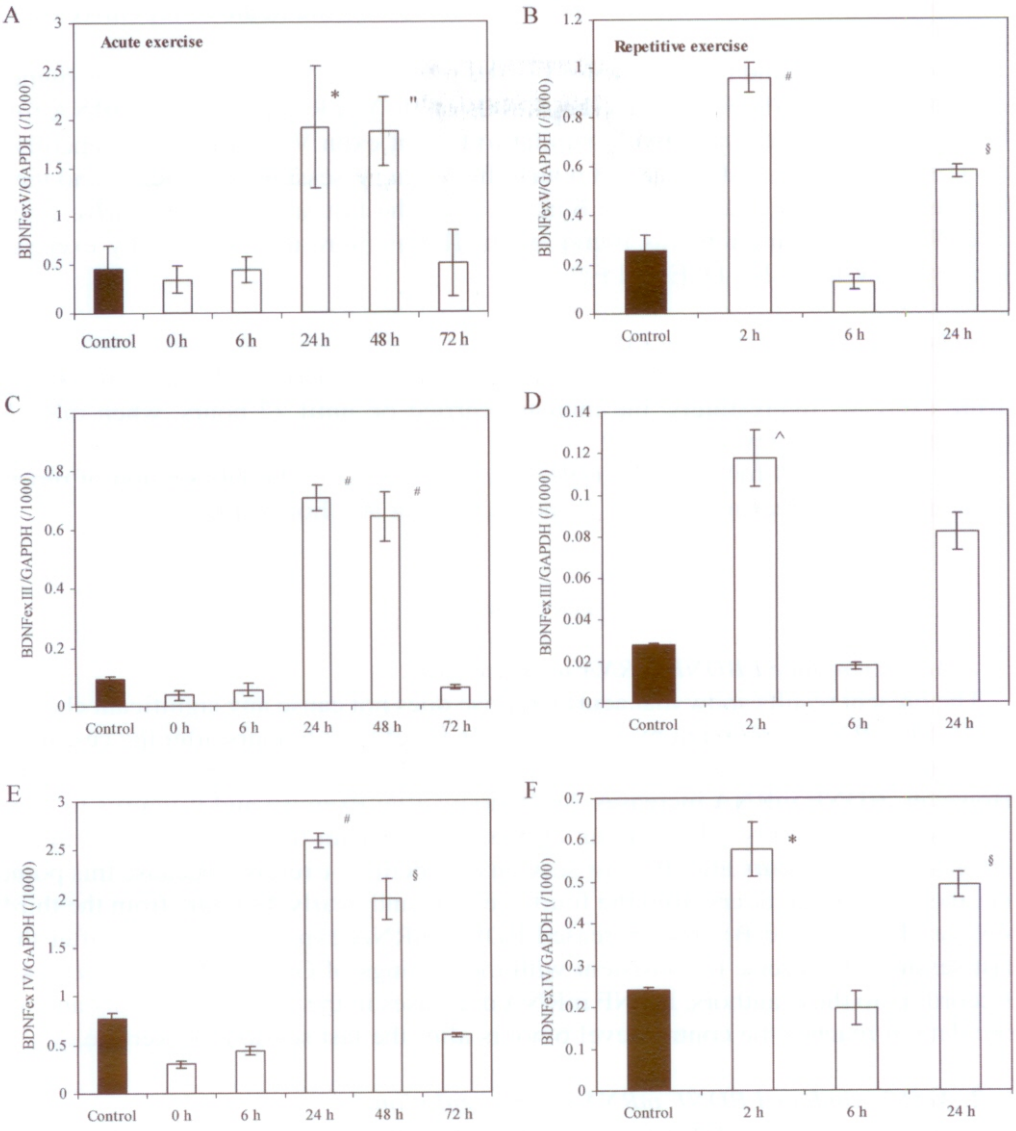

Fig. 2. – *Levels of BDNF mRNA expression after exercise.*

Total mRNA expression (exon V) after acute (A) and repetitive (B) exercise, respectively. Quantitative expression of BDNF exons III (C and D) and IV (E and F) after acute (C and E) and repetitive (D and F) exercise. In y axes values of BDNF/GAPDH ratio x 1000 are indicated. N = 3-5 for each group; samples were tested in triplicate. ANOVA: $p < 0.001$ for all panels. Tukey's t-Test: * $p < 0.001$, " $p < 0.003$, # $p < 0.0002$, $ $p < 0.02$ and ^ $p < 0.005$ vs controls.

3. Levels of the different BDNF splice variants following acute or repetitive exercise.

Exon IV was found to be expressed in the muscle at a level about 8 times higher than that of exon III (Fig. 2C and E). Levels of III-V mRNA and IV-V mRNA followed a time course qualitatively similar to that of exon V after acute or repetitive exercise: they increased 24 and 48 hours after a single session of exercise, and they increased 2 and 24 hours, but not 6 hours, after the last session of repetitive exercise. When up-regulation was found, exon III was more up-regulated by exercise than exon IV (Fig. 2C, D, E and F).

4. Levels of BDNF protein expression.

The muscle BDNF protein level progressively increased in the days following acute exercise. Interestingly, the increase carried on until 72 hours, when mRNA returned to control levels.

The protein level followed the mRNA time course after the last session of repetitive exercise, increasing at 2 and 24 hours, but not at 6 hours (Fig. 3).

DISCUSSION

1. Activity-induced BDNF mRNA up-regulation.

The present results show that acute exercise does not cause any significant change in BDNF mRNA expression by *soleus* muscle for at least 6 hours after the cessation of exercise. This result is consistent with the findings of Gomez-Pinilla *et al.* (14). However, BDNF mRNA increases 24-48 hours after exercise and it returns to control levels at 72 hours. This finding allows us to explain the quantitatively similar increase found 2 hours after the last session of repetitive exercise, because this point in time is nearly 24 hours from the fourth session and nearly 48 hours from the third session (Fig. 2A and B). The increased BDNF mRNA expression 2 hours after the last session of exercise is consistent with the findings of Gomez-Pinilla *et al.* (14). According to these authors, BDNF mRNA decreases in the subsequent hours and, in our data, it reaches the control level 6 hours after the last session of exercise.

2. Activity-induced BDNF mRNA down-regulation.

If the high level of mRNA found 2 hours after the last session of repetitive exercise is consistent with the up-regulation due to previous session, the subsequent transient decrease to the control level observed 6 hours after the last session cannot be expected without making an additional hypothesis. The two findings together – the up-regulation during the second day after a single session of exercise and the transient fall of up-regulation 6 hours after the last session of repetitive exercise – suggest that the last session of exercise induces a transient down-regulation. The fact that a decrease in the BDNF mRNA level does not occur after an acute exercise seems to suggest that the down-regulation concerns the share over-expressed on the second day after the exercise session, but not the basal expression. The observed

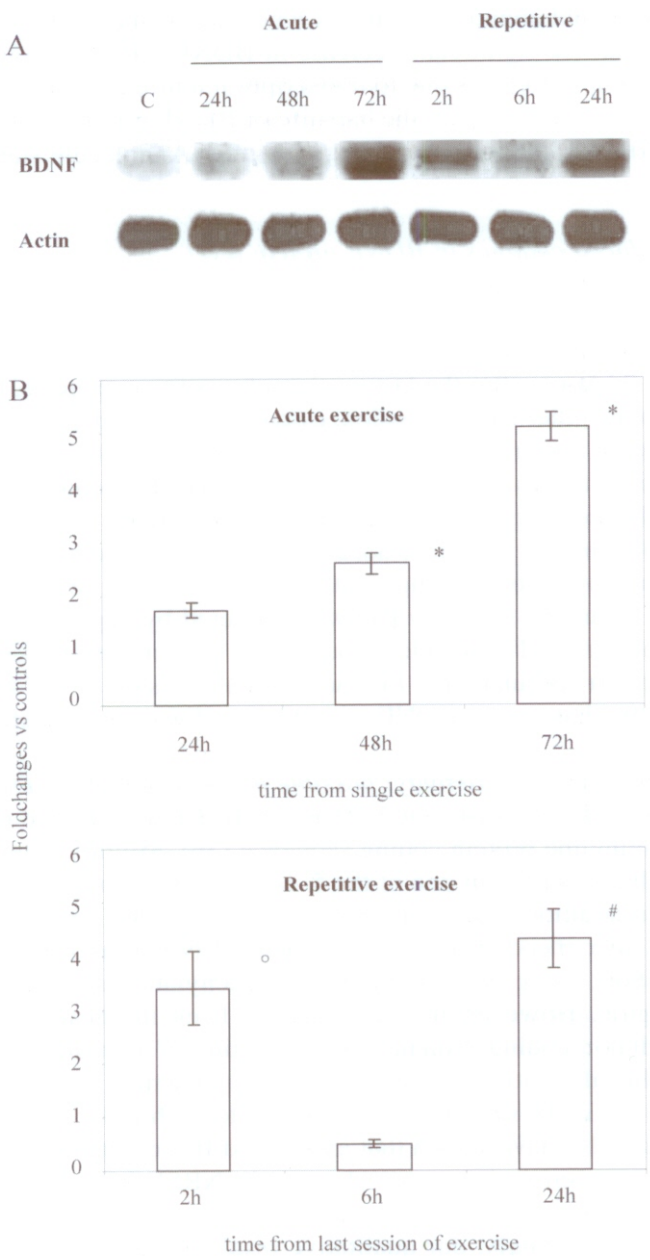

Fig. 3. – *(A): immunoblot of muscle protein extracts separated on 15% SDS-PAGE with anti-BDNF.*

Results are representative of 4 independent experiments. Anti-actin blots shown as loading controls. Bands were detected using enhanced chemioluminescence reagents (see Methods).
(B): densitometric analyses of BDNF blots (mean ± SEM) of muscles from acutely and repetitively exercised rats.
Relative changes in band optical densities (arbitrary units) were normalized for the control band in each series. ANOVA: $p < 0.001$. Tukey's t-Test: * $p < 0.001$, ° $p < 0.02$, # $p < 0.002$ vs. controls.

down-regulation is not found at 24 hours when we again observed a high BDNF level. The idea that the significant decrease in BDNF mRNA 6 hours after the last session of repetitive exercise is due to transcription inhibition following the last session of exercise is consistent with the half-life of BDNF mRNA, which is thought to be about 2 hours (4, 31). However changes in mRNA molecule stability cannot be ruled out (7).

3. Activity-induced changes in BDNF protein level.

BDNF protein level progressively increased until 72 hours after a single session of exercise when mRNA was found to return to the control level, showing the long life of the protein within the cell after translation. The protein level was found to be enhanced 2 and 24 hours after the last session of repetitive exercise, but it fell to the control level 6 hours after the last session, essentially following the mRNA level time course. This finding does not confirm the long life of the protein within the cell suggested by data from the acutely trained group (Fig. 3B). To reconcile these apparently conflicting results we suggest that repetitive exercise speeds up the BDNF secretory pathway, not allowing the protein to accumulate within the cell for a long time. This hypothesis is supported by findings obtained in neuronal cells, where BDNF is released via the regulated secretory pathway triggered by depolarization (3, 25), but when it dimerizes with NT-4, its release occurs via the constitutive secretory pathway (18). Hence, we can speculate that the increased expression of NT-4 by muscle following activity (9) changes the BDNF exocytosis pathway and therefore its rate.

4. Exon-specific effects of activity: possible mechanisms of induction.

The BDNF gene has at least four 5' exons (I, II, III and IV) and one common 3' exon (V) containing the protein coding sequence (36). Multiple alternative splicing is possible in which a different one of the 5' non-coding exons is spliced to the coding sequence. In addition, each transcript can have either a longer or a shorter 3'-UTR, which is also encoded on exon V. Each mRNA transcript can therefore be either long or short, using two alternative polyadenylation sites located within the untranslated region. However, this fact has not been implicated in regulation of expression. Each non-coding exon has its own distinct 5'-flanking regulatory region, thus each of the alternative transcripts is independently regulated, and BDNF expression in a cell is the sum of expression due to each individual promoter.

Timmusk *et al.* (37) have shown that exon I and II promoters contain a neuronal-restrictive silencer element/repressor element-1 (NRSE/RE1) to which a type of silencer protein binds to prevent the expression of neuron-specific genes in non-neuronal cells (32). Accordingly, our qualitative results revealed that only splice variants III-V and IV-V are detectable by PCR in *soleus* muscle. These data are consistent with the recent findings of Liu *et al.* (23), who have shown the presence of exons III and IV (that they named IV and V) and the absence of exons I and II in skeletal muscle. Their exon III is not expressed in skeletal muscle, whereas exon VI is detectable at 50 cycles of PCR, but does not seem to be quantitatively relevant, compared to our III and IV, in determining total BDNF expression.

We reported that the exon IV is always more expressed, but the expression of exon III is relatively more enhanced after acute or repetitive exercise. These different effects of exercise on the expression of the two exons suggest that the mechanisms of regulation are different and, at least in part, independent. Most studies on BDNF gene regulation have been carried out on nerve cells, and therefore we have to base our speculation on the possible mechanisms underlying the activity-driven changes in BDNF expression in muscle on these experimental models. BDNF expression is controlled by $[Ca^{2+}]_i$ (10). Promoters of exons III and IV contain widely expressed transcriptional regulatory elements that recognize calcium-responsive transcription factors (CaRF), cAMP/calcium-responsive binding proteins (CREB) and methyl-CpG binding protein 2 (MeCP2) (5, 35). Moreover, it should be considered that promoter III has two other sequences responsible for Ca^{2+} signal responsiveness, CaRE1 and CaRE2 (5, 7). These findings are consistent with the fact that exon III is more responsive than exon IV to $[Ca^{2+}]_i$ transients occurring during muscle activity. Paradoxically, an increase of $[K^+]_o$ (used as a depolarizing stimulus to induce Ca^{2+} influx) over 25 mM causes a decrease in transcriptional activity until it is fully depressed (34). It is interesting to relate this finding to our observation of a transient down-regulation 6 hours after the last session of repetitive exercise.

5. Possible functions of activity-regulated muscle BDNF expression.

Since muscle fibers are well known to be the source of BDNF in uninjured skeletal muscle (22), it is likely that they are also the main responsible for activity-dependent BDNF expression.

Given the above-mentioned consideration and the fact that BDNF serum level changes are not known to be associated with muscle exercise, muscle-derived BDNF might have an autocrine/paracrine role. Muscle fibers, motoneurons, endothelial cells and Schwann cells all express TrkB receptors (16, 20, 39). Microvascular reorganization in skeletal muscles appears as a consequence of prolonged exercise (33) and BDNF is known to promote vascularization by recruitment of local endothelial cells (20). These findings, together with our results showing BDNF up-regulation in exercised muscle, suggest that BDNF might be involved in muscle vascularization remodeling induced by activity. Moreover, TrkB receptors are involved in clustering of acetylcholine receptors and nerve terminal reorganization (9, 16), suggesting that muscle activity may be involved in the maintenance of the neuromuscular junction structure.

SUMMARY

Brain derived growth factor (BDNF) gene of rat has a complex structure: at least four 5' untranslated exons regulated by different promoters and one 3' exon containing the encoding region. BDNF is expressed by skeletal muscles in an activity-dependent manner. In this study, BDNF mRNA was analysed by RT-PCR in the soleus muscle following a single (acute) session of running or a training of five days

of running (repetitive exercise). Moreover, the expression of the exons was quantitatively analysed by real time RT-PCR. Finally, muscle BDNF protein level was evaluated by western blotting. BDNF mRNA was found to increase over the second day after acute exercise; on the other hand, two peaks (2 and 24 hours after the last session, respectively) in BDNF mRNA level were found after repetitive exercise, but it was similar to that of controls 6 hours after the last session. BDNF protein level progressively increased also after the mRNA went back to the basal level, so suggesting that it cumulates within the cell after acute exercise, whereas it followed the mRNA level time course after repetitive exercise. These results point to the following conclusions: BDNF mRNA is up-regulated by activity, but this response is delayed to the second day after acute exercise; repetitive exercise transiently depresses the expression of BDNF mRNA, so that the over-expression due to the previous day's exercise completely disappears 6 hours after the last exercise session.

Acknowledgements – This work was supported by grant from Istituto di Riabilitazione "S. Stefano", Potenza Picena, Italy.

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