Exercise-Induced Changes in c-Fos Protein Levels in Skeletal Muscle of Trained and Untrained Rats

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Abstract

We investigated the effect of an acute bout of endurance exercise on c-Fos protein levels in the extensor digitorum longus muscle of trained and untrained rats. Fifty rats were equally divided into a trained and an untrained group. Rats of the trained group ran on a treadmill 45 min/day for 5 days. On the sixth day, 5 rats were killed without exercise, while the remaining 20 ran as above and were killed 0, 3, 6, and 12 h post-exercise (5 rats at each time point). In the untrained group, 5 rats were killed without exercise, while the remaining 20 ran as above only once and were killed at the same time points as the trained group. Western blotting demonstrated no significant changes in c-Fos protein levels in the untrained group. On the contrary, in the trained group, there was a significant increase at 6 and 12 h compared to 3 h post-exercise. The levels of the protein in the trained rats were above the corresponding levels in the untrained ones at all time points, although these differences reached statistical significance only immediately, 6 h and 12 h post-exercise. These results show that trained skeletal muscle exhibits increased levels of c-Fos, probably as a cumulative result of changes occurring during recovery from each exercise bout, and greater c-Fos response after acute endurance exercise compared to untrained skeletal muscle.

Key words
Contractile activity · gene expression · recovery · treadmill running.

Introduction

Skeletal muscle exhibits a remarkable capacity for long-term adaptations to endurance exercise, which are mediated by changes in gene expression. Despite the profound effects of endurance exercise on muscle phenotype, little is known about the intracellular mechanisms that link the "exercise signals" to the modulation of gene expression [1,43].

c-Fos is an intracellular immediate-early protein, which is usually induced very early in response to a variety of extracellular signals. c-Fos, in complex with a member of the Jun family, constitutes activator protein-1 (AP-1), a transcription factor implicated in cell proliferation, differentiation and transformation [38]. AP-1 has also been associated with mitochondrial biogenesis [25], responses to myocellular injury [27], and apoptotic cell death [38], all of which take place in skeletal muscle after endurance exercise [25,27,42]. Additionally, AP-1 is thought to regulate the expression of certain genes [3,6,24,26,41,44] known to be induced in response to exercise or increased contractile activity in general [22–24,29,40]. However, the role of AP-1 and, hence, c-Fos in the cellular adaptations taking place in skeletal muscle after exercise is largely unknown.

In recent years, many studies have reported that modified contractile activity alters the expression of the c-*fos* gene in skeletal muscle of animals and humans [2,5,12,14,19,28,30,33,35,39,43,48,50,51]. Most of these studies have used animal models that do not mimic human physical activity adequately and have presented results at the mRNA level

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immediately after the end of contractile activity. To our knowledge, only two relevant studies utilized an animal model that closely mimics human physical exercise, i.e., treadmill running [14,30], and one used humans [39]. Of these studies, only the latter measured c-Fos protein, which is important, since changes in mRNA levels after increased contractile activity are not always reflected on protein levels [7,36]. Moreover, there are no published data on the effect of repeated exercise bouts on c-Fos protein. Considering also the fact that acute exercise in rats trained for 5 days did not increase c-*fos* mRNA, contrary to untrained animals [30], it seemed interesting to examine the temporal expression pattern of c-Fos after acute endurance exercise as a step towards defining its role in the adaptations to modified contractile activity. Therefore, the aims of the present research were to investigate: (i) the effect of an acute bout of endurance exercise on c-Fos protein levels in the extensor digitorum longus (EDL) muscle of untrained rats, (ii) the effect of 5 days of training on c-Fos protein levels, and (iii) the effect of acute endurance exercise on c-Fos protein levels in rats trained for 5 days.

Materials and Methods

Animals

Fifty male Wistar rats (9 weeks old, weighing 250 – 280 g) were supplied by the Theagenion Cancer Hospital (Thessaloniki, Greece) and housed in the same hospital. The rats lived in groups of five per cage at 22 °C, on a 12 : 12-h light-dark cycle, and were allowed free access to water and rodent chow. The rats were maintained according to the European Union guidelines for the care and use of laboratory animals.

Exercise protocol

All rats that exercised (45 out of the 50) were habituated to the exercise protocol by running on a motor-driven treadmill at a speed of 12 m/min, at 0% grade for 5 min before the first exercise bout. The rats were equally divided into a trained and an untrained group. The rats of the trained group ran on the treadmill at a speed of 20 m/min, at 0% grade for 45 min/day for 5 days. The exercise intensity chosen corresponds to 55–60% of maximal oxygen consumption [4]. The 5-day protocol used – with minor differences – was found capable of eliciting significant adaptations in rat skeletal muscle of rats of similar age [15,16]. On the sixth day, 5 rats were killed without exercise by exposure to ether, while the remaining 20 ran as above and were killed 0, 3, 6, and 12 h post-exercise (5 rats at each time point). In the untrained group, 5 rats were killed without exercise, while the remaining 20 ran according to the same protocol only once and were killed at the same time points as above (5 rats at each time point). Mild electrical shocks (0.8–1.0 mA) were used sparingly to motivate the animals to run. All procedures took place at the same time of the day (12 : 00 – 13 : 00).

Muscle dissection

The EDL muscle was surgically removed from the right hind limb of each animal and was immediately immersed in liquid nitrogen. This muscle belongs to the family of dorsiflexors of the ankle joint and is activated during the swing phase of the step cycle in the rat as measured by electromyography [45]. Furthermore, many studies on rats have used this muscle and reported significant diverse adaptations to treadmill training [13,20,31].

Protein extraction

For analysis, the frozen muscle was pulverized with mortar and pestle in liquid nitrogen and stored at –80 °C until analysis. The muscle powder was homogenized in RIPA buffer containing 0.5% sodium deoxycholate, 1% Nonidet P40, 0.1% SDS, 10 mmol/L sodium orthovanadate, 10 mg/mL aprotinin, and 10 mg/mL phenylmethysulfonyl fluoride in PBS (10 mmol/L sodium phosphate, 138 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4). The homogenate was then centrifuged at 12,000 × g for 15 min. Homogenization and centrifugation were performed at 4 °C. Total protein in the supernatant was assayed using a commercially available kit (Sigma, St. Louis, MO) based on the Bradford method.

Electrophoresis

Protein extracts were prepared for electrophoresis by adjusting the protein concentration to 3 µg/µL with RIPA buffer, followed by the addition of 5 µL of sample buffer (250 mmol/L Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 6.25% 2-mercaptoethanol and 0.06% bromphenol blue) to 10 µL of extract. Proteins were separated in SDS-polyacrylamide pre-cast gels with a 10 – 20% gradient (Owl Separation Systems, Portsmouth, NH). Kaleidoscope prestained molecular weight markers (Bio-Rad, Richmond, CA) were included in each gel. Electrophoresis was performed in a P8DS electrophoresis apparatus (Owl Separation Systems) at 100 V and stopped when the dye front reached the bottom of the gels.

Western blotting

Proteins were transferred from the gels to polyvinylidene fluoride membranes (Millipore, Bedford, MA) by using a Mini Trans-Blot Cell (Bio-Rad) at 100 V for 1 h. After transfer, blots were blocked overnight with PBS containing 0.1% Tween-20 (PBS-T) and 5% bovine serum albumin (Sigma). The membranes were then incubated with a rabbit polyclonal antibody against c-Fos, which recognizes amino acid residues 4 – 17 (Ab-2; Oncogene Research Products, Cambridge, MA), in a 1 : 2500 dilution with PBS-T for 1 h. This was followed by an one-hour incubation with a horseradish peroxidase-conjugated anti-rabbit antibody (Amersham, Buckinghamshire, England) in a 1 : 5000 dilution with PBS-T. Finally, blots were developed using the Super Signal® West Pico Chemiluminescence Substrate (Pierce, Rockford, IL). Developed bands were photographed with a DC 120 camera (Kodak, New York, NY) and quantified by a computerized densitometric image-analysis software (Biosure, Athens, Greece). To account for gel-to-gel variation, data were normalized according to control samples that were included in all gels [11,52].

Statistical analysis

Data are expressed as the mean ± SEM. c-Fos levels were compared through two-way (training × time) ANOVA. Post-hoc pairwise comparisons were performed through simple main effects. The level of statistical significance was set at $\alpha = 0.05$.

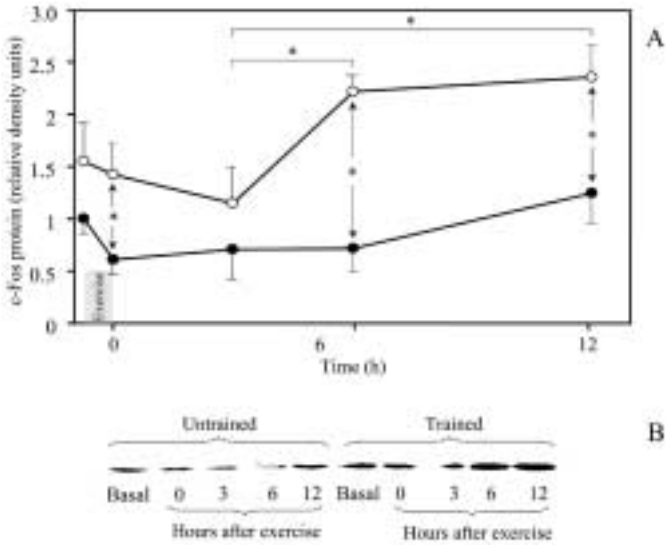

Fig. **1** **A** Time course of changes in the amount of c-Fos protein relative to total protein in the EDL muscle of trained for 5 days (o) and untrained rats (●) in response to acute exercise. The mean optical density of the bands corresponding to untrained sedentary rats was set as one. Error bars denote SEM. Asterisks denote significant differences ($P < 0.05$) between different time points in the same group or between the two groups at the same time point. See Results for exact P values. **B** A representative Western blot of c-Fos protein.

Results

The exercise-induced changes in c-Fos protein levels in the EDL muscle of trained for 5 days and untrained rats are presented in Fig. **1**. Western blots demonstrated a biphasic change in both groups, consisting of a decrease followed by an increase. The interaction was not significant, but both the main effects of training and time were ($p < 0.001$ and $p = 0.002$, respectively). There were no significant differences between time points in the untrained group, but, in the trained group, the values at 6 and 12 h after exercise were significantly higher than the one at 3 h ($p = 0.016$ and 0.004, respectively). Interestingly, both groups exhibited the highest level of c-Fos (25–51 % above the corresponding basal level) 12 h after exercise. The levels of the protein in the trained rats were above the corresponding levels in the untrained ones at all time points, although these differences reached statistical significance only immediately ($p = 0.013$), 6 h ($p < 0.001$), and 12 h ($p < 0.001$) after exercise.

Discussion

The present study aimed at examining the effect of acute endurance exercise on c-Fos protein levels in trained and untrained rats. To our knowledge, this is the first attempt to study the response of c-Fos protein to training. Most of the studies on the effect of modified contractile activity on c-*fos* expression have presented results solely at the mRNA level [2,5,12,14,19,30,33,35,43,48,50,51]. Moreover, the majority of these studies have used animal models that do not mimic human exercise closely enough to allow for an extrapolation of their results to humans [2,5,12,19,33,35,43,48,50,51]. Hence, we used treadmill running as the means to modify the contractile activity of our experimental animals, since this model is thought to imitate human physical activity [7].

In both trained and untrained animals, we found a somewhat unexpected kinetics of c-Fos protein, characterized by initial reduction and later induction (though not fully justified statistically). This biphasic progress is difficult to interpret, since data relevant to protein kinetics during recovery are scanty, owing to the fact that most studies have only determined protein levels immediately post-exercise. Nevertheless, it has become apparent in recent years that changes in gene expression may indeed occur during recovery from exercise (e.g., [34,37]). Indeed, Booth et al. [8] have argued that frequently more than one time-point are needed to ensure the direction of gene expression after acute exercise. As for the effect of exercise specifically on c-Fos protein kinetics, we were able to detect only one study [39] in which the protein was measured at multiple time points during recovery (up to 3 h after treadmill running). In that study, the researchers found an increase of c-Fos protein in human vastus lateralis muscle during recovery from treadmill running. On the other hand, one study has reported diminished levels of c-*fos* mRNA and c-Fos protein in human trabecular meshwork cells shortly after mechanical stretch, followed by increases after stretch release [49]. Furthermore, Neufer et al. [33] reported that, although c-*fos* mRNA decreased immediately after 7 days of intermittent low-frequency electrical stimulation of the tibialis anterior muscle of rabbits, it transiently increased 2 – 8 h later. It is interesting to note that c-Fos showed a biphasic (decrease followed by increase) response also in spermatocytes exposed to a drug causing apoptosis [46]. The biphasic response of c-Fos found in our study emphasises the value of measuring gene expression at multiple time-points after exercise and implies that erroneous conclusions may be drawn from single measurements.

Both groups of rats exhibited the highest level of c-Fos protein 12 h after exercise (though not significantly higher than baseline). This pattern may underlie the mechanism of increased c-Fos protein levels in the trained rats. As far as we know, this is the first demonstration that brief training can increase the levels of c-Fos protein. Increased levels of c-Fos have been also reported after continuous low-frequency electrical stimulation of tibialis anterior [28] and latissimus dorsi muscle of rabbits [35].

Using an experimental procedure similar to the one in the present study, Murakami et al. [30] found increases in c-*fos* mRNA in soleus muscle following treadmill running in untrained but not in trained for 5 days rats. To reconcile these findings with ours, we have to assume that the increase in c-Fos protein occurs through greater efficiency of translation and/or post-translational modifications.

Another finding of our study was the greater c-Fos response of trained vs untrained skeletal muscle to acute endurance exercise. What caused this greater response and what is its biological significance? One of the main elements of the c-*fos* promoter is the serum response element (SRE), which is occupied by either a heterodimer of the serum response factor (SRF) and the ternary complex factor or a homodimer of two SRF [9,47]. A series of studies have shown that SRF expression, at both the mRNA and

protein levels, is increased during overload-induced hypertrophy of skeletal muscle of chicken [10], rooster [17] and rat [21]. Moreover, a recent study found the activity of the kinase that activates SRF to be significantly increased after short-term wheel running [18]. These observations suggest that, after repeated bouts of exercise, the "signals" of a subsequent bout may be transferred to target genes (e.g., c-*fos*) more efficiently because of an enlargement of the "exercise signalling pathway" (part of which, in this example, may be the SRF). The probable physiological value of the observed greater exercise-induced increase of c-Fos in trained rats compared to untrained ones is that c-*fos* expression becomes more sensitive to environmental changes, a situation that would be advantageous for the response to a subsequent exercise session.

The fact that, according to the present study, exercise increased the c-Fos protein levels, combined with the reported induction of c-*jun* after acute endurance exercise [1,30,39], might contribute to a higher content of Fos/Jun heterodimers in exercising muscle and thus increased AP-1 binding activity. Actually, Hollander et al. [24], using electrophoretic mobility shift assay, reported upregulation of AP-1 binding activity after a single bout of exercise in rat skeletal muscle. Increases in AP-1 binding activity can lead to subsequent alterations in the expression of AP-1-dependent genes. These include cytochrome *c* [3], skeletal α-actin [6], manganese-containing superoxide dismutase [24], myosin light chains [26], vascular endothelial growth factor [41], and lactate dehydrogenase [44], which are known to be induced in skeletal muscle during or after exercise [22–24,29,40]. In addition, it is probable that c-Fos plays a role in the apoptotic events taking place in skeletal muscle after exercise [42], since it may be a component of the regulatory pathway leading to apoptosis [38]. We have to mention that most of the above studies try to ascribe a role to c-Fos based on time sequences of intracellular events. However, caution is warranted in interpreting these findings, since the time course of activation of c-Fos and downstream targets suggests, but in no way demonstrates, a link between them in response to modified contractile activity.

In conclusion, our findings suggest that the skeletal muscle of rats trained for a rather brief period of 5 days exhibited increased levels of c-Fos protein, probably to account for an increased need of this protein during periods of intense physical activity. Furthermore, the present results support the hypothesis that adaptations of skeletal muscle to endurance training may be the cumulative effect of transient changes in gene expression during recovery from individual exercise bouts [32]. However, it remains to be determined whether the product of the gene studied is required for downstream adaptive events in skeletal muscle during and/or after exercise.

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