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Measures of Growth Processes and Myogenesis in Glycolytic and Oxidative Muscle Fibers in Rats after Indirect Electrical Stimulation

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We report here a comparative study of activity in signal pathways and gene expression in the red (RGM) and white (WGM) parts of the gastrocnemius muscle in rats after series of short (1 sec) tetanic contractions evoked by stimulation of the motor nerve at a frequency of 100 Hz and an amplitude sufficient to activate all the motor units of the muscle. At 2 h after stimulation, WGM showed more marked increases in the level of ERK1/2 phosphorylation than RGM, though increases in AMPK phosphorylation were no different. Furthermore, the increases in MyoD and myogenin mRNA in WGM were significantly greater than those in RGM, while the effects of stimulation on expression of the IGF-1, MaFbx, and MuRF genes were weak and similar in WGM and RGM. There was also an increase in the content of myostatin mRNA in RGM. Thus, glycolytic muscle fibers in WGM display more marked regulatory hypertrophic-type shifts than the oxidative muscle fibers making up RGM.

Keywords: rat, gastrocnemius muscle, glycolytic and oxidative muscle fibers, ERK1/2, MyoD, myogenin, myostatin.

Skeletal muscles in mammals consist of oxidative and glycolytic muscle fibers (MF), the former (mainly types I and IIA) being responsible for maintaining posture or performing slow low-intensity movements and the latter (type IIB in rodents) being recruited in rapid movements developing significant muscular force [30]. These types of MF differ significantly in terms of the expression levels of many proteins controlling anabolic and catabolic processes [8, 14, 16, 33]. It has been suggested that oxidative MF, as compared with glycolytic, show higher levels of expression of the genes responsible for both the synthesis and degradation of muscle proteins [33].

It is well known that metabolic shifts in skeletal muscles are fundamentally dependent not only on the properties of their constituent MF, but also on the loading pattern [5, 28]. In conditions of voluntary muscle activity, it is impossible for all muscle fibers to be loaded identically over long periods of time, as oxidative MF are recruited at lower forces than glycolytic [30]. Conversely, transcutaneous stimulation of the motor nerve first activates glycolytic motor units, because their motor axons are thicker and have lower stimulation thresholds. Thus, the question of how metabolic shifts differ depending on the intrinsic characteristics of MF requires model studies on animals. In particular, simultaneous recruitment of all motor units of a muscle can be achieved by stimulation of the nerve at an intensity definitively exceeding the stimulation thresholds of all the motor axons in the nerve. In the present study, this approach was applied to the medial head of the rat gastrocnemius muscle, in which different types of MF are distributed nonuniformly. The internal (red) part of this muscle (RGM) contains 20–25% type I MF, about 50% type IIA MF, and a total of 1% type IIB MF, while the superficial (white) part (WGM) contains virtually no oxidative MF and around 90% type IIB MF [7, 10].

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The aim of the present work was to conduct a comparative analysis of the activity of the signal pathways and gene expression profiles in rat RGM and WGM after series of intense contractions evoked by motor nerve stimulation. The authors investigated measures of the activity of the anabolic pathways (levels of ERK1/2 phosphorylation and levels of IGF-1 and myostatin gene expression)and catabolic pathways (levels of AMPKα_1/α_2 phosphorylation and levels of E3-ubiquitin ligase, MAFbx, and MuRF-1 gene expression), as well as the activation and differentiation of satellite cells (levels of MyoD and myogenin gene expression).

Methods

Experiments were performed on eight male Wistar rats weighing 466 ± 15 g. The experimental protocol was approved by the Biomedical Ethics Committee of the Institute of Medical-Biological Problems, Russian Academy of Sciences.

Recording of muscle contraction force on motor nerve stimulation. The method was based on that of MacIntosh et al. [23]. Rats were anesthetized with urethane (1.2 g/kg, i.p.) and placed on a heated platform to maintain rectal temperature at 36–38°C. Electrical stimulation of the sciatic nerve was performed by applying a cuff bearing electrodes of an original design; all branches of the nerve were transected apart from those running to the medial head of the gastrocnemius muscle. The nerve was stimulated with square-wave impulses (amplitude 2 V, duration 0.1 msec) produced using an ESL-2 (Russia) electrostimulator fitted with a specially constructed controller. Spike amplitude was set at twice the level producing maximal contraction, ensuring recruitment of all motor units of the muscle. The stimulation protocol included a series of 50 short (duration 1 sec) trains of impulses separated by 17-sec intervals; the frequency of impulses within trains was 100 Hz. This protocol imitates the training loads directed at developing muscle hypertrophy. Muscle contraction force was measured using a tensometric probe, whose signal was amplified and recorded on computer using an E14-140 ADC (L-Card, Russia) and the PowerGraph 3.3 program (DiSoft, Russia). Preloading was established at a level optimum for developing muscle force (50 g).

At 2 h after stimulation, rats were rapidly euthanased and the medial heads of the gastrocnemius muscle on the stimulated and contralateral (unstimulated) limbs were excised. Published data show that this time was sufficient for developing marked shifts in the expression of the genes regulating muscle biogenesis [27]. In addition, the maximal changes in signal cascades were seen for several hours after electrical stimulation, while recovery of the initial level took days [26]. Tissue fragments from the surface (white) and deep (red) parts of the muscle were rapidly frozen in liquid nitrogen and stored at –70°C until analysis.

Assessment of quantities of phosphorylated ERK and AMPK by western blotting. Frozen muscle fragments were ground with a cryotome (Leica, Germany) and dissolved in iced lysis buffer RIPA (Santa Cruz, USA) containing proteinase and phosphatase inhibitors (50 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 20 μg/ml aprotinin, 50 μg/ml leupeptin, 20 μg/ml pepstatin, 1 mM PMSF); insoluble particles were removed by centrifugation at 10000 *g* (+4°C) for 10 min. Protein concentrations in samples were measured by the bicinchoninic acid method. Aliquots containing 20 μg of total protein were separated by electrophoresis in 10% polyacrylamide gels in the presence of SDS followed by transfer to nitrocellulose membranes (Santa Cruz, USA) using a Mini-Protean Tetra Cell and a Mini Trans-Blot (BioRad, USA). Transfer efficiency was assessed by staining membranes with Ponceau S, and membranes were blocked in 5% defatted milk (Applichem, Germany) solution for 1 h. Primary antibodies were polyclonal antibodies to AMPKα_1/α_2 (Cell Signaling, diluted 1:500) and phospho-AMPKα_1/α_2 (Thr172) (Santa Cruz, USA, diluted 1:100), as well as monoclonal antibodies to ERK1/2 and phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling, both diluted 1:500); incubation was overnight at +4°C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at +25°C (Cell Signaling, diluted 1:5000) and then treated with ECL reagent solution (1.25 mM luminol, 0.2 mM coumaric acid, 100 mM Tris-HCl pH 8.5, and 0.009% hydrogen peroxide) and luminescence was assessed using medical x-ray films (Kodak, USA). Signal levels were evaluated densitometrically in ImageJ (USA). Protein contents were expressed as percentages of the group mean for WGM from the contralateral limb.

Assessment of mRNA contents by quantitative polymerase chain reaction (PCR). Experiments were performed as described previously [1]. Direct and reverse primer sequences for reference (β-actin, RPLP0) and study genes [IGF-1 (all isoforms), myostatin, MyoD, myogenin, MaFbx, MuRF] were taken from previously published articles [1, 15, 20, 33] and primers were synthesized at Evrogen (Russia).

RNA was extracted using an RNeasy Mini kit (Qiagen, Germany) with subsequent spectrophotometric measurement of concentrations (SmartSpec Plus, BioRad, USA). The absence of RNA degradation was checked by agarose gel electrophoresis. Samples with uniform RNA concentrations were treated with DNAse I (Fermentas, USA) and samples of 100 ng of RNA were used for synthesizing cDNA using an OT-1 kit (Sintol, Russia).

Quantitative PCR was performed in a Rotor Gene 6000 amplifier (Corbett Research, Australia). The amplification program consisted of initial denaturation at 95°C (10 min), 40 PCR cycles 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec), and final incubation at 72°C for 10 min. Melting curves for all PCR products were analyzed over the temperature range 50–95°C and the sizes of amplified fragments were determined by agarose gel electrophoresis. Sample contamination by genomic DNA was monitored by running PCR for each gene using samples not subjected to reverse transcription.

TABLE 1. Baseline Contents of Proteins (ERK1/2 and AMPK) and Gene mRNAs (rows 3–8) in the Two Parts of the Medial Head of the Gastrocnemius Muscle in the Absence of Electrical Stimulation of the Motor Nerve

No.	Names of proteins and genes	White part	Red part	p(U)
1	ERK1/2	1.00 ± 0.16	1.00 ± 0.16	0.721
2	AMPK α_1/α_2	1.00 ± 0.12	0.85 ± 0.09	0.234
3	IGF-1	1.00 ± 0.11	0.73 ± 0.05	**0.028**
4	MyoD	1.00 ± 0.13	0.44 ± 0.05	**0.001**
5	Myogenin	1.00 ± 0.28	7.00 ± 1.45	**0.0002**
6	MaFbx	1.00 ± 0.14	0.85 ± 0.14	0.382
7	MuRF	1.00 ± 0.25	1.08 ± 0.12	0.328
8	Myostatin	1.00 ± 0.22	0.05 ± 0.01	**0.0002**

Note. Data are presented as proportions of the group means for WGM; p(U) is probability, Mann–Whitney test.

Quantities of mRNA for the genes of interest relative to the quantity of reference gene mRNA (geometric means for both genes) were assessed using the $\Delta\Delta C_t$ method. Baseline mRNA contents (unstimulated muscles) were expressed as proportions of the group mean for WGM. Changes in gene expression due to stimulation were determined as the ratio of the mRNA content in the corresponding muscles (RGM or WGM) in the stimulated and contralateral limbs.

Results were analyzed statistically using the nonparametric Mann–Whitney test (comparison of different muscles) and Wilcoxon test (effects of stimulation). Differences were regarded as statistically significant at $p < 0.05$. Data in the text, Figures, and Tables are presented as mean ± error of the mean, n = group size.

Results and Discussion

Data from comparison of the basal characteristics of WGM and RGM (tissues from the contralateral limb) are shown in Table 1. The content of ERK1/2 protein did not differ in the two parts of the gastrocnemius muscle (see Table 1). Published data on total ERK1/2 levels in fibers of different types are contradictory. Thus, for example, two studies [3, 34] showed higher ERK1/2 levels in the soleus muscle, which consists mainly of type I MF, as compared with the extensor digitorum longus muscle (20% type I MF and 40% type IIB MF [10]. However, data from other authors indicate that ERK1/2 expression levels did not differ in different types of MF [32]. The AMPK content also showed no difference between WGM and RGM (see Table 1), and an analogous result was reported by Lee-Young et al. [22].

At the same time, the contents of mRNA for many genes differed in WGM and RGM: lower levels of myogenin expression and higher levels of MyoD, IGF, and myostatin expression were seen in WGM than RGM. Levels of E3-ubiquitin ligase expression (MaFbx, MuRF) showed no difference between the two parts of the gastrocnemius muscle (see Table 1). The results described above are in good agreement with our previous data [1] and data reported by other authors [14, 16, 33].

During stimulation, the amplitude of tetanic muscle contractions decreased more than twofold by the end of the stimulation cycle (Fig. 1). This indicates that intense muscle contraction led to muscle fatigue, which is linked with significant metabolic shifts in muscle fibers and decreases in their energy potential [17]. Accumulation of metabolic products in muscles can affect the contractile apparatus of muscle fibers and can also lead to decreases in their excitability and impairment to neuromuscular transmission, these changes appearing earlier in glycolytic fibers than in oxidative fibers [2, 4, 25].

Muscle fatigue was reflected in changes in the levels of phosphorylation of regulatory kinases and gene expression (Figs. 2 and 3). Stimulation increased the levels of ERK1/2 phosphorylation in both RGM and WGM, though the content of phosphorylated ERK1/2 in WGM was significantly greater than that in RGM (Fig. 2, *A*); the total ERK1/2 contents in these tissues were not different (see Table 1). ERK1/2 phosphorylation is known to lead to activation of the cell cycle and cell proliferation. In skeletal muscle, this applies to satellite cells, whose proliferation is induced by intense loading and/or damage to muscle [36]. ERK1/2 activation also leads to suppression of apoptosis and prevents cell death, including in skeletal muscles [29, 35].

Proteins of the IGF family are powerful ERK1/2 activators in skeletal muscle [36]. The increase in ERK1/2 phosphorylation seen in our studies may at least partly be due to the action of IGF-1, whose expression level increases in both parts of the muscle on loading (Fig. 3). The role of IGF in the development of skeletal muscle hypertrophy due to strength training has been well studied. Muscle injury or physical loading induce rapid increases in the expression of one of the isoforms of IGF-1, i.e., MGF, which leads to activation of satellite cells. IGF also stimulates protein synthesis and the operation of the glycolytic pathway and inhibits E3-ubiquitin ligases MAFbx and MuRF, which are involved in protein degradation [19, 36]. Thus, the increase in the IGF-1 mRNA expression level seen here is evidence

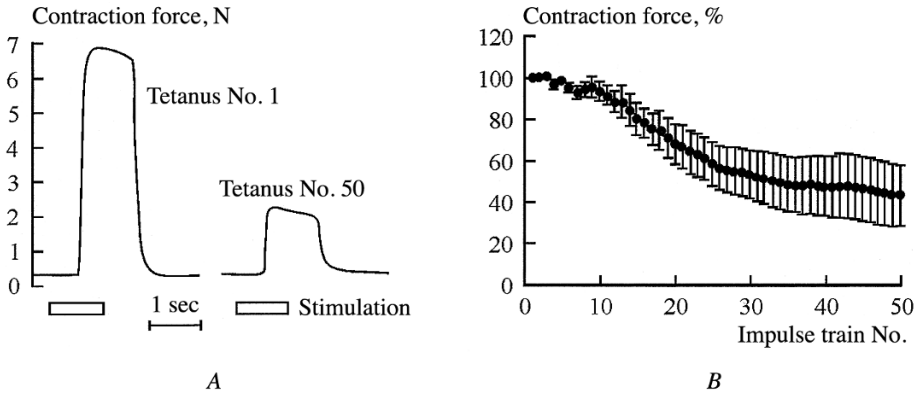

Fig. 1. Decreases in contraction strength of the medial head of the gastrocnemius muscle during sequential contractions induced by stimulation of the motor neve with trains of impulse (intratrain impulse frequency 100 Hz, train duration 2 sec, interval duration 17 sec). *A*) Traces of first and subsequent tetanic contractions in one experiment; *B*) decrease in mean contraction force during tetanus using 50 sequential nerve stimuli (mean data for eight experiments, % of force in response to first tetanus, which was 7.5 ± 0.3 N).

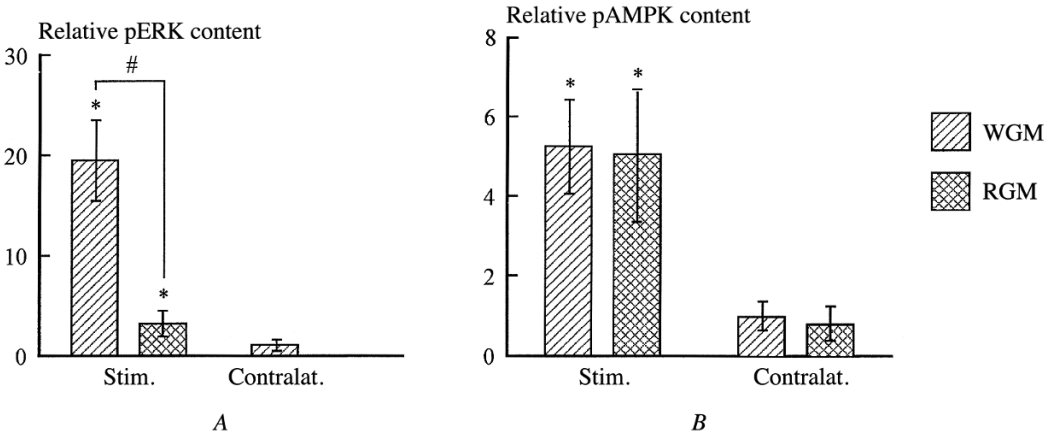

Fig. 2. Changes in the contents of phospho-ERK1/2 (*A*) and phospho-AMPK α_1/α_2 (*B*) in the two parts of the medial head of the gastrocnemius muscle from rats after series of stimuli applied to the motor nerve. Stim – Stimulated muscle; Contralat – contralateral muscle (unstimulated); WGM – white part of gastrocnemius muscle; RGM – red part of gastrocnemius muscle. Data are presented as proportions of the group means for the WGM of the contralateral limb. $^{*}p < 0.05$ compared with data for the contralateral limb (Wilcoxon test); $^{\#}p < 0.05$ between WGM and RGM (Mann–Whitney test).

of processes linked with muscle tissue regeneration and hypertrophy.

However, on the background of comparable changes in IGF-1 expression levels (Fig. 3), increases in ERK1/2 phosphorylation in WGM were significantly greater (Fig. 2, *A*), pointing to more marked ERK1/2-dependent regulation of growth processes in glycolytic MF as compared with oxidative MF. This ability of glycolytic MF may be linked with the greater basal level of IGF-1 production (see Table 1) or with higher IGF-1 sensitivity. The effects of other factors promoting ERK1/2 activation cannot be excluded. For example, activation of the ERK pathway is known to occur in conditions of elevated intracellular Ca^{2+} concentrations [31], contraction of fast glycolytic MF being linked with more significant increases in the Ca^{2+} concentration than slow oxidative MF [6].

The level of phosphorylation of another kinase, AMPK, also increases as a result of stimulation (Fig. 2, *B*), though in contrast to ERK1/2, there was no difference between RGM and WGM. Increased AMPK phosphorylation reflects activation of this signal pathway after series of muscle contractions, probably as a result of decreases in the energy potential of MF (increased AMP/ATP ratio). It should be noted that this signal pathway leads to activation of catabolic processes in MF: previous studies have demonstrated that it promotes increases in the level of E3-ubiquitin ligase expression [21], which provides damaged proteins with access to proteasomes for degradation. Increases in the expression

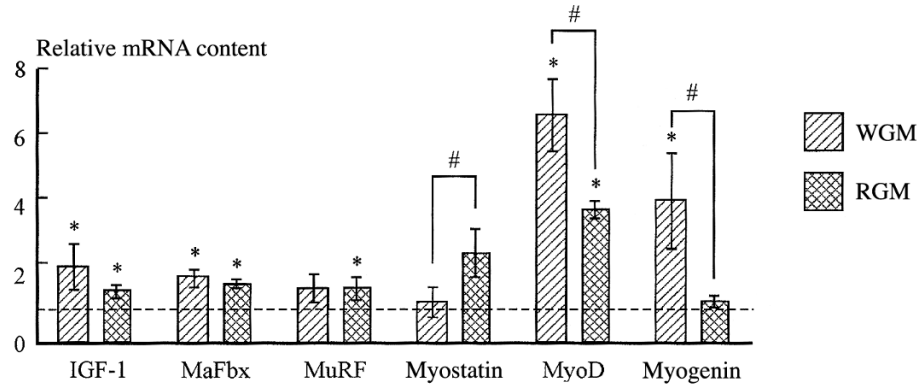

Fig. 3. Changes in gene mRNA contents in the two parts of the medial head of the gastrocnemius muscle from rats after series of stimuli applied to the motor nerve. WGM – white part of gastrocnemius muscle; RGM – red part of gastrocnemius muscle. Data are presented as ratio of mRNA contents in the corresponding tissues of the stimulated and contralateral limbs. $^{*}p < 0.05$ compared with data for the contralateral limb (Wilcoxon test); $^{\#}p < 0.05$ between WGM and RGM (Mann–Whitney test).

of these genes in muscles is generally seen in states leading to muscle atrophy, such as muscle denervation, diabetes mellitus, and cancer [11]. In our experiments, stimulation was also followed by increases in the expression levels of MAFbx and MuRF, though these were very moderate and there was no difference between RGM and WGM (Fig. 3). In all probability, brief (duration 1 sec) tetanic muscle contraction, even after multiple repetition, did not lead to marked activation of catabolic processes. Previous studies have shown that E3-ubiquitin ligase expression increases by factors of tens in muscle atrophy [13], while strength training produces increases by factors of less than 10 [12]. A moderate increase in E3-ubiquitin ligase activity is required for salvaging of proteins damaged by intense muscle operation and does not lead to MF atrophy, as is it compensated for by an increase in the rate of protein synthesis.

Myostatin, in contrast to IGF-1, is a negative regulator of protein synthesis and MF size [14, 33]. In WGM, stimulation did not lead to any change in myostatin expression, while in RGM there was a tendency to an increase in its expression in stimulated muscle as compared with the contralateral muscle ($p = 0.097$, Fig. 3) and the relative content of myostatin mRNA in RGM was greater than that in WGM 2 h after stimulation ended (Fig. 3). On the one hand, such an increase in myostatin expression in RGM seems paradoxical, as muscle hypertrophy after strength training is usually linked with decreased myostatin expression, particularly in glycolytic type II MF [14]. It is possible that the increase in myostatin expression after loading is a distinguishing feature of oxidative type I MF, which have a low basal level of myostatin expression (see Table 1). It is of note that an increase in myostatin mRNA expression after physical loading can occur in the heart [24].

Finally, we will consider post-stimulus changes in MyoD and myogenin mRNA expression – these are transcription factors which regulate satellite cell differentiation during embryonic development and muscle regeneration [36]. Various muscle injuries, including those occurring as a result of intensive strength training, activate satellite cell proliferation, which is accompanied by increases in MyoD expression. Myogenin expression increases later, at the stage at which myoblasts differentiate into muscle fibers. Satellite cell activity is among the causes of muscle hypertrophy [36]. Despite the fact that complete regeneration of muscle fibers requires several weeks, increases in MyoD and myogenin expression in satellite cells are seen within the first few days after injury or physical loading [19]. MyoD and myogenin expression is also seen in the nuclei of mature MF [18], where they can operate as transcription factors supporting high levels of expression of muscle-specific proteins [14].

As shown in Fig. 3, increases in the level of MyoD expression were seen in both parts of the muscle 2 h after stimulation, more extensively in WGM than RGM, while myogenin expression increased only in WGM. These changes may reflect more marked activation of satellite cell proliferation and differentiation in WGM, which consists of fast glycolytic MF; this suggestion is consistent with published data [9]. One cause of the more significant increase in the levels of MyoD and myogenin mRNA expression in WGM than RGM (at least in satellite cells) may be the difference in IGF-1 expression (see Table 1). These changes in the expression levels of these genes many also be related to acceleration of protein synthesis in MF. Thus, the changes in MyoD and myogenin described here in response to stimulation may lead to MF hypertrophy. The more significant changes in MyoD and myogenin expression in WGM than RGM (Fig. 3) are evidence of more marked hypertrophy-related changes occurring in WGM.

Conclusions

Our studies show that high-frequency electrical stimulation of the motor nerve, which leads to simultaneous

activation of all the motor units of the muscle at the beginning of stimulation, is accompanied by different changes in the levels of ERK1/2 phosphorylation and gene expression (myostatin, MyoD, and myogenin) in the two parts of the gastrocnemius muscle, consisting predominantly of glycolytic (WGM) and oxidative (RGM) MF. Glycolytic MF, as compared with oxidative, show more marked increases in ERK1/2 phosphorylation and levels of MyoD and myogenin expression despite the fact that during stimulation they can undergo more intense inactivation than oxidative MF as a result of the effects of muscle metabolites on the mechanisms of neuromuscular transmission and electromechanical linkage. These results provide evidence of earlier or more marked activation of satellite cells, and also increases in the rate of protein synthesis, in glycolytic MF, which may lead to more marked hypertrophy of these fibers than of oxidative MF. These data are consistent with views of post-work muscle hypertrophy consisting mainly of fast glycolytic MF.

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