Contractile Activity Modifies Fru-2,6-P₂ Metabolism in Rabbit Fast Twitch Skeletal Muscle

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Modification of muscular contractile patterns by denervation and chronic low frequency stimulation induces structural, physiological, and biochemical alterations in fast twitch skeletal muscles.

Fructose 2,6-bisphosphate is a potent activator of 6-phosphofructo-1-kinase, a key regulatory enzyme of glycolysis in animal tissues. The concentration of Fru-2,6-P₂ depends on the activity of the bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2), which catalyzes the synthesis and degradation of this metabolite. This enzyme has several isoforms, the relative abundance of which depends on the tissue metabolic properties. Skeletal muscle expresses two of these isoforms; it mainly contains the muscle isozyme (M-type) and a small amount of the liver isozyme (L-type), whose expression is under hormonal control. Moreover, contractile activity regulates expression of muscular proteins related with glucose metabolism. Fast twitch rabbit skeletal muscle denervation or chronic low frequency stimulation can provide information about the regulation of this enzyme. Our results show an increase in Fru-2,6-P₂ concentration after 2 days of denervation or stimulation. In denervated muscle, this increase is mediated by a rise in liver PFK-2/FBPase-2 isozyme, while in stimulated muscle it is mediated by a rise in muscle PFK-2/FBPase-2 isozyme. In conclusion, our results show that contractile activity could alter the expression of PFK-2/FBPase-2.

Skeletal muscle is a heterogeneous tissue, which responds differentially to a large variety of stimuli (1, 2). Modifications of basal contractile activity, induced by denervation or chronic low frequency stimulation, promote an adaptive response. These adaptations are reflected in muscle phenotype; i.e. there are variations in muscle type fiber composition, characterized by different biochemical and physiological properties.

One consequence of denervation or chronic low frequency stimulation in rabbit fast twitch skeletal muscle is the phenotypic transition from fast twitch to slow twitch fiber type (3–6). These changes involve variations in enzyme activities and, in some cases, isozyme expression. Fast twitch fibers are characterized by the predominance of anaerobic glycolysis, while in slow twitch fibers aerobic glycolysis is the main pathway by which energy demands are supplied during contraction. Thus, glucose metabolism is involved in this process.

The rate-limiting enzyme of glycolysis is 6-phosphofructo-1-kinase (PFK-1), which is regulated by a large number of modulators. The bisphosphorylated sugar fructose 2,6-bisphosphate (Fru-2,6-P₂) has been identified as a potent positive effecter of this enzyme in several mammalian tissues (7). Moreover, modifications of Fru-2,6-P₂ by factors such as hormonal or contraction pattern have been related with variations of the glycolytic rate (8–11).

The intramuscular concentration of Fru-2,6-P₂ is controlled by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2), which is responsible for the synthesis and degradation of this metabolite. This enzyme has several isoforms, the relative abundance of which depends on the metabolic properties of the tissue. These isozymes differ in size, kinetics, and immunologic properties as well as in their response to phosphorylation by protein kinases. Skeletal muscle expresses two of these isoforms. It mainly contains muscle isozyme (M-type), and a small amount of the liver isozyme (L-type) (12–16). Both isoforms are encoded by the same gene, as a result of alternative splicing, whose expression is under hormonal control (17).

Moreover, contractile activity has a regulatory effect on some proteins related with glucose metabolism in fast twitch skeletal muscle. The absence of contractions induces an increase in GLUT-1 (18) and HK-II (19, 20), while an increase in contractile activity induces a rise in GLUT-4 (21) and HK-II (22). These studies highlight the importance of muscle contraction in the regulation of protein expression related with glucose metabolism.

Here we examine the influence of contractile activity on Fru-2,6-P₂ metabolism and its regulation through PFK-2/FBPase-2. We measured the changes in this enzyme induced by denervation or by chronic low frequency stimulation at 10 Hz in fast twitch rabbit skeletal muscle.

EXPERIMENTAL PROCEDURES

Chemicals—[γ-³²P]ATP (5000 Ci/mmoll), [α-³²P]dCTP (3000 Ci/ mmol), and Hybond N filters were purchased from Amersham Pharmacia Biotech. The random priming DNA labeling kit was from Roche Molecular Biochemicals. Enzymes and biochemical reagents were from either Roche Molecular Biochemicals or Sigma. All other reagents were of analytical grade.

Treatment of Animals—Adult White New Zealand rabbits weighing between 3.0 and 3.5 kg were fed ad libitum and housed in animal quarters with a 12-h light, 12-h dark cycle. For denervation studies, animals were anesthetized by intramuscular injection of 20 mg/kg...
ketamine (Ketolar, Parke-Davis) and a 2-ml solution of 0.5% azepromazine (Calmoneose, Smithkline Beecham), the peroneal nerve was unilaterally severed, and 10–15 mm of the distal stump was removed. Finally, the proximal part of the nerve was ligated in the Biceps femoris muscle to avoid reinnervation. The contralateral control leg was sham-operated.

For stimulation studies, under the same anesthesia protocol as in denervated animals, 1 week before the onset of stimulation period, electrodes were implanted laterally to the peroneal nerve as described elsewhere (23). This allowed the continuous stimulation at 10-Hz trains (trains of pulses of 18 mA for 0.15 ms) for 24 h/day.

After various periods of denervation or stimulation (1, 2, 4, 7, or 14 days), right (control) and left (denervated or stimulated) tibialis anterior (TA) muscles were carefully exposed. Both muscles were removed simultaneously by two researchers and quickly frozen with aluminum clamps precooled in liquid N2. The frozen samples were stored at −80 °C until analysis.

**Assay of Metabolites**—Samples of frozen muscle were powdered in a stainless steel percussion mortar precooled in liquid N2 and used for the measurement of metabolites. Glycogen was extracted from about 10 mg of muscle by alkaline treatment. The hydrolysis and measurement of glucose produced were carried out using the anthrone method (24). ATP, glucose 6-phosphate (Glc-6-P) and fructose 6-phosphate (Fru-6-P) were extracted by acid treatment (10 mg in 20 ml of 0.25 N NaOH). After neutralization and centrifugation, aliquots of the extracts were used. Measurements were carried out using enzymatic methods with fluorimetric techniques (25).

For measuring fructose 2,6-bisphosphate (Fru-2,6-P2), about 30 mg of frozen muscle powder was homogenized in 10 volumes of 50 ml NaOH and kept at 90 °C for 10 min. The extract was neutralized with 250 mM sodium acetate (pH 4.0), and the soluble material was used for determination of Fru-2,6-P2 (26).

**Partial Purification of 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase**—Muscle samples were homogenized in 5 volumes of 20 mM TES, 100 mM KCl, 1 mM diithiothreitol, 5 mM EDTA, 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 2.5 mg/ml leupeptin, pH 7.5. The crude extracts were fractionated with 6% (w/v) polyethylene glycol 6000 and centrifuged at 26,000 × g for 30 min. After supernatants were fractionated again with 15% (w/v), polyethylene glycol 6000 and centrifuged in the same conditions described before. Finally, the pellets were resuspended in a half volume of initial tissue weight with homogenizing medium and used to measure PFK-2/FBPase-2 activity (27).

**Enzyme Assays**—PFK-2 activity was measured as described by El-Maghrabi et al. (28). Partially purified (6–21% PEG) TA muscle PFK-2 activity was assayed at 30 °C in 0.1 ml of 20 mM TES, 10 mM KCl, 50 mM MgCl2, 25 mM KH2PO4, 0.5 mM EDTA, 5 mM EGTA, 1.2 mM phenylmethylsulfonyl fluoride, 2.5 mg/ml leupeptin, 25 mM ATP, 25 mM Fru-6-P under optimal conditions and 0.5 mM ATP, 0.1 mM Fru-6-P under suboptimal conditions. The reaction was stopped by adding 5 ml of 2.5 N NaOH, and samples were held at 90 °C for 10 min, after which time samples were used for assay of Fru-2,6-P2.

FBPase-2 activity was measured by the production of [32P]Fru-6-P from [2,2-32P]Fru-6-P2, which was synthesized as described by El-Maghrabi et al. (28). The reaction was carried out at 30 °C in 50 ml of Hepes buffer (pH 7.5) containing 50 mM KH2PO4, 5 mM MgCl2, 2 mM EDTA, 1 mM diithiothreitol, 2 mM MgCl2, 5 mM ATP, 15 mM dithiothreitol, and 0.25 mM [2-32P]Fru-2,6-P2 (200,000 cpm/assay). The product of the reaction, fructose 6-phosphate, was removed by an enzymatic system consisting of 0.1 ml NADP+, 9 units/ml phosphoglucoisomerase, and 4 units/ml glucose 6-phosphate dehydrogenase. The reaction was stopped by the addition of 1 volume of 0.1 M NaOH. Blanks did not exceed 0.2% of the applied radioactivity. Treatment of PFK-2 with the Catalytic Subunit of Cyclic AMP-dependent Protein Kinase—Partial purified PFK-2 from control, denervated, and stimulated TA muscle were incubated at 30 °C for 20 min with the catalytic subunit of cyclic AMP-dependent protein kinase (1 milliunit/ml) in a final volume of 0.1 ml containing 100 mM Hepes (pH 7.1), 1 mM diithiothreitol, 0.1% bovine serum albumin. The reaction was started by the addition of 1 ml ATP-Mg2+ and stopped by the addition of 5 ml EDTA. Samples were removed and assayed for PFK-2 activity (28, 29).

**Western Blot Analysis of PFK-2/FBPase-2**—Control, denervated, and stimulated muscle PFK-2/FBPase-2 were partially purified by PEG fractionation followed by SDS-polyacrylamide gel electrophoresis in a 10% acrylamide gel. Proteins were then transferred to a nitrocellulose membrane (Immobilon-P, polyvinylidene difluoride, Millipore Corp.). For immunodetection, two antibodies were used: polyclonal antiserum MCL-2 (30) against L-type and M-type PFK-2/FBPase-2 isoforms and polyclonal antiserum CL against t-type PFK-2/FBPase-2 isoform (31). Both antibodies were a generous gift from Dr. Hue. MCL-2 and CL IgG were produced in rabbits. Primary antibody was preincubated for 1 h at 4 °C with a secondary antibody (peroxidase-conjugated anti-rabbit). To prevent an excess of secondary antibody, a third IgG, against a maize protein that is not present in mammalian tissue, was added and incubated for 1 h. Later, this mixture was used for incubating the membrane (2 h) and for detecting PFK-2/FBPase-2 by enhanced chemiluminescence, (ECL, Amersham Pharmacia Biotech). A control test of rabbit blood ruled out any non-specific disturbance attributable to saturation of the secondary with the tertiary antibody.

**RNA Isolation and Northern Blot Analysis**—Total RNA from muscle was extracted using the acid guanidinium thiocyanate-phenol/chloroform method as described by Chomczynski and Sacchi (32). All samples had a 260/280 absorbance ratio above 1.7.

After quantification, total RNA (15–30 µg) was denatured at 65 °C in the presence of formamide, formaldehyde, and ethidium bromide (33) to allow the visualization of RNA. RNA was separated on a 1.2% agarose/formaldehyde gel and blotted on Hybond N filters. The RNA in gels and filters was visualized with ethidium bromide and photographed by UV transillumination to ensure the integrity of RNA, to check the loading of equivalent amounts of total RNA, and to confirm proper transfer. Radiograms were exposed to X-ray film for 2 h at −80 °C.

**Results**

**Effects of Muscle Denervation or Stimulation on Muscle Fructose-2,6-bisphosphate Level**—Fru-2,6-P2 levels were determined at different times after TA muscles were denervated or stimulated. Both treatments increased Fru-2,6-P2 (Fig. 1).

In denervated muscle, a significant increase occurred 2 days after nerve section, and it persisted throughout the 14 days of the experiment. At the end of this period Fru-2,6-P2 concentration was approximately 3–4-fold higher than the control value. Chronic low frequency stimulation also increased Fru-2,6-P2. By day 1, there was a significant increase, which persisted at day 4. The peak at day 4 was approximately 4–5-fold higher than control. As stimulation continued, the concentration declined to control values at day 30 (results not shown). Fru-2,6-P2 values in control remained constant throughout the experiment.

Because levels of Fru-2,6-P2 in skeletal muscle are controlled by substrate availability (12), its precursors (Glc-6-P, Fru-6-P, ATP) were studied. Denervation or stimulation had different effects on the intracellular concentration of these metabolites (Table I).

In denervated muscle, Glc-6-P and Fru-6-P decreased by more than 50% at day 4, and their concentration remained significantly lower than that of control muscle. ATP showed a significant decrease at day 4, which persisted for 14 days.

In stimulated muscle, although Glc-6-P was already lower on the first day, Fru-6-P followed the same pattern as in denervated muscle. However, stimulated muscle these metabolites
return to control values at day 14. ATP was constant throughout this period.

**Effects of Denervation or Stimulation on PFK-2 and FBPase-2 Activities**—In order to explain the increases observed in Fru-2,6-P₂ after denervation or stimulation of muscle, we measured the enzyme activity for the synthesis and degradation of this bisphosphorylated metabolite, using partial purified (6–21% PEG) TA muscle homogenate.

Denervation and stimulation increased PFK-2 activity (Fig. 2A). In denervated muscle, kinase activity increased significantly at day 1, reaching the highest value at day 2. PFK-2 activity then decreased rapidly to control values. The pattern of change in stimulated muscle was similar to the changes in denervated muscle. Kinase activity increased significantly at day 1, reached a maximum at day 4, and declined thereafter to control values. The response of FBPase-2 activity depended on contractile activity throughout this period.

**Effects of Denervation or Stimulation on the Quantity of PFK-2/FBPase-2 Protein**—In order to determine the relative amounts of PFK-2/FBPase-2 protein, we used polyclonal antiserum MCL-2 (30), which recognizes L- and M-type isoforms. The signal intensity from denervated muscle (Fig. 5A, lane D) was 10% of control muscle (lane C). In contrast, the signal from stimulated muscle (Fig. 5A, lane S) increased 2.5-fold.

To determine the L-type isoform, we used the polyclonal antiserum CL raised against the L-type isozone (31). The signal from denervated muscle (lane D) was twice as strong as that signal from control muscle (lane C) (Fig. 5B).

**DISCUSSION**

We examined the influence of contractile activity on Fru-2,6-P₂ concentration and the influence of PFK-2/FBPase-2 activity on fast twitch skeletal muscle. To this end we chose two situations: (i) denervated fast twitch skeletal muscle, which is characterized by the absence of contractions, and (ii) chronic low frequency stimulated fast twitch skeletal muscle, which is characterized by increased contractile activity.

Our results show that both conditions induced an increase in Fru-2,6-P₂ and a modification of the L- and M-type PFK-2/FBPase-2 isozone expression. Moreover, we found these changes may depend on contractile activity. Denervation provoked an increase in L-type isozone, while chronic low frequency stimulation induced an increase in M-type isozone.

It has been found that Fru-2,6-P₂ concentration is dependent on substrate availability (7); however, the lack of variations in Fru-6-P and ATP in our experiments suggested that Glc-6-P, Fru-6-P, and ATP were not responsible for the increase in Fru-2,6-P₂. Because Fru-2,6-P₂ concentration is also dependent on synthesis/degradation equilibrium, we focused our attention on the study of PFK-2/FBPase-2 activities.

In denervated muscle, the increased PFK-2 activity and the concomitant reduction in FBPase-2 could thus explain the increase in Fru-2,6-P₂. The PFK-2/FBPase-2 ratio increased after denervation from 0.3 to 2.4. This change toward values characteristic of liver-type isozone was interpreted as resulting from increased L-type isozone synthesis. Since there is evidence that L-type could be differentiated from M-type by the inhibitory effect of sn-glycerol-3-phosphate and by the lower kinase activity after phosphorylation by cyclic AMP-dependent protein kinase.
protein kinase (35), we proposed using these characteristics to examine the possible change from M-type to L-type. Our findings are consistent with an increase in L-type isozyme. This possibility was supported by an increased signal in Western blot analysis, with specific L-PFK-2/FBPase-2 antibody. In conclusion, denervation elicited a change from M-type to L-type in fast twitch skeletal muscle.

In stimulated muscle, the increase in Fru-2,6-P₂ was coincident with increases in kinase activity, which confirms previous results (11). Moreover, stimulation induces increases in FBPase-2. The PFK-2/FBPase-2 ratio therefore remained constant throughout the stimulation period. This ratio was similar to values described elsewhere in skeletal muscle from rat (14). The lack of variation in this ratio indicated that the isozyme type was not modified. This was confirmed by the absence of sn-glycerol-3-phosphate inhibition (Fig. 3) and the lack of effect of the catalytic subunit of cyclic AMP-dependent protein kinase on kinase activities (Table II). Since isozyme expression did not appear to have changed, the increase in kinase and phosphatase activity was attributed to a rise in M-type PFK-2/FBPase-2.

![FIG. 2. Time course of PFK-2/FBPase-2 activities. PFK-2/FBPase-2 activities were measured from extract treated with PEG 6–21% under conditions described under “Experimental Procedures.” A, the time course of PFK-2 activity; B, the time course of FBPase-2 activity; C, the ratio PFK-2/FBPase-2 from denervated (○), stimulated (■), or control muscle (□). Each point shows the mean ± S.E. for 4–6 samples. * significant difference (p < 0.05) between zero point and values after the onset of stimulation.](image)

![FIG. 3. Effects of sn-glycerol 3-phosphate on PFK-2 activity from control, denervated, and stimulated rabbit tibialis anterior muscle. Partially purified PFK-2 from control (○), 2-day denervated (●), and 2-day stimulated (■) muscles were assayed in suboptimal conditions in the presence of 0.1 mM fructose 6-phosphate and 0.5 mM ATP-Mg²⁺ and with the indicated concentrations of sn-glycerol 3-phosphate at pH 7.1. Each point shows the mean ± S.E. for 4–6 samples. * significant difference (p < 0.05) between levels of control and denervated or stimulated muscles.](image)

**TABLE I**

| Metabolite concentration after denervation or stimulation | 0 days | 1 day | 2 days | 4 days | 7 days | 14 days |
|----------------------------------------------------------|--------|-------|--------|--------|--------|--------|
| Glc-6-P Denervated                                       | 1.23 ± 0.09 | 0.98 ± 0.17 | 1.06 ± 0.11 | 0.38 ± 0.07* | 0.24 ± 0.01* | 0.38 ± 0.05* |
| Fru-6-P Denervated                                       | 0.192 ± 0.02 | 0.188 ± 0.05 | 0.197 ± 0.04 | 0.073 ± 0.01* | 0.046 ± 0.01* | 0.071 ± 0.01* |
| ATP Denervated                                           | 5.71 ± 0.26 | 6.53 ± 0.17 | 6.36 ± 0.25 | 2.79 ± 0.18* | 3.06 ± 0.08* | 3.42 ± 0.23* |

*Statistically significant differences between control (0 days) and denervated or stimulated TA muscle (p < 0.05).
Contractile Activity and PFK-2/FBPase-2

TABLE II
Effects of treatment of PFK-2 with the catalytic subunit of cAMP
Protein kinase

A

| Tissue PEG fraction | Specific activity of PFK-2 | Decrement |
|---------------------|---------------------------|-----------|
| No catalytic subunit | Plus catalytic subunit    | %         |
| Control             | 0.326 ± 0.097             | 0.341 ± 0.053 |
| 2-Day stimulated    | 0.773 ± 0.197             | 0.766 ± 0.267 |
| 2-Day denervated    | 1.516 ± 0.188             | 0.745 ± 0.091 |

* Statistically significant differences between incubations in presence or absence of catalytic subunit (p < 0.001).

FIG. 4. Expression of PFK-2/FBPase-2 mRNA on control, denervated, and stimulated rabbit tibialis anterior muscle. Total RNA was purified from denervated and stimulated muscles were partially purified by polyethylene glycol fractionation, as described under “Experimental Procedures.” SDS-polyacrylamide gel electrophoresis (40 μg of protein/lane) was then analyzed by Western blot using polyclonal antisera MCL-2 (30) against L-type and M-type PFK-2/FBPase-2 isozymes (A) and polyclonal antisera CL against L-type PFK-2/FBPase-2 isozyme specifically (31). A liver sample was used as a positive control of L-type (lane L) (B). Control muscle; lane D, 2-day denervated muscle; lane S, 2-day-stimulated muscle.

FIG. 5. Western blot analysis of PFK-2/FBPase-2 in control, denervated, and stimulated rabbit tibialis anterior muscle. Samples of control, denervated, and 2-day stimulated muscles were partially purified by polyethylene glycol fractionation, as described under “Experimental Procedures.” SDS-polyacrylamide gel electrophoresis (40 μg of protein/lane) was then analyzed by Western blot using polyclonal antisera MCL-2 (30) against L-type and M-type PFK-2/FBPase-2 isozymes (A) and polyclonal antisera CL against L-type PFK-2/FBPase-2 isozyme specifically (31). A liver sample was used as a positive control of L-type (lane L) (B). C, control muscle; lane D, 2-day denervated muscle; lane S, 2-day-stimulated muscle.

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