Muscle contractile activity increases fatty acid metabolism and transport and FAT/CD36

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1Department of Kinesiology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1; and 2Department of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, New York 11749-8661

Bonen, Arend, David J. Dyck, Azeddine Ibrahimi, and Nada A. Abumrad. Muscle contractile activity increases fatty acid metabolism and transport and FAT/CD36. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E642–E649, 1999.—We have examined whether 1) fatty acid (FA) uptake, 2) FA transporter expression, and 3) FA metabolism are increased when the oxidative capacity of skeletal muscle is increased. The oxidative capacities of red and white tibialis anterior and extensor digitorum longus muscles were increased via chronic stimulation (10 Hz, 24 h/day for 7 days). The contralateral muscles served as controls. After 7 days of increased muscle activity 1) palmitate uptake by giant sarcolemmal vesicles was increased twofold (P < 0.05), 2) the expression of FA translocase (FAT)/CD36 was increased at both the mRNA (3.2–to 10-fold) and protein (3.4-fold) levels, and 3) palmitate oxidation and esterification into triacylglycerols and phospholipids were increased 1.5–, 2.7–, and 1.7-fold, respectively (P < 0.05). These data show that when the oxidative capacity of muscle is increased, there is a parallel increase in the rate of FA transport and FA transporters at the sarcolemmal membrane, which is associated with the enhanced expression of the membrane transporter FAT/CD36.

LIPIDS, and in particular circulating fatty acids (FAs), are important substrates for skeletal muscle metabolism (35, 40). The uptake and oxidation of circulating FA by muscle increase rapidly when the energy demands are increased by exercise (40). Because contraction of isolated muscles also increases the uptake and metabolism of palmitate (8), it would be expected that the uptake of FAs is regulated at the level of the muscle, possibly involving the protein-mediated uptake of FAs in this tissue (7).

Exercise training increases the oxidative capacity of muscle and its reliance on lipids as an energy source (40). However, there is no information on whether training also enhances the membrane transport of FA, although such an increase would constitute an important adaptive mechanism to provide more FA for the working muscle. An increase in FA transport would be required if transport was rate limiting for FA metabolism, as has been suggested to be the case in isolated adipocytes (3). Increases in the transport rates of some circulating substrates (glucose and lactate) have been shown when the oxidative capacity of muscle is increased after a period of chronically increased muscle activity (e.g., exercise training and chronic muscle stimulation). These increased transport rates of glucose and lactate are due to the increased expression of their respective transport proteins glucose transporter isoform 4 (GLUT-4) (17, 29) and monocarboxylate transporter-1 (MCT-1) (5, 26).

Because of the lipid environment of the plasma membrane, it had been assumed that FAs simply traverse the sarcolemma by passive diffusion, and as a result, their membrane uptake cannot be regulated. However, strong and compelling evidence has been presented over the last decade to support the existence of a carrier-mediated component of long-chain FAs in adipocytes, (3), hepatocytes (36), cardiac myocytes (6), and skeletal muscle membrane vesicles (7). Several candidate FA transport proteins have been identified in the past few years. A FA binding protein associated with the plasma membrane (FABPpm) has been isolated by affinity chromatography of solubilized membrane proteins (16). Two integral membrane proteins, FA translocase (FAT) FAT/CD36 (1) and FA transport protein (FATP) (32), have also been identified by affinity-labeling and expression cloning, respectively. Expression of each of these transporters increases FA uptake rates (1, 16, 32). Although the tissue distribution of these transporters differs significantly, they are coexpressed in many tissues including skeletal muscle (6, 7, 39).

Expression of FAT is higher in muscle with a predominance of red oxidative as opposed to white glycolytic fibers (7). This difference also parallels differences in the protein-mediated FA uptake by vesicles obtained from red and white muscle (7). In addition, FAT increases significantly under conditions where FA utilization is increased, such as during muscle development (41) or when the animal is fed a high-fat diet (10), suggesting it plays an important role in muscle adaptation to a change in metabolic needs.

In this report, we have examined whether increasing the oxidative capacity of muscle, and consequently its reliance on lipid utilization (23), can be associated with concurrent increases in expression of FAT/CD36 and in FA transport into giant sarcolemmal vesicles. Remarkably rapid (≤7 days) and reproducible increases in muscle oxidative capacities and in the expression of substrate transporters can be obtained by chronically increasing muscle contractile activity with electrical stimulation of the peroneal nerve (10 Hz, 24 h/day) (17, 25, 26, 28). This experimental model also offers a number of advantages. First, only selected muscles in...
one hindlimb are made to contract [i.e., red (R) and white (W) tibialis anterior (TA) and extensor digitorum longus (EDL)] while the same contralateral hindlimb muscles from the same animal serve as the control. Second, adaptive responses in metabolically heterogeneous muscles, such as the TA and WTA, can be examined under the same conditions. Third, the EDL can be used in vitro to examine changes in muscle lipid metabolism (9). Finally, giant sarcolemmal vesicles can be obtained from chronically stimulated and nonstimulated (control) muscles to examine changes in substrate transport (24). In the present study, we have examined the effects of chronically increased muscle activity on FAT/CD36 mRNA and protein levels and on FA transport into giant sarcolemmal vesicles. In addition, changes in the oxidation and esterification of palmitate were examined in isolated chronically stimulated and control EDL muscles in vitro.

METHODS

In these experiments, we used male Sprague-Dawley rats (~250 g). All procedures were approved by the committee on animal care at the University of Waterloo. Animals were provided with rat chow and water ad libitum. They were kept on a reverse 12-h light (6 PM to 6 AM) and 12-h dark cycle (6 AM to 6 PM).

Chronic Electrical Stimulation of Rat Muscles

Muscles were stimulated as previously described by our laboratory (17, 24, 26). Briefly, in anesthetized rats, stainless steel electrodes were sutured to underlying muscles on either side of the peroneal nerve. These electrodes were passed subcutaneously from the thigh and exteriorized at the back of the neck, where they were attached to a miniature electronic stimulator. After we waited at least 5 days to allow animals to recover from surgery and regain preoperative weight, stimulus pulses (10 Hz, 50 µs duration) were initiated. The peroneal nerve, which innervates the TA and EDL muscles, was stimulated for 24 h/day for 7 days. Thereafter, animals were subdivided into two groups of animals. In one group, palmitate metabolism was determined in incubated control and chronically stimulated EDL muscles, and FAT/CD36 protein and mRNA levels were measured in chronically stimulated and control TA and WTA muscles (n = 5 rats). In another group of rats (n = 18 rats), the muscles within each treatment were pooled (3-4 rats/pool) for the purposes of preparing giant sarcolemmal vesicles from chronically stimulated muscles for the purpose of measuring palmitate uptake. All animals were euthanized early during their dark cycle (9–10 AM).

Lipid Metabolism in EDL Muscles

Palmitate metabolism was determined in isolated EDL muscles with procedures described elsewhere (8, 9). Briefly, muscles were incubated for 1 h at 30°C in 1.5 ml of Krebs-Henseleit buffer (95% O2-5% CO2, pH 7.4), containing 4% FA-free BSA, 10 mM glucose, and [9,10-3H]palmitate (3 µCi/vial; 1 nM). Tritiated H2O produced from the oxidation of [9,10-3H]palmitate was separated from the labeled substrate by adding 2.5 ml of 2:1 chloroform-methanol to a 0.5-ml aliquot of incubation medium, followed by the addition of 1 ml of 2 M KCl-HCl. The solution was mixed and centrifuged at 3,000 g for 5 min. The aqueous phase (1 ml) was removed and treated again with chloroform-methanol and KCl-HCl. A final 0.5-ml aqueous aliquot was removed for liquid scintillation counting.

To determine palmitate incorporated into intramuscular lipid pools during the incubation period, muscles were blotted, weighed, and placed in plastic centrifuge tubes. Lipids were then extracted exactly as we have described in detail in previous studies (8, 9). Once extracted, 50 µl of each sample were spotted in its own lane on an oven-dried silica gel plate (Silica Gel GF, 250 µm; Analtech, Newark, DE). Lipids were separated by thin-layer chromatography as previously detailed (8, 9). Thereafter, the individual lipid bands were marked on the plate with a scalpel and scraped into vials for liquid scintillation counting.

FA Uptake by Giant Sarcolemmal Vesicles

Our laboratory recently characterized palmitate uptake by giant sarcolemmal vesicles obtained from skeletal muscles (7). Giant sarcolemmal vesicles were prepared as previously described by our laboratory (7, 24) and others (18, 30). Briefly, chronically stimulated and control rat hindlimb muscles were obtained. Because of the tissue needs for this procedure, control and chronically stimulated muscles from 3-4 rats were combined. The muscles obtained were cut into thin layers (~1-3 mm thick) and incubated for 1 h at 34°C in 140 mM KCl-10 mM MOPS (pH 7.4), 150 U/ml collagenase, and aprotinin (0.01 µg/ml). The muscles were then washed with KCl-MOPS and 10 mM EDTA, and the supernatant was collected. Percoll (final concentration 16%) and aprotinin were added to the supernatant. This supernatant was placed at the bottom of a density gradient consisting of a 3-ml middle layer of 4% Nycodenz (wt/vol) and a 1-ml KCl-MOPS upper layer. The samples were spun at 60 g for 45 min at room temperature. After centrifugation, the vesicles were harvested from the interface of the two upper solutions. The vesicles were diluted in KCl-MOPS and recentrifuged at 800 g for 30 min.

FA Transport

Palmitate uptake was measured by addition of unlabeled (Sigma) and radiolabeled (Amersham) 0.3 µCi [9,10-3H]palmitate and 0.06 µCi [14C]mannitol (Amersham) in a 0.1% BSA KCl-MOPS solution to 40 µl of vesicles (~80 µg protein). The reaction was carried out at room temperature for 10 s, unless otherwise specified. Palmitate uptake was terminated by addition of 1.4 ml of ice-cold KCl-MOPS-2.5 mM HgCl and 0.1% BSA. The sample was quickly centrifuged at maximal speed in a microfuge for 1 min. The supernatant was discarded, and radioactivity was determined in the tip of the tube. Non-specific uptake was measured by adding the stop solution to the membrane before the addition of the isotopes.

FAT/CD36 Protein Analysis

Vesicle samples (50 µg protein) were solubilized and electrophoresed according to Laemmli (20). This was followed by transfer to a nitrocellulose membrane (Duralose, Stratagene). The membrane was incubated for 2 h with a 1:500 dilution of the monoclonal anti-CD36 antibody, washed, and then incubated with a 1:10,000 dilution of secondary antibody labeled with horseradish peroxidase. Immune complexes were detected with enhanced chemiluminescence (ECL; Amersham) according to directions of the manufacturer. FAT/CD36 protein band densities were obtained by scanning the blots on a densitometer connected to a Macintosh LC computer with appropriate software.
RNA Analysis

RNA was prepared with the RNA STAT-60 kit 9 (Tel-Test). Red and white muscle tissues were removed from liquid nitrogen and homogenized manually (Kontes glass homogenizer; Teflon pestle B) into 1 ml of RNA STAT-60 buffer. RNA was electrophoresed on a denaturing agarose gel, transferred to Hybond N+ membranes (Amersham), and hybridized with ~10^6 counts·min^{-1}·ml^{-1} of randomly primed ^32P-labeled DNA probes as previously described (15). Membranes were exposed to Hyperfilm (Kodak) at −75°C. mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

Muscle Fiber Composition and Enzyme Determinations

Stimulated and control muscles were analyzed for muscle fiber composition as previously described (27). For this purpose, a small portion of freshly excised muscle was embedded in Tissue-Tek optimum cutting temperature compound (Miles) and frozen in isopentane that was cooled in liquid nitrogen. Serial cross sections (10 µm) were stained for myofibrillar ATPase (at pH 10.3), NADH, and GAPDH. Muscle fibers were classified according to the method of Peter et al. (27).

Statistics

The data were analyzed with paired t-tests using a commercially available statistical package (STATVIEW). All data are reported as means ± SE.

RESULTS

After we implanted the stainless steel wires around the peroneal nerve, the animals lost ~10% of their body weight in the first three days after surgery. Thereafter, they began gaining weight, and at the end of the 7 days of chronic stimulation (14 days after surgery), muscle weights were greater than before surgery. This is similar to results reported previously by our laboratory with this experimental model (17, 24, 26).

With 7 days of chronic muscle stimulation, the fiber composition of the three types of skeletal muscles was not markedly altered, except that in the RTA there was a small increase in the fast oxidative glycolytic fibers at the expense of the fast glycolytic fibers (Table 1). No other changes in muscle fiber composition were observed in either the chronically stimulated WTA or EDL muscles (Table 1). In a previous study (24), our laboratory showed that the oxidative capacities of chronically stimulated muscles are markedly increased as indicated by a two- to threefold increase in citrate synthase activity of red and white muscles. This was confirmed in several muscles examined in the present studies (data not shown).

Table 1. Muscle fiber composition of control and 7-day chronically stimulated rat hindlimb muscles

<table>
<thead>
<tr>
<th>Muscle</th>
<th>%Slow Oxidative</th>
<th>%Fast Oxidative Glycolytic</th>
<th>%Fast Glycolytic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Stimulated</td>
<td>Control</td>
</tr>
<tr>
<td>RTA</td>
<td>11.3 ± 0.9</td>
<td>9.5 ± 1.1</td>
<td>60.8 ± 2.4</td>
</tr>
<tr>
<td>WTA</td>
<td>0</td>
<td>0</td>
<td>23.5 ± 3.6</td>
</tr>
<tr>
<td>EDL</td>
<td>3.5 ± 0.7</td>
<td>2.2 ± 0.7</td>
<td>36.2 ± 4.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals. RTA, red tibialis anterior; WTA, white tibialis anterior; EDL, extensor digitorum longus. *Significant difference vs. control, P < 0.05.

FAT mRNA and FAT/CD36 Protein in Red and White Muscles

The abundance of FAT mRNA (Fig. 1) was greater in red muscle than in white muscle (Fig. 1). The plasma membrane FAT/CD36 protein content was also greater in vesicles obtained from red muscles compared with those obtained from white muscles (Fig. 1). This confirms recent observations in these tissues (7).

Effects of Chronic Stimulation on FAT mRNA, FAT/CD36 Protein, and Palmitate Uptake

FAT mRNA. After 7 days of chronic muscle stimulation, the abundance of FAT mRNA was increased 3-fold (P < 0.05) in the RTA and 8.7-fold (P < 0.05) in the WTA muscles (Fig. 2). FAT mRNAs in RTA and WTA muscles were similar after 7 days of chronic stimulation (P > 0.05; Fig. 2).

FAT/CD36 protein and palmitate uptake. Because tissue requirements for giant vesicle preparation are high, it was necessary to pool the EDL and the red and white portions of TA muscles from four animals to obtain sufficient material to perform one transport assay and a Western blot in control and stimulated muscles. Thus, for these measurements, we compared only control and stimulated muscles.

The FAT/CD36 protein in giant vesicles obtained from chronically stimulated muscle was increased 3.4-fold (Fig. 3). Concurrently, the uptake of palmitate by giant sarcolemmal vesicles was increased twofold in chronically stimulated muscles (Fig. 4).

Palmitate Metabolism in EDL Muscle In Vitro

After 7 days of chronic electrical stimulation, the oxidation of palmitate in resting EDL muscle was increased 1.5-fold (P < 0.05; Table 2). Palmitate incorporation into the triacylglycerols and phospholipids was also increased 2.5- (P < 0.05) and 1.7-fold (P < 0.05), respectively (Table 2). Similar increases were observed in the mono- (1.9-fold; P < 0.05) and diacylglycerol (1.4-fold; P < 0.05; data not shown).

Comparison of Increase in Palmitate Uptake in Giant Vesicles and Palmitate Metabolism in Intact Muscle

Total palmitate uptake was determined by summing the palmitate oxidation and palmitate incorporation into the various lipid pools (i.e., phospholipids and tri-, di-, and monoacylglycerols) in the EDL muscles. This showed that total palmitate uptake by the intact,
chronically stimulated EDL muscle was increased 1.9fold (P < 0.05). When we compared this relative (%) increase in palmitate metabolism with the relative increase in palmitate transport in the chronically stimulated muscles, the changes were identical (Fig. 5).

DISCUSSION

The present study showed that chronic stimulation of skeletal muscle increased palmitate oxidation and esterification. A novel and significant finding is the demonstration that this was associated with an increase in FA transport, measured in sarcolemmal vesicles, and with an increase in the FA transporter FAT/CD36. This strongly suggests that increasing the muscle capacity for oxidizing FA requires a concurrent increase in membrane FA transport, and that FA transport may be the rate-limiting factor for FA metabolism.

Our laboratory (7) recently provided convincing evidence that there is a FA transport system in giant sarcolemmal vesicles obtained from skeletal muscle. The transport characteristics measured in these sarcolemmal vesicles are generally consistent with the properties of FA transport determined earlier in multiple cell types (2, 3, 6, 22, 37), except that FA metabolism and esterification are completely absent in giant sarcolemmal vesicles, whereas in hepatocytes, cardiac myocytes, and adipocytes, FA metabolism is still present (2, 3, 6, 22, 37).

In this study, we document increases in sarcolemmal FA transport under conditions known to enhance the total oxidative capacity of the muscle (i.e., chronic muscle stimulation; for review see Ref. 28). This suggests that membrane transport of FA in muscle is sensitive to the metabolic requirements of this tissue and that FA transport can be regulated in a manner similar to that of other substrates, like glucose or lactate. Indeed, our laboratory previously showed that chronic stimulation of muscle increases GLUT-4 (17) and MCT-1 transport proteins (26), resulting in proportional increases in glucose (17) and lactate transport (26). The present data imply that the transport step limits the rate of FA utilization so that under conditions where FA metabolism is enhanced, FA transport would need to be upregulated. Two additional pieces of evidence lend support to this interpretation. First, the observation that FA transport was higher in vesicles obtained from muscles with a predominance of oxidative fibers compared with those from muscles with glycolytic fibers (7) argues for a necessary correlation between the respective capacities of transport and metabolism. Second, chronic stimulation of muscle increased FA metabolism in general and not only FA oxidation. As shown in Table 2, palmitate esterification
into triglycerides and phospholipids was also significantly increased in chronically stimulated muscle. This would suggest that increases in FA uptake can modulate FA metabolism, which has its rate limited by the FA supply. In line with this, the twofold increase in membrane palmitate uptake could quantitatively account for the increased capacity in FA metabolism in chronically stimulated intact muscle (see Ref. 14 for review). The present study demonstrates that an increased capacity to transport palmitate across the sarcolemmal membrane is also an important mechanism that can provide additional lipids inside the muscle cell.

The present data do not rule out the possibility that muscle stimulation might have resulted in upmodulation of a variety of intracellular proteins involved in FA binding (cytosolic FABP) and metabolism (fatty-acyl-CoA synthase and FA-esterifying enzymes). However, it is important to stress that changes in FA metabolism did not contribute to the increase in FA transport measured in sarcolemmal vesicles, in which all the palmitate taken up is recovered as nonmetabolized palmitate (7).

The increased expression of FAT/CD36 in muscle after chronic stimulation supports the interpretation that this protein could have accounted for the increase measured in palmitate transport. FAT/CD36 has been identified by labeling with inhibitors of FA transport in adipocytes (11–13). Its tissue distribution (1) and the regulation of its gene expression by FA (4, 34) are significant.

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consistent with its postulated role in FA binding and/or transport (1). FAT/CD36 expression in fibroblasts was shown to induce a saturable, phloretin-sensitive FA transport component (15). Levels of FAT/CD36 mRNA were shown to be higher in oxidative than in glycolytic muscle (7, 41) in adult as opposed to fetal heart and increased during heart development when FA utilization increased (41). These findings suggested an association in skeletal muscle between FAT/CD36 expression and the capacity for FA metabolism. The present study, by documenting that muscle stimulation induced parallel increases in FA metabolism and transport and FAT/CD36 expression, demonstrated this association directly.

FAT/CD36 protein expression in giant sarcolemmal vesicles from stimulated muscle was increased 3.4-fold, and this increase apparently reflected a pretranslational mechanism, as there was a parallel increase in FAT mRNA. The increase in FAT/CD36 protein levels was comparable in magnitude to that measured in FA transport rates, suggesting it contributed a significant fraction of the measured FA uptake. However, the data do not rule out that increases in other proteins implicated in FA transport could have occurred, and they may have contributed to the increased FA transport. In addition to FAT/CD36, two other membrane proteins, FATP (32) and FABPpm (16, 38), have been implicated in FA transport and are expressed in muscle (1, 6, 7). Whether FABPpm and FATP are increased with chronically increased muscle activity remains to be established. One report has shown an increase in human muscle FABPpm after exercise training (19). Thus both FAT/CD36 (present study) and FABPpm (19) are increased when muscle activity is increased chronically.

We also have some preliminary evidence to suggest that these two proteins may interact with each other to facilitate the uptake of FA (21).

Table 2. Palmitate oxidation and esterification into intramuscular triacylglycerol and phospholipid pools in control and chronically stimulated rat EDL muscles

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Chronically Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation</td>
<td>58.6 ± 13.5</td>
<td>88.6 ± 10.1*</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>72.1 ± 19.5</td>
<td>176.7 ± 34.0*</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>44.1 ± 9.4</td>
<td>74.0 ± 19.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 individual muscles/group. Measurements in control and chronically stimulated muscles were obtained at rest. *Significant difference vs. control, P < 0.05.

Fig. 5. Comparison of relative increases induced by chronic stimulation in palmitate metabolism in incubated EDL muscles and palmitate transport into giant sarcolemmal vesicles. Control is 100%, and chronically stimulated muscle data are expressed relative to this control. Palmitate metabolism in extensor digitorum longus (EDL) is sum of palmitate oxidation and palmitate incorporation into phospholipids, tri-, di-, and monoacylglycerols.
into giant sarcolemmal vesicles (i.e., heart > red > white muscle). Although these differing transport rates are highly correlated with the presence of FABP<sub>pm</sub> and FAT/CD36 in giant vesicles, the FATP content in these vesicles is negatively correlated with the FA transport rates (21). This suggests that FABP<sub>pm</sub> and FAT/CD36 are the key FA transport proteins in heart and muscle, but that perhaps FATP is not critical for the uptake of FA in these tissues. FATP may, however, be important in other tissues.

In summary, our studies have shown that increasing the oxidative capacities of rat skeletal muscle via chronic electrical stimulation increases the rate of palmitate transport presumably via an increase in the putative FA transporter FAT/CD36. This adaptation is highly functional, because the import of greater quantities of long-chain FAs enables the muscle to increase its metabolism of FAs. Such an increase in long-chain FA metabolism was demonstrated in the chronically stimulated EDL. Notably, the increase in palmitate transport into vesicles and palmitate metabolism by intact muscle were almost identical. This suggests that FA transport may be a rate-limiting step for FA metabolism in muscle.

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