Fatty acid binding protein facilitates sarcolemmal fatty acid transport but not mitochondrial oxidation in rat and human skeletal muscle

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The transport of long-chain fatty acids (LCFAs) across mitochondrial membranes is regulated by carnitine palmitoyltransferase I (CPTI) activity. However, it appears that additional fatty acid transport proteins, such as fatty acid translocase (FAT)/CD36, influence not only LCFA transport across the plasma membrane, but also LCFA transport into mitochondria. Plasma membrane-associated fatty acid binding protein (FABPpm) is also known to be involved in sarcolemmal LCFA transport, and it is also present on the mitochondria. At this location, it has been identified as mitochondrial aspartate amino transferase (mAspAT), despite being structurally identical to FABPpm. Whether this protein is also involved in mitochondrial LCFA transport and oxidation remains unknown. Therefore, we have examined the ability of FABPpm/mAspAT to alter mitochondrial fatty acid oxidation. Muscle contraction increased ($P < 0.05$) the mitochondrial FAT/CD36 content in rat (+22%) and human skeletal muscle (+33%). By contrast, muscle contraction did not alter the content of mitochondrial FABPpm/mAspAT protein in either rat or human muscles. Electrotransfecting rat soleus muscles, *in vivo,* with FABPpm cDNA increased FABPpm protein in whole muscle (+150%; $P < 0.05$), at the plasma membrane (+117%; $P < 0.05$) and in mitochondria (+80%; $P < 0.05$). In these FABPpm-transfected muscles, palmitate transport into giant vesicles was increased by +73% ($P < 0.05$), and fatty acid oxidation in intact muscle was increased by +18% ($P < 0.05$). By contrast, despite the marked increase in mitochondrial FABPpm/mAspAT protein content (+80%), the rate of mitochondrial palmitate oxidation was not altered ($P > 0.05$). However, electrotransfection increased mAspAT activity by +70% ($P < 0.05$), and the mitochondrial FABPpm/mAspAT protein content was significantly correlated with mAspAT activity ($r = 0.75$). It is concluded that FABPpm has two distinct functions depending on its subcellular location: (a) it contributes to increasing sarcolemmal LCFA transport while not contributing directly to LCFA transport into mitochondria; and (b) its primary role at the mitochondria level is to transport reducing equivalents into the matrix.

The cellular transport of long-chain fatty acids (LCFAs) across the plasma membrane has long been thought to occur via passive diffusion. However, in recent years a substantial body of literature has emerged indicating that LCFA uptake into muscle cells occurs via a protein-mediated process (for review see Bonen *et al.* 2002; Luiken *et al.* 2004; Koonen *et al.* 2005). A number of proteins have been shown to facilitate the uptake of LCFAs into parenchymal cells, including fatty acid translocase, the homologue of human CD36, 88 kDa) (Abumrad *et al.* 1993), a family of fatty acid transport proteins (FATP1–6, 63–70 kDa) (Schaffer & Lodish, 1994; Hirsch *et al.* 1998; Gimeno *et al.* 2003) and plasma membrane-associated fatty acid binding protein

(FABPpm, 43 kDa) (Stremmel *et al.* 1985; Schwieterman *et al.* 1988; Isola *et al.* 1995). Whereas little is known about the regulation of FATP1–6 in muscle tissue, considerable evidence has accumulated to indicate that FAT/CD36 and FABPpm are important in regulating the uptake of LCFAs into cardiac and skeletal muscle.

Recently, FAT/CD36 was also found to be present in mitochondrial membranes of skeletal muscle (Campbell *et al.* 2004; Bezaire *et al.* 2006). In rodents, electrically induced muscle contraction (30 min) increased the mitochondrial content of FAT/CD36 (Campbell *et al.* 2004). Similar results have been observed in mitochondria isolated from human muscle after 2 h of moderate-intensity exercise (∼60% maximal O_2 consumption rate) (Holloway *et al.* 2006). In both rodents and humans, the muscle contraction-induced increases in muscle mitochondrial FAT/CD36 content were associated with increases in fatty acid oxidation in isolated mitochondria (Campbell *et al.* 2004; Holloway *et al.* 2006). In addition, when FAT/CD36 was blocked by sulfo-*N*-succinimidyloleate (SSO), a specific inhibitor of FAT/CD36, mitochondrial fatty acid oxidation was almost completely inhibited (∼90%) in mitochondria obtained from either resting or exercised muscle (Campbell *et al.* 2004; Bezaire *et al.* 2006; Holloway *et al.* 2006). These studies do not negate the well-known role of carnitine palmitoyltransferase (CPTI) activity in mitochondrial fatty acid oxidation. However, it appears that FAT/CD36 works in conjunction with CPTI, as together these two proteins predict rates of mitochondrial fatty acid oxidation ($r = 0.90$; Bezaire *et al.* 2006), and FAT/CD36 and CPTI are colocalized in mitochondria (Campbell *et al.* 2004; Schenk & Horowitz, 2006).

Whether FABPpm, another LCFA transport protein, also contributes to regulating mitochondrial LCFA oxidation is not known. However a series of studies have shown that FABPpm and mitochondrial aspartate aminotransferase (mAspAt) are identical proteins (Berk *et al.* 1990; Stump *et al.* 1993; Bradbury & Berk, 2000), and are found at the plasma membrane and on mitochondria, respectively (Cechetto *et al.* 2002). To date it is known that plasma membrane FABPpm contributes to regulating fatty acid transport (Schwieterman *et al.* 1988; Stump *et al.* 1993; Zhou *et al.* 1995) while in mitochondria, mAspAt catalyses the following reversible reaction: glutamate + oxaloacetate ⇌ aspartate + 2-oxoglutarate (Lehninger *et al.* 1993). Transfecting 3T3 fibroblasts (Isola *et al.* 1995) or skeletal muscle (Clarke *et al.* 2004) with mAspAt cDNA increased plasmalemmal FABPpm content and increased the rates of LCFA transport (Isola *et al.* 1995; Clarke *et al.* 2004), as well as the rates of LCFA oxidation in muscle (Clarke *et al.* 2004). Whether the up-regulation of LCFA oxidation was due to the increased influx of LCFA into the muscle and/or to an increase in FABPpm/mAspAt content at the mitochondria was not determined. Furthermore, there

is strong evidence suggesting that FABPpm collaborates with FAT/CD36 to transport LCFAs across the plasma membrane (Luiken *et al.* 1999), and there may also be a similar role for FABPpm/mAspAt in mitochondria where FAT/CD36 is also present. Thus, it is important to resolve whether FABPpm/mAspAt has a role in both plasma membrane LCFA transport and in mitochondrial LCFA oxidation.

Because we have previously shown that up-regulation of LCFA oxidation in contracting muscles involves the translocation of FAT/CD36 to the mitochondria (Campbell *et al.* 2004; Holloway *et al.* 2006), we first examined whether FABPpm/mAspAt was also translocated to the mitochondria during electrically induced muscle contraction in a rat muscle and after 2 h of cycling exercise in human muscle. In addition, we also transfected rat muscle with mAspAt cDNA to examine the effects of FABPpm/mAspAt up-regulation on the rates of plasma membrane LCFA transport, and on the rates of LCFA oxidation in intact muscle and in isolated mitochondria. Finally, we also examined the effects of FABPpm/mAspAt up-regulation on mAspAT activity in whole muscle and isolated mitochondria.

Methods

Electrical stimulation of rat muscle

Female Sprague–Dawley rats ($n = 5$, weighing ∼175 g) were used in the contraction experiments. Animals were housed in a climate- and temperature-controlled room, on a 12–12 h reverse light–dark cycle. Rat chow and water were provided *ad libitum*. The study was approved by the University of Guelph Animal Ethics Committee.

To ascertain whether mitochondrial FABPpm/mAspAT content was altered in response to muscle contraction in a similar manner to FAT/CD36, rat hindlimbs were acutely (30 min) stimulated as we have previously described (Bonen *et al.* 2000; Campbell *et al.* 2004). Briefly, while under anaesthesia (intraperitoneal injections of sodium pentobarbital, 6 mg (100 g body weight)$^{-1}$; MTC Pharmaceuticals, Cambridge, ON, Canada), a small incision was made in one hindlimb, and stimulating electrodes placed on the exposed sciatic nerve. The contralateral muscle in the same animal acted as a resting control. Muscle contraction consisted of stimulating the sciatic nerve in one hindlimb (10 V, 100 Hz, 100 ms trains, 20 tetani min^{-1} for 30 min; Grass S48 Stimulator, Astro-Medical Inc., Longueuil, QC, Canada), as we have reported previously (Campbell *et al.* 2004). Immediately after stimulation, the contracting soleus and the contralateral resting soleus were removed for mitochondrial isolation as described below, and used for Western blotting analysis.

Human exercise experiments

Twelve healthy, recreationally active, individuals volunteered for this study ($n = 9$ males; $n = 3$ females; age, 22 ± 1 year; weight, 77 ± 4 kg; body mass index, 24 ± 1 kg m^{-2}; peak O_2 consumption rate ($\dot{V}_{O_2 peak}$), 49 ± 2 ml min^{-1} (kg body mass)$^{-1}$. Subjects were fully informed of the purpose of the experiments and of any possible risk before giving written consent to participate. The study was approved by the University of Guelph Ethics Committee.

Participants cycled for 2 h at $\sim$60% $\dot{V}_{O_2 peak}$ on a cycle ergometer (LODE Instrument, Groningen, the Netherlands), following a 12 h overnight fast. Pulmonary O_2 uptake was measured with a metabolic cart (SensorMedics V_{max} model, CA, USA). Prior to exercising, both legs were prepared for muscle biopsies of the vastus lateralis muscle. Ventilatory and muscle samples were obtained before and after 2 h of cycling. Muscle samples were obtained under local anaesthesia (2% lignocaine (lidocaine) without adrenaline (epinephrine)) using the percutaneous needle biopsy technique described by Bergstrom (1975). Immediately following tissue sampling, visible fat and connective tissue were dissected free from the muscle and the samples were blotted to remove excess blood. The tissue was used for the immediate isolation of mitochondria and for the determination of selected proteins with Western blotting.

Rat solei electrotransfection

Female Sprague–Dawley rats (weighing $\sim$175 g) were used in these experiments.

During surgery, rats were maintained unconscious by inhalation of the anaesthetic isoflorane (AErane; Baxter, Deerfield, IL, USA) at 2–2.5% in pure O_2 at a flow rate of 2 l min^{-1}. Electrotransfection of FABPpm/mAspAt was performed as we have previously described (Clarke *et al.* 2004). Briefly, the control limb was left 'intact' and the experimental lower hindlimb was shaved and sterilized with iodine solution. A 1.5 cm incision was made laterally along the left hindlimb parallel to the tibia and extending to the Achilles tendon. The soleus muscle was exposed and 100 μl 0.45% saline solution containing 250 μg plasmid cDNA (provided by Dr A. Iriarte, University of Missouri, USA) was injected using a 27 gauge needle. The plasmid contained the open reading frame of FABPpm under the control of the CMV promotor as we have previously described (Clarke *et al.* 2004), with the addition of a FLAG epitope (Sigma, St Louis, MO, USA), inserted at the C-terminus by PCR methods. The modified FABPpm–FLAG was subcloned into pcDNA3.1 between *Eco*RI and *Xba*I sites. Immediately following the cDNA injection, electroporation of the intact soleus muscle was performed as we have previously described

(Clarke *et al.* 2004) (eight electric pulses, 200 V cm^{-1}, 1 Hz, 20 ms in duration) (ECM 830 Square Wave Electroporator; BTX, Holliston, MA, USA) using Tweezertrodes (BTX). After the pulse was delivered, the overlying superficial muscle was sutured, and the skin incision was closed. The animals were randomly divided into three groups 7 days after this procedure. The first group was used for the analysis of whole-muscle measurements (Western blot analysis, palmitate transport, oxidation and incorporation into diacylglycerol (DAG) and triacylglycerol (TAG) pools) ($n = 12$). The second group was used to isolate vesicles ($n = 12$). Because of tissue requirements, the soleus muscles of eight animals were pooled to create one sample for each independent fatty acid transport assay. The third group was used for isolating mitochondria ($n = 5$). Because of tissue requirements, the soleus muscles of two animals were pooled to create one sample for mitochondrial analysis (i.e. $n = 1$ is two rats), thus 10 rats were used to create five data points. However, prior to pooling for mitochondrial isolations, a small section of muscle ($\sim$10 mg) was removed for determining the enzymatic characteristics of each animal (see below). Additional experiments were conducted with an empty vector to ensure that the transfection procedures did not alter any of the measured parameters.

Preparation of giant vesicles

Giant vesicles from pooled control and electrotransfected solei muscles were generated as previously described (Bonen *et al.* 1998, 2000, 2004; Luiken *et al.* 2001; Luiken *et al.* 2002*b*; Koonen *et al.* 2002; Steinberg *et al.* 2002). Briefly, the tissues 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), aprotinin (30 μg ml^{-1}, Sigma) and collagenase (type VII, 150 units ml^{-1}) in a shaking water bath. At the end of the incubation, the supernatant fraction was collected and the remaining tissue was washed with KCl/MOPS and 10 mM EDTA which resulted in a second supernatant fraction. Both supernatant fractions were pooled, and Percoll (G.E. Healthcare, Aurora, OH, USA), and aprotinin were added to final concentrations of 3.5% (v/v) and 10 μg ml^{-1}, respectively. The resulting suspension was placed at the bottom of a density gradient consisting of a 3 ml middle layer of 4% Nycodenz (w/v) and a 1 ml KCl/MOPS upper layer. This sample was centrifuged at 60 g for 45 min at room temperature (25°C). Subsequently, the vesicles were harvested from the interface of the upper and middle layer, diluted in KCl/MOPS and re-centrifuged at 12 000 g for 5 min. The pellet was resuspended in KCl/MOPS.

Palmitate uptake by giant vesicles

Palmitate uptake studies were performed as we have previously described (Bonen *et al.* 1998, 2004; Luiken

et al. 2001, 2002*c*; Koonen *et al.* 2002; Steinberg *et al.* 2002). Fatty acid-free bovine serum albumin (BSA; MP Biomedicals, Solon, OH, USA), [9,10-^{3}H]palmitate (Perkin Elmer, Wellesley, MA, USA) and [^{14}C]mannitol (Perkin Elmer) were used in the experiments. For palmitate transport measurements, 40 μl 0.1% BSA in KCl/MOPS, containing unlabelled (15 μM) and radio-labelled [^{3}H]palmitate (0.3 μCi) and [^{14}C]mannitol (0.06 μCi), was added to 40 μl vesicle suspension. The incubation was carried out for 15 s. Palmitate uptake was terminated by addition of 1.4 ml ice-cold KCl/MOPS, 2.5 mM HgCl$_2$ and 0.1% BSA. The sample was then centrifuged in a microfuge at 12 000 r.p.m. (13 000*g*) for 2 min. The supernatant was discarded, and radioactivity was determined in the tip of the tube.

Whole-muscle palmitate metabolism

In control and electrotransfected soleus muscle we examined the metabolism of palmitate. For these measurements we used our previously published methods (Dyck *et al.* 1997, 2000, 2001). Briefly, soleus muscle strips were preincubated for 20 min (30°C), followed by a 60 min incubation period (30°C). The incubation medium contained 0.5 mM palmitate, and 5 mM glucose, as well as 2 μCi [^{14}C]palmitate (G.E. Healthcare). Palmitate oxidation was determined by acidifying the incubation medium to release the ^{14}CO$_2$, which was trapped by benzethonium hydroxide (Sigma). To determine the incorporation of palmitate into DAG and TAG pools, muscle was homogenized, spotted on silica gel plates (Silica Gel GF, 250 mm; Analtech, Newark, DE, USA), and resolved in solvent (60 : 40 : 4, heptane/isopopylether/acetic acid) for 45 min. Plates were air-dried, sprayed with dichlorofluorescein dye and visualized under long-wave ultraviolet light. Individual lipid bands were quantified against known standards, and scraped into vials for liquid scintillation counting.

Isolation of mitochondria from skeletal muscle

Differential centrifugation was used to obtain pure and intact mitochondria containing both intermyofibrillar (IMF) and subsarcelommal (SS) fractions (Campbell *et al.* 2004). All procedures were identical to those that we have previously published (Bezaire *et al.* 2006; Holloway *et al.* 2006). Briefly, muscle (~300 mg) was homogenized with a tight-fitting Teflon pestle. The homogenate was centrifuged at 800 *g* for 10 min, to separate the SS and IMF mitochondria. The IMF mitochondria were treated with a protease (0.025 ml g^{-1}; Sigma) for exactly 5 min to digest the myofibrils. Further centrifugation was used to remove the myofibrils, and recombine the IMF with the SS mitochondria. The combined samples were centrifuged

twice at 10 000 *g* for 10 min. The pellet was resuspended in 1 μl buffer per milligram of tissue. Following the oxidation measurements, the remaining mitochondria were further purified using a Percoll gradient for Western blotting analysis. Samples were centrifuged at 20 000 *g* for 1 h and the mitochondrial layer was removed. The Percoll was removed from the sample by further centrifuging at 20 000 *g* for 5 h.

Mitochondrial palmitate oxidation

Labelled CO$_2$ production from palmitate oxidation and acid-soluble trapped ^{14}C were measured during state 4 respiration following a 30 min incubation of viable mitochondria in a sealed system, as previously described by us (Campbell *et al.* 2004; Bezaire *et al.* 2006; Holloway *et al.* 2006). Briefly, viable mitochondria (100 μl) were added to a system containing a pre-gassed modified Krebs–Ringer solution supplemented with 5 mM ATP, 1 mM NAD$^+$, 0.5 mM DL-carnitine, 0.1 mM coenzyme A, 25 μM cyto-chrome C and 0.5 mM malate. A microcentrifuge tube containing 500 μl 1 M benzethonium hydroxide inserted into a 1.5 ml centrifuge tube, was placed in the system to capture ^{14}CO$_2$ produced during the oxidation reaction. The system was then sealed with a rubber cap and further sealed with parafilm. The reaction was initiated by the addition of a 6 : 1 palmitate–BSA complex (containing 10 μCi [1-^{14}C]palmitate, for a final palmitate concentration of 77 μM) administered by syringe through the rubber cap. The reaction continued for 30 min at 37°C and was terminated with the addition of ice-cold 12 N perchloric acid by syringe through the rubber cap.

A fraction of the reaction medium was removed through the cap and analysed for isotopic fixation. Gaseous CO$_2$ produced from oxidation of [1-^{14}C]palmitate was measured by acidifying the remaining reaction mixture. Liberated ^{14}CO$_2$ was trapped by the benzethonium hydroxide over a 90 min incubation period at room temperature. The microcentrifuge tube containing the ^{14}CO$_2$ was put in a scintillation vial, and radioactivity was determined.

Mitochondrial viability

Electrotransfection was performed on five rats (weighing ~250 g), and mitochondria isolated from both electro-transfected and contralateral control limbs as outlined above. Using a Clark-type electrode, mitochondrial respiration rates were measured in state 3 and state 4 conditions, and the respiratory control ratios (RCRs) were calculated for both control and electrotransfected limbs to ensure the isolation procedure yielded intact mitochondria, and that electrotransfection did not alter the viability. Briefly, following the isolation procedure outlined above, 500 μg (~50 μl) mitochondria was added

to 1500 μl oxygraph medium containing (mM): mannitol 225, sucrose 75, Tris-base 10, K_2HPO_4 10, EDTA 0.1 and $MgCl_2$ 0.8; pH 7.0. State 3 respiration was measured in the presence of 5 mM pyruvate, 2 mM malate and 300 μM ADP, and subsequently, state 4 respiration was measured following the consumption of the ADP. The RCR was calculated as the ratio between state 3 and state 4 respiration rates (Chavez *et al.* 1995; Mogensen & Sahlin, 2005; Sahlin *et al.* 2007).

β-Hydroxyacyl-CoA dehydrogenase, citrate synthase and aspartate aminotransferase activities

A portion of the soleus muscle ($\sim$10 mg) was immediately homogenized in 100 μl/mg of a 100 mM potassium phosphate buffer (Bergmeyer, 1974*b*) and used for the measurements of β-hydroxyacyl-CoA dehydrogenase (β-HAD), citrate synthase (CS) and mAspAT activities. Total muscle β-HAD activity was measured in Tris-HCl buffer (50 mM Tris-HCl, 2 mM EDTA, 250 μM NADH, pH 7.0) and 0.04% Triton X-100. The reaction was started by addition of 100 μM acetoacetyl-CoA and absorbance was measured at 340 nm over a 2 min period (37°C) (Bergmeyer, 1974*b*).

CS activity was determined in isolated mitochondria as well as in aliquots of homogenized whole muscle. CS activity in intact mitochondria was determined by first assaying the extramitochondrial fraction in the suspension (1 : 20 dilution) and then assaying the total CS activity of the suspension (1 : 20 dilution) after lysing the mitochondria with 0.04% Triton X-100 and repeated freeze-thawing. The net difference provided a measure of the viability of the mitochondria, and when compared to the total muscle CS activity provided a measure of the mitochondria recovered during our isolation procedure. The CS activity was assayed spectrophotometrically at 37°C by measuring the disappearance of NADH at 412 nm (Bergmeyer, 1974*b*).

Mitochondrial aspartate aminotransferase activity was also measured in isolated mitochondria and in aliquots of homogenized whole muscle. Mitochondria and homogenate were repeatedly freeze-thawed prior to analysis. Maximal measurements were determined in a 75 mM phosphate buffer (75 mM phosphate, 60 mM 2-oxoglutarate, 6 U malate dehydrogenase, 0.25% Triton X-100, and excess NADH; adjusted to optimize spectrophotometer sensitivity range). The reaction was started by addition of 400 mM aspartate and absorbance was measured at 340 nm over a 2 min period (25°C) (Bergmeyer, 1974*a*).

Western blotting

Whole-muscle crude membranes were generated as we have previously described (Bonen *et al.* 1998, 2000; Luiken *et al.* 2001), analysed for total protein (bicinchoninic

(BCA) protein assay), and 25 μg denatured protein was loaded for Western blotting. The plasma membrane content of fatty acid transporters was determined by generating vesicles (as described above), analysing for total protein (BCA protein assay), and loading 10 μg denatured protein for Western blotting. Purified isolated mitochondrial fractions were analysed for total protein (BCA protein assay) and 25 μg denatured protein from each sample was loaded for Western blotting. All proteins were separated by electrophoresis on an 8% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The FABPpm polyclonal antibody used was produced in our laboratories and has been used previously in our work (Bonen *et al.* 1998, 2000; Luiken *et al.* 2001). The MO-25 antibody used to detect FAT/CD36 was produced by N. N. Tandon (Thrombosis Research Laboratory, Rockville, Maryland, USA). Commercially available antibodies were used to detect cytochrome c oxidase IV (Cox-IV; Invitrogen, Burlington, ON, Canada), sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA2) (Sigma) and Glut-4 (Chemicon International Inc., Temecula, CA, USA). In addition, an epitopic FLAG-tag (Sigma), specific to a six amino acid sequence inserted at the N-terminus of FABPpm, was used to detect *de novo* synthesis as a result of the cDNA electrotransfected into soleus muscles. An internal control of previously extracted crude membranes from rat was used in each gel. Blots were quantified using chemiluminescence and the Syngene 2 ChemiGenius bioimaging system (Syngene, Cambridge, UK).

Statistics

All data are presented as the means $\pm$ s.e.m. All data were compared using a paired t test. Associations between variables were investigated using Pearson correlation analyses. Statistical significance was accepted at $P < 0.05$.

Results

Effects of contraction on mitochondrial FABPpm content

Initially we examined whether muscle contraction or exercise increased the mitochondrial content of FABPpm/mAspAt, as we have previously shown for FAT/CD36 in both rat (Campbell *et al.* 2004) and human muscle (Holloway *et al.* 2006). In accordance with our previous work (Campbell *et al.* 2004), electrical stimulation increased FAT/CD36 content on rat mitochondria by +22% ($P < 0.05$, Fig. 1*C*). By contrast, electrical stimulation did not alter rat mitochondrial FABPpm content (Fig. 1*A*). Similarly, 2 h of cycling at $\sim$60% $\dot{V}_{O_2peak}$, increased mitochondrial FAT/CD36 content in human muscle by +33% ($P < 0.05$, Fig. 1*D*),

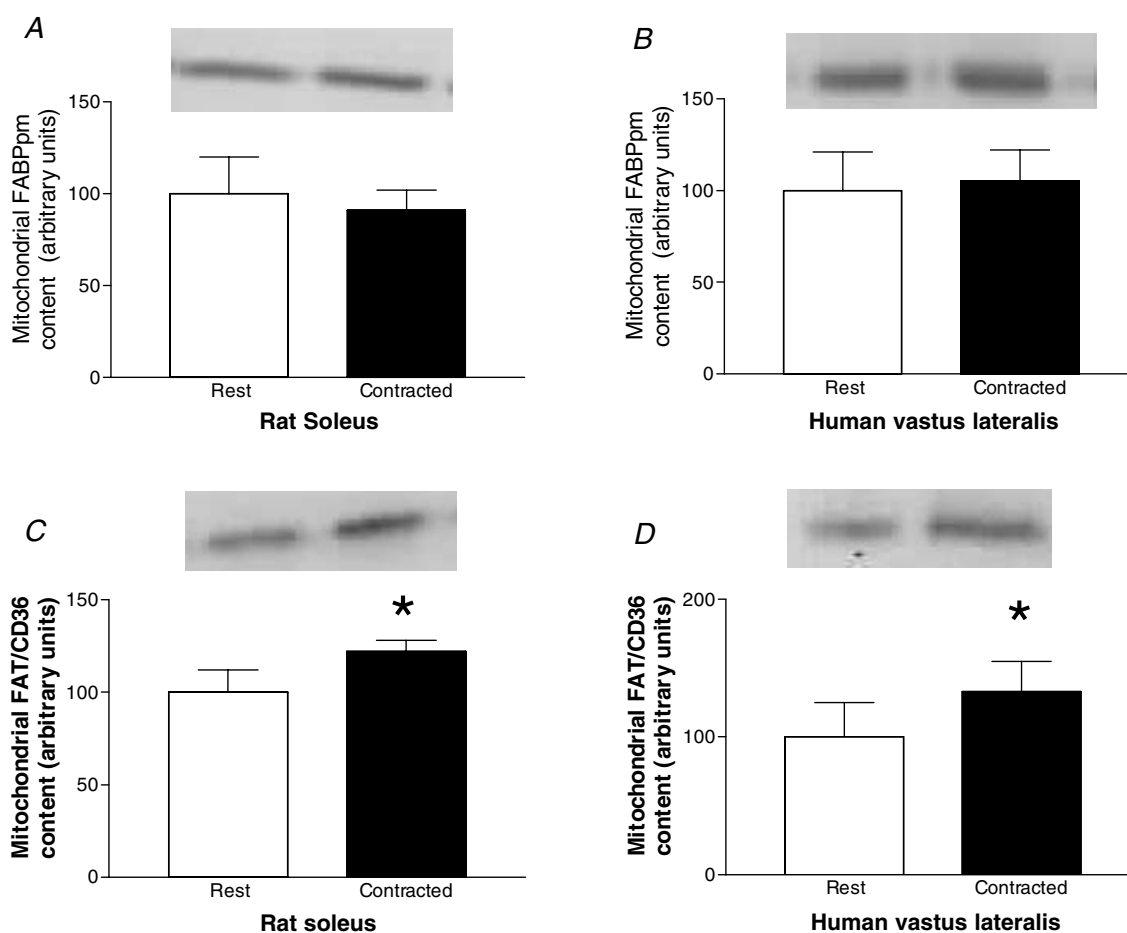but did not alter mitochondrial FABPpm/mAspAt content (Fig. 1*B*).

Effects of FABPpm/mAspAT electrotransfection on plasma membrane proteins, palmitate transport into giant vesicles and palmitate oxidation in intact soleus muscle

While the contraction-induced up-regulation of FAT/CD36 has been linked to increased rates of mitochondrial LCFA oxidation (Campbell *et al.* 2004; Holloway *et al.* 2006), the lack of change in mitochondrial FABPpm/mAspAt during muscle contraction suggests that this protein is not involved with fatty acid oxidation in mitochondria. To examine this further we investigated the effects of up-regulating FABPpm/mAspAt on rates of

LCFA transport and fatty acid oxidation in intact muscle and in isolated mitochondria.

When FABPpm/mAspAt was transfected into soleus muscles, there was a +150% increase in muscle FABPpm/mAspAT content ($P < 0.05$, Fig. 2*A*), and a +117% increase in the plasmalemmal FABPpm content ($P < 0.05$, Fig. 2*B*). The content of FAT/CD36, another fatty acid transporter, was not altered with transfection in either whole muscle or at the plasma membrane (data not shown). Transfection increased ($P < 0.05$) the rate of palmitate transport into giant sarcolemmal vesicles by +73% (Fig. 2*C*).

In intact soleus muscle, there was a somewhat smaller increase in the rate of palmitate oxidation following transfection (+18%, $P < 0.05$, Fig. 3*A*), while the rates of palmitate incorporation into DAG (Fig. 3*B*) or TAG pools were not altered (Fig. 3*C*).

Figure 1. Effects of muscle contraction on the content of mitochondrial FABPpm/mAspAT and FAT/CD36
Values are means ± s.e.m., expressed in arbitrary units. *A*, mitochondrial FABPpm/mAspAT content in rat soleus muscle at rest, and following 30 min of electrical stimulation ($n = 5$). *B*, mitochondrial FABPpm/mAspAT content in human vastus lateralis muscle at rest, and following 2 h of cycling at ~60% peak O$_2$ consumption rate ($\dot{V}_{O_2peak}$) ($n = 12$). *C*, mitochondrial FAT/CD36 content in rat soleus muscle at rest, and following 30 min of electrical stimulation ($n = 5$). *D*, mitochondrial FAT/CD36 content in human vastus lateralis muscle at rest, and following 2 h of cycling at ~60% $\dot{V}_{O_2peak}$ ($n = 7$). *Significantly different ($P < 0.05$) from rest.
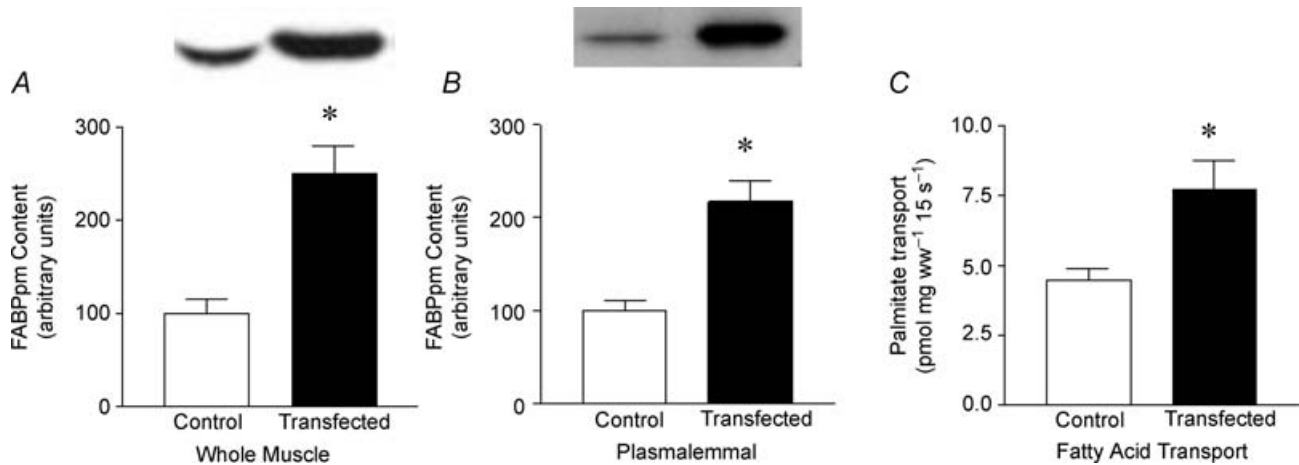
Figure 2. Effects of electrotransfection with FABPpm cDNA on soleus muscle
Values are means ± S.E.M.; protein contents expressed in arbitrary units, and transport expressed in pmol (mg wet weight)$^{-1}$ 15 s^{-1} ($n = 12$). A, whole-muscle FABPpm protein content. B, plasmalemmal FABPpm protein content. C, palmitate transport into giant sarcolemmal vesicles. ww, wet weight. *Significantly different ($P < 0.05$) from control.

Electrotransfecting FABPpm/mAspAT in mitochondria

Next we determined whether the electrotransfection of FABPpm/mAspAt into muscle increased FABPpm/mAspAt content in mitochondria, and whether this altered the rates of palmitate oxidation and/or aspartate aminotransferase activities in these organelles. The mitochondrial isolation procedure yielded intact mitochondria as the RCR values were similar those to previously published (Chavez *et al.* 1995; Mogensen & Sahlin, 2005; Sahlin *et al.* 2007), as were the mitochondrial recovery and viability as calculated from CS values (see Methods) (Bezaire *et al.* 2006; Bruce *et al.* 2006; Holloway *et al.* 2006). In addition, control and electrotransfection RCR values were not different

(10.4 ± 1.9 *versus* 10.0 ± 1.4, respectively; $P = 0.84$). The absence of SERCA (110 kDa) and Glut-4 (45 kDa) proteins, and the presence of Cox-IV (22 kDa), FAT/CD36 (88 kDa) and FABPpm (43 kDa) (Fig. 4) indicated that the isolation procedures successfully yielded highly purified mitochondria without contamination from other sources.

A FLAG-tag specific for the exogenous expression of FABPpm (43 kDa) was only found on the purified mitochondrial extracts obtained from the FABPpm/mAspAT electrotransfected muscles (Fig. 4). Electrotransfection of FABPpm/mAspAT into soleus muscle increased the mitochondrial FABPpm/mAspAT protein content by +80% ($P < 0.05$, Fig. 5A), without altering mitochondrial FAT/CD36 (Fig. 5B) or COX-IV
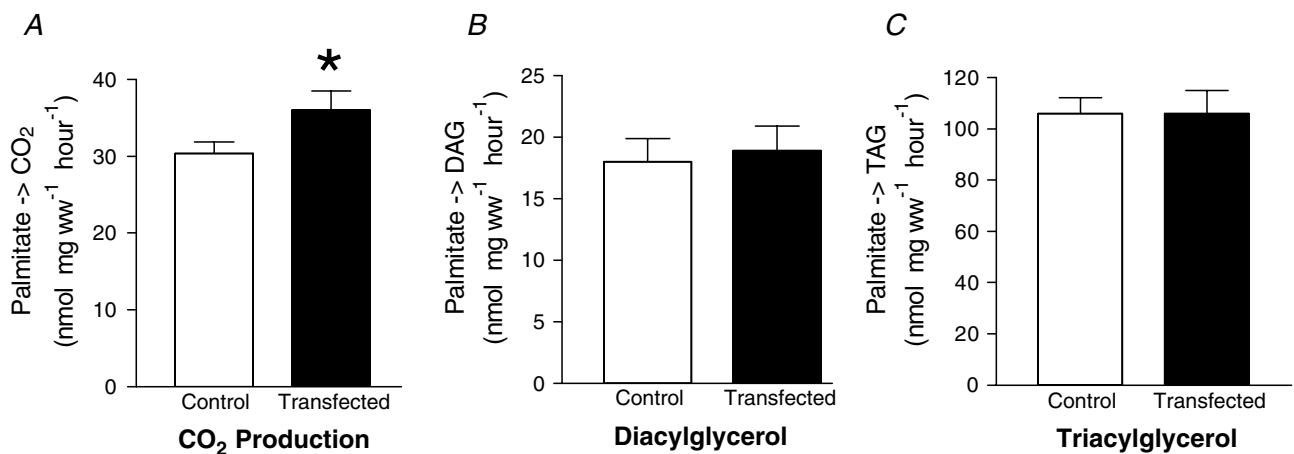
Figure 3. Effects of electrotransfection with FABPpm cDNA on the fate of palmitate in soleus muscle
Values are means ± S.E.M., expressed in nmol mg wet weight^{-1} hour^{-1} ($n = 12$). A, palmitate incorporation into the diacylglycerol pool. B, palmitate incorporation into triacylglycerol pool. C, palmitate oxidation. ww, wet weight. *Significantly different ($P < 0.05$) from control.
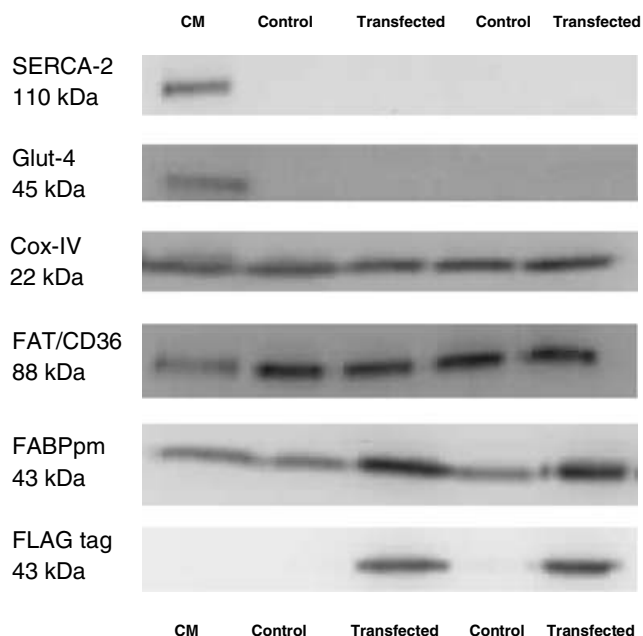
Figure 4. Representative Western blots performed on mitochondria isolated from control and electrotransfected solei

(Fig. 5*C*) contents. In addition, control experiments with an empty vector did not alter mitochondrial FABPpm/mAspAT, FAT/CD36 or Cox-IV contents (data not shown).

Mitochondrial palmitate oxidation and enzymatic activity of FABPpm/mAspAT

Although electrotransfection increased FABPpm/mAspAT content on mitochondria by +80%, isolated mitochondrial palmitate oxidation rates were not altered (Fig. 5*D*). By contrast, FABPpm/mAspAT electro-transfection resulted in a +90% increase ($P < 0.001$) in whole-muscle mAspAT activity (Fig. 6*A*), and a +70% increase ($P < 0.001$) in isolated mitochondrial mAspAT activity (Fig. 6*B*). Whole-muscle β-HAD activity (Fig. 6*C*) and whole-muscle CS activity (Fig. 6*D*) were not altered. In control experiments with an empty vector, mAspAT activity was not altered (data not shown). There was a significant correlation ($r = 0.75$) between mAspAT activity and mitochondrial FABPpm protein levels (Fig. 7).
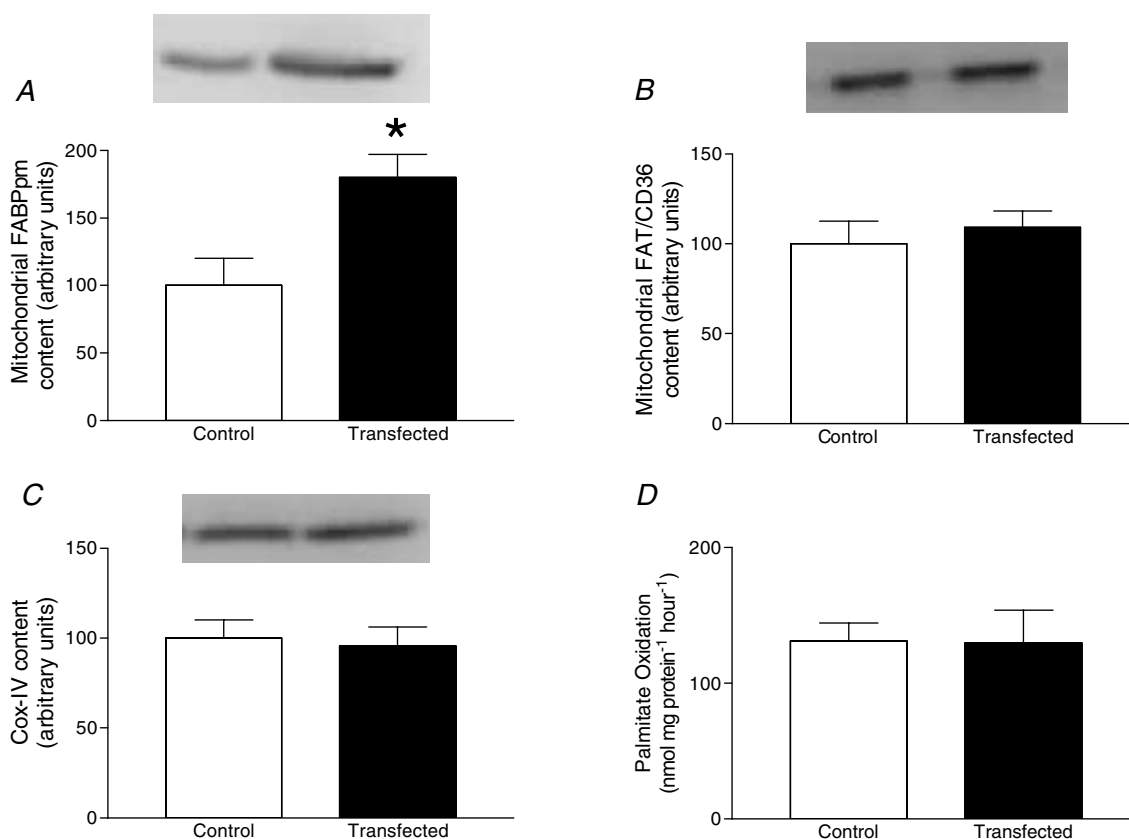
Figure 5. Effects of electrotransfection with FABPpm cDNA on the content of soleus muscle mitochondrial proteins, and the ability of mitochondria to oxidize palmitate
Values are means ± s.e.m., expressed in arbitrary units ($n = 5$). *A*, mitochondrial FABPpm/mAspAT protein content. *B*, mitochondrial FAT/CD36 protein content. *C*, Cox-IV protein content. *D*, isolated mitochondrial palmitate oxidation, expressed in nmol mg protein^{-1} hour^{-1}. *Significantly different ($P < 0.05$) from control.

Discussion

Studies by Berk and colleagues demonstrated that FABPpm and mAspAt are identical proteins (Berk *et al.* 1990; Stump *et al.* 1993; Bradbury & Berk, 2000), that are located at the plasma membrane and the mitochondria, respectively. We previously reported that FABPpm/mAspAT overexpression increased not only the rates of LCFA transport into muscle but it also increased the rates of palmitate oxidation in whole muscle (Clarke *et al.* 2004). This raised the spectre that FABPpm might have a role in regulating fatty acid oxidation at the mitochondrial level, as we have repeatedly demonstrated for FAT/CD36 (Campbell *et al.* 2004; Bezaire *et al.* 2006; Holloway *et al.* 2006). The present work has shown that (i) unlike for FAT/CD36, muscle contraction in rats and exercise in humans do not increase mitochondrial FABPpm/mAspAT content, whereas (ii) the up-regulation of this protein in muscle stimulates the rate of fatty acid transport but (iii) fails to alter the rate of fatty acid oxidation in

isolated mitochondria. Hence (iv) the FABPpm-induced up-regulation of fatty acid oxidation in whole muscle would seem to be solely related to the increased rate of plasmalemmal fatty acid transport. Finally (v) over-expression of FABPpm/mAspAT markedly increased the activity of aspartate aminotransferase. Thus, these studies have shown that despite the fact that FABPpm and mAspAt are identical proteins (Berk *et al.* 1990; Stump *et al.* 1993), they have quite distinct metabolic functions depending on their subcellular location. Specifically, the protein appears to function as a LCFA transport protein on the plasma membrane, and as an enzyme associated with the mitochondrial membranes involved in the transport of reducing equivalents into mitochondria.

Although LCFA transport was originally viewed as a purely passive process, it has become increasingly apparent that LCFA transport proteins are involved in the regulation of cellular fatty acid uptake and metabolism. Research at the level of the plasma membrane, specifically pertaining to FAT/CD36 and FABPpm proteins, has provided insight
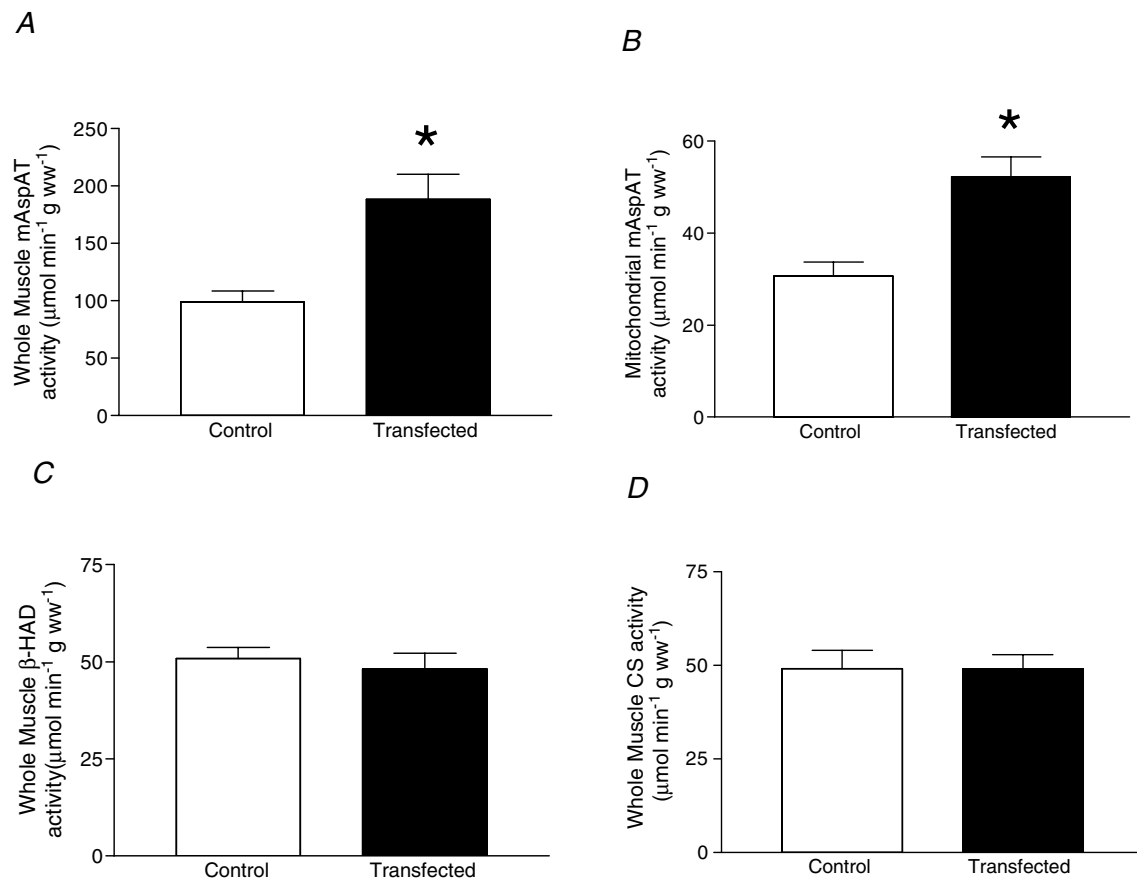
Figure 6. Effects of electrotransfection with FABPpm cDNA on mitochondrial enzymatic activity
Values are means ± s.e.m., expressed in μmol min^{-1} g wet weight^{-1}. *A*, mitochondrial aspartate aminotransferase (mAspAT) activity in homogenates ($n = 10$). *B*, mitochondrial aspartate aminotransferase activity (mAspAT) in isolated mitochondria ($n = 5$). *C*, homogenate β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity ($n = 10$). *D*, homogenate citrate synthase (CS) activity ($n = 10$). ww, wet weight. *Significantly different ($P < 0.05$) from control.

into the regulation of LCFA transport. It has been shown that FAT/CD36 and FABPpm proteins are up-regulated whenever fatty acid oxidation is chronically increased, such as during 7-day low-frequency muscle stimulation in rodents (Bonen *et al.* 1999; Koonen *et al.* 2004) and exercise training in humans (Tunstall *et al.* 2002), or in insulin-deficient animals (Luiken *et al.* 2002*a*). In these studies, the rates of fatty acid transport are concomitantly altered as a result of changes in FAT/CD36 and FABPpm protein expression and their appearance at the plasma membrane (Bonen *et al.* 1999; Luiken *et al.* 2002*a*; Koonen *et al.* 2004). In addition, rates of fatty acid transport can also be altered when these transporter proteins are redistributed to the plasma membrane in the absence of any change in their protein expression, which not only occurs acutely but also chronically. A translocation of these LCFA transporter proteins from a low-density microsomal pool to the plasma membrane has been observed following muscle contraction (Bonen *et al.* 2000) and AMP kinase activation (Luiken *et al.* 2003; Chabowski *et al.* 2005), thereby facilitating a very rapid up-regulation of fatty acid transport into muscle cells. A similar chronic translocation ('relocation') was found in muscle from obese Zucker rats (Luiken *et al.* 2001) and in obese and type 2 diabetic humans (Bonen *et al.* 2004). Taken altogether it is apparent that FAT/CD36 and FABPpm can influence fatty acid metabolism both acutely and chronically by altering the rate of LCFA entry into parenchymal cells.

Recently, FAT/CD36 has been shown to translocate to the mitochondria during exercise, in both rat and human skeletal muscle, increasing the ability of mitochondria to oxidize palmitate (Campbell *et al.* 2004; Holloway *et al.* 2006), and hence providing another level of regulation in fatty acid oxidation. At the level of the plasma membrane, the acute regulation of FAT/CD36 and FABPpm has in the past appeared similar (Chabowski *et al.* 2005; Chabowski

et al. 2006). However, the results of the current work suggest that the acute regulation of these proteins may be different at the level of the mitochondria. These results support the hypothesis that FAT/CD36 content on mitochondria increases following muscle contraction (Campbell *et al.* 2004; Holloway *et al.* 2006), while contraction does not alter mitochondrial FABPpm protein content, in either rats or in human skeletal muscle, and represents a much less flexible system.

As fatty acid oxidation appeared to increase during exercise without alterations in mitochondrial FABPpm/mAspAT content, a series of studies were designed to determine the role of FABPpm/mAspAT at the mitochondrial level. Electrotransfection of FABPpm cDNA into soleus muscle has previously been shown to increase rates of plasma membrane palmitate transport and whole-muscle palmitate oxidation, independent of changes in FAT/CD36 content (Clarke *et al.* 2004). In the present study we have replicated these findings, and investigated the role of mitochondrial FABPpm/mAspAT. Because FABPpm is located both on the plasma membrane and mitochondria (Stump *et al.* 1993; Cechetto *et al.* 2002), it was not known whether the increase in palmitate oxidation following electrotransfection of FABPpm resulted solely from enhanced rates of fatty acid transport across the plasma membrane, or whether FABPpm/mAspAT also played a role in fatty acid oxidation in mitochondria. The novel finding in the current study is that a +80% increase in mitochondrial FABPpm/mAspAT protein content failed to alter mitochondrial palmitate oxidation. This indicates that FABPpm/mAspAT, unlike FAT/CD36 (Campbell *et al.* 2004; Bezaire *et al.* 2006; Holloway *et al.* 2006; Schenk & Horowitz, 2006), is not involved in regulating fatty acid oxidation in mitochondria. Therefore, as whole-muscle oxidation was increased, without an increase in the ability of mitochondria to oxidize palmitate, increases in plasma membrane fatty acid transport and mass action are responsible for the observed increase in whole-muscle oxidation.

While others have suggested that FABPpm and mAspAT are structurally identical proteins, but responsible for different functions as a result of their subcellular compartmentation (Stump *et al.* 1993; Isola *et al.* 1995), we are the first to directly establish this. The increase in both the content of FABPpm at the plasma membrane and rate of fatty acid transport across the plasma membrane following electrotransfection, further supports the belief that FABPpm functions as a fatty acid transporter at this level (Isola *et al.* 1995; Luiken *et al.* 1999; Turcotte *et al.* 1999; Turcotte *et al.* 2000; Clarke *et al.* 2004). Altering the mitochondrial FABPpm/mAspAT protein presumably did not affect transport into mitochondria, because oxidation remained constant. However, increasing the mitochondrial content
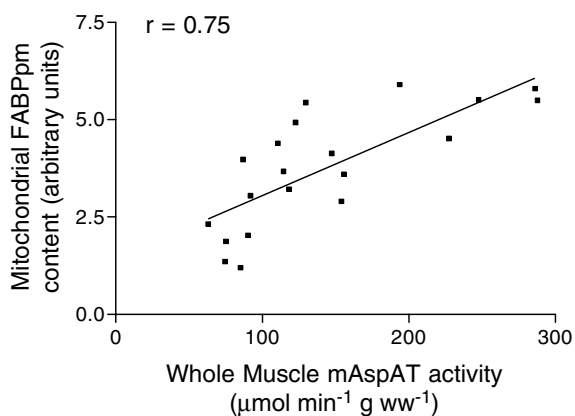
Figure 7. Pearson correlation calculated between mitochondrial FABPpm/mAspAT content and mAspAT activity in homogenates
$n = 20$ (10 control and 10 transfected) muscles.

of FABPpm/mAspAT increased mAspAT enzymatic activity. This evidence strongly suggests that FABPpm contributes to transporting fatty acids across plasma membranes, as well as enzymatically regulating the rate of the following reaction (glutamate + oxaloacetate $\rightleftharpoons$ aspartate + 2-oxoglutarate) in mitochondria (Lehninger *et al.* 1993). These different functions for the same protein may be explained by a differential post-translational modification of the protein depending on the subcellular location, or a co-functioning with other proteins (such as FAT/CD36 at the plasma membrane as previously suggested; Luiken *et al.* 1999).

In summary, we measured mitochondrial FABPpm/mAspAT content in contracting rat and human skeletal muscle, and have demonstrated an inability of FABPpm to translocate to mitochondria during contraction, suggesting that it does not regulate mitochondrial fatty acid oxidation. In addition, we have used electro-transfection to independently alter the content of FABPpm in rat soleus muscle on both the plasma membrane and mitochondria. We propose that the FABPpm-mediated up-regulation of fatty acid oxidation occurs via an increased rate of fatty acid transport across the plasma membrane, and thus mass action accounts for their increased mitochondrial oxidation. It is well known that another fatty acid transport protein, FAT/CD36, can have different functions in different tissues (for review see Febbraio *et al.* 2001), and the current novel finding is that FABPpm has diverse metabolic functions depending on its subcellular compartmentalization. Functionally, in muscle, it appears that FABPpm is a fatty acid transport protein on the plasma membrane, and FABPpm/mAspAT is a functional enzyme at the level of the mitochondria, facilitating the transport of reducing equivalents into the mitochondrial matrix.

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Acknowledgements