A null mutation in skeletal muscle FAT/CD36 reveals its essential role in insulin- and AICAR-stimulated fatty acid metabolism

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1Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada; 2Department of Molecular Genetics, Maastricht University, Maastricht, The Netherlands; 3Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Cleveland, Ohio; and 4Department of Biochemical Physiology and Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands

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Bonen A, Han X-X, Habets DD, Febbraio M, Glatz JF, Luiken JJ. A null mutation in skeletal muscle FAT/CD36 reveals its essential role in insulin- and AICAR-stimulated fatty acid metabolism. Am J Physiol Endocrinol Metab 292: E1740–E1749, 2007. —Fatty acid translocase (FAT)/CD36 is involved in regulating the uptake of long-chain fatty acids into muscle cells. However, the contribution of FAT/CD36 to fatty acid metabolism remains unknown. We examined the role of FAT/CD36 on fatty acid metabolism in perfused muscles (soleus and red and white gastrocnemius) of wild-type (WT) and FAT/CD36 null (KO) mice. In general, in muscles of KO mice, 1) insulin sensitivity and 5-aminomimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) sensitivity were normal, 2) key enzymes involved in fatty acid oxidation were altered minimally or not at all, and 3) except for an increase in soleus muscle FATP1 and FATP4, these fatty acid transporters were not altered in red and white gastrocnemius muscles, whereas plasma membrane-bound fatty acid binding protein was not altered in any muscle. In KO muscles perfused under basal conditions (i.e., no insulin, no AICAR), rates of hindquarter fatty acid oxidation were reduced by 26%. Similarly, in oxidative but not glycolytic muscles, the basal rates of triacylglycerol esterification were reduced by 40%. When muscles were perfused with insulin, the net increase in fatty acid esterification was threefold greater in the oxidative muscles of WT mice compared with the oxidative muscles in KO mice. With AICAR-stimulation, the net increase in fatty acid oxidation by hindquarter muscles was 3.7-fold greater in WT compared with KO mice. In conclusion, the present studies demonstrate that FAT/CD36 has a critical role in regulating fatty acid esterification and oxidation, particularly during stimulation with insulin or AICAR.

perfusion; palmitate; esterification; oxidation; fatty transport proteins 1 and 2; plasma membrane-bound fatty acid binding protein; 5-amino-imidazole-4-carboxamide-1-β-d-ribofuranoside

IN RECENT YEARS it has been established that fatty acid transport into metabolically active tissues such as heart and skeletal muscle occurs, in part, via a protein-mediated process (4, 7–9, 32, 33). Although a number of fatty acid transporters have been identified, it appears that fatty acid translocase (FAT/CD36 and plasma membrane-bound fatty acid binding protein (FABPPm) are two key fatty acid transporters. Upregulation of FAT/CD36 and FABPPm, either after 7 days of chronic muscle contraction or after exposure of cardiac myocytes to insulin or 5-amino-imidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), increases the rates of fatty acid transport (1, 5, 11, 13, 27). Conversely, when FAT/CD36 and FABPPm are concurrently downregulated, the rates of fatty acid transport into resting muscle are decreased (27, 44). The content of plasmalemmal FAT/CD36, but not FABPPm, is increased in insulin-resistant skeletal muscle, and this correlates with intramuscular triacylglycerol accumulation (9, 31). Despite these associations of FAT/CD36 and FABPPm with fatty acid transport and metabolism, the relative importance of these two fatty acid transporters is not known. Indirect evidence suggests that FAT/CD36 may be the more critical fatty acid transporter, given that the relative increase in plasmalemmal FABPPm, when overexpressed in vivo, did not scale well with the increased rate of fatty acid transport (14), and independent overexpression of FAT/CD36 and FABPPm suggested that FAT/CD36 has a greater capacity to upregulate fatty acid transport than FABPPm (36). Collectively, these studies suggest that FAT/CD36 has a key role in fatty acid metabolism.

Whether fatty acid metabolism is largely determined by the altered activity/capacity of selected enzymes involved in fatty acid esterification and/or oxidation or by the FAT/CD36-mediated influx of fatty acids is unclear. Based on studies conducted under basal conditions in skeletal muscle of FAT/CD36 null and overexpressing mice, there is conflicting evidence as to whether this transporter is important in regulating fatty acid metabolism (15, 26). For example, incorporation of iodinated fatty acid analogs into triacylglycerols was reduced in heart, skeletal muscle, and adipocytes of FAT/CD36 null mice (15). However, the converse was not observed when FAT/CD36 was overexpressed, since basal rates of skeletal muscle fatty acid oxidation and esterification were not altered despite a twofold increase in FAT/CD36 (26). These studies appear to suggest that FAT/CD36-mediated rates of fatty acid transport may only have limited effects on basal rates of fatty acid metabolism. However, when FAT/CD36-overexpressing muscles were stimulated to contract, it appeared that the resulting upregulation of fatty acid oxidation was closely linked to the increased FAT/CD36-mediated flux (26). Subsequent studies have shown that FAT/CD36 may be induced to translocate from an endosomal pool to the plasma membrane within minutes by muscle contraction (7, 46), by insulin (12, 33, 34), and by AICAR (12, 32). Therefore, in previous studies (15), the role of FAT/CD36 in regulating fatty acid metabolism may...
have been underestimated when examined only under basal conditions and not during metabolic challenges. Along similar lines, the critical role of the glucose transporter GLUT4 in mediating glucose transport only became evident when GLUT4 null muscles were challenged with insulin or muscle contraction, since basal rates of glucose transport did not differ in wild-type and GLUT4 null mice (39, 45). Thus, to establish unequivocally the role of FAT/CD36 in fatty acid metabolism, it is necessary to examine fatty acid metabolism in FAT/CD36 null mice during selected metabolic challenges. This may also reveal whether fatty acid transport is rate limiting under different physiological conditions, as has been shown for GLUT4 (21), since FAT/CD36 can be induced to translocate to the plasma membrane by selected metabolic stimuli (7, 12, 32–34, 46).

In the present studies we have examined the rates of fatty acid esterification and oxidation in perfused hindlimb muscles of wild-type and FAT/CD36 null mice under basal conditions as well as during metabolic challenges with insulin and with AICAR. Each of these metabolic challenges normally induces the translocation of FAT/CD36 and thereby markedly increases the rates of fatty acid transport (7, 11, 12, 32–34). In addition, insulin stimulates skeletal muscle fatty acid esterification (19, 33), whereas AICAR stimulates fatty acid oxidation by activating AMP kinase (12, 32, 42). The present studies have shown that FAT/CD36 is critically important for supporting the increase in 1 fatty acid esterification when challenged with insulin and 2 fatty acid oxidation when challenged with AICAR.

METHODS

Materials

Antibodies against FAT/CD36, fatty acid transport proteins 1 and 4 (FATP1 and FATP4; Santa Cruz Biotechnology, Santa Cruz, CA), carnitine palmitoyltransferase 1 (CPT1; Cedarlane, Burlington, ON, Canada), cyclooxygenase-4 (COX-4; Invitrogen, Burlington, ON, Canada), AMP-activated protein kinase (AMPKα2; Upstate, Lake Placid, NY), phosphorylated acetyl-CoA carboxylase (ACC, Ser79; Cell Signaling Technology, Danvers, MA), and phosphorylated AMPK (Thr172; Upstate) were obtained from commercial sources. Anti-FABPpm was a gift from Dr. J. Calles-Escandon (Wake Forest University, Winston-Salem, NC). Bovine serum albumin and reagents for enzyme analyses were obtained from Sigma-Aldrich (St. Louis, MO). [1-14C]palmitate and D-3H]glucose were obtained from Amersham Biosciences (St.signin, UK).

Animals

FAT/CD36 null mice were generated by targeted homologous recombination and crossed back six times to the C57Bl/6 background (20). Separate groups of wild-type (WT) and FAT/CD36 null (KO) mice were bred on site at the University of Guelph. Mice (males only, 21 ± 0.6 wk old; weight: WT, 36.6 ± 1.0 g and KO, 36.2 ± 1.4 g) were housed in a temperature (20°C)-regulated environment with a reversed 12:12-h light-dark cycle. Mice had access to standard laboratory chow and water ad libitum. Approval for all experiments was provided by the committee on animal care at the University of Guelph.

Hindlimb Perfusion

Palmitate esterification and oxidation were examined during 1) basal (no insulin, no AICAR) conditions, 2) insulin stimulation, and 3) AICAR stimulation in perfused hindlimb muscles of fed WT and KO mice. These hindlimb perfusion procedures were similar to those our group has reported previously in rats (23, 29, 33), with only minor modifications to accommodate the much smaller mice. In addition, to validate the hindlimb perfusion procedure for mice, we conducted a preliminary experiment (n = 7). These pilot studies demonstrated that the perfused mouse hindlimb perfusion preparation was very stable, because 1) ATP and ADP concentrations were not altered during the perfusion compared with freshly harvested muscle, and 2) pH, PCO2, and PO2 of the venous effluent were also unaltered (data not shown).

For the present studies, fed WT and KO mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (65 mg/kg body wt) and surgically prepared for perfusion as has been done previously in rats (23, 29, 33). In all experiments, the hindquarters of mice were preperfused (20 min) and perfused (60 min) under identical conditions with a Krebs-Henseleit buffer (i.e., 95% O2 and 5% of CO2, pH 7.4, 37°C, 7 mM glucose, 0.5 mM palmitate, 4% bovine serum albumin, 3 ml/min flow rate, 100-ml recirculating volume). During the 20-min preperfusion period, the venous outflow was discarded. During the experiments (60 min), the perfusion medium (100 ml) was supplemented with 30 μCi, [1-14C]palmitate, and 40 μCi D-3H]glucose. In three separate groups of WT and KO mice, muscles were perfused under basal conditions, with insulin (20 mU/ml), or with AICAR (2 mM). For the respective insulin and AICAR experiments, insulin (20 mU/ml) and AICAR (2 mM) were included during the preperfusion period. In all experiments (basal, insulin, and AICAR), arterial and venous perfusate samples were collected every 5 min for the last 30 min and kept in tightly sealed vials for later analysis of 14CO2. After 60 min of perfusion, the experiments were stopped. Soleus and red and white gastrocnemius muscles were quickly harvested, rapidly frozen in liquid nitrogen, and stored at −80°C until analyzed for palmitate accumulation in the intramuscular phospholipids and di- and triacylglycerol depots.

CO2 Production

Because fatty acid oxidation cannot be measured in individual hindlimb perfused muscles, the measurement of palmitate oxidation was performed across the perfused hindquarter, using procedures that our group has reported previously (33). Briefly, 0.5 ml of both arterial and venous perfusate samples were introduced into a tightly capped, sealed vial containing 1 M H2SO4 (1 ml). Liberated 14CO2 was captured in benzethonium hydroxide and kept in a small Eppendorf tube securely centered in the vial. After 60 min, the tube containing benzethonium hydroxide with the released 14CO2 was quickly transferred to a scintillation vial and counted for radioactivity. Standard calculations based on flow rate, timing of the sample, arteriovenous differences in 14CO2, and specific activity of palmitate were used to calculate CO2 production across the perfused hindquarter (33). Palmitate oxidation was adjusted for radioactive label fixation (18, 48). For these purposes, acid-soluble 14C products in hindlimb muscles were obtained during the extraction of the lipids from the hindlimb muscles. This method has been widely used in isolated muscles (3, 5, 9, 18, 19, 37, 44) and in studies with fatty acid oxidation by isolated mitochondria (10, 28).

Palmitate Incorporation into Skeletal Muscle Phospholipids, Diacylglycerols, and Triacylglycerols

In soleus and red and white gastrocnemius muscles, palmitate incorporation into intramuscular phospholipids and tri- and diacylglycerols was determined using thin-layer chromatography as previously described in detail by our group (5, 18, 19, 33, 37). Rates of palmitate incorporation into the muscles were calculated based on the specific activity of the palmitate in the perfusion experiments.
Fatty Acid Uptake by Skeletal Muscle

It was unrealistic to determine rates of fatty acid transport into giant vesicles prepared from individual muscles, because that would have required pooling of individual muscles from ~30 mice for each independent experiment (i.e., ~1,080 mice in total for n = 6 per condition). Therefore, we have determined the rates of fatty acid uptake during the hindlimb perfusion. For these purposes, we calculated from the sum of palmitate esterification (i.e., palmitate incorporation into phospholipids + di- + triacylglycerol) in each muscle. From this the total, palmitate esterification by the mouse hindquarter was calculated, taking into account the muscle mass and the fiber composition of mouse hindlimb muscles (Bonen A, unpublished data). This was then summed with the palmitate oxidized by the hindlimb muscles. Our group (29) has previously used a similar approach to determine glucose transport by the rat hindquarter based on data from individual muscles with different fiber types. In that study (29), the fold increments in glucose transport determined in the total hindlimb musculature corresponded closely with the fold increase in the insulin- and contraction-induced GLUT4 translocation in pooled hindlimb muscles.

Determination of Glycogen Synthesis

The rate of D-[^3H]glucose incorporation into muscle glycogen was based on procedures that Bonen and colleagues (6, 25) have previously used in isolated muscles. Briefly, frozen muscle samples were boiled in 1 N NaOH (0.3 ml) for 5 min and cooled on ice. A portion (10 μl) of the hydrolyzed muscle was used to determine protein concentration and the remainder was added in 0.5 ml of 30% KOH saturated with Na2SO4. Thereafter, the solution was boiled for another 5 min, and 0.5 ml of glycogen (40 mg/ml in 30% KOH) was added to act as a carrier. Glycogen was precipitated from muscle with 3 ml of 95% ethanol in H2O at 20°C for 1 h. The precipitate was recovered with centrifugation at 4°C. The supernatant fraction was discarded, and the pellet was dissolved with 1 ml of H2O and washed with 3 ml of 95% ethanol. Glycogen was pelleted with centrifugation at 4°C.

Table 1. Circulating concentrations of glucose, insulin, and fatty acids in wild-type and FAT/CD36 null mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose, mM</th>
<th>Insulin, pg/ml</th>
<th>Fatty Acids, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>7.5±0.3</td>
<td>190±20</td>
<td>0.52±0.04</td>
</tr>
<tr>
<td>FAT/CD36 null</td>
<td>8.0±0.4</td>
<td>220±30</td>
<td>0.70±0.03*</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 7–10 mice. *P < 0.05, FAT/CD36 null vs. wild type.

Fig. 1. Fatty acid transport proteins fatty acid translocase (FAT/CD36 (A), plasma membrane-bound fatty acid binding protein (FABPpm; B), fatty acid transport protein (FATP1) (C), and FATP4 (D) in soleus (SOL) and red (RG) and white gastrocnemius (WG) muscles of wild-type (WT) and FAT/CD36 null (KO) mice. Data are means ± SE; n = 8 for each muscle in each group. ND, not detected. *P < 0.05, KO vs. WT muscle. **P < 0.05, RG vs. SOL in WT or KO mice. ***P < 0.05, WG vs. RG in WT or KO mice.
After washing (4×), the glycogen pellet was dissolved in 1 ml of H₂O, transferred to a scintillation vial, and counted for radioactivity.

**Blood Sampling and Analyses**

Blood samples were obtained from the inferior vena cava during the surgical preparation of the mice. Samples were analyzed for glucose (Sigma-Aldrich), insulin (Linco, St. Charles, MO), and fatty acids (Wako Chemicals, Richmond, VA) using commercially available procedures.

**Enzyme Activities and Protein Expression**

Muscles obtained from fed WT and KO mice were analyzed for fatty acid transporters (FAT/CD36, FABPpm, FATP1, and FATP4), CPT1, COX-4, AMPKα2, and phosphorylated ACC and AMPK with the use of standard preparations and Western blotting procedures as our group has previously reported (7, 10–12). The activities of β-hydroxyacyl-CoA dehydrogenase (β-HAD) and citrate synthase (CS) were also analyzed in muscle homogenates using well-known standard enzymatic procedures described by Lowry and Passonneau (30).

**Statistics**

The data were analyzed using repeated-measures analyses of variance (ANOVA), i.e., WT vs. KO mice, with repeated measures on muscles within each group. Post hoc tests were performed with the Fisher’s least-squares difference procedure only when a significant F ratio was found in the ANOVA. Statistical significance was accepted at P < 0.05. All data are reported as means ± SE.

**RESULTS**

**Glucose, Insulin, and Fatty Acids**

In the fed WT and KO mice, there were no differences in circulating glucose or insulin concentrations (P > 0.05; Table 1). In contrast, circulating fatty acids were 35% higher in fed KO mice compared with fed WT mice (P < 0.05; Table 1). Others have found that glucose concentrations are lower in overnight-fasted KO mice compared with WT mice (22) and that circulating fatty acid concentrations were not different in fed KO and WT mice (15).

**Comparisons among metabolically heterogeneous muscles in WT mice**. In general, fatty acid transport proteins (Fig. 1), selected mitochondrial enzymes (Fig. 2), and insulin-stimulated rates of glycogenesis (Fig. 3) were greatest in the oxidative muscles (soleus and red gastrocnemius). Conversely, AMPKα2 protein and AICAR-stimulated AMPK phosphorylation were greatest in the fast-twitch red and white gastroc-
nemius muscles (Fig. 4, A and B), whereas AICAR-induced ACC phosphorylation was lower in these muscles (Fig. 4, C).

With respect to fatty acid metabolism, rates of palmitate incorporation into phospholipids (Fig. 5, A–C) and diacylglycerols (Fig. 5, D–F) were greater in oxidative muscles (soleus and red gastrocnemius) than in white gastrocnemius muscle (P < 0.05). Phospholipid synthesis was not altered by insulin or AICAR stimulation in any muscle (Fig. 5, A–C), whereas insulin-mediated diacylglycerol formation was increased (P < 0.05) to a similar extent in all muscles (Fig. 5, A–C).

A large quantity of palmitate was incorporated into intramuscular triacylglycerols. In WT mice, basal rates of triacylglycerol formation in soleus muscle were 1.5- and 10-fold greater than in the red and white gastrocnemius muscles, respectively (P < 0.05; Fig. 5, G–I). Insulin stimulated the rate of palmitate incorporation into triacylglycerols in the soleus (≈80%) and red gastrocnemius muscles (≈59%) (P < 0.05; Fig. 5, G and H).

Comparisons between WT and KO mice. The different metabolic characteristics of the three muscles in WT mice were also generally observed among the muscles of KO mice. However, ablation of FAT/CD36 also resulted in marked differences in respective muscles of WT and KO mice.

Fatty Acid Transport and Mitochondrial Proteins

FAT/CD36 protein was not present in muscles of the KO mice. However, a compensatory increase occurred in FATP1 (+12%, Fig. 1C) and FATP4 (+57%, Fig. 1D) in soleus muscle only (P < 0.05). No differences in β-HAD activity were observed between respective muscles of KO and WT mice. However, in KO mice, CPT1 (soleus, +39%; red gastrocnemius, +20%; P < 0.05) and COX-4 (soleus, +18%) were increased (P < 0.05), whereas CS activity (soleus, −23%) was lower (P < 0.05).

Sensitivity to Insulin-Stimulated Glycogenesis

We assessed whether insulin sensitivity of glucose metabolism was maintained in muscles of FAT/CD36 null mice. In muscles of KO and WT mice, both the basal and the markedly increased insulin-stimulated rates of glycogenesis were similar (Fig. 3), and no differences were found in GLUT4 protein in muscles of WT and KO mice (P > 0.05; data not shown).

AMPKα2 and Effects of AICAR on Phosphorylation of AMPK and ACC

In KO soleus muscle, AMPKα2 protein was increased (37%) compared with WT soleus muscle (P < 0.05; Fig. 4A), but the basal AMPK phosphorylation state was greater in red and white gastrocnemius of KO mice (P < 0.05; Fig. 4B). As in WT mice, AICAR also increased the phosphorylation of AMPK in red and white gastrocnemius muscles of KO mice (P < 0.05; Fig. 4B).

Under basal conditions ACC phosphorylation was increased in soleus (117%) and white gastrocnemius muscles (94%)...
of KO mice \((P < 0.05, \text{Fig. 4C})\). With AICAR stimulation, the ACC phosphorylation attained was similar in the respective muscles in WT and KO mice \((P < 0.05; \text{Fig. 4C})\).

**Palmitate Uptake and Incorporation into Intramuscular Phospholipids and Di- and Triacylglycerols**

**Palmitate uptake.** Under basal perfusion conditions, palmitate uptake was reduced in KO mice \((-23\%, P < 0.05; \text{Fig. 6})\). In WT mice, insulin \((+60\%)\) and AICAR \((+77\%)\) both stimulated palmitate uptake \((P < 0.05; \text{Fig. 6})\). In contrast, in KO mice there was only a small increase in insulin \((21\%)\)- and AICAR-stimulated \((+13\%)\) palmitate uptake \((\text{Fig. 6})\).

**Phospholipids.** No differences in palmitate incorporation into phospholipids were found between WT and KO mice \((P > 0.05, \text{Fig. 5, A–C})\).

**Diacylglycerols.** Under basal conditions, rates of palmitate incorporation into diacylglycerols did not differ in respective WT and KO muscles \((P > 0.05, \text{Fig. 5})\). AICAR stimulated palmitate incorporation into diacylglycerols in all WT \((58–79\%, P < 0.05; \text{Fig. 5})\) and KO muscles \((42–77\%, P < 0.05; \text{Fig. 5})\), except white gastrocnemius muscle in KO mice. The insulin-stimulated increase was far greater in WT muscles \((+92 \text{ to } +234\%)\) than in KO muscles \((+52 \text{ to } +69\%, P < 0.05; \text{Fig. 5})\).

**Triacylglycerols.** As in WT mice, a large proportion of palmitate was synthesized into triacylglycerols. However, in KO mice, basal rates of triacylglycerol formation were reduced in soleus \((-36\%)\) and red gastrocnemius \((-41\%, P < 0.05; \text{Fig. 5})\). The AICAR-stimulated rates of triacylglycerol formation were greater in WT muscles than in the respective KO muscles \((P < 0.05, \text{Fig. 5})\).
Insulin stimulated the rate of palmitate incorporation into triacylglycerols in soleus (+80%) and red gastrocnemius (+59%) of WT mice (P < 0.05; Fig. 5). However, in KO mice, a much lower insulin stimulation occurred in soleus (+39%) and red gastrocnemius muscles (+27%).

**Palmitate Oxidation**

The basal rates of palmitate oxidation were reduced by 26% in KO mice compared with WT mice (P < 0.05, Fig. 7). Insulin reduced the rate of palmitate oxidation in both WT and KO mice (P < 0.05, Fig. 7). Although AICAR stimulated the rate of palmitate oxidation in WT mice (+100%), the AICAR-induced increase (+38%) in palmitate oxidation in KO mice was severely blunted (P < 0.05; Fig. 7).

**Net Increase in Insulin-Stimulated Esterification and AICAR-Stimulated Oxidation**

It is well known that the primary effect of insulin is the stimulation of palmitate incorporation into intramuscular triacylglycerols and that the primary effect of AICAR is the stimulation of fatty acid oxidation. To better appreciate the inhibition of these stimuli in FAT/CD36 null mice, we have compared the net increases in the insulin-stimulated triacylglycerol esterification (Fig. 8A) and in the AICAR-stimulated palmitate oxidation (Fig. 8B). The absolute increase in insulin-stimulated triacylglycerol esterification (Δ = insulin − basal) was threefold lower in the soleus and red gastrocnemius of KO mice (P < 0.05; Fig. 8A). The absolute increase in AICAR-stimulated palmitate oxidation (Δ = AICAR − basal) was 3.7-fold lower in KO compared with WT mice (P < 0.05; Fig. 8B).

**DISCUSSION**

In the present study we have examined the effects of ablating the fatty acid transporter FAT/CD36 on palmitate metabolism in three metabolically heterogeneous types of skeletal muscle under basal conditions as well as during metabolic challenges with insulin and with AICAR. The present studies demonstrate that palmitate metabolism is markedly downregulated under basal conditions in FAT/CD36 null mice. This becomes even more apparent when muscles are challenged either with insulin or with AICAR. These metabolic effects are most pronounced in oxidative types of skeletal muscle, which have a greater capacity for fatty acid esterification and oxidation (18) and in which FAT/CD36 is most abundantly expressed (8, 35). Thus, in skeletal muscle, it appears that FAT/CD36 has a key regulatory role in fatty acid esterification and oxidation.

**Fatty Acid Uptake by Skeletal Muscle**

In recent years work in our laboratory has shown that fatty acid uptake into cardiac myocytes (32–35) and skeletal muscle (7, 8, 27, 33, 44) is a highly regulated, protein-mediated process. Early work suggested that FAT/CD36 was likely a key fatty acid transporter (8, 26, 35), which could be induced to translocate from an endosomal depot to the plasma membrane by insulin (12, 33, 34), muscle contraction (7, 32, 46), oligomycin (16, 32), and AICAR (12, 33, 32). In the present study we found that both insulin and AICAR stimulated fatty acid uptake in muscle of WT mice but that in muscles of FAT/CD36 null mice, fatty acid uptake was impaired under basal as well as insulin- and AICAR-stimulating conditions. It also appears that, relative to FAT/CD36, other fatty acid transporters (FABPpm, FATP1, and FATP4) contribute only minimally to increasing fatty acid uptake, despite the fact that FABPpm can be induced to translocate by AICAR (12). Insulin-induced translocation of FATP1 remains controversial (12, 38, 43, 49). Altogether, it appears that 1) FAT/CD36 is important for maintaining basal rates of fatty acid uptake, and 2) FAT/CD36 translocation to the plasma membrane is critically important for increasing the uptake of fatty acids when muscle is stimulated by insulin or by AICAR. This has important consequences for fatty acid esterification and oxidation.

**Fatty Acid Metabolism in WT Muscle**

The differences in palmitate esterification and oxidation in metabolically heterogeneous muscles of WT mice parallel...
observations among similar muscles in rats (18). The responses to insulin and AICAR in muscles of WT mice concur with previous observation by our group in rat skeletal muscle in which insulin also stimulated fatty acid esterification (19, 33) and both contraction (17, 50) and AICAR (41, 42) stimulated fatty acid oxidation via AMPK activation.

Since the perfusion medium contains glucose, the reduction in skeletal muscle fatty acid oxidation during perfusion with insulin, observed in this and other studies (19), is attributable to the insulin-mediated increase in glucose transport into muscle (47, 50). The increased intracellular availability of glucose results in higher malonyl-CoA levels, which inhibit CPT1 and reduce the rates of fatty acid oxidation (47, 50).

The AICAR-induced increase in palmitate incorporation in triacylglycerols and glucose into glycogen is not without precedence in other situations in which rates of substrate oxidation are increased. For example, during muscle contraction, our group also found a net increase in palmitate incorporation into intramuscular triacylglycerols (17). Similarly, during treadmill exercise, our group observed an increase in glucose incorporation into glycogen (25). Under these conditions the uptake of substrates (palmitate and glucose) is known to be increased, and most of the palmitate and glucose taken up is oxidized. However, some is also seen to be synthesized into triacylglycerol (present study and Ref. 17) and glycogen (present study and Ref. 25). Despite this, these depots are concomitantly increased. For example, during muscle contraction, our group also found a net increase in palmitate incorporation into intramuscular triacylglycerols (17). Similarly, during treadmill exercise, our group observed an increase in glucose incorporation into glycogen (25).

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The intracellular signaling and enzymatic machinery that is required to respond to either insulin or contraction-like stimuli do not differ appreciably in muscles of WT and KO mice. This makes the FAT/CD36 null model very suitable for determining the role of FAT/CD36 in fatty acid metabolism under basal as well as insulin- and AICAR-stimulating conditions.

Ablation of FAT/CD36 Accounts for Impaired Basal Rates of Fatty Acid Metabolism

The present study has shown that under basal conditions, fatty acid esterification and oxidation are diminished in FAT/CD36 null mice. This is largely attributable to the ablation of FAT/CD36, because a compensatory upregulation of other known fatty acid transporters (FABPpm, FATP1, FATP4) was not observed in either the red or white gastrocnemius muscles of FAT/CD36 null mice. However, there was an upregulation of FATP1 and FATP4 in the soleus muscle of FAT/CD36 KO mice, but these FATP1 and FATP4 increases appeared to have little effect, since they failed to normalize fatty acid esterification in the absence of FAT/CD36 in soleus muscle. In addition, the reduction in the basal rates of fatty acid oxidation by the hindlimb muscles of FAT/CD36 null mice is also associated with the ablation of FAT/CD36, despite the presence of other fatty acid transporters (FABPpm, FATP1, FATP4). Therefore, the evidence strongly indicates that reductions in the basal rates of palmitate esterification (~−40%) and oxidation (~−26%) in skeletal muscle of FAT/CD36 null mice are the consequence of a reduced FAT/CD36-mediated fatty acid uptake.

FAT/CD36 is Critical for Upregulating Insulin- and AICAR-Stimulated Rates of Fatty Acid Metabolism

It is known that insulin stimulates palmitate uptake and esterification in skeletal muscle (19, 33). In the present study, the insulin-stimulated increases in the rate of fatty acid esterification into red muscle of FAT/CD36 mice were threefold...
lower in FAT/CD36 null mice. In the highly glycolytic white gastrocnemius muscle, in which fatty acid metabolism is already very low, FAT/CD36 ablation had minimal impact. Since FAT/CD36 appears to be the primary fatty acid transporter that can be induced to translocate by insulin (12, 33, 34), and since in muscles of FAT/CD36 null mice the postreceptor insulin signaling machinery and the capacity for esterification in FAT/CD36 muscles are intact [i.e., normal insulin-stimulated rates of glycogenesis (present study) and insulin sensitivity (22), and normal DGAT and long-chain acyl-CoA synthetase activities (15)], it is highly likely that the impaired insulin stimulation of triacylglycerol formation in muscles of FAT/CD36 null mice is attributable to the marked reduction in palmitate transport into the muscle. This alone would limit substrate availability for palmitate esterification. Therefore, the present results strongly indicate that under normal conditions, the insulin-induced translocation of FAT/CD36 is responsible for the bulk of the fatty acids that are esterified.

FAT/CD36 ablation has a minimal effect on fatty acid incorporation into phospholipids, since under basal and stimulation conditions there were no differences between WT and KO mice. It may well be that preservation of membrane integrity is central to maintaining cellular viability, and therefore, the magnitude of the reduction in intracellular fatty acids associated with the ablation of FAT/CD36 may not be sufficient to reduce phospholipid synthesis.

Upregulation of fatty acid oxidation also has a large FAT/CD36-component, since the net change in AICAR-stimulated fatty acid oxidation was severely blunted in FAT/CD36 null mice. The modest upregulation of fatty acid oxidation may be attributable to the AMPK-stimulated translocation of FABPpm, since both AICAR (12) and muscle contraction (Han X-X and Bonen A, unpublished observations) induce the translocation of this transporter to the plasma membrane. As noted above, the signaling machinery and enzymatic capacity for fatty acid oxidation appeared to be essentially normal in FAT/CD36 null muscles. Previously, it has been shown that muscle contraction (7) and activation of AMPK, by AICAR (12) or oligomycin (32), upregulated fatty acid oxidation, and this also induces the translocation of FAT/CD36 from an intracellular depot to the plasma membrane, thereby increasing the rate of fatty acid transport. (12, 32). Therefore, the severe limitation in the ability to upregulate fatty acid oxidation in FAT/CD36 null mice would seem to be attributable to the reduced transport rate of fatty acids due to the ablation of FAT/CD36. This would limit fatty acid availability for oxidation.

There is, however, a caveat to our present findings. In recent studies it has been observed that FAT/CD36 is also present in mitochondria (2, 10, 24, 40), where it is associated with CPT1 (40) and appears to contribute to regulation of fatty acid oxidation in skeletal muscle (2, 10, 24, 40). Hence, we cannot ignore the possibility that the absence of FAT/CD36 at the mitochondrial membrane may also have contributed to a reduced fatty acid oxidation in FAT/CD36 null mice. It is therefore important to determine whether the impaired fatty acid oxidation in muscle of FAT/CD36 null mice is largely attributable to a reduction in the rate of FAT/CD36-mediated fatty acid transport or to the absence of FAT/CD36 at the mitochondrion. Nevertheless, given the substantial impairment in the rate of insulin-stimulated esterification in FAT/CD36 null mice, which is most likely attributable to an impaired rate of palmitate transport, it would seem that a substantial component of the impaired fatty acid oxidation in FAT/CD36 null mice is also attributable to the impaired rate of fatty acid transport. Therefore, until the role of mitochondrial contribution of FAT/CD36 has been determined, it seems reasonable to propose that under normal conditions, the AICAR-stimulated translocation of FAT/CD36 is responsible for the bulk of the fatty acids that are oxidized.

In summary, the present study has shown that ablation of FAT/CD36 impairs basal as well as insulin- and AICAR-stimulated rates of palmitate esterification and oxidation in mouse skeletal muscles. FAT/CD36 ablation likely limits the quantity of fatty acids available for esterification and oxidation, particularly during metabolic challenges such as stimulation with insulin and with AICAR.

REFERENCES


FATTY ACID METABOLISM IN FAT/CD36 NULL MICE