FAT/CD36 is localized in sarcolemma and in vesicle-like structures in subsarcolemma regions but not in mitochondria

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Abstract The primary aim of the present study was to investigate in which cellular compartments fatty acid translocase CD36 (FAT/CD36) is localized. Intact and fully functional skeletal muscle mitochondria were isolated from lean and obese female Zucker rats and from 10 healthy male individuals. FAT/CD36 could not be detected in the isolated mitochondria, whereas the mitochondrial marker F_1ATPase-β was clearly detected using immunoblotting. Lack of markers for other membrane structures indicated that the mitochondria were not contaminated with membranes known to contain FAT/CD36. In addition, fluorescence immunocytochemistry was performed on single muscle fibers dissected from soleus muscle of lean and obese Zucker rats and from the vastus lateralis muscle from humans. Costaining against FAT/CD36 and MitoNEET clearly show that FAT/CD36 is highly present in sarcolemma and it also associates with some vesicle-like intracellular compartments. However, FAT/CD36 protein was not detected in mitochondrial membranes, supporting the biochemical findings. Based on the presented data, FAT/CD36 seems to be abundantly expressed in sarcolemma and in vesicle-like structures throughout the muscle cell. However, FAT/CD36 is not present in mitochondria in rat or human skeletal muscle. Thus, the functional role of FAT/CD36 in lipid transport seems primarily to be allocated to the plasma membrane in skeletal muscle.—Jeppesen, J., M. Morgensen, C. Prats, K. Sahlin, K. Madsen, and B. Kiens. **FAT/CD36 is localized in sarcolemma and in vesicle-like structures in subsarcolemma regions, but not in mitochondria. *J. Lipid Res.* 2010.** 51: **1504–1512.**

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Recent findings in rodent and human skeletal muscle suggest that the plasma membrane protein fatty acid translocase CD36 (FAT/CD36) is located in the mitochondrial outer membrane (1–6). These findings lead to the idea that FAT/CD36 could be important in regulating long-chain fatty acid (LCFA) transport into mitochondria (7). Elucidating the possible role of mitochondrial FAT/CD36 content could be of great importance, because decreased mitochondrial membrane FAT/CD36 content could reduce mitochondrial LCFA uptake and oxidation, leading to accumulation of LCFA derivatives, which have been linked to the development of insulin resistance by inhibition of key regulatory signaling molecules in the insulin signaling cascade (8–10). Initially, it was suggested that FAT/CD36 interacted with carnitine palmitoyltransferase 1 (CPT 1), the mitochondrial enzyme that catalyzes the first step in β-oxidation of LCFA, in regulation of LCFA entry into mitochondria. This was based on the observation that the addition of sulfo-N-succinimidyl esters (SSO), a FAT/CD36 inhibitor (11, 12), reduced CPT 1 activity by ~50% (1). However, other studies have shown a ~90%

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Abbreviations: CPT 1, carnitine palmitoyltransferase 1; CS, citrate synthase; FABPc, cytosolic fatty acid binding protein; FAT/CD36, fatty acid translocase CD36; LCFA, long-chain fatty acid; PVDF, polyvinylidene difluoride; mM SOD/SOD2, manganese superoxide dismutase; SSO, sulfo-N-succinimidyl ester; VDAC, voltage-dependent anion channel.

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decrease in palmitoyl-carnitine oxidation when blocking FAT/CD36 by SSO in isolated mitochondria (3) despite unchanged CPT 1 activity. These findings suggest a role for FAT/CD36 in mitochondrial fatty acid oxidation independent of CPT 1 (3, 6). In contrast, recent data from King et al. (13) showed no differences in maximal ADP-stimulated respiration in mitochondria isolated from skeletal and cardiac muscle from both wild-type and CD36 null mice. This was observed when they used palmitate (CPT 1 and long-chain acyl-CoA synthase dependent), palmitate-CoA (CPT 1 dependent), or palmitoyl-carnitine (CPT 1 independent) as substrates. Furthermore, a similar decrease in mitochondrial respiration (state 3) was observed when skeletal muscle mitochondria isolated from wild-type mice and CD36 null mice were incubated with SSO, demonstrating that this compound is unspecific for FAT/CD36, at least in mitochondria (13). These findings furthermore suggest that FAT/CD36 might not be essential to preserve mitochondrial ability to oxidize LCFA. In addition, previous attempts to detect intracellular FAT/CD36 in human (14), rat (15), and mouse (16) muscle cross-sections using microscopy approaches have only been able to detect FAT/CD36 in plasma membranes. Interestingly, Keizer et al. (17) detected small intracellular FAT/CD36 structures using fluorescence immunohistochemistry; however, when costaining with the mitochondrial marker cytochrome C, no FAT/CD36 staining in mitochondria was observed, supporting the view that FAT/CD36 is not present in mitochondria in the basal state. Considering the contradictory reports in the literature, the question arises as to whether or not FAT/CD36 is part of the mitochondrial outer membrane. The primary aim of the present study was therefore to investigate in which cellular compartments FAT/CD36 is localized in skeletal muscle. To do so, biochemical and morphological approaches were used on isolated mitochondria preparations from both human and rat skeletal muscles.

EXPERIMENTAL PROCEDURES

Animals

Female lean and obese Zucker rats (Harlan, Shaw's Farm, Blacktorn, Bicestor, UK) were used in this study. The obese phenotype develops extensive liver and muscular insulin resistance in contrast to the lean Zucker rats (18). Rats were housed for at least 1 week in our animal facilities before experiments. All animals were housed in box cages and maintained in a temperature-controlled room (22 ± 1°C) with a 12:12 h light/dark cycle. The rats were provided unrestricted access to food and water. Housing and husbandry practices were in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals". The lean and obese Zucker rats were used for experiments at the age of 16 weeks and were studied in the fed state. Animals were anesthetized using isoflurane (4%), and resting soleus muscles were removed. After experiments, the animals were euthanized by cervical dislocation. All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the European Convention for the Protection of Vertebrate Animals used for Experiments and other Scientific Purposes (council of Europe no. 123, Strasbourg, France, 1985).

Human subjects

Ten healthy male subjects volunteered for the study. Their age, weight, and height [mean and (range)] were: 26 (21–31) years, 81 (65–98) kg, and 184 (179–190) cm, respectively, as previously described (19). Subjects were informed of the purpose and potential risks of the experiments before being enrolled into the study. The project was approved by the local ethics committee at the Odense University Hospital (VF 20030129).

After arriving in the laboratory in the fasted state, the subjects rested in the supine position for 20 min. A muscle biopsy, using a modified Bergström needle with suction, was obtained from the vastus lateralis muscle under local anesthesia (2–3 ml 20 mg ml^{-1} Carbocain, AstraZeneca AB, Sweden) of the skin and fascia.

Isolation of muscle mitochondria

Mitochondria were isolated from the Zucker rat soleus muscles and from biopsies obtained from human vastus lateralis muscle. The muscle pieces were trimmed free of visible connective tissue, weighed, and placed in ice-cold isolation medium. Part of the muscle (5–10 mg) was frozen in liquid nitrogen and stored at −80°C for later determinations of protein expression. Mitochondria were isolated according to a technique previously described in detail (20). Briefly, muscle was finely minced and rinsed thoroughly with isolation medium (100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, 1 mM KH_2PO_4, 0.1 mM EGTA, and 0.2% BSA, pH 7.40) incubated for 2 min with 0.2 mg ml^{-1} bacterial protease (Nagarse; EC 3.4.21.62, Type XXVII, Sigma Chemical Co.) and homogenized for 2 min in an ice-cooled glass homogenizer with a motor-driven (180 rpm) Teflon pestle (radial clearance 0.15 mm). The homogenate was diluted with 3 volumes of protease free isolation medium and centrifuged at 750 *g* for 10 min. The supernatant was centrifuged at 10,000 *g* for 10 min and the pellet washed free from the lighter fluffy layer, suspended in the isolation medium, and again centrifuged (7,000 *g* for 3 min). The final pellet was suspended in a suspension medium (about 0.5 µl/mg initial muscle) containing (225 mM mannitol, 75 mM sucrose, 10 mM Tris, 0.1 mM EDTA, pH 7.40). A small aliquot of the isolated mitochondria were frozen in liquid nitrogen and stored at −80°C for later protein marker characterization analysis.

Mitochondrial respiratory activity

Mitochondrial oxygen consumption was measured polarographically using a Clark-type electrode (DW1 oxygraph, Hansatech Instruments, King's Lynn, Norfolk, UK) in an oxygraph at 25°C in the mitochondria isolated from lean and obese Zucker rat soleus muscle as well as mitochondria isolated from human vastus lateralis muscle. Respiration was measured in 300 µl oxygraph medium [225 mM mannitol, 75 mM sucrose, 10 mM Tris, 10 mM KCl, 10 mM K_2HPO_4, 0.1 mM EDTA, 0.8 mM $MgCl_2 \cdot (6H_2O)$, pH 7.0]. State 3 respiration [with ADP (0.3 mM)] and state 4 respiration (without ADP) were determined with pyruvate (5 mM) + L-malate (2 mM) and palmitoyl-L-carnitine (10 µM) + L-malate (2 mM). Respiration was, as described previously (21), expressed relative to the activity of citrate synthase (CS) to determine the intrinsic mitochondrial function. The mitochondrial P/O ratio was calculated as a measure of mitochondrial integrity. To assure that the outer mitochondrial membrane was intact in the mitochondria preparation, further experiments were performed on three obese and three lean Zucker rats. Mitochondria were isolated as described above, and mitochondrial oxygen consumption was measured using palmitoyl-CoA (5 µM) + l-malate (2 mM) + L-carnitine (2 mM) as substrate. Palmitoyl-CoA is a substrate for the outer mitochondrial membrane protein CPT 1. If the outer mitochon-

drial membrane is removed during the isolation procedure, ADP-stimulated respiration (state 3 respiration) using palmitate-CoA as substrate should be minute and not higher than the basal respiration (state 2 respiration, with substrates but without ADP). Mitochondrial oxygen consumption using palmitoyl-L-carnitine (10 μM) + L-malate (2 mM) as substrate was performed at the same time with the same amount of mitochondrial-rich solution to assure that the mitochondria were well coupled and functional.

Fluorescence immunostaining of single muscle fiber

To investigate whether FAT/CD36 was present in skeletal muscle mitochondria, single muscle fibers were obtained from soleus muscle of four lean and four obese Zucker rats and from vastus lateralis muscle from four male individuals. Muscles were immersed in cold Krebs-Henseleit bicarbonate buffer containing procaine hydrochloride (1 g/L) for 5 min and then fixed with 2% formaldehyde supplemented with 0.15% picric acid during 30 min at room temperature and 3.5 h at 4°C. After isolation of at least 20 single muscle fibers per muscle these were coimmunostained for FAT/CD36 and MitoNEET, a marker for mitochondrial outer membrane (22), as previously described (23). FAT/CD36 and MitoNEET were immunodetected using specific polyclonal antibodies (FAT/CD36: RnD systems, UK and MitoNEET: kindly donated by Dr. Philipp E. Scherer). Secondary antibodies conjugated with Alexa 488 or Alexa 568 (Invitrogen, UK) were used. All antibodies were diluted in 50 mM glycine, 0.25% BSA, 0.03% saponin, and 0.05% sodium azide in phosphate-buffered saline. Between incubation periods, muscle fibers were washed with the same buffer, but the last wash was performed with phosphate-buffered saline. Negative controls for each of the staining conditions were performed by staining without primary antibody or without primary and secondary antibodies. Muscle fibers were mounted in Vectashield mounting medium and analyzed. Confocal images were collected with a TCS SP2 microscope (Leica) using a Plan-Apo ×63/1.32 oil objective at 20°C. Imaging settings were set so that no signal was detected in the respective negative controls and a low fraction of pixels showed saturation intensity values when imaging the stained samples. Confocal z-stack images were collected from the surface to the center of muscle fibers, spaced 0.35 μm apart in the z-plane. Images were analyzed using Metamorph software (Universal Imaging Corp.).

Tissue preparation

Muscle samples were homogenized while on ice (i.e., 0°C) in a buffer (pH 7.4) containing 10% glycerol, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% nonidet P-40, 20 mM β-glycerolphosphate, 10 mM sodium flouride, 2 mM EDTA, 2 mM PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 2 mM sodium orthovanadate, and 3 mM benzamidine using a polytron homogenizer (PT 1200, Kinematic) until no visible particles remained. The homogenates were mixed end over end at 4°C for 60 min and then centrifuged at 16,000 *g* for 20 min at 4°C. The cleared supernatant (lysate) was collected and stored at −80°C for further analysis. The isolated mitochondria suspensions used for protein marker characterization were resuspended in ice-cold buffer (pH 7.4) to a final concentration of 10% glycerol, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% nonidet P-40, 20 mM β-glycerolphosphate, 10 mM sodium flouride, 2 mM EDTA, 2 mM PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 2 mM sodium orthovanadate, and 3 mM benzamidine. The isolated mitochondria suspensions were mixed end over end at 4°C for 60 min. The mitochondria suspensions were stored at −80°C until further analysis. Protein concentrations of lysates and mitochondria suspensions were determined in triplicates by the bicinchoninic acid method using BSA standards (Pierce Biotechnology Inc., Rockford, IL) and bicinchoninic acid assay reagents (Pierce technology). A maximal coefficient of variance of 5% was accepted between replicates.

Immunoblotting

Lysates and mitochondria suspensions were heated, 5 min at 96°C, in Laemmli's buffer before being subjected to SDS-PAGE and semi-dry immunoblotting. Polyvinylidene difluoride (PVDF) membranes were incubated with primary antibodies for anti-Caveolin 3 and anti-Caveolin 1 (BD Transduction Laboratories, CA), anti-manganese superoxide dismutase (mM SOD/SOD2) (Upstate Biological, NY), anti-voltage-dependent anion channel (VDAC) (RnD Systems, Minneapolis, MN), anti-SERCA1 and anti-GLUT4 (Affinity Bio Reagents Inc., CO), anti-F_1-ATPase-β (Santa Cruz Biotechnology, CA) and anti-Perilipin (Progen Biotechnik, Germany). The following noncommercial antibodies were used: anti-H-FABPc (cytosolic fatty acid binding protein) and anti-CD36 (MO25 clone) (kindly provided by Prof. Jan Glatz, University of Maastricht, The Netherlands). Appropriate horseradish peroxidase-conjugated secondary antibodies were used (DAKO, Glostrup, Denmark). Antigen antibody complexes were visualized using enhanced chemiluminescence (ECL+, Amersham Biosciences, Little Chalfont, UK) and quantified by a Kodak Image Station E440CF (Kodak, Glostrup, Denmark).

Statistics

Data are presented as mean ± SEM. One-way ANOVA was performed to test for differences between the lean and obese Zucker rats. Tukey's post hoc test was applied when there were differences between means. In all cases, $P = 0.05$ was used as the level of significance.

RESULTS

Integrity and purity of mitochondrial isolation

The isolated mitochondria were probed using antibodies detecting proteins known to be located in certain subcellular compartments or organelles. The integrity and purity of the isolated mitochondria from lean and obese Zucker rats, and from human vastus lateralis muscle, were confirmed by the presence of VDAC and F_1-ATPase-β, markers of the outer and inner mitochondrial membrane, respectively (24, 25) (**Fig. 1**), and by the presence of the mitochondria matrix protein Mn SOD (26) (Fig. 1). Furthermore, the isolated mitochondria preparations did not contain Caveolin 3, a marker of sarcolemma (14); Caveolin 1, a marker of endothelial cells and adipocyte plasma membrane (14); or SERCA1, a marker of sarcoplasmatic reticulum (27) (Fig. 1). In addition, the preparations were free of perilipin, a marker of adipocytes (28), and the cytosolic FABPc, a marker of cytosolic soluble proteins (29) (Fig. 1). There were no differences in marker protein distribution between the mitochondria isolated from the lean or obese Zucker rats or mitochondria isolated from human vastus lateralis muscle.

Functionality of the isolated mitochondria: respiration and P/O ratios

To insure that the isolated mitochondria were intact and to further examine the functional quality of the isolated mitochondria, respiration measurements were performed.

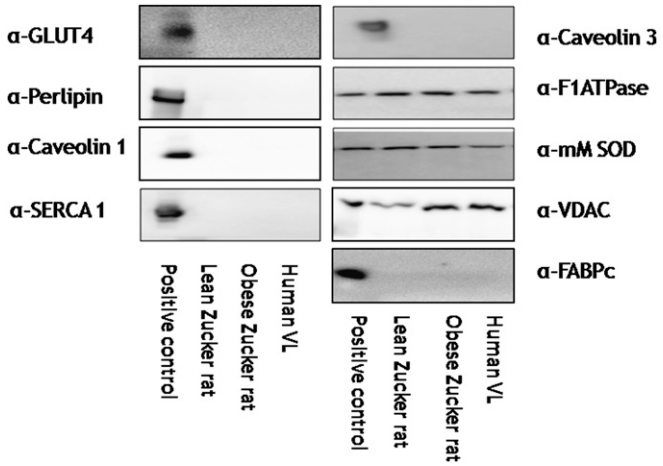

Fig. 1. Characterization of isolated mitochondria from skeletal muscle. Mitochondria isolated from lean and obese Zucker rats (n = 8) and human vastus lateralis muscle (VL) (n = 10) were characterized using immunoblotting techniques. Equal protein amounts of lysate (positive control) and mitochondria preparation (all other lanes) were resolved by SDS-PAGE and were immunoblotted using antibodies specific for subcellular protein markers: GLUT4, perilipin, Caveolin 1, SERCA 1, Caveolin 3, F_1ATPase-β, VDAC, Mn SOD (SOD2), and FABPc. For all protein markers, except perilipin, rat skeletal muscle lysate was used as positive control. Postive control for perilipin was rat fat tissue lysate. Images of representative immunoblots are shown. α-: anti-.

The mitochondrial P/O ratio from the lean and obese Zucker rats were 2.40 ± 0.05 vs. 2.37 ± 0.03, respectively, and 2.65 ± 0.06 in mitochondria isolated from biopsies obtained from human vastus lateralis muscle, indicating that mitochondria were fully intact.

There was no difference in maximal ADP-stimulated respiration (state 3) between the lean and obese Zucker rats (pyruvate + malate: 157.3 ± 9.0 vs. 164.0 ± 8.1 nmol O_2 min^{-1} U CS^{-1}, palmitoyl-L-carnitine + malate: 112.1 ± 3.4 vs. 109.2 ± 2.5 nmol O_2 min^{-1} U CS^{-1}) (**Fig. 2**). In addition, we measured state 3 respirations in a subgroup of Zucker rats using palmitoyl-L-carnitine and palmitoyl-CoA as substrate. ADP-stimulated respiration using palmitoyl-CoA was 6.4 ± 2.3- and 6.1 ± 1.4-fold higher than basal respiration in the lean and obese rats, respectively, assuring the presence of the outer mitochondrial membrane. There was no significant difference between obese and lean animals. ADP-stimulated respiration with palmitoyl-CoA as substrate was significantly lower (40%) than respiration with palmitoyl-L-carnitine.

FAT/CD36 is not detectable by immunoblotting in the isolated mitochondria

FAT/CD36 protein content in isolated mitochondrial membranes was investigated using SDS-PAGE and immunoblotting. Equal protein amounts of lysate and mitochondria preparations were resolved by SDS-PAGE and were immunoblotted using specific antibodies. FAT/CD36 protein was not detected in the mitochondrial fractions isolated from both lean and obese Zucker rats or from human vastus lateralis muscle (**Fig. 3**). The same PVDF membranes were immunoblotted against F_1-ATPase-β protein, a marker of mitochondrial membranes, showing that F_1-ATPase-β was present in all mitochondrial fractions (Fig. 3).

Fluorescence immunocytochemistry

Single muscle fibers from female Zucker rat soleus muscle and from male human vastus lateralis muscle were subjected to immunocytochemistry to visualize the cellular localization of FAT/CD36, as well as the distribution of the mitochondrial protein MitoNEET, in skeletal muscle (**Figs. 4** and **5**). Single muscle fibers from rat soleus (Fig. 4) and human vastus lateralis (Fig. 5) both presented a vesicle-like dotted pattern of intracellular FAT/CD36 distribution throughout the muscle fibers, being especially abundant at the subsarcolemmal region. The FAT/CD36 distribution was clearly different from the distribution pattern of mitochondrial networks (Figs. 4 and 5). These results clearly show that in the basal state, FAT/CD36 is not located in the outer membrane of mitochondria. The merged higher magnification images of FAT/CD36 and mitochondrial intracellular distribution in rat soleus muscle fibers (Fig. 4C–E) and in human vastus lateralis (Fig. 5A, right panel) show no colocalization between FAT/CD36 and the outer mitochondrial membrane marker MitoNEET. If a colocalization was apparent, it should be visualized as a yellow color. These results support the present biochemical data from immunoblotting of purified isolated mitochondria (Fig. 3). Further studies are needed to identify the exact intracellular compartment with which FAT/CD36 associates.

Total protein expression

There were no differences between the lean and obese Zucker rats in total protein expression of the lipid binding proteins FAT/CD36, FABPpm, Caveolin 3, and Caveolin 1 (**Fig. 6**). Furthermore, the mitochondrial proteins F_1-ATPase-β and Cytochrome C did not differ between the lean and obese rats (**Fig. 7**).

DISCUSSION

The main finding in the present study was that a major fraction of muscle FAT/CD36 was found in the sarcolemma and subsarcolemma region as small vesicle-like structures, while the rest was associated with some vesicle-like structures distributed homogeneously throughout the muscle fibers. Interestingly, in contrast to other recent reports, we clearly show that FAT/CD36 was not detectable in human or rat skeletal muscle mitochondria in the basal state, using both a biochemical and an immunocytochemical approach. FAT/CD36 showed the same intracellular distribution in soleus muscle from female Zucker rats and in vastus lateralis from human male subjects.

In the present study, we carefully isolated mitochondria from Zucker rat soleus muscle and from human vastus lateralis muscle. We also demonstrated that the isolated mitochondria preparations were free of cellular membranes where FAT/CD36 is known to be abundantly expressed,

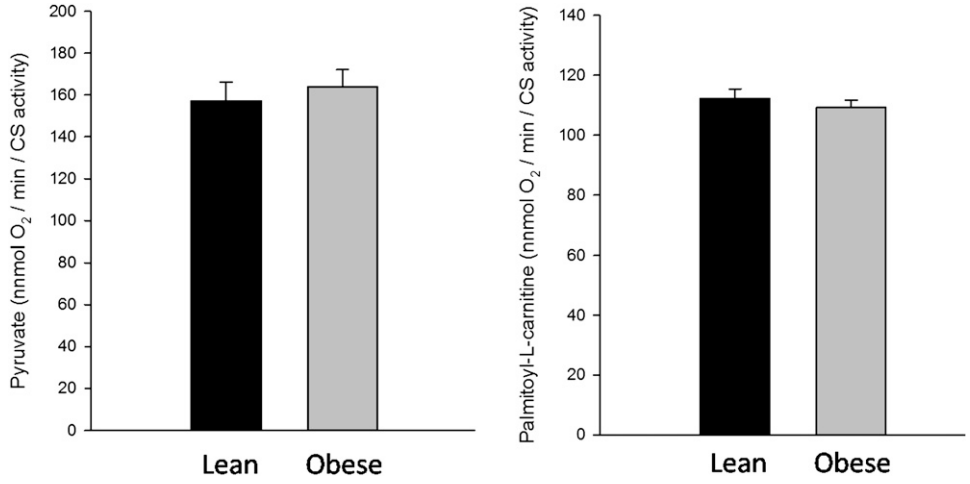

Fig. 2. Mitochondrial oxygen consumption in isolated mitochondria from lean and obese Zucker rats. The functional quality of the isolated mitochondria was addressed by respiration measurements. There was no difference in maximal ADP-stimulated respiration (state 3) using pyruvate + malate or palmitoyl-L-carnitine + malate as substrates between the lean (black bars) and obese (gray bars) Zucker rats (n = 8). Data are mean ± SEM.

such as skeletal muscle plasma membrane (14), endothelial cell surface membranes (30), and adipocyte plasma membranes (28, 31) (Fig. 1). In addition, we also demonstrated that the isolated mitochondria were intact, visualized by the presence of the specific outer and inner mitochondria membrane proteins VDAC and F_1-ATPase-β, respectively (25, 32). This indicates that the mitochondrial outer membrane structure, in which FAT/CD36 has been suggested to be localized (1), was intact (Fig. 1). To further investigate the integrity of the isolated mitochondria, mitochondrial oxygen consumption was measured (maximal ADP-stimulated respiration, state 3) using both palmitoyl-L-carnitine (CPT 1 independent) and palmitoyl-CoA (CPT 1 dependent) as substrates. Both substrates were usable for oxidation in the isolated mitochondria, showing that the isolated mitochondria were intact and with fully functional CPT 1.

It was previously reported from studies in female Sprague-Dawley rat gastrocnemius muscle (1) and in vastus lateralis from human female and male individuals (3–5) that FAT/CD36 was present in isolated mitochondria (1, 3–5). The present findings strongly indicate that FAT/CD36 is not present in skeletal muscle mitochondria from Zucker rats and from human male subjects. Considering the controversial findings in rat muscle, one could speculate that they could be due to rat strain differences, because the Zucker rat strain has a different muscle fiber type distribution compared with other rat models (33, 34) and humans (35). However, this does not explain the contradictory findings in human skeletal muscle FAT/CD36 intracellular distribution. Similar to the findings in the rat soleus muscle, FAT/CD36 was undetectable in isolated mitochondria from male human vastus lateralis muscle (Fig. 3). Thus, the findings in Zucker rats do not seem to be a genotype phenomenon. The absence of FAT/CD36 in mitochondria isolated from both Zucker rats and male human muscle were confirmed using fluorescence immunocytochemistry in single muscle fibers, where colocalization between FAT/CD36 and the mitochondrial outer membrane marker, MitoNEET (22), was not apparent (Figs. 4 and 5). These findings are in agreement with a previous study using immunohistochemical techniques in which no costaining of FAT/CD36 and the mitochondrial protein cytochrome C was reported, despite detection of some intracellular FAT/CD36 protein (17). The reason for the discrepancy between the present study and others whether FAT/CD36 protein is localized in isolated mitochondria is unclear. The differences could, however, be ascribed to technical differences in the procedure to isolate mitochondria. It should be recognized that in the isolation procedure, it is difficult to avoid contaminants from other cellular compartments. In the present study, we have carefully characterized our mitochondrial

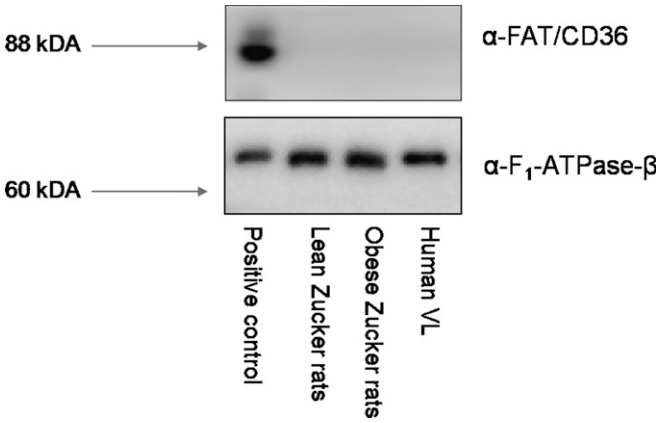

Fig. 3. FAT/CD36 protein expression in isolated mitochondria from lean and obese Zucker rat soleus muscle (n = 8) and human vastus lateralis muscle (VL) (n = 10) was analyzed using immunoblotting. FAT/CD36 was not detected in the isolated mitochondria but in the control (lysate) samples. On the same PVDF membrane, the β-subunit of F_1-ATPase, a mitochondrial marker protein, was detected. Rat skeletal muscle lysate was used as positive control. Images of representative immunoblots are shown. α-: anti-.

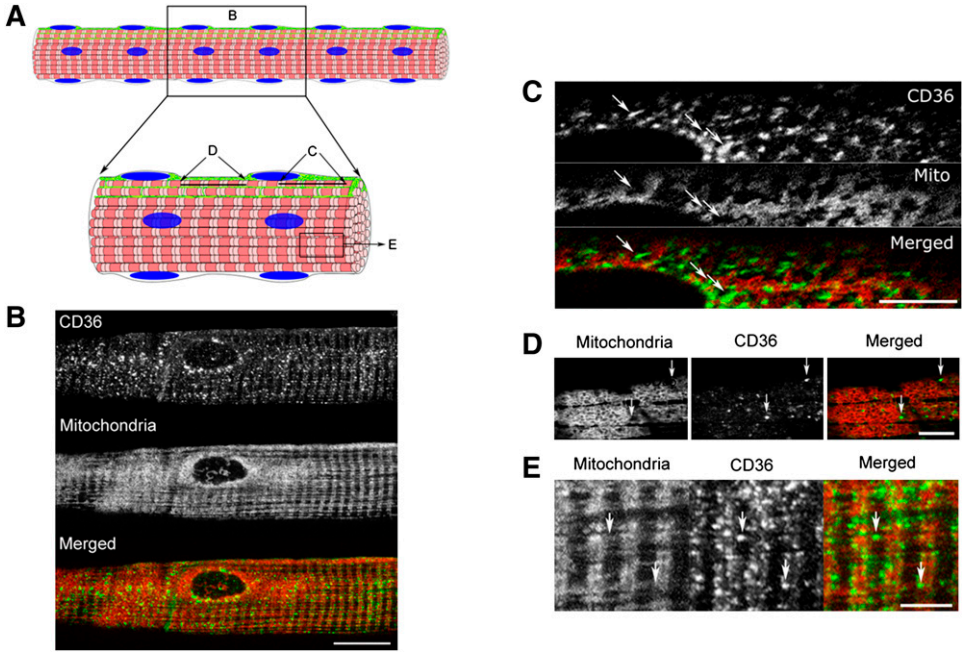

Fig. 4. Soleus muscle from 4 lean and 4 obese Zucker rats were fixed for confocal immunoflourescence microscopy and single muscle fibers were coimmunostained for FAT/CD36 (green) and MitoNEET (red) a marker for the mitochondrial outer membrane. A: A cartoon of a single muscle fiber is presented to give an overview and indicate the regions represented by the different images presented. B: FAT/CD36 and mitochondria distribution all throughout a rat soleus muscle fiber. Bar: 10 μm. Representative images of FAT/CD36 and mitochondria distribution in subsarcolemma perinuclear (C), subsarcolemmal (D), and intramyofibrillar (E) are shown. Bars: 5 μm. Note that in none of the analyzed areas does FAT/CD36 distribution show any resemblance or colocalization with mitochondria distribution.

preparation both from Zucker rat soleus muscle and human vastus lateralis muscle using a number of different marker proteins to verify that our mitochondria did not contain membranes from other cellular organelles. Furthermore, the results on isolated mitochondria demonstrate a tight coupling between ADP and respiration and efficiency (P/O ratio) within the expected range. This verifies that the isolated mitochondria had a high integrity and were function-

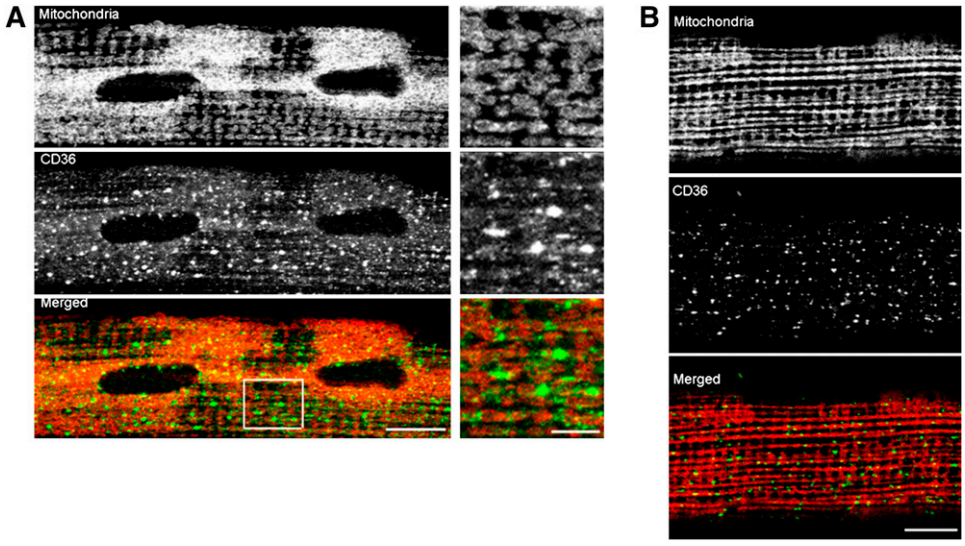

Fig. 5. Vastus lateralis muscle biopsies from four young male subjects were fixed for confocal immunoflourescence microscopy and single muscle fibers were coimmunostained for FAT/CD36 (green) and MitoNEET (red) as a marker for mitochondria outer membrane. A: Representative image of subsarcolemmal FAT/CD36 and mitochondria distribution are shown. Bar: 10 μm. Note in the magnified right panel images (bar: 2.5 μm) that no colocalization is present between FAT/CD36 and MitoNEET. B: Representative images of intermyofibrillar FAT/CD36 and mitochondria distribution are shown. Bar: 7.5 μm. Note the presence of FAT/CD36 positive structures, which do not colocalize with mitochondria, all throughout the muscle fiber.

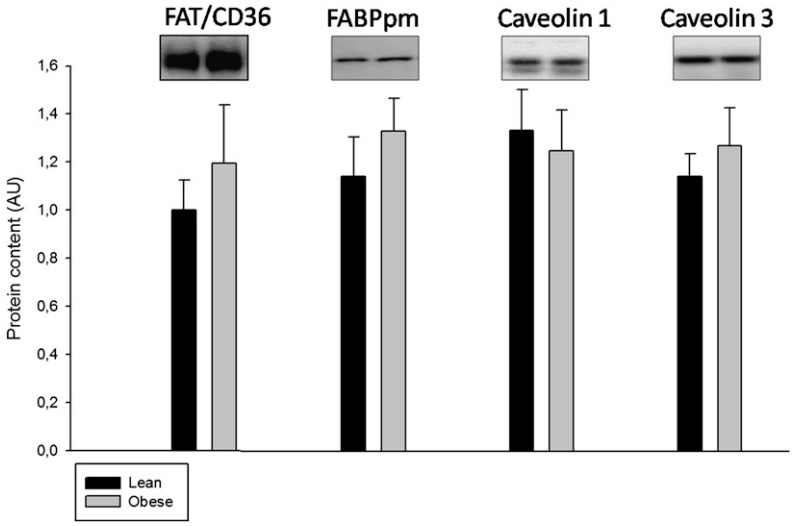

Fig. 6. Lysate proteins from lean and obese Zucker rat soleus muscle (n = 8) were resolved by SDS-PAGE and membranes were immunoblotted using antibodies specific against FAT/CD36, FABPpm, Caveolin 3, and Caveolin 1. There were no differences between the lean (black bars) and obese (gray bars) Zucker rats in any of the analyzed lipid binding proteins ($P > 0.05$). Data are mean ± SEM. Representative images of immunoblots are shown.

ally intact. We have not been able to find similar control measurements in the studies where evidence for functional mitochondrial FAT/CD36 has been presented (1–5, 7). Several proteomic investigations have clearly shown that the purification approaches may suffer from the problem of copurification, in particular with proteins associated with other cellular membrane fractions (36). Therefore, it is very difficult to distinguish novel mitochondrial proteins from those that are just contaminants of the preparation. Although FAT/CD36 was recently identified by tandem mass spectrometry in a preparation of functional mitochondria isolated from human skeletal muscle (37), the presence of FAT/CD36 in the preparation was considered contamination based on extensive bioinformatic analysis. Consistently, FAT/CD36 was not identified as a protein with mitochondrial localization in mass spectrometry-based analysis of isolated mitochondria from 14 different tissues in mouse (38). All together, these observations combined with the present findings (Figs. 1, 3, 4, and 5) strongly suggest that FAT/CD36 is not present in skeletal muscle mitochondria, at least not in the resting metabolic state. It is likely that previous reports of FAT/CD36 in mitochondrial preparations could be due to contamination with other membrane fractions.

There is substantial evidence that supports a functional and dynamic role of FAT/CD36 (39–43) and other lipid binding proteins (44–47) in regulation of transsarcolemmal fatty acid transport in skeletal muscle. Furthermore, the role of FAT/CD36 in LCFA plasma membrane transport is supported by studies reporting decreased LCFA uptake in cardiac muscle from humans who exhibit partial or total deficiency in FAT/CD36 protein (48, 49) and in cardiac and skeletal muscle from CD36 null mice (50, 51). In the present study, we did not observe differences in total FAT/CD36 and FABPpm protein expression between lean and obese Zucker rats (Fig. 6), which is in accordance with some (5, 52) but not all studies (53). The dynamic role of FAT/CD36 (i.e., translocation from intracellular compartments to the cell surface upon stimulation) has been intensely debated, because no comprehensive evidence has identified a clear intracellular FAT/CD36 pool. In the present study, we show a clear intracellular FAT/CD36 pool in skeletal muscle from both Zucker rats and male human vastus lateralis muscle (Figs. 4 and 5).

It has been suggested that the obesity and type 2 diabetes-related decrease in skeletal muscle fatty acid oxidation capacity is due to a decrease in skeletal muscle mitochondrial content (54, 55) or a dysfunction of the individual mitochondria (56–58). In the present study, we did not observe differences in mitochondrial oxygen consumption between the lean and the insulin-resistant obese Zucker rats

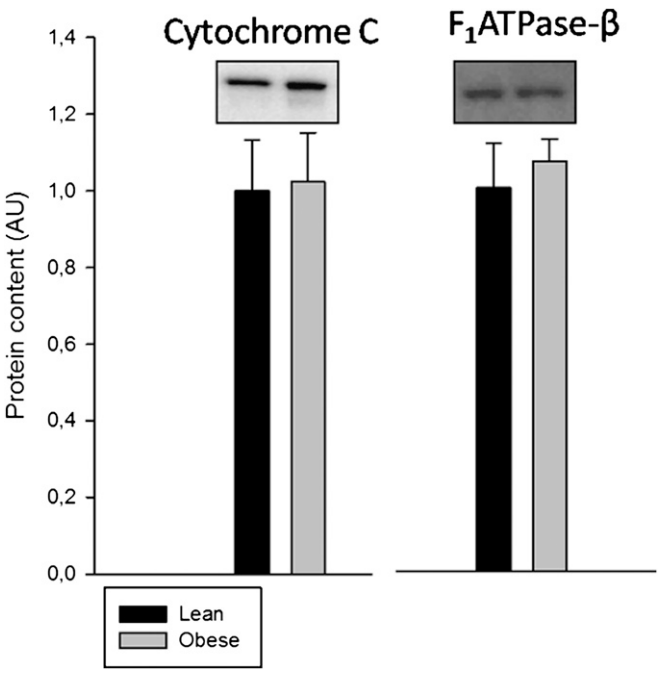

Fig. 7. Protein expression of mitochondrial marker proteins. Lysate proteins from lean and obese Zucker rat soleus muscle (n = 8) were resolved by SDS-PAGE and membranes were immunoblotted using antibodies specific against the mitochondrial proteins F_1-ATPase-β and Cytochrome C. There were no differences between the lean (black bars) and obese (gray bars) Zucker rats ($P > 0.05$). Data are mean ± SEM. Representative images of immunoblots are shown.

using pyruvate and palmitoyl-L-carnitine (CPT 1 independent) (Fig. 2) or palmitoyl-CoA (CPT 1 dependent) as substrates, showing functional and intact mitochondria. Furthermore, we did not observe differences in total protein expression of the mitochondria marker proteins F_1-ATPase-β and Cytochrome C between the lean and obese Zucker rats (Fig. 7), which suggests that mitochondrial function and content in the obese Zucker rats was unchanged compared with their lean controls.

In summary, we have in the present study demonstrated a clear distribution of FAT/CD36 protein in muscle fibers. Moreover, our data show that FAT/CD36 is not present in skeletal muscle mitochondria from Zucker rats and human male subjects. FAT/CD36 protein was found to be abundantly expressed in the sarcolemma and subsarcolemmal region as small vesicle-like structures, while some FAT/CD36 was associated with some vesicle-like structures distributed homogeneously all throughout the muscle fibers. FAT/CD36 showed the same intracellular distribution in both soleus muscle from female Zucker rats and in vastus lateralis from human male subjects. Thus, our data do not support a functional role for FAT/CD36 in mitochondrial fatty acid oxidation. The different findings between this study and others are most likely due to methodologically differences in mitochondria isolation procedure and therefore major methodological concerns have to be taken into account to avoid mitochondria preparations being contaminated with other cellular membrane fractions containing FAT/CD36 protein.

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