

In obese rat muscle transport of palmitate is increased and is channeled to triacylglycerol storage despite an increase in mitochondrial palmitate oxidation

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Holloway GP, Benton CR, Mullen KL, Yoshida Y, Snook LA, Han X-X, Glatz JFC, Luiken JJFP, Lally J, Dyck DJ, Bonen A. In obese rat muscle transport of palmitate is increased and is channeled to triacylglycerol storage despite an increase in mitochondrial palmitate oxidation. *Am J Physiol Endocrinol Metab* 296: E738–E747, 2009. First published January 13, 2009; doi:10.1152/ajpendo.90896.2008.—Intramuscular triacylglycerol (IMTG) accumulation in obesity has been attributed to increased fatty acid transport and/or to alterations in mitochondrial fatty acid oxidation. Alternatively, an imbalance in these two processes may channel fatty acids into storage. Therefore, in red and white muscles of lean and obese Zucker rats, we examined whether the increase in IMTG accumulation was attributable to an increased rate of fatty acid transport rather than alterations in subsarcolemmal (SS) or intermyofibrillar (IMF) mitochondrial fatty acid oxidation. In obese animals selected parameters were upregulated, including palmitate transport (red: +100%; white: +51%), plasmalemmal FAT/CD36 (red: +116%; white: +115%; not plasmalemmal FABPpm, FATP1, or FATP4), IMTG concentrations (red: ~2-fold; white: ~4-fold), and mitochondrial content (red +30%). Selected mitochondrial parameters were also greater in obese animals, namely, palmitate oxidation (SS red: +91%; SS white: +26%; not IMF mitochondria), FAT/CD36 (SS: +65%; IMF: +65%), citrate synthase (SS: +19%), and β -hydroxyacyl-CoA dehydrogenase activities (SS: +20%); carnitine palmitoyltransferase-I activity did not differ. A comparison of lean and obese rat muscles revealed that the rate of change in IMTG concentration was eightfold greater than that of fatty acid oxidation (SS mitochondria), when both parameters were expressed relative to fatty transport. Thus fatty acid transport, esterification, and oxidation (SS mitochondria) are upregulated in muscles of obese Zucker rats, with these effects being most pronounced in red muscle. The additional fatty acid taken up is channeled primarily to esterification, suggesting that upregulation in fatty acid transport as opposed to altered fatty acid oxidation is the major determinant of intramuscular lipid accumulation.

fatty acid translocase; fatty acid transport proteins 1 and 4; plasma membrane-associated fatty acid-binding protein; glucose transport

OBESITY IS A STRONG RISK FACTOR for the development of insulin resistance. Although the exact mechanisms underlying the etiology of insulin resistance remain unknown, it is widely believed that alterations in fatty acid metabolism that result in the increased accumulation of intramuscular lipids play a prominent role (reviewed in Ref. 49). Specifically, diacylglyc-

erol (DAG; Ref. 46) and ceramides (25, 45) impair the insulin-stimulated activation of insulin receptor substrate-1 and Akt, two important intermediates in the insulin-signaling cascade. The mechanisms that contribute to the increased concentration of intramuscular lipids remain uncertain. However, this may result from an imbalance between fatty acid delivery and utilization, possibly as a result of 1) a decreased rate of fatty acid oxidation (28, 29) relative to their uptake by muscle, and/or 2) an increase in the rate of fatty acid transport into muscle (8, 24) that exceeds fatty acid oxidation.

Although there is an increase in circulating fatty acids in obesity, it has been proposed that fatty acid oxidation is decreased in the skeletal muscle of obese individuals (29). Such decrements can occur as a result of a reduction in the number of mitochondria and/or an intrinsic dysfunction in mitochondrial fatty acid oxidation. Indeed, a variety of indirect measurements, including imaging techniques (28, 40), biochemical enzymatic analysis (9, 27–29), and magnetic resonance spectroscopy (43), have suggested that the number of mitochondria is reduced in the skeletal muscle of obese, insulin-resistant, and type 2 diabetic humans. In addition, an indirect assessment of the capacity for mitochondrial fatty acid oxidation suggested an intrinsic dysfunction within mitochondria (28). It was also proposed that these defects were more pronounced in subsarcolemmal (SS) than intermyofibrillar (IMF) mitochondria (41). However, such a selective reduction in SS mitochondrial fatty acid oxidation (41) remains to be shown, since rates of fatty acid oxidation were not impaired in pooled, isolated mitochondria obtained from the muscles of either obese humans (1, 27, 39) or obese rodents (48).

Fatty acid transport into skeletal muscle is predominantly a protein-mediated process, involving several fatty acid transport proteins, including fatty acid translocase (FAT/CD36), plasma membrane-associated fatty acid-binding protein (FABPpm), and fatty acid transport proteins (FATP1 and 4; for review see Ref. 5). In obese humans (8) and in rodent models of obesity (24, 35), the rates of fatty acid transport into skeletal muscle are upregulated and are positively associated with skeletal muscle triacylglycerol concentrations and plasmalemmal FAT/CD36 (8, 35) and possibly FATP4 (1). Whether these increased rates of fatty acid

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transport exceeded the rates of fatty acid oxidation in SS and/or IMF mitochondria is not known.

Since recent studies (1, 9, 27, 39) have shown that mitochondrial fatty acid oxidation is not impaired in insulin-resistant muscle, it appears that other mechanisms account for the increased intramuscular lipid accumulation. Koves et al. (33) proposed a novel mechanism of fatty acid-induced insulin resistance, whereby lipid "oversupply" to the mitochondria, either by high-fat feeding or genetic manipulations, induced insulin resistance. This model would suggest that in insulin-resistant animals an increased rate of fatty acid transport into muscle exceeds the rate of fatty acid oxidation. Given that rates of fatty acid transport are increased in obese skeletal muscle (8, 24, 35), this upregulated fatty acid delivery would appear to be the means whereby additional fatty acids are provided to the mitochondria. In addition, the excess influx of fatty acids may be sufficiently large so as to overwhelm mitochondrial fatty acid oxidation, and hence, the unoxidized fatty acids accumulate within intramuscular lipid depots. Thus it appears that intramuscular lipid accumulation is not simply the result of reduced rates of fatty acid oxidation.

The foregoing studies indicate that the understandings concerning the dysregulation of fatty acid metabolism in insulin-resistant skeletal muscle are incomplete. It is unknown to what extent changes in fatty acid uptake, oxidation, and esterification are altered in skeletal muscle in relation to each other. We hypothesized that all facets of skeletal muscle fatty acid metabolism were upregulated in skeletal muscles of Zucker obese rats and that the increased triacylglycerol concentration is attributable to an inability to upregulate fatty acid oxidation sufficiently to cope with a large influx of protein-mediated fatty acid transport. This novel view is at odds with much of the prevailing notion that triacylglycerol accumulation is attributable to a reduced rate of fatty acid oxidation. To test our hypothesis, we examined, in red and white skeletal muscles from lean and obese Zucker rats, mitochondrial density, triacylglycerol concentrations, rates of palmitate transport and esterification, rates of palmitate oxidation in SS and IMF mitochondria, the activities of selected mitochondrial enzymes, mitochondrial DNA (mtDNA), and the expression and subcellular distribution of fatty acid transporters (FAT/CD36, FABPpm, FATP1, and FATP4). The novel observations in our work are that in obese muscles 1) mitochondrial content and 2) all facets of fatty acid metabolism were increased, including the rates of fatty acid transport, esterification, and oxidation in SS mitochondria, while fatty acid oxidation was unaltered in IMF mitochondria. Therefore, the concurrent increase in triacylglycerol concentrations cannot be attributed to reductions in fatty acid oxidation but likely results from the markedly increased rate of FAT/CD36-mediated fatty acid transport, which exceeds the capacity for the increased rate of fatty acid oxidation.

METHODS

Animals. Female lean (226 ± 3 g) and obese Zucker rats (349 \pm 7 g, Charles River) were housed in a climate controlled room (12:12-h light-dark cycle). Rat chow and water were provided ad libitum. This study was approved by the University of Guelph Animal Ethics Committee.

Glucose transport in perfused skeletal muscle. Rates of basal and insulin-stimulated 3-O-methyl- ^3H glucose (3-O-MG) transport were determined in perfused rat hindlimb muscles as previously described

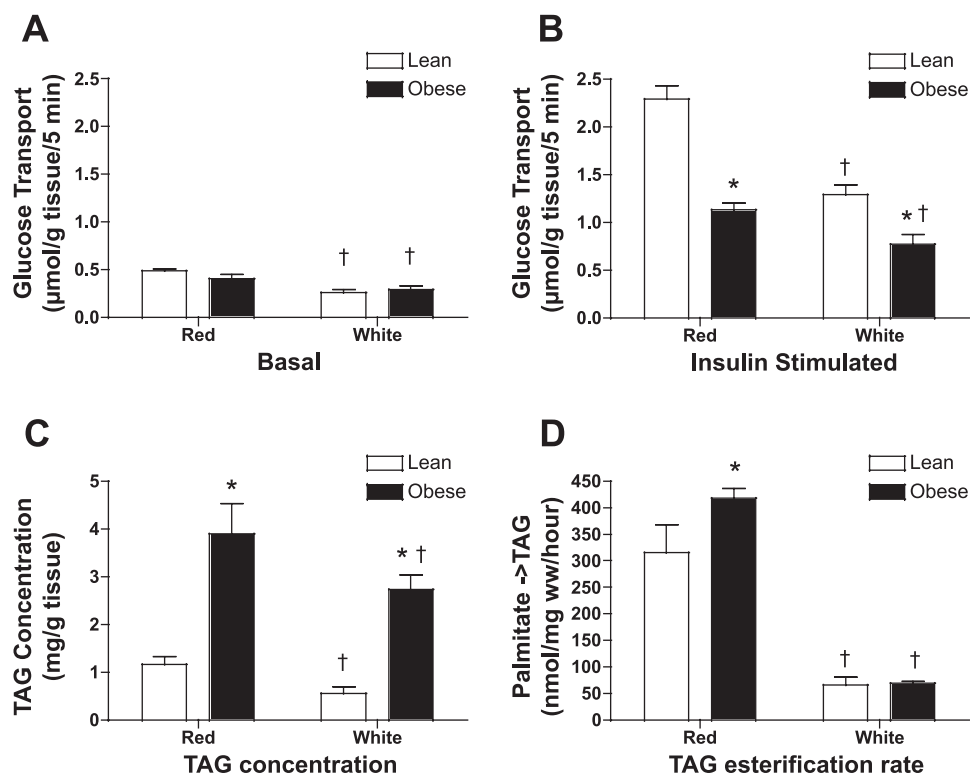
(3). Briefly, the animals were surgically prepared under anesthesia (65 mg pentobarbital sodium/100 g body wt). The cell-free perfusate consisted of a Krebs-Henseleit buffer, 2 mM pyruvate, and 4% BSA under constant gassing (95% O_2 -5% CO_2). Initially (5 min), the venous outflow was discarded and the perfusion flow was adjusted to 18 ml/min. Thereafter, muscles were preperfused, either without (basal) or with insulin (Humulin-R; Eli-Lilly, Toronto, ON, Canada) for 20 min. Thereafter, 3-O-MG, corresponding to the concentration at which the maximal rate of glucose transport occurs (40 mM, 10 μCi 3-O-MG; Amersham Life Science, Oakville, ON, Canada), was added to the perfusion reservoir. In all experiments, mannitol (2 mM, 10 μCi [^{14}C]mannitol; Amersham Life Science) was used as an extracellular space marker. Since 3-O-MG transport increases linearly for 30 and 15 min for basal and insulin-stimulated conditions, respectively (A. Bonen and X.-X. Han, unpublished data), perfusions for the basal conditions were performed for 20 min to accumulate sufficient counts in the muscles, whereas perfusion with insulin was performed for 9 min. Upon completion of the perfusion, the muscles from both hindlimbs were immediately excised, blotted for excess liquid on paper, and frozen in liquid N_2 . Subsequently, muscle samples were boiled with 1 N NaOH for 15 min and chilled on ice. Thereafter, 200 μl of aqueous solution as well as perfusate sample were counted in a liquid scintillation counter. Determinations of 3-O-MG transport rate were performed using standard calculations.

Fatty acid esterification and intramuscular triacylglycerol concentrations. Rates of palmitate incorporation into triacylglycerol depots were determined in perfused rat hindlimb muscles as we have described in detail previously (24). Briefly, in all experiments, the hindquarter of rats were preperfused (gassed continuously with 95% O_2 -5% of CO_2 , at 37°C) with 0.1% BSA (Sigma-Aldrich, St. Louis, MO) in Krebs-Henseleit buffer (pH 7.4) for 10 min, after which the outflow was discarded. During the experimental period, muscles from the hindlimbs were perfused (recirculating mode) for 60 min at a flow rate of 18 ml/min pH 7.4, with Krebs-Henseleit buffer containing 6 mM glucose, [^{14}C]palmitate (1 mM, 0.1 $\mu\text{Ci}/\text{ml}$; Amersham Life Science), and 4% BSA. At the point that the experiments were stopped (60 min), muscles were quickly harvested, rapidly frozen in liquid nitrogen, and stored at -80°C until analyzed. The rate of palmitate esterification was determined using thin layer chromatography. For this purpose, muscle (50 mg) was homogenized (Polytron; Kinematica, Brinkmann, Littau-Lucerne, Switzerland) in 2 ml 1:1 chloroform-methanol on ice for 2×15 s at speed of 8 separated by a 15-s interval. Solvent solution was recovered by centrifugation at 6,000 g for 10 min at 4°C and transferred to a new tube. This solution was washed with 2 ml H_2O , and the lipid containing chloroform phase was separated from the aqueous phase with another centrifugation step. Thereafter, 500 μl of the chloroform phase was dried under nitrogen and samples were reconstituted with 100 μl of 2:1 chloroform-methanol (vol:vol). Samples (50 μl) were then spotted onto 250-mm silica gel plates and resolved (60:40:3; heptane:isopropylether:acetic acid) for 50 min. Afterwards, the plate was air dried and sprayed with chlorofluorescein dye (0.02% wt/vol in ethanol). The triacylglycerol lipid band was visualized under long-wave ultraviolet light against standards that were included with each separation. The silica gel powder of the individual band was carefully scraped off the plate and transferred into scintillation vials for determining the radioactive counts.

We also determined the intramuscular triacylglycerol concentration in muscles of lean and obese rats. For this purpose, muscles were harvested and the same thin layer chromatography separation procedure described above was used. The resulting triacylglycerol bands were quantified against known standards.

Preparation of giant sarcolemmal vesicles and rates of fatty acid transport. Fatty acid transport was determined in giant sarcolemmal vesicles. These were prepared from the red and white portions of the quadriceps and gastrocnemius muscles, as previously described (7, 8, 35, 37). Briefly, muscle 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

Fig. 1. Basal (A) and insulin stimulated glucose transport (B), intramuscular triacylglycerol content (C), and rate of palmitate esterification (D) in red and white muscles of lean and obese Zucker rats. Data are means \pm SE; $n = 5-6$ for lean and obese animals. * $P < 0.05$, obese vs. lean animals. † $P < 0.05$, white vs. red muscle.



(pH 7.4), aprotinin (30 $\mu\text{g/ml}$), and collagenase type VII (150 U/ml; Sigma-Aldrich) 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 (Sigma-Aldrich), which resulted in a second supernatant fraction. Both supernatant fractions were pooled, and Percoll (Sigma-Aldrich), KCl, and aprotinin were added to final concentrations of 3.5% (vol/vol), 28 mM, and 10 $\mu\text{g/ml}$, respectively. The resulting suspension was placed at the bottom of a density gradient consisting of a 3-ml middle layer of 4% Nycodenz

(wt/vol; Sigma-Aldrich) and a 1-ml KCl-MOPS upper layer. This sample was centrifuged at 60 g for 45 min at room temperature. Subsequently, the vesicles were harvested from the interface of the upper and middle layers, diluted in KCl-MOPS, and recentrifuged at 12,000 g for 5 min.

Rates of palmitate transport into giant vesicles were determined as we have previously described (20–22). Briefly, 40 μl of 0.1% BSA in KCl-MOPS, containing unlabeled (15 μM) and radiolabeled 0.3 μCi [^3H]palmitate and 0.06 μCi [^{14}C]mannitol, were added to 40 μl of

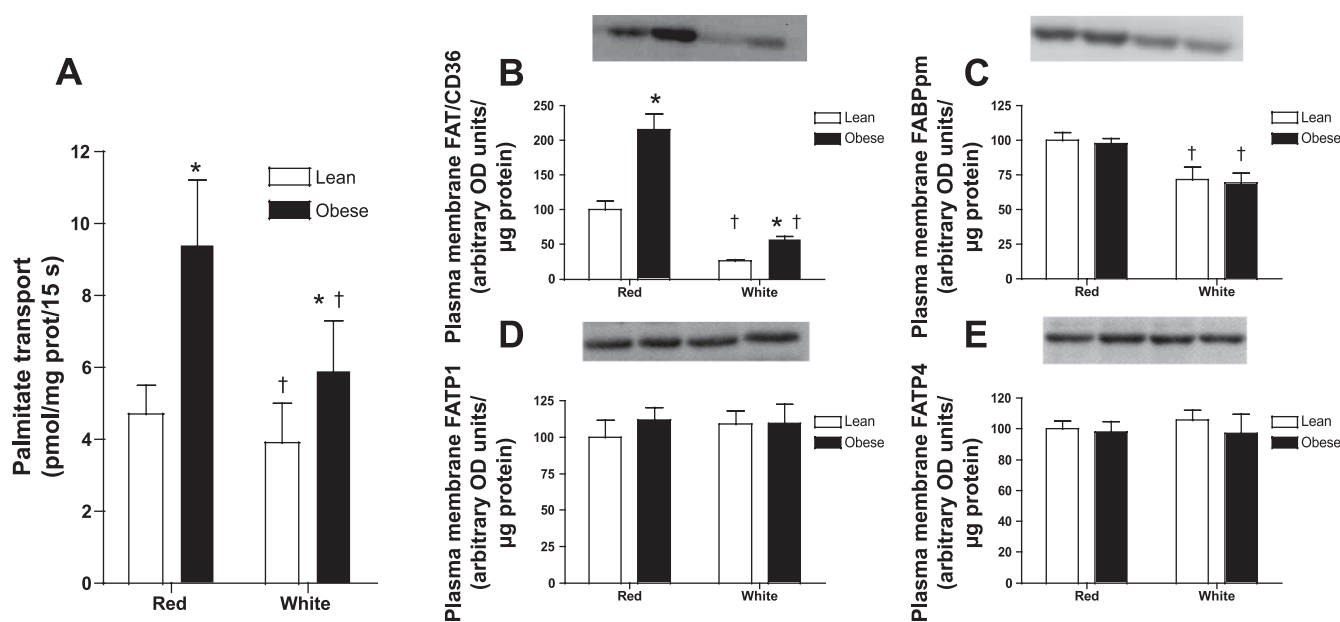


Fig. 2. Rates of palmitate transport (A) and plasma membrane fatty acid translocase (FAT/CD36; B), plasma membrane-associated fatty acid-binding protein (FABPpm; C), fatty acid transport proteins 1 (FATP1; D), and FATP4 (E) in red and white muscles of lean and obese Zucker rats. Data are means \pm SE; $n = 5$ for lean and obese animals. * $P < 0.05$, obese vs. lean animals. † $P < 0.05$, white vs. red muscle. OD, optical density.

Table 1. Fatty transport protein expression in muscle of lean and obese Zucker rats

Transporter	Red Muscle		White Muscle	
	Lean	Obese	Lean	Obese
FAT/CD36	100±8.6	101.0±15.0	43.5±2.5*	58.7±6.7*,†
FABPpm	100±8.7	90.0±7.7	60.9±5.8*	53.0±6.6*
FATP1	100±7.4	99.1±9.2	39.2±4.1*	43.1±4.6*
FATP4	100±12.7	108.3±15.8	39.6±8.7*	57.5±9.0*

Data are means ± SE (arbitrary optical density units/μg protein; $n = 7-9$). FAT/CD36, fatty acid translocase; FABPpm, plasma membrane-associated fatty acid-binding protein; FATP1 and FATP4, fatty acid transport protein 1 and 4. * $P < 0.05$, white vs. red muscle. † $P < 0.05$, white obese vs. white lean muscle.

vesicle suspension. The incubation was carried out for 15 s. Palmitate uptake was terminated by addition of 1.4 ml of ice-cold KCl-MOPS, 2.5 mM HgCl₂, and 0.1% BSA. The sample was then quickly centrifuged, and the supernatant fraction was discarded. Thereafter, radioactivity was determined in the remaining pellet. Nonspecific uptake was measured by addition of the stop solution before addition of the radiolabeled palmitate solution.

Isolation of mitochondria from skeletal muscle. Differential centrifugation was used to obtain both SS and IMF mitochondrial fractions, as we have previously described (4, 14, 26). Briefly, muscle was homogenized (Teflon pestle) and centrifuged (800 g) to separate the SS and IMF mitochondria. Subsequently, IMF mitochondria were treated with protease (Subtilisin A; Sigma-Aldrich; 0.25 μg protease/mg wet wt muscle) for exactly 5 min and centrifuged to remove myofibrils. Recovery of both SS and IMF mitochondria was accomplished by subsequent centrifugation (10,000 g).

Carnitine palmitoyltransferase I activity and mitochondrial palmitate oxidation. Carnitine palmitoyltransferase I (CPT I) activity and palmitate oxidation were measured in isolated SS and IMF mitochondria. Briefly, the forward radioisotope assay was used for the determination of CPT I activity {75 μM palmitoyl-CoA (L-[³H]carnitine)}, as described by McGarry et al. (38) and as we have previously

reported (2, 3). Palmitate oxidation was measured in a sealed system, as we have described previously (4, 26). Briefly, mitochondria were added to a preagitated modified Krebs Ringer buffer, 37°C. The reaction (30 min) was initiated by addition of a 6:1 palmitate:BSA complex (75 μM palmitate). Oxidation was determined from trapping of the ¹⁴CO₂ produced and the ¹⁴C label released from acidified buffer (3, 4, 14, 26).

Enzyme activities and mitochondrial DNA. Activities of citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) were determined in muscle homogenates and isolated mitochondria using standardized procedures (3, 4). mtDNA was determined in the red and white portions of the tibialis anterior muscle using real-time PCR, as we have previously reported (3). Briefly, total DNA was isolated using DNeasy blood and tissue kit (Qiagen), and real-time PCR was performed using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). mtDNA primers were designed using the rat mitochondrial genome sequence (Genbank accession number NC_001665) within the NADH dehydrogenase subunit 5 gene: forward, 5'-GCAGCCA-CAGGAAAATCCG-3'; and reverse, 5'-GTAGGGCAGAGACGG-GAGTTG-3'. Primers measuring genomic content were designed within the solute carrier family 16, member1 gene sequence on chromosome 2 (Genbank accession number NC_005101): forward, 5'-TAGCTGGATCCCTGATGCGA-3'; and reverse, 5'-GCATCA-GACTTCCCAGCTTCC-3'. Relative mtDNA content was calculated by the ΔΔCT method using genomic DNA content as an internal standard with 7500 System SDS Software Version 1.2.1.22 (Applied Biosystems).

Western blotting. Selected proteins were identified in red and white muscle homogenates and on the plasma membrane of red and white muscle giant vesicles using Western blotting as previously described (6-8, 32, 35, 36, 44). Antibodies against FABPpm and FAT/CD36 were the same as used previously (3, 24). Commercially available antibodies were used to detect cytochrome *c* oxidase complex IV (COXIV; Invitrogen, Burlington, ON, Canada), diacylglycerol acyltransferase (Santa Cruz Biotechnology, Santa Cruz, CA), and FATP1 and -4 (Santa Cruz Biotechnology). Blots were quantified using chemiluminescence and the ChemiGenius 2 Bioimaging system (SynGene, Cambridge, UK). Ponceau staining was used to confirm equal loading.

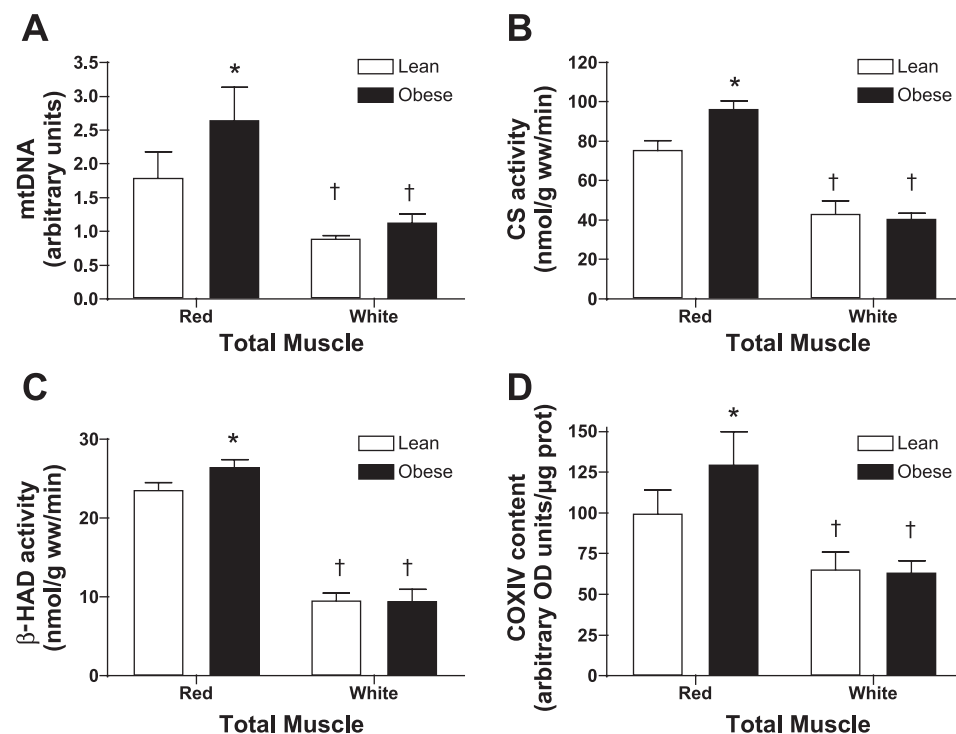
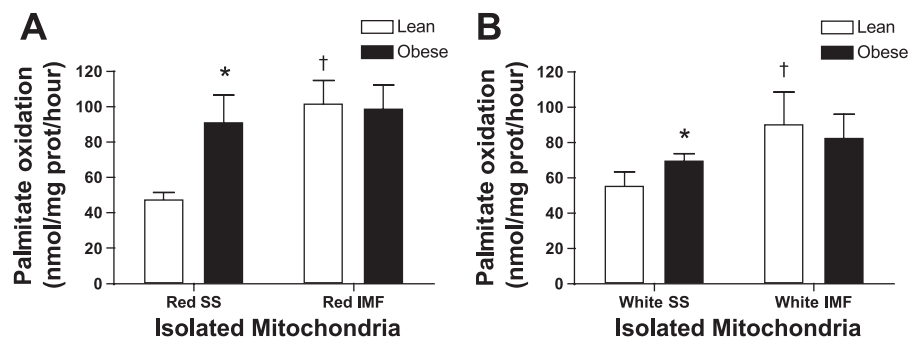


Fig. 3. Mitochondrial DNA (A), citrate synthase (CS) activity (B), β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity (C), and cytochrome *c* oxidase complex IV (COXIV) protein (D) in red and white muscles of lean and obese Zucker rats. Data are means ± SE; $n = 5$ for lean and obese animals. * $P < 0.05$, obese vs. lean animals. † $P < 0.05$, white vs. red muscle.

Fig. 4. Palmitate oxidation in isolated subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria isolated from red (A) and white muscles (B) of lean and obese Zucker rats. Data are means \pm SE; $n = 5$ for lean and obese animals. * $P < 0.05$, obese vs. lean animals. † $P < 0.05$, IMF vs. SS.



Statistics. All data are presented as the means \pm SE. A two-way analysis of variance was used, and when appropriate a Fisher's least significant difference post hoc analysis was used. To compare relationships between selected parameters, least squares regression analyses were used.

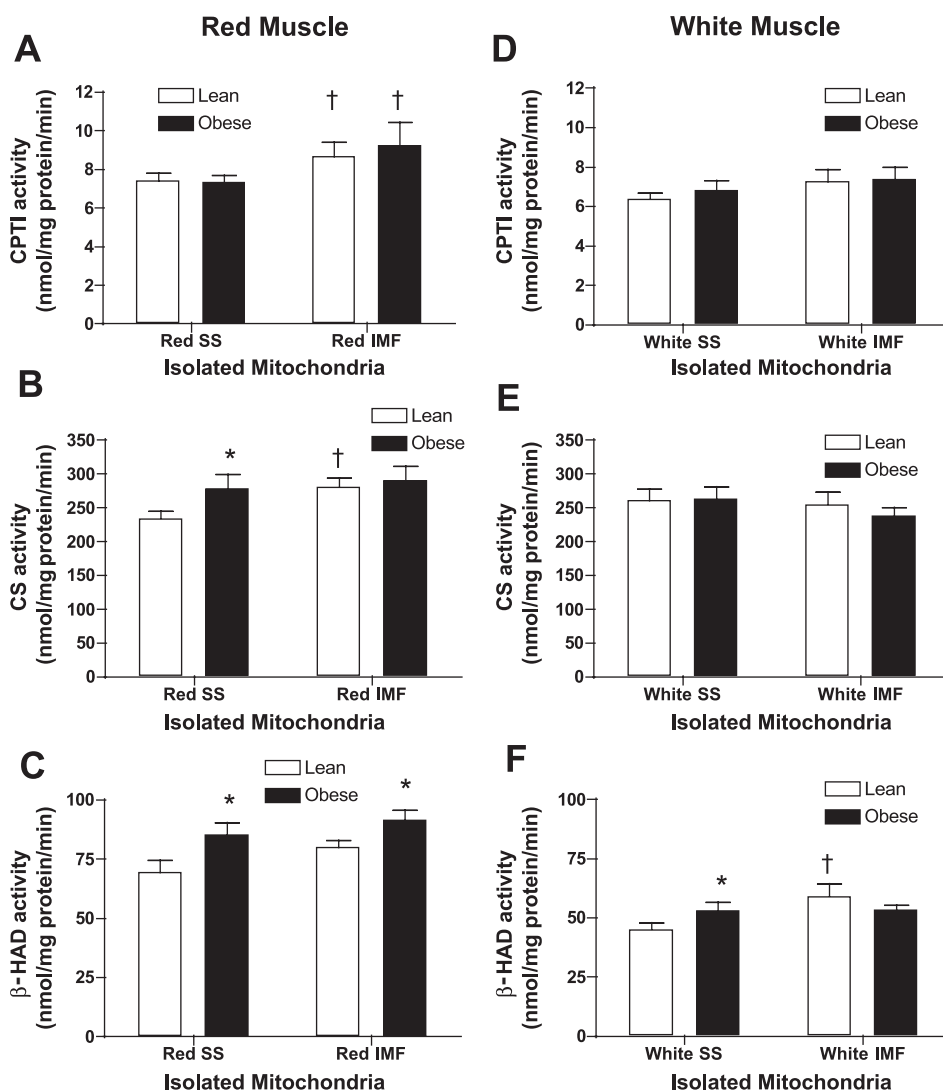
RESULTS

Glucose transport rates. Basal rates of glucose transport, although lower in white compared with red muscle, did not differ between lean and obese animals ($P > 0.05$; Fig. 1A).

Insulin-stimulated glucose transport was lower in both red (-51%) and white muscles (-48%) of the obese rats ($P < 0.05$; Fig. 1B).

Triacylglycerol concentration and esterification rates. Triacylglycerol concentrations were lower in white than in red muscles of both lean and obese animals. However, in obese rats, triacylglycerol concentrations were higher in both red (~ 2 -fold) and white muscles (~ 4 -fold; $P < 0.05$; Fig. 1C). In addition, the rate of palmitate incorporation into triacylglycerol

Fig. 5. Enzymatic activities of carnitine palmitoyltransferase I (CPT I; A and D), CS (B and E), and β -HAD (C and F) in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria isolated from red and white muscles of lean and obese Zucker rats. Data are means \pm SE; $n = 11$ for lean and obese animals, except β -HAD ($n = 6$). * $P < 0.05$, obese vs. lean animals. † $P < 0.05$, IMF vs. SS.



was greater (+32%) in the red muscle of obese rats ($P < 0.05$; Fig. 1D). Protein expression (arbitrary optical density unit/ μg protein) of diacylglycerol acyltransferase was very high in red lean (100 ± 5.2) and obese muscles (151 ± 6.1 ; $P < 0.05$) compared with white muscles (lean: 4.4 ± 1.3 ; obese: 6.8 ± 0.83) and differed relatively much more (25–34-fold) between red and white muscles than either the rates of palmitate incorporation into intramuscular triacylglycerol or triacylglycerol concentrations.

Muscle fatty acid transport and fatty acid transport protein expression and plasmalemmal content. In obese Zucker rats, the rate of palmitate transport was higher in both red (+100%) and white (+51%) skeletal muscles compared with lean animals ($P < 0.05$; Fig. 2A). Differences between red and white muscle were apparent in both lean and obese animals (red > white, $P < 0.05$; Fig. 2A).

The protein expression of FAT/CD36, FABPpm, FATP1, and FATP4 was all 40–60% lower in white muscles compared with red muscles ($P < 0.05$; Table 1). Except for white muscle FAT/CD36, there were no differences in the fatty acid transport protein expression in the respective red and white muscles of lean and obese Zucker rats ($P > 0.05$; Table 1). In contrast, the plasmalemmal FAT/CD36 content was considerably up-regulated in both red (+116%) and white (+115%) skeletal muscles of obese compared with lean animals ($P < 0.05$; Fig. 2B), whereas the plasmalemmal content of the other fatty acid transport proteins (Fig. 2, C–E) did not differ in respective red and white muscles of lean and obese animals ($P > 0.05$). Moreover, fiber type differences (red > white) were only observed for plasmalemmal FAT/CD36 and FABPpm ($P < 0.05$; Fig. 2, B and C).

Muscle mitochondrial content and enzymes. In obese red muscles, mtDNA (+47%), CS (+27%) and β -HAD (+12%) activities, and COXIV (+30%) protein expression were higher (Fig. 3, A, B, C, and D, respectively). In contrast, none of these parameters differed in the white muscles of lean and obese animals.

Palmitate oxidation in isolated SS and IMF mitochondria. In lean animals, the rates of palmitate oxidation were greater in IMF compared with SS mitochondria in red (+113%; Fig. 4A) and white muscle (+63%; Fig. 4B). IMF mitochondrial palmitate oxidation did not differ in lean and obese muscle (Fig. 4, A and B). In contrast, in SS mitochondria from obese animals the rates of palmitate oxidation were markedly greater in both red (+91%) and white (+26%) muscle ($P < 0.05$; Fig. 4, A and B) and were similar to the rates in the respective IMF mitochondria.

Enzyme activities in isolated SS and IMF mitochondria. In red muscle, 1) CPT I activity was greater in lean and obese IMF mitochondria (+22%) relative to SS mitochondria, 2) CS activity was increased in obese SS mitochondria (+19%), and 3) β -HAD activity was greater in obese SS (+23%) and IMF (+14%) mitochondria (Fig. 5, A–C). In white muscles, no differences in enzyme activities were found between lean and obese animals (Fig. 5, D and E), except that β -HAD activity was greater in obese SS mitochondria (+18%; Fig. 5F).

COXIV and FAT/CD36 in isolated SS and IMF mitochondria. In red and white muscle, COXIV protein content was not different in SS and IMF mitochondria from lean and obese animals (Fig. 6, A and B). However, in obese rats, mitochondrial FAT/CD36 was greater in red muscle (SS: +68%; IMF:

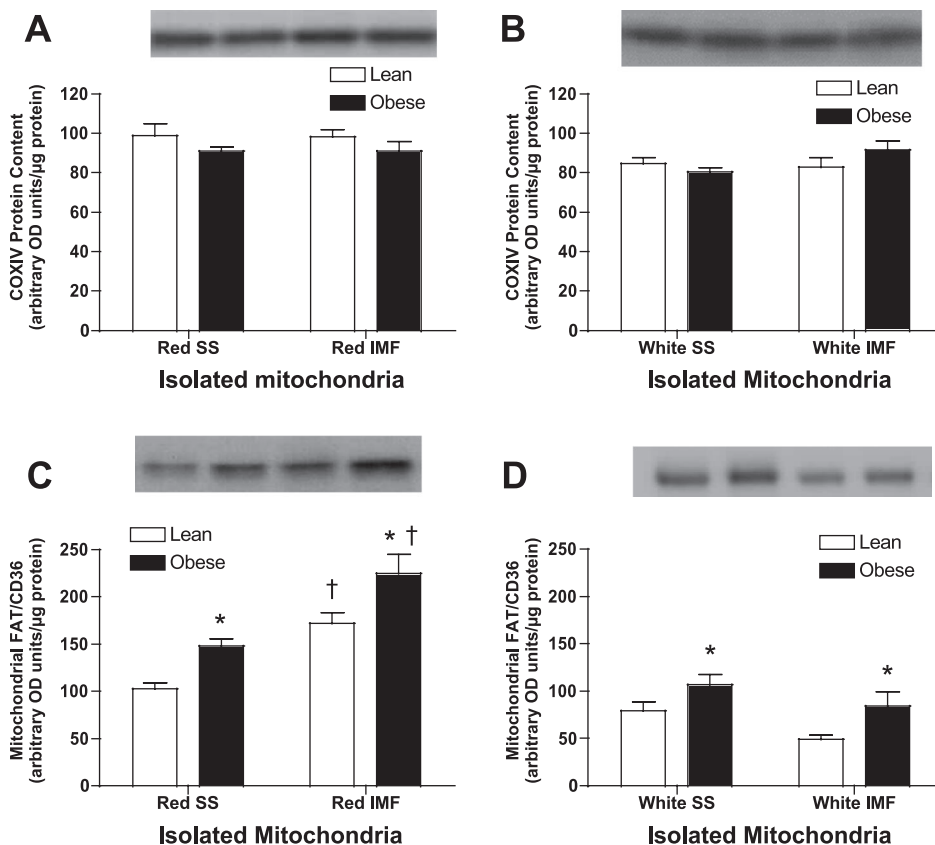


Fig. 6. COXIV (A and B) and fatty acid translocase FAT/CD36 (C and D) proteins in isolated SS and IMF mitochondria. Data are means \pm SE; $n = 5$ –6 for lean and obese animals, except β -HAD ($n = 6$). * $P < 0.05$, obese vs. lean animals. † $P < 0.05$, IMF vs. SS.

+52%) and white muscle mitochondria (SS: +62%; IMF: +79%; Fig. 6, C and D).

Skeletal muscle triacylglycerol concentrations and mitochondrial fatty acid oxidation rates are positively associated with rates of fatty acid transport. When muscles from lean and obese animals were compared, fatty acid transport rates into muscle giant vesicles were positively associated with plasma membrane FAT/CD36 ($r = 0.91$; $P < 0.05$; Fig. 7A), intramuscular triacylglycerol content ($r = 0.95$; Fig. 7B), and palmitate oxidation rates in SS ($r = 0.94$; Fig. 7C) but not IMF (data not shown) mitochondria. We also compared the rates of fatty acid transport directly with intramuscular triacylglycerol concentrations and rates of palmitate oxidation (Fig. 7D). For this purpose, these latter data were both normalized to lean white muscle (=100%), so as to allow direct comparisons between their relative changes with rates of fatty acid transport. These comparisons revealed that in relation to fatty acid transport there was a considerably greater response in triacylglycerol accumulation than in fatty acid oxidation (SS mitochondria), as the slopes of the regression lines differed eightfold (Fig. 7D).

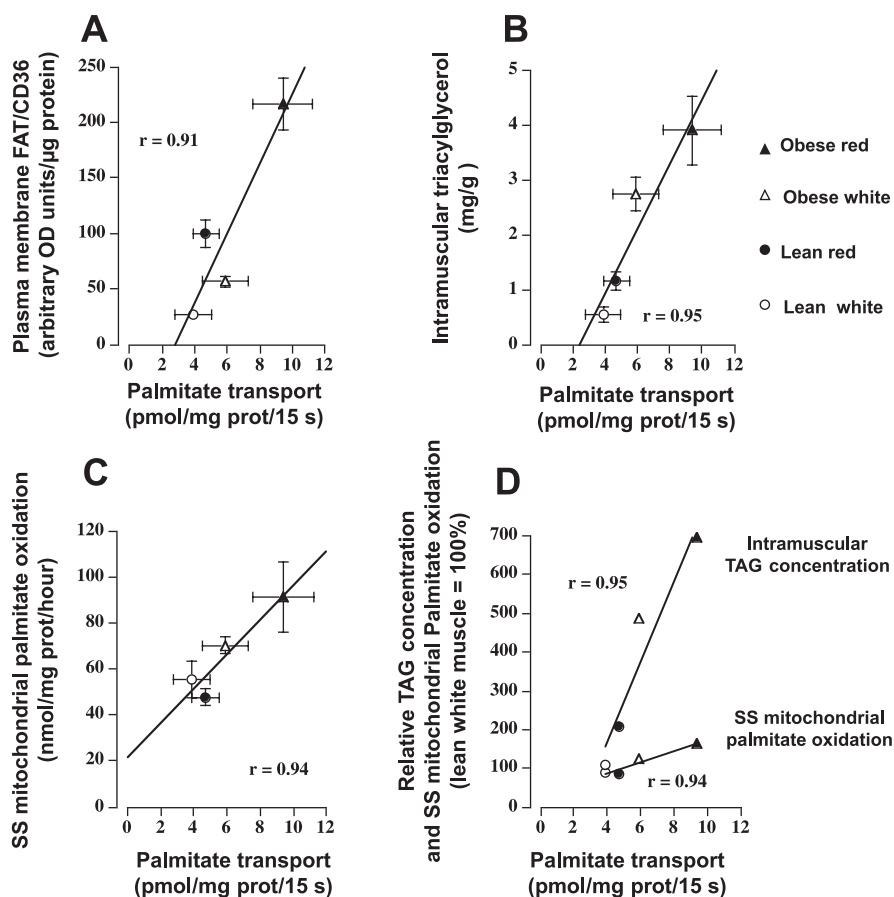
DISCUSSION

We have examined the basis for the increase in intramuscular triacylglycerol accumulation in skeletal muscles of obese Zucker rats. The novel observations are that 1) skeletal muscle mitochondrial content (red muscle), as well as 2) fatty acid oxidation in SS mitochondria (red and white muscle) are both increased, while 3) fatty acid oxidation in IMF mitochondria is normal, yet, at the same time 4) intramuscular triacylglycerol

accumulation has increased. Our work strongly suggests that the increased presence of plasmalemmal FAT/CD36 facilitates an increased rate of fatty acid transport into red and white obese muscles, a process that overwhelms the upregulated capacity to oxidize fatty acids in these muscles. Hence, the additional fatty acids taken up are esterified at a greater rate and intramuscular triacylglycerol concentrations are increased. This suggests that the regulation of fatty acid transport represents a key therapeutic target.

Plasma membrane fatty acid transport and transporters. In agreement with previous studies in muscles of obese rats (24, 47) and obese humans (8), the rate of fatty acid transport into muscles of obese Zucker rats was increased. FAT/CD36 and FABPpm traffic between intracellular depots and the plasma membrane and an increase in their plasmalemmal content increase the rate of fatty acid transport (6, 24, 50). The functional roles of FATP1 and FATP4 are still uncertain, as FATP1 but not FATP4 appears to have a fatty acid transport role (34), although others dispute this (18). Among these fatty acid transporters only the plasmalemmal content of FAT/CD36 was upregulated in obese Zucker rat muscle in agreement with previous observations in the muscles (24) and hearts (16) of these animals. Taken altogether, these observations suggest that the increased plasmalemmal FAT/CD36 accounted for the increased rate of fatty acid transport into obese muscle. We have previously shown in skeletal muscle (24) and in the heart (16) that this increase in sarcolemmal FAT/CD36 in obese Zucker rats is attributable to a permanent redistribution of this protein from its intracellular depot to the plasma membrane

Fig. 7. Comparison of palmitate transport rates with plasmalemmal FAT/CD36 (A), intramuscular triacylglycerol concentrations (B), and SS palmitate oxidation rates (C) in red and white skeletal muscles from lean and obese Zucker rats. Relative differences (%) in triacylglycerol concentrations and SS palmitate oxidation rates were compared against differences in palmitate transport rates in red and white muscles of lean and obese animals (D); for these purposes the mean data in lean, white muscles were set to 100% and all other means were expressed relative to this. Data for IMF palmitate oxidation rates were not plotted, as these did not differ among muscles and groups (see Fig. 4). Regression lines: intramuscular triacylglycerol (%) = $106 \times$ palmitate transport - 260; SS mitochondrial palmitate acid oxidation (%) = $13.3 \times$ palmitate transport + 40. Data are redrawn from Fig. 1, A and B, and Fig. 4A and data presented in RESULTS (triacylglycerol concentrations). Data are means \pm SE.



(24), as our present observations and previous work (8, 24, 35) show that the protein expression of FAT/CD36 is not altered. This implies that the intracellular signaling and/or trafficking of FAT/CD36 is altered in muscles of obese animals. Physiological stimuli known to induce FAT/CD36 translocation (insulin, muscle contraction, and AMPK activation) in lean animals fail to do so in the heart (16) and skeletal muscle of obese animals (24). These observations for FAT/CD36 parallel, in part, the observations for GLUT4 in obese skeletal muscle in which GLUT4 protein expression is not altered and insulin-stimulated GLUT4 translocation is impaired (31). However, unlike GLUT4 (10–12), the contraction-stimulated FAT/CD36 translocation is also impaired in these animals (24). The disrupted FAT/CD36 signaling/trafficking machinery in obese Zucker rat muscle remains to be identified.

Mitochondrial fatty acid oxidation is not impaired in muscles of obese Zucker rats. Mitochondrial dysfunction in fatty acid oxidation can result from 1) a reduction in the number of mitochondria, and/or 2) a reduction in mitochondrial intrinsic activity. Indirect evidence of mitochondrial fatty acid oxidation (28, 41) in skeletal muscle of obese, insulin-resistant humans has been taken to indicate that this would contribute to the increase in skeletal muscle triacylglycerol concentrations (28). However, the current study supports more recent work that indicates that insulin resistance can be present without reductions in muscle mitochondrial content (13) and that high-fat diets provoke insulin resistance while increasing mitochondrial content and fatty acid oxidation (23, 48). It has also been shown that fatty acid oxidation, when measured directly, is not impaired in samples of pooled SS and IMF mitochondria isolated from skeletal muscle of obese and type 2 diabetic individuals, (9, 27, 39). However, Ritov et al. (41) have speculated that the dysfunction in fatty acid oxidation in obesity likely occurred in SS mitochondria, which have a lower capacity for fatty acid oxidation (present study and Ref. 15) and which are less abundant (~25% of total mitochondrial pool; Ref. 17). However, the present study has shown in both red and white muscles of obese Zucker rats that 1) fatty acid oxidation was not altered in IMF mitochondria, and 2) fatty acid oxidation was increased, not decreased, in SS mitochondria. Thus our work (current study and Ref. 27) and that of others (9, 23, 39, 48) indicate that in animal models of obesity (23, 48) and in human obesity (9, 27, 39), there is little evidence to support the view that impaired mitochondrial fatty acid oxidation accounts for the increased intramuscular triacylglycerol concentrations.

Although mitochondrial fatty acid oxidation is thought to be regulated by CPT I, the CPT I data in the present study cannot account for the higher rates of fatty acid oxidation in SS mitochondria of obese animals, nor for differences in rates of fatty acid oxidation between IMF and SS mitochondria. While increases in the activities of CS and β -HAD for the most part displayed similar patterns to the changes in palmitate oxidation, neither of these two enzymes are thought to be rate limiting for fatty acid oxidation and are therefore likely not responsible for the observed increase in fatty acid oxidation rates in SS mitochondria. Taken altogether, these data suggest additional mechanisms contribute to the regulation of mitochondrial fatty acid oxidation.

Recently, FAT/CD36 has been found on mitochondrial membranes in rat (14), mouse (30), and human skeletal muscle

(4, 42). This fatty acid transport protein has been implicated in regulating mitochondrial fatty acid oxidation rates since 1) mitochondrial FAT/CD36 is increased during muscle contraction (14, 26), 2) the amount of mitochondrial FAT/CD36 during exercise correlates with mitochondrial function (26), 3) FAT/CD36 coimmunoprecipitates with CPT I (14, 42), and 4) rosiglitazone increases mitochondrial fatty acid oxidation and FAT/CD36 without altering CPT I activity (2). In the present study, we found that mitochondrial FAT/CD36 is increased in SS mitochondria of obese animals in parallel with the increased rates of SS mitochondrial fatty acid oxidation rates. However, FAT/CD36 was also increased in the IMF fraction of obese red and white muscle, without a concurrent upregulation of mitochondrial fatty acid oxidation. This may indicate that FAT/CD36 is not involved in regulating mitochondrial fatty acid oxidation. However, in studies with FAT/CD36 null mice mitochondrial fatty acid oxidation is reduced (G. P. Holloway and A. Bonen, unpublished observations). This recent work

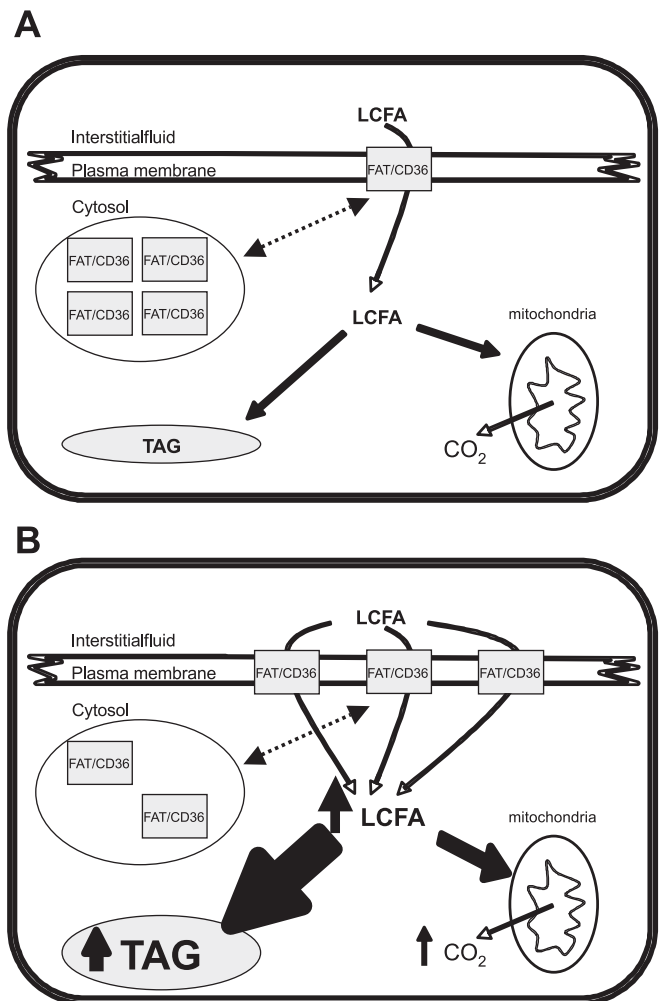


Fig. 8. Schematic representation of intramuscular triacylglycerol (TAG) accumulation in muscles of obese Zucker rats despite a concurrent increase in mitochondrial fatty acid oxidation. In this scheme, based on the present experimental results, a greater portion of the available FAT/CD36 is permanently relocated to the plasma membrane, which allows for an increased rate of fatty acid uptake into muscle. Since the increased rate of fatty acid uptake exceeds the capacity for their increased oxidation, intramuscular triacylglycerol depots are increased. A: lean animals. B: obese animals. LCFA, long-chain fatty acid.

and the present study suggest that additional factor(s) besides CPT I and FAT/CD36 are involved in regulating mitochondrial fatty acid oxidation. Taken altogether, it appears that FAT/CD36 may contribute, in an unknown manner, to the regulation of mitochondrial fatty acid oxidation in lean and obese muscle.

Imbalance between upregulated fatty acid transport and upregulated mitochondrial fatty acid oxidation increases intramuscular triacylglycerol. It is apparent that there is an increased capacity for fatty acid oxidation in muscles of obese animals, since mitochondrial content is increased and fatty acid oxidation is increased or unchanged in SS and IMF mitochondria, respectively. Yet, intramuscular triacylglycerol is seen to accumulate. Its accumulation, rather than the rate of palmitate incorporation into triacylglycerol, was correlated with the rate of fatty acid transport, presumably since the radiolabeled palmitate pool turns over more rapidly than intramuscular triacylglycerol and therefore serves only as an index of intramuscular triacylglycerol content (19, 21). Taken altogether, our results suggest that the markedly increased rate of fatty acid transport into insulin-resistant muscle exceeds the capacity for fatty acid oxidation, and therefore, more of these fatty acids that have entered the muscle are esterified, as we have shown schematically (Fig. 8). This notion is further supported by the observation that as palmitate transport is increased, the rate of intramuscular triacylglycerol accumulation occurs at much greater rate than the compensatory increase in the rate of SS mitochondrial fatty acid oxidation (Fig. 7D). This also supports, in part, the mechanism proposed by Koves et al. (33), namely, that in insulin-resistant muscle the excess lipids available to mitochondria exceed the capacity for their oxidation. We recognize that the comparisons in the isolated mitochondria do not take into account the ~30% increase in mitochondrial content in muscles of obese Zucker rats. However, this small difference between lean and obese animals would not negate our conclusion of a much greater relative (%) change (~8-fold) in intramuscular triacylglycerol accumulation. Thus it appears that a key event in raising intramuscular lipid concentrations is the increase in fatty acid transport, mediated by the increase in plasmalemmal FAT/CD36 (present study). In this manner, an increased delivery of fatty acids into muscle can overwhelm the increased capacity for their oxidation, which results in the accumulation of intramuscular triacylglycerol.

In conclusion, we have found in the muscles of obese Zucker rats that 1) the rate of plasma membrane fatty acid transport is increased, this is most likely attributable to the permanent relocation of FAT/CD36, but not FABPpm, FATP1, or FATP4, to the plasma membrane; 2) mitochondrial density is increased; and 3) SS mitochondrial fatty acid oxidation is increased, while that of IMF mitochondria is normal. We acknowledge that the experiments were performed primarily in vitro, in which contributions from additional factors such as other metabolites, hormones, and cytokines that are present in vivo are not taken into account. Nevertheless, it is difficult to ascribe a reduction in fatty acid oxidation in the muscle of obese animals as being at the root of the increase in intramuscular lipid accumulation that is associated with insulin resistance in obese skeletal muscle. Our study suggests that the increased intramuscular accumulation of lipids is attributable to an increased rate of fatty acid transport that exceeds the concurrently increased capacity for mitochondrial fatty acid oxidation.

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