CD36 in Myocytes Channels Fatty Acids to a Lipase-Accessible Triglyceride Pool That Is Related to Cell Lipid and Insulin Responsiveness

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High levels of intramyocellular triglycerides are linked to insulin resistance and reflect conditions in which fatty acid uptake exceeds the myocyte oxidative capacity. CD36 facilitates fatty acid uptake by myocytes, and its level is increased in diabetic muscle. We examined whether high CD36 levels would increase lipid content and susceptibility of myocytes to fatty acid–induced insulin resistance. C2C12 myoblasts with stable fivefold overexpression of CD36 (+CD36) were generated and differentiated into myotubes. CD36 expression increased palmitate uptake, oxidation, and lipid incorporation but had no effect on cell triglyceride content. Importantly, glycerol release increased fourfold, indicating enhanced triglyceride turnover and suggesting that CD36 promotes futile cycling of fatty acids into triglyceride. When +CD36 myotubes were incubated with excess palmitate, CD36 enhancement of glycerol release was blunted, triglyceride content increased above wild-type cells, and insulin resistance of glucose metabolism was observed. In contrast to palmitate, oleate-treated +CD36 cells exhibited enhanced glycerol release and no alteration in triglyceride content or insulin responsiveness. Furthermore, increased expression of hormone-sensitive lipase was measured with CD36 expression and with oleate treatment. In conclusion, high futile cycling of fatty acids is important for maintaining low triglyceride content and insulin responsiveness of myocytes. The findings provide a new perspective related to the etiology of lipid accumulation and insulin resistance in myocytes. *Diabetes* 53: 2209–2216, 2004

Abnormalities of muscle fatty acid utilization with atypical accumulation of intramuscular triglycerides are common in type 2 diabetes and obesity (1) and have been strongly linked to insulin resistance and lipotoxicity (2). The mechanisms underlying the increased muscle triglyceride levels are not well defined but are thought to be associated with conditions in which fatty acid uptake is increased, exceeding the muscle oxidative capacity. It is well known that fatty acid uptake by muscle is regulated by local factors in addition to fatty acid availability since it does not simply reflect blood fatty acid levels (3). For example, during contraction, muscle fatty acid uptake increases disproportionately, exceeding what would be expected from increased blood supply and fatty acid delivery.

There is now substantial evidence to indicate that transfer across the sarcolemma plays an important role in determining the rate of muscle fatty acid uptake. Several membrane proteins have been implicated in this process (4–6). Among these, CD36 (also called "FAT" for "fatty acid translocase") has been the most studied in vivo, where it was shown to facilitate a major fraction of muscle fatty acid uptake (7,8). CD36 is recruited to the plasma membrane by muscular contraction (8,9), enhancing fatty acid uptake and oxidation by the exercising muscle. In CD36-deficient mice (7) and humans (10), muscle has reduced fatty acid uptake despite high blood fatty acids, whereas muscle with CD36 overexpression exhibits enhanced fatty acid uptake and oxidative capacity during contraction (11).

Presumably, increasing CD36 level and fatty acid uptake would lead to increases in myocyte triglyceride and could enhance susceptibility to fatty acid–induced lipotoxicity and/or insulin resistance. Increased levels of CD36 in muscle of diabetic (12) and obese (9) mice have been reported, suggesting that abnormal levels or function of the protein may contribute to the pathogenesis of these diseases. Other findings, however, suggest that muscle CD36 expression may have a protective effect. For example, CD36 is essential for the beneficial effects of pioglitazone on lipid metabolism and for the drug action to increase muscle insulin sensitivity (13,14). Pioglitazone also prevents in parallel the decrease in muscle CD36 and the development of insulin resistance that follow Intralipid administration to rats (15). Such observations suggest that increasing CD36 may preferentially channel fatty acid to oxidation and not triglyceride formation or may promote fatty acid efflux as a cellular feedback mechanism that tends to limit lipid accumulation.

The objective of the present study was to examine how increasing CD36 level impacts lipid accumulation and insulin responsiveness of muscle cells independent of other factors likely to play a role in vivo. Such information is important for understanding the metabolic consequences of changes in muscle CD36 expression, such as

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ASM, acid soluble metabolite; DGAT, diacylglycerol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; HSL, hormone-sensitive lipase; KRH, Krebs-Ringer solution buffered with 10 mmol/l HEPES.

TABLE 1
List of primers used for quantitative PCR of CD36, MyoD1, myogenin, and HSL

	Sense	Antisense
rCD36	5′-CCGTTATTGGTGCTGTCCTGGC-3′	5′-AAGAGACAGTGCTGTCCTTGGG-3′
mMyoD1	5′-GCGACTCAGATGCATCCAGCC-3′	5′-GCGACTCTGGTGGTGCATCTG-3′
mMyogenin	5′-GCAATGCACTGGAGTTCGGTC-3′	5′-GCCTTATGTGAATGGGGAGTG-3′
mHSL	5′-GCGGCTGTCTAATGTCTTTGCA-3′	5′-CCTTCATCACCCTCGAAGAAGA-3′

those observed with diet (16), diabetes (17), and widely used antidiabetic drugs (14,15).

RESEARCH DESIGN AND METHODS

Dulbecco's modified Eagle's medium (DMEM) and FBS were from Invitrogen (Carlsbad, CA) and Roche Molecular Biochemicals (Indianapolis, IN). [9,10(n)-^{3}H]- or [1-^{14}C]-fatty acids and [U-^{14}C]-D-glucose were from ICN Biochemicals (Costa Mesa, CA). Monoclonal CD36 antibody was from Cascade Biosciences (Winchester, MA). Other antibodies and fatty acid–free BSA were from Sigma (St. Louis, MO). Immobilon-P membranes for Western blots were from Millipore (Billerica, MA). Primers for quantitative PCR were from Invitrogen.

C2C12 cells with stable expression of CD36. Rat CD36 cDNA was cloned into the *Eco*RI site of the pBizeoneo retroviral vector as described (18). Briefly, bosc23 envelope-expressing packaging cells were transfected at 70% confluence with 8 μg of the pBizeoneo-CD36 vector. After 8 h, cells were washed with PBS and incubated (48 h) in fresh medium. The medium with the recombinant virus was filtered (0.45 μm Nalgene filters) and used to infect C2C12 myoblasts at 25–50% confluence. After 6 h, cells were washed in PBS, grown (48 h) in fresh medium, and replated (1:5) in the presence of 0.4 mg/ml geneticin to select Stable populations.

Cell culture. Wild-type and CD36-expressing C2C12 (+CD36) myoblasts were grown until 80% confluent in DMEM, low glucose (5 mmol/l) supplemented with 8% FBS, 200 units/ml penicillin, 50 μg/ml streptomycin, and 0.4 mg/ml geneticin. Myotube differentiation was induced by switching to medium with 1% FBS (differentiation medium). Cells were used for assays when fully differentiated (usually 5 days).

Plasma membrane purification and Western blot analysis. Myotubes were washed three times, scraped into 2 ml of ice-cold PBS, and centrifuged (1,500*g*, 3 min). Cell pellets were homogenized (Dounce, 50 strokes) in 1.25 ml of buffer A (20 mmol/l Tris, pH 7.4, 5 mmol/l EDTA, 250 mmol/l sucrose) with 1 mmol/l phenylmethylsulfonyl fluoride, 20 μg/ml aprotenin, and 10 μg/ml leupeptin. After a low spin (1,200*g*, 10 min), the clear supernatant was centrifuged at 16,000*g* for 30 min. The pellet resuspended in 0.2 ml of buffer B (20 mmol/l Tris, 5 mmol/l EDTA, pH 7.4) was layered on 1.3 ml of 30% sucrose and centrifuged (100,000*g* for 1 h). The membrane fraction was then obtained by centrifuging the supernatant at 30,000*g* for 30 min and was suspended in buffer B.

For Western blots, 10 μg of protein were mixed with loading buffer containing 50 mmol/l dithiotreithiol, heated at 95°C for 5 min, and subjected to SDS-PAGE. The separated proteins were transferred to an immobilon P membrane and incubated with CD36 antibody (1:1,000) overnight at 4°C in buffer containing 20 mmol/l Tris, 137 mmol/l NaCl, pH 7.6 and 5% dry milk. Blots were washed three times in buffer with 0.1% Tween 20 and incubated with the secondary antibody (anti-mouse IgG horseradish peroxidase, 1:5,000) for 1 h at room temperature. Immunodetection was by the luminol reagent (Santa Cruz, CA).

Fatty acid uptake. Myotubes were switched to serum-free DMEM for 2 h before beginning the experiments. Cells were washed three times with 2 ml of a Krebs-Ringer solution buffered with 10 mmol/l HEPES (KRH), pH 7.4, with 0.1% BSA, and once with 2 ml KRH. KRH (1 ml) containing 40 μmol/l BSA was added, and uptake was started by addition of 1 ml of transport buffer (KRH with 80 μmol/l ^{3}H-palmitate; 0.5 μCi/ml; fatty acid–to–BSA ratio = 2) at room temperature for the indicated times. The reaction was stopped by aspirating the medium and washing three times with ice-cold KRH/0.5% BSA. Cells were lysed in 1 ml of 0.1N NaOH for 30 min, and aliquots were taken for scintillation counting (Beckman LS3801) and protein determination (BioRad Assay; Life Science Research, Hercules, CA). For experiments testing the effects of preincubation with fatty acids, oleate or palmitate were added to myotubes kept in differentiation medium. The preincubation medium contained 1.5 μmol/l with 0.4 μmol/l BSA. At the end of the preincubation, cells were washed three times with KRH buffer and processed as indicated. Fatty acid–treated cells did not exhibit any morphological signs of toxicity. Protein concentration, determined for dishes with cells exposed or not exposed to fatty acid, was identical, indicating no cell death during the preincubation or experimental periods. In addition, expression of several genes of lipid metabolism (carnitine palmitoyl transferase-1 [CPT-1], stearoyl-CoA desaturase-1 [SCD-1], and diacylglycerol acyltransferase [DGAT]) was measured and found not to be altered by fatty acid treatment.

Fatty acid oxidation and lipid incorporation. Myotubes were switched to serum-free medium (2 h) before the start of the experiment. Fatty acid oxidation was assayed as by Muoio et al. (19). Briefly, cells were washed three times in KRH with 40 μmol/l BSA and incubated for 2 h in 1 ml of the same buffer containing ^{14}C-palmitate or -oleate (1 μCi/80 μmol/l). CO_2 trapping was overnight at 30°C with gentle shaking (19). The acid soluble metabolite (ASM) fraction was obtained by perchloric acid precipitation of the medium. Oxidation rates (CO_2+ASM) were expressed as nmol/l · mg protein^{-1} · 2 h^{-1}.

Fatty acid incorporation into cell lipid was determined as previously described (19,20). Cells were washed three times at 4°C with PBS/0.5% BSA and twice with PBS and scraped into 500 μl of the same buffer. Aliquots were taken for Folch extraction and for protein measurement. Folch extracts were analyzed by thin-layer chromatography on silica gel 60 Å plates (Whatman, Clifton, NJ) using a two-solvent system (21). The first solvent (diethyl ether/benzene/ethanol/acetic acid 40:50:2:0.2) was run up to three-quarters of the plate. Plates were air dried, briefly heated to remove traces of acetic acid, and run in the second solvent (diethyl ether:hexane 6:94) to 1 cm from the top. Plates were again dried at 60°C for 30 min. Spots corresponding to major lipid fractions, identified by standards run simultaneously and visualized by iodine vapors, were scraped and counted.

Triglyceride content. Folch extracts were evaporated and redissolved in isopropanol. Triglyceride mass was measured using GPO-Trinder (Sigma) and related to cell protein.

Glycerol and fatty acid release. Myotubes were washed and then incubated (3 h) in KRH with 2% BSA and 5 mmol/l glucose. Glycerol release was evaluated using the Triglycerides Trinder Reagent A. For fatty acid efflux, washed myotubes were incubated (4 min) in KRH with 80 μmol/l ^{3}H-palmitate (0.5 μCi/ml; fatty acid–to–BSA ratio = 2). They were washed three times and switched to 1 ml KRH with 2% BSA. At indicated times, medium was aspirated and the cells lysed in 0.1N NaOH. Aliquots of the lysate were counted by scintillation spectroscopy and assayed for protein.

Glycogen synthesis. Myotubes were incubated (2 h) in KRH with 5 mmol/l glucose and D-[U-^{14}C]-glucose (1 μCi/ml). Glycogen formation was assayed according to Berti et al. (22). Washed cells were scraped into 1 mol/l KOH (300 μl) and heated (100°C, 10 min), and aliquots were taken for measuring protein. Saturated Na_2SO_4 solution (40 μl) was added and glycogen precipitated (−70°C, 30 min) with ice-cold acetone (700 μl). Pellets (20,000*g*, 30 min) were washed with acetone, dissolved in 100 μl water, and mixed with scintillant for counting.

Quantitative PCR. Total RNA was prepared using the High-Pure RNA kit from Roche Diagnostics. Complementary DNA was synthesized from 10 μg RNA with Superscript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR (qPCR) was performed (23) using GeneAmp 9700 (Applied Biosystems, Foster City, CA) in the presence of SYBR Green I (Sigma-Aldrich) with *Taq*DNA polymerase (Roche Diagnostics).

Intron-spanning oligonucleotides were used (Table 1). Annealing temperatures were optimized to obtain only one DNA product using a T-Gradient thermal cycler (Biometra, Göttingen, Germany). Amplicon mass at cycle threshold was obtained by comparison to a standard curve (log-linear over 7 orders of magnitude) generated for each run. Postrun melting-curve analysis confirmed amplicon identity.

Statistics. Results are expressed as means ± SD. Differences between cells or treatments were tested for statistical significance using the unpaired Student's *t* test.

RESULTS

Generation of C2C12 myoblasts stably overexpressing CD36. C2C12 cells, derived from the mouse myoblast line established by Yaffe and Saxel (24), are extensively used for in vitro study of muscle metabolism because they

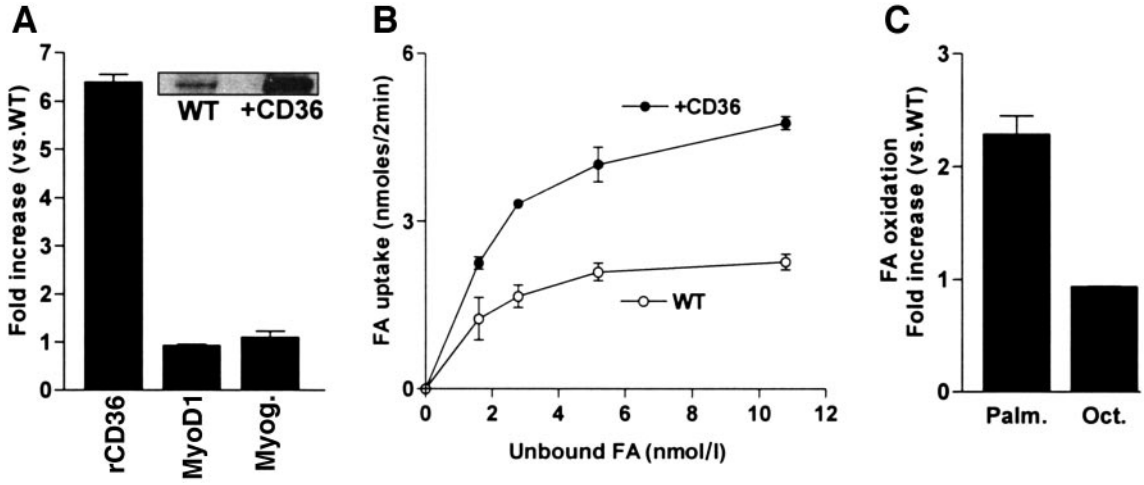

FIG. 1. CD36 expression on fatty acid uptake, oxidation, and differentiation markers. *A*: Quantitative PCR analysis of CD36, MyoD1, and myogenin (Myog.) mRNA and a Western blot of CD36 levels in plasma membranes from wild-type (WT) and CD36 (+CD36) myotubes. *B*: Dependence of uptake (2 min) on unbound fatty acid concentration. *C*: Fatty acid oxidation was determined from production of $^{14}CO_2$ and of ^{14}C-ASMs. Cells were switched to a serum-free medium, then incubated with ^{14}C-palmitate (Palm.) or ^{14}C-octanoate (Oct.) in KRH buffer containing BSA (fatty acid–to–BSA ratio = 2). Data shown for *B* and *C* are means ± SD of triplicates from two different experiments. *$P < 0.05$.

differentiate rapidly into contractile myotubes. Cells with stable CD36 overexpression were obtained by retroviral infection. Levels of CD36 mRNA and protein (Fig. 1*A*) were fivefold higher in infected (+CD36) versus wild-type myotubes. Differentiation was accelerated by 8–12 h in +CD36 cells, which were usually plated 1 day ahead of wild-type cells. Cells were used for experiments only when fully differentiated. At that point, wild-type and +CD36 cells expressed similar levels of differentiation markers (MyoD1 and myogenin) (Fig. 1*A*).

Fatty acid uptake and oxidation. To evaluate whether the expressed CD36 protein was functional, we compared fatty acid transport in wild-type and +CD36 myotubes. Palmitate uptake was reproducibly higher in +CD36 than in wild-type myotubes. Dependence of uptake rates on unbound fatty acid concentration (K_m = 2 nmol/l) was similar in wild-type and +CD36 cells (Fig. 1*B*), indicating that the ectopic protein was functioning like the endogenous one.

Free fatty acid oxidation was measured from the sum of CO_2 production and accumulation of ASMs (reflecting incompletely oxidized fatty acid) (25). ASMs accounted for two-thirds of oxidation and paralleled CO_2 production (data not shown), as reported by Veerkemp et al. (25). Palmitate oxidation (Fig. 1*C*) was 2.4-fold higher in +CD36 than in wild-type myotubes. Dependence of oxidation on unbound fatty acids exhibited a K_m of 2 nmol/l (data not shown), which was similar to the uptake K_m and in line with previous studies (26). Uptake of the short-chain fatty acid octanoate is not facilitated by CD36, and its oxidation was not increased in +CD36 cells, suggesting that the increased oxidation reflected the CD36-enhanced uptake.

Fatty acid incorporation into lipids, triglyceride content, and breakdown. To investigate whether CD36 expression affected fatty acid metabolism, fatty acid partitioning, triglyceride mass, and glycerol release were examined. Palmitate incorporation into triglyceride was increased by 40% in +CD36 myotubes, but there was no increase in triglyceride mass (Fig. 2*A*). Glycerol release was about fourfold higher in +CD36 cells, indicating increased triglyceride breakdown. CD36 also increased fatty acid efflux, as there was more rapid loss of cellular radioactivity from +CD36 versus wild-type cells preincubated with ^{3}H-palmitate and switched to KRH with BSA (Fig. 2*C*). We compared expression of several genes of fatty acid metabolism in wild-type and +CD36 cells (data not shown). There was no significant change in expression of SCD-1 or CPT-1. Expression of DGAT was comparable for isoforms 1 and 2 and similar for wild-type and +CD36 cells. In contrast, mRNA for hormone-sensitive lipase (HSL) was increased twofold in +CD36 cells (Fig. 2*B*). The data suggested enhanced futile cycling of the fatty acids in +CD36 cells, where both triglyceride synthesis and breakdown appeared increased.

Exposure to a high concentration of palmitate or oleate. To examine if a combination of excess fatty acids and high CD36 levels would induce accumulation of myocellular lipid and insulin resistance, myotubes were incubated for 36 h with palmitate or oleate (Fig. 3*A* and *B*). Preincubation with either fatty acid did not alter levels of retrovirally expressed CD36 as determined by qPCR (data not shown).

In the case of palmitate, fatty acid uptake (data not shown) and palmitate oxidation (Fig. 3*A*) were still twofold higher in +CD36 than in wild-type cells. Label recovery into mono-, di-, and tri-glycerides almost doubled (Table 2). +CD36 myotubes accumulated more mass triglycerides than wild-type cells (Fig. 3*A*). The effect of CD36 to increase glycerol release was blunted (Fig. 3*A*), indicating that it was less efficient in inducing triglyceride breakdown after palmitate exposure. This did not reflect a negative effect on HSL since its expression (Fig. 3*A*) was unchanged compared with +CD36 cells not preincubated with palmitate.

As palmitate and oleate are processed differently by muscle (27), we examined the metabolic effects of preincubation with oleate. Oleate uptake (data not shown) and oxidation (Fig. 3*B*) were higher in +CD36 than in wild-type cells, similar to findings with palmitate. However, in contrast to palmitate, glycerol release was increased by

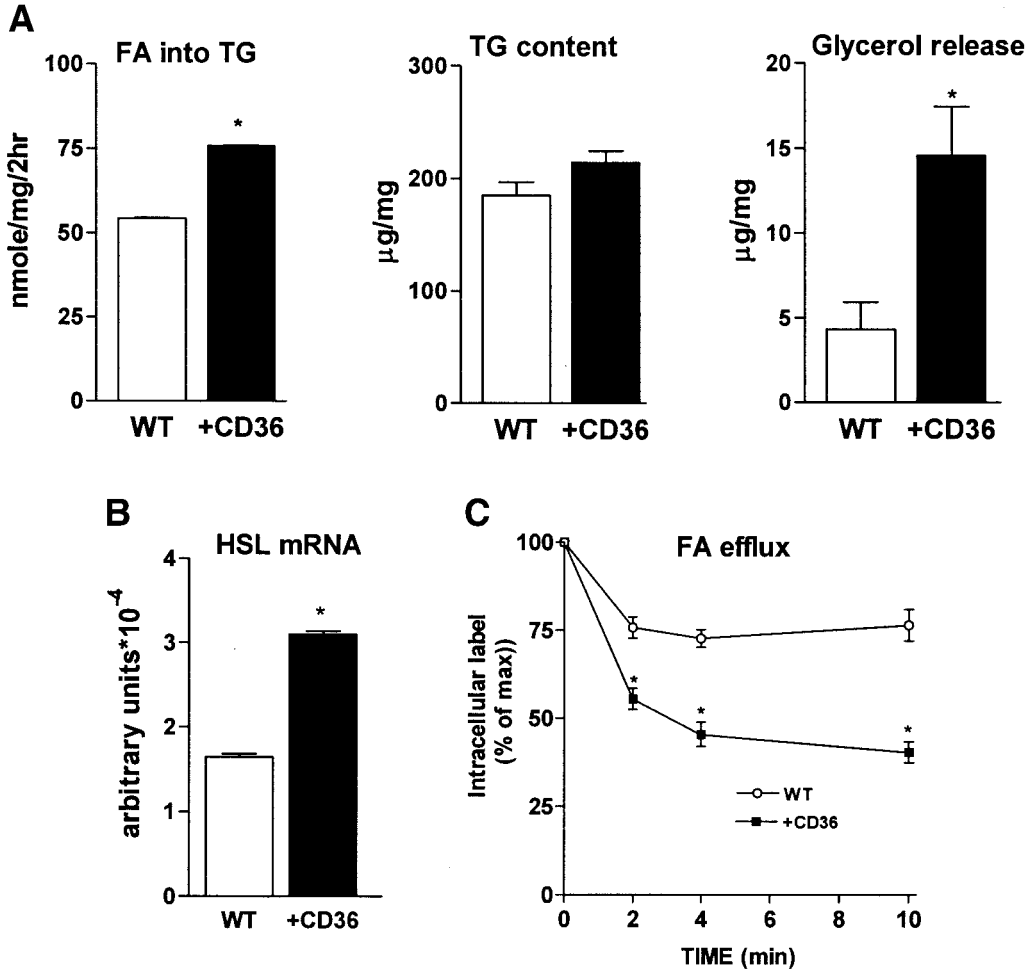

FIG. 2. Fatty acid incorporation into triglycerides, triglyceride content, glycerol release, and fatty acid efflux in wild-type and +CD36 myotubes. *A*: Differentiated myotubes were incubated for 2 h with ^{14}C-palmitate in KRH buffer (fatty acid–to–BSA ratio = 2). Label recovery into triglycerides was determined from thin-layer chromatography of lipid extracts of washed cells. Alternatively, myotubes were assayed for triglyceride content and glycerol release. For glycerol release, cells were washed and incubated for 3 h in KRH with 2% BSA and 5 mmol/l glucose. *B*: HSL mRNA abundance was determined by RT-PCR. Data are means ± SD from two separate experiments with triplicate determinations. *C*: Cells were preincubated (4 min) with ^{3}H-palmitate, quickly washed, and incubated in KRH with 2% BSA. At the indicated times, cells were lysed in 0.1N NaOH and aliquots were counted. Cell radioactivity is expressed as percentage of initial radioactivity at the end of the preincubation. Data shown are means ± SD of triplicates from two different experiments. *$P < 0.05$.

250% in +CD36 cells (Fig. 3*B*). In line with this, HSL mRNA was up threefold and there was no increase in triglyceride content (Fig. 3*B*). Also, in contrast to palmitate, +CD36 cells incubated with oleate did not accumulate fatty acid label into diglycerides and triglycerides (Table 2).

Incubation with either fatty acid did not alter levels of several key genes of fatty acid metabolism. Under our experimental conditions, no significant differences were observed in the mRNA abundance for CPT-1, DGAT-1 and -2, or SCD-1 (data not shown).

Glycogen synthesis and its response to insulin. Increases in fatty acid oxidation in muscle cells are associated with suppression of glucose metabolism and with resistance to stimulation by insulin (28). As shown in Fig. 4, basal rates of glycogen synthesis were decreased in +CD36 cells, but their stimulation by insulin was unaltered and even slightly enhanced (up by 1.6- and 1.8-fold in wild-type and +CD36 cells, respectively). For both wild-type and +CD36 cells (Fig. 4*A*), preincubation with fatty acid inhibited (~50%) basal glycogen synthesis. However, while palmitate-treated wild-type cells responded to insulin (rates increased 1.4-fold), +CD36 cells did not (Fig. 4*B*). In contrast to palmitate, oleate-treated +CD36 cells responded to insulin with a doubling of glycogen synthesis.

DISCUSSION

This study examined the effects of excessive fatty acid uptake on myocyte lipid and insulin responsiveness in C2C12 muscle cells with stable CD36 overexpression.

CD36 expression on futile cycling of fatty acids. High CD36 expression increased fatty acid uptake and utilization by myotubes. More important, it promoted triglyceride breakdown and futile cycling of the fatty acids, so the increased uptake did not result in lipid accumulation since triglyceride turnover was also enhanced. The most likely interpretation of these data are that CD36 targets fatty acids to a lipase-accessible triglyceride pool because (as shown in Fig. 2*C*) 55% of cell fatty acid radioactivity could be released in +CD36 cells versus 25% in wild-type cells. This ability of CD36 may represent a property shared with other members of the CD36 family. Recent work by

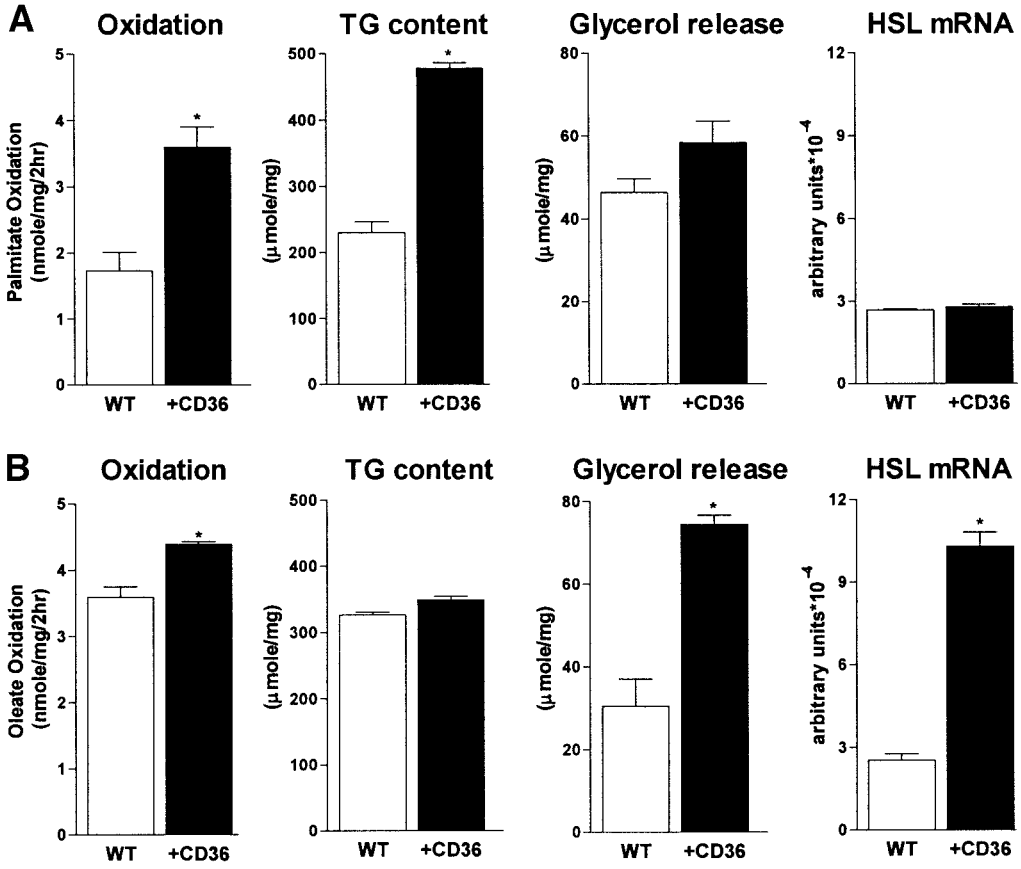

FIG. 3. Effect of palmitate and oleate preincubation on fatty acid oxidation, triglyceride content, glycerol release, and HSL mRNA. Wild-type and +CD36 myotubes were preincubated for 36 h with 750 μmol/l palmitate (*A*) or 750 μmol/l oleate (*B*), washed, and assayed for fatty acid uptake and oxidation and for triglyceride content and glycerol release as described for Figs. 1 and 2. Data are means ± SD of triplicates from four different experiments. *$P < 0.05$. HSL mRNA abundance was determined by RT-PCR. Data are means ± SD from two separate experiments with triplicate determinations.

Connelly et al. (29) documented that scavenger receptor BI directs cholesteryl ester to a compartment where it is efficiently hydrolyzed. As a result, targeting these receptors would alter both cellular uptake and processing of fatty acids and cholesterol and may provide more comprehensive strategies to modify cellular lipid metabolism.

HSL expression was induced in +CD36 cells and in wild-type cells exposed to fatty acids (Fig. 2*B* vs. Fig. 3*B*), suggesting a cellular feedback mechanism to limit lipid accumulation when fatty acid uptake is increased. Changes in HSL expression were in line with glycerol release and inversely related to triglyceride mass. This suggests that HSL induction may be involved in the increased futile cycling of fatty acids in +CD36 cells and that the lipase may act on the newly formed triglyceride pool targeted by CD36-facilitated uptake.

HSL has been reported to be important for muscle triglyceride metabolism (30). However, there is little information related to the mechanisms controlling triglyceride turnover in this tissue. It is not known whether lipid-associated droplets may regulate HSL translocation in muscle, as in adipose and steroidogenic tissues, or whether lipases other than HSL play a significant role (31, 32).

Interference with futile fatty acid cycling may represent a potential mechanism for the negative cellular effects of high palmitate, which in contrast to oleate induces lipotoxicity (33,34). As shown in Fig. 5, there was a significant, negative correlation ($r = -0.57$) between the relative contents of labeled diglycerides and triglycerides in the case of oleate, indicating efficient conversion or hydrolysis of newly made diglycerides (labeled) into triglycerides. This correlation did not hold for palmitate-treated cells, where, in addition, there was accumulation of palmitate label into mono- and diglycerides. This may reflect the apparently 50% lower affinity of DGAT for palmitate compared with oleate (35,36) and serve to limit synthesis of triglycerides rich in saturated fatty acids, which are less favorable for the cell. Thus, excess palmitate would lead to accumulation of diglycerides, monoglycerides, and possibly other upstream derivatives, such as ceramides. Increases in ceramide levels have been reported in C2C12 cells exposed to palmitate and were postulated to inhibit insulin signaling and responsiveness (28).

Accumulation of palmitate metabolites such as monoacylglycerols and unesterified fatty acids (Table 2) may also exert product inhibition on HSL (37) and explain the decreased efficiency of glycerol release in palmitate-treated cells.

Etiology of myocyte triglyceride accumulation. Decreases in fatty acid oxidation, such as with deficiencies in specific mitochondrial enzymes (38) or increases in fatty acid uptake as a result of oversupply, are associated with lipid accumulation and cell dysfunction (39) and are a

TABLE 2
Effect of preincubation with palmitate or oleate on the recovery of ^{14}C- palmitate or ^{14}C-oleate into cellular mono-, di-, and triglycerides, and free fatty acids by wild-type and CD36-overexpressing (+CD36) myotubes

	Wild type	CD36
	nmol/l · mg protein^{-1} · 2 h^{-1}	
Monoglycerides		
Oleate	1.02 ± 0.07	1.14 ± 0.05
Palmitate	1.93 ± 0.09	3.97 ± 0.14*
Diglycerides		
Oleate	1.53 ± 0.03	1.92 ± 0.05
Palmitate	2.43 ± 0.01	4.72 ± 0.04*
Triglycerides		
Oleate	61.91 ± 1.43	68.97 ± 1.38*
Palmitate	36.91 ± 0.07	70.42 ± 0.55*
Free fatty acids		
Oleate	3.16 ± 0.11	2.49 ± 0.16*
Palmitate	4.12 ± 0.29	3.00 ± 0.01*

After a 36-h preincubation in medium supplemented with 750 μmol/l palmitate or oleate, cells were washed and incubated for 2 h in KRH buffer containing 80 μmol/l ^{14}C-palmitate or ^{14}C-oleate (1 μCi/ml) bound to 40 μmol/l albumin and with 5 mmol/l glucose. Label recovery into mono- di-, and triglycerides and into free fatty acids was determined from thin-layer chromatography of lipid extracts of washed cells. *$0.02 < P < 0.05$ vs. wild type.

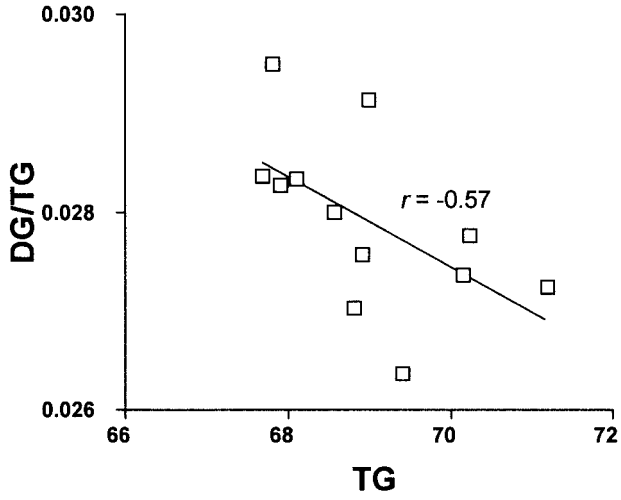

FIG. 5. *A*: Negative correlation between the label recovered into diglycerides (DG) and triglycerides (TG) in C2C12 myotubes preincubated with oleate. Data are from four experiments and show a decrease in the relative amount of diglyceride to triglyceride label in oleate-treated cells ($P < 0.05$, $n = 12$). No such correlation was observed with palmitate ($P < 0.6$, $n = 11$).

frequent manifestation of diabetes (40) and obesity (1,41). There is a strong negative correlation between intramuscular triglyceride and insulin responsiveness of glucose utilization (1,40–42) with the mechanism involving down-regulation of membrane transport (43,44) and insulin signaling (45). However, the etiology of lipid accumulation in the myocyte remains unclear. The data with myotubes preincubated with excess oleate indicate that increases in fatty acid uptake, oxidation, and incorporation into lipid do not lead to insulin resistance of glucose metabolism because futile cycling of the fatty acid is efficient. Glycogen synthesis in oleate-treated +CD36 myotubes (Fig. 4) exhibited good insulin responsiveness despite the fact that basal glycogen formation was halved by the increased fatty acid supply. CD36 expression in muscle would be beneficial for insulin responsiveness because it increases futile cycling of the fatty acid and minimizes, under high fatty acid supply, the accumulation of fatty acid metabolites and triglycerides. However, when metabolism of the fatty acids is impaired, high levels of CD36 would have a negative effect. Our data suggest that a major reason for the different insulin responsiveness of palmitate- versus oleate-treated cells is that palmitate metabolism does not favor enhanced diglyceride and triglyceride turnover and results in accumulation of both species. Accumulation of other inhibitory intermediates such as ceramides may also be promoted as a result of the slower turnover of neutral lipids in palmitate-saturated cells. It is well known that diglycerides and ceramides exert negative effects on insu-

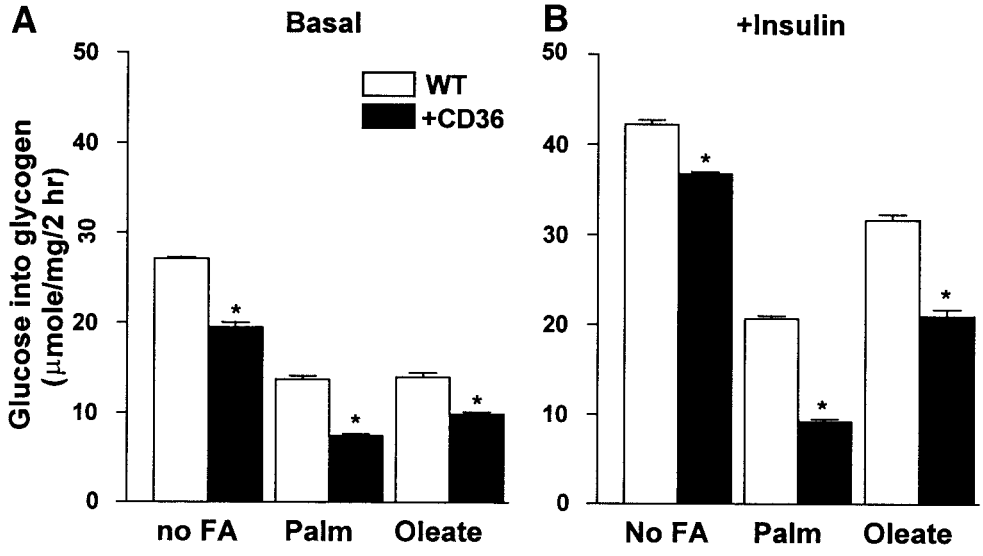

FIG. 4. Effects of palmitate and oleate on insulin stimulation of glycogen synthesis. Cells were treated with each fatty acid as described in Fig. 3 and incubated with D-[U-^{14}C]-glucose in the presence or absence of 100 nmol/l insulin. *A*: Basal rates of glycogen synthesis for control (no fatty acid), palmitate-, and oleate-preincubated wild-type and +CD36 myotubes. *B*: Glycogen synthesis for the same conditions shown in *A* but in response to 100 nmol/l insulin. Data are means ± SD of triplicates from three different experiments. *$P < 0.05$.

lin signaling (28,45,46). In general, the findings indicate that enhanced futile cycling of exogenous fatty acids is important for maintaining low lipid levels and insulin responsiveness of myocytes. This may help explain the reported beneficial effects of diets rich in monounsaturated fatty acids as opposed to saturated fatty acids, especially in populations with high prevalences of insulin resistance (47).

In summary, the findings provide a new perspective with respect to the etiology of lipid accumulation and insulin resistance in muscle cells by demonstrating the protective effect of futile fatty acid cycling into triglycerides. Increasing fatty acid uptake (CD36 expression) will only lead to negative effects if this futile cycling is interfered with, as in the case of palmitate or with inherent defects of fatty acid metabolism. Interestingly, futile cycling of triglycerides in adipocytes mediated by thiazolidinedione induction of glycerol kinase has been described. It was postulated to contribute to the action of thiazolidinedione to enhance insulin sensitivity by promoting fatty acid esterification and decreasing fatty acid release from adipocytes (48). In the myocyte, insulin sensitivity would be enhanced by a cycle operating in the opposite direction to enhance fatty acid release from the cell and to limit fatty acid incorporation into triglycerides. In line with this, thiazolidinediones, which strongly induce muscle CD36 expression (13,14), could enhance insulin sensitivity in part by promoting futile fatty acid cycling in the myocyte.

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