Oxidation of intramyocellular lipids is dependent on mitochondrial function and the availability of extracellular fatty acids

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SKELETAL MUSCLE INSULIN RESISTANCE is a central pathophysiological feature of type 2 diabetes (T2D) mellitus, and increasing evidence has shown that it is associated with increased intramyocellular lipid (IMCL) content. The precise molecular mechanisms underlying this relationship are not known but may involve the accumulation of lipid intermediates that either interfere with the insulin-signaling pathway and/or induce inflammatory pathways (1, 12, 22). The accumulation of IMCLs and lipid intermediates may be due to a mismatch between lipid storage, lipolysis, and oxidation (28). Elevation of plasma fatty acid concentration, so-called lipid overflow, may enhance IMCL accumulation and induce insulin resistance, as shown in lipid infusion studies (6, 36). Several animal models show that an improved fat oxidation can prevent high-fat diet-induced insulin resistance (8, 24). More specifically a reduced capacity to oxidize IMCLs may contribute to intracellular accumulation of lipids and lipid intermediates. It is unknown to what extent lipid overflow (i.e., increased extracellular fatty acid availability), mitochondrial function, and the oxidation of intracellular lipids contribute to IMCL accumulation.

Multiple studies have shown that fatty acid handling is impaired in skeletal muscle of obese, impaired glucose-tolerant, and T2D individuals (3, 9, 10, 25, 26), which may be due to a primary genetic component. A promoter polymorphism in the gene for the fatty acid transporter CD36 was associated with a decreased insulin sensitivity and an increased prevalence of T2D (11). Despite significant weight loss, a lack of improvement in the ability to increase fat oxidation during β-adrenergic stimulation in obese subjects was observed (4), and under similar conditions the ability to increase fat oxidation during exercise in T2D individuals remained impaired (5). A reduced ability to stimulate fat oxidation during exercise (26) and to switch between lipids and carbohydrates for fuel after a meal (9) was already present in “prediabetic” impaired glucose-tolerant subjects. In vitro experiments with myotubes established from diabetic patients show a lower baseline palmitate oxidation during acute palmitate exposure relative to cultured myotubes from body mass index-matched controls (19, 20, 40). Whether a primary defect in IMCL oxidation may play a role in the development of diabetes is unknown.

To study both primary and secondary impairments in fatty acid handling, cultured human myotubes represent a well-characterized in vitro model of skeletal muscle. The advantages of using human myotubes is that the extracellular environment can be controlled precisely and kept constant over time, which allows for studying the importance of the intrinsic characteristics without systemic homeostatic regulatory components from the nervous and endocrine systems. It has been used previously to study the separate contributions of intracellular and extracellular substrates on total substrate oxidation (13). Moreover, previous studies in these cultured myotubes have shown that the insulin-resistant phenotype is conserved (15, 18). In the present study, we took advantage of this model to investigate the effects of extracellular fatty acids, glucose, and mitochondrial uncoupling [carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)] on oxidation of intracellular lipids (ICLOX) and to compare these effects in primary
myotubes established from healthy lean with those from obese T2D individuals.

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM-Glutamax), DMEM without phenol red (Sigma D5030), non-heat-inactivated fetal calf serum (FCS), and penicillin-streptomycin-amphotericin B were purchased from GIBCO Invitrogen (GIBCO Life Technologies, Paisley, UK). Bovine serum albumin (BSA; essentially fatty acid free), l-carnitine, Dulbecco’s phosphate-buffered saline (DPBS; with Mg²⁺ and Ca²⁺), oleic acid (OA), extracellular matrix (ECM) gel, and HEPES were purchased from Sigma (St. Louis, MO). Ultrasor G was purchased from Ciphergen (Cergy-Saint-Christophe, France), and insulin (Actrapid) was purchased from Novo Nordisk ( Bagsvaerd, Denmark). [1-14C]OA (55 mCi/mmole) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [1-14C]OA (55 mCi/mmol) was purchased from Molecular Probes (Invitrogen, Carlsbad, CA). All other chemicals used were of standard commercial high-purity quality.

Characterization of the donors. To establish primary myotube cultures, muscle biopsies were taken from M. vastus lateralis by needle biopsy according to Bergstrom (2) in 17 Caucasian participants, of which eight were lean healthy glucose-tolerant controls (5 males, 4 females) without family history of T2D and nine were obese T2D subjects with T2D (5 males, 4 females). The characteristics of the participants are shown in Table 1. Initially, nine lean controls were included, but from one of the lean subjects (female) the myotubes showed insufficient growth for reliable measurements. Subjects remained from strenuous physical activity ≥3 days before metabolic investigations. Diabetic subjects were treated with diet alone or in combination with sulfonylurea, metformin, or insulin, which was changed every 2–3 days. Experiments were performed after 7 days of differentiation, and preincubation with fatty acid was started after 5 days.

Cell culture. Muscle cell cultures were established as described previously (16, 17). In brief, muscle biopsy tissue was minced and washed, and cells were dissociated for 60 min by three treatments with 0.05% trypsin-EDTA. The harvested cells were pooled, and FCS was added to stop trypsinization. The isolated satellite cells (muscle precursor cells) were proliferated and stored in liquid nitrogen until further use. For experiments, cells were grown on 12-, 24-, or 96-well plates with DMEM-Glutamax (5.5 mM glucose), 2% FCS, 2% Ultrasor G, P/S, and amphotericin B until 70–80% confluence. Myoblast differentiation to myotubes was then induced by changing medium to DMEM-Glutamax supplemented with 2% FCS, P/S, 1.25 µg/ml amphotericin B, and 25 µM insulin. The cells were cultured in a humidified 5% CO₂ atmosphere at 37°C, and the medium was changed every 2–3 days. Experiments were performed after 7 days of differentiation, and preincubation with fatty acid was started after 5 days.

Experimental setup. In the present study, a crossover design was used as explained in Fig. 1. After 5 days of differentiation, the cells were pretreated with 100 µM OA for 2 days (48 h) and then treated acutely for 4 h with varying concentrations of OA (5 or 100 µM; f or F, respectively) and glucose (0.1 or 5.0 mM; g or G, respectively). These concentrations were chosen on the basis of uptake rates rather
than on imitation of plasma values. The acute treatments were chosen to mimic various physiological conditions for skeletal muscle cells, such as the fed state [high glucose supply, low free fatty acid (FFA) availability] and fasting [high FFA uptake, low glucose transport], and other combinations to study the response on lipid oxidation (e.g., adaptability to increased fatty acid availability and suppressibility of glucose on fatty acid oxidation when being changed from low to high concentrations).

During the 4-h acute experiment, the production of $^{14}$CO$_2$ was measured as described under the heading substrate oxidation assay. To distinguish between $^{14}$CO$_2$ production from intracellular (ICL-OX) or extracellular sources (ECLOX), labeling with [1-14C]OA was applied with subsequent capture of liberated $^{14}$CO$_2$ for 4 h at 37°C, using a 0.1 M NaOH (200 l/well). The cells were washed twice with PBS after pretreatment, before addition of acute medium for CO$_2$ trapping.

**Substrate oxidation assay.** Substrate oxidation was monitored by incubating cells grown on 96-well plates (CellBIND) with [1-14C]OA, with subsequent capture of liberated $^{14}$CO$_2$ for 4 h at 37°C, using a “sandwich” clamp instrument, as described previously (41). Radioactivity in the filter wells was counted in a scintillation counter for multwell plates (MicroBeta; PerkinElmer). Immediately after $^{14}$CO$_2$ trapping, the cells were washed twice with PBS and harvested with 0.1 M NaOH (200 l/well). Protein was determined by Coomassie reagent (7). For total (cell-associated) accumulation of [14C]OA, 50 lM cell homogenate/sample was added to scintillation fluid and counted (MicroBeta).

**Scintillation proximity assay.** Radiolabeled substrates taken up and accumulated by adherent cells were concentrated close to the scintillator embedded in the plastic bottom of each well and thus provided a stronger signal than the radiolabel dissolved in the medium alone (41). Measurements of OA uptake by scintillation proximity assay (SPA) were performed in DME-medium, using 96-well ScintiPlates (http://www.perkinelmer.com for SPA technology; Perkin-Elmer) as described above without phenol red, with an additional 100 lM OA (1.5–2.2 lM/ml [1-14C]OA) bound to BSA at a ratio of 2:5:1.

**Lipid distribution.** Myotubes grown on 12-well plates were pretreated with [1-14C]OA (100 lM) for 48 h. After the acute experiment (4 h), cells were washed twice with PBS, harvested in distilled water (200 l), and stored at −20°C for extraction of cellular lipids (19). Briefly, the homogenized cell fraction was mixed with 20 volumes of chloroform-methanol (2:1), and FCS (30 l) was added as a lipid carrier. After 30 min, four volumes of 0.9% NaCl (pH 2) was added and the mixture was centrifuged (1,000 g, 5 min). The organic phase was evaporated under a nitrogen stream at 40°C. The residual lipid extract was redissolved in 150 l of n-hexane and separated by thin-layer chromatography (TLC) using hexane-diethyl ether-acetic acid (65:35:1) as mobile phase. The lipid bands were visualized with iodine and excised, and radioactivity was quantified by liquid scintillation. The lipid classes were identified with standards.

**Determination of lipolysis.** Cells grown on 96-well plates (CellBIND) were pretreated with [1-14C]OA (1.5 lM/ml, 100 lM) for 48 h to prelabel the endogenous triacylglycerol (TAG) pool. Following preincubation, myotubes were incubated for 4 h in DPBS with 2% BSA (fatty acid free) and 10 lM triacsin C to block fatty acid recycling into the TAG pool and oxidation. Lipolysis was performed under the gf (0.1 mM glucose/5 lM OA) and GF (5.0 mM glucose/100 lM OA) conditions. At the end of incubation, medium was collected and cells harvested for scintillation counting. Protein was determined by Coomassie reagent (7).

**Mitochondrial uncoupling.** Cells grown on 96-well plates were exposed to pretreatment and acute treatment as described under experimental setup, with the exception of now using gf, GF (0.1 mM glucose/100 lM OA), and GF as acute conditions. During the acute experiment, myotubes were incubated in the presence and absence of 1 lM FCCP, a mitochondrial protonophore. OA uptake and oxidation were measured as described under substrate oxidation assay.

**Live imaging of lipid droplets and mitochondria.** Myotubes were cultured and pretreated with 100 lM OA for 48 h using 24-well glass bottom plates (MatTek) with ECM coating. Myotubes were incubated with Bodipy 493/503 (2 lM/ml) for 5 min to stain lipid droplets (LDs), with MitoTracker Red FM (100 nM) for 15 min to stain mitochondria, and with Hoechst (2.5 lM/ml) for 15 min to stain the nuclei. Automated image acquisition was performed with an Olympus Scan^R platform (Olympus IX81 inverted fluorescence microscope with a computer-controlled motorized stage) equipped with a temperature and CO$_2$ enrichment incubator for long-term live imaging. We used a ×20 objective and standard filter sets with excitation window for Hoechst (350 nm), Bodipy 493/503 (488 nm), and MitoTracker Red FM (594 nm) and a triple band filter for emission. Images were taken in 25 positions/well, 2 wells/donor, and each image was the background-subtracted maximal intensity projection from 12 images taken in z-direction (1 lM apart), where one image contained 52 ± 2 nuclei on average. Scan^R software was used for automated image analysis, using edge detection algorithm for object segmentation to quantify the number of nuclei, density, diameter, and number of LDs and total mitochondrial mass (total intensity of MitoTracker Red).

**Presentation of data and statistics.** All values are reported as means ± SE. The value n represents the number of different donors used. Linear mixed models were used to compare the differences between conditions with within-donor variation and simultaneously compare differences between groups with between-donor variation. The linear mixed models include all observations in the statistical analyses and at the same time take into account that not all observations are independent. Correlations are presented as Spearman’s correlation coefficient (r). Clinical data were compared with two-tailed, unpaired Student’s t-test. The statistical analyses were performed with SPSS version 12 (SPSS, Chicago, IL). P < 0.05 was considered significant.

**RESULTS**

**Experimental setup: fatty acid uptake, accumulation, and prelabeling of myotubes.** Isotopic equilibrium of the intracellular lipid pool of myotubes. Isotopic equilibrium of the intracellular lipid pool (label entering the pool equals label leaving the pool) reached a plateau after ~48 h (Fig. 2A). Lipid accumulation measured as the cell-associated radioactive activity and as the number of LDs (Fig. 3) per nucleus was not significantly different between myotubes from lean and obese T2D subjects (Fig. 2, B and C). The average LD sizes were 1.7 ± 0.1 and 1.8 ± 0.1 lM^2 for T2D and lean, respectively (P = 0.31). The density of LDs was measured as fluorescence intensity per LD, showing no significant differences between T2D and lean subjects (data not shown). Furthermore, intracellular lipid distribution was assessed by TLC in myotubes from lean subjects (n = 4) after 48 h of pretreatment with labeled OA and 4 h of acute experiments (see Fig. 1 for conditions). Most of the cell-associated lipids were represented by triacylglycerols (53–61%), FFA (25–33%), and to a lesser extent diacylglycerols (6–7%), phospholipids (2–4%), and cholesterol esters (3–4%). There was no significant effect of the different acute treatments on intracellular lipid distribution.

**Mitochondrial mass by live imaging.** After 48-h treatment with 100 lM OA, mitochondrial mass, measured as total intensity of MitoTracker Red FM per nucleus, was not different between myotubes from the two donor groups (fluorescence intensity: 611 ± 72 × 10^3 AU in T2D and 482 ± 34 × 10^3 AU in lean, P = 0.14; for images, see Fig. 3).

**Oxidation of lipids.** In myotubes from obese T2D subjects, the ICL-OX (Fig. 4A), ECL-OX (Fig. 4C), and total lipids (sum of
ICLOX and ECLOX; Fig. 4D) were systematically reduced in all four of the acute conditions compared with myotubes from lean subjects. Also, ICLOX adjusted for differences in total ICL content (cell-associated radioactivity) was systematically reduced in T2D myotubes (Fig. 4B). This indicates that a general defect in lipid oxidation can be extended to a reduced oxidation of intracellular lipids. Increasing the concentration of extracellular fatty acid markedly increased extracellular lipid oxidation (ECLOX) and total lipid oxidation (TLOX) (Fig. 4, C and D). The increase in ECLOX and TLOX upon increased fatty acid availability was smaller in myotubes from obese T2D subjects compared with myotubes from lean subjects at both 0.1 (g) and 5.0 (G) mM extracellular glucose (P < 0.05; Fig. 4, E and F, respectively). Cell-associated OA from the acute incubations was not different between myotubes from the two donor groups (data not shown).

Effects of extracellular fatty acids, glucose, and mitochondrial uncoupling on ICLOX. Figure 5 shows the relative change in substrate oxidation upon increases in glucose and fatty acid availability, taking the “gf” condition as the reference. Figure 5A shows that in the presence of extracellular fatty acids the ICLOX was lower (in the 100-μM condition “F”) compared with the 5-μM condition “I” (P < 0.001) in myotubes from lean individuals (−32%) and in myotubes from T2D individuals (−46%). Figure 5B shows that a higher concentration of glucose has a similar but smaller effect and significantly suppressed ICLOX in the T2D cells (−24%, P < 0.05; Fig. 5B), but not in lean cells (−12%). Overall, the suppressive effects of extracellular OA and glucose on intracellular lipid utilization were significantly stronger in myotubes established from obese T2D subjects (P = 0.002). Figure 5C shows the effects after mitochondrial respiration was increased by addition of the mitochondrial uncoupler FCCP. The suppressive effect of increased extracellular fatty acid availability was still present when mitochondrial oxidative capacity (electron transport chain flux) was increased by FCCP. Figure 5C also shows that increased mitochondrial oxidative respiration by FCCP restored ICLOX in the presence of increased extracellular fatty acid availability (gf/F+CPP and GF/F+CPP) to the level of low fatty acid availability (gf/F−CPP), at least in myotubes from lean subjects. In the presence of FCCP, ICLOX was increased significantly for both groups at all acute conditions examined. The relative effect of FCCP on ICLOX is presented in Fig. 5D. The uncoupling effect of FCCP overall was 14% lower in myotubes from T2D compared with lean donors (P = 0.04; Fig. 5D), suggesting a primary reduced mitochondrial capacity despite comparable mitochondrial mass.

Correlations with in vivo characteristics. To examine whether intrinsic characteristics of the myotubes in vitro were reflected by the phenotype of the donor in vivo, lipid oxidation...
Results represent means ± SE, with each donor (n = 8–9) quantified from 8 wells/condition. *P < 0.05 for T2D vs. lean myotubes. A: CO₂ production over 4 h from intracellular lipids (ICL). *Group effect, P = 0.002; overall differences between treatments, P = 0.08. Post hoc analysis with Bonferroni correction for “gf” vs. “GF,” P = 0.02. B: fractional oxidation of intracellular lipids (ICLOX), i.e., the ICLs that are oxidized in relation to the total fatty acid availability in the cell [fatty acid oxidized/cell-associated fatty acids + fatty acids oxidized]. *Group effect, P = 0.002; overall differences between treatments not significant. C: CO₂ production over 4 h from extracellular lipids (ECL). *Group effect, P = 0.014; overall differences between treatments, P < 0.001. D: total CO₂ production (from intracellular and extracellular fatty acids). *Group effect, P = 0.02; overall differences between treatments, P = 0.003. E: bars represent the difference in ECLOX at high fatty acid availability in the “F” condition (100 μM OA) and low fatty acid availability in the “F” condition (5 μM OA). Glucose availability is indicated by “g” (0.1 mM glucose) or “G” (5 mM glucose). Lean vs T2D; *P = 0.04. F: bars represent the difference in total lipid oxidation (TLOX) at high fatty acid availability in the F condition (100 μM OA) and low fatty acid availability in the F condition (5 μM OA). Glucose availability is indicated by g (0.1 mM glucose) or G (5 mM glucose). Lean vs. T2D; *P = 0.03. Differences between the 0.1 and 5.0 mM glucose were not significant (g vs. G), P > 0.50.

The findings of the present study suggest that elevated extracellular fatty acids suppress the oxidation of intramyocellular lipids. This is not because of an effect of extracellular lipids on ICL lipolysis. Mitochondrial uncoupling can counter the effect and increase ICLOX to basal levels under conditions of high extracellular fatty acid availability. A reduced oxidation of ICL and reduced mitochondrial mass were shown to contribute independently to accumulation of intramyocellular lipids. Furthermore, the capacity to oxidize ICL and to increase ECLOX upon increased fatty acid availability is reduced in myotubes established from obese T2D individuals despite comparable mitochondrial mass. The finding that lipid oxidation in vitro was inversely related to fasting RQ in vivo supports the idea that the primary impairments observed in vitro are relevant for the in vivo phenotype.

An important observation is that when fatty acid availability is high, the oxidation of intracellular lipids is decreased. In general, most fatty acids that are taken up in skeletal muscle are shuttled toward oxidation (Fig. 8A). In subjects with a reduced capacity for lipid oxidation, a larger part of the fatty acids taken up may be shuttled toward storage (Fig. 8B). In the fasting to the insulin-stimulated state (metabolic flexibility). The correlations were studied in the total group (n = 17). The in vivo fasting RQ was consistently inversely associated with outcomes for in vitro lipid oxidation, including the ICLOX under all conditions and the ECLOX under increased OA (100 μM) conditions (gF and GF) (Spearman \( r_{\text{ALL}} = -0.59, P_{\text{ALL}} < 0.05 \)). Examples of these associations are given in Fig. 6, A and B, representing the association between in vivo fasting RQ with in vitro ICLOX or in vitro total lipid oxidation, respectively.

**Lipolysis.** Lipolysis of intracellular prelabeled lipids was measured in myotubes from lean subjects to study whether a product inhibition of lipolysis by extracellular fatty acids could explain the reduction in ICLOX. It was found that basal lipolysis of ICL (in the presence of triacsin C) was not different between the gf (180 ± 26 nmol/mg protein) and GF (169 ± 28 nmol/mg protein) conditions, respectively. Over 4 h, the amount of OA released from ICL was 45% of the total OA present in the cells (data not shown). This suggests that the availability of ICL-derived fatty acids is not directly limiting ICLOX.

**ICLOX, mitochondrial function, and IMCL accumulation.** To identify which factors regulate ICLOX and cell-associated OA accumulation, correlations were studied (Fig. 7). Since there is a broad variation in the cellular phenotype and a large degree of overlap in these parameters, the groups were analyzed together. In linear mixed-model analyses, taking into account the repeated measurements of the cell-associated OA accumulation and ICLOX under the various acute conditions, the accumulation of OA was inversely related to mitochondrial mass \( (P < 0.001) \) and ICLOX \( (P = 0.003) \). Both factors contributed independently. Mitochondrial mass was not directly related to ICLOX in the group as a whole but was strongly related in myotubes from lean subjects only (Fig. 7A). Furthermore, an increased intramyocellular OA accumulation was strongly related to a larger LD size \( (\text{Spearman } r = 0.66, P = 0.02, n = 12; \text{Fig. } 7B) \).

**DISCUSSION**

parameters (ICLOX, ECLOX, total CO₂ production) and lipid accumulation (cell-associated lipids after pretreatment and acute conditions) were studied in relation to in vivo substrate metabolism, represented by fasting RQ, RQ during the hyperinsulinemic euglycemic clamp, and the change in RQ from the
case of an increased availability of extracellular fatty acids, the oxidation of extracellular fatty acids is increased due to a mass effect (Fig. 8C). This is demonstrated in the present in vitro experiment (Fig. 4C) as well as in vivo in high-fat feeding studies (32, 33). However, we show that at the same time the oxidation of fatty acids derived from ICL is suppressed. In this way, increased extracellular fatty acid availability, also called lipid overflow, may enhance intramyocellular lipid accumulation despite an increase in ECL_{OX} (Fig. 8C). From a physiological point of view, this implies a phenotype in the initial phases of obesity that displays increased fat oxidation in parallel with increased IMTG and possibly increased insulin resistance. Other in vivo observations support the hypothesis that the oxidation of ICLs is increased when fatty acid availability is reduced and vice versa. Several human in vivo stable isotope studies have investigated the use of TAG for fuel (35, 36).

Fig. 5. Effects of glucose, OA, and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) on the oxidation of intracellular lipids. The effects of extracellular substrates and FCCP on the oxidation of intracellular lipids are expressed relative to the f or gf condition, which is set to 100%. Results represent means ± SE, with each donor (n = 8–9) quantified from 4–8 wells/condition. For further explanation of abbreviations, see legend to Fig. 1. For absolute values, see Fig. 4A. A: increased fatty acid availability is indicated by going from f (5 μM OA) to F (100 μM OA). Pairwise comparisons were performed with Bonferroni correction: gf vs. gF, P < 0.001; Gf vs. GF, P < 0.001. *P < 0.05 vs. the respective treatment set to 100%; aP < 0.05 vs. lean subjects. B: increased glucose availability is indicated by going from g to G. Pairwise comparisons were performed with Bonferroni correction: gf vs. gF, P = 0.04; gF vs. GF, P = 0.14. *P < 0.05 vs. the respective treatment set to 100%. No differences between groups were found. C: oxidation of intracellular lipids with (+) and without (−) the mitochondrial uncoupler FCCP (1 μM) in gF and GF relative to the gf condition without FCCP. Open bars, lean without added FCCP; black bars, T2D without added FCCP; hatched bars, lean myotubes in the presence of FCCP; checkered bars, T2D myotubes in the presence of FCCP. aP < 0.05 vs. basal gf condition; n = 6 donors/group, with each donor quantified from 4 wells/condition. *P < 0.05 for T2D vs. lean.

Fig. 6. Correlation between fasting respiratory quotient (RQ) in vivo and lipid oxidation in the gF condition in the myotubes in vitro. A low RQ approaching 0.7 indicates a relatively high fat oxidation, whereas a high RQ approaching 1.0 indicates a low fat oxidation. • Obese T2D subjects; ○ lean subjects. r, Spearman correlation coefficient; n = 17. For further explanation of abbreviations, see legend to Fig. 1. A: correlation between ICLOX in the gF condition in vitro and fasting whole body RQ. B: correlation between total lipid oxidation in the gF condition in vitro and fasting whole body RQ.
During exercise, an initial increase in the utilization of TAG-derived fatty acids was observed. For a while after the start of exercise, plasma FFA availability was increased due to increased epinephrine-induced lipolysis, and this was paralleled by a decrease in TAG-derived FFA oxidation in skeletal muscle. Inhibition of adipose tissue lipolysis with acipimox decreased FFA availability from adipose tissue and increased the oxidation of TAG-derived fatty acids during rest and exercise (38). What these studies further support is that, under restricted fatty acid availability, the contribution of ICLOX is relevant for total fat oxidation. In human myotubes, both exogenous and endogenous lipid sources are contributors to total lipid oxidation (13).

Differences in the oxidation of IMCL may be caused by differences in IMCL lipolysis. Some time ago, studies by Wicklmayr et al. (42) suggested that lipolysis of intramyocellular TAG, and therefore availability of fatty acids from the intracellular lipid pool, is to a lesser extent under hormonal control and may be more regulated by substrate supply. It was suggested that product inhibition of hormone-sensitive lipase by long-chain fatty acyl-CoA could lead to a decrease in IMCL oxidation. In the present study, no differences in total lipolysis were observed between high and low FFA availability, and thus the effect of extracellular OA on ICLOX could not be explained by changes in the ICL lipolysis rate.

When comparing myotubes from obese T2D individuals to lean controls, the oxidation of both intracellular and extracellular lipids was reduced, and the T2D myotubes showed a lower ability to increase ECLOX and total lipid oxidation upon increased fatty acid availability. It is suggested that the capacity to increase fatty acid oxidation in adaptation to a high-fat intake may be important to maintain insulin sensitivity, since it may limit the formation of potential lipotoxic intermediates (1, 12, 22, 23, 28). In this study, a lower ICLOX as well as a lower mitochondrial mass were independent factors related to a higher cell-associated lipid accumulation. A lower lipid oxidation, represented by a high-fasting RQ at the whole body level, predisposed Pima Indians and nonobese men to obesity and insulin resistance (34, 43). In myotubes established from young healthy subjects, the capacity to increase fat oxidation in vitro was positively related to insulin sensitivity in vivo (37). The increase in fat oxidation in vitro was also related to a change in RQ during a hyperinsulinemic euglycemic clamp. In the present study, this correlation with a change in RQ could not be replicated. Nevertheless, we also observed that a low lipid oxidation in vitro, represented by a high RQ and measured during fasting, was reflected by a low lipid oxidation in vitro.
in particular in myotubes from lean subjects. These observations suggest that the characteristics of the in vivo phenotype are to a certain extent carried over to the myotubes in vitro by either genetic or epigenetic mechanisms.

A molecular explanation for the impaired lipid oxidation in myotubes established from obese T2D subjects may be found in reduced mitochondrial function or number. Several studies demonstrate a reduced mitochondrial mass and function in muscle of lean, insulin-resistant offspring of T2D individuals (27, 31), which may be related to a downregulation of peroxisome proliferator-activated receptor-γ coactivator-1α, as shown in some (30) but not all (27) studies. In the present study, mitochondrial mass was not related to ICLOX in the myotubes established from obese T2D individuals. Moreover, the uncoupling effect of FCCP in myotubes from T2D subjects was slightly reduced compared with lean subjects. These findings support a reduced mitochondrial capacity in primary cultured myotubes. This is in line with a previous report where the uncoupling effect of FCCP in myotubes from T2D subjects was reduced compared with lean, whereas the mitochondrial mass was not different (14, 29).

In the present study, we compared myotubes established from lean subjects with myotubes from obese T2D subjects to optimize our conditions for finding significant differences. A limitation of the study is that myotubes established from obese nondiabetic subjects were not studied. Very recently, we showed that myotubes established from obese subjects express a reduced oxidation of endogenous lipids, whereas myotubes established from obese T2D subjects express a reduced oxidation of both endogenous and exogenous lipids (13).

In conclusion, extracellular lipid overflow may inhibit the oxidation of IMCL, but this could not be explained by changed lipolysis of IMCL in our study. Mitochondrial mass and the rate of ICLOX contributed independently to IMCL accumulation. Increased mitochondrial uncoupling by FCCP rescued the reduction in ICLOX. This implicates that improved mitochondrial function and a reduction in circulating fatty acids, e.g., by reduced adipose tissue lipolysis, may help to oxidize IMCL-drial function and a reduction in circulating fatty acids, e.g., by cultured myotubes. This is in line with a previous report where reduced adipose tissue lipolysis, may help to oxidize IMCL.

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DISCLOSURES

None of the authors have a conflict of interest to declare.

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