Muscle lipid incorporation is retained in vitro

Reduced incorporation of fatty acids into triacylglycerol in myotubes from obese individuals with type 2 diabetes

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Abstract

Altered skeletal muscle lipid metabolism is a hallmark feature of type 2 diabetes (T2D). Here we investigated muscle lipid turnover in T2D versus BMI-matched controls and examined if putative in vivo differences would be preserved in the myotubes.

Male obese T2D individuals (T2D) (n=6) and their BMI-matched controls (C) (n=6) underwent a hyperinsulinemic-euglycemic clamp, VO₂max test, DXA scan, underwater weighing and muscle biopsy of v. lateralis. ¹⁴C-palmitate and ¹⁴C-oleate oxidation rates and incorporation into lipids were measured in muscle tissue, as well as in primary myotubes.

Palmitate oxidation (C: 0.99 ± 0.17, T2D: 0.53 ± 0.07nmol/mg protein; P=0.03) and incorporation of fatty acids (FAs) into triacylglycerol (TAG) (C: 0.45 ± 0.13, T2D: 0.11 ± 0.02nmol/mg protein; P=0.047) were significantly reduced in muscle homogenates of T2D. These reductions were not retained for palmitate oxidation in primary myotubes (P=0.38); however, incorporation of FAs into TAG was lower in T2D (P=0.03 for oleate and P=0.11 for palmitate), with a strong correlation of TAG incorporation between muscle tissue and primary myotubes (r=0.848, P=0.008).

Our data indicate that the ability to incorporate FAs into TAG is an intrinsic feature of human muscle cells that is reduced in individuals with T2D.

Key words: skeletal muscle, primary myotubes, type 2 diabetes, lipid metabolism, obesity

Abbreviations: ASMs; acid-soluble metabolites; DAG, diacylglycerol; DXA, dual energy x-ray absorptiometry; FAs, fatty acids; FFM, fat-free mass; OB, obese; PLIN, perilipin; TAG, triacylglycerol; T2D, type 2 diabetes; VO₂max, maximal aerobic capacity.

DISCLOSURE STATEMENT: The authors have nothing to disclose.
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Introduction

Perturbations in lipid metabolism are associated with insulin resistance and type 2 diabetes (T2D) (1). It has been proposed that an increased lipid supply, altered lipid partitioning and a reduced capacity for skeletal muscle fat oxidation could contribute to intramyocellular lipid (IMCL) accumulation, lipotoxicity and insulin resistance (2-4). However, whether these are inherited characteristics of T2D, or simply the consequence of altered lifestyle and obesity, remains an ongoing debate. In that context, studies in human primary myotubes are of interest, as cell autonomous models are devoid of direct environmental influences. Using such a model, Hulver et al. (5) demonstrated that myotubes of non-diabetic severely obese individuals retained abnormal lipid partitioning (i.e. elevated triacylglycerol to blunted fatty acid oxidation ratio; TAG/FAO) (5). Interestingly, Aguer et al. (6) showed that T2D subjects had increased IMCL content in muscle tissue, and that this elevation was maintained in primary myotubes from these subjects when compared with obese non-diabetic controls, indicating that increased IMCL content is preserved in vitro. However, the underlying reason for this was not examined. Elevated FA uptake could contribute to an increased IMCL content observed in obesity and T2D. Some studies show no difference in uptake of FAs (7; 8), while others show increased FA uptake in skeletal muscle of obese and T2D individuals (9). Likewise, studies in primary human myotubes also showed inconsistent results (6; 10; 11). Taken together, it is unclear whether disturbances that exist in skeletal muscle tissue lipid metabolism of T2D individuals are preserved in the myotubes. If these disturbances do persist in the myotubes, are they related to any aspects of the in vivo metabolic phenotype of the donors themselves?

As aberrant lipid metabolism is a central feature of obesity and T2D, the goal of the present study was to investigate whether disturbances exist in intramyocellular FA metabolism in
Muscle lipid incorporation is retained *in vitro* skeletal muscle of obese T2D individuals compared with BMI- and age-matched normoglycemic controls and to examine whether disturbances in lipid metabolism are retained *in vitro* in the myotubes established from these donors. Our study is a unique combination of *ex vivo* and *in vitro* analyses of lipid metabolism in human skeletal muscle, coupled with detailed *in vivo* clinical phenotyping to assess insulin sensitivity and aerobic capacity. We measured *ex vivo* FA metabolism in skeletal muscle tissue and established primary myotubes from these donors for *in vitro* studies. For comparison, we measured FA metabolism in skeletal muscle tissue of young, lean males. The myotubes were used to investigate lipid turnover (FA uptake, oxidation and storage) using two different long-chain FAs. We show that FA incorporation into triacylglycerol (TAG) is impaired in T2D muscle and in the myotubes established from these T2D donors, indicating that this perturbation is inherent to the T2D muscle cell.
Research Design and Methods

Participants. Twelve obese males with type 2 diabetes (T2D, n=6) and BMI- and age-matched males (Control) participated. Participants with T2D were diagnosed at least 1 year prior to the study, non-insulin dependent, had well-controlled diabetes (HbA1c < 7.8%; 62mmol/mol; 177 mg/dL) and no diabetes-related co-morbidities. Medication use (Metformin only or Metformin plus sulfonylureas) was stable for at least 6 months. Control participants had no family history of diabetes. We included data from 16 young, lean males that participated in another (unpublished) study. The studies were approved by the Medical Ethical Committee of Maastricht University. All participants gave written informed consent, performed a maximal aerobic capacity test (VO2max) (12) and underwent DXA or hydrostatic weighing for body composition (13).

Hyperinsulinemic-euglycemic clamp. To measure peripheral insulin sensitivity, a two-step hyperinsulinemic-euglycemic clamp was performed according to DeFronzo et al. (14). Briefly, after an overnight fast, a blood sample was drawn to measure glucose, insulin and free fatty acids (FAs). Step 1 was initiated with an insulin infusion at 10mU/m2/min for 4h with variable co-infusion of 20% glucose. Step 2 consisted of a 2h insulin infusion at 40mU/m2/min (20% variable glucose). The M-value was calculated as the glucose infusion rate and corrected for fat-free mass (FFM).

Muscle biopsy. Muscle biopsies were taken from the vastus lateralis according to Bergström (15) and processed same day for ex vivo assays and cell culture. Remaining tissue was stored at -80°C for future analyses.

Primary muscle cell cultures. Primary skeletal muscle cell cultures were established as previously described (16). Briefly, satellite cells were isolated and grown in media supplemented with 16% Fetal Bovine Serum (FBS) at 37°C, 5%CO2.
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**Real Time quantitative RT-PCR.** Total RNA was isolated from ~20mg of muscle tissue as previously described (17). Primers and probes are shown in Supplemental Table 3. Real Time qRT-PCR were performed as one-step reactions (18) as previously described (19). All expression data were normalized by dividing the target gene by the internal control gene.

**Western blots.** Western blots were performed using antibodies directed against PLIN2 and PLIN5 (Progen, Germany), PLIN3 (Santa Cruz, Germany), ATGL (Cell Signaling, USA), and sr-actin (Sigma, USA). We were unable to detect PLIN2 in myotubes. Secondary antibodies contained a fluorescent tag (IRdye). Protein quantification was performed on an Odyssey Infrared Imaging system (LI-COR Biotechnology, USA).

**Oil-red-O staining.** Fresh muscle cryosections were stained for IMCL by Oil-Red-O as described previously (20) and expressed per cell surface area.

**14C-labeled ex vivo palmitate oxidation and lipid incorporation.** Palmitate oxidation was determined by measuring production of $^{14}$CO$_2$ and acid soluble metabolites ($^{14}$C-ASMs) in skeletal muscle homogenates containing 250mM sucrose, 10mM Tris-HCl, 1mM EDTA and 2mM ATP. Reactions were initiated with 0.2mM palmitate and 0.0175mM [1-$^{14}$C] palmitate and terminated with 70% perchloric acid. CO$_2$ was trapped in 1N NaOH (21; 22).

*All in vitro experiments described below were performed in triplicates per participant and normalized to protein content.*

**In vitro $^{14}$C-palmitate oxidation and lipid incorporation.** Palmitate oxidation was measured as production of $^{14}$CO$_2$ and acid-soluble metabolites ($^{14}$C-ASMs) from [1-$^{14}$C]-palmitate (1µCi/ml), non-labeled palmitate (100 µM) and 1mM carnitine after a 3h. Lipids were extracted from the myotubes and lipid incorporation was measured by thin layer chromatography. Bands corresponding to TAG and DAG were quantified by liquid scintillation as previously described (16).
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\textit{In vitro pulse-chase 14C-oleate experiments.} Pulse chase experiments were adapted from Koves et al. (23). Muscle cells were grown and differentiated in 24-well plates. αMEM supplemented with 500µM oleate (2µCi per ml [1-14C]-oleic acid) complexed to BSA (1.25%) was added for 24hr on day 6 of differentiation (pulse). Cell samples were collected after the 24 hour ‘pulse’, lipids extracted and lipid species separated using thin layer chromatography (16). These samples represent lipid synthesis capacity. The ‘chase’ media was supplemented with 1mM carnitine and collected after 3 hours to measure oxidation (14CO$_2$ and 14C-ASMs) of the endogenously-labeled lipids.

\textit{In vitro fatty acid uptake.} FA uptake was measured by incubating myotubes with FBS-free DMEM containing 0.2µCi/mL [1-14C]-palmitate and 20µM non-labeled palmitate. Cells were incubated at 37°C for 4 minutes and lysed in 0.1M NaOH. Lysates were counted by liquid scintillation.

\textit{In vitro triacylglycerol levels.} Intracellular triacylglycerol levels in differentiated myotubes were measured using the method of Schwartz and Wolins (24).

\textit{DGAT activity assay.} Determination of DGAT activity was performed in cellular homogenates of the human myotubes as previously described (25). DGAT1 inhibitor was provided by Dr. Robert V. Farese, Jr. (26). Data are presented as the rate of formation of 14C-TAG.

\textit{Statistics.} Results are presented as mean ± SEM. Statistical analyses were performed using SPSS version 16.0 for MacOS 16.0 (SPSS, Chicago, IL). Statistical comparisons between conditions were performed using unpaired T-tests. In the case of \textit{in vitro} lipid incorporation and corresponding protein expressions, a one-sided unpaired T-test was performed since a reduced incorporation in T2D myotubes was not plausible based on the \textit{ex vivo} data of lipid.
Muscle lipid incorporation is retained *in vitro* incorporation. Pearson’s correlation coefficients were used to describe the linear association between variables. P<0.05 was considered statistically significant.
Results

In vivo

Clinical characteristics of individuals with type 2 diabetes, obese controls and young, lean subjects

Clinical characteristics are presented in Table 1. By definition, fasting plasma glucose levels were significantly higher in individuals with T2D compared with obese controls (C) (C: 5.22 ± 0.15, T2D: 7.13 ± 0.39mmol/L). Fasting circulating (FAs) were not different between groups (P=0.76). Skeletal muscle insulin sensitivity (M-value at 40mU/m²/min insulin) was lower, albeit not significantly, in T2D compared with obese controls (C: 32.8 ± 4.9, T2D: 20.5 ± 3.0µmol/kgFFM/min, P=0.05). Aerobic capacity was significantly lower in individuals with T2D compared with controls (VO₂max; C: 31.5 ± 0.8, T2D: 25.5 ± 1.8ml/min/kgFFM, P<0.05). As expected, clinical characteristics of young, lean subjects were significantly different from obese controls and individuals with T2D.

Ex vivo

Intramuscular lipid content, fiber type and fatty acid metabolism

Intramuscular lipid content (IMCL) was similar between obese controls and T2D (Figure 1). We then investigated fatty acid oxidation rates in skeletal muscle homogenates using an exogenously supplied long-chain fatty acid (LCFA; 14C-palmitate). Palmitate oxidation to 14CO₂ (“complete oxidation”) was significantly reduced in individuals with T2D (C: 0.099 ± 0.017, T2D: 0.053 ± 0.007nmol/mg protein, P=0.03; Figure 2A). Acid-soluble metabolites (ASMs; “incomplete oxidation”) were not different between the two groups (P=0.86; Figure 2B). Thus, the ratio of complete to incomplete oxidation (CO₂:ASMs), which is indicative of a more efficient FA metabolism, was also reduced in the T2D group (C: 0.12 ± 0.01, T2D: 0.07 ± 0.01, P<0.01;
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Figure 2C). The ratio of palmitate CO$_2$:ASMs trended to correlate positively with VO$_2$max ($r=0.537$, $P=0.072$) and inversely with fasting plasma glucose levels ($r=-0.566$, $P=0.055$) (Supplemental Figure 1). The same measurements performed in skeletal muscle tissue from young, lean subjects revealed significantly higher “complete” oxidation compared with both groups (Figures 2A-C).

We next measured the incorporation of $^{14}$C-palmitate into lipids in skeletal muscle homogenates. While incorporation of $^{14}$C-palmitate into the total lipid pool was similar between the two groups (Figure 2D), we did observe decreased incorporation of $^{14}$C-palmitate into triacylglycerols (TAG) in individuals with T2D (C: $0.045 \pm 0.013$, T2D: $0.011 \pm 0.002$nmol/mg protein, $P=0.047$; Figure 2E), with similar incorporation into diacylglycerols (DAG) (Figure 2F). This may indicate a blunted ability of the skeletal muscle of individuals with T2D to efficiently store and retain the lipids in the TAG pool.

Lean subjects showed significantly higher incorporation of palmitate into total lipids and a trend for higher TAG incorporation when compared with T2Ds (Figures 2D-E). We then measured the expressions of the lipid droplet coat proteins PLIN5, PLIN3 and PLIN2, as well as the lipolytic protein ATGL in individuals with T2D and obese controls. PLIN5 trended to be elevated in the T2D group (C: $4.42 \pm 1.07$, T2D: $9.38 \pm 2.14$au, $P=0.065$; Figure 6A). Interestingly, PLIN5 protein was inversely associated with \textit{ex vivo} (tissue) palmitate oxidation (CO$_2$:ASMs vs. PLIN5: $r=0.69$, $P=0.0122$; data not shown). No significant differences were observed between the two groups for PLIN3 ($P=0.185$), PLIN2 ($P=0.145$) and ATGL ($P=0.775$) expressions (Figures 6B-D).

\textit{In vitro}

\textbf{Exogenous fatty acid metabolism}
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In contrast to $^{14}$C-palmitate oxidation in muscle homogenates, \textit{in vitro} $^{14}$C-palmitate oxidation to CO$_2$ was not significantly lower in individuals with T2D ($P=0.38$; Figure 3A). Furthermore, oxidation to ASMs was not significantly different ($P=0.59$; Figure 3B), and consequently neither was the ratio of CO$_2$ to ASMs ($P=0.38$; Figure 3C). Moreover, \textit{in vitro} $^{14}$C-palmitate oxidation to CO$_2$ did not correlate with the \textit{ex vivo} $^{14}$C-palmitate oxidation ($P=0.721$; data not shown), suggesting that the capacity to oxidize palmitate to CO$_2$ is not an intrinsic property of the myotubes established from these obese T2D and non-diabetic individuals.

$^{14}$C-palmitate incorporation into the total lipid pool was lower in myotubes established from individuals with T2D (C: 5.84 ± 1.46, T2D: 3.28 ± 0.27nmol/mg protein, $P=0.058$; Figure 3D). Similarly, $^{14}$C-palmitate incorporation into TAG trended to be reduced in T2D myotubes ($P=0.085$; Figure 3E); however, DAGs ($P=0.145$; Figure 3F) were not significantly lower in individuals with T2D. \textit{In vitro} $^{14}$C-palmitate incorporation into TAG strongly correlated with \textit{ex vivo} $^{14}$C-palmitate incorporation into TAG ($r=0.848$, $P=0.008$; data not shown). We next determined $^{14}$C-palmitate uptake and DGAT activity in primary myotubes to investigate if a reduced FA uptake and/or reduced enzymatic activity might underlie the reduced incorporation of FAs into TAG. Palmitate uptake ($P=0.85$; Figure 3G) and DGAT activity (C: 9.28 ± 0.92, T2D: 7.69 ± 1.17nmol/h/mg protein, $P=0.31$; Figure 3H) did not significantly differ between groups.

\textbf{Endogenous fatty acid metabolism}

It has been suggested that endogenous, rather than exogenous, IMCL oxidation is reduced in myotubes from obese or obese T2D subjects compared with lean subjects (27). Therefore, we examined $^{14}$C-oleate oxidation after a 24h pulse with 500µM oleate (in the absence of carnitine to label the endogenous lipid pool). Oleate was chosen instead of palmitate because of the
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lipotoxic effects of palmitate on myotubes (28; 29). Confirming the lower $^{14}$C palmitate incorporation into TAG, incorporation of $^{14}$C-oleate into the total lipid pool after 24 hours was significantly lower (1.7-fold) in myotubes from individuals with T2D (C: 16.3 ± 2.40, T2D: 9.5 ± 0.78nmol/mg protein, P=0.02; Figure 4A). Furthermore, $^{14}$C-oleate incorporation into TAG was significantly lower in myotubes from individuals with T2D (C: 2.24 ± 0.47, T2D: 0.94 ± 0.25nmol/mg protein, P=0.03; Figure 4B). $^{14}$C-oleate incorporation into DAG was not significantly different between the two groups (P=0.31; Figure 4C).

Following a 24h incubation with $^{14}$C-oleate, oxidation was initiated by addition of carnitine and measured after 3 hours. Interestingly, even despite a reduced $^{14}$C-oleate incorporation into TAG in myotubes from individuals with T2D, the oxidation of endogenous $^{14}$C-oleate to CO$_2$ was not significantly different between the groups (P=0.384; Figure 4D). Likewise, oxidation to ASMs was similar between the two groups (C: 0.070 ± 0.012, T2D: 0.058 ± 0.015nmol/mg protein, P=0.543; Figure 4E). Consequently, no differences were observed in the ratio of CO$_2$ to ASMs (P=0.49; Figure 4F). Thus, in concert with the oxidation rates of exogenous $^{14}$C-palmitate, oxidation of the endogenously labeled $^{14}$C-oleate pool was similar in primary myotubes from both groups.

Of interest, in vivo basal glucose concentration was inversely related to in vitro incorporation of the endogenously-labeled lipid [$^{14}$C-oleate] into the total lipid pool ($r$=-0.615, P=0.033, Figure 5A), as well as TAG ($r$=-0.580, P=0.048, Figure 5B). Insulin sensitivity was also positively associated with $^{14}$C-oleate incorporation in the total lipid pool ($r$=0.602, P=0.038, Figure 5C).

It is important to note that there was no difference in the basal myocellular lipid content between the two donor groups, and thus no label dilution effect on the metabolic assays (data not shown).
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Finally, we measured the expressions of the lipid droplet coat proteins, PLIN5 and PLIN3, as well as the lipolytic protein ATGL before and after 24 hours of a 400µM oleate load (Figures 6E-G). We were unable to detect PLIN2 protein in the myotubes. In contrast to the *ex vivo* findings, PLIN5 was not different between the groups in the basal condition. However, PLIN5 trended to increase with the fatty acid load in the control group only (C, P=0.073 vs. T2D, P=0.468; Figure 6E). PLIN3 and ATGL protein expressions were not different between groups in the basal condition, nor did they significantly change in response to the oleate load (Figure 6F-G). Interestingly, PLIN5 protein (post-oleate load) was significantly associated with M-value at 40mU of insulin (r=0.61, P=0.035; data not shown). We further examined if differences between groups were present in mRNA expression of genes involved in lipid metabolism (Supplemental Table 1) in skeletal muscle tissue and/or primary myotubes. Although no significant differences were observed, PGC1α mRNA was significantly associated with increased palmitate and oleate incorporation into TAG in the myotubes, as well as DGAT activity; while PGC1α mRNA in the muscle tissue was significantly associated with tissue PLIN5 mRNA (Supplemental Table 2).
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**Discussion**

Obesity and type 2 diabetes (T2D) are associated with ectopic lipid accumulation in tissues such as skeletal muscle (1; 30-33). IMCL content inversely correlates with peripheral insulin sensitivity (34-37), suggesting that fat accumulation leads to insulin resistance. However, recent studies have shown that lipid intermediates—and not total IMCL \textit{per se}—are the true culprits of the development of insulin resistance (38; 39). Recent studies even suggest that increasing the TAG storage capacity, specifically in skeletal muscle, may be beneficial (40-42). While these studies employed different methods (i.e. genetic manipulations and exercise), they clearly demonstrated a beneficial effect of an increased TAG storage on skeletal muscle insulin sensitivity. Here, we investigated the oxidative and storage capacities of both skeletal muscle tissue and myotubes established from satellite cells of obese individuals with T2D and their BMI-matched non-diabetic controls. We show that oxidation and incorporation of exogenously supplied long-chain FAs (LCFAs, palmitate) into TAG is significantly blunted in muscle tissue from individuals with T2D. Importantly, we demonstrate that this blunted FA oxidative capacity is not retained in primary myotubes from these patients. However, myotubes of individuals with T2D do show reduced incorporation of exogenous palmitate into TAG, and this is significantly related to the impaired palmitate incorporation into TAG observed \textit{ex vivo}. Moreover, incorporation of oleate into the total neutral lipid pool, specifically the TAG pool, after prolonged FA incubation in the absence of carnitine supplementation is significantly reduced in the myotubes from the individuals with T2D. Since the absence of carnitine prevents FA oxidation, this measurement mainly reflects unidirectional TAG synthesis. Therefore, we conclude that lower lipid incorporation is an intrinsic metabolic characteristic of skeletal muscle of obese individuals with T2D compared with BMI-matched controls.
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We show a blunted “complete” oxidation of palmitate in the skeletal muscle tissue of obese individuals with T2D compared with their BMI-matched controls and young, lean subjects. As there were no differences in the acid-soluble metabolites (ASMs), the ratio of complete to incomplete oxidation was also reduced in T2Ds. These findings extend those of Hulver et al., who demonstrated aberrant exogenous FA oxidation in the skeletal muscle of obese and severely obese (BMI, 53.8 ± 3.5 kg/m²) individuals compared with lean controls (43). In this context, our data imply that T2D is associated with reduced tissue FA oxidation independent of BMI. Interestingly though, we did not observe significant differences in oxidation of exogenous palmitate to CO₂ and ASMs between myotubes of obese individuals with T2D and their BMI-matched controls. We then examined if FA oxidation from endogenous lipid pools was compromised in the myotubes of these individuals with T2D. After an overnight loading of the myotube lipid pool with oleate, no differences in endogenous FA oxidation rates between obese and T2D myotubes were observed. This contrasts with the findings of Gaster et al. (27) who demonstrated that complete oxidation of endogenous, but not exogenous, FAs was reduced in T2D myotubes compared with obese controls. One major difference between our studies and those of Gaster et al. is that in our studies we included subjects that were marginally obese (although all had a BMI >30 kg/m²), resulting in a lower average BMI compared with the previous study by Gaster et al. (27). Another potential explanation is that these two studies compare distinct subgroups of the obese population that may have been exposed to different environmental factors which could uniquely impact a varied genetic or epigenetic background. Our data suggest that a reduced myocellular fat oxidative capacity in individuals with T2D may not be an intrinsic characteristic, but rather a consequence of their environment.
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It was recently shown that one bout of exercise improves the capacity to store TAG in muscle and thereby prevents lipid-induced insulin resistance (42). Moreover, by use of *in vivo* infusion of \(^{13}\)C-palmitate, Bergman et al. (44) showed that palmitate incorporation into skeletal muscle TAG was increased in endurance trained athletes compared with sedentary controls. Here we found that incorporation of palmitate into TAG, but not into DAG, was blunted in skeletal muscle of individuals with T2D compared with BMI-matched controls and young, lean subjects. Collectively, these reports indicate that increased channeling of FAs towards storage in the form of TAG in skeletal muscle is associated with an improved metabolic profile. In contrast to the reduced oxidative capacity in the muscle tissue of the individuals with T2D, which was not retained in the myotubes, reduced incorporation of FAs into TAG was retained in the myotubes of individuals with T2D. By reducing lipid intermediate accumulation, a high capacity to channel FAs towards intramyocellular neutral lipid (i.e. TAG) storage may protect against lipid-induced insulin resistance. Thus, a reduced rate of TAG storage in T2D may be an important factor in the development of lipid-induced insulin resistance in these individuals. In support of this hypothesis, an enhanced capacity of the myotubes to incorporate endogenously labeled oleate into the total lipid pool, and specifically TAG, was associated with reduced basal glucose concentration and levels and insulin sensitivity.

The reason for the retained aberrant lipid incorporation capacity in T2D is so far unknown. We investigated if skeletal muscle FA uptake may underlie these findings. Skeletal muscle FA uptake is a controversial topic, with some investigators finding no difference in uptake of FAs (7; 45), while others show increased FA uptake in skeletal muscle of obese and T2D individuals (9). Here we could not detect differences in short-term FA uptake rates in cultured myotubes of individuals with T2D compared with BMI-matched controls. Reduced TAG incorporation in the
Muscle lipid incorporation is retained in vitro setting of unaltered FA uptake, oxidation or DAG incorporation might indicate that the FAs are incorporated into other lipid species not measured here that may (i.e. ceramides, etc.) (46) or may not yet be associated with insulin resistance and T2D. In addition, we could not detect differences in mRNA levels of lipid metabolism genes, nor in enzymatic activity of DGAT, between T2D and obese controls. However, we did observe significantly positive associations of PGC1α mRNA with DGAT activity and the capacity to incorporate both palmitate and oleate into TAG in the myotubes. These relationships support our previous findings that highlight the role of PGC1α in the regulation of intramuscular lipid droplet programming in mice and humans (47). Finally, we focused on expression levels of the lipid droplet coat proteins PLIN5, PLIN3 and PLIN2, as well as the lipolytic protein ATGL, in both muscle tissue and myotubes because we previously showed in animal studies that overexpressing PLIN2 or PLIN5 increases TAG storage capacity and results in prevention from high-fat induced insulin resistance (40; 48). Here we found that PLIN5 was elevated in the muscle tissue of the T2D individuals, but not in the myotubes. This is surprising given that the tissue lipid levels were not different between the two groups, and that PLIN5 has been associated with insulin sensitivity in humans (38). However, PLIN5 protein expression was not different between the two groups of myotubes in the basal state, which is in line with the similar lipid levels observed in these myotubes. Moreover, PLIN5 protein levels increased after an overnight oleate load in the BMI-matched myotubes, but not in those myotubes derived from T2D individuals. These data are consistent with the increased neutral lipid and TAG storage observed in these myotubes from the BMI-matched controls. In support of the notion that an increased TAG storage capacity is protective against the development of insulin resistance in conditions of increased lipid supply, we demonstrate that PLIN5 protein levels (post-oleate load) in the myotubes were positively related to insulin
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sensitivity. In the context of the similar lipid levels in the tissue and cells, it is not surprising that the expressions of PLIN2 and PLIN3 were not different between the two groups. However, when taken altogether, it is clear that future studies should investigate the intrinsic pathways in muscle lipid turnover (and its associated proteins) in order to identify the critical regulation sites.

In summary, our data show that skeletal muscle tissue lipid oxidation and FA incorporation into TAG are perturbed in obese individuals with T2D compared with their BMI-matched controls, but that only the disturbances in TAG incorporation are conserved in cultured myotubes from these individuals. Our results are consistent with the view that lipid turnover has a significant impact on insulin sensitivity and glucose homeostasis. Future studies using primary human muscle cell models with muscle-specific modulations of the lipid turnover pathways may help to unravel the specific regulation sites of skeletal muscle and whole-body energy metabolism in vivo. These findings could have profound implications for how we utilize precision medicine to treat, manage and prevent type 2 diabetes moving forward.
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Author Contributions

L.M.S. researched data; contributed to the study concept, design, analysis and interpretation of the data and wrote the manuscript. M.B. researched data; contributed to the study concept, design, analysis and interpretation of the data; and wrote the manuscript; B.B. researched data and reviewed and edited the manuscript; T.VdW. researched data and reviewed and edited the manuscript; L.B. researched data and reviewed and edited the manuscript; G.S. researched data; E.M-K. researched data; T.O.E. researched data; A.L. researched data; M.K.C.H. contributed to the study concept, design, analysis and interpretation of the data; and reviewed and edited the manuscript; P.S. contributed to the study concept, design, analysis and interpretation of the data; and reviewed and edited the manuscript.
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References

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Figure legends

Figure 1. Intramyocellular neutral lipid content measured by Oil-red-O staining in combination with an immunofluorescence staining against slow myosin heavy chain to determine fiber type. Mean ± SEM.

Figure 2. Ex vivo $^{14}$C-palmitate metabolism. (A) $^{14}$C-palmitate oxidation to CO$_2$. (B) $^{14}$C-palmitate oxidation to ASMs. (C) Ratio CO$_2$:ASMs. (D) Total $^{14}$C-palmitate incorporation into lipids. (E) $^{14}$C-palmitate incorporation into TAG. (F) $^{14}$C-palmitate incorporation into DAG. *P<0.05 vs. Control, **P<0.01 vs. Control, #P<0.05 vs. T2D, ##P<0.01 vs. T2D Error bars represent SEM.

Figure 3. In vitro exogenous $^{14}$C-palmitate metabolism. (A) $^{14}$C-palmitate oxidation to CO$_2$. (B) $^{14}$C-palmitate oxidation to ASMs. (C) Ratio CO$_2$:ASMs. (D) Total $^{14}$C-palmitate incorporation into lipids. (E) $^{14}$C-palmitate incorporation into TAG. (F) $^{14}$C-palmitate incorporation into DAG. (G) $^{14}$C-palmitate uptake. (H) DGAT activity. -/-: basal condition. +D1i: with DGAT1 inhibitor. *P<0.05 Error bars represent SEM.

Figure 4. In vitro $^{14}$C-oleate pulse-chase metabolism. Primary myotubes were loaded with 400µM oleate for 24h and then pulsed in the presence of 1mM carnitine for 3h. (A-C) Incorporation of $^{14}$C-oleate was measured after 24h incubation with 400µM oleate in the absence of carnitine. (A) Total lipid synthesis. (B) TAG synthesis. (C) DAG synthesis. Oxidation rates were measured during the 3h pulse period. (D) $^{14}$C-oleate oxidation to CO$_2$. (E) $^{14}$C-oleate oxidation to ASMs. (F) Ratio CO$_2$:ASMs. *P<0.05. Error bars represent SEM.

Figure 5. Correlations between in vivo measures of glucose and insulin sensitivity and in vitro endogenous lipid incorporation. Primary myotubes were loaded with 400µM oleate for 24h and
Muscle lipid incorporation is retained *in vitro*

then pulsed in the presence of 1mM carnitine for 3h. Incorporation of $^{14}$C-oleate was measured after 24h incubation with 400μM oleate in the absence of carnitine. Basal plasma glucose levels were inversely related to (A) total lipid synthesis and (B) TAG synthesis. Insulin sensitivity (M-value at 40 mU/m$^2$/min insulin infusion rate) was positively associated with (C) total lipid synthesis.

**Figure 6.** *Ex vivo* and *in vitro* protein expressions. Lipid droplet coating proteins, PLIN5 (A), PLIN3 (B) and (C) PLIN2, as well as the lipolytic protein ATGL (D), were measured in the muscle tissues of the Control and T2D individuals. Primary myotubes were loaded with 400μM oleate for 24h and then harvested for protein. (E-G) Protein expressions were measured before (C) and after the 24h incubation with 400μM oleate in the absence of carnitine (OA). (E) PLIN5, (F) PLIN3 and (G) ATGL. Sr-Actin was used as an internal control for all western blots. Error bars represent SEM.
Muscle lipid incorporation is retained *in vitro*

Table 1. Clinical Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>T2D</th>
<th>Young, Lean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>54.7 ± 4.1^A</td>
<td>58.3 ± 2.1^A</td>
<td>23.8 ± 1.5^B</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>31.2 ± 0.3^A</td>
<td>30.8 ± 0.3^A</td>
<td>21.4 ± 0.4^B</td>
</tr>
<tr>
<td>%Fat</td>
<td>32.8 ± 3.0^A</td>
<td>33.0 ± 2.0^A</td>
<td>14.3 ± 1.2^B</td>
</tr>
<tr>
<td>VO_{2max} (ml/min/kgFFM)</td>
<td>31.5 ± 0.8^A</td>
<td>25.5 ± 1.8^A</td>
<td>51.7 ± 1.9^B</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.22 ± 0.15^B</td>
<td>7.13 ± 0.39^A</td>
<td>5.15 ± 0.14^B</td>
</tr>
<tr>
<td>FAs (mmol/L)</td>
<td>0.78 ± 0.39</td>
<td>0.62 ± 0.17</td>
<td>-</td>
</tr>
<tr>
<td>M-value (µmol/kgFFM/min)*</td>
<td>32.8 ± 4.9^A</td>
<td>20.5 ± 3.0^A</td>
<td>73.6 ± 4.1^B</td>
</tr>
</tbody>
</table>

N=6 (Control), N=6 (T2D), N=16 (Young, Lean). Data are presented as mean ± SEM.

FAs were not measured in the Young, Lean cohort. T2D, Type 2 Diabetes; BMI, body mass index; FAs, free fatty acids

*M-value, skeletal muscle insulin sensitivity at an insulin infusion rate of 40mU/m^2/min

^A,B Significant differences between groups (one-way ANOVA).
Palmitate oxidation to CO$_2$ (nmol/mg protein)

Palmitate incorporation into total neutral lipids (nmol/mg protein)

Ratio CO$_2$:ASMs

Palmitate incorporation into TAG (nmol/mg protein)

Palmitate incorporation into DAG (nmol/mg protein)
A. OA incorporation into total lipids (nmol/mg protein)

B. TAG synthesis (nmol/mg protein)

C. DAG synthesis (nmol/mg protein)

D. Oleate oxidation to CO2 (nmol/mg protein)

E. Oleate oxidation to ASMs (nmol/mg protein)

F. Ratio CO2:ASMs
**A**  
C-oleate incorporation into total lipids (nmol/mg protein) vs glucose (mmol/L)  
- $r = -0.615$  
- $P = 0.033$

**B**  
C-oleate incorporation into TAG (nmol/mg protein) vs glucose (mmol/L)  
- $r = -0.580$  
- $P = 0.048$

**C**  
C-oleate incorporation into total lipids (nmol/mg protein) vs M-value ($\mu$mol/kg/min)  
- $r = 0.602$  
- $P = 0.038$
A

![Graph A]

VO₂ max (ml/min/kg)

PA oxidation ratio CO₂:ASM

r = 0.537
P = 0.072

B

![Graph B]

Glucose (mmol/L)

PA oxidation ratio CO₂:ASM

r = -0.566
P = 0.055
Statement of Justification

These Supplemental Materials are pertinent to providing additional information in the form of data tables and figures that enhance the understanding of the manuscript as a whole.

**Supplemental Figure 1.** Correlation of the ratio $^{14}$C-palmitate oxidation to CO$_2$:ASMs with (A) the VO$_{2}^{\text{max}}$ and (B) the fasting plasma glucose levels in obese individuals with T2D and BMI-matched controls.

**Supplemental Table 1. mRNA Expressions of Key Lipid Metabolism Genes in Human Skeletal Muscle Tissue and Myotubes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>T2D</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGL</td>
<td>1.35 ± 0.23</td>
<td>1.42 ± 0.21</td>
<td>0.8381</td>
</tr>
<tr>
<td>HSL</td>
<td>3.04 ± 0.59</td>
<td>3.54 ± 0.54</td>
<td>0.5515</td>
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<tr>
<td>DGAT1</td>
<td>1.52 ± 0.26</td>
<td>1.48 ± 0.23</td>
<td>0.9258</td>
</tr>
<tr>
<td>SCD1</td>
<td>0.02 ±0.01</td>
<td>0.03 ± 0.01</td>
<td>0.5615</td>
</tr>
<tr>
<td>PLIN5</td>
<td>3.88 ± 0.65</td>
<td>4.63 ± 0.57</td>
<td>0.4186</td>
</tr>
<tr>
<td>PGC1α</td>
<td>6.08 ± 0.55</td>
<td>5.15 ± 0.51</td>
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<tr>
<td>PPARδ</td>
<td>0.88 ± 0.10</td>
<td>0.83 ± 0.10</td>
<td>0.7169</td>
</tr>
<tr>
<td>CPT1β</td>
<td>4.11 ± 0.67</td>
<td>4.49 ± 0.61</td>
<td>0.6805</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>T2D</th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Mean ± SEM</td>
<td>Control Mean ± SEM</td>
<td>p-value</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>--------------------</td>
<td>---------</td>
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<tr>
<td>ATGL</td>
<td>0.75 ± 0.17</td>
<td>0.70 ± 0.17</td>
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<tr>
<td>HSL</td>
<td>0.35 ± 0.07</td>
<td>0.28 ± 0.06</td>
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<tr>
<td>DGAT1</td>
<td>0.90 ± 0.17</td>
<td>0.77 ± 0.16</td>
<td>0.5878</td>
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<tr>
<td>SCD1</td>
<td>1.03 ± 0.37</td>
<td>0.88 ± 0.37</td>
<td>0.7867</td>
</tr>
<tr>
<td>PLIN5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PGC1α</td>
<td>0.05 ± 0.04</td>
<td>0.03 ± 0.03</td>
<td>0.6978</td>
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<tr>
<td>PPARδ</td>
<td>1.14 ± 0.24</td>
<td>0.96 ± 0.23</td>
<td>0.6093</td>
</tr>
<tr>
<td>CPT1β</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.9021</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. N=6 per group. T2D, Type 2 Diabetes; ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; DGAT1, diacylglycerol acyl-transferase 1. SCD1, stearoyl-CoA desaturase 1; PLIN5, perilipin 5; PGC-1α, peroxisome proliferator activated receptor coactivator-1 alpha; PPARδ, peroxisome proliferator activated receptor delta; CPT1β, carnitine palmitoyltransferase 1 beta. All data are normalized to an internal control gene, RPL26, ribosomal protein L26. “-” means that the mRNA expression was not detectable.
Supplemental Table 2. Associations of PGC1α mRNA Expressions with Key Lipid Metabolism Endpoints in Human Skeletal Muscle Tissue and Myotubes

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Lipid Metabolism Endpoint</th>
<th>$r$</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>PGC1α (myotubes)</td>
<td>PA incorporation into TAG (myotubes)</td>
<td>0.5818</td>
<td>0.0604</td>
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<tr>
<td>PGC1α (myotubes)</td>
<td>OA incorporation into TAG (myotubes)</td>
<td>0.6091</td>
<td>0.0467</td>
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<tr>
<td>PGC1α (myotubes)</td>
<td>DGAT activity (myotubes)</td>
<td>0.6573</td>
<td>0.0202</td>
</tr>
<tr>
<td>PGC1α (muscle tissue)</td>
<td>PLIN5 mRNA (muscle tissue)</td>
<td>0.7000</td>
<td>0.0165</td>
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</tbody>
</table>

Data are simple correlations among all 12 participants (Control and T2D). T2D, Type 2 Diabetes; PGC1α, peroxisome proliferator activated receptor coactivator-1 α; DGAT1, diacylglycerol acyl-transferase 1. PLIN5, perilipin 5; All mRNA data are normalized to an internal control gene, RPL26, ribosomal protein L26.

Due to formatting, Supplemental Table 3 is in a separate file.
### Supplemental Table 3. Oligonucleotide sequences for primer/probe sets used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward primer</th>
<th>Probe</th>
<th>Reverse primer</th>
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</thead>
<tbody>
<tr>
<td>ATGL</td>
<td>NM_020376.2</td>
<td>CCACGGCGCTGGTCA</td>
<td>TTGGCACACGCCCTACCCAGG</td>
<td>GGGCCCTTCTTAGATACCTCAATGA</td>
</tr>
<tr>
<td>HSL</td>
<td>NM_005357.2</td>
<td>ACGCTGCATAAGGGATGCTT</td>
<td>AGTTACGGCTGCAATCCGGGC</td>
<td>CCTGCTCTGTTGCGTTTGTAGT</td>
</tr>
<tr>
<td>DGAT1</td>
<td>NM_012079</td>
<td>CGTGAAGCTACCCGGACAAATC</td>
<td>ACCTACCGCGATCTCTACTACTTCCTTCACC</td>
<td>AAAGTTGAGCTGATACACAGAAGG</td>
</tr>
<tr>
<td>SCD1</td>
<td>NM_005063</td>
<td>TGGCATTCAGAAATGATGTCTATG</td>
<td>CGTGACCACCGTGCACACCA</td>
<td>GGAATTATGAGATAGCATGATG</td>
</tr>
<tr>
<td>PLIN5</td>
<td>NM_001013706.2</td>
<td>GAGCCATGCTGATGATGTTGTA</td>
<td>TGGATCACTTCTTGCCATGACAGC</td>
<td>CAGTGGCAGAGCTCTTC</td>
</tr>
<tr>
<td>PGC1α</td>
<td>NM_013261.3</td>
<td>TGCTGAAGAGAAAGTGAGCGATTATTTG</td>
<td>CATGTAGAATTGGCAGTGGAAA</td>
<td>AGGTGAAGATGTAATACGTGATGAG</td>
</tr>
<tr>
<td>PPARδ</td>
<td>NM_006238.3</td>
<td>TCTACAATGCTACCTGAAAAACTTC</td>
<td>ACATGACAAAAAGAAGGGCCCAGG</td>
<td>GGCTTTGGGCGTGAGGAT</td>
</tr>
<tr>
<td>CPT1β</td>
<td>NM_152247.1</td>
<td>CCAGAGCGACACCCCAAT</td>
<td>CATCTGTACTAGGGCAAGGCCACCT</td>
<td>CTGCAATCATGAGAAAACCTCATAG</td>
</tr>
</tbody>
</table>

For all gene expression assays the ribosomal protein L26 gene, RPL26, was used as the internal control. ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; DGAT1, diacylglycerol acyltransferase 1; SCD1, stearoyl-CoA desaturase 1; PLIN5, perilipin 5; PGC-1α, peroxisome proliferator activated receptor coactivator-1 alpha; PPARδ, peroxisome proliferator activated receptor delta; CPT1β, carnitine palmitoyltransferase 1 beta.