

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

Citation for published version (APA):

Sparks, L. M., Bosma, M., Brouwers, B., van de Weijer, T., Bilet, L., Schaart, G., Kornips, E., Eichmann, T. O., Lass, A., Hesselink, M. K., & Schrauwen, P. (2014). Reduced incorporation of Fatty acids into triacylglycerol in myotubes from obese individuals with type 2 diabetes. *Diabetes*, 63(5), 1583-1593. <https://doi.org/10.2337/db13-1123>

Document status and date:

Published: 01/05/2014

DOI:

[10.2337/db13-1123](https://doi.org/10.2337/db13-1123)

Document Version:

Publisher's PDF, also known as Version of record

Document license:

Taverne

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Lauren M. Sparks,^{1,2} Madeleen Bosma,¹ Bram Brouwers,¹ Tineke van de Weijer,¹ Lena Bilet,¹ Gert Schaart,³ Esther Moonen-Kornips,¹ Thomas O. Eichmann,⁴ Achim Lass,⁴ Matthijs K.C. Hesselink,³ and Patrick Schrauwen¹



Reduced Incorporation of Fatty Acids Into Triacylglycerol in Myotubes From Obese Individuals With Type 2 Diabetes

Diabetes 2014;63:1583–1593 | DOI: 10.2337/db13-1123

Altered skeletal muscle lipid metabolism is a hallmark feature of type 2 diabetes (T2D). We investigated muscle lipid turnover in T2D versus BMI-matched control subjects (controls) and examined whether putative *in vivo* differences would be preserved in the myotubes. Male obese T2D individuals ($n = 6$) and BMI-matched controls ($n = 6$) underwent a hyperinsulinemic-euglycemic clamp, $\text{VO}_{2\text{max}}$ test, dual-energy X-ray absorptiometry scan, underwater weighing, and muscle biopsy of the vastus lateralis. ^{14}C -palmitate and ^{14}C -oleate oxidation rates and incorporation into lipids were measured in muscle tissue as well as in primary myotubes. Palmitate oxidation (controls: $0.99 \pm 0.17 \text{ nmol/mg protein}$; T2D: $0.53 \pm 0.07 \text{ nmol/mg protein}$; $P = 0.03$) and incorporation of fatty acids (FAs) into triacylglycerol (TAG) (controls: $0.45 \pm 0.13 \text{ nmol/mg protein}$; T2D: $0.11 \pm 0.02 \text{ nmol/mg protein}$; $P = 0.047$) 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 ($r = 0.848$, $P = 0.008$). The 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.

Perturbations in lipid metabolism are associated with insulin resistance and type 2 diabetes (T2D) (1). An increased lipid supply, altered lipid partitioning, and reduced capacity for skeletal muscle fat oxidation have been proposed to contribute to intramyocellular lipid (IMCL) accumulation, lipotoxicity, and insulin resistance (2–4). However, whether these characteristics of T2D are inherited or simply the consequence of an altered lifestyle and obesity remains an ongoing debate. In that context, studies in human primary myotubes are of interest because cell autonomous models are devoid of direct environmental influences. Hulver et al. (5) used such a model to demonstrate that myotubes of nondiabetic, severely obese individuals retain abnormal lipid partitioning (i.e., elevated triacylglycerol [TAG]-to-blunted fatty acid [FA] oxidation ratio) (5). Of note, 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 compared with obese non-diabetic control subjects, indicating that increased IMCL content is preserved *in vitro*. However, the underlying reason for this finding was not examined. Elevated FA uptake could contribute to an increased IMCL content observed in obesity and T2D. Some studies showed no difference in uptake of FAs (7,8), whereas others showed increased FA uptake in skeletal muscle of obese and T2D

¹Department of Human Biology, NUTRIM—School for Nutrition, Toxicology and Metabolism, Departments of Human Biology, Maastricht University Medical Center, Maastricht, the Netherlands

²Translational Research Institute for Metabolism and Diabetes, Florida Hospital, Orlando, FL

³Department of Human Movement Sciences, NUTRIM—School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Center, Maastricht, the Netherlands

⁴Institute of Molecular Biosciences, University of Graz, Graz, Austria

Corresponding author: Patrick Schrauwen, p.schrauwen@maastrichtuniversity.nl.

Received 19 July 2013 and accepted 26 January 2014.

Clinical trial reg. no. NCT01298375, clinicaltrials.gov.

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db13-1123/-DC1>.

L.M.S., M.B., M.K.C.H., and P.S. contributed equally to this work.

© 2014 by the American Diabetes Association. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

individuals (9). Likewise, studies in primary human myotubes showed inconsistent results (6,10,11). Taken together, whether disturbances that exist in skeletal muscle tissue lipid metabolism of T2D individuals are preserved in the myotubes is unclear. If these disturbances persist in the myotubes, are they related to any aspects of the in vivo metabolic phenotype of the donors?

Because aberrant lipid metabolism is a central feature of obesity and T2D, the goal of the current study was to investigate whether disturbances exist in intramyocellular FA metabolism in skeletal muscle of obese T2D individuals compared with BMI- and age-matched normoglycemic control subjects and to examine whether disturbances in lipid metabolism are retained in vitro in the myotubes established from these donors. This 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 TAG is impaired in T2D muscle and in the myotubes established from T2D donors, indicating that this perturbation is inherent in the T2D muscle cell.

RESEARCH DESIGN AND METHODS

Participants

Twelve obese males with T2D ($n = 6$) and BMI- and age-matched males (controls) participated. Participants with T2D were given a diagnosis at least 1 year before the study, were noninsulin dependent, had well-controlled diabetes ($\text{HbA}_{1c} < 7.8\%$; 62 mmol/mol; 177 mg/dL), and had no diabetes-related comorbidities. Medication use (metformin only or metformin plus sulfonylureas) was stable for at least 6 months. Controls had no family history of diabetes. We included data from 16 young, lean males who 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 ($\text{VO}_{2\text{max}}$) (12), and underwent dual-energy X-ray absorptiometry 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 FA levels. Step 1 was initiated with an insulin infusion at 10 mU/m²/min for 4 h with variable coinfusion of 20% glucose. Step 2 consisted of a 2-h insulin infusion at 40 mU/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 biopsy specimens were taken from the vastus lateralis according to Bergstrom (15) and processed the 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% FBS at 37°C and 5% CO₂.

Real-Time Quantitative RT-PCR

Total RNA was isolated from ~ 20 mg of muscle tissue as previously described (17). Primers and probes are shown in Supplementary Table 1. Real-time quantitative RT-PCR was 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, Heidelberg, Germany), PLIN3 (Santa Cruz Biotechnology, Heidelberg, Germany), ATGL (Cell Signaling Technology, Danvers, MA), and sr-actin (Sigma-Aldrich, St. Louis, MO). We were unable to detect PLIN2 in myotubes. Secondary antibodies contained a fluorescent tag (IRDye; LI-COR, Lincoln, NE). Protein quantification was performed on an Odyssey Infrared Imaging system (LI-COR).

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 ¹⁴CO₂ and acid-soluble metabolites (¹⁴C-ASMs) in skeletal muscle homogenates containing 250 mmol/L sucrose, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, and 2 mmol/L ATP. Reactions were initiated with 0.2 mmol/L palmitate and 0.0175 mmol/L [1-¹⁴C]-palmitate and terminated with 70% perchloric acid. CO₂ was trapped in 1N NaOH (21,22). All in vitro experiments described next were performed in triplicate per participant and normalized to protein content.

In Vitro ¹⁴C-Palmitate Oxidation and Lipid Incorporation

Palmitate oxidation was measured as production of ¹⁴CO₂ and ¹⁴C-ASMs from [1-¹⁴C]-palmitate (1 $\mu\text{Ci}/\text{mL}$), non-labeled palmitate (100 $\mu\text{mol}/\text{L}$), and 1 mmol/L carnitine after 3 h. Lipids were extracted from the myotubes, and lipid incorporation was measured by thin-layer chromatography. Bands corresponding to TAG and diacylglycerol

(DAG) were quantified by liquid scintillation as previously described (16).

In Vitro Pulse-Chase ^{14}C -Oleate Experiments

Pulse-chase experiments were adapted from Koves et al. (23). Muscle cells were grown and differentiated in 24-well plates. α -Minimum essential medium supplemented with 500 $\mu\text{mol/L}$ oleate (2 $\mu\text{Ci/mL}$ [$1-^{14}\text{C}$]-oleic acid) complexed to BSA (1.25%) was added for 24 h on day 6 of differentiation (pulse). Cell samples were collected after the 24-h pulse, lipids extracted, and lipid species separated using thin-layer chromatography (16). These samples represent lipid synthesis capacity. The chase media were supplemented with 1 mmol/L carnitine and collected after 3 h to measure oxidation ($^{14}\text{CO}_2$ and ^{14}C -ASMs) of the endogenously labeled lipids.

In Vitro FA Uptake

FA uptake was measured by incubating myotubes with FBS-free Dulbecco's modified Eagle's medium containing 0.2 $\mu\text{Ci/mL}$ [$1-^{14}\text{C}$]-palmitate and 20 $\mu\text{mol/L}$ nonlabeled palmitate. Cells were incubated at 37°C for 4 min and lysed in 0.1 mol/L NaOH. Lysates were counted by liquid scintillation.

In Vitro TAG Levels

Intracellular TAG levels in differentiated myotubes were measured using the method of Schwartz and Wolins (24).

Acyl-CoA:DAG Acyltransferase Activity Assay

Determination of acyl-CoA:DAG acyltransferase (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 ^{14}C -TAG.

Statistics

Results are presented as mean \pm SEM. Statistical analyses were performed using SPSS version 16.0 for MacOS 16.0 (IBM Corp., Chicago, IL). Statistical comparisons between conditions were performed using unpaired *t* tests. In the case of in vitro lipid incorporation and corresponding protein expressions, a one-sided unpaired *t* test was performed because a reduced incorporation in T2D myotubes was not

plausible based on the ex vivo data of lipid incorporation. Pearson 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 T2D; 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 than in obese controls (controls: $5.22 \pm 0.15 \text{ mmol/L}$; T2D: $7.13 \pm 0.39 \text{ mmol/L}$). Fasting circulating FAs were not different between groups ($P = 0.76$). Skeletal muscle insulin sensitivity (M-value at 40 $\text{mU/m}^2/\text{min}$ insulin) was lower, albeit not significantly, in T2D than in obese controls (controls: $32.8 \pm 4.9 \mu\text{mol/kg FFM/min}$; T2D: $20.5 \pm 3.0 \mu\text{mol/kg FFM/min}$; $P = 0.05$). Aerobic capacity ($\text{VO}_{2\text{max}}$) was significantly lower in individuals with T2D than in controls (controls: $31.5 \pm 0.8 \text{ mL/min/kg FFM}$; T2D: $25.5 \pm 1.8 \text{ mL/min/kg FFM}$; $P < 0.05$). 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 FA Metabolism

Intramuscular lipid content (IMCL) was similar between obese controls and individuals with T2D (Fig. 1). We then investigated FA oxidation rates in skeletal muscle homogenates using an exogenously supplied long-chain FA (^{14}C -palmitate). Palmitate oxidation to $^{14}\text{CO}_2$ (complete oxidation) was significantly reduced in individuals with T2D (controls: $0.099 \pm 0.017 \text{ nmol/mg protein}$; T2D: $0.053 \pm 0.007 \text{ nmol/mg protein}$; $P = 0.03$) (Fig. 2A). ASMs (incomplete oxidation) were not different between the two groups ($P = 0.86$) (Fig. 2B). Thus, the ratio of complete-to-incomplete oxidation ($\text{CO}_2:\text{ASMs}$), which indicates a more efficient FA metabolism, was also reduced in the T2D group (controls: 0.12 ± 0.01 ; T2D: 0.07 ± 0.01 ; $P < 0.01$) (Fig. 2C). The palmitate CO_2 :

Table 1—Clinical characteristics

	Control (<i>n</i> = 6)	T2D (<i>n</i> = 6)	Young, lean (<i>n</i> = 16)
Age (years)	$54.7 \pm 4.1^{\text{A}}$	$58.3 \pm 2.1^{\text{A}}$	$23.8 \pm 1.5^{\text{B}}$
BMI (kg/m^2)	$31.2 \pm 0.3^{\text{A}}$	$30.8 \pm 0.3^{\text{A}}$	$21.4 \pm 0.4^{\text{B}}$
% fat	$32.8 \pm 3.0^{\text{A}}$	$33.0 \pm 2.0^{\text{A}}$	$14.3 \pm 1.2^{\text{B}}$
$\text{VO}_{2\text{max}}$ ($\text{mL}/\text{min/kg FFM}$)	$31.5 \pm 0.8^{\text{A}}$	$25.5 \pm 1.8^{\text{C}}$	$51.7 \pm 1.9^{\text{B}}$
Glucose (mmol/L)	$5.22 \pm 0.15^{\text{B}}$	$7.13 \pm 0.39^{\text{A}}$	$5.15 \pm 0.14^{\text{B}}$
FAs (mmol/L)	0.78 ± 0.39	0.62 ± 0.17	—
M-value ($\mu\text{mol/kg FFM/min}$)*	$32.8 \pm 4.9^{\text{A}}$	$20.5 \pm 3.0^{\text{A}}$	$73.6 \pm 4.1^{\text{B}}$

Data are mean \pm SEM. FAs were not measured in the young, lean cohort. ^{A,B,C}Significant differences between groups, $P < 0.05$ (one-way ANOVA). *M-value, skeletal muscle insulin sensitivity at an insulin infusion rate of 40 $\text{mU/m}^2/\text{min}$.

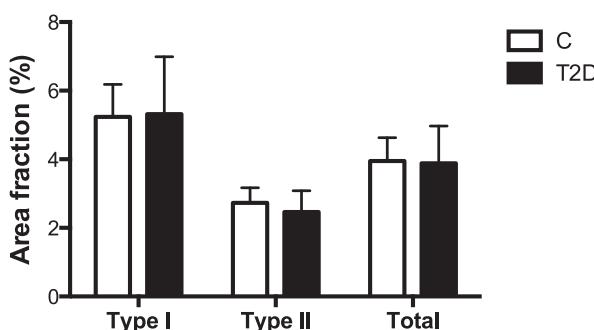


Figure 1—Intramyocellular neutral lipid content measured by Oil Red O staining in combination with an immunofluorescence staining against slow myosin heavy chain to determine fiber type. Data are mean \pm SEM. C, controls.

ASMs correlated positively with $\text{VO}_{2\text{max}}$ ($r = 0.537$, $P = 0.072$) and inversely with fasting plasma glucose levels ($r = -0.566$, $P = 0.055$) (Supplementary Fig. 1). The same measurements performed in skeletal muscle tissue from young, lean subjects revealed significantly higher complete oxidation compared with both groups (Fig. 2A–C).

We next measured the incorporation of ^{14}C -palmitate into lipids in skeletal muscle homogenates. Although incorporation of ^{14}C -palmitate into the total lipid pool was similar between the two groups (Fig. 2D), we observed decreased incorporation of ^{14}C -palmitate into TAGs in individuals with T2D (controls: 0.045 ± 0.013 nmol/mg protein; T2D: 0.011 ± 0.002 nmol/mg protein; $P = 0.047$) (Fig. 2E), with similar incorporation into DAGs (Fig. 2F). This finding 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 compared with T2D (Fig. 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 was elevated in the T2D group (controls: 4.42 ± 1.07 arbitrary units [au]; T2D: 9.38 ± 2.14 au; $P = 0.065$) (Fig. 6A). Of note, PLIN5 protein was inversely associated with 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 (Fig. 6B–D).

In Vitro

Exogenous FA Metabolism

In contrast to ^{14}C -palmitate oxidation in muscle homogenates, in vitro ^{14}C -palmitate oxidation to CO_2 was not significantly lower in individuals with T2D ($P = 0.38$) (Fig. 3A). Furthermore, oxidation to ASMs was not significantly different ($P = 0.59$) (Fig. 3B) and, consequently, neither was CO_2 :ASMs ($P = 0.38$) (Fig. 3C). Moreover, in

vitro ^{14}C -palmitate oxidation to CO_2 did not correlate with the 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 nondiabetic individuals.

^{14}C -palmitate incorporation into the total lipid pool was lower in myotubes established from individuals with T2D (controls: 5.84 ± 1.46 nmol/mg protein; T2D: 3.28 ± 0.27 nmol/mg protein; $P = 0.058$) (Fig. 3D). Similarly, ^{14}C -palmitate incorporation into TAG was reduced in T2D myotubes ($P = 0.085$) (Fig. 3E); however, DAGs were not significantly lower in individuals with T2D ($P = 0.145$) (Fig. 3F). In vitro ^{14}C -palmitate incorporation into TAG strongly correlated with 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 whether a reduced FA uptake and/or reduced enzymatic activity might underlie the reduced incorporation of FAs into TAG. Palmitate uptake ($P = 0.85$) (Fig. 3G) and DGAT activity did not significantly differ between groups (controls: 9.28 ± 0.92 nmol/h/mg protein; T2D: 7.69 ± 1.17 nmol/h/mg protein; $P = 0.31$) (Fig. 3H).

Endogenous FA 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 24-h pulse with 500 $\mu\text{mol/L}$ oleate (in the absence of carnitine to label the endogenous lipid pool). Oleate was chosen instead of palmitate because of the 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 h was significantly lower (1.7-fold) in myotubes from individuals with T2D (controls: 16.3 ± 2.40 nmol/mg protein; T2D: 9.5 ± 0.78 nmol/mg protein; $P = 0.02$) (Fig. 4A). Furthermore, ^{14}C -oleate incorporation into TAG was significantly lower in myotubes from individuals with T2D (controls: 2.24 ± 0.47 nmol/mg protein; T2D: 0.94 ± 0.25 nmol/mg protein; $P = 0.03$) (Fig. 4B). ^{14}C -oleate incorporation into DAG was not significantly different between the two groups ($P = 0.31$) (Fig. 4C).

Following a 24-h incubation with ^{14}C -oleate, oxidation was initiated by the addition of carnitine and measured after 3 h. 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 ($P = 0.384$) (Fig. 4D). Likewise, oxidation to ASMs was similar between the two groups (controls: 0.070 ± 0.012 nmol/mg protein; T2D: 0.058 ± 0.015 nmol/mg protein; $P = 0.543$) (Fig. 4E). Consequently, no differences were observed in CO_2 :ASMs ($P = 0.49$) (Fig. 4F). Thus, in concert with the oxidation rates of exogenous ^{14}C -palmitate, oxidation of the endogenously

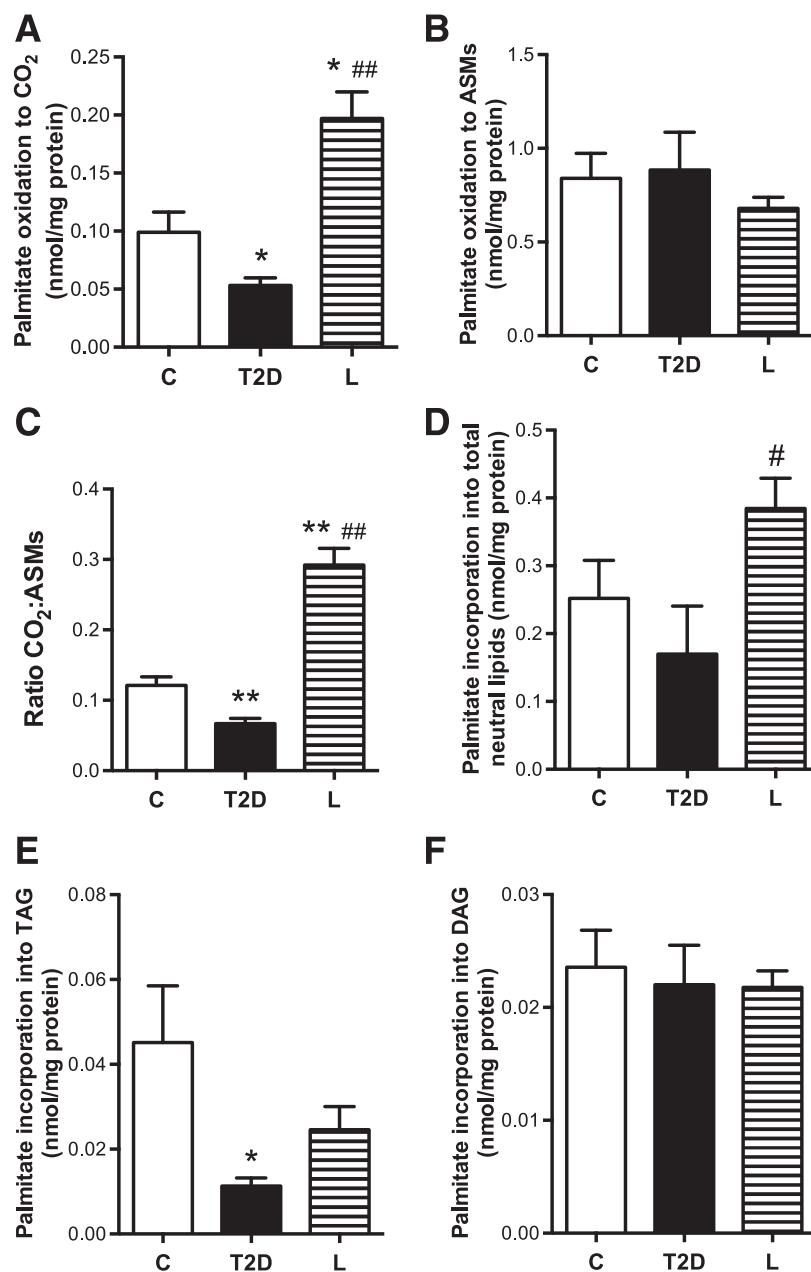


Figure 2—Ex vivo ^{14}C -palmitate metabolism. *A*: ^{14}C -palmitate oxidation to CO_2 . *B*: ^{14}C -palmitate oxidation to ASMs. *C*: CO_2 :ASMs. *D*: ^{14}C -palmitate incorporation into total neutral lipids. *E*: ^{14}C -palmitate incorporation into TAG. *F*: ^{14}C -palmitate incorporation into 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. C, control; L, young, lean.

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 ($r = -0.615$, $P = 0.033$) (Fig. 5A) as well as TAG ($r = -0.580$, $P = 0.048$) (Fig. 5B). Insulin sensitivity was also positively associated with ^{14}C -oleate incorporation in the total lipid pool ($r = 0.602$, $P = 0.038$) (Fig. 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 was observed (data not shown).

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 h of a 400 $\mu\text{mol/L}$ oleate load (Fig. 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 increased with the FA load in the control group only (controls: $P = 0.073$; T2D: $P = 0.468$) (Fig. 6E). PLIN3 and ATGL protein expressions were not different between groups in the basal condition or significantly changed in response to the oleate load (Fig. 6F–G). Of note, PLIN5 protein

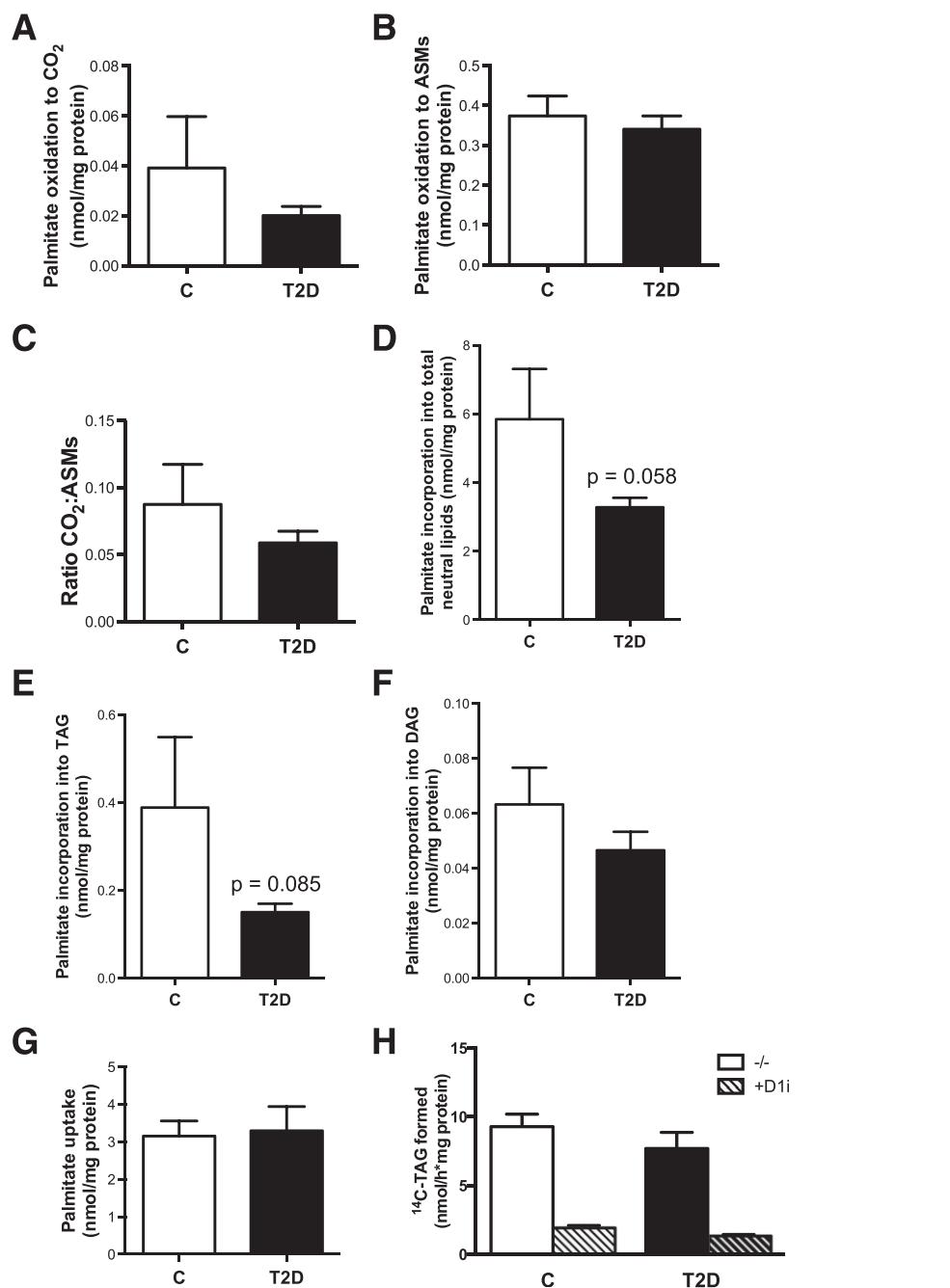


Figure 3—In vitro exogenous ^{14}C -palmitate metabolism. *A*: ^{14}C -palmitate oxidation to CO_2 . *B*: ^{14}C -palmitate oxidation to ASMs. *C*: CO_2 : ASMs. *D*: ^{14}C -palmitate incorporation into total neutral lipids. *E*: ^{14}C -palmitate incorporation into TAG. *F*: ^{14}C -palmitate incorporation into DAG. *G*: ^{14}C -palmitate uptake. *H*: DGAT activity. Error bars represent SEM. $-/-$, basal condition; C, control; +D1i, with DGAT1 inhibitor.

(postoleate load) was significantly associated with the M-value at 40 mU of insulin ($r = 0.61$, $P = 0.035$) (data not shown). We further examined whether differences between groups were present in the mRNA expression of genes involved in lipid metabolism (Supplementary Table 2) in skeletal muscle tissue and/or primary myotubes. Although no significant differences were observed, PGC1 α mRNA was significantly associated with increased palmitate and oleate incorporation into TAG in the myotubes as

well as with DGAT activity, whereas PGC1 α mRNA in the muscle tissue was significantly associated with tissue PLIN5 mRNA (Supplementary Table 3).

DISCUSSION

Obesity and 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

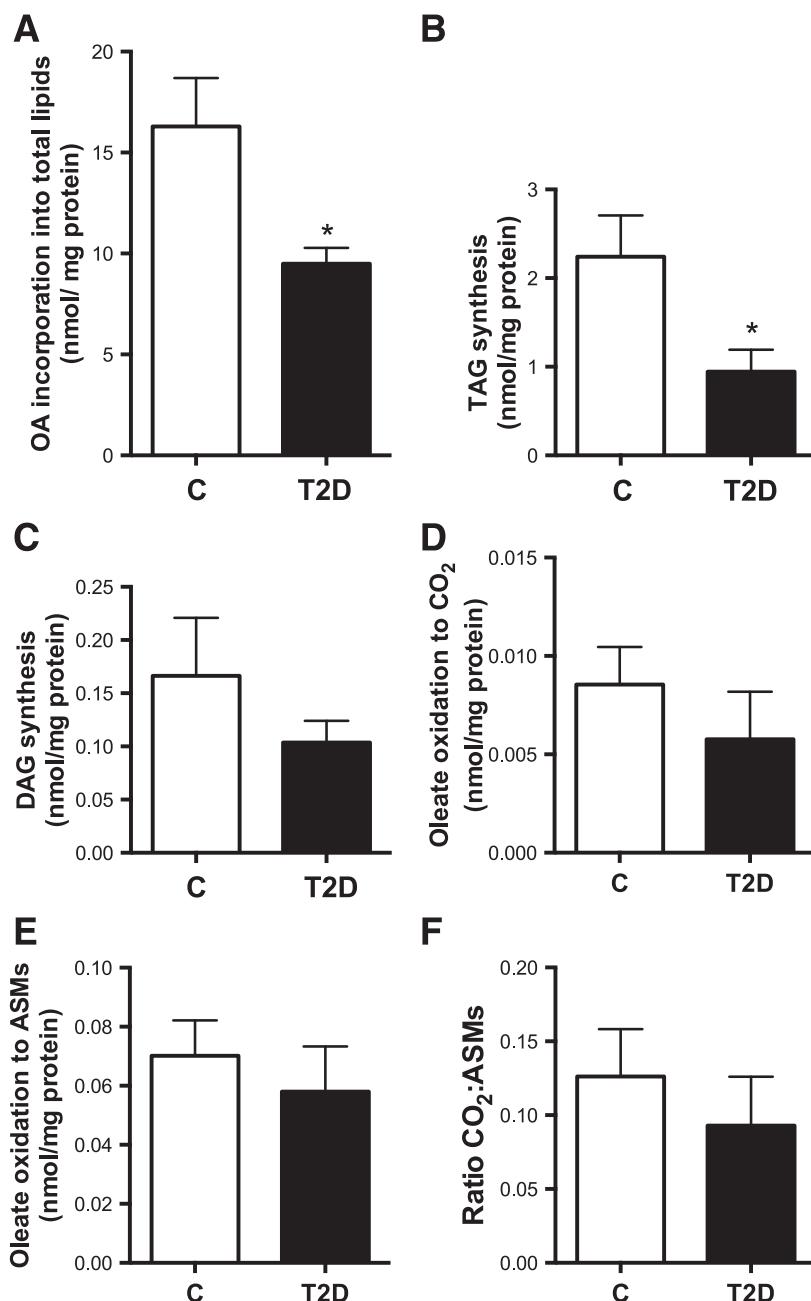


Figure 4—In vitro ^{14}C -oleate pulse-chase metabolism. Primary myotubes were loaded with 400 $\mu\text{mol/L}$ oleate for 24 h and then pulsed in the presence of 1 mmol/L carnitine for 3 h. A–C: Incorporation of ^{14}C -oleate was measured after 24-h incubation with 400 $\mu\text{mol/L}$ oleate in the absence of carnitine. A: Total lipid synthesis. B: TAG synthesis. C: DAG synthesis. Oxidation rates were measured during the 3-h pulse period. D: ^{14}C -oleate oxidation to CO_2 . E: ^{14}C -oleate oxidation to ASMs. F: $\text{CO}_2:\text{ASMs}$. * $P < 0.05$. Error bars represent SEM. C, controls; OA, oleate in the absence of carnitine.

leads to insulin resistance. However, studies have shown that lipid intermediates—not total IMCL per se—are the true culprits of the development of insulin resistance (38,39). Other studies have even suggested that increasing the TAG storage capacity, specifically in skeletal muscle, may be beneficial (40–42). Although these studies used different methods (i.e., genetic manipulations, exercise), they clearly demonstrated a beneficial effect of increased TAG storage on skeletal muscle insulin

sensitivity. In the current study, we investigated the oxidative and storage capacities of both skeletal muscle tissue and myotubes established from satellite cells of obese individuals with T2D and BMI-matched nondiabetic controls. We show that oxidation and incorporation of exogenously supplied long-chain FAs (palmitate) into TAG is significantly blunted in muscle tissue from individuals with T2D. Of importance, we demonstrate that this blunted FA oxidative capacity is not retained

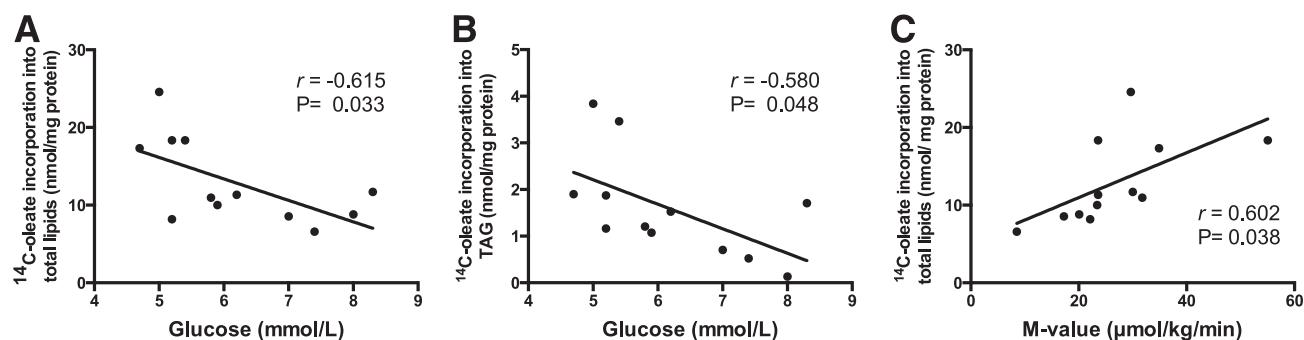


Figure 5—Correlations between in vivo measures of glucose and insulin sensitivity and in vitro endogenous lipid incorporation. Primary myotubes were loaded with $400 \mu\text{mol/L}$ oleate for 24 h and then pulsed in the presence of 1 mmol/L carnitine for 3 h. Incorporation of ^{14}C -oleate was measured after 24-h incubation with $400 \mu\text{mol/L}$ oleate in the absence of carnitine. Basal plasma glucose levels were inversely related to total lipid synthesis (A) and TAG synthesis (B). Insulin sensitivity (M-value at a $40 \text{ mU/m}^2/\text{min}$ insulin infusion rate) was positively associated with total lipid synthesis (C).

in primary myotubes from these patients. However, myotubes of individuals with T2D show reduced incorporation of exogenous palmitate into TAG, and this is significantly related to the impaired palmitate incorporation into TAG observed 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 individuals with T2D. Because the absence of carnitine prevents FA oxidation, this measurement mainly reflects

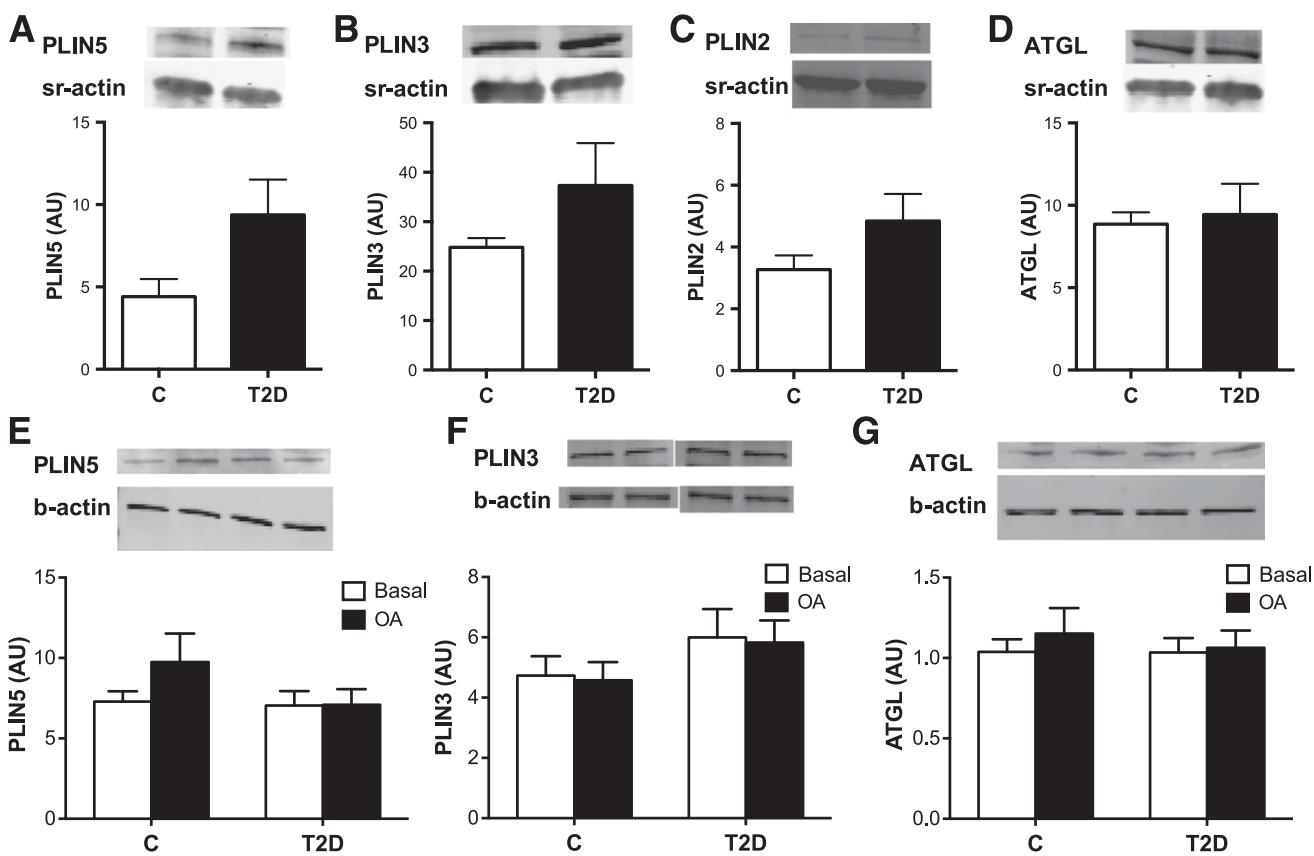


Figure 6—Ex vivo and in vitro protein expressions. Lipid droplet coating proteins PLIN5 (A), PLIN3 (B), and PLIN2 (C) as well as the lipolytic protein ATGL (D) were measured in the muscle tissues of the control and T2D individuals. Primary myotubes were loaded with $400 \mu\text{mol/L}$ oleate for 24 h and then harvested for protein. E–G: Protein expressions were measured before (Basal) and after the 24-h incubation with $400 \mu\text{mol/L}$ oleate in the absence of carnitine (OA). E: PLIN5. F: PLIN3. G: ATGL. Sr-actin was used as an internal control for all Western blots. Error bars represent SEM. C, controls.

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.

We show a blunted complete oxidation of palmitate in the skeletal muscle tissue of obese individuals with T2D compared with BMI-matched controls and young, lean subjects. Because there were no differences in the ASMs, the ratio of complete to incomplete oxidation was also reduced in individuals with T2D. These findings extend those of Hulver et al. (43), who demonstrated aberrant exogenous FA oxidation in the skeletal muscle of obese and severely obese ($\text{BMI } 53.8 \pm 3.5 \text{ kg/m}^2$) individuals compared with lean control subjects. In this context, the current data imply that T2D is associated with reduced tissue FA oxidation independent of BMI. However, we did not observe significant differences in oxidation of exogenous palmitate to CO_2 and ASMs between myotubes of obese individuals with T2D and BMI-matched controls. We then examined whether 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. These findings contrast those of Gaster (27), who demonstrated that complete oxidation of endogenous, but not exogenous, FAs was reduced in T2D myotubes compared with those from obese control subjects. One major difference between the current study and Gaster is that we included subjects who were marginally obese (although all had a $\text{BMI} > 30 \text{ kg/m}^2$), resulting in a lower average BMI than that in Gaster. Another potential explanation is that these two studies compared distinct subgroups of the obese population that may have been exposed to different environmental factors, which could uniquely affect a varied genetic or epigenetic background. The current data suggest that a reduced myocellular fat oxidative capacity in individuals with T2D may not be an intrinsic characteristic but, rather, a consequence of environment.

One bout of exercise has been shown to improve the capacity to store TAG in muscle and, thereby, prevent 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 athletes compared with sedentary control subjects. In the current study, 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 toward 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 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 toward 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 insulin sensitivity.

The reason for the retained aberrant lipid incorporation capacity in T2D is so far unknown. We investigated whether 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 (7,8) and others showing increased FA uptake in skeletal muscle of obese and T2D individuals (9). In the current study, 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 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) (45) 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 or in enzymatic activity of DGAT between T2D and obese controls. However, we did observe significantly positive associations of PGC1 α 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 PGC1 α in the regulation of intramuscular lipid droplet programming in mice and humans (23). 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 have previously shown in animal studies that overexpressing PLIN2 or PLIN5 increases TAG storage capacity and results in the prevention of high-fat-induced insulin resistance (40,46). In the current study, we found that PLIN5 was elevated in the muscle tissue of the T2D individuals but not in their myotubes. This finding 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 myotubes derived from T2D individuals. These data are consistent with the increased neutral lipid and TAG storage observed in 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 (postoleate load) in myotubes were positively related to insulin sensitivity. In the context of 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 together, future studies should investigate the intrinsic pathways in muscle lipid turnover (and its associated proteins) to identify the critical regulation sites.

In summary, the data show that skeletal muscle tissue lipid oxidation and FA incorporation into TAG are perturbed in obese individuals with T2D compared with BMI-matched controls but that only the disturbances in TAG incorporation are conserved in cultured myotubes from these individuals. The 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 use precision medicine to treat, manage, and prevent T2D in the future.

Acknowledgments. The authors thank the study participants; Denis Dahlmans and Lucas Lindeboom of the Department of Human Biology, Maastricht University, for assistance with the *in vivo* measurements; and Anne Gemmink of the Department of Human Movement Sciences, Maastricht University, for assistance with the *ex vivo* measurements.

Funding. M.B. is financially supported by the NUTRIM—School for Nutrition, Toxicology and Metabolism and the Graduate School VLAG. This work received support from Austrian Science Fund grant P25193 (to A.L.). This work also was partially funded by a Vici grant (918.96.618) for innovative research from the Netherlands Organisation for Scientific Research (to P.S.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. L.M.S. and M.B. researched data, contributed to the study concept and design and data analysis and interpretation, and wrote the manuscript. B.B., T.v.d.W., and L.B. researched data and reviewed and edited the manuscript. G.S., E.M.-K., T.O.E., and A.L. researched data. M.K.C.H. and P.S. contributed to the study concept and design and data analysis and interpretation and reviewed and edited the manuscript. P.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in poster form at the 73rd Scientific Sessions of the American Diabetes Association, Chicago, IL, 21–25 June 2013.

References

- McGarry JD. What if Minkowski had been ageusic? An alternative angle on diabetes. *Science* 1992;258:766–770
- Kelley DE, Goodpaster B, Wing RR, Simoneau JA. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* 1999;277:E1130–E1141
- Morino K, Petersen KF, Shulman GI. Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. *Diabetes* 2006;55(Suppl. 2):S9–S15
- Bosma M, Kersten S, Hesselink MKC, Schrauwen P. Re-evaluating lipotoxic triggers in skeletal muscle: relating intramyocellular lipid metabolism to insulin sensitivity. *Prog Lipid Res* 2012;51:36–49
- Hulver MW, Berggren JR, Carper MJ, et al. Elevated stearoyl-CoA desaturase-1 expression in skeletal muscle contributes to abnormal fatty acid partitioning in obese humans. *Cell Metab* 2005;2:251–261
- Aguer C, Mercier J, Man CY, et al. Intramyocellular lipid accumulation is associated with permanent relocation *ex vivo* and *in vitro* of fatty acid translocase (FAT)/CD36 in obese patients. *Diabetologia* 2010;53:1151–1163
- Bruce CR, Anderson MJ, Carey AL, et al. Muscle oxidative capacity is a better predictor of insulin sensitivity than lipid status. *J Clin Endocrinol Metab* 2003;88:5444–5451
- Pelsers MM, Tsintzas K, Boon H, et al. Skeletal muscle fatty acid transporter protein expression in type 2 diabetes patients compared with overweight, sedentary men and age-matched, endurance-trained cyclists. *Acta Physiol (Oxf)* 2007;190:209–219
- Bonen A, Parolin ML, Steinberg GR, et al. Triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal FAT/CD36. *FASEB J* 2004;18:1144–1146
- Aguer C, Foretz M, Lantier L, et al. Increased FAT/CD36 cycling and lipid accumulation in myotubes derived from obese type 2 diabetic patients. *PLoS One* 2011;6:e28981
- Gaster M, Rustan AC, Aas V, Beck-Nielsen H. Reduced lipid oxidation in skeletal muscle from type 2 diabetic subjects may be of genetic origin: evidence from cultured myotubes. *Diabetes* 2004;53:542–548
- Kuijpers H, Verstappen FTJ, Keizer HA, Geurten P, van Kranenburg G. Variability of aerobic performance in the laboratory and its physiologic correlates. *Int J Sports Med* 1985;6:197–201
- Siri WE. The gross composition of the body. *Adv Biol Med Phys* 1956;4:239–280
- DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 1979;237:E214–E223
- Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* 1975;35:609–616
- Sparks LM, Moro C, Ukporevbo B, et al. Remodeling lipid metabolism and improving insulin responsiveness in human primary myotubes. *PLoS One* 2011;6:e21068
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156–159
- Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;25:169–193
- Sparks LM, Xie H, Koza RA, et al. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. *Diabetes* 2005;54:1926–1933
- Koopman R, Schaart G, Hesselink MK. Optimisation of Oil Red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol* 2001;116:63–68
- Kim J-Y, Koves TR, Yu G-S, et al. Evidence of a malonyl-CoA-insensitive carnitine palmitoyltransferase I activity in red skeletal muscle. *Am J Physiol Endocrinol Metab* 2002;282:E1014–E1022
- Sparks LM, Johannsen NM, Church TS, et al. Nine months of combined training improves *ex vivo* skeletal muscle metabolism in individuals with type 2 diabetes. *J Clin Endocrinol Metab* 2013;98:1694–1702
- Koves TR, Sparks LM, Kovalik JP, et al. PPAR γ coactivator-1 α contributes to exercise-induced regulation of intramuscular lipid droplet programming in mice and humans. *J Lipid Res* 2013;54:522–534
- Schwartz DM, Wolins NE. A simple and rapid method to assay triacylglycerol in cells and tissues. *J Lipid Res* 2007;48:2514–2520

25. Eichmann TO, Kumari M, Haas JT, et al. Studies on the substrate and stereo/regioselectivity of adipose triglyceride lipase, hormone-sensitive lipase, and diacylglycerol-O-acyltransferases. *J Biol Chem* 2012;287:41446–41457
26. Stone SJ, Levin MC, Zhou P, Han J, Walther TC, Farese RV Jr. The endoplasmic reticulum enzyme DGAT2 is found in mitochondria-associated membranes and has a mitochondrial targeting signal that promotes its association with mitochondria. *J Biol Chem* 2009;284:5352–5361
27. Gaster M. Reduced lipid oxidation in myotubes established from obese and type 2 diabetic subjects. *Biochem Biophys Res Commun* 2009;382:766–770
28. Watt MJ, Hoy AJ, Muoio DM, Coleman RA. Distinct roles of specific fatty acids in cellular processes: implications for interpreting and reporting experiments. *Am J Physiol Endocrinol Metab* 2012;302:E1–E3
29. Aas V, Rokling-Andersen M, Wensaas AJ, Thoresen GH, Kase ET, Rustan AC. Lipid metabolism in human skeletal muscle cells: effects of palmitate and chronic hyperglycaemia. *Acta Physiol Scand* 2005;183:31–41
30. Chen YD, Golay A, Swislocki ALM, Reaven GM. Resistance to insulin suppression of plasma free fatty acid concentrations and insulin stimulation of glucose uptake in noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 1987;64:17–21
31. Falholt K, Jensen I, Lindkaer Jensen S, et al. Carbohydrate and lipid metabolism of skeletal muscle in type 2 diabetic patients. *Diabet Med* 1988;5:27–31
32. Reaven GM. Banting Lecture 1988. Role of insulin resistance in human disease. *Diabetes* 1988;37:1595–1607
33. Reaven GM, Hollenbeck C, Jeng CY, Wu MS, Chen YD. Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes* 1988;37:1020–1024
34. Krssak M, Falk Petersen K, Dresner A, et al. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a ¹H NMR spectroscopy study. *Diabetologia* 1999;42:113–116
35. Moro C, Galgani JE, Luu L, et al. Influence of gender, obesity, and muscle lipase activity on intramyocellular lipids in sedentary individuals. *J Clin Endocrinol Metab* 2009;94:3440–3447
36. Pan DA, Lillioja S, Kriketos AD, et al. Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* 1997;46:983–988
37. Perseghin G, Scifo P, De Cobelli F, et al. Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a ¹H-¹³C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* 1999;48:1600–1606
38. Amati F, Dubé JJ, Alvarez-Carnero E, et al. Skeletal muscle triglycerides, diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained athletes? *Diabetes* 2011;60:2588–2597
39. Kovacs TR, Ussher JR, Noland RC, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* 2008;7:45–56
40. Bosma M, Hesselink MKC, Sparks LM, et al. Perilipin 2 improves insulin sensitivity in skeletal muscle despite elevated intramuscular lipid levels. *Diabetes* 2012;61:2679–2690
41. Liu L, Shi X, Choi CS, et al. Paradoxical coupling of triglyceride synthesis and fatty acid oxidation in skeletal muscle overexpressing DGAT1. *Diabetes* 2009;58:2516–2524
42. Schenk S, Horowitz JF. Acute exercise increases triglyceride synthesis in skeletal muscle and prevents fatty acid-induced insulin resistance. *J Clin Invest* 2007;117:1690–1698
43. Hulver MW, Berggren JR, Cortright RN, et al. Skeletal muscle lipid metabolism with obesity. *Am J Physiol Endocrinol Metab* 2003;284:E741–E747
44. Bergman BC, Perreault L, Hunerdosse DM, Koehler MC, Samek AM, Eckel RH. Increased intramuscular lipid synthesis and low saturation relate to insulin sensitivity in endurance-trained athletes. *J Appl Physiol (1985)* 2010;108:1134–1141
45. Boon J, Hoy AJ, Stark R, et al. Ceramides contained in LDL are elevated in type 2 diabetes and promote inflammation and skeletal muscle insulin resistance. *Diabetes* 2013;62:401–410
46. Bosma M, Sparks LM, Hooiveld GJ, et al. Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression and intramyocellular lipid content without compromising insulin sensitivity. *Biochimica Biophys Acta* 2013;1831:844–852