

# Fatty Acid Incubation of Myotubes From Humans With Type 2 Diabetes Leads to Enhanced Release of $\beta$ -Oxidation Products Because of Impaired Fatty Acid Oxidation

## Effects of Tetradecylthioacetic Acid and Eicosapentaenoic Acid

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**OBJECTIVE**—Increased availability of fatty acids is important for accumulation of intracellular lipids and development of insulin resistance in human myotubes. It is unknown whether different types of fatty acids like eicosapentaenoic acid (EPA) or tetradecylthioacetic acid (TTA) influence these processes.

**RESEARCH DESIGN AND METHODS**—We examined fatty acid and glucose metabolism and gene expression in cultured human skeletal muscle cells from control and type 2 diabetic individuals after 4 days of preincubation with EPA or TTA.

**RESULTS**—Type 2 diabetes myotubes exhibited reduced formation of CO<sub>2</sub> from palmitic acid (PA), whereas release of  $\beta$ -oxidation products was unchanged at baseline but significantly increased with respect to control myotubes after preincubation with TTA and EPA. Preincubation with TTA enhanced both complete (CO<sub>2</sub>) and  $\beta$ -oxidation of palmitic acid, whereas EPA increased only  $\beta$ -oxidation significantly. EPA markedly enhanced triacylglycerol (TAG) accumulation in myotubes, more pronounced in type 2 diabetes cells. TAG accumulation and fatty acid oxidation were inversely correlated only after EPA preincubation, and total level of acyl-CoA was reduced. Glucose oxidation (CO<sub>2</sub> formation) was enhanced and lactate production decreased after chronic exposure to EPA and TTA, whereas glucose uptake and storage were unchanged. EPA and especially TTA increased the expression of genes involved in fatty acid uptake, activation, accumulation, and oxidation.

**CONCLUSIONS**—Our results suggest that 1) mitochondrial dysfunction in diabetic myotubes is caused by disturbances downstream of fatty acid  $\beta$ -oxidation; 2) EPA promoted accumulation of TAG, enhanced  $\beta$ -oxidation, and increased glucose

oxidation; and 3) TTA improved complete palmitic acid oxidation in diabetic myotubes, opposed increased lipid accumulation, and increased glucose oxidation. *Diabetes* 58:527–535, 2009

**T**ype 2 diabetes is characterized by hyperglycemia, reduced ability to oxidize fat, and accumulation of triacylglycerol (TAG) in skeletal muscle fibers. The increased deposition of intramyocellular TAG (imTAG) has received special interest, because several studies have demonstrated a positive association between insulin resistance and imTAG storage (1,2). Accumulation of imTAG depends on the availability and uptake of fatty acids, the rate of fatty acid oxidation, and the rate of synthesis and hydrolysis of TAG. Increased availability of plasma free fatty acid (FFA) during lipid infusion or high-fat feeding is associated with development of insulin resistance and accumulation of imTAG in vivo (3). Moreover, studies have shown impaired capacity for fatty acid oxidation in skeletal muscle from insulin-resistant/type 2 diabetic individuals (4,5), and reduced mitochondrial fatty acid oxidation in skeletal muscle and myotubes is associated with increased deposition of imTAG (6–8). Fatty acids may promote insulin resistance via intracellular intermediates such as acyl-CoA, diacylglycerol (DAG), and ceramides, interfering with insulin signaling and glucose metabolism (9).

Previous studies have demonstrated positive effects on skeletal muscle insulin sensitivity of mono- and polyunsaturated fatty acids (PUFAs) compared with saturated fatty acids (10–12). Very long-chain  $\omega$ -3 fatty acids, including eicosapentaenoic acid (EPA), may protect against skeletal muscle insulin resistance caused by high-fat feeding in vivo (1,13). PUFAs may also promote increased TAG accumulation without impairing insulin-stimulated glucose uptake in myotubes (10,11). The sulfur-substituted fatty acid analog tetradecylthioacetic acid (TTA) is a pan-peroxisome proliferator-activated receptor (pan-PPAR) activator that reduces plasma lipids and enhances hepatic fatty acid oxidation in rodents (14). Dual and pan-PPAR agonists are currently being developed for treatment of type 2 diabetes (15), and TTA has been shown to improve glucose metabolism in insulin-resistant rats (16) and to stimulate mitochondrial proliferation in rat

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skeletal muscle (17). We have recently demonstrated that TTA may increase fatty acid oxidation in human myotubes similar to the PPAR $\delta$ -specific agonist GW501516 (18).

Skeletal muscle metabolism is influenced by physical activity, hormonal status, and muscle fiber type, rendering it difficult to determine the impact of EPA and TTA on basal and insulin-stimulated intermediary metabolism. Cultured human myotubes display the morphological, metabolic, and biochemical properties of adult skeletal muscle (19) and offer a unique model to distinguish between genetic and environmental factors in the etiology of insulin resistance (20). We and others have reported several potential intrinsic deficiencies in myotubes from individuals with type 2 diabetes, including lower basal palmitate oxidation (21) and impaired insulin-stimulated glucose metabolism (20,22). It is unknown whether EPA or TTA may improve insulin resistance or other characteristics of type 2 diabetes, such as decreased lipid oxidation in myotubes.

To identify the potential effects of EPA and TTA on the intermediary energy metabolism and insulin resistance, we compared the effect of TTA, EPA, and oleic acid in myotubes established from obese individuals with type 2 diabetes and obese healthy subjects.

## RESEARCH DESIGN AND METHODS

Dulbecco's modified Eagle's medium (DMEM)-Glutamax, FCS, Ultrosor G, penicillin-streptomycin-amphotericin B, and trypsin-EDTA were obtained from Gibco, Invitrogen (Paisley, U.K.). 2-[ $^3$ H(G)]deoxy-D-glucose (6.00 Ci/mmol) and D-[ $^{14}$ C(U)]glucose (5 mCi/mmol) were purchased from PerkinElmer, NEN (Boston, MA). [ $^{14}$ C]acetate (55 mCi/mmol), [ $^{14}$ C]PA (55 mCi/mmol), and D-[ $^{14}$ C]glucose (55 mCi/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). Palmitic acid, oleic acid, BSA (essentially fatty acid free), cytochalasin B, and extracellular matrix gel were purchased from Sigma-Aldrich (St. Louis, MO). Insulin Actrapid was from Novo-Nordisk (Bagsvaerd, Denmark). Agilent Total RNA Isolation Mini kit was purchased from Matriks (Oslo). The primers, SYBR Green and TaqMan reverse-transcription reagents kit were obtained from Applied Biosystems (Warrington, U.K.), and the protein assay kit was purchased from BioRad (Copenhagen). EPA was purchased from NU-Chek-Prep, Ida kjemi (Ås, Norway). TTA and [ $^{14}$ C]TTA were provided by R.K.B.

**Biopsies.** Skeletal muscle biopsies were obtained from eight obese type 2 diabetic patients ( $49.2 \pm 1.8$  years) and eight obese control subjects ( $50.3 \pm 1.8$  years) taken from musculus vastus lateralis in the fasted state by needle biopsy under local anesthesia. Only sedentary subjects were recruited. Diabetic individuals were treated either with diet alone or in combination with sulfonylurea, metformin, or insulin, which was withdrawn 1 week before taking the biopsies. The patients had no diabetic complications apart from simple retinopathy. The control subjects had normal glucose tolerance and no family history of type 2 diabetes. The groups were matched with respect to age and BMI but differed by fasting plasma glucose concentrations ( $5.5 \pm 0.2$  vs.  $10.4 \pm 0.7$  mmol/l,  $P < 0.001$ ,  $t$  test), fasting serum insulin levels ( $50.5 \pm 5.6$  vs.  $96.1 \pm 12.5$  pmol/l,  $P < 0.05$ ), glucose disappearance rate during euglycemic-hyperinsulinemic clamp ( $280.0 \pm 34.8$  vs.  $121.1 \pm 23.2$  mg  $\cdot$  min $^{-1}$   $\cdot$  m $^{-2}$ ,  $P < 0.001$ ), and A1C ( $5.4 \pm 0.1$  vs.  $7.6 \pm 0.6\%$ ,  $P < 0.05$ ). All subjects gave written informed consent, and the local ethics committee of Funen and Vejle County approved the study.

**Cell culture and fatty acid incubation.** Myotubes were established from satellite cells (19). Human myoblasts from control and type 2 diabetic subjects were differentiated to myotubes at a physiological concentration of insulin (25 pmol/l) and glucose (5.5 mmol/l) for 4 days. Myotubes were then exposed to BSA (40  $\mu$ mol/l), oleic acid (100  $\mu$ mol/l), TTA (100  $\mu$ mol/l), or EPA (100  $\mu$ mol/l) for another 4 days. Stock solutions of fatty acid sodium salts (6 mmol/l) and BSA (2.4 mmol/l) were heated to 45°C and rapidly mixed (molar ratio of 2.5:1). Only optically clear solutions were used. There were no differences between the myotubes after preincubation with fatty acids as evaluated by microscopic inspection searching for floating cells or cells with lipid droplets. The myotubes had similar protein content independent of incubation medium.

### Palmitic acid uptake and distribution of radiolabel in lipids

**Palmitic acid uptake.** Myotubes were incubated in DMEM supplemented with 0.24 mmol/l BSA, 0.5 mmol/l L-carnitine, 20 mmol/l HEPES, [ $^{14}$ C]PA (0.5

$\mu$ Ci/ml, 0.6 mmol/l), and 25 pmol/l or 1.0  $\mu$ mol/l insulin for 4 h, to study basal and insulin-mediated uptake and lipid distribution of palmitate.

**De novo lipid synthesis.** Myotubes preincubated with fatty acids were further incubated 4 h in medium containing [ $^{14}$ C]acetate (3–4  $\mu$ Ci/ml, 100  $\mu$ mol/l). After incubation, myotubes were placed on ice, washed three times with 1 ml PBS, scraped twice with 250  $\mu$ l distilled water, and homogenized. The cell homogenates were assayed for protein and extracted for lipids, and the radiolabeled lipids were separated by thin-layer chromatography (21) and quantified by liquid scintillation. Total lipid uptake is the sum of oxidation and storage products.

### Measures of fatty acid oxidation

**Acid-soluble metabolites.** Myotubes were incubated in DMEM supplemented with 0.24 mmol/l BSA, 0.5 mmol/l L-carnitine, [ $^{14}$ C]PA (0.5  $\mu$ Ci/ml, 0.6 mmol/l), and 25 pmol/l or 1.0  $\mu$ mol/l insulin for 4 h to study cellular release of excess  $^{14}$ C-palmitic acid-derived acid-soluble metabolites (ASMs) to the media. The incubation media were transferred to new tubes and assayed for labeled ASMs, which mainly are byproducts of  $\beta$ -oxidation remaining in solution after precipitation of the radiolabeled fatty acid with perchloric acid (PCA) (23).

**CO $_2$  and total oxidation.** Myotubes were incubated in DMEM supplemented with 0.24 mmol/l BSA, 0.5 mmol/l L-carnitine, 20 mmol/l HEPES, [ $^{14}$ C]PA (0.5  $\mu$ Ci/ml, 0.6 mmol/l), and 25 pmol/l insulin for 4 h to study CO $_2$  formation (21). PCA was added to the cells to measure both intra- and extracellular (total) ASMs from palmitic acid. Thus, total palmitic acid oxidation is the sum of CO $_2$  and total ASMs.

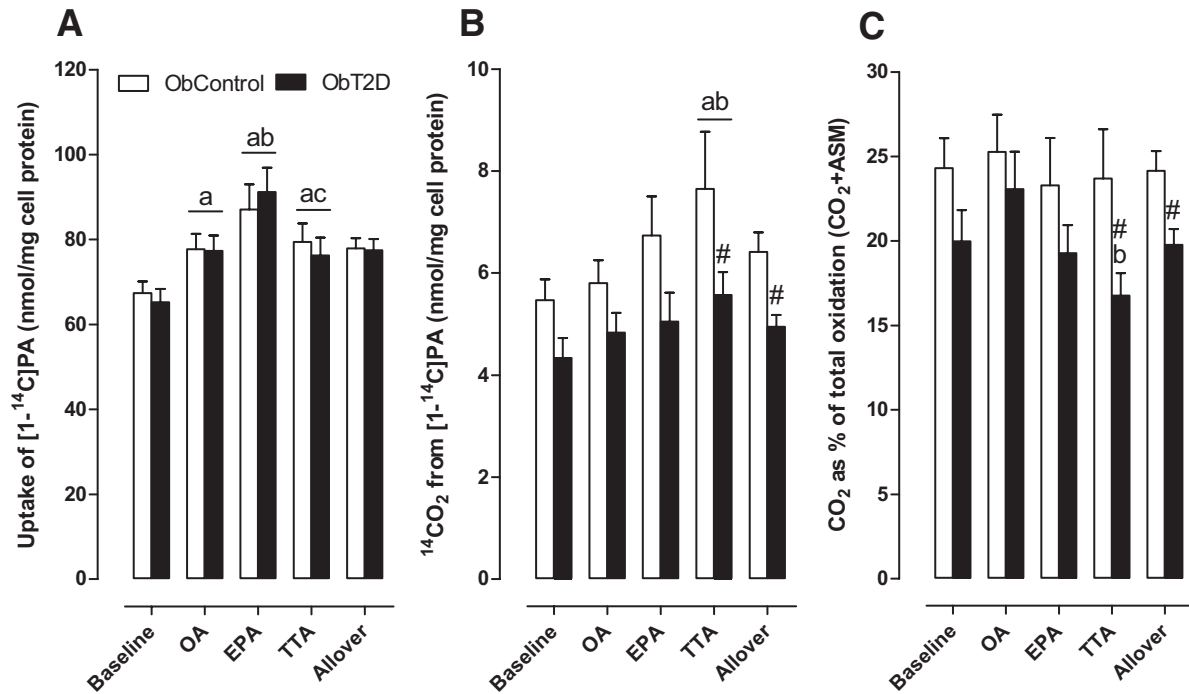
**Glucose metabolism.** Glucose uptake, glycogen synthesis, and glucose oxidation were determined as previously described (7).

**RNA isolation and real-time RT-PCR.** Myotubes were washed and centrifuged to a pellet before total RNA was isolated by Total RNA Isolation Mini kit (Agilent), according to the supplier protocol. Total RNA (0.05  $\mu$ g/ $\mu$ l) was reversely transcribed with a TaqMan reverse-transcription reagents kit (7). Quantitative RT-PCR was performed using an ABI PRISM 7000 Detection System. DNA expression was determined by SYBR Green (7), and primers for uncoupling protein 2 (*UCP2*), fatty acid translocase (*CD36/FAT*), carnitine palmitoyl transferase (*CPT1a*, *CPT2*), fatty acid binding protein 3 (*FABP3*), long-chain acyl-coenzyme A synthetase 1 (*ACSL1*), *ACSL3*, mitochondrial trifunctional protein  $\beta$ -subunit (*HADHB*), acyl-coenzyme A dehydrogenase medium-chain (*ACADM*), *DGAT1*, *DGAT2*, hexokinase II (*HKII*), *GLUT1*, *GLUT4*, actin- $\beta$  (*ACTB*), and ribosomal protein, large, P0 (*RPLP0*) (supplemental Table 1, available in an online appendix at <http://dx.doi.org/10.2337/db08-1043>) were designed using Primer Express (Applied Biosystems). In addition, expression of *CPT1b*, citrate synthase (*CS*), malate dehydrogenase 2 (*MDH2*), *ACC2*, *UCP3*, stearoyl-coenzyme A desaturase 1 (*SCD1*), *PGC1a*, *ACTB*, and *RPLP0* (supplemental Table 1) were analyzed with the inventoried TaqMan gene expression assays of Applied Biosystems on a 7900HT Fast Real-Time PCR system with 96-well block module following standard protocols. The transcription levels were normalized to *ACTB* and *RPLP0*.

**Analysis of acyl-CoA fractions.** Cells were harvested by centrifugation and washed twice with 10 mmol/l PCA and stored at  $-80^\circ\text{C}$  until long-chain fatty acyl-CoA (LCFA-CoA) extraction. Heptadecanoyl-CoA (33 pmol) and H $_2$ O were added to a final volume of 800  $\mu$ l before addition of 3 ml chloroform:methanol (2:1). Cells were then homogenized with an Ultraturax homogenizer. Chloroform (1 ml) and H $_2$ O (1 ml) were added and vortexed vigorously. After centrifugation (3,000 rpm/10 min/4°C), the interphase was recovered and dried under a stream of N $_2$ . The LCFA-CoAs were further extracted and derivatized to fluorescent acyl etheno-CoA esters using 0.5 mol/l chloroacetaldehyde, 0.5% SDS, and 0.15 mol/l citrate, pH 4 (200  $\mu$ l), and then separated and analyzed by reverse-phase chromatography (24). Peak areas were integrated using Chromeleon version 6.50 (Dionex) and quantified relative to the internal heptadecanoyl-CoA standard. Identification of individual LCFA-CoA was performed using standard LCFA-CoA mixtures. The detection level of our methods was 0.5 pmol/l.

**Scintillation proximity assay.** We applied this method because it provides accurate and reproducible cell data with limited use of resources for quite extensive analyses (25). In short, radiolabeled substrates taken up and accumulated by adherent cells are concentrated close to the scintillator embedded in the plastic bottom of each well and thus provide a stronger signal than the radiolabel dissolved in the medium alone (25). Myotubes were preincubated with 100  $\mu$ mol/l oleic acid for 4 days with addition of 100  $\mu$ mol/l TTA the last 24 h. Then myotubes were washed with PBS, medium containing [ $^{14}$ C]PA (1  $\mu$ Ci/ml, 100  $\mu$ mol/l) was added, and myotubes were incubated up to 4 h. Fatty acid was bound to BSA at a ratio of 2.5:1.

**Statistical analysis.** The effect of group and incubations was evaluated by linear mixed models analysis in SPSS (v12.0.1), monitoring group and treatment effects based on all available data from each experiment and taking into account the correlation between measurements/myotubes from the same subjects. Some acyl-CoA values were below detection limit and were assigned



**FIG. 1.** Palmitate uptake and oxidation. Differentiated myotubes from type 2 diabetic or control subjects were preincubated with 40  $\mu\text{mol/l}$  BSA, 100  $\mu\text{mol/l}$  oleic acid, 100  $\mu\text{mol/l}$  TTA, or 100  $\mu\text{mol/l}$  EPA for 4 days and incubated with 0.6  $\text{mmol/l}$   $[1-^{14}\text{C}]\text{PA}$  for 4 h. The panels show palmitate uptake, which is the sum of total recovered  $\text{CO}_2$ , ASMs, and total lipids (A); oxidation to  $\text{CO}_2$  (B);  $\text{CO}_2$  from palmitic acid as percentage of total oxidation (C). Results present means  $\pm$  SE ( $n = 8$ ). a,  $P \leq 0.05$  vs. baseline; b,  $P \leq 0.05$  vs. oleic acid; c,  $P \leq 0.05$  vs. EPA; # $P \leq 0.05$  vs. ObControl, analyzed by linear mixed models statistics (SPSS), which also calculated the allover results based on all available data. T2D, type 2 diabetes.

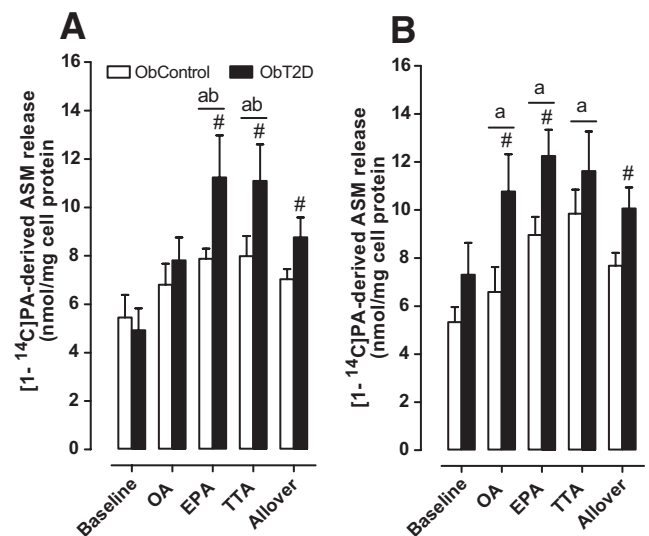
the value 0.5  $\text{pmol/l}$  in accordance to the detection level. Nonparametric statistical analyses (Mann-Whitney and Wilcoxon's signed-rank test) were performed on the acyl-CoA data for evaluation of group and treatment differences, respectively. A  $P$  value  $\leq 0.05$  was considered significant.

## RESULTS

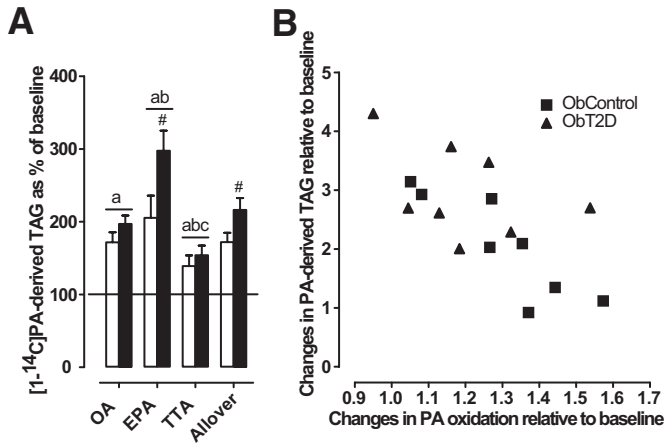
**Effect of fatty acids on uptake and oxidation of fatty acids.** We observed enhanced uptake of  $^{14}\text{C}$ -palmitic acid in cultured myotubes from controls and type 2 diabetic subjects after 4 days of preincubation with oleic acid, EPA, or TTA (Fig. 1A), showing that fatty acid availability is important for cellular lipid uptake. By adding 1  $\mu\text{mol/l}$  insulin acutely,  $^{14}\text{C}$ -palmitic acid accumulation was further enhanced by 15–30% (data not shown;  $P < 0.05$ ). There was an allover significant 20% reduction in  $^{14}\text{C}$ -palmitic acid-derived  $\text{CO}_2$  released from type 2 diabetes vs. control myotubes (Fig. 1B), indicating that type 2 diabetes cells exhibit reduced complete palmitic acid oxidation. However, after incubation with TTA, control and type 2 diabetes myotubes showed enhanced  $^{14}\text{CO}_2$  production from  $^{14}\text{C}$ -palmitic acid compared with baseline or preincubation with oleic acid (Fig. 1B), suggesting that TTA may improve mitochondrial fatty acid oxidation. The percentage of palmitic acid that was oxidized completely to  $\text{CO}_2$  compared with total oxidation [sum of complete ( $\text{CO}_2$ ) plus incomplete fatty acid oxidation (ASMs)] was lower in type 2 diabetes myotubes, especially after preincubation with TTA (Fig. 1C). Preincubation with EPA and TTA enhanced  $^{14}\text{C}$ -palmitic acid-derived ASMs recovered in the media, and the response was significantly stronger in type 2 diabetes myotubes (EPA 125% and TTA 128%) compared with control myotubes (EPA 45% and TTA 47%; Fig. 2A). In the presence of insulin, preincubation with EPA, TTA, and oleic acid enhanced ASM release, with a stronger allover response in type 2 diabetes compared with control myotubes (Fig. 2B). We also observed

similar effects of EPA and TTA on ASMs derived from  $^{14}\text{C}$ -oleic acid, with significantly stronger release from type 2 diabetes than control myotubes in the presence of insulin (data not shown).

**Accumulation of TAG.** Preincubation of myotubes with oleic acid and EPA enhanced  $^{14}\text{C}$ -palmitic acid accumulation into lipids compared with baseline by 23–32% and 29–53%, respectively (results not shown;  $P < 0.05$ ). The



**FIG. 2.** Palmitate-derived ASMs release to the medium. Differentiated myotubes from type 2 diabetes or control subjects were preincubated with fatty acids as described in Fig. 1 and then acutely incubated with 0.6  $\text{mmol/l}$   $[1-^{14}\text{C}]\text{PA} \pm 1 \mu\text{mol/l}$  insulin for 4 h. The panels show ASMs released from the cells, derived from  $^{14}\text{C}$ -palmitic acid without (A) and with (B) 1  $\mu\text{mol/l}$  insulin. Results represent means  $\pm$  SE ( $n = 6-7$ ). a,  $P \leq 0.05$  vs. baseline; b,  $P \leq 0.05$  vs. oleic acid; # $P \leq 0.05$  vs. ObControl, analyzed by linear mixed models statistics (SPSS), which also calculated the allover results based on all available data.



**FIG. 3.** Changes in TAG accumulation from <sup>14</sup>C-palmitic acid relative to baseline (A) and negative correlation between changes in <sup>14</sup>C-palmitic acid oxidation and TAG accumulation after preincubation with EPA (B). Differentiated myotubes from type 2 diabetic or control subjects were preincubated with fatty acids as described in Fig. 1 and then acutely incubated with [1-<sup>14</sup>C]PA (0.6 mmol/l) for 4 h. The panels show changes in <sup>14</sup>C-palmitic acid-derived TAGs as percentage of baseline (BSA) (A) and a negative correlation between changes in total <sup>14</sup>C-palmitic acid oxidation and TAG accumulation in myotubes preincubated with EPA ( $r = -0.69, P < 0.001$ ). Results represent means  $\pm$  SE ( $n = 8$ ). a,  $P \leq 0.05$  vs. baseline; b,  $P \leq 0.05$  vs. oleic acid; c,  $P \leq 0.05$  vs. EPA; #  $P \leq 0.05$  vs. ObControl, analyzed by linear mixed models statistics (SPSS), which also calculated the allover results based on all available data.

enhanced lipid accumulation might be explained by a strong increment in TAG accumulation after preincubation with oleic acid (7.4 and 7.9 nmol/mg cell protein, control, and type 2 diabetes, respectively) and EPA (10.9 and 17.5 nmol/mg cell protein, control, and type 2 diabetes, respectively) compared with baseline (results not shown;  $P < 0.05$ ). The relative increase in TAG accumulation was significantly higher in type 2 diabetes than control myotubes after preincubation with EPA (Fig. 3A). Preincubation with TTA did not increase total <sup>14</sup>C-palmitic acid lipid accumulation, although radiolabeled TAG was slightly increased compared with baseline but reduced compared with oleic acid and EPA (Fig. 3A). Similar effects of oleic acid, EPA, and TTA on lipid synthesis were observed using <sup>14</sup>C-oleic acid (data not shown). Moreover, preincubation

with EPA caused a negative correlation between relative change in total oxidation and TAG synthesis from <sup>14</sup>C-palmitic acid ( $r = -0.69, P < 0.005$ ) (Fig. 3B), but not with oleic acid or TTA (results not shown).

**Gene expression related to mitochondrial function and lipid metabolism.** There were no differences between the two groups in expression of several genes important for mitochondrial function and fatty acid metabolism (Table 1). Preincubation with fatty acids increased the expression of *CPT1- $\alpha/\beta$*  (1.5- to 2-fold) and *HADHB* (30%). However, *UCP2* and *-3* were moderately increased with respect to baseline by 43 and 24%, respectively, only after preincubation with TTA.

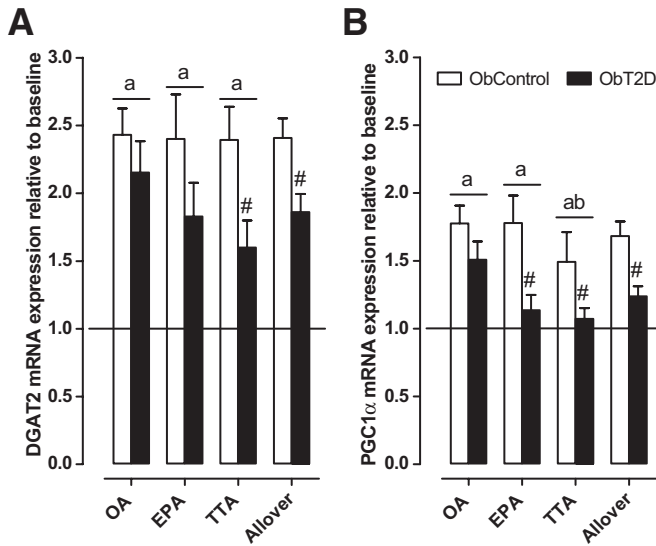
Expression of *CD36/FAT*, *ACSL*, and *FABP3* was increased after preincubation with TTA more so than oleic acid and EPA (Table 1). Preincubation with TTA also promoted a 2.5-fold induction in *SCD1* compared with the other fatty acids (Table 1). DAG acyltransferase 2 (*DGAT2*), responsible for the final step in TAG synthesis, was increased similarly by all fatty acids in control myotubes, whereas type 2 diabetes myotubes displayed a reduced response, especially during incubation with TTA (Fig. 4A). The nuclear transcription factor PPAR $\gamma$  coactivator  $\alpha$  (*PGC1 $\alpha$* ) had a similar expression pattern to *DGAT2*, with enhanced expression after fatty acid preincubation in control cells and reduced response in type 2 diabetes myotubes (Fig. 4B).

**Acyl-CoA fractions.** Total acyl-CoA was increased relative to baseline after preincubation with TTA, whereas preincubation with EPA reduced acyl-CoA by >40% in type 2 diabetes myotubes but significantly less in control cells (Fig. 5A). Preincubation with EPA reduced C18:1-CoA by an average of 61% (not shown;  $P < 0.05$ ) and C16:0-CoA by 46–55% (Fig. 5B) relative to baseline, whereas C16:1-CoA was markedly increased (Fig. 5C). EPA also substantially reduced the level of C18:2-CoA relative to baseline in type 2 diabetes myotubes, to a level significantly lower than in control myotubes (Fig. 5D). Preincubation with oleic acid reduced C16:0-CoA by 71–82% (Fig. 5B) and increased C18:1-CoA by an average of 75% (not shown;  $P < 0.05$ ) relative to baseline. TTA-CoA and C16:0-CoA had similar retention times and therefore

**TABLE 1**  
Gene expression relative to baseline

	Baseline (Ct no.)	Oleic acid	EPA	TTA
<i>CPT1<math>\alpha</math></i>	1.00 (27)	2.25 $\pm$ 0.09 <sup>a</sup>	2.24 $\pm$ 0.11 <sup>a</sup>	2.23 $\pm$ 0.11 <sup>a</sup>
<i>CPT1<math>\beta</math></i>	1.00 (33)	1.71 $\pm$ 0.10 <sup>a</sup>	1.42 $\pm$ 0.07 <sup>ab</sup>	1.78 $\pm$ 0.15 <sup>ac</sup>
<i>CPT2</i>	1.00 (30)	1.08 $\pm$ 0.03 <sup>a</sup>	1.04 $\pm$ 0.03	1.05 $\pm$ 0.03
<i>ACADM</i>	1.00 (28)	1.08 $\pm$ 0.08	1.05 $\pm$ 0.08	0.97 $\pm$ 0.06
<i>HADHB</i>	1.00 (24)	1.32 $\pm$ 0.06 <sup>a</sup>	1.38 $\pm$ 0.06 <sup>a</sup>	1.31 $\pm$ 0.06 <sup>a</sup>
<i>CS</i>	1.00 (26)	1.10 $\pm$ 0.04 <sup>a</sup>	0.99 $\pm$ 0.03 <sup>b</sup>	1.12 $\pm$ 0.04 <sup>ac</sup>
<i>MDH2</i>	1.00 (25)	1.04 $\pm$ 0.02	1.00 $\pm$ 0.02 <sup>b</sup>	1.04 $\pm$ 0.02 <sup>a</sup>
<i>UCP2</i>	1.00 (28)	1.14 $\pm$ 0.10	0.84 $\pm$ 0.06 <sup>ab</sup>	1.43 $\pm$ 0.08 <sup>abc</sup>
<i>UCP3</i>	1.00 (34)	1.11 $\pm$ 0.07	0.96 $\pm$ 0.06 <sup>b</sup>	1.24 $\pm$ 0.07 <sup>ac</sup>
<i>CD36/FAT</i>	1.00 (29)	2.05 $\pm$ 0.11 <sup>a</sup>	2.01 $\pm$ 0.10 <sup>a</sup>	4.41 $\pm$ 0.32 <sup>abc</sup>
<i>ACSL1</i>	1.00 (25)	1.25 $\pm$ 0.04 <sup>a</sup>	1.21 $\pm$ 0.05 <sup>a</sup>	1.13 $\pm$ 0.04 <sup>ab</sup>
<i>ACSL3</i>	1.00 (24)	1.51 $\pm$ 0.11 <sup>a</sup>	1.63 $\pm$ 0.09 <sup>a</sup>	2.01 $\pm$ 0.13 <sup>abc</sup>
<i>FABP3</i>	1.00 (25)	1.33 $\pm$ 0.06 <sup>a</sup>	1.56 $\pm$ 0.06 <sup>ab</sup>	2.75 $\pm$ 0.26 <sup>abc</sup>
<i>SCD1</i>	1.00 (24)	0.88 $\pm$ 0.07	1.05 $\pm$ 0.08 <sup>b</sup>	2.50 $\pm$ 0.26 <sup>abc</sup>
<i>DGAT1</i>	1.00 (34)	1.00 $\pm$ 0.13	1.29 $\pm$ 0.21	1.26 $\pm$ 0.11 <sup>a</sup>

Data are means  $\pm$  SE ( $n = 16$ ). Individual results were normalized to baseline (set to 1; average baseline Ct numbers for the investigated genes are shown in brackets, giving an impression of relative expression levels). <sup>a</sup> $P \leq 0.05$  vs. baseline; <sup>b</sup> $P \leq 0.05$  vs. oleic acid; <sup>c</sup> $P \leq 0.05$  vs. EPA analyzed by linear mixed models statistics (SPSS).



**FIG. 4.** Gene expression of *DGAT2* and *PGC1 $\alpha$* . Panels show changes in *DGAT2* (A) and *PGC1 $\alpha$*  (B) gene expression relative to baseline (set to 1) for control and type 2 diabetes myotubes. Cells were preincubated as described in RESEARCH DESIGN AND METHODS. Results represent means  $\pm$  SE ( $n = 8$ ). a,  $P \leq 0.05$  vs. baseline; b,  $P \leq 0.05$  vs. oleic acid; #  $P \leq 0.05$  vs. ObControl, analyzed by linear mixed models statistics (SPSS), which also calculated the allover results based on all available data.

appeared in the same peak on the chromatogram. The relative increase in acyl-CoA after preincubation with TTA (Fig. 5A) could be ascribed to a marked increase in the combined TTA/C16:0-CoA peak compared with C16:0-CoA at baseline (Fig. 5B). Interestingly, C20:4-CoA was decreased in type 2 diabetes compared with control myotubes after fatty acid preincubations and significantly after TTA (Fig. 5E).

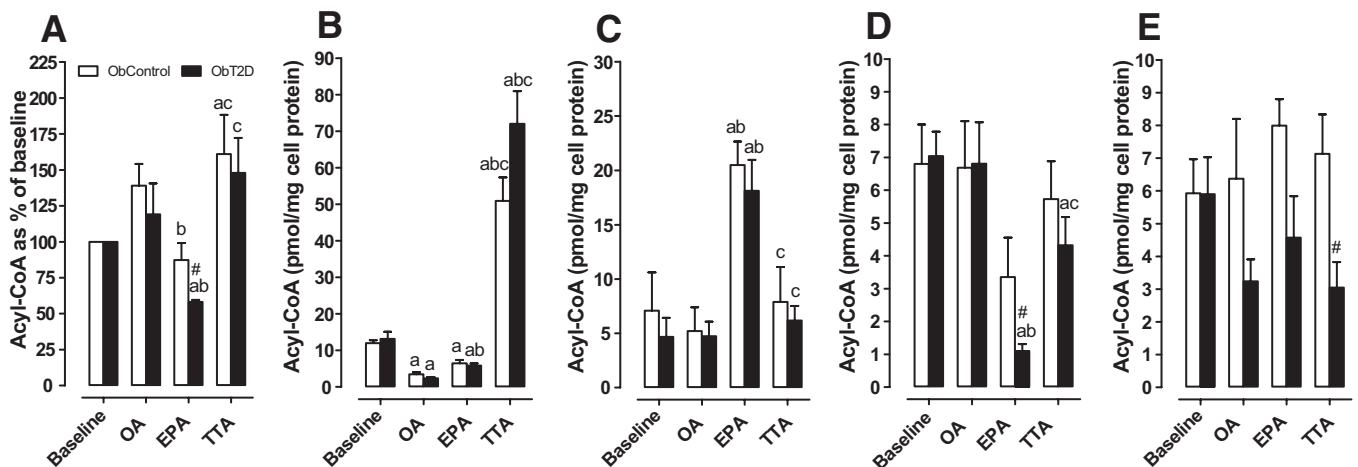
**Glucose metabolism.** Diabetic myotubes displayed an allover (basal and insulin-stimulated) 10% decreased glucose oxidation compared with control cells (results not shown;  $P < 0.05$ ). Basal and insulin-mediated glucose oxidation was enhanced after preincubation with EPA (24%) and TTA (28%) in myotubes from both groups (results not shown,  $P < 0.05$ ). Glucose uptake and glycogen synthesis, however, were mostly unaffected by preincubations with fatty acids (data not shown). Thus,

preincubation with EPA and TTA may have reduced nonoxidized glucose metabolites like lactate and pyruvate, accounting for the increased glucose oxidation. Moreover, the mRNA expression of *GLUT4*, *GLUT1*, and *HKII* were unchanged after fatty acid preincubations (results not shown).

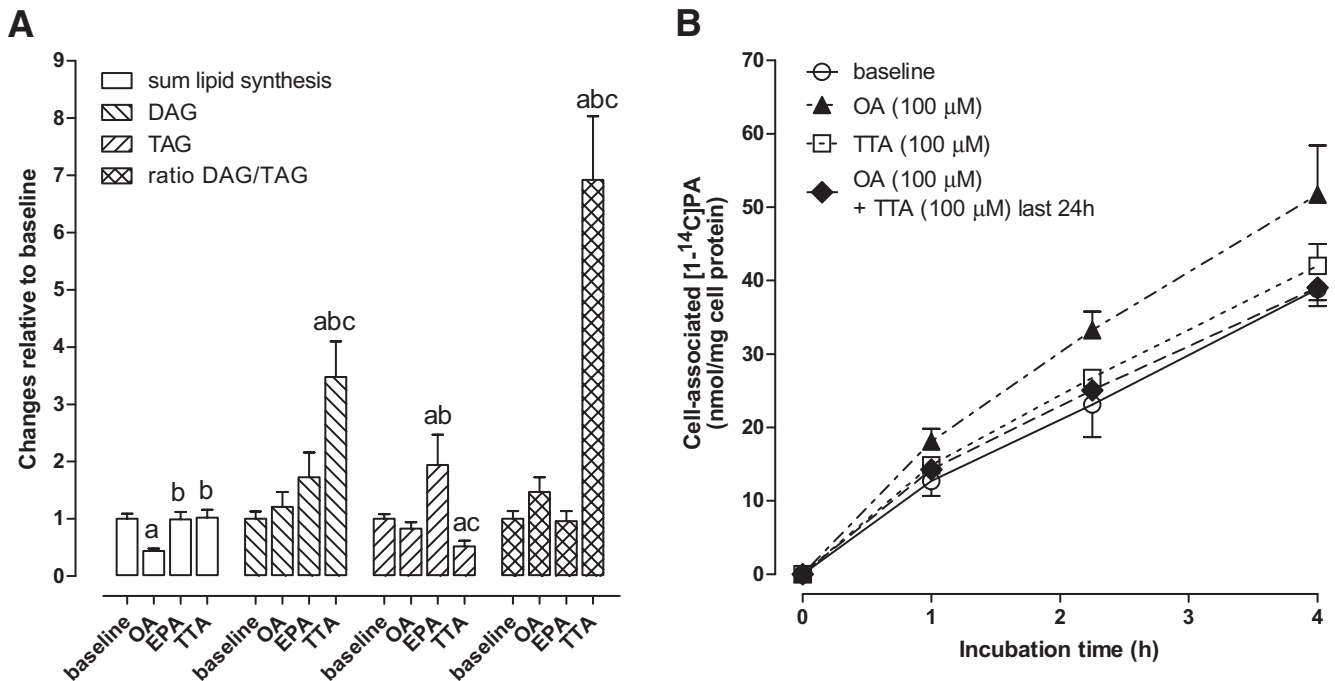
**TTA inhibits the oleic acid-mediated increase of cell-associated  $^{14}\text{C}$ -palmitic acid.** We monitored incorporation of  $^{14}\text{C}$ -TTA into complex cellular lipids and found that only a minimal amount was recovered in the TAG fraction, whereas most was found in phospholipids (not shown). De novo synthesis of fatty acids from  $^{14}\text{C}$ -acetate was similar after 4 days of preincubation of myotubes with TTA compared with baseline (Fig. 6A), but the ratio between radioactivity recovered in DAG and TAG was sevenfold higher after TTA (Fig. 6A). To further elucidate the impact of TTA on cellular fatty acid accumulation, we monitored the time course for cell-associated  $^{14}\text{C}$ -palmitic acid by scintillation proximity assay (SPA). As expected, 4 days of preincubation with oleic acid caused enhanced cell-associated  $^{14}\text{C}$ -palmitic acid (Fig. 6B). TTA itself caused no change compared with baseline, and addition of TTA for the last 24 h of the oleic acid preincubation period was sufficient to inhibit the oleic acid-mediated increase in cell-associated  $^{14}\text{C}$ -palmitic acid (Fig. 6B). Together, these data suggest that TTA or TTA-CoA may reduce fatty acid-stimulated TAG synthesis, possibly by inhibiting DGAT activity as also suggested previously in rat livers (26).

## DISCUSSION

Cultured human myotubes is the most similar cell system to intact skeletal muscle that can be modulated ex vivo. Compared with rodent models, they express the right genetic background and the specific skeletal muscle phenotype. The extracellular environment can be controlled precisely and kept relatively constant over time, without interference by systemic homeostatic compensatory mechanisms. We and others have reported several potential intrinsic deficiencies in myotubes from individuals with type 2 diabetes (20–22). These differences appear most obvious when comparing myotubes established from lean with obese type 2 diabetic subjects but are not always



**FIG. 5.** Cellular acyl-CoA relative to baseline. Differentiated myotubes were preincubated as described in Fig. 1 before the cells were harvested for measurement of acyl-CoA. A: shows total acyl-CoA relative to baseline; and B–E: display C16:0-CoA (and TTA-CoA where preincubated with TTA), C16:1-CoA, C18:2-CoA, and C20:4-CoA, respectively. Results represent means  $\pm$  SE ( $n = 6–8$ ). a,  $P \leq 0.05$  vs. baseline; b,  $P \leq 0.05$  vs. oleic acid; c,  $P \leq 0.05$  vs. TTA. #  $P \leq 0.05$  vs. ObControl. Evaluated by nonparametric Mann-Whitney and Wilcoxon's signed-rank test.



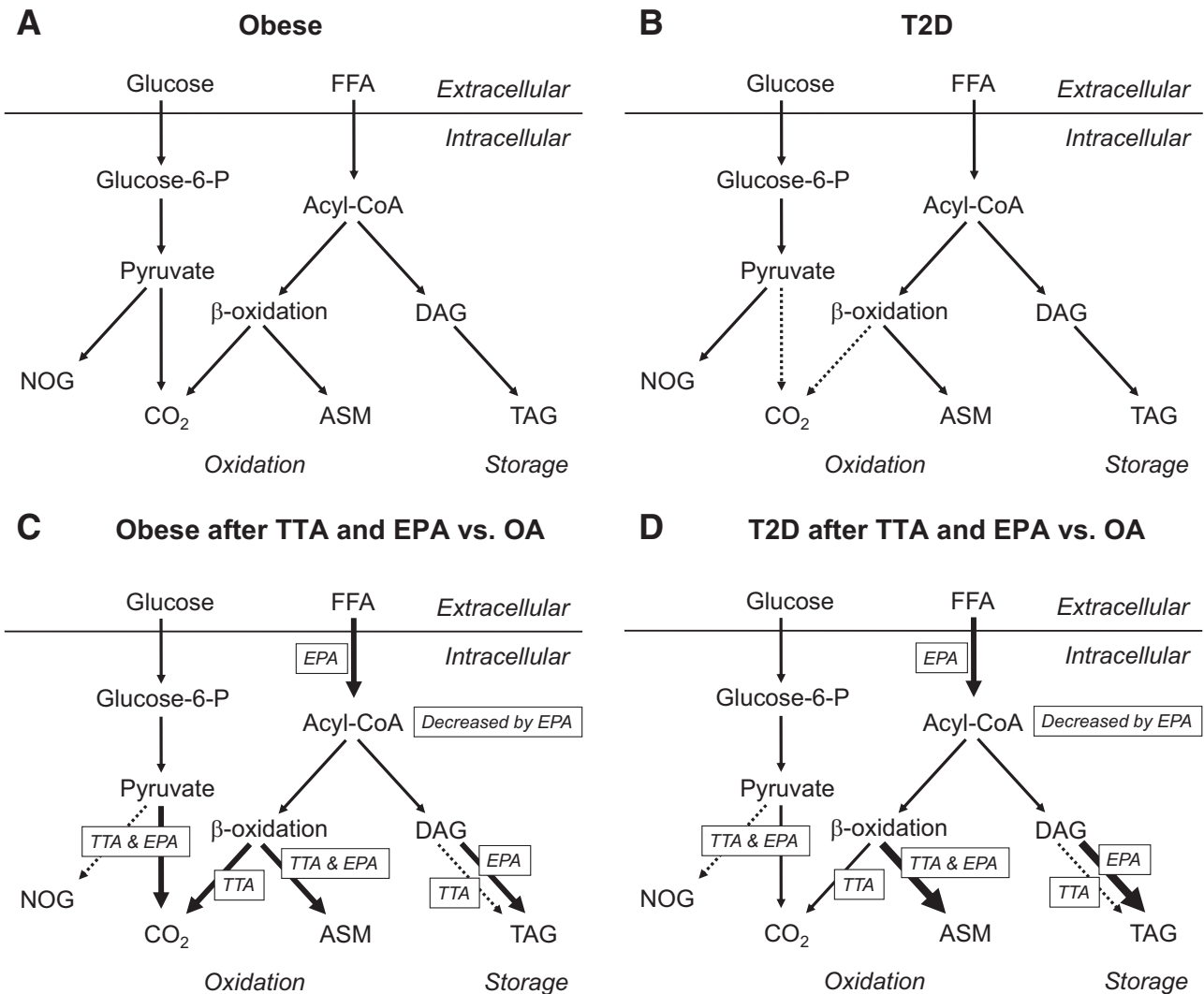
**FIG. 6.** Partitioning of de novo synthesized fatty acids in DAG and TAG (A) and effect of TTA on oleic acid-mediated increase in cell-associated  $^{14}\text{C}$ -palmitic acid (B). A: Distribution of de novo synthesized  $^{14}\text{C}$ -acetate-derived fatty acids after 4 h in complex lipids (DAG and TAG) after 4-day preincubation of human myotubes from healthy donors with 40  $\mu\text{mol/l}$  fatty acid-free BSA (baseline), 100  $\mu\text{mol/l}$  oleic acid, 100  $\mu\text{mol/l}$  EPA, or 100  $\mu\text{mol/l}$  TTA ( $n = 4$ ). B: Cell-associated  $^{14}\text{C}$ -palmitic acid measured by SPA in human myotubes from healthy donors preincubated 4 days with 40  $\mu\text{mol/l}$  BSA (baseline), 100  $\mu\text{mol/l}$  oleic acid, 100  $\mu\text{mol/l}$  TTA, or 100  $\mu\text{mol/l}$  oleic acid + 100  $\mu\text{mol/l}$  TTA the last day ( $n = 4$ ). Results represent means  $\pm$  SE. a,  $P \leq 0.05$  vs. baseline; b,  $P \leq 0.05$  vs. oleic acid; c,  $P \leq 0.05$  vs. EPA, analyzed by linear mixed models statistics (SPSS).

significant when comparing obese with obese type 2 diabetes (27). We studied the effects of EPA and TTA on the intermediary energy metabolism and insulin resistance in myotubes established from obese subjects with type 2 diabetes and obese healthy subjects. We have previously demonstrated reduced capacity for complete oxidation of labeled palmitic acid to  $\text{CO}_2$  in myotubes from obese type 2 diabetic subjects compared with obese control myotubes (21), and this dysfunction was reproduced in our present study. Here, we observed that  $\text{CO}_2$  production from labeled palmitic acid was reduced for diabetic myotubes (Fig. 7A vs. B). Furthermore, the release of fatty acid  $\beta$ -oxidation products (ASMs) was increased by type 2 diabetes compared with control myotubes after preincubation with fatty acids (Fig. 7C vs. D). Our data suggest that mitochondrial dysfunction in type 2 diabetes myotubes is probably caused by disturbances downstream of fatty acid  $\beta$ -oxidation leading to excess production and release of ASMs during increased influx and/or  $\beta$ -oxidation of fatty acids. In support for this, several studies have suggested a mitochondrial dysfunction in the etiology of insulin resistance and type 2 diabetes (4,6,28). Moreover, increased levels of  $\beta$ -oxidation products have been found in both murine skeletal muscle and plasma, probably reflecting mitochondrial overload due to excess availability of fatty acids (29,30).

Excess FFA taken up by myotubes may either be oxidized or stored as TAG. *PGC1 $\alpha$*  is an important regulator of muscle oxidative capacity, mitochondrial content, and fiber type (31,32), and insulin resistance may be negatively associated with *PGC1 $\alpha$*  expression in skeletal muscle (33,34). In the present study, gene expression of *PGC1 $\alpha$*  was increased in response to fatty acids, but the relative response was blunted in diabetic myotubes compared with control cells. In C2C12 myotubes, *PGC1 $\alpha$*

expression was enhanced after 4 h but reduced after 16 h of incubation with palmitic acid (35), whereas unsaturated fatty acids like oleic acid and linoleic acid enhanced *PGC1 $\alpha$*  expression in human myotubes (36). Our data suggest that the reduced capacity for complete mitochondrial fatty acid oxidation observed for type 2 diabetes myotubes might be related to suboptimal responses in expression of *PGC1 $\alpha$* , e.g., when the myotubes are exposed to fatty acids. *DGAT2*, which is involved in TAG formation (37), showed a similar expression pattern to *PGC1 $\alpha$* , and the expression of these genes correlated well ( $r = 0.81$ ,  $P < 0.0001$ ). Reduced *DGAT2* gene expression after preincubation with fatty acids in diabetic cells, together with impaired complete fatty acid oxidation, may contribute to enhanced levels of potentially lipotoxic intracellular fatty acid intermediates and might play a role in lipid-induced insulin resistance.

Preincubation of myotubes with EPA led to increased production of ASMs, whereas TTA increased both ASMs and  $\text{CO}_2$  production (Fig. 7C and D). Increased formation of  $\beta$ -oxidation intermediates may cause mitochondrial stress and induce skeletal muscle insulin resistance (30,38). However, we did not observe any detrimental effects of chronic preincubation with either EPA or TTA on insulin-stimulated glucose metabolism in human myotubes. This does not exclude any such link, because we did not coincide with fatty acids during the glucose experiments that might be a prerequisite for negative effects to occur. Nevertheless, Power et al. (39) showed that supplementation with L-carnitine could ameliorate rather than worsen insulin resistance in mice, despite increased levels of  $\beta$ -oxidation products in plasma and skeletal muscle. Furthermore, preincubation with EPA and TTA increased glucose oxidation, suggesting improved instead of reduced capacity for glucose handling (Fig. 7C and D).



**FIG. 7.** Changes in energy metabolism in human myotubes after preincubation with fatty acids. We observed a reduced oxidative metabolism ( $\text{CO}_2$  formation) of fatty acids and glucose in obese type 2 diabetes myotubes compared with obese control cells (A vs. B). Incubation with EPA or TTA promoted increased  $\beta$ -oxidation in diabetic cells (C vs. D). EPA enhanced lipid uptake, accumulation of TAG (especially in diabetic cells), and fatty acid  $\beta$ -oxidation in both groups of myotubes (C and D). TTA “normalized” complete lipid oxidation in diabetic myotubes to baseline level in control cells (A and B vs. D) and opposed increased lipid accumulation (C and D). Glucose oxidation was enhanced after chronic exposure to EPA and TTA, reducing nonoxidized glucose (NOG; C and D). NOG consists mostly of lactate and pyruvate. dotted arrows, reduced; fortified arrows, increased; boxes labeled with EPA and TTA, effects of preincubation.

Preincubation of myotubes with the naturally occurring fatty acids enhanced  $^{14}\text{C}$ -palmitic acid uptake in line with previous observations (8,10), and EPA was more effective than oleic acid (Fig. 7C and D). Preincubation with oleic acid, and especially EPA, also promoted increased incorporation of  $^{14}\text{C}$ -palmitic acid into TAG, and the response to EPA was more pronounced in diabetic myotubes (Fig. 7C vs. D). We observed a negative correlation between total fatty acid oxidation and TAG synthesis in the presence of EPA, suggesting that EPA may redirect excess fatty acids into TAG. Storage of excess fatty acids such as TAG may protect against cell damage due to neutralization of lipotoxic intermediates (40,41). Furthermore, EPA reduced the total amount of acyl-CoA, in particular C18:2-CoA, and the reduction was greatest for diabetic myotubes. Increased levels of acyl-CoA and especially C18:2-CoA have been associated with insulin resistance (42,43). Moreover, EPA markedly increased the level of C16:1-CoA, which has recently been shown to function as a lipokine improving peripheral insulin action (44) and glucose metabolism in L6 rat myotubes (45). In addition,

incubation of myotubes from normal lean subjects with EPA improved glucose uptake, despite an increased incorporation of  $^{14}\text{C}$ -oleic acid into TAG (10). In rats, EPA also improved insulin resistance while increasing imTAG levels (46).

Preincubation with TTA increased complete oxidation of  $^{14}\text{C}$ -palmitic acid to  $\text{CO}_2$  to a similar degree in control and diabetic myotubes. Thus, TTA “restored” complete palmitic acid oxidation in diabetic cells to baseline level of control myotubes (Fig. 7A and B vs. D). The uptake of palmitic acid was increased after preincubation with TTA but did not lead to similar accumulation of  $^{14}\text{C}$ -palmitic acid into TAG compared with preincubation with oleic acid or EPA. The reduced TAG accumulation may reflect enhanced fatty acid oxidation, and we have data showing increased TAG accumulation from  $^{14}\text{C}$ -palmitic acid after TTA preincubation when myotubes were acutely coincubated with the CPT1 inhibitor etomoxir (A.J.W., A.C.R., unpublished observations). Alternatively, TTA-CoA may inhibit DGAT activity (26), as also suggested by the markedly increased cellular DAG-to-TAG recovery ratio of

$^{14}\text{C}$ -acetate in the presence of TTA, without changes in lipid synthesis. Furthermore, we observed that  $^{14}\text{C}$ -TTA itself could not be efficiently incorporated into TAG in human myotubes and that coinubation with TTA prevented the oleic acid-induced increase in cell-associated  $^{14}\text{C}$ -palmitic acid. Moreover, myotubes preincubated with TTA had enhanced levels of acyl-CoA and TTA-CoA probably accounted for most of the difference because 3-thia fatty acids cannot undergo  $\beta$ -oxidation (14). However, preincubation of myotubes with TTA neither impaired insulin-stimulated glucose uptake nor glycogen synthesis, whereas glucose oxidation was increased in cells from both donor groups (Fig. 7C and D).

TTA may enhance mitochondrial proliferation in skeletal muscle (17), and activation of PPAR nuclear receptors probably mediates much of the effects of TTA on gene expression (16,18,26). Because there were no differences in induction of *CPT1* expression after preincubation with TTA relative to oleic acid and EPA, the enhanced  $\beta$ -oxidation after TTA may be explained by the twofold higher induction of *CD36/FAT* expression. CD36 may facilitate fatty acid transport across the plasma membrane in response to contraction and insulin (47), but CD36 may also reside on the outer mitochondrial membrane (48) in colocalization with *CPT1* to facilitate mitochondrial fatty acid influx (48,49). In rats, the PPAR $\gamma$  agonist rosiglitazone increased skeletal muscle fatty acid oxidation and mitochondrial CD36 amount, but not *CPT1* protein expression or activity (49), which is in line with our observations. Part of the increased oxidation of  $^{14}\text{C}$ -palmitic acid to  $\text{CO}_2$  after preincubation with TTA may be related to the increased *UCP2* expression and mitochondrial uncoupling (50). Thus, preincubation with the synthetic fatty acid analog TTA enhanced complete fatty acid and glucose oxidation in diabetic myotubes and opposed the increased lipid accumulation observed after preincubation with oleic acid and especially EPA.

In summary, type 2 diabetes myotubes seem to have an impaired mitochondrial capacity for fatty acid oxidation and glucose (Fig. 7A vs. B), linked to defects downstream of fatty acid  $\beta$ -oxidation, leading to increased release of ASMs during high fatty acid availability (Fig. 7C vs. D). Reduced response of PGC $\alpha$  to fatty acids in diabetic myotubes may be associated with reduced mitochondrial function and reduced DGAT activity, creating lipid intermediates and consequently insulin resistance. TTA and EPA might execute beneficial effects by increasing complete fatty acid oxidation and TAG formation, respectively (Fig. 7C and D), thereby improving overall energy metabolism and fatty acid handling in type 2 diabetes skeletal muscle.

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