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Impaired fatty acid metabolism in type 2 diabetic skeletal muscle cells is reversed by PPARγ agonists

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Cha, Bong-Soo, Theodore P. Ciaraldi, Kyong-Soo Park, Leslie Carter, Sunder R. Mudaliar, and Robert R. Henry. Impaired fatty acid metabolism in type 2 diabetic skeletal muscle cells is reversed by PPARγ agonists. *Am J Physiol Endocrinol Metab* 289: E151–E159, 2005. First published February 22, 2005; doi:10.1152/ajpendo.00141.2004.—The impact of type 2 diabetes on the ability of muscle to accumulate and dispose of fatty acids and triglycerides was evaluated in cultured muscle cells from nondiabetic (ND) and type 2 diabetic (T2D) subjects. In the presence of 5 μM palmitate, T2D muscle cells accumulated less lipid than ND cells (11.5 ± 1.2 vs. 15.1 ± 1.4 nmol/mg protein, $P < 0.05$). Chronic treatment (4 days) with the peroxisome proliferator-activated receptor-γ (PPARγ) agonist troglitazone increased palmitate accumulation, normalizing uptake in T2D cells. There were no significant differences between groups with regard to the relative incorporation of palmitate into neutral lipid species. This distribution was also unaffected by troglitazone treatment. β-Oxidation of both long-chain (palmitate) and medium-chain (octanoate) fatty acids in T2D muscle cells was reduced by ~40% compared with ND cells. Palmitate oxidation occurred primarily in mitochondrial (~40–50% of total) and peroxisomal (20–30%) compartments. The diabetes-related defect in palmitate oxidation was localized to the mitochondrial component. Both palmitate and octanoate oxidation were stimulated by a series of thiazolidinediones. Oxidation in T2D muscle cells was normalized after treatment. Troglitazone increased the mitochondrial component of palmitate oxidation. Skeletal muscle cells from T2D subjects express defects in free fatty acid metabolism that are retained in vitro, most importantly defects in β-oxidation. These defects can be corrected by treatment with PPARγ agonists. Augmentation of fatty acid disposal in skeletal muscle, potentially reducing intramyocellular triglyceride content, may represent one mechanism for the lipid-lowering and insulin-sensitizing effects of thiazolidinediones.

type 2 diabetes mellitus; fatty acid oxidation; thiazolidinediones; mitochondria

THE METABOLIC PROFILE OF TYPE 2 DIABETES includes impaired glucose metabolism and insulin resistance; these are frequently combined with dyslipidemia as well as insulin resistance for control of free fatty acid (FFA) and triglyceride (TG) metabolism. Members of the thiazolidinedione class of antidiabetic drugs have been shown to ameliorate aspects of this dyslipidemia, as well as improve glucose metabolism (27). Because FFAs are the major fuel source for skeletal muscle in the fasting and exercising states, impairments in FFA metabolism in skeletal muscle would be expected to have a major impact on energy homeostasis. Indeed, the literature contains reports that both obese and type 2 diabetic individuals oxidize lower than normal amounts of fatty acids in muscle in the fasting state (5, 33, 37, 38, 40, 59).

If not oxidized, FFA can accumulate as TG in skeletal muscle, potentially impacting insulin action. A strong inverse correlation between intramyocellular TG (IMTG) content and insulin action has been established across a wide weight range of nondiabetic and type 2 diabetic subjects (reviewed in Refs. 26, 35). It has been postulated that it is the accumulation of intermediates of fatty acid metabolism, and not just IMTG itself, that contributes to the development of insulin resistance (22, 67). The processes involved in this TG accumulation in muscle are numerous, including the mass action effect of elevated circulating FFA levels due to augmented adipose tissue lipolysis in obese and diabetic individuals (16). Also contributing to this accumulation is the fact that obese individuals favor fatty acid esterification and storage over oxidation in skeletal muscle (59).

Lipid metabolism is a highly complex and tightly regulated process. FFAs are provided in the circulation by both release from lipoproteins, by the action of lipoprotein lipase, and lipolysis in adipose tissue. A key regulated process after that is FFA entry into cells. FFAs have been shown to diffuse passively across the cell membrane (reviewed in Ref. 25). In addition, it has been proposed that a portion of FFA uptake is protein mediated (reviewed in Ref. 8). Once inside the muscle cell, FFAs are either incorporated into lipids by esterification for storage and structural purposes or oxidized in mitochondria and peroxisomes. After production of fatty acid CoA derivatives by acetyl-CoA synthase, entry of long-chain fatty acid CoA into mitochondria is mediated by carnitine palmitoyltransferase I (CPT I), a crucial regulatory point in lipid and glucose metabolism (47), whereas medium-chain fatty acids enter the mitochondria independently of CPT I (47).

To investigate the nature of lipid metabolism in skeletal muscle of type 2 diabetic patients, independent of the hyperglycemia, hyperinsulinemia, and hyperlipidemia present in vivo, we utilized human skeletal muscle cells in culture. When differentiated, cultured human skeletal muscle cells display many of the morphological, biochemical, and metabolic characteristics of mature skeletal muscle (28). Most importantly, cells from diabetic subjects display defects in glucose and fatty acid metabolism that are reflective of the subject's in vivo metabolic status (19, 20, 28, 29). Muscle cells from diabetic subjects also display insulin resistance for the control of glucose metabolism (15, 19). In this study, multiple steps in

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FFA metabolism were compared between nondiabetic and type 2 diabetic muscle cells before and after treatment with agonists for the peroxisome proliferator-activated receptor-γ (PPARγ). These include de novo synthesis, incorporation into lipids, and β-oxidation to evaluate potential mechanisms that could contribute to increased levels of fatty acids and/or intermediates in insulin-resistant muscle.

MATERIALS AND METHODS

Subjects. Thirty patients with type 2 diabetes and 39 nondiabetic subjects provided muscle tissue for the studies. Of the type 2 diabetic patients, 22 were treated with oral antidiabetic agents (glipizide and metformin), 3 with insulin in combination with an oral agent, and 5 with diet alone. Diabetic patients had their medication withheld on the morning of biopsy. Nondiabetic subjects were not taking any medications known to influence glucose and lipid metabolism. Glucose tolerance was determined in all subjects after a 75-g oral glucose tolerance test. In vivo insulin action was determined by a 3-h euglycemic (5.0–5.5 mM) hyperinsulinemic (1,800 pmol·m^{-2}·min^{-1}) clamp; the glucose disposal rate was determined during the last 30 min of the clamp (62). Subjects did not exercise for at least 24 h before the procedure. Clinical characteristics of the subjects are summarized in Table 1. On average, the diabetic subjects were older and more obese than the nondiabetic subjects. However, there were no associations between either age or body-mass index and in vitro measures of FFA oxidation (not shown). In addition, matching subjects within the groups for age and obesity did not alter the in vitro results and conclusions; therefore, the entire nondiabetic population was included. The experimental protocol was approved by the Committee on Human Investigation of the University of California, San Diego. Informed, written consent was obtained from all subjects after explanation of the protocols.

Materials. Cell culture materials were purchased from Irvine Scientific (Irvine, CA) except for skeletal muscle basal medium, which was obtained from Clonetics (San Diego, CA). [9,10-^{3}H]palmitate, D-[U-^{14}C]glucose, and [^{14}C]acetate were obtained from NEN Life Science Products (Boston, MA). [8-^{3}H]octanoate was supplied by American Radiolabeled Chemicals (St. Louis, MO). Unlabeled palmitate octanoate and FFA-free BSA were purchased from Sigma (St. Louis, MO). Reagents for electrophoresis, as well as the AG-1X8 resin, were obtained from Bio-Rad (Richmond, CA). A polyclonal antibody against human muscle-specific CPT I (mCPT I) was purchased from Alpha Diagnostics (San Antonio, TX); phosphoserine (S79)-specific antibody against acetyl-CoA carboxylase (ACC) was purchased from Upstate (Lake Placid, NY). Anti-rabbit IgG complexed to horseradish peroxidase and Hyperfilm were from Amersham (Arlington Heights, IL). SuperBlock and SuperSignal chemiluminescent substrate kits were obtained from Pierce (Rockford, IL). Troglitazone was a kind gift from Dr. Alan Saltiel (formally of Pfizer Parke-Davis Pharmaceuticals, Ann Arbor, MI), rosiglitazone was a gift from Dr. Steven Smith (GlaxoSmithKline), and pioglitazone was supplied by Dr. A. Kozai (Takeda Pharmaceuticals America, Lincolnshire, IL).

Table 1. *Subject characteristics*

	Nondiabetic	type 2 Diabetic
No. of subjects (women/men)	39 (16/23)	30 (4/26)
Age, yr	44±2	53±2*
BMI, kg/m^2	29.0±1.1	34.7±1.5*
Fasting glucose, mM	5.1±0.1	9.6±0.6*
Fasting insulin, pM	68±6	208±33*
Hb A_{1c}, %	5.5±0.1	8.7±0.4*
GDR, mg·kg^{-1}·min^{-1}	10.06±0.47	5.69±0.34*
TG, mg/dl	111±10	188±20*
FFA, mM	0.391±0.026	0.487±0.039*

Values are means ± SE. BMI, body mass index; FFA, free fatty acids; GDR, glucose disposal rate determined from hyperinsulinemic (300 mU·m^{-2}·min^{-1}), euglycemic (5.0–5.5 mM) clamp procedure; Hb A_{1c}, hemoglobin A1 glycosylate; TG, triglyceride. *$P < 0.025$ vs. nondiabetic.

Muscle biopsy and cell culture. Needle biopsies of the vastus lateralis muscle were completed before insulin infusion. The methods for muscle cell isolation and growth have been described in detail previously (28). At 80–90% confluence, cells were fused for 4 days in α-MEM containing 2% FBS, 1% fungibact, 100 U/ml penicillin, and 100 μg/ml streptomycin. Fusion medium was changed every other day. Approximately 90% of the cells take on the multinucleated morphology characteristic of mature, differentiated myotubes (28); fibroblasts were not detected in the cultures. When indicated, 11.5 μM troglitazone, 10 μM rosiglitazone, or 10 μM pioglitazone was added for 4 days at the initiation of fusion and differentiation. The doses of agents were determined in preliminary studies such that maximal stimulation of palmitate oxidation was provided. Agents were dissolved in DMSO (final concentration of 0.05%). This treatment protocol does not alter the extent of differentiation of myocytes into mature myotubes (data not shown).

Measurement of FFA oxidation. The procedure for assaying palmitate and octanoate oxidation is a modification of a method established for adherent cells (56). Cells were incubated in serum-free media containing substrate ([9,10-^{3}H]palmitic acid or [8-^{3}H]octanoic acid, 0.2 μCi, final concentration of 5 μM) in a 95% O_2-5% CO_2 incubator at 37°C for 3 h. After incubation, a 100-μl aliquot of the culture medium was placed over an ion-exchange resin, and the column was washed twice with 0.75 ml of water. Intact FFA (charged state) was retained by the resin, whereas ^{3}H released during oxidation was incorporated into water, which passes freely through the resin column.

To learn more about the sites of palmitate oxidation in diabetic muscle, we exploited the differential sensitivity of long-chain fatty acid CoA uptake across the organelle membrane for inhibition by etomoxir (3), using this property to discriminate between the mitochondrial and peroxisomal pathways of FFA β-oxidation. The oxidation of [^{3}H]palmitate measured in untreated cells would be total oxidation. The portion of palmitate oxidation occurring in the mitochondria is irreversibly inhibited by a low concentration of etomoxir (1 μM). The peroxisomal component of palmitate oxidation is reversibly inhibited by a high dose (50 μM) of etomoxir. Residual palmitate oxidation is considered to occur by other, undefined processes.

Extraction and analysis of cellular lipids. Myotubes were incubated with [^{3}H]palmitate (as described for the FFA oxidation assay), [^{14}C]glucose (as described for measurement of glucose incorporation into glycogen; see below), or [^{14}C]acetate and washed, and then lipids were extracted with $CHCl_3$-MeOH (2:1). Parallel wells of cells were solubilized with 0.1 N NaOH to determine total cell-associated radioactivity. The extracted lipids were sampled for determination of radioactivity or separated by TLC in heptane-isopropyl ether-acetic acid (60:40:3). Spots were visualized with rhodamine solution under UV light and excised, and radioactivity was determined. Lipid species were identified by comigration with commercially available standards.

Glycogen synthesis. Glucose incorporation into muscle glycogen was determined as previously described (29) from the incorporation of D-[U-^{14}C]glucose (0.5 μCi, final glucose concentration of 5 mM) in the absence or presence of maximal (33 nM) insulin concentrations for 2 h. Results are expressed as nanomoles of glucose converted to glycogen per milligram of protein per hour. Total cellular protein was determined by the Bradford method (10).

Cell protein extraction. Extraction buffer (20 mM Tris·HCl, 145 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton X-100, 0.5% Nonidet *P*-40, 200 μM sodium vanadate, 200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin, 10 μg/ml aprotinin, 100 mM NaF, and 40 mM sodium pyrophosphate, pH 7.4) was added to confluent monolayers of fused myotubes, which had been treated for 4 days. Cells were scraped into tubes and solubilized by incubating for 30 min on

ice with frequent mixing; this was followed by centrifugation at 14,000 *g* for 10 min at 4°C. We analyzed the supernatant fluid (total cell lysate) for protein content using the Bradford method (10).

Western blotting. The method for Western blot analysis was described previously (28). Equal amounts of total extracted cell proteins were loaded and separated on 10% SDS-PAGE gels and then transferred to a nitrocellulose membrane (63). All samples from the same individual set of cells were analyzed on the same gel. The secondary antibody for mCPT I and ACC serine phosphorylation was anti-rabbit IgG conjugated with horseradish peroxidase. We detected immunocomplexes using an enhanced chemiluminescence kit according to the manufacture's instructions, followed by autoradiography. Quantification was performed with a scanning laser densitometer (Scan Analysis; Biosoft, Cambridge, UK). A sample of human skeletal muscle protein was included on each gel to serve as a control for intergel variability.

Statistical analyses. Statistical significance was evaluated using Student's *t*-test, and two-tailed *P* values were calculated. Paired analysis was performed for comparisons of chronic effects of each agonist. Significance was accepted at the $P < 0.05$ level. Because of the limited number of cells available, we did not conduct every study in each subject. Clinical characteristics of the subsets of subjects studied in the different experiments did not differ from the average of the total group; this held true for both nondiabetic and diabetic subjects.

RESULTS

Lipid synthesis. De novo synthesis was the first aspect of lipid metabolism evaluated in cultured muscle cells. Glucose incorporation into total lipid (lipogenesis), defined as incorporation into organic extractable material, was modest, representing only ~1% of the amount of glucose incorporated into glycogen over the same period (Table 2). Lipogenesis from glucose was not acutely stimulated by insulin and was similar in nondiabetic and type 2 diabetic muscle cells. This behavior differs from that of glycogenesis, which was reduced in diabetic muscle and is acutely insulin responsive (Table 2). Lipogenesis from acetate was considerably greater than from glucose (Table 2) and was also not insulin responsive. There was a tendency for lipogenesis from acetate to be elevated in diabetic muscle cells ($P < 0.1$).

Fatty acid accumulation and esterification. After incubation of muscle cells with 5 μM palmitate for 3 h, palmitate accumulation by muscle cells from diabetic subjects was reduced to 70% of the value in nondiabetic cells (Fig. 1). This result is consistent with our recent finding that carrier-mediated palmitate uptake is impaired in diabetic muscle cells (66). Treatment with the PPARγ agonist troglitazone increased palmitate accumulation in cells from both groups, but the difference was statistically significant only in the type 2 diabetic cells. After treatment, the significant difference between groups was lost; impaired palmitate accumulation in diabetic muscle was restored to the level seen in untreated nondiabetic cells. After incubation with [^{3}H]palmitate, essentially all (98–99%) of the cell-associated radioactivity was present in the lipid extract. One half (50%) of the lipid-associated radioactivity migrated in the chromatographic solvent used, which was selected to separate neutral lipid species; the balance remained at the origin. Of the radioactivity that migrated, 65–75% was present in a peak identified as TG, with the remainder in diacylglycerol, monoacylglycerol, cholesterol esters, and other, unidentified forms (Fig. 2). The relative distribution of [^{3}H]palmitate between these lipid species did not differ significantly between nondiabetic and diabetic muscle, although there was a tendency toward greater incorporation into diacylglycerol in type 2 diabetic cells. Troglitazone treatment did not significantly alter the relative pattern of palmitate incorporation into lipid species (data not shown).

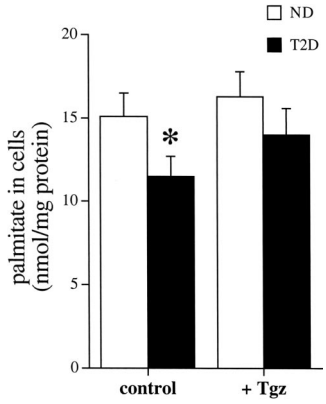

Fig. 1. Palmitate accumulation by human skeletal muscle cells. Myotubes were incubated with [^{3}H]palmitate (5 μM final concentration) for 3 h at 37°C and washed, and cell-associated radioactivity was determined. Cells were treated with troglitazone (Tgz, 11 μM) for 4 days. Results are averages + SE; $n = 4$ for nondiabetic (ND) and 3–7 for type 2 diabetic (T2D). *$P < 0.05$ vs. ND.

Table 2. *Glycogen and lipid synthesis in human skeletal muscle cells*

Substrate and Treatment	Nondiabetic		type 2 Diabetic	
	Glycogen	Lipid	Glycogen	Lipid
Glucose				
Basal	4.42±0.65	0.039±0.008	2.70±0.33*	0.034±0.008
+Insulin	7.78±1.48†	0.035±0.007	5.10±1.21†	0.035±0.007
Acetate				
Basal		0.14±0.04		0.26±0.08
+Insulin		0.098±0.01		0.26±0.08

Values are averages ± SE; $n = 8$ nondiabetic subjects and 6 diabetic subjects. Results are presented as nmol of substrate incorporated into glycogen or lipid after 2-h exposure to glucose (5 mM) or acetate (1 mM) + glucose, normalized to protein content (1 mg). Insulin (33 nM) was present, when indicated, during the 2-h period. *$P < 0.05$ vs. nondiabetic. †$P < 0.05$ vs. basal.

Fatty acid oxidation. The major fate of fatty acids in the muscle cell is oxidation. The oxidation of palmitate in type 2 diabetic muscle cells was impaired, compared with nondiabetic muscle (Fig. 3*A*). In a subset of subjects, oxidation of the medium-chain fatty acid octanoate was measured in parallel with that of palmitate. Even when substrate concentrations were matched (5 μM), muscle cells oxidized less octanoate (Fig. 4*A*): 41 ± 5% and 47 ± 5% of the amount of palmitate oxidized in nondiabetic and type 2 diabetic cells, respectively. Both palmitate and octanoate oxidation were reduced in type 2 diabetic muscle cells (Fig. 4*A*) and to a similar extent, ~40% lower than nondiabetic samples.

As reported previously by our laboratory (13), ~50% of palmitate oxidation in nondiabetic muscle occurs in mitochondria (Fig. 3*B*). The difference in palmitate oxidation between the diabetic and nondiabetic groups is seen in the mitochondrial component (Fig. 3*B*). Peroxisomal oxidation of palmitate

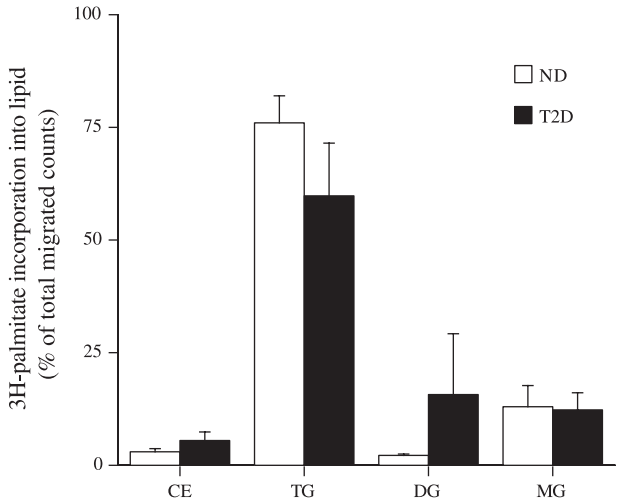

Fig. 2. Palmitate incorporation into lipid in human muscle cells. Myotubes were incubated with [^{3}H]palmitate as described in Fig. 1, and lipids were extracted and analyzed as described in MATERIALS AND METHODS. Lipid species were identified by comigration with commercial standards: CE, cholesterol esters; TG, triglycerides; DG, diglycerides; MG, monoglycerides. Results are presented as % of total counts that migrated from the origin in that spot, determined for each individual set of cells. Results are averages + SE; $n = 3$ for ND and 4 for T2D.

is similar in normal and diabetic muscle. Although total palmitate oxidation was lower in the subsets of subjects studied for this analysis compared with the values for each entire group, the differences between nondiabetic and type 2 diabetic cells were still present for both mitochondrial and total palmitate oxidation (Fig. 3*B*).

Chronic treatment (4 days) of muscle cells with several different thiazolidinediones resulted in stimulation of both palmitate (Fig. 5*A*) and octanoate (Fig. 4*B*) oxidation. Because the responses of palmitate oxidation to troglitazone rosiglitazone and pioglitazone were similar, indicating that this is a class effect, the results for the different thiazolidinediones were combined for each subject group. Octanoate oxidation was measured only in pioglitazone-treated cells. With treatment, palmitate oxidation in diabetic cells did not differ from the activity in control nondiabetic cells; thiazolidinedione treatment normalized long-chain fatty acid oxidation in diabetic muscle. The same was true for octanoate oxidation. The increase in palmitate oxidation in response to treatment was due entirely to a change in the mitochondrial component (Fig. 5*B*) in both nondiabetic and diabetic muscle cells.

Regulation of protein expression and phosphorylation. Because the rate-controlling step for mitochondrial β-oxidation of long-chain fatty acids in mitochondria is transit across the outer mitochondrial membrane (47), mediated by CPT I, regulation of the expression of mCPT I was evaluated. mCPT I protein was readily detectable in cultured muscle cells (Fig. 6*A*). The extent of protein expression was similar in nondiabetic and diabetic muscle cells (Fig. 6*B*). Because the responses of mCPT I protein to both troglitazone (11 μM) and pioglitazone (10 μM) were comparable (not shown), the results for the two agents were combined. Thiazolidinedione treatment resulted in a modest decrease in mCPT I protein expression: 88 ± 9% of control in nondiabetic (P = not significant) and 73 ± 9% in type 2 diabetic ($P < 0.05$) cells.

mCPT I is also under allosteric control by malonyl-CoA, the product of a reaction catalyzed by ACC (57). Because ACC is deactivated by serine phosphorylation, this property was evaluated under the same conditions as mCPT I expression. In the control state, there was no difference in ACC phosphorylation between nondiabetic and type 2 diabetic cells (Fig. 7). Pioglitazone treatment resulted in an increase in ACC phosphorylation; this difference did not attain statistical significance in either individual group ($P = 0.058–0.060$) but was highly significant when the groups were pooled ($P < 0.025$).

DISCUSSION

type 2 diabetes is recognized as a disease of disorders of both glucose and lipid metabolism. Circulating FFA levels are elevated in diabetic individuals, due in large part to elevated basal lipolysis from adipose tissue depots, together with a reduced ability of insulin to suppress stimulated lipolysis (reviewed in Ref. 43). The critical importance of elevated circulating FFA levels in diabetic and obese individuals was demonstrated in both rodents and humans, where FFA infusion was shown to generate whole body insulin resistance (6, 7, 18, 23). Although the impact of circulating FFA is major, recent attention has focused on the influence of fat accumulated within muscle, termed intramyocellular lipid or IMTG, on

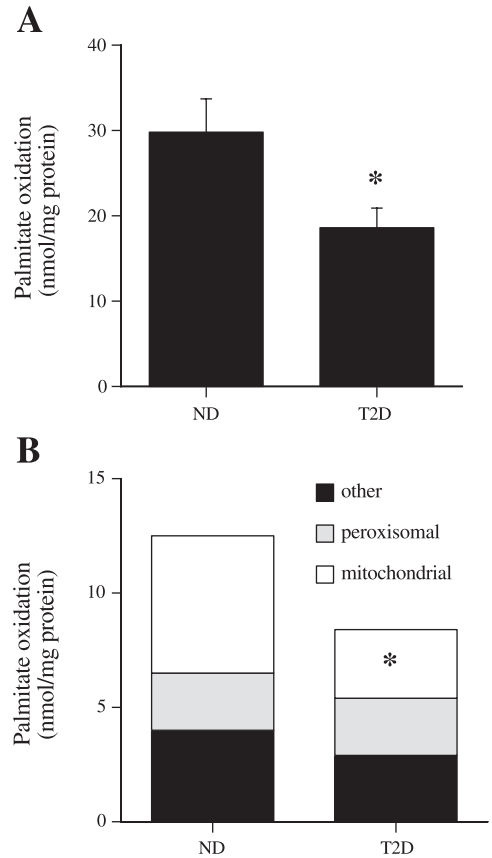

Fig. 3. Palmitate oxidation in human muscle cells. Myotubes were incubated with [^{3}H]palmitate (5 μM final concentration) for 3 h at 37°C. Products of oxidation were monitored as ^3H_2O released to the media. *A*: absolute rates of total palmitate oxidation in untreated cells. Results are average + SE; $n = 39$ for ND and $n = 30$ for T2D. *B*: components of palmitate oxidation, as defined in the text. Results are averages; $n = 5$ for ND and 9 for T2D. *$P < 0.05$ vs. ND.

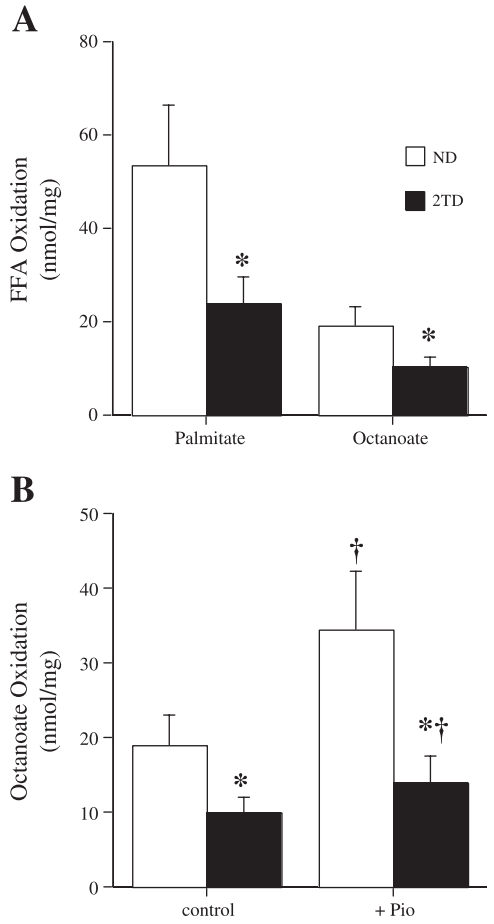

Fig. 4. Octanoate oxidation in human muscle cells. Myotubes were incubated with [^{3}H]octanoate (5 μM final concentration) or palmitate for 3 h at 37°C. *A*: absolute rates of palmitate and octanoate oxidation measured in the same subjects' cells. Results are averages + SE; $n = 6$ for ND and $n = 9$ for T2D. FFA, free fatty acid. *B*: regulation of octanoate oxidation. Cells were treated for 4 days in the absence (control) or presence of pioglitazone (+Pio, 10 μM) before assay. Results are averages + SE; $n = 6$ for ND and 9 for T2D. *$P < 0.05$ vs. ND. †$P < 0.05$ vs. control for same individual.

insulin action and glucose metabolism. Multiple investigators have reported strong associations between IMTG and the presence of insulin resistance in diabetic and nondiabetic individuals (5, 6, 34, 65). However, this relationship is not absolute; there is a paradox for endurance-trained athletes, who are highly insulin sensitive, because they also display high IMTG (21). Thus factors other than the total amount of IMTG, such as the accumulation of fatty acid intermediates (1, 26, 67), may contribute to insulin resistance.

There could be several reasons for excess IMTG and fatty acid intermediates in diabetic and obese individuals. One would be the mass action effect of increased substrate delivery to muscle, from the elevated circulating FFA levels. There could also be a change in the ultimate fate of FFA, with greater esterification and storage and less disposal through oxidation. Both behaviors have been observed in diabetic (4, 9, 37) and obese (31) individuals. What is uncertain is how much of this behavior of diabetic muscle is acquired from the hyperlipidemic and dysmetabolic environment present in vivo, whether this is perhaps potentially reversible, and whether this is perhaps an intrinsic property of diabetic muscle. It is these questions that we addressed in human skeletal muscle cells maintained in culture under defined conditions. Our group (28, 49) and others (19, 42) have reported that muscle cells from type 2 diabetic subjects maintain, at least in part, defects in glucose uptake, glycogen synthesis, and insulin signaling that are reflective of the behaviors observed in vivo. Less is known about lipid metabolism under these circumstances, although it has recently been reported that cultured skeletal muscle cells from type 2 diabetic subjects do display impaired palmitate oxidation (20), in agreement with the present results.

De novo lipid synthesis in cultured skeletal muscle was found to be modest, and not insulin responsive, whether as acetate incorporation into FFA or glucose formation of the glycerol backbone of TGs (Table 2). The latter is not surprising, as muscle, unlike adipose tissue under most conditions, contains glycerol kinase (24) and can reuse glycerol released by lipolysis. When extracellular FFA levels are matched between nondiabetic and diabetic muscle, as is possible in the cultured cell system, diabetic muscle cells accumulate less palmitate, at least at low substrate levels. This finding is in agreement with our laboratory's recent report (66), where we found the protein-mediated component of FFA uptake to be reduced in diabetic muscle cells, and with in vivo results from limb balance and flux studies that demonstrated defects in FFA uptake in diabetic muscle (5) when differences in substrate levels were controlled for. The incorporation of palmitate into neutral lipid species in diabetic muscle cells is reduced to the same extent as total accumulation, suggesting that esterification may be intact. It is interesting to note that there is a tendency for there to be relatively more diacyglycerol present in diabetic muscle (Fig. 2), although this difference did not attain statistical significance. This may be of importance because it has been suggested that, more so than total IMTG, it may be the presence of specific lipid metabolites, such as ceramide or diacyglycerol, that modulate insulin action (1, 22, 26, 67).

The major observation of our present work is that the oxidation of both palmitate and octanoate is significantly impaired in diabetic muscle cells. Although the diabetic subjects studied were, on average, older and more obese than the nondiabetic subjects, there were no significant associations between these two parameters and rates of fatty acid oxidation in muscle cells. These results in cultured myotubes are in agreement with in vivo data (45, 37); by matching FFA levels, both acutely and chronically, between the nondiabetic and diabetic groups, we show that defects in FFA oxidation are likely an intrinsic property of diabetic muscle, although additional impairments may be acquired from the hyperglycemic, hyperinsulinemic, and hyperlipidemic environment present in vivo. A similar in vitro finding for palmitate oxidation has recently been reported (20). The magnitude of the reduction in palmitate oxidation (~40%) is greater than that seen for total uptake. The net result of such a difference would suggest that, in diabetic muscle, a greater proportion of FFA entering the cell would be stored, leading to increased IMTG and intermediates, which would be consistent with observations made in both in vivo (1, 55), at least in obesity, and in vitro (20) systems. It must be noted that the present observations were made when FFA levels in the media were the same for nondiabetic and diabetic muscle cells. The contribution of the diabetes-related defect in FFA oxidation to increased IMTG

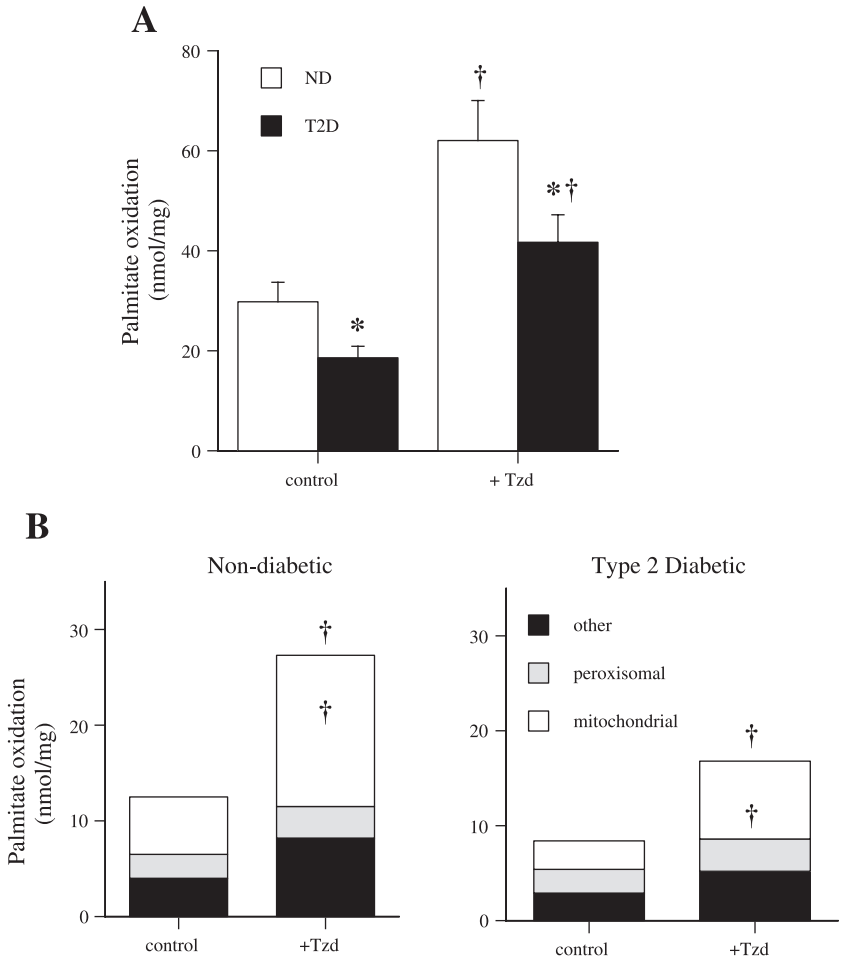

Fig. 5. Regulation of palmitate oxidation in skeletal muscle cells. Cells were treated for 4 days, during fusion/differentiation with Tzd (10–11 μM) before assay of palmitate oxidation. *A*: total oxidation. Results are averages + SE; $n = 25–37$ for ND and 23–30 for T2D. *B*: components of oxidation. Results are averages; $n = 5$ for ND and 9 for T2D. *$P < 0.05$ vs. ND. †$P < 0.05$ vs. control for same individual.

would be accentuated by the elevated substrate supply available to diabetic muscle in vivo (Table 1). We have previously shown (13) that a major portion (~50%) of the β-oxidation of palmitate occurs in the mitochondria. The impairment in FFA oxidation in diabetic muscle cells appears to be localized to the mitochondrial component. The finding that defects exist for both palmitate and octanoate oxidation indicates that there are perturbations in common aspects of FFA metabolism. This behavior would be consistent with other observations, such as the finding that the activities of enzymes involved in mitochondrial oxidation of fatty acids are reduced in muscle biopsies from diabetic subjects (11, 36, 58), as are the expression of many mitochondrial genes of oxidative metabolism (53).

The fate of exogenous FFA between β-oxidation and incorporation into storage forms such as mono-, di-, and triacylglycerol is subject to multiple levels of control. The key control point for mitochondrial β-oxidation of long-chain fatty acids is passage of long-chain fatty acyl-CoA esters across the outer mitochondrial membrane, mediated in skeletal muscle by mCPT I, whereas medium-chain fatty acids like octanoate do not require CPT I to enter the mitochondria (47). Conversely, the committed rate-limiting step for glycerolipid synthesis is performed by glycerol 3-phosphate acyltransferase (61). Impaired palmitate oxidation in diabetic muscle cells could be the result of defects in the expression and/or function of mCPT I, a reciprocal increase in glycerol-3-phosphate acyltransferase activity, or a decreased oxidative capacity of the mitochondria;

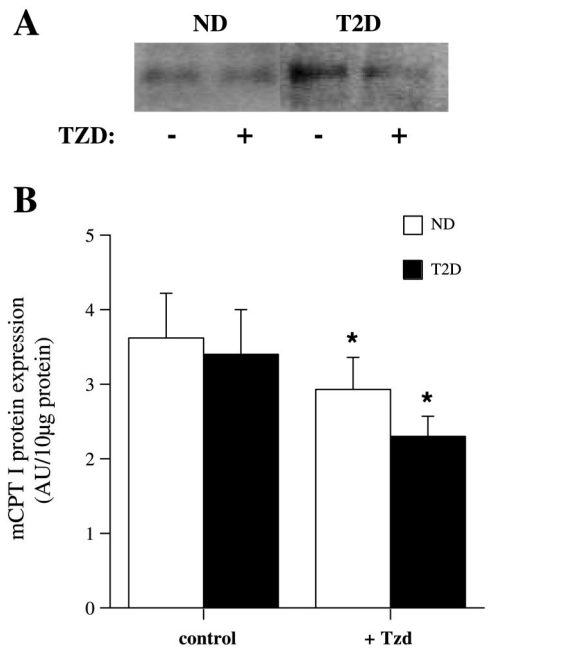

Fig. 6. Regulation of muscle-specific carnitine palmitoyltransferase (mCPT I) protein expression in muscle cells. *A*: representative Western blot. *B*: quantitation of expression. Results from Tgz and Pio treatments were combined for each group. Results are averages + SE; $n = 9$ for ND and 8 for T2D. AU, arbitrary units. *$P < 0.05$ vs. control.

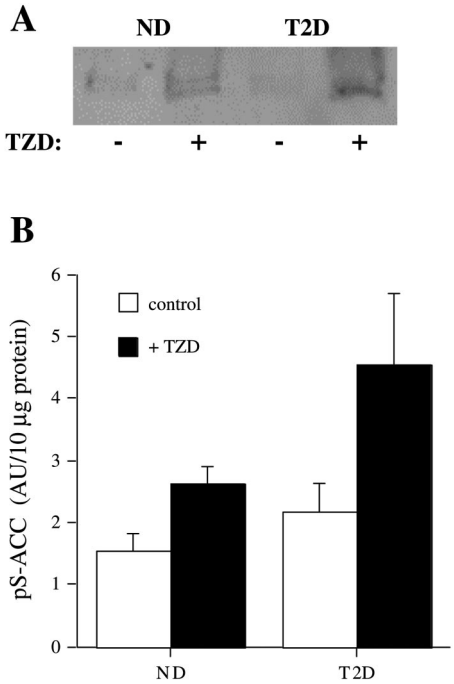

Fig. 7. Regulation of acetyl-CoA carboxylase (ACC) serine phosphorylation (pS) in muscle cells. *A*: representative Western blot. *B*: quantitation. Results are averages + SE; $n = 4$ each for ND and T2D.

there is in vivo evidence for this latter possibility (36, 54, 58), which would be consistent with the shared impairments in long- and medium-chain FFA oxidation reported here. CPT I activity has been found to be reduced in muscle of insulin-resistant obese individuals (33); however, the impact of type 2 diabetes on CPT I activity in muscle is unknown, although it was recently reported that the mRNA expression of CPT I is normal in diabetic muscle (17). This last observation would suggest that any reduction in CPT I activity may be acquired in nature, due primarily to allosteric factors, as is also indicated by the fact that physiological hyperglycemia with hyperinsulinemia is able to decrease long-chain fatty acid oxidation even in healthy subjects (55). Reduced FFA oxidation is accompanied by lower functional CPT I activity and elevations in the muscle content of malonyl-CoA (55). This observation is crucial because CPT I activity is under well-defined allosteric control, where the lipogenic precursor malonyl-CoA inhibits CPT I activity and subsequent FFA oxidation (55, 57). Because malonyl-CoA is synthesized by ACC, inactivating phosphorylation of ACC would reduce malonyl-CoA levels, ameliorating the inhibition of CPT I and permitting increased FFA oxidation (57). The importance of allosteric regulation would also be consistent with the present results in cultured muscle cells, where there were no differences between nondiabetic and type 2 diabetic muscle cells with regard to mCPT I protein expression (Fig. 6), similar to the behavior in muscle tissue (17); rather, control may lay at the level of CPT I activity and malonyl-CoA concentrations in the vicinity of the mitochondria. Although malonyl-CoA levels were not measured directly in our study, the tendency for increased phosphorylation of ACC in muscle cells after thiazolidinedione treatment (Fig. 7) would be expected to lower malonyl-CoA levels. In addition, the presence of reductions in medium-chain fatty acid oxidation indicates that diabetes-related defects in FFA oxidation occur distal to the action of CPT I as well. The ability of thiazolidinedione treatment to improve long-chain fatty acid oxidation could involve effects at any or all of these steps.

Consequences of a diabetes-related impairment in FFA oxidation in skeletal muscle could include increased lipid storage in muscle, elevated IMTG and fatty acid metabolism intermediates, and diversion of excess FFA to other tissues (4). Elevated intracellular lipid contents of muscle and liver have been strongly associated with insulin resistance (34, 65), although lipid accumulation in the pancreas has been shown to lead to β-cell toxicity (41). Thiazolidinedione treatment of diabetic individuals has been shown to reduce both circulating FFA levels (46, 48) and IMTG (46) and improve insulin sensitivity, even while increasing subcutaneous adipose tissue mass (39, 46). Many of these effects have been attributed to an increase in adipose tissue differentiation and diversion of lipid stores from other tissues (liver, muscle, and β-cell) to this expanding adipose tissue mass, termed the lipid steal hypothesis (45, 64). These responses would also be consistent with a more active role in skeletal muscle to reduce IMTG by increasing fatty acid oxidation, thereby disposing of a portion of the stored lipid and intermediates, as suggested by the present results. It is most likely that both mechanisms, the lipid steal and increased disposal in muscle, are contributing to the thiazolidinedione-induced changes in circulating and tissue FFA content and the resulting improvement in insulin action. Similar to the situation in vivo, thiazolidinedione treatment of human skeletal muscle cells has been shown to improve both insulin signaling (32) and insulin responsiveness (51). The fact that thiazolidinedione treatment ameliorates both defective FFA oxidation and insulin resistance, in vivo and in vitro, indicates a close relationship, possibly causal, between these two aspects of the metabolic phenotype of diabetic muscle.

Thiazolidinediones are known to be selective agonists for the nuclear receptor PPARγ (60). PPARγ protein is present in skeletal muscle (44) and muscle cells (1, 53), although not to the same level as in adipose tissue (44). Evidence in cultured muscle cells (2, 51, 66), transgenic animals depleted of adipose tissue (12, 14), and muscle-specific knockouts of PPARγ (30, 50) supports a role for direct effects of PPARγ in muscle on control of metabolism. Although troglitazone, rosiglitazone, and pioglitazone display differing affinities for PPARγ (60), the fact that maximally effective doses of the three compounds have similar effects on palmitate oxidation suggests that this response is a family effect, most likely mediated through PPARγ.

In summary, skeletal muscle cells from type 2 diabetic subjects express intrinsic defects in FFA metabolism that are retained in vitro, including impaired accumulation and defects in β-oxidation. These defects can be corrected by treatment with PPARγ agonists. The abilities of PPARγ-activating agents to improve glucose tolerance and insulin action may involve effects on both glucose and lipid metabolism in skeletal muscle in addition to the well-characterized actions in adipose tissue.

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