

# Increased Malonyl-CoA Levels in Muscle From Obese and Type 2 Diabetic Subjects Lead to Decreased Fatty Acid Oxidation and Increased Lipogenesis; Thiazolidinedione Treatment Reverses These Defects

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Increased accumulation of fatty acids and their derivatives can impair insulin-stimulated glucose disposal by skeletal muscle. To characterize the nature of the defects in lipid metabolism and to evaluate the effects of thiazolidinedione treatment, we analyzed the levels of triacylglycerol, long-chain fatty acyl-coA, malonyl-CoA, fatty acid oxidation, AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), malonyl-CoA decarboxylase, and fatty acid transport proteins in muscle biopsies from nondiabetic lean, obese, and type 2 subjects before and after an euglycemic-hyperinsulinemic clamp as well as pre- and post-3-month rosiglitazone treatment. We observed that low AMPK and high ACC activities resulted in elevation of malonyl-CoA levels and lower fatty acid oxidation rates. These conditions, along with the basal higher expression levels of fatty acid transporters, led accumulation of long-chain fatty acyl-coA and triacylglycerol in insulin-resistant muscle. During the insulin infusion, muscle fatty acid oxidation was reduced to a greater extent in the lean compared with the insulin-resistant subjects. In contrast, isolated muscle mitochondria from the type 2 subjects exhibited a greater rate of fatty acid oxidation compared with the lean group. All of these abnormalities in the type 2 diabetic group were reversed by rosiglitazone treatment. In conclusion, these studies have shown that elevated malonyl-CoA levels and decreased fatty acid oxidation are key abnormalities in insulin-resistant muscle, and, in type 2 diabetic patients, thiazolidinedione treatment can reverse these abnormalities. *Diabetes* 55:2277-2285, 2006

**A**bnormal skeletal muscle lipid metabolism is a characteristic of insulin resistance and can influence gene expression and intracellular signaling, leading to energy imbalance, impaired insulin action, and the metabolic syndrome (1). In humans, 70–80% of insulin-stimulated glucose transport occurs in skeletal muscle, and the flux of intramyocellular fatty acids to lipogenesis or oxidation is a key determinant of glucose disposal. One of the factors regulating fatty acid oxidation is malonyl-CoA (2). Increased malonyl-CoA levels inhibit mitochondrial fatty acid uptake and oxidation, promoting triacylglycerol synthesis (3). Insulin activates acetyl-CoA carboxylase (ACC), leading to production of malonyl-CoA from acetyl-CoA, thus favoring lipogenesis (2). Cellular level of malonyl-CoA is a key factor in energy homeostasis. Malonyl-CoA levels are decreased by activation of AMP-activated protein kinase (AMPK), which inactivates ACC through phosphorylation, and by malonyl-CoA decarboxylase (MCD), which degrades malonyl-CoA. Previous studies correlating decreased AMPK activity and increased malonyl-CoA levels with the metabolic syndrome have been extensively reviewed by Ruderman and Prentki (4).

The other key players in the regulation of fatty acid oxidation are fatty acid transporters, such as fatty acid transport protein 1 (FATP1) and FATP4, as well as the fatty acid translocator membrane glycoprotein 36 (FAT/CD36) (5,6). The levels of expression of these proteins have been negatively correlated with insulin sensitivity (5–7). In muscle cells, FAT/CD36 has been shown to redistribute between the cell surface and endosomal compartments in response to insulin (8). Although FATP1 and -4 have been shown to undergo similar insulin-stimulated translocation in fat cells (9), FATP1 does not seem to respond to insulin the same way in muscle cells (10,11). Thus, it is important to assess the contribution of these proteins to the abnormal lipid metabolism that occurs in muscle tissues from insulin-resistant patients.

Thiazolidinediones are widely used antidiabetic drugs (12). Because insulin sensitivity in rodents rendered insulin resistant by high-fat diet or lipid infusion can be improved by thiazolidinedione treatment (13,14), it is evident that thiazolidinediones can reverse the abnormal lipid metabolism brought about by such nutritional stress (15,16). This situation is reminiscent of that observed with the overexpression of the MCD gene or depletion of genes

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ACC, acetyl-CoA carboxylase; ALBP, adipocyte lipid-binding protein; AMPK, AMP-activated protein kinase; FATP, fatty acid transport protein; FAT/CD36, fatty acid translocator membrane glycoprotein 36; MCD, malonyl-CoA decarboxylase; PPAR, peroxisome proliferator-activated receptor.

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TABLE 1  
Characteristics of study subjects

Parameters	Nondiabetic	Obese	Type 2 diabetic
Age (years)	47.25 ± 4.8	43.5 ± 4.0	53.08 ± 2.47
Weight (kg)	75.2 ± 5.4	96.7 ± 4.0	102.1 ± 7.6
BMI (kg/m <sup>2</sup> )	24.4 ± 1.2	33.3 ± 1.06*	36.93 ± 1.95*
Fasting plasma glucose (mg/dl)	91.7 ± 2.16	92.0 ± 1.8	184.23 ± 16.3†
Glucose infusion rate (mg · kg <sup>-1</sup> · min <sup>-1</sup> )	9.9 ± 0.93	7.0 ± 0.37‡	1.68 ± 0.42§
Plasma insulin (pmol/l)	66.7 ± 10	125.36 ± 26	315 ± 78¶
Plasma free fatty acids (mmol/l)	346 ± 36	400 ± 30	750 ± 120#
Plasma triacylglycerol (mg/dl)	88.75 ± 6.8	128.6 ± 21**	271 ± 75.1††

Data are means ± SE. A total of 8 nondiabetic (lean), 8 obese, and 13 type 2 diabetic subjects were involved in this study. All *P* values were determined by comparing with nondiabetic (lean) subjects. \**P* < 0.05, †*P* < 0.005, ‡*P* < 0.05, §*P* < 0.002, ||*P* < 0.02, ¶*P* < 0.02, #*P* < 0.02, \*\**P* = 0.05, ††*P* < 0.05.

for ACC2, FAT/CD36, and FATP1 because, in each case, protection against high-fat diet-induced insulin resistance was achieved (7,17–19). Therefore, thiazolidinediones may correct abnormal lipid metabolism by modulating the functions of one or more of these genes. Thiazolidinediones can also influence lipid metabolism in muscle indirectly through enhanced production of circulating adiponectin from adipose tissue (20,21), which in turn can lead to AMPK activation (20,22,23). Because genetic deletion of peroxisome proliferator-activated receptor-γ (PPARγ; the target for thiazolidinedione action) in muscle per se can create insulin resistance and block thiazolidinedione-induced muscle insulin sensitization in mice (24), it appears that the overall outcome of thiazolidinedione treatment is determined by the combined effects on muscle and adipose tissues (25).

To evaluate the dysregulation of lipid and malonyl-CoA metabolism in insulin-resistant human muscle, we have analyzed lipid products, FATPs, and the activities of enzymes involved in malonyl-CoA metabolism in human muscle biopsies obtained from nondiabetic lean, obese, and type 2 diabetic subjects before and after hyperinsulinemic-euglycemic clamps. The type 2 diabetic patients were also treated for 3 months with rosiglitazone to determine whether the insulin-sensitizing effects of rosiglitazone are related to lowered malonyl-CoA levels and improved lipid metabolism.

## RESEARCH DESIGN AND METHODS

A total of 8 nondiabetic lean male subjects, 8 obese male subjects, and 13 type 2 diabetic subjects (12 male and 1 postmenopausal female) participated in the

TABLE 2  
Clinical characteristics of type 2 subjects treated with rosiglitazone

Parameters	Baseline	After 3 months of treatment
Age (years)	54.5 ± 2.37	—
Weight (kg)	106.35 ± 9.3	108.38 ± 9.8
BMI (kg/m <sup>2</sup> )	36.7 ± 3.15	—
Glucose disposal, <i>R<sub>d</sub></i> (mg · kg <sup>-1</sup> · min <sup>-1</sup> )	3.54 ± 0.56	5.81 ± 1.0
Glucose infusion rate (mg · kg <sup>-1</sup> · min <sup>-1</sup> )	1.15 ± 0.46	3.94 ± 1.05
Fasting plasma glucose (mg/dl)	162.5 ± 13.9	142.8 ± 13.6*
Hepatic glucose output (mg · kg <sup>-1</sup> · min <sup>-1</sup> )	2.39 ± 0.24	1.87 ± 0.16*
Plasma cholesterol (mg/dl)	200.3 ± 22.85	207 ± 18.47
Plasma LDL cholesterol (mg/dl)	132.33 ± 15.74	139.67 ± 10.42
Plasma HDL cholesterol (mg/dl)	34.83 ± 2.56	32.67 ± 2.39
Plasma triacylglycerol (mg/dl)	165.3 ± 36.9	164.6 ± 40.5
HbA <sub>1c</sub> (%)	7.5 ± 0.4	7.2 ± 0.33

Data are means ± SE. Of the 13 type 2 diabetic patients, 6 were treated with rosiglitazone for 3 months, and clinical parameters were determined before and after treatment. All *P* values are based on comparison between numbers obtained before and after rosiglitazone treatment of type 2 diabetic subjects. \**P* < 0.03, pre- vs. posttreatment.

study. Of the 13 type 2 diabetic subjects, 6 were treated with rosiglitazone (4 mg twice a day) for 3 months. The lean and obese subjects as well as 7 of the 13 type 2 diabetic patients who did not receive rosiglitazone treatment have been part of a previous study (26). The clinical characteristics of the subjects are given in Tables 1 and 2. With regard to the medication history of the subjects, duration of the clamp, infusion rate, and levels of insulin and glucose achieved during the clamp, the information is the same as in the previous publication (26). Briefly, insulin (Humulin; Eli Lilly, Indianapolis, IN) was infused for 300 min at a rate of 80 mU/m<sup>2</sup> per min to achieve a level of 1–1.2 nmol/l. A variable infusion of 20% glucose was delivered to achieve a level of 5 mmol/l. The procedures for hyperinsulinemic-euglycemic clamps and leg muscle biopsies were described previously in detail (14,20).

**Reagents.** All buffer components, phosphatase inhibitors, and lipids were purchased from Sigma (St. Louis, MO). Antibody against ACC as well as SAMS peptide were purchased from Upstate Biotechnology (Charlottesville, VA). The antiserum against FATP1 was a gift from Dr. A. Stahl, Palo Alto Medical Foundation and Stanford University School of Medicine (Palo Alto, CA). Anti-adipocyte lipid-binding protein (ALBP) antibody was obtained from Cayman Chemical (Ann Arbor, MI). Goat anti-FATP4, rabbit anti-FAT/CD36, and horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat secondary antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies to phospho-AMPK, phospho-ACC, and AMPK were from Cell Signaling Technology (Beverly, MA). Purified fatty acid synthase was received as a gift from Dr. S. Smith, Children Hospital (Oakland, CA).

**Tissue fractionation, mitochondria, and membrane preparation.** Muscle samples (30–40 mg) were dissected to remove visible fatty and connective tissues. Muscle samples were powdered under liquid nitrogen, homogenized by Polytron in 2 ml ice-cold homogenization buffer, and centrifuged at 800g for 15 min at 4°C as described previously (26). The supernatant was saved in ice. To extract intermyofibrillar mitochondria, the pellet was resuspended in 2 ml homogenization buffer, homogenized again in a Potter-Elvehjem glass homogenizer (10 passes), and then centrifuged at 800g for 10 min. The first and second supernatants were combined, and the resulting homogenates were subjected to bicinchoninic acid-based protein assay (Pierce, Rockford, IL), aliquoted, snap-frozen in liquid nitrogen, and stored at –80°C.

We used ~40 mg of tissue for the preparation of plasma membrane, endosomal membrane, and mitochondrial fractions by adapting the protocols published by Stahl et al. (9) and Kim et al. (27). Mitochondrial preparations obtained by following the protocol of Kim et al. (27) were suitable for functional assays, such as malonyl-CoA-sensitive fatty acid oxidation (28). Mitochondria obtained from subsarcolemmal and intermyofibrillar fractions were combined and used for our studies. For verifying the quality of the preparations, we carried out immunoblotting for cytochrome C oxidase IV subunit, a mitochondrial marker; transferrin receptor, an endosomal marker; sodium-potassium ATPase  $\alpha$ -1, a plasma membrane marker; and calcium ATPase, a marker for sarcoplasmic reticulum. Mitochondrial integrity was judged by comparing citrate synthase activities (29) in the homogenates, mitochondrial pellets, and postmitochondrial supernatants.

Cell fractions were mixed with Laemmli buffer containing 10 mmol/l 2-mercaptoethanol and saved at  $-20^{\circ}\text{C}$ . Immunoblotting of the membranes after PAGE, enhanced chemiluminescence detection, and quantitation of the signals by densitometry were carried out following standard protocols. Equal amounts of protein (25–50  $\mu\text{g}$ ) were loaded per lane for Western blotting.

**[1- $^{14}\text{C}$ ]palmitate oxidation in homogenate and in isolated mitochondria.** The released  $^{14}\text{CO}_2$  and radiolabeled acid-soluble materials produced from oxidation of [1- $^{14}\text{C}$ ]palmitate were measured by adapting a method published by Kim et al. (27) and described by Koves et al. (28) for viable mitochondria. In brief, 40  $\mu\text{l}$  of the homogenate (100  $\mu\text{g}$  protein) or mitochondrial suspension (50  $\mu\text{g}$  protein) was preincubated at  $30^{\circ}\text{C}$  for 15 min inside a vial with a conical insert. A 160- $\mu\text{l}$  reaction mixture, as described by Koves et al. (28), was then added to the preincubated suspension, followed by 0.1  $\mu\text{Ci}$  [1- $^{14}\text{C}$ ]palmitate (0.5  $\mu\text{Ci/ml}$ , final concentration 200  $\mu\text{mol/l}$ ) as albumin complex (fatty acid and albumin dilution of 5:1). After 60 min of incubation, the reactions were stopped with  $\text{HClO}_4$ , released radiolabeled  $\text{CO}_2$  was trapped in an organic solvent mixture, and the radioactivity in the trapped  $\text{CO}_2$  and acid-soluble materials in the acidified supernatant was determined as described by Glatz et al. (30). These two radioactivities were combined, and the amount of oxidized palmitate (in nmol) was calculated from the specific activity corrected for endogenous long-chain fatty acyl-coA, assuming that the average palmitate content of nonpolar muscle lipid is 32% (31). Dilution of the specific activity of exogenous radiolabeled palmitate in the incubations attributable to the increase in long-chain fatty acyl content in type 2 samples was minimized to 6–8% by the addition of a 10-fold excess of palmitate. The results are presented as nanomoles of palmitate oxidized per microgram protein.

**Assay for malonyl-CoA, triacylglycerol, and long-chain fatty acyl-coA.** We added 1 ml 6% perchloric acid to a 20-mg portion of muscle biopsies that had been ground and cleaned under liquid nitrogen. The mixture was homogenized by a Polytron and centrifuged at 5,000g for 10 min at  $4^{\circ}\text{C}$ . A 0.5-ml aliquot of the supernatant was neutralized with 2 mol/l  $\text{KHCO}_3$  to pH 7.0 and centrifuged at 2,000g for 10 min at  $4^{\circ}\text{C}$ . The clear supernatant was subjected to malonyl-CoA assay, using the protocol of McGarry et al. (32). The remaining half of the first supernatant was subjected to lipid extraction and purification following the protocol of Bligh and Dyer (33). The extracted lipids were subjected to assay for triacylglycerol, using a kit from Wako Chemicals (Richmond, VA). The acid-insoluble precipitates were washed with diethyl ether and water, adjusted to pH 11.5 in the presence of dithiothreitol, heated at  $55^{\circ}\text{C}$  to hydrolyze thioester bonds of long-chain fatty acyl-coA, and assayed for released CoA, following the procedure described by Antinozzi et al. (34). **Activity assays for AMPK, ACC, and MCD.** AMPK activity was assayed by two methods. The phosphorylation of AMPK was assayed by immunoblotting with anti-phospho-AMPK antiserum (Cell Signaling). Total activity of AMPK (phosphorylation and AMP induced) was assayed by immunoprecipitation of total AMPK followed by a kinase reaction in which synthetic SAMS peptide was phosphorylated with [ $\gamma$ - $^{32}\text{P}$ ]ATP by the immunobeads. The protocol described by Winder and Hardie was followed (35). The antibody used for immunoprecipitation recognizes both AMPK- $\alpha$ 1 and - $\alpha$ 2 isoforms.

ACC activity was measured by immunoblotting for phosphorylated and total ACC. Activity for MCD was assayed by degradation of [ $^{14}\text{C}$ ]malonyl-CoA to [ $^{14}\text{C}$ ]acetyl-CoA, which was then converted to [ $^{14}\text{C}$ ]acetyl-carnitine by carnitine-acetyltransferase and separated from the reaction mixture by mini Dowex-resin (8X  $\text{Cl}^-$ ) column chromatography. The procedure described by Kerner and Hoppel (36) was followed.

**Statistical calculations.** Results are the means  $\pm$  SE. Significance of a difference between two groups was determined by ANOVA and paired two-tailed *t* tests.  $P \leq 0.05$  was considered significant.

## RESULTS

**Regulation of malonyl-CoA levels.** Malonyl-CoA is a key metabolic intermediate that regulates fatty acid oxi-

dation and the size of the cytosolic pool of fatty acyl-CoA. As seen in Fig. 1, we measured intramyocellular malonyl-CoA levels in the control and insulin-resistant subjects in the basal state and in the insulin-stimulated state at the end of the glucose clamp period. In the type 2 diabetic subjects, studies were repeated after a 3-month period of rosiglitazone treatment.

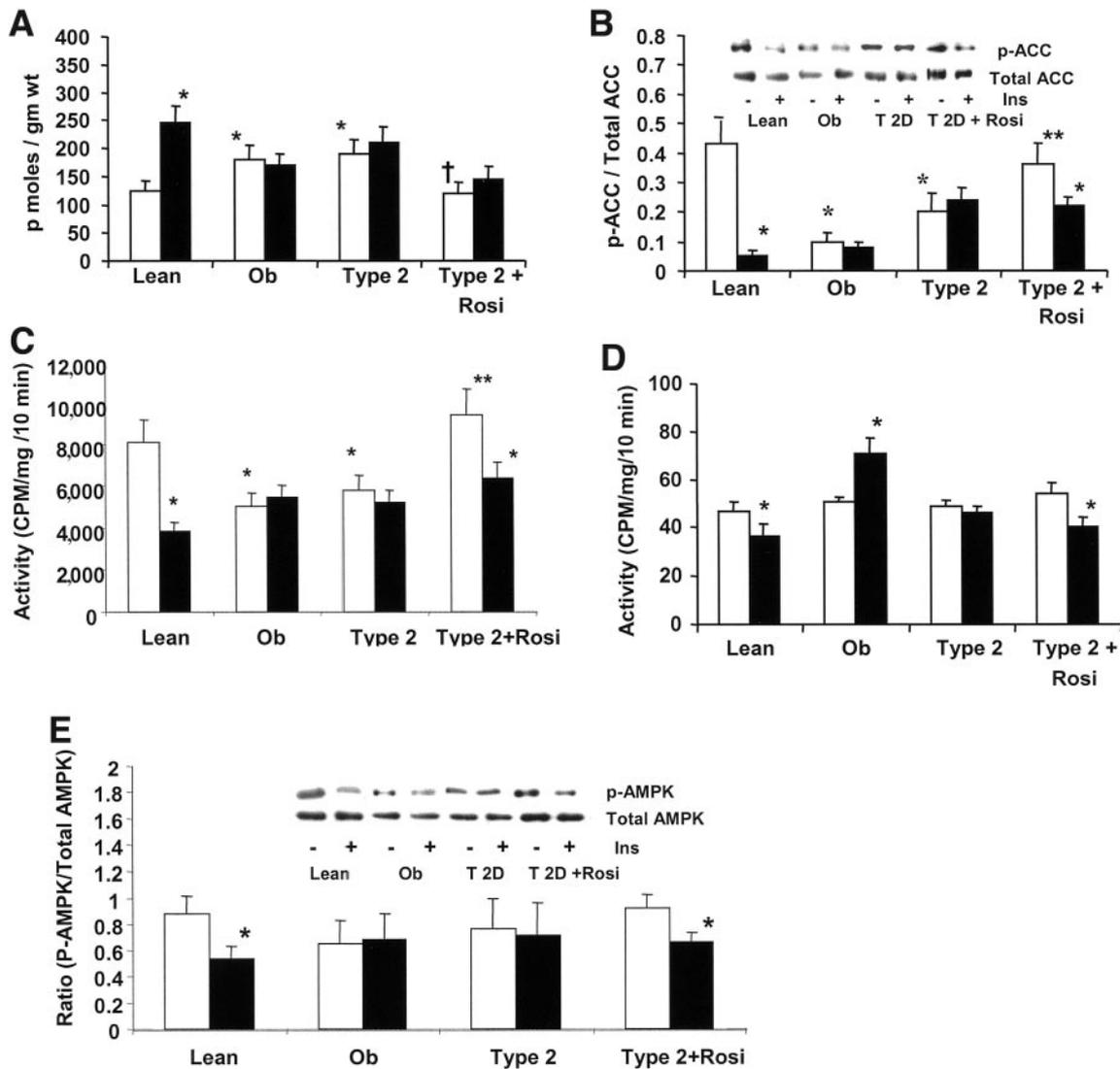
As shown in Fig. 1A, insulin stimulation had a robust effect to increase malonyl-CoA levels in the lean subjects. In the insulin-resistant subjects, basal malonyl-CoA levels were elevated, and insulin stimulation was without further effect to increase these levels. In the type 2 diabetic subjects, after rosiglitazone treatment, basal malonyl-CoA levels fell to normal, whereas the effect of insulin to increase malonyl-CoA was still substantially attenuated.

Malonyl-CoA is produced by activated ACC, and insulin is known to activate ACC by mediating its dephosphorylation. We measured these events in the muscle samples and found (Fig. 1B) that in the basal state, >40% of ACC was in the phosphorylated inactive form in the lean subjects. Additionally, in these subjects, insulin stimulation had a marked effect to dephosphorylate and activate ACC. In the insulin-resistant obese and type 2 diabetic subjects, basal ACC levels were already highly activated, and no further effect of insulin was noted. After rosiglitazone treatment, the type 2 diabetic subjects showed restoration of basal phospho-ACC levels toward normal, with a substantial recovery of the insulin effect to dephosphorylate ACC (Fig. 1B). These results are fully consistent with the role of ACC to convert acetyl-CoA to malonyl-CoA.

Intracellular malonyl-CoA levels can be decreased by decarboxylation of malonyl-CoA to acetyl-CoA through the enzyme MCD. In the normal subjects, we found that insulin stimulation had a modest effect to decrease MCD activity. In the insulin-resistant subjects, basal MCD levels were indistinguishable from control subjects, but no effect of insulin to decrease MCD was observed. In fact, a paradoxical increase in MCD occurred in the obese subjects. During the clamped insulin-stimulated state, the low phospho-ACC levels in the obese subjects (representing high ACC activity) apparently offset the higher MCD activity in the obese group, leading to lower malonyl-CoA levels compared with the lean subjects. After rosiglitazone treatment, the effect of insulin to reduce MCD activity was restored to normal (Fig. 1D).

**AMP kinase activity.** ACC is one of the targets of AMP kinase, and by mediating ACC phosphorylation, AMP kinase decreases ACC activity. We found that insulin had a substantial effect to inhibit AMP kinase activities in the lean control muscle samples but not in the insulin-resistant subjects (Fig. 1C), providing a further explanation for the insulin-induced activation of ACC. In contrast to our results, Kuhl et al. (37) did not find a correlation between the decrease in the levels of phospho-ACC and that of phospho-AMPK. In their study with lean male and female subjects (four of each), insulin treatment decreased phospho-ACC levels without affecting phospho-AMPK levels.

In the insulin-resistant groups, the combined activities of AMPK- $\alpha$ 1 and - $\alpha$ 2 in the preclamp state were reduced compared with lean control subjects. When the level of phospho-AMPK was normalized to total AMPK protein levels, a similar trend was observed, but the decrease in phosphorylation of AMPK in the insulin-resistant groups was not statistically significant (Fig. 1E). After 3 months of rosiglitazone treatment in the type 2 diabetic patients, basal levels of AMPK activities were completely normal-



**FIG. 1.** Malonyl-CoA (A) and phospho-ACC (B) levels, AMPK (C) and MCD (D) activities, and phospho-AMPK levels (E) in muscle from lean, obese (Ob), and type 2 diabetic subjects before and after hyperinsulinemic-euglycemic clamp. A group of type 2 patients were also treated with rosiglitazone (Rosi) for 3 months. At the end of the treatment period, muscle biopsies were taken before and after the clamp. Equal amounts of proteins were loaded for Western blotting, and the signals for phospho-ACC were normalized against total ACC after densitometric scan. A: \* $P < 0.03$  vs. lean basal, † $P < 0.05$  vs. type 2 basal. B: \* $P < 0.01$  vs. lean basal, \*\* $P < 0.05$  vs. type 2 basal. C: \* $P < 0.05$  vs. lean basal, \*\* $P < 0.02$  vs. type 2 basal. D: \* $P < 0.05$  vs. corresponding basal. E: \* $P < 0.01$  vs. corresponding basal. □, basal; ■, insulin. Ins, insulin; T 2D, type 2 diabetes.

ized, and the inhibitory effect of insulin on AMP kinase activity was largely restored.

Taken together, these results show increased basal malonyl-CoA levels in skeletal muscle from insulin-resistant obese type 2 diabetic subjects accompanied by a complete loss of the acute effects of insulin to increase malonyl-CoA levels. These results are likely explained by the coordinated directional effects of insulin resistance and acute insulin treatment on ACC, MCD, and AMP kinase. It would appear that the marked differences in ACC activity outweigh the smaller differences in MCD activity, resulting in higher basal malonyl-CoA levels. Interestingly, in the type 2 diabetic patients, thiazolidinedione treatment partially rescued all of these abnormalities. **Intramyocellular triacylglycerol and long-chain fatty acyl-coA.** The chronically elevated intramyocellular malonyl-CoA levels seen in the insulin-resistant subjects would be expected to favor lipogenesis at the expense of lipid oxidation. Evidence for this lipogenic state is seen in Fig. 2, which shows increased basal levels of intramyocel-

lular triacylglycerol in the obese and type 2 diabetic subjects compared with the lean control subjects. After rosiglitazone treatment, the intramyocellular triacylglycerol levels in the type 2 diabetic patients showed a tendency to decrease, but the change was not significant. Insulin stimulation had a modest effect to acutely increase intramyocellular triacylglycerol that was lost in the insulin-resistant subjects. Long-chain fatty acyl-CoA levels follow a parallel pattern, with increased basal long-chain fatty acyl-CoA concentrations in the insulin-resistant groups compared with the control subjects and restoration of long-chain fatty acyl-CoA levels to normal in the rosiglitazone-treated type 2 diabetic subjects.

**Fatty acid oxidation in skeletal muscle from untreated and rosiglitazone-treated type 2 diabetic subjects.** We also assessed the oxidative state of these skeletal muscle samples by measuring fatty acid oxidation in whole-muscle homogenates as well as in isolated muscle mitochondria before and after thiazolidinedione treatment in the type 2 diabetic subjects.

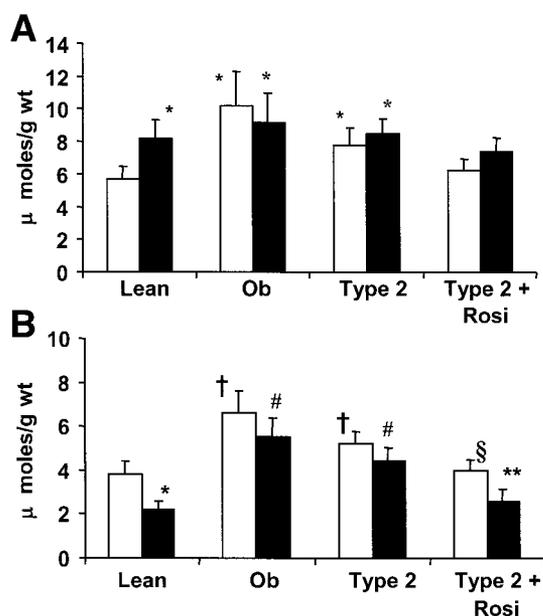


FIG. 2. Intramyocellular triacylglycerol (A) and long-chain fatty acyl-coA (B) levels in human muscle. Source of tissues are same as in Fig. 1. A: \* $P < 0.05$  vs. lean basal. B: \* $P < 0.01$  vs. lean basal, † $P < 0.05$  vs. lean basal, ‡ $P < 0.03$  vs. lean insulin treated, § $P < 0.05$  vs. type 2 basal, \*\* $P < 0.05$  vs. type 2 basal. □, basal; ■, insulin. Ob, obese.

**Studies in whole-muscle homogenates.** As demonstrated in Fig. 3, basal fatty acid oxidation rates in whole-muscle homogenates were relatively high in the lean subjects with a marked effect of insulin to decrease fatty acid oxidation, consistent with the action of insulin to increase malonyl-CoA. In type 2 diabetic subjects, basal fatty acid oxidation rates were significantly reduced with no significant further effect of insulin. This decrease in oxidation (30–35%) was over and above the small dilution of specific activity of the exogenously labeled fatty acid attributable to increased content of long-chain fatty acyl in type 2 muscle. After rosiglitazone treatment, basal fatty acid oxidation rates were partially restored to normal, and the insulin effect to lower fatty acid oxidation was restored. These results support previous findings about reduced oxidative capacity in insulin-resistant tissues (38,39).

**Studies in muscle mitochondria.** A different picture emerged on measurement of fatty acid oxidation in mitochondria. Here, we observed an increase in mitochondrial fatty acid oxidation capacity in type 2 diabetic subjects compared with control subjects, and this increase was returned to normal by rosiglitazone treatment. It is of interest that when cytosolic constituents (e.g., malonyl-CoA) are factored out, by performing direct measurements of fatty acid oxidation in mitochondria, an increased, rather than a decreased, rate of fatty acid oxidation was noted in the untreated type 2 diabetic subjects. The increased mitochondrial fatty acid oxidation may reflect an intrinsic mitochondrial abnormality. To further assess the possibility of an intrinsic abnormality within mitochondria leading to an enhanced capacity for fatty acid oxidation in the mitochondrial preparations from the untreated type 2 diabetic subjects, similar measurements were performed after the addition of exogenous malonyl-CoA to the mitochondria fractions. As seen in Fig. 4, the addition of malonyl-CoA has the expected effect to inhibit mitochondrial-mediated palmitate oxidation in all groups.

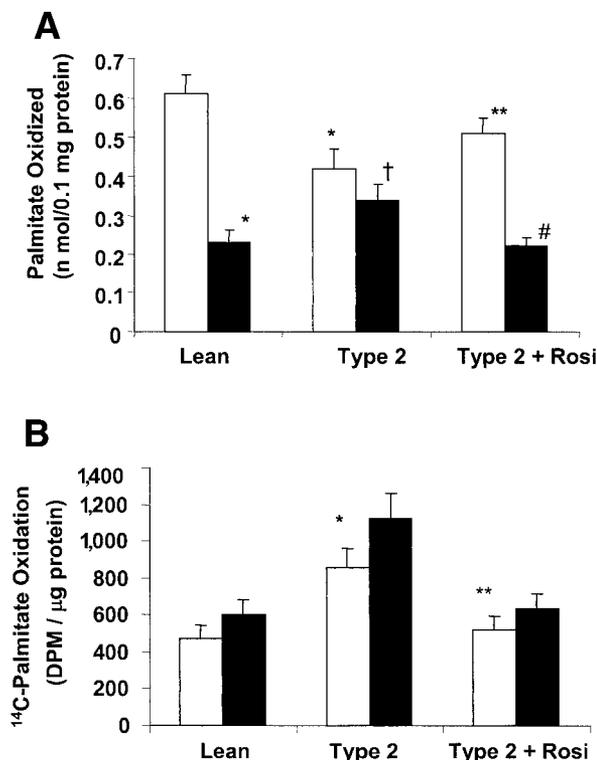


FIG. 3. [1-<sup>14</sup>C]palmitate oxidation in tissue homogenates (A) and in isolated mitochondria (B) from muscle biopsies obtained from subjects as described in Fig. 1. A: \* $P < 0.02$  vs. lean basal, † $P < 0.05$  vs. lean insulin treated, \*\* $P < 0.05$  vs. type 2 basal, ‡ $P < 0.05$  vs. type 2 insulin treated. B: \* $P < 0.01$  vs. lean basal, \*\* $P < 0.02$  vs. type 2 basal. □, basal; ■, insulin. DPM, disintegrations per minute; Rosi, rosiglitazone.

However, oxidation rates were still elevated in the type 2 diabetic group compared with lean control subjects and were restored to normal after rosiglitazone treatment.

**Fatty acid transporter/translocator.** Another control point for muscle fatty acid oxidation relates to the ability of muscle cells to take up fatty acids through fatty acid transporters/translocators. Therefore, we measured FAT/CD36 and FATP4 in muscle homogenates (Fig. 5A) plasma membrane and microsomal fractions obtained in both the basal and insulin-stimulated state (Fig. 5B–D). In the lean

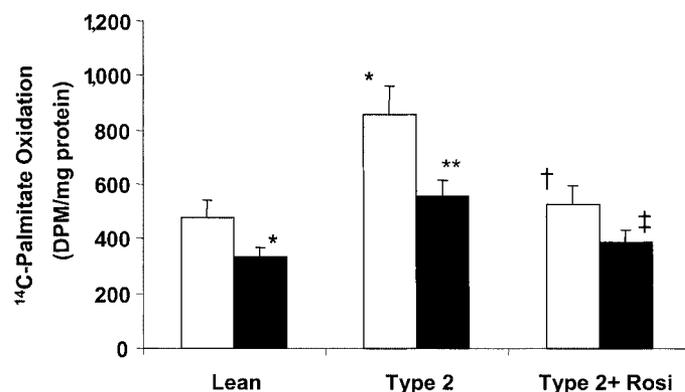
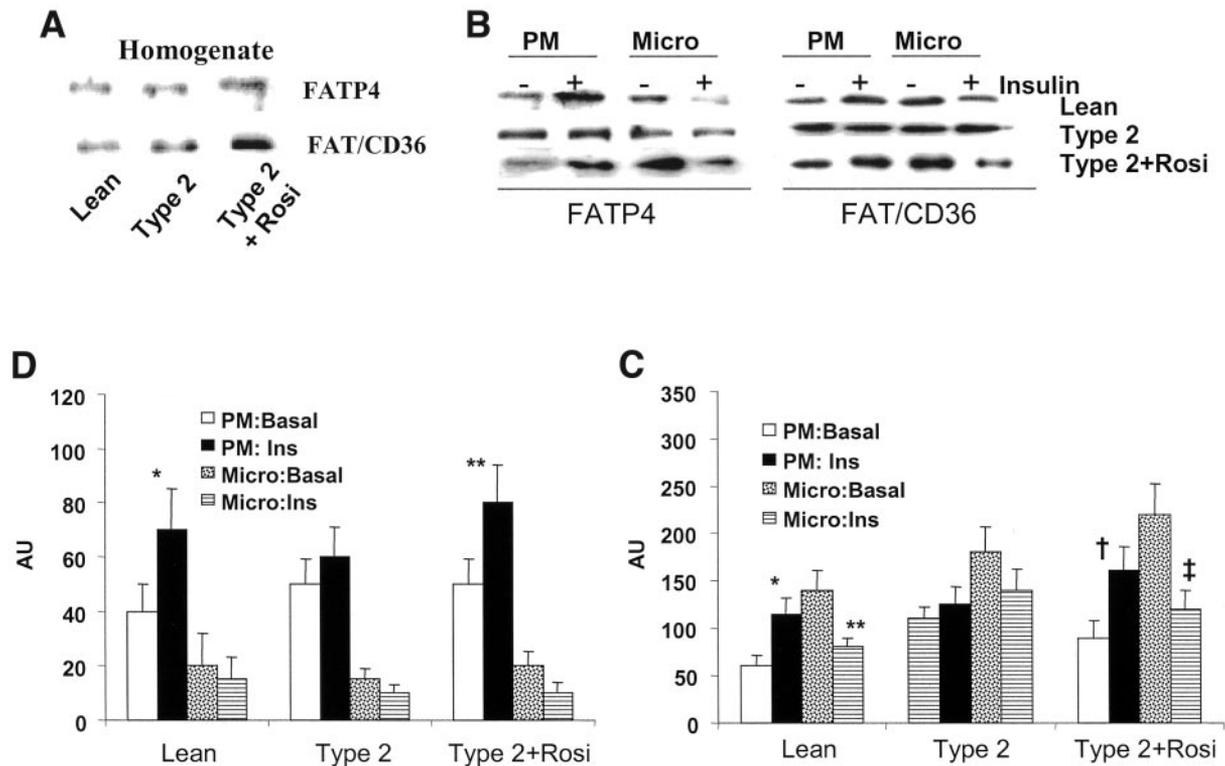


FIG. 4. [1-<sup>14</sup>C]palmitate oxidation in isolated mitochondria in the presence or absence of malonyl-CoA. Mitochondria were isolated from muscle biopsies from subjects as described in Fig. 1. Mitochondria were preincubated with buffer or 25  $\mu\text{mol/l}$  malonyl-CoA for 15 min before adding labeled palmitate. \* $P < 0.01$  vs. lean basal, \*\* $P < 0.05$  vs. lean insulin treated, † $P < 0.03$  vs. type 2 basal, and ‡ $P < 0.05$  vs. type 2 insulin treated. □, basal; ■, plus malonyl-CoA. DPM, disintegrations per minute; Rosi, rosiglitazone.



**FIG. 5.** Expression and insulin-stimulated translocation of fatty acid transporters in muscle membranes before and after treatment with rosiglitazone. The immunoblots representing expression of FATP4 and FAT/CD36 in muscle homogenates (**A**) and insulin-stimulated translocation of FATP4 and FAT/CD36 from microsomal compartment to plasma membrane (**B**) are shown. The signals were subjected to densitometric scan, and the areas under the curves (AU) were compared. Results are presented as histograms showing insulin-stimulated translocation of FAT/CD36 (**C**) and FATP4 (**D**) from microsome to plasma membrane. **C:** \* $P < 0.02$  vs. lean basal plasma membrane, \*\* $P < 0.05$  vs. lean basal microsome, † $P < 0.03$  vs. basal plasma membrane from type 2 diabetes plus rosiglitazone, and ‡ $P < 0.05$  vs. basal microsome from type 2 diabetes plus rosiglitazone. **D:** \* $P < 0.01$  vs. lean basal plasma membrane, \*\* $P < 0.05$  vs. basal plasma membrane from type 2 diabetes plus rosiglitazone. Ins, insulin; Micro, microsomal compartment; PM, plasma membrane; Rosi, rosiglitazone.

subjects, insulin had the expected effect to increase translocation of these two proteins from the microsomes to the plasma membrane, but this insulin effect was absent in the untreated type 2 diabetic subjects. Furthermore, basal levels of FAT/CD36 expression were substantially elevated in plasma membrane fractions of the type 2 diabetic subjects, and a modest increase in the FATP4 content was also noted. FATP1 levels were not different between any of the subject groups (data not shown). After rosiglitazone treatment, basal fatty acid levels of FAT/CD36 and FATP4 remained elevated, but the effect of insulin to induce translocation to the plasma membrane fraction was completely restored (Fig. 5C and D). This is consistent with the view that fatty acid uptake is increased in insulin-resistant type 2 diabetic subjects, but that the intracellular fatty acids are preferentially directed toward lipogenesis rather than oxidation.

It should be mentioned that this increase in expression in muscle is unlikely to be caused by contaminating adipocytes in muscle biopsies because thiazolidinedione-stimulated expression of FAT/CD36 was also observed in human muscle cells in culture, where there should not be any fat cells present (40). Previously, Loviscach et al. (41) assessed expression of the adipocyte-specific protein ALBP in human muscle biopsies and found that the expression level was below detection. We also performed Western blot analysis for ALBP content in the muscle biopsies and found a small signal averaging 3% of that from human fat cells.

## DISCUSSION

In these studies, we have examined intracellular fatty acid metabolism and mitochondrial oxidative capacity in skeletal muscle samples in normal subjects, obese nondiabetic subjects, and patients with type 2 diabetes. Malonyl-CoA is a key intracellular sensor and regulator of intracellular fatty acid flux. The major finding in this article is that there is a decrease in AMP kinase activity and an increase in ACC activity in insulin-resistant muscle that results in elevated intracellular levels of malonyl-CoA. In turn, increased cytosolic malonyl-CoA inhibits flux of fatty acids into the mitochondria, favoring lipogenesis, which is fully consistent with our findings that fatty acid oxidation is decreased in whole-tissue homogenates from skeletal muscle of insulin-resistant subjects, whereas lipogenesis, as measured by long-chain fatty acyl-coA and intramyocellular triacylglycerol, is increased. This was accompanied by increases in the concentration of FAT/CD36 and FATP4 in plasma membranes from the insulin-resistant groups, consistent with increased uptake of fatty acids into muscle. Because, for the most part, the defects appear to be expressed equally in the obese subjects and in type 2 diabetic subjects (who were also obese), we conclude that these differences from lean control subjects are caused by insulin resistance/obesity rather than hyperglycemia/diabetes. Finally, when the type 2 diabetic subjects were treated for 3 months with rosiglitazone, the various defects in fatty acid and mitochondrial metabolism reverted toward normal.

Our data show that insulin-resistant subjects consistently display increased intracellular malonyl-CoA levels in skeletal muscle. This is most likely caused by the coordinate effects we have observed on AMP kinase and ACC. Because ACC2 is predominantly expressed in muscle and heart (42), our measurement of malonyl-CoA likely reflects the activity primarily of ACC2. Previous reports did not find decreased AMPK activity in insulin-resistant human muscle (43–45), whereas skeletal muscle from obese rats did display decreased AMPK and increased ACC activities (46). In studies with obese rat muscle, Lessard et al. (46) demonstrated that the decrease in AMPK activity and phospho-ACC levels were normalized after rosiglitazone treatment, similar to what we observed in our study. As mentioned above, obesity, rather than type 2 diabetes, correlated well with the decreased AMP kinase activity we have reported. In this regard, previous studies have reported no change in skeletal muscle AMP kinase activity in type 2 diabetic subjects compared with control subjects. However, in two of these previous studies, the control group and type 2 diabetic groups were either both lean (44) or both obese (45), so that no differences in adiposity existed between the control and type 2 diabetic subjects. Thus, these results do not disagree with our own data because we found that differences in obesity correlated more closely with decreased AMP kinase activity than did the presence of diabetes. A third study found no change in AMP kinase activity, but it compared lean and obese nondiabetic female subjects (43). Although we cannot fully reconcile that study with our own results, it should be noted that in the report by Steinberg et al. (43), the muscle biopsies were obtained after general anesthesia, and most of the subjects were premenopausal female subjects, whereas none of ours were. It is possible that either of these factors could have influenced the results.

Although we did not measure adiponectin levels in our subjects, we have previously reported (20), as have others (21,47), that adiponectin levels are lower in type 2 diabetic patients and in obese subjects (48) compared with lean control subjects. In addition, Chen et al. (49) and Bruce et al. (50) have provided evidence both in muscle strips and in skeletal muscle cultures that muscle obtained from obese and obese type 2 diabetic subjects is resistant to the effects of adiponectin to stimulate AMP kinase activity. Therefore, we suggest the possibility that decreased adiponectin levels and/or adiponectin resistance might contribute to the reduced AMP kinase activity observed in the skeletal muscle samples obtained from the insulin-resistant groups.

Our findings support the concept that malonyl-CoA can direct intracellular fatty acid flux away from mitochondrial oxidation and toward cytosolic lipogenesis. These findings are consistent with previous reports because other studies have shown diminished basal fatty acid oxidation in insulin-resistant muscle (16,27,51,52). We also found that although fatty acid oxidation was inhibited by the insulin infusion, oxidation rates were not suppressed to normal values in the muscle tissue from the type 2 diabetic subjects, consistent with previous reports (51,53). The elevated malonyl-CoA levels lead to reduced fatty acid oxidation and accumulation of long-chain fatty acyl-CoAs and triglycerides in muscle from insulin-resistant subjects. Although it is unlikely that intracellular triglycerides can cause insulin resistance, previous work from various laboratories, as extensively reviewed by Ruderman and Prentki (4), have observed a correlation between accumu-

lation of malonyl-CoA and increased lipid esterification leading to diacylglycerol accumulation. These studies suggest that increased levels of malonyl-CoA promote insulin resistance by decreasing fatty acid oxidation and increasing diacylglycerol-sensitive protein kinase C activity, which in turn can attenuate insulin action. In insulin-resistant muscle, insulin is not able to suppress fatty acid oxidation further to the level of control muscle (1,51–53). These observations are in general agreement with the “metabolic inflexibility” hypothesis proposed by Kelley et al. (54), which suggests that insulin-resistant muscle tissue does not flexibly switch fuel oxidation from fat to glucose in response to insulin and meals. As a result, fat oxidation remains higher and glucose oxidation remains lower in insulin-resistant muscle compared with insulin-sensitive muscle.

Interestingly, when mitochondria were prepared and studied *in vitro* in the absence of cytosolic factors, an increased capacity for fatty acid oxidation in the type 2 diabetic state was observed. As would be expected, addition of exogenous malonyl-CoA to the incubated muscle homogenates suppressed fatty acid oxidation; however, residual mitochondrial fatty acid oxidation in the diabetic subjects was still higher than in lean groups. This indicates that fatty acid oxidation in mitochondria from the type 2 subjects is less sensitive to inhibition by malonyl-CoA. It is possible that while reduced electron transport activity inside mitochondria diminishes ATP production in type 2 diabetic muscle (38), fatty acid oxidation continues at a higher rate, leading to increased production of lipid peroxides and reactive oxygen species. This could have subsequent deleterious effects through oxidative damage (39,55,56). Regardless of the biochemical mechanism of these defects in fatty acid oxidation, they were reversed by rosiglitazone treatment.

Fatty acid transporters/translocators play a key role in fatty acid metabolism, and our studies demonstrated that in the basal state, the concentrations of FATP4 and FAT/CD36 were elevated in the plasma membrane fractions of the type 2 diabetic subjects. We also showed that insulin stimulation leads to an increase in plasma membrane FATP4 and FAT/CD36, with a corresponding decrease in the microsomal fraction. Importantly, this effect of insulin to translocate FATP4 and FAT/CD36 from microsomes to the plasma membrane was absent in the insulin-resistant type 2 diabetic subjects, representing other aspects of decreased insulin sensitivity. Even though the insulin-stimulated translocation to the plasma membrane is blunted in type 2 diabetic muscle, the overall content of these proteins at the cell surface remains higher than in lean muscle, consistent with increased facilitative fatty acid uptake and triacylglycerol accumulation.

Interestingly, after the type 2 diabetic subjects were treated for 3 months with rosiglitazone, the ability of insulin to mediate translocation of FATP4 and FAT/CD36 was completely restored. Although rosiglitazone treatment restored translocation of FATP4 and FAT/CD36, it also lowered malonyl-CoA levels, and the net effect was to increase fatty acid oxidation.

Taken together, treatment of insulin-resistant subjects with rosiglitazone causes removal of extracellular fatty acids by elevating cell surface transporter levels and diverting intracellular long-chain fatty acyl-coA to mitochondria, resulting in enhanced fatty acid oxidation. As such, these findings represent an additional manifestation of the insulin-resistant state and a new feature of the

generalized insulin-sensitizing effects of thiazolidinediones. In an attempt to place these effects in the total context of insulin resistance and thiazolidinedione-induced insulin sensitization in these patients, it should be noted that rosiglitazone treatment normalized basal phospho-ACC levels, AMPK activity, and malonyl-CoA levels, with near normalization of muscle fatty acid oxidation. It is possible that thiazolidinediones cause mitochondrial remodeling via PPAR $\gamma$  coactivator-1 $\alpha$  (which requires AMPK activity), increasing energy expenditure in muscle cells, as was already shown in adipocytes by Wilson-Fritch et al. (57). With regard to thiazolidinedione-induced changes in mRNA expression for fat-metabolizing enzymes and FAT/CD36, earlier reports suggested that thiazolidinedione effects were significant in adipose but not muscle tissues of type 2 diabetic patients (58). However, thiazolidinedione treatment caused increased mRNA expression of FAT/CD36 in diabetic rat muscle (59) and directly in cultured human muscle cells from type 2 diabetic patients (40). In cultured human muscle cells, where contaminating adipocytes cannot be present, thiazolidinediones increased both mRNA and protein expression of FAT/CD36 and restored fatty acid oxidation in type 2 diabetic muscle (16,40). Because it is not obvious what contributed to these differences in results, further study is required to understand the full role of thiazolidinediones in lipid metabolism in human muscle.

Even though thiazolidinediones cause improved insulin sensitivity in type 2 diabetic patients, only 20–45% of the defects in insulin sensitivity are corrected (Table 2), and they remain quite insulin resistant compared with nondiabetic subjects (14). This would suggest that the reported defects in fatty acid metabolism only account for a portion of the total insulin resistance in these subjects.

In summary, we have found that malonyl-CoA levels are increased in skeletal muscle homogenates from insulin-resistant obese and type 2 diabetic subjects, which could be largely explained by increased ACC and decreased AMP kinase activity. This increase in malonyl-CoA levels directs intracellular fatty acid flux away from oxidation toward long-chain fatty acyl-CoA and triglyceride synthesis. This was associated with decreased fatty acid oxidation in muscle homogenates from the insulin-resistant subjects and a reduced sensitivity of mitochondria preparations to malonyl-CoA-induced inhibition of fatty acid oxidation. Interestingly, all of these defects are reverted toward normal after 3 months of rosiglitazone treatment in the type 2 diabetic subjects.

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