Reduced Lipid Oxidation in Skeletal Muscle From Type 2 Diabetic Subjects May Be of Genetic Origin

Evidence From Cultured Myotubes

Michael Gaster,1 Arild C. Rustan,2 Vigdis Aas,2 and Henning Beck-Nielsen1

Insulin resistance in skeletal muscle in vivo is associated with reduced lipid oxidation and lipid accumulation. It is still uncertain whether changes in lipid metabolism represent an adaptive compensation at the cellular level or a direct expression of a genetic trait. Studies of palmitate metabolism in human myotubes established from control and type 2 diabetic subjects may solve this problem, as genetic defects are preserved and expressed in vitro. In this study, total uptake of palmitic acid was similar in myotubes established from both control and type 2 diabetic subjects under basal conditions and acute insulin stimulation. Myotubes established from diabetic subjects expressed a primary reduced palmitic acid oxidation to carbon dioxide with a concomitantly increased esterification of palmitic acid into phospholipids compared with control myotubes under basal conditions. Triacylglycerol (TAG) content and the incorporation of palmitic acid into diacylglycerol (DAG) and TAG at basal conditions did not vary between the groups. Acute insulin treatment significantly increased palmitate uptake and incorporation of palmitic acid into DAG and TAG in myotubes established from both study groups, but no difference was found in myotubes established from control and diabetic subjects. These results indicate that the reduced lipid oxidation in diabetic skeletal muscle in vivo may be of genetic origin; it also appears that TAG metabolism is not primarily affected in diabetic muscles under basal physiological conditions. Diabetes 53:542–548, 2004

Type 2 diabetes is characterized by hyperglycemia, hyperinsulinemia, reduced ability to oxidize fat, and accumulation of triacylglycerol (TAG) within skeletal muscle fibers. Impaired glucose transport and glycogen synthesis are well documented in insulin-resistant subjects (1–3), but lipid metabolism is less clearly understood. Using the leg-balanced technique, Kelley and Simoneau (4) have demonstrated a diminished uptake of plasma free fatty acids (FFAs) and a reduced lipid oxidation rate of fatty acids in the postabsorptive state in type 2 diabetic patients. Mensink et al. (5) in a recent study showed that FFA uptake and oxidation are also diminished in subjects with impaired glucose tolerance. Furthermore, in obese versus lean subjects, the FFA oxidation rate and enzyme activities are reduced (6), and there is also a reduced oxidative capacity in muscle fibers from obese and type 2 diabetic subjects (7). Recently, Kelly et al. (8) demonstrated that mitochondria size and activity are reduced in obese and type 2 diabetic subjects and are correlated with the degree of insulin resistance. Several studies have found an increased accumulation of TAG in muscle fibers of obese and type 2 diabetic subjects (7,9,10). Intracellular TAG content correlates inversely with insulin resistance (11–15). Lowering of the TAG content by weight loss or training is followed by improved insulin sensitivity. Despite all these studies, it is still uncertain whether the reduced lipid oxidation and accumulation of TAG represent an adaptive compensation at the cellular level and/or are a direct expression of a primary genetic trait. In this context, cultures of human myotubes offer an excellent model for performing studies under standardized conditions. First, satellite cultures express traits known from in vivo muscles (16–20), and second, they allow the genetic and adaptive processes to be differentiated (21). Recently, we described the optimized conditions for satellite culture proliferation and differentiation (19), which have been shown to be suitable for studying the regulation of glycogen synthase activity and the differentiation between induced and primary defects in glucose metabolism in diabetic and normal myotubes (20,21). In the present study, we measured palmitic acid uptake, oxidation, and incorporation into complex cellular lipids in myotubes established from type 2 diabetic subjects and matched control subjects to clarify whether myotubes established from the diabetic subjects expressed inherited defects in fatty acid metabolism.

From the 1Department of Endocrinology, Odense University Hospital, Odense, Denmark; and the 2Department of Pharmacology, School of Pharmacy, University of Oslo, Oslo, Norway.

Address correspondence and reprint requests to Michael Gaster, MD, PhD, Department of Endocrinology, Odense University Hospital, DK-5000, Odense, Denmark. E-mail: michael.gaster@ouh.fyns-amt.dk.

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ASM, acid-soluble metabolite; CPT, carnitine palmitoyltransferase; DAG, diacylglycerol; DMEM, Dulbecco’s modified Eagle’s medium; FFA, free fatty acid; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol.
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RESEARCH DESIGN AND METHODS

Dulbecco’s modified Eagle’s medium (DMEM), FCS, Ultraser G, penicillin-streptomycin-amphotericin B, and trypsin-EDTA were obtained from Life Technology (Scotland, U.K.). [1-14C]palmitic acid (2064.6 MBq/mmol) was purchased from Du Pont-NEN (Boston, MA). The protein assay kit was purchased from BioRad (Copenhagen, Denmark). Palmitic acid, BSA (essentially fatty acid free), L-carnitine, and ECM-gel were purchased from Sigma (St. Louis, MO). Insulin (Actrapid) was obtained from Novo Nordisk ( Bagsvaerd, Denmark).

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TABLE 1
Clinical characteristics of study subjects

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Type 2 diabetic</th>
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<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age (years)</td>
<td>51.1 ± 2.0</td>
<td>50.4 ± 1.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.6 ± 0.9</td>
<td>31.1 ± 1.1</td>
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<tr>
<td>HbA1c (%)</td>
<td>5.1 ± 0.1</td>
<td>6.9 ± 0.5*</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>5.5 ± 0.1</td>
<td>10.3 ± 1.1*</td>
</tr>
<tr>
<td>Fasting serum insulin (pmol/l)</td>
<td>40 ± 5</td>
<td>69 ± 9*</td>
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</tbody>
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Data are means ± SE. *P < 0.05 for diabetic vs. control subjects.

RESULTS
Clinical data for the control and type 2 diabetic patients are given in Table 1. Myotubes established from control and diabetic subjects did not differ in appearance under phase-contrast microscopy (Fig. 1). Lipid metabolism in differentiated myotubes established from control and diabetic subjects was studied using [1-14C]palmitic acid. The cells were incubated with 0.6 mmol/l palmitic acid for 4 h, and the cellular level of free palmitic acid, the incorpora-

FIG. 1. Phase contrast appearance of human myotube cultures. Human satellite cell cultures were established, grown, and differentiated for 8 days under physiological conditions (5.5 mmol/l glucose, 25 pmol/l insulin) as described in RESEARCH DESIGN AND METHODS. Morphological appearance was investigated by phase contrast microscopy. The culture contains many multinucleated myofibres. Magnification ×200.
Palmitate incorporation into cellular lipids.

Incorporation of palmitic acid into complex cellular lipids, and palmitic acid oxidation were measured.

**Total fatty acid uptake.** Total fatty acid uptake (sum of cell-associated lipids and oxidized palmitic acid) was similar in cells from control and diabetic subjects under basal conditions (131.7 ± 14.7 vs. 153.6 ± 8.9 nmol/mg cell protein; P = 0.16) and acute insulin stimulation (171.7 ± 19.7 vs. 186.1 ± 11.5 nmol/mg cell protein; P = 0.18) (Fig. 2).

**Palmitate incorporation into cellular lipids.** Incorporation of palmitic acid into various lipid classes showed that phospholipids were significantly increased under basal conditions (68.0 ± 6.6 vs. 50.0 ± 5.6 nmol/mg protein; P = 0.05), but not under acute insulin stimulation (68.3 ± 5.8 vs. 54.2 ± 5.5 nmol/mg protein; P = 0.16) in diabetic compared with control myotubes (Fig. 3A and B). The incorporation of palmitate into TAG, diacylglycerol (DAG), cholesterol ester, and cellular free palmitate did not show significant differences between control and diabetic myotubes. Palmitate-labeled total cellular lipids were not significantly increased in diabetic myotubes under basal conditions (133.4 ± 10.7 vs. 107.6 ± 12.1 nmol/mg protein; P = 0.08) or acute insulin stimulation (166.5 ± 11.8 vs. 146.3 ± 17.4 nmol/mg protein; P = 0.18) (Fig. 3A and B).

**Fatty acid oxidation.** Oxidation of palmitate was determined by the measurement of ASMs in the cell medium and by the trapping of CO2. Production of CO2 was significantly decreased (by 26%) in myotubes established from type 2 diabetic patients under basal conditions (4.3 ± 0.5 vs. 5.9 ± 0.6 nmol/mg protein; P = 0.03) as well as under acute insulin stimulation (4.3 ± 0.5 vs. 5.9 ± 0.4 nmol/mg protein; P = 0.03) (Fig. 3A and B). ASM and total palmitate oxidation (sum of ASM and CO2) were not significantly decreased in myotubes established from diabetic subjects whether under basal conditions or acute insulin stimulation (Fig. 4A and B). To study the importance of CPT-1 for palmitate oxidation under basal conditions, myotubes were treated with the CPT-1 inhibitor etomoxir (1 μmol/l). Production of CO2 under basal conditions was significantly decreased by etomoxir in both diabetic myotubes (3.5 ± 0.3 vs. 4.3 ± 0.5 nmol/mg protein; P < 0.005) and control myotubes (4.6 ± 0.4 vs. 5.9 ± 0.6 nmol/mg protein; P < 0.005). There was no significant difference between the groups (3.5 ± 0.3 [diabetic] vs. 4.6 ± 0.4 [control] nmol/mg protein; P = 0.11) in the presence of etomoxir. However, etomoxir tended to de-

**FIG. 2.** Uptake of palmitic acid by myotubes established from control and type 2 diabetic (T2D) subjects. Differentiated myotubes (day 8) were exposed to [1-14C]palmitic acid (2.0 μCi/ml, 0.6 mmol/l), 5.5 mmol/l glucose, and 25 pmol/l or 1 μmol/l insulin in serum-free DMEM for 4 h to determine total palmitate uptake under basal and acute insulin stimulation (see RESEARCH DESIGN AND METHODS). Total palmitate uptake was determined as the sum of cell-associated free palmitic acid, palmitic acid–labeled lipids, and oxidized palmitic acid. Data are means ± SE, n = 8 and 10 for control and diabetic myotubes, respectively.

**FIG. 3.** Palmitate incorporation into cellular lipids in myotubes established from control and type 2 diabetic (T2D) subjects. Differentiated myotubes (day 8) were exposed to [1-14C] palmitic acid (2.0 μCi/ml, 0.6 mmol/l), 5.5 mmol/l glucose, and 25 pmol/l or 1 μmol/l insulin in serum-free DMEM for 4 h to determine incorporation of palmitate into various cellular lipids under basal conditions (A) and acute insulin stimulation (B), as described in RESEARCH DESIGN AND METHODS. Data are means ± SE, n = 8 and 10 for control and diabetic myotubes, respectively. *P ≤ 0.05 for diabetic versus control myotubes.
crease CO₂ production more in control than in diabetic myotubes (24.3 ± 3.2 vs. 14.7 ± 3.0%; P = 0.06).

**Acute insulin stimulation.** Figure 5 shows the effect of acute insulin (1 μmol/l) treatment on palmitate metabolism. Acute insulin stimulation significantly increased incorporation of labeled palmitate into TAG by 96 (P = 0.01) and 84% (P = 0.005) and incorporation into DAG by 24 (P = 0.01) and 31% (P = 0.005) for control and diabetic myotubes, respectively. There was no significant effect of insulin on the other lipid classes examined or on oxidation of palmitate (Fig. 5). Total palmitate uptake was increased by 30 (P = 0.01) and 21% (P = 0.007) for control and diabetic myotubes, respectively. However, the insulin responses were not different between the groups. In diabetic as well as control myotubes, about 80% of the insulin-mediated palmitic acid uptake was incorporated into the TAG fraction.

**DISCUSSION**

The main finding of the present study was that myotubes established from type 2 diabetic subjects expressed a primary reduced complete palmitate oxidation with a concomitant increase in the esterification of palmitic acid into phospholipids. Palmitic acid incorporation into DAG and TAG did not differ between the groups, whether under basal conditions or acute insulin stimulation, and TAG content in myotubes established from control and type 2 diabetic subjects did not differ significantly. These results indicate that the reduced lipid oxidation in diabetic muscle observed in vivo may be of genetic origin and that TAG metabolism may not be primarily affected in diabetic muscles under basal physiological conditions.

Previously, it has been described that intracellular TAG is increased (7,9,10) and that oxidation of lipids is reduced in muscle fibers from obese and type 2 diabetic subjects (4,6,27,31); however, the mechanism responsible for these changes has not yet been identified. To gain further insight into this mechanism, we investigated whether these alterations could be partially explained by inherited factors using a model of cultured human myotubes. The myotube model is based on the assumption that preconditioning in vivo is not a serious problem when it comes to acquired
Irreversible defects as a consequence of chronic hyperinsulinemia, hyperlipidemia, and/or hyperglycemia before satellite cell isolation. The contribution of previous metabolic influences may be rather small as isolated quiescent satellite cells are allowed to replicate for weeks in a new environment. In this study, we compared a group of obese control subjects with obese type 2 diabetic subjects to minimize the contribution of adaptive processes, given that the subjects differed only by diabetes-promoting genes.

The reduced lipid oxidation found in this study can be explained by 1) reduced fatty acid (lipid) uptake, 2) reduced entry of acyl-CoA into the mitochondria, 3) reduced number of mitochondria or altered morphology, and/or 4) reduced fatty acid β-oxidation and carbon dioxide formation. In the literature, there is evidence for all these mechanisms. Using the leg-balanced technique, Kelley and Simoneau (4) demonstrated a diminished uptake of plasma fatty acids and lipid oxidation rate in the postabsorptive state in type 2 diabetic patients. More recently, Mensink et al. (5) showed that FFA uptake and oxidation are diminished in subjects with impaired glucose tolerance. Rasmussen et al. (28) showed that malonyl-CoA can regulate CPT-1 activity and fat oxidation in human skeletal muscle. However, Bävenholm et al. (29) did not find any differences in malonyl-CoA concentration in muscle in obese control subjects and type 2 diabetic subjects. Kelley et al. (8) have demonstrated a decreased size and impaired functional capacity of mitochondria in type 2 diabetic subjects. He et al. (7) demonstrated through immunohistochemical studies that muscle fibers from obese and type 2 diabetic subjects express a reduced oxidative capacity. However, in all these studies, it was not possible to differentiate between primary and adaptive consequences. With our model system, which consisted of cultivated human myotubes and was devoid of other environmental factors, we found no difference in fatty acid uptake but did observe a reduced palmitate oxidation in myotubes established from type 2 diabetic subjects compared with those from control subjects. Etomoxir inhibited palmitic acid oxidation to a lesser extent in diabetic myotubes compared with control myotubes, although not significantly, indicating that the CPT-1 activity is reduced or CPT-1 is already inhibited by an increased malonyl-CoA concentration in diabetic myotubes. However, recently Bävenholm et al. (29) showed that changes in muscle malonyl-CoA concentration were similar in obese control subjects and type 2 diabetic subjects, thereby implying that the CPT-1 number may be different. Kelley et al. (8) found that the function of mitochondria is impaired in diabetic muscle, meaning that CPT-1 could be affected. The decreased fatty acid oxidation in diabetic myotubes could also be explained by an increased ongoing hydrolysis of endogenous TAG. This possibility seems unlikely as lipid oxidation did not increase under acute insulin stimulation when we observed an increased palmitic acid uptake and TAG synthesis. Altogether, our study showed that fatty acid oxidation and possibly the mitochondrial function seemed to be reduced in diabetic myotubes compared with control myotubes, suggesting that the reduced lipid oxidation in vivo is, at least partially, of primary origin.

Several studies have found that lipid uptake is reduced in diabetic muscles under fasting conditions (4,27,30,31). Moreover, subjects with impaired glucose tolerance express a reduced fatty acid uptake (5,32). However, lipid uptake did not seem to be primarily affected under basal conditions or acute insulin stimulation in our study, indicating that lipid uptake in vivo was decreased as an adaptive reaction. The mechanism could be a combination of an increased TAG content in diabetic muscle fibers and an increased rate of lipolysis based on mass action, thereby increasing the intracellular FFA concentration and diminishing the FFA uptake from the plasma. In line with this, Blaak et al. (31) described an increased release of glycerol from the forearm muscle of type 2 diabetic subjects.

Increased intramyocellular TAG has been described for obese and type 2 diabetic subjects (7,9,10). We measured the TAG content and the incorporation of palmitate into DAG and TAG at basal conditions and could not show any significant difference between the groups. Moreover, acute insulin stimulation increased palmitic acid incorporation into DAG and TAG to the same extent as it did in myotubes established from control and diabetic subjects. These data imply that the increased TAG accumulation seen in diabetic muscle fibers in vivo is an adaptive event. It has been speculated that reduced oxidation may be related to an increased TAG accumulation, i.e., defects in CPT-1 are associated with TAG accumulation (33). In this study, we provided the first evidence that myotubes established from type 2 diabetic subjects expressed primary reduced palmitic acid oxidation. Fatty acid uptake was not decreased in myotubes established from type 2 diabetic subjects compared with control subjects under basal conditions, implying a concomitant increase in the esterification of palmitic acid. We found that palmitic acid incorporation into phospholipids, but not incorporation into TAG in diabetic versus control myotubes, was significantly increased. Acute insulin stimulation increased palmitate uptake, and the palmitic acid taken up was incorporated into DAG and TAG to the same extent in myotubes established from both control and diabetic subjects, without increasing palmitic acid oxidation. This means that the accumulation of TAG in human myotubes under basal conditions and acute insulin stimulation was not dependent on a reduced lipid oxidation in myotubes precultured under physiological conditions. It has been observed that inhibition of CPT-1 for 4 weeks in rats significantly increased TAG accumulation and muscle insulin resistance (34). Thus, it could be speculated that TAG accumulation would occur if the culturing period were extended to 4 weeks with/without a diabetes-inducing environment (e.g., high glucose, high insulin, high FFA concentrations), either on the basis of a long-lasting lower function of lipid oxidation or an increase in malonyl-CoA by the insulin resistance—inducing environment.

Acute insulin stimulation increased palmitic acid esterification into DAG and TAG in diabetic and control myotubes, but without showing any differences between the groups. The increase in TAG and DAG under insulin stimulation was fully explainable by the increased palmitic acid uptake, suggesting that the induced TAG synthesis under insulin stimulation was completely dependent on exogenous FFA. Phospholipid metabolism seemed to not
be sensitive to acute insulin stimulation, indicating that it was determined by endogenous processes and that the increased esterification of palmitic acid into phospholipids in diabetic myotubes could be genetically determined. In this study, we described how phospholipid esterification from palmitic acid is increased and oxidation is reduced under basal conditions, suggesting that palmitic acid oxidation could be associated with the incorporation of palmitic acid into phospholipids in type 2 diabetic myotubes. It has been observed that diets rich in saturated fatty acids decrease insulin sensitivity, whereas replacement of part of the fat by polyunsaturated fatty acids (PUFAs) prevents this effect (15,35). The content of PUFAs in phospholipids seems to correlate with insulin resistance, suggesting that the PUF content of phospholipids may modulate insulin action (15,36). Pan et al. (37) showed in Pima Indians that the fatty acid profile of skeletal muscle phospholipids was closely linked to insulin action, with a higher proportion of saturated lipids being associated with insulin resistance. We found an increased incorporation of palmitic acid (saturated) into phospholipids in diabetic myotubes, suggesting that these myotubes could be more insulin resistant than control myotubes.

In several in vitro studies, it has been shown that insulin-mediated glycogen synthesis and glucose transport are genetically affected in myotubes established from type 2 diabetic subjects (16,18,21,38). We now added reduced fatty acid oxidation and increased palmitic acid esterification to this picture. The evidence is that human myotubes established from type 2 diabetic subjects express multiple inherited defects in both glucose and lipid metabolism, suggesting an insulin-resistant phenotype.

In conclusion, we have shown that myotubes established from type 2 diabetic subjects express a reduced fatty acid oxidation with a concomitant increase in the esterification of palmitate into phospholipids. Basal and insulin-mediated TAG synthesis did not vary significantly in control and diabetic myotubes precultured under basal physiological conditions.

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