Differential Utilization of Saturated Palmitate and Unsaturated Oleate

Evidence From Cultured Myotubes

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We recently described a primarily reduced palmitate oxidation in myotubes established from type 2 diabetic subjects, whereas triacylglycerol (TAG) accumulation seemed to be adaptive. However, it is still uncertain whether these changes are similar for saturated and unsaturated fatty acids and whether high concentrations of glucose and/or insulin may change this picture. Studies of palmitic acid and oleic acid metabolism in human myotubes established from control and type 2 diabetic subjects under conditions of acute high concentrations of insulin and/or glucose may solve these questions. Total oleic acid and palmitic acid uptake in myotubes was increased during acute insulin stimulation ($P < 0.01$) but not under acute, high-glucose concentrations, and no differences were found between the groups. Type 2 diabetic myotubes expressed a reduced palmitic acid oxidation to carbon dioxide ($P \leq 0.04$), whereas oleic acid oxidation showed no differences between myotubes from both groups. High glucose concentrations decreased oleic acid oxidation ($P \leq 0.03$). Lipid distribution was not different in diabetic and control myotubes when palmitic acid and oleic acid incorporation into cellular lipids was compared. Myotubes that were exposed to palmitic acid showed an increased palmitic acid incorporation into diacylglycerol (DAG) and TAG compared with myotubes that were exposed to oleic acid ($P < 0.05$) expressing an increased intracellular free fatty acid (FFA) level ($P < 0.05$). Lipid distribution was not affected by high glucose, whereas insulin increased FFAs, DAG, and TAG ($P < 0.05$). De novo lipid synthesis from glucose in both diabetic and control myotubes was of the same magnitude independent of glucose and insulin concentrations. These results indicate that palmitic acid and oleic acid are utilized in the same pattern in diabetic and control myotubes even though palmitic acid oxidation is primarily reduced in diabetic cells. Palmitic acid and oleic acid are handled differently by myotubes: Palmitic acid seems to accumulate as DAG and TAG, whereas oleic acid accumulates as intracellular FFAs. These observations indicate that oleic acid is preferable as fatty acid as it accumulates to a lesser extent as DAG and TAG than palmitic acid. Neither acute hyperglycemia nor de novo lipid synthesis from glucose seems central to the TAG accumulation in obesity or type 2 diabetes. *Diabetes* 54:648–656, 2005

T ype 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 individuals (1–3), but lipid metabolism is less clearly understood. A majority of in vivo studies that have investigated the importance of free fatty acids (FFAs) or TAG suggest that increased plasma FFA and TAG concentrations are associated with insulin resistance (4–12). Increased availability of plasma FFA has been associated with insulin resistance through several experiments showing that insulin resistance can be induced within hours by lipid infusion (11–14) or within weeks by feeding rats a high-fat diet (15–17). An inverse correlation between insulin sensitivity and intramyotubular TAG has been shown in muscle tissue (4,5,9,18). Moreover, TAG accumulation can be induced by lipid infusion and in high-fat–fed rats (19). The capacity to oxidize lipids also seems reduced (20,21). We recently showed that complete palmitate oxidation was reduced in diabetic myotubes and that TAG synthesis was not primarily affected (22). Our observations have introduced the following new questions. First, do diabetic myotubes also reduce oxidation for other FFAs? Second, will hyperglycemia further decrease FFA oxidation (23), as hyperglycemia may increase the intracellular content of malonyl-CoA, an inhibitor of carnitine palmitoyltransferase I (CPT-I) responsible for transport of long-chain acyl-CoA into the mitochondria? In line with this, Turcotte et al. (24) showed that increased malonyl-CoA levels induced by high carbohydrate availability is associated with a decreased lipid oxidation in rat muscles. Third, will other FFAs express a different lipid distribution than unsaturated FFAs? Fourth, can lack of TAG accumulation in diabetic myotubes under basal, physiological conditions be explained by a missing high-glucose environment? Glucose oversupply is associated

TABLE 1
Clinical characteristics

	Control subjects	Type 2 diabetic subjects
n	10	10
Age (years)	51.1 ± 2.0	50.4 ± 1.6
BMI (kg/m^2)	29.6 ± 0.9 (24.6–33.9)	31.1 ± 1.1 (26.7–37.0)
HbA$_{1c}$ (%)	5.1 ± 0.1	6.9 ± 0.5*
Fasting plasma glucose (mmol/l)	5.5 ± 0.1	10.3 ± 1.1*
Fasting serum insulin (pmol/l)	40 ± 5	69 ± 9*

Data are means ± SE and range. *$P < 0.05$ for type 2 diabetic vs. control subjects.

with an increase in muscle TAG (25). And fifth, can the TAG accumulation in diabetic muscles be based on de novo synthesis of lipids? High glucose concentrations have been shown to induce de novo lipogenesis in rat myotubes (26). Palmitate (C16:0) and oleate (C18:1) are the most prevalent saturated and unsaturated FFAs in the circulation. To answer the above questions, we measured cellular palmitic acid and oleic acid uptake and their oxidation and incorporation into complex cellular lipids in myotubes established from obese type 2 diabetic subjects and matched control subjects under conditions with and without high glucose and/or insulin concentrations.

RESEARCH DESIGN AND METHODS

Dulbecco's modified Eagle's medium (DMEM), FCS, Ultroser G, penicillin-streptomycin-amphotericin B, and trypsin-EDTA were obtained from Life Technology (Scotland, U.K.). [1-^{14}C]palmitic acid, [1-^{14}C]oleic acid, and D-[^{14}C(U)]glucose were purchased from DuPont, NEN Life Science Products (Boston, MA). Protein assay kit was purchased from BioRad (Copenhagen, Denmark). Palmitic acid, oleic acid, BSA (essentially fatty acid-free), L-carnitine, and ECM gel were purchased from Sigma Chemical Co. (St. Louis, MO). Insulin Actrapid was from Novo Nordisk (Bagsvaerd, Denmark). Thin-layer chromatography plates (Silica gel) were from Merck (Darmstadt, Germany). The scintillation liquids Instagel, Ultima Gold, and Hionic were from Packard Bioscience Company (Groningen, The Netherlands).

Ten middle-aged obese type 2 diabetic patients and 10 matched middle-aged obese control subjects who had a BMI of ~30 kg/m^2 participated in the study (Table 1). Only sedentary male individuals were recruited. None of the diabetic patients received insulin treatment. The patients were without diabetic complications apart from simplex retinopathy. Muscle biopsies were obtained from *m. vastus lateralis* in the fasting state, according to Bergström (27). All participants gave written informed consent, and the local ethics committee of Funen and Vejle County approved the study.

Cell cultures were established as described in connection with our previous studies (28,29). In brief, muscle tissue was minced, washed, and dissociated for 60 min by three treatments using 0.05% trypsin-EDTA. The harvested cells were pooled, and FCS was added as a protease inhibitor. The cells obtained were seeded for upscaling on ECM gel–coated dishes after 30 min of preplating. Cell cultures were established in DMEM medium supplemented with 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml amphotericin B. After 24h, cell debris and nonadherent cells were removed by changing the growth medium to DMEM supplemented with 2% FCS, 2% Ultroser G, 50 units/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml amphotericin B. Cells were subcultured twice before final seeding. At 75% confluence the growth medium was replaced by basal medium (DMEM supplemented with 2% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml amphotericin B, and 25 pmol/l insulin) to induce differentiation. The cells were cultured in humidified 5% CO$_2$ atmosphere at 37°C, and the medium was changed every 2–3 days.

Human myotubes established from control subjects and type 2 diabetic subjects were allowed to differentiate under physiological conditions of insulin (25 pmol/l) and glucose (5.5 mmol/l) for 8 days. All myotube cultures were used for analysis on day 8 after onset of differentiation. Myotubes were rinsed twice and incubated for 4 h with radiolabeled 0.6 mmol/l palmitic acid or oleic acid with either 5.5 or 20 mmol/l glucose and supplemented with either 25 pmol/l or 1 μmol/l insulin to clarify the impact of acute high insulin and/or glucose concentrations on the oleic acid and palmitic acid metabolism in myotubes established from control or type 2 diabetic subjects. To eval-uate the de novo lipid synthesis, we studied the formation of lipids from [^{14}C]glucose under the same experimental conditions.

Analysis

Fatty acid oxidation. Cells were cultured in 12.5-cm^2 flasks and differentiated as described above. Myotubes were exposed to DMEM supplemented with 0.24 mmol/l fatty acid-free albumin (BSA), 0.5 mmol/l L-carnitine, 20 mmol/l HEPES, either [1-^{14}C]oleic acid (2.0 μCi/ml, 0.6 mmol/l) or [1-^{14}C] palmitic acid (2.0 μCi/ml, 0.6 mmol/l) with 5.5 or 20 mmol/l glucose, and 25 pmol/l or 1 μmol/l insulin (22). After CO$_2$ trapping, the incubation media were transferred to new tubes and assayed for labeled β-oxidation products (acid-soluble metabolites [ASMs]) (30). The protein content of each sample was determined as described previously (31).

Lipid profile. Cells were cultured in six-well plates and differentiated as described above. Myotubes were exposed to DMEM supplemented with 0.24 mmol/l fatty acid-free albumin (BSA), 0.5 mmol/l L-carnitine, 20 mmol/l HEPES, either [1-^{14}C]oleic acid (2.0 μCi/ml, 0.6 mmol/l) or [1-^{14}C]palmitic acid (2.0 μCi/ml, 0.6 mmol/l) with 5.5 or 20 mmol/l glucose, and 25 pmol/l or 1 μmol/l insulin and analyzed as previously described (22). Cell-associated lipids were extracted with chloroform:methanol as described (32). A total of 400 μl of cell homogenate was mixed with 8 ml of chloroform:methanol (2:1 vol/vol), and FCS (30 μl) was added as a carrier. After 30 min, 1.6 ml of 0.9% NaCl (pH 2) was added and the mixture was centrifuged (1,000g, 5 min). The organic phase was evaporated under a nitrogen steam at 40°C. The residual lipid extract was redissolved in 200 μl of n-hexane and separated by thin-layer chromatography using hexane:diethylether:acetic acid (65:35:1) as the mobile phase. The bands were visualized with iodine, excised, and counted by liquid scintillation. Because no difference was found for lipid parameters between the groups, the pooled data are presented.

Incorporation of [^{14}C]glucose into lipids. Extraction of lipids was also carried out after incubation of myotubes with D-[^{14}C(U)]glucose for 4 h. Myotubes were exposed to DMEM supplemented with 0.24 mmol/l fatty acid-free albumin (BSA), 0.5 mmol/l L-carnitine, 20 mmol/l HEPES, 5.5 or 20 mmol/l glucose [D-^{14}C(U) 2.0 μCi/ml], and 25 pmol/l or 1 μmol/l insulin. On the basis of a very low incorporation, we determined the overall glucose incorporation into lipids by counting the total lipid extract washed with a water phase that contained 0.5 mol/l glucose to displace all glucose labeled (32).

Statistical analysis. Data in text, tables, and figures are given as means ± SE for all experiments run in duplicate or triplicate. The number of different contributing myotube cultures in each experiment is indicated in the figure legends. Statistical analyses were performed with INSTAT 2.01 (GraphPad, San Diego, CA). Student's t test was used for unpaired and the paired t test was used for paired comparisons. $P \leq 0.05$ was considered significant.

RESULTS

Clinical data are given in Table 1. Myotubes that were established from control and type 2 diabetic subjects did not differ in appearance under phase-contrast microscopy or during oleic acid or palmitic acid exposure (data not shown).

Total fatty acid uptake. Total fatty acid uptake (sum of cell-associated lipids and oxidized palmitic acid or oleic acid) was similar in control and diabetic myotubes under basal conditions and during acute insulin stimulation with or without high glucose concentrations (Table 2). The total palmitate uptake did not differ from the total oleate uptake under the experimental conditions examined. Palmitate uptake was insulin sensitive for both diabetic

TABLE 2
Total FFA uptake

	Control myotubes	Type 2 diabetic myotubes	Overall
Palmitic acid			
G-Ins	165.3 ± 20.4*	174.9 ± 10.2*	170.4 ± 11.1*
G	130.3 ± 14.9	127.8 ± 7.7	129.0 ± 8.0
HG-Ins	179.0 ± 13.7*	183.7 ± 12.7*	181.4 ± 9.5*
HG	155.5 ± 9.9†	141.8 ± 8.2	148.3 ± 6.6
Oleic acid			
G-Ins	151.8 ± 9.3	144.1 ± 19.0	147.9 ± 10.2*
G	141.7 ± 8.3	126.7 ± 12.6	134.2 ± 7.6
HG-Ins	165.8 ± 14.3	144.0 ± 13.8*	154.9 ± 10.2*
HG	152.2 ± 13.7	111.6 ± 12.5	131.9 ± 10.9

Data are means ± SE (nmol/mg protein). G-Ins, acute insulin stimulation; G, basal conditions; HG-Ins, both high glucose and high insulin concentration; HG, high glucose concentration. *$P < 0.05$ vs. corresponding condition without insulin; †$P < 0.05$ vs. corresponding condition without high glucose.

and control myotubes; oleate uptake expressed the same tendency but reached no significance under all conditions. However, both overall palmitate and oleate uptake was insulin sensitive, whereas acute high glucose concentration was not important for the total palmitate or oleate uptake.

Palmitic acid and oleic acid incorporation into cellular lipids. Palmitic acid and oleic acid incorporation into various lipid classes in control and type 2 diabetic myotubes is shown in Table 3. We demonstrated no differences in palmitic acid or oleic acid incorporation when comparing diabetic and control myotubes; therefore, we pooled the data from both groups. Our study showed that oleic acid was found mainly in phospholipids, as free oleic acid and to a lesser extent as TAG, whereas palmitic acid was incorporated into phospholipids and TAG and to a lesser extent found as cellular free palmitic acid. Palmitic acid incorporation into diacylglycerol (DAG) and TAG was significantly increased compared with oleic acid incorporation. Acute insulin stimulation significantly increased palmitic acid and oleic acid incorporation into DAG and TAG, and it furthermore increased the cellular level of free palmitic acid or oleic acid, respectively. In contrast to this, high glucose concentrations seemed to be of minor importance for the oleic acid and palmitic acid incorporation into complex lipids, e.g., palmitic acid incorporation into TAG. Despite the different palmitic acid and oleic acid incorporations into cellular lipid classes, the total amount of oleic acid–labeled and palmitic acid–labeled cellular lipids was not significantly different under the various conditions examined.

Fatty acid oxidation. Palmitic acid and oleic acid oxidation was determined by ASM measurement and formation of CO_2 from the myotubes. Production of CO_2 from palmitic acid was significantly decreased by 33% in myotubes from type 2 diabetic subjects under basal conditions (9.5 ± 1.3 vs. 6.4 ± 0.7 nmol/mg protein; $P = 0.04$) as well as during acute insulin stimulation and/or with high glucose concentration (Fig. 1A). Although insulin and high glucose concentrations seemed to increase palmitic acid oxidation, this could not be statistically verified for either diabetic or control myotubes. For evaluating whether

insulin and high glucose concentrations had an impact on palmitic acid oxidation, the CO_2 production values under the various conditions in each experiment were normalized by the average value from the single-exposure condition, and the data from control and diabetic myotubes were pooled. However, insulin and high glucose concentrations showed no significant effects on CO_2 production (data not shown). CO_2 production from oleic acid was not significantly different between myotubes from type 2 diabetic subjects and control subjects under basal conditions (8.6 ± 1.3 vs. 7.1 ± 1.2 nmol/mg protein; $P = 0.65$) or under acute insulin stimulation and/or with high glucose concentration (Fig. 1B). We pooled the two groups to increase the sensitivity to detect the impact of insulin and high glucose on oleic acid oxidation. High glucose concentration significantly reduced oleic acid oxidation to CO_2 in myotubes both with (7.7 ± 1.0 vs. 7.0 ± 0.8 nmol/mg protein; $P < 0.01$) and without insulin stimulation (7.8 ± 0.9 vs. 6.8 ± 0.6 nmol/mg protein; $P = 0.03$). Insulin stimulation increased β-oxidation (ASM) of palmitic acid in both diabetic and control myotubes, whereas high glucose concentrations did not affect palmitic acid oxidation (Fig. 2A). In contrast, ASMs from oleic acid were reduced in

TABLE 3
FFA distribution

	Palmitic acid control + type 2 diabetic myotubes	Oleic acid control + type 2 diabetic myotubes
FFA		
G-Ins	28.4 ± 2.1*†	56.6 ± 6.9†
G	25.6 ± 1.7*	40.7 ± 6.7
HG-Ins	29.7 ± 2.2*†	60.6 ± 7.5†
HG	27.8 ± 1.6*	46.4 ± 8.1
DAG		
G-Ins	7.0 ± 0.4*†	5.4 ± 0.7†
G	5.5 ± 0.4*	3.8 ± 0.6
HG-Ins	7.5 ± 0.5*†	5.5 ± 1.0†
HG	6.2 ± 0.5*	3.7 ± 0.7
TAG		
G-Ins	53.9 ± 4.5*†	9.0 ± 2.3†
G	25.6 ± 2.4*	4.8 ± 1.3
HG-Ins	51.5 ± 4.5*†	8.3 ± 2.4†
HG	30.2 ± 3.0*‡	4.5 ± 1.3
Phospholipids		
G-Ins	51.2 ± 2.6	48.4 ± 3.6
G	47.4 ± 2.6	48.4 ± 2.8
HG-Ins	51.9 ± 2.6	50.5 ± 3.1
HG	51.0 ± 3.1	49.0 ± 3.8
Cholesterol ester		
G-Ins	1.9 ± 0.1*	1.4 ± 0.2
G	1.7 ± 0.2	1.5 ± 0.2
HG-Ins	1.9 ± 0.2	1.6 ± 0.2
HG	1.9 ± 0.2	1.4 ± 0.2
Sum lipid		
G-Ins	142.4 ± 7.9†	119.7 ± 8.6†
G	105.7 ± 5.8	100.1 ± 7.7
HG-Ins	142.4 ± 8.2†	126.4 ± 9.0†
HG	117.1 ± 5.6	105.0 ± 10.9

Data are means ± SE (nmol/mg protein). G-ins, acute insulin stimulation; G, basal conditions; Hg-ins, both high glucose and high insulin concentration; HG, high glucose concentration. *$P < 0.05$ vs. corresponding condition during oleic acid exposure; †$P < 0.05$ vs. corresponding condition without insulin; ‡$P < 0.05$ vs. corresponding condition without high glucose.
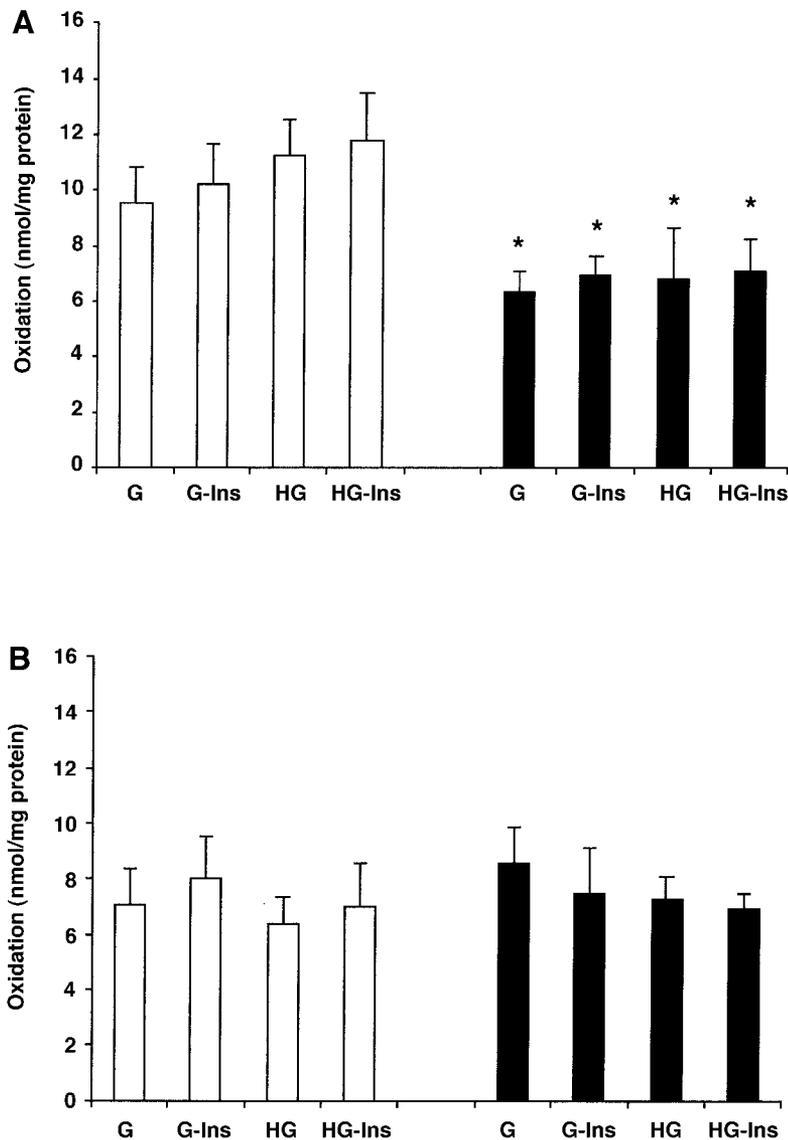
FIG. 1. Oxidation of palmitic acid (*A*) and oleic acid (*B*) to CO_2 in myotubes that were established from control and type 2 diabetic subjects. Differentiated myotubes (day 8) were exposed to [1-^{14}C]palmitic acid (2.0 µCi/ml, 0.6 mmol/l) or [1-^{14}C]oleic acid (2.0 µCi/ml, 0.6 mmol/l), 5.5 mmol/l glucose, and 25 pmol/l insulin or supplemented with 1 µmol/l insulin, 20 mmol/l glucose, or both in serum-free DMEM for 4 h to determine palmitate or oleate oxidation under basal (G), acute insulin stimulation (G-Ins), high glucose concentration (HG), or both high glucose and high insulin concentration (HG-Ins), as described in RESEARCH DESIGN AND METHODS. Results are shown from control (□) and diabetic myotubes (■). Data are shown as means ± SE; *n* = 10 for control and diabetic myotubes, respectively. *$P \leq 0.05$ for type 2 diabetic vs. control myotubes.

diabetic myotubes that were exposed to high glucose concentrations, whereas control myotubes were not affected by insulin or high glucose (Fig. 2*B*).

Acute insulin stimulation. The effect of acute insulin (1 µmol/l) treatment of palmitic acid and oleic acid metabolism in myotubes is shown in Fig. 3. Total palmitic acid uptake was increased by 30% ($P < 0.01$) and oleic acid by 17% ($P < 0.01$). Insulin-stimulated palmitic acid uptake was significantly increased compared with oleic acid uptake ($P < 0.01$). Acute insulin stimulation significantly increased the incorporation of labeled palmitic acid into FFAs, DAG, and TAG and stimulated total palmitic acid oxidation, whereas acute insulin stimulation increased only the incorporation of labeled oleic acid into FFAs, DAG, and TAG. The palmitic acid oxidation and incorporation into TAG were significantly increased compared with corresponding findings for oleic acid. However, the insulin-mediated FFA increase for oleic acid was significantly increased compared with palmitic acid. Approximately 65% of the insulin-mediated palmitic acid uptake was found in the TAG fraction, whereas 30% of the insulin-mediated oleic acid was incorporated into TAG. Analyzing the impact of acute high glucose–mediated

changes in palmitic acid and oleic acid metabolism revealed no impact on palmitic acid or oleic acid metabolism apart from oleic acid oxidation being sensitive to high glucose and palmitic acid oxidation not being sensitive to high glucose (data not shown).

De novo lipid synthesis from glucose. We also investigated whether glucose incorporation into lipids varied between myotubes established from control and type 2 diabetic subjects under basal physiological conditions. No significant differences were found between control and diabetic myotubes in the total amount of [^{14}C]glucose-labeled cellular lipids (Fig. 4). De novo lipogenesis was low in myotubes as its order of magnitude was ~2–3% of what was taken up during the same time when myotubes were exposed to oleic acid or palmitic acid. Neither acute high glucose nor high insulin concentrations increased de novo lipogenesis from glucose in diabetic and control myotubes.

DISCUSSION

The main finding of the present study is that palmitic acid and oleic acid are identically utilized in diabetic and control myotubes, but palmitic acid oxidation is reduced
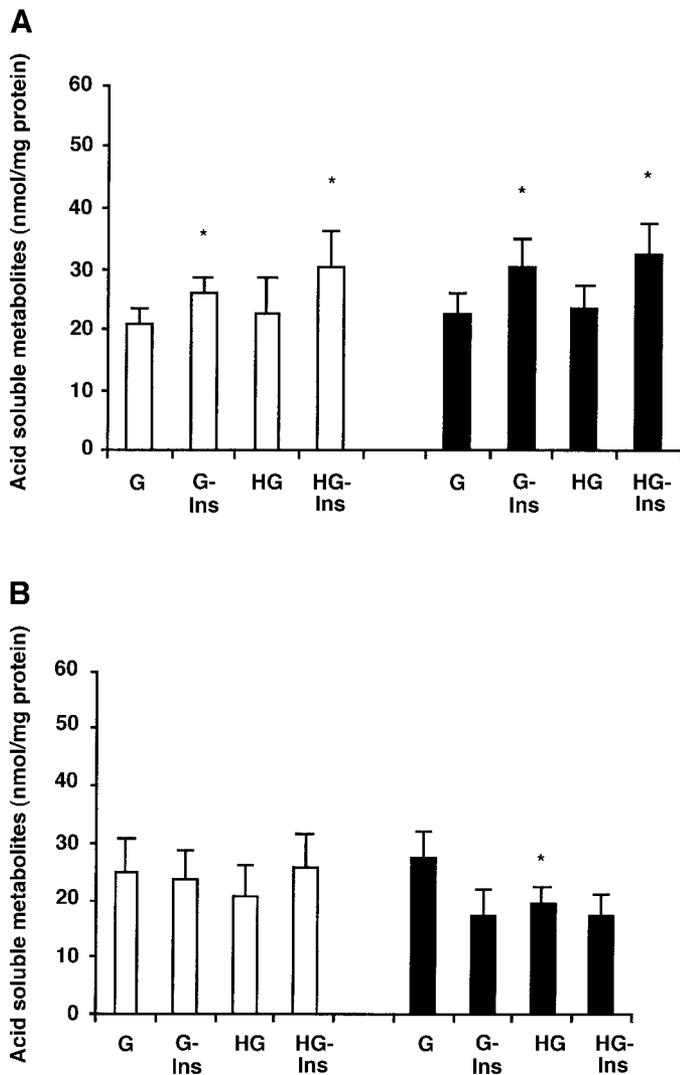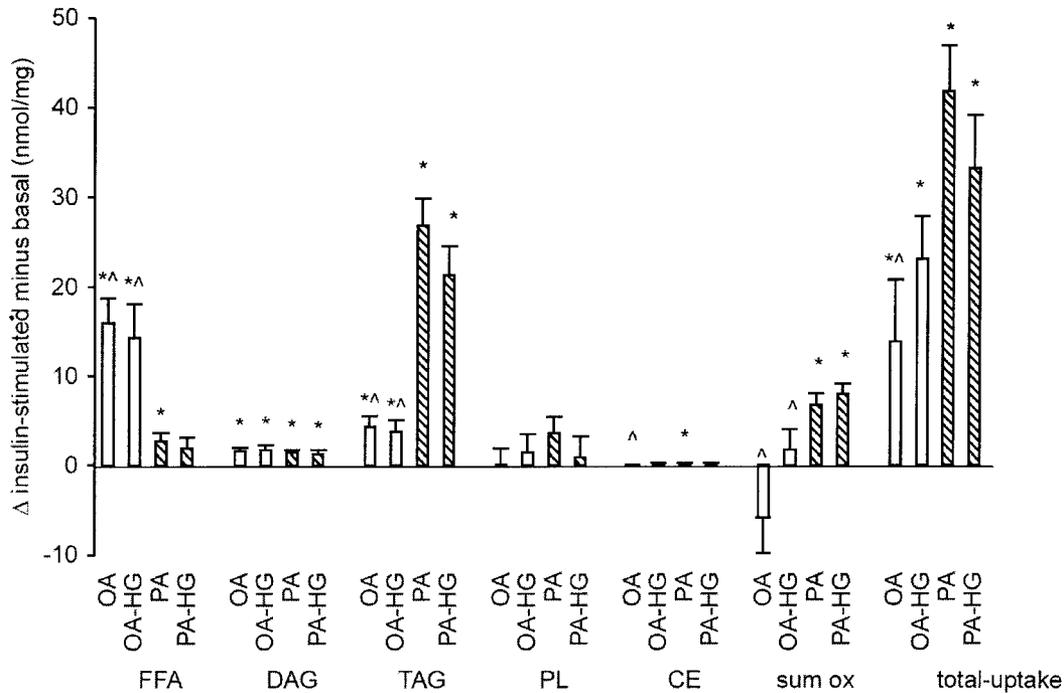
A

B

FIG. 2. Oxidation of palmitic acid (*A*) and oleic acid (*B*) to ASMs in myotubes established from control and type 2 diabetic subjects. Differentiated myotubes (day 8) were exposed to [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid (2.0 μCi/ml, 0.6 mmol/l), 5.5 mmol/l glucose, and 25 pmol/l insulin or supplemented with 1 μmol/l insulin, 20 mmol/l glucose, or both in serum-free DMEM for 4 h to determine palmitate oxidation as ASMs under basal (G), acute insulin stimulation (G-Ins), high glucose concentration (HG), or both high glucose and high insulin concentration (HG-Ins), as described in RESEARCH DESIGN AND METHODS. Results are shown from control (□) and diabetic myotubes (■) as means ± SE; *n* = 10 for control and diabetic myotubes. *P ≤ 0.05 vs. corresponding basal condition without insulin or corresponding condition without high glucose.

in diabetic myotubes. Palmitic acid and oleic acid, however, are handled differently in myotubes: Palmitic acid seems to accumulate as DAG and TAG, whereas oleic acid accumulates as intracellular FFAs. Lipid metabolism seems unaffected by high glucose concentrations, but Randle's inverse cycle seems active in myotubes that are exposed to oleic acid. De novo lipid synthesis from glucose does not seem to be a major contributor to increased intracellular lipid levels in obese and obese diabetic muscles. These observations indicate that unsaturated oleic acid is preferable as fatty acid in dietary fat as it accumulates as DAG and TAG to a lesser extent than palmitic acid. Moreover, the present study further supports that changes in lipid metabolism in vivo are adaptive, except for the reduced palmitic acid oxidation.

We measured total oleic acid and palmitic acid uptake in myotubes that were established from control and type 2 diabetic subjects under basal conditions and during acute insulin stimulation with and without high glucose but were not able to show any significant difference between myotubes from both groups. This indicates that lipid uptake is not primarily affected, thus suggesting that the reduced lipid uptake in diabetic muscles (33–36) in vivo is decreased as an adaptive reaction. Absolute rates of fatty acid uptake differ by up to 2 orders of magnitude between myotubes and in vivo muscle (11,23), which may be explained by the higher surface-to-cell volume ratio in myotubes.

Muscle fibers from obese and type 2 diabetic subjects express a reduced lipid oxidation (33,34,36,37), but the responsible mechanism has not yet been identified (please see discussion by Gaster et al. [22] for more details). We recently showed that myotubes established from type 2 diabetic subjects express a primarily reduced palmitic acid oxidation, indicating that the reduced lipid oxidation in diabetic skeletal muscle in vivo may be of genetic origin. In the present study, we confirmed that palmitic acid oxidation to CO_2 is reduced in diabetic myotubes. In contrast to palmitic acid, oleic acid was not oxidized differently in diabetic and control myotubes, thus suggesting that diabetic myotubes express a specific defect associated with type 2 diabetes. This observation is surprising as we also expected diabetic myotubes to express a lower oleic acid oxidation. The reason for this finding is not clear, but it may be based on differences in palmitic acid and oleic acid oxidation fluxes. Oleic acid oxidation in both control and diabetic myotubes reached the palmitic acid oxidation level only in diabetic myotubes, thus suggesting that the oleic acid flux may not be sufficient to detect an impaired FFA oxidation. To support this hypothesis, oleic acid was found not to accumulate into DAG and TAG to the same extent as palmitic acid, probably as a result of differences in substrate specificity (see later in DISCUSSION). Siddosis et al. (23) showed that oleic acid combined with high glucose caused reduced oleic acid oxidation. The responsible mechanism seems to be high glucose concentrations increasing the concentration of malonyl-CoA and thereby inhibiting the entrance of oleic acid into the mitochondria. This mechanism is designated Randle's inverse cycle. Guillet-Deniau et al. (26) reported that acute glucose exposure to rat myotubes upregulated acetyl-CoA carboxylase, the enzyme that is responsible for malonyl-CoA synthesis, through a sterol-regulatory-element-binding-protein-1c–dependent mechanism. In the present study, high glucose concentrations attenuated the oleic acid oxidation in myotubes but was less pronounced compared with in vivo (23). Palmitic acid oxidation was not sensitive to high glucose concentrations. Moreover, palmitic acid oxidation was insulin sensitive in both control and diabetic myotubes, but this pattern was not found for oleic acid oxidation. At present, we do not know which enzymes could express specificity for palmitic acid and at the same time also be selectively insulin sensitive to palmitic acid. The explanation for this discrepancy is not obvious, but it may indicate that the regulatory mechanism could be different for various FFAs or modified by the single FFA. Further studies are needed to clarify this issue.

FIG. 3. Effects of acute insulin stimulation on palmitic acid (PA) and oleic acid (OA) metabolism in myotubes established from control and type 2 diabetic subjects. Differentiated myotubes (day 8) were exposed to [1-^{14}C]PA or [1-^{14}C]OA (2.0 μCi/ml, 0.6 mmol/l), 5.5 mmol/l glucose, and 25 pmol/l insulin or supplemented with 1 μmol/l insulin, 20 mmol/l glucose, or both in serum-free DMEM for 4 h to determine palmitate and oleate metabolism under basal (G), acute insulin stimulation (G-Ins), high glucose concentration (HG), or both high glucose and high insulin cencentration (HG-Ins), as described in RESEARCH DESIGN AND METHODS. The acute insulin effect was calculated as the absolute difference between insulin-stimulated and basal values. Sum ox, acid soluble metabolites and CO_2 accumulation; total uptake, sum of cell-associated lipids and sum ox; OA and PA, exposure to oleate (□) or palmitate (▨), respectively. Data are shown as means ± SE (n = 20). Significant insulin effect: *P = 0.05 and ^P = 0.05 vs. corresponding value for PA.

Extracellular FFAs cross the sarcolemma either by diffusion or mediation by different transporters such as CD36/FAT, FATP-1, and FABPpm (38). Intracellular FFAs are activated by acyl-CoA synthases to long-chain acyl-CoA consequently entering numerous pathways, i.e., incorporation into complex lipids, oxidation, or cholesterol esterification. Certain lipid metabolites have been associated with insulin resistance, long-chain acyl-CoA (LCA-CoA), DAG, and ceramides (a palmitate derivative). They are second messengers and may affect lipid and glucose metabolism directly (39–41). We found that FFAs were increased in myotubes that were exposed to oleic acid during 4 h compared with palmitic acid, a time span

known not to induce insulin resistance and suggesting that myotubes may tolerate high FFA concentrations. The intracellular FFA level seems to depend on acute insulin stimulation, thus suggesting that either FFA uptake or activation by acyl-CoA synthases may be insulin sensitive. CD36/FAT translocation by insulin has been described (42) and indicated that insulin-mediated FFA uptake may happen through an increased CD36/FAT expression or translocation into the plasma membrane. Recently, Chabowski et al. (43) showed for cardiac cells that insulin regulates the protein expression of the LCFA transporter FAT/CD36 but not FABPpm via the phosphatidylinositol 3-kinase/protein kinase B (PKB) insulin-signaling pathway.
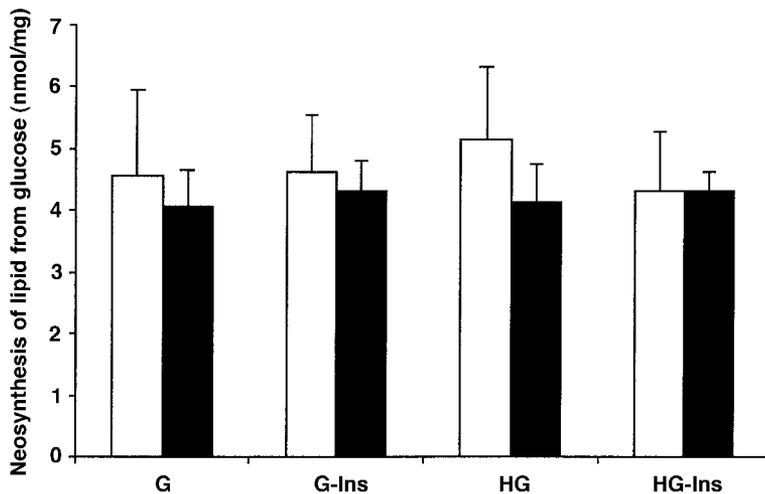
FIG. 4. Lipogenesis in myotubes that were established from control and type 2 diabetic subjects. Differentiated myotubes (day 8) were exposed to [1-^{14}C]glucose (2.0 μCi/ml, 5.5 mmol/l) and 25 pmol/l insulin or supplemented with 1 μmol/l insulin or 20 mmol/l glucose or both in serum-free DMEM for 4 h to determine de novo lipid synthesis (lipogenesis) under basal (G), acute insulin stimulation (G-Ins), high glucose concentration (HG), or both high glucose and high insulin concentration (HG-Ins), as described in RESEARCH DESIGN AND METHODS. Results are shown for control (□) and diabetic myotubes (■) as means ± SE; n = 4 for control and diabetic myotubes.

LCA-CoAs are key metabolites in lipid metabolism and play a role in regulating other cellular processes and are inversely correlated with insulin action (19). Very little is known about oleic acid and its CoA derivative compared with palmitic acid and palmitoyl-CoA. It has been shown that palmitic acid can exert effects on insulin-signaling pathways without converting into palmitoyl-CoA (44). FFA-induced insulin resistance is associated with an impairment of insulin receptor substrate-1 tyrosine phosphorylation and insulin receptor substrate-1–associated phosphatidylinositol 3-kinase activation (12,45,46). Hexokinase II in human skeletal muscle catalyzing the phosphorylation of glucose to glucose-6-phosphate (G6P) is allosterically inhibited by palmitoyl-CoA as well as other LCA-CoAs (47,48). G6P is an important intermediate metabolite at the branch point of the glycogen synthesis and glycolysis pathways; hence, altered G6P concentrations may affect the rates of these processes. Ceramide is a palmitate derivative and a second messenger produced by sphingomyelinase activation, and it is known to be increased in insulin-resistant cell systems (40,49). Studies have shown that PKB activation can be reduced in the presence of ceramide (41).

Palmitic acid seems to be incorporated into DAG to a higher extent than oleic acid in myotubes, suggesting that various FFAs may be utilized differently in myotubes, which is in line with the study by Montell et al. (50). DAG has been shown to block insulin signaling and glucose uptake and glycogen synthesis in cultured cells (50–52). DAG from palmitic acid was associated with activation of protein kinase C, which has been shown to desensitize insulin stimulation of glucose uptake in human skeletal muscle cells (50). Recently, DAG was accumulated in C2C12 myotubes that were exposed to palmitate and other saturated FFAs but not when exposed to oleic acid (52), with corresponding abolished insulin-mediated glycogen synthesis for palmitic acid but not for oleic acid (52); the mechanism seems to be mediated through PKB and ceramides (52). Thus, even though oleic acid accumulates to a higher extent as FFAs, the palmitic acid accumulation or its derivatives seem to be associated with a more insulin-resistant promoting potential through palmitic acid, palmitoyl-CoA, palmitic acid incorporated into DAG, and ceramides than oleic acid.

Recently, we described that TAG accumulation during palmitic acid exposure was not primarily affected in myotubes that were established from control and type 2 diabetic subjects (22). In the present study, we measured the oleic acid and palmitic acid incorporation into TAG under basal conditions and during acute insulin stimulation, but we were not able to show any significant differences between myotubes that were established from control and diabetic subjects. Moreover, high glucose concentrations had no impact either. On the basis of our results, the increased TAG accumulation in obese and type 2 diabetic muscle fibers in vivo seems to be an adaptive event, with the magnitude possibly depending on the FFA composition of the diet. Palmitic acid also seems to be incorporated into TAG in myotubes to a higher extent than oleic acid. This observation is surprising and unexpected. The equal oleic acid and palmitic acid incorporation into phospholipids and the increased palmitic acid incorpora-

tion into TAG in the same myotubes with nearly equal total fatty acid uptake suggests that the overall enzyme system for TAG biosynthesis may be present. The first committed step of glycerolipid biosynthesis is the acylation of glycerol-3-phosphate catalyzed by glycerol-3-phosphate acyltransferase (GPAT) (rev. in 53,54). Two isoforms have been described: a microsomal and a mitochondrial isoform (mtGPAT). The mtGPAT has 3- to 10-fold higher activity with palmitoyl-CoA than oleoyl-CoA. In the present study, we found that palmitic acid was incorporated five to six times more abundantly in TAG than oleic acid, thus suggesting that myotubes synthesize glycerolipids through mtGPAT. In line with this, oleic acid incorporation in DAG was also reduced in myotubes but not to the same extent. In contrast, incorporation of oleate into phospholipids was not impaired. The reason for this could be that the reacylation pathway seems functionally separated from glycerolipid synthesis, as triacsin (55), a fungal-derived competitive inhibitor of LCA-CoA synthetases (ACS, isoforms 1 and 4), seems to inhibit glycerolipid biosynthesis and de novo phospholipid synthesis, whereas incorporation of oleate into phospholipids through reacylation was unaffected. A reduced flux through the pathways for FFA storage may increase the intracellular FFA and acyl-CoA levels, as observed in the present study.

Lipogenesis has been suspected to contribute to the increased TAG in obesity and type 2 diabetes, but the presence of fatty acid synthase—the rate-limiting step for synthesis of fatty acids—has not been possible to detect because humans are supplied by exogenous lipids that suppress lipogenesis. We observed that myotubes that were established from control and type 2 diabetic subjects both expressed de novo lipogenesis under conditions of no exogenous lipid, which was equal and not affected by either insulin stimulation or high glucose concentrations. Lipogenesis was only at the level of a few percent of the FFA amount taken up during the same time period, and it did not seem important to the TAG differences in vivo between obese control and type 2 diabetic subjects. Thus, conversion of labeled glucose into lipids denoted that de novo lipogenesis can take place in myotubes, but this does not seem to be the explanation for the increased intramyotubular TAG in obese and type 2 diabetic subjects.

We studied the utilization of oleic acid and palmitic acid in myotubes under basal conditions and under acute insulin stimulation with and without high glucose and were able to show that palmitic acid and oleic acid are metabolized differently. Palmitic acid exposure seems associated with DAG and TAG accumulation and a reduced lipid oxidation in diabetic myotubes, whereas myotubes that are exposed to oleic acid express an increased intracellular FFA level. The differences in oleic acid and palmitic acid metabolism in human myotubes may be explained by differences in substrate specificity of their different enzymes. Even though oleic acid accumulates to a higher extent as FFAs, the palmitic acid accumulation or its derivatives seem to be associated with a more insulin-resistant promoting potential than for oleic acid. Lipid metabolism seems unaffected by high glucose concentration, except that Randle's inverse cycle is active in myotubes that are exposed to oleic acid. De novo lipid synthesis from glucose does not seem central to the TAG

accumulation in obesity or type 2 diabetes. On the basis of our observations, most of the alteration described in lipid metabolism in type 2 diabetes in vivo seems to be adaptive apart from a primarily reduced palmitic acid oxidation. We recommend a reduced lipid intake and substituting palmitic acid and possibly other saturated FFAs by oleic acid or other unsaturated FFAs to reduce accumulation of metabolites, which promotes insulin resistance. Further studies are needed in which the combined effect of oleic acid and palmitic acid is examined to clarify whether the combined presence may change glucose and lipid metabolism in diabetic myotubes.

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