Original Article

Mitochondrial Respiration Is Decreased in Skeletal Muscle of Patients With Type 2 Diabetes

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We tested the hypothesis of a lower respiratory capacity per mitochondrion in skeletal muscle of type 2 diabetic patients compared with obese subjects. Muscle biopsies obtained from 10 obese type 2 diabetic and 8 obese nondiabetic male subjects were used for assessment of 3-hydroxy-Acyl-CoA-dehydrogenase (HAD) and citrate synthase activity, uncoupling protein (UCP)3 content, oxidative stress measured as 4-hydroxy-2-nonenal (HNE), fiber type distribution, and respiration in isolated mitochondria. Respiration was normalized to citrate synthase activity (mitochondrial content) in isolated mitochondria. Maximal ADP-stimulated respiration (state 3) with pyruvate plus malate and respiration through the electron transport chain (ETC) were reduced in type 2 diabetic patients, and the proportion of type 2X fibers were higher in type 2 diabetic patients compared with obese subjects (all $P < 0.05$). There were no differences in respiration with palmitoyl-L-carnitine plus malate, citrate synthase activity, HAD activity, UCP3 content, or oxidative stress measured as HNE between the groups. In the whole group, state 3 respiration with pyruvate plus malate and respiration through ETC were negatively associated with A1C, and the proportion of type 2X fibers correlated with markers of insulin resistance ($P < 0.05$). In conclusion, we provide evidence for a functional impairment in mitochondrial respiration and increased amount of type 2X fibers in muscle of type 2 diabetic patients. These alterations may contribute to the development of type 2 diabetes in humans with obesity.
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Type 2 diabetes is characterized by insulin resistance in major metabolic tissues such as skeletal muscle, liver, and adipose tissue, as well as failure of the pancreatic β-cells to compensate for this abnormality (1). Skeletal muscle is the major site of glucose disposal in response to insulin and, correspondingly, the major site of insulin resistance in type 2 diabetes (1,2). Despite extensive research, the mechanisms underlying insulin resistance are not fully understood. Indeed, several abnormalities have been identified in insulin-resistant muscle including impaired insulin activation of glycogen synthase (1,3), impairment of the proximal components of the insulin signaling cascade (2), and increased intramuscular triglyceride content (4,5). Another important component of insulin resistance appears to be a decreased ability of insulin to regulate fuel utilization (6–8). In insulin-resistant subjects, this impaired ability to switch from lipid to carbohydrate oxidation in response to insulin has been described as "metabolic inflexibility" of skeletal muscle (8).

Being the site of fuel oxidation, the mitochondrion has gained increasing interest in type 2 diabetes research during the last decade. Several studies have indicated a role for mitochondrial dysfunction in the pathogenesis of insulin resistance and type 2 diabetes. This includes reports of a decreased leg lipid oxidation in type 2 diabetes and obesity (9) and a strong negative correlation between leg lipid oxidation and insulin resistance (10–13). Furthermore, microarray analysis has revealed a coordinated downregulation of genes involved in mitochondrial oxidative phosphorylation in type 2 diabetic patients and high-risk individuals (14–16), and proteomic analysis has demonstrated a decreased content of the ATP synthase β-subunit in type 2 diabetic patients (17). These findings are supported by reports of a decreased activity of the electron transport chain (ETC) in muscle mitochondria in type 2 diabetes and obesity (18,19) and in vivo studies using magnetic resonance spectroscopy showing impaired mitochondrial ATP production in insulin-resistant offspring of type 2 diabetic patients (20).

Nevertheless, it remains to be fully clarified whether mitochondrial dysfunction in type 2 diabetes and obesity is caused by a reduced content of mitochondria, a reduced functional capacity, or a combination of both. In a study by Kelley et al. (18), it was reported that the ratio between the activities of NADH:O_2 oxidoreductase and citrate synthase (a marker of mitochondria content) in skeletal muscle did not differ between lean and obese nondiabetic subjects and type 2 diabetic patients. In a more recent study from

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ETC, electron transport chain; FFA, free fatty acid; HAD, 3-hydroxy-Acyl-CoA-dehydrogenase; HNE, 4-hydroxy-2-nonenal; HOMA-IR, homeostasis model assessment of insulin resistance; RCI, respiratory control index; ROS, reactive oxygen species; UCP, uncoupling protein.

the same research group, ETC activity was measured as the activity of succinate oxidase in the subsarcolemmal and intermyofibrillar subpopulations of muscle mitochondria (19). Total ETC activity corrected for mitochondrial content (mitochondrial DNA) was reduced in both type 2 diabetes and obesity compared with lean subjects. Moreover, ETC activity in the subsarcolemmal mitochondria fractions was reduced in type 2 diabetic patients compared with obese subjects, but this was not corrected for mitochondrial content (19). Thus, it is still unclear whether the respiratory capacity per mitochondrion is reduced in type 2 diabetic patients compared with obese nondiabetic subjects.

An essential feature of the mitochondrion is the coupling between substrate oxidation and phosphorylation of ADP through generation and utilization of the proton gradient over the inner mitochondrial membrane. Previous studies have suggested a possible link between insulin resistance and the coupling of these systems (21). The functionality of the mitochondrion can be assessed by measuring respiration in isolated mitochondria from human skeletal muscle under controlled conditions without the influence of circulating hormones and substrates (22). Here, the intactness of the inner membrane is sustained, enabling an interaction between substrate oxidation and phosphorylation. By using different substrate combinations, different enzymatic pathways can be included and excluded. The mitochondrial respiratory rate will thereby describe the functionality and interaction between these pathways. By relating the mitochondrial respiration to citrate synthase activity, the respiratory capacity per mitochondrion can be assessed. To our knowledge, no previous studies have investigated the respiratory function of the mitochondrion in skeletal muscle of type 2 diabetic patients.

The present study was undertaken to investigate the role of mitochondrial dysfunction in insulin resistance in skeletal muscle of patients with type 2 diabetes. To account for the effects of fiber type composition, mitochondrial content, obesity, and oxidative stress induced by hyperglycemia, we measured the respiratory capacity per mitochondrion, oxidative stress as 4-hydroxy-2-nonenal (HNE), and fiber type distribution in skeletal muscle of type 2 diabetic patients and well-matched obese, healthy subjects.

RESEARCH DESIGN AND METHODS

Ten patients with type 2 diabetes and eight healthy control male subjects, matched according to age and BMI, participated in the study (Table 1). All subjects were obese and sedentary and did not participate in any kind of physical training. Type 2 diabetic patients were treated with either diet alone or diet in combination with sulfonylurea, metformin, or insulin. Medication was withdrawn 1 week before the study. The patients were all GAD65 antibody negative and without signs of diabetic retinopathy, neuropathy, nephropathy, or macrovascular complications. The control subjects had normal glucose tolerance and no family history of diabetes. All subjects had normal results on screening blood tests of hepatic and renal function. All subjects gave written informed consent, and the study was approved by the local ethics committee of Funen and Vejle County and was performed in accordance with the Declaration of Helsinki. Participants were instructed to avoid vigorous exercise for 48 h before the study, which was carried out after a 10-h overnight fast. Fasting blood samples were analyzed for glucose (Glucose Analyzer II; Beckman Instruments, Fullerton, CA), A1C, free fatty acids (FFAs) (Wako Chemicals, Neuss, Germany), triglycerides, total cholesterol, insulin, and C-peptide (Wallac, Turku, Finland). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting values of serum insulin and plasma glucose (23).

TABLE 1
Clinical, metabolic, and muscle characteristics

	Type 2 diabetic subjects	Control subjects	*P*
Age (years)	55 ± 2	53 ± 2	0.36
BMI (kg/m^2)	31 ± 1	32 ± 1	0.77
Weight (kg)	99 ± 4	106 ± 6	0.36
Plasma glucose (mmol/l)	11.5 ± 1.6	5.8 ± 0.1	0.006
A1C (%)	7.4 ± 0.4	5.5 ± 0.1	0.001
C-peptide (pmol/l)	1312 ± 167	929 ± 97	0.08
Insulin (pmol/l)	100 ± 24	76 ± 12	0.41
FFA (mmol/l)	0.6 ± 0.1	0.6 ± 0.1	0.73
Total cholesterol (mmol/l)	5.7 ± 0.3	5.4 ± 0.3	0.60
Triglyceride (mmol/l)	1.4 ± 0.2	2.9 ± 1.1	0.28
HOMA-IR	8.0 ± 1.7	3.3 ± 0.51	0.03
Citrate synthase activity	104 ± 8	110 ± 11	0.63
HAD activity	160 ± 13	161 ± 11	0.95
UCP3	1.4 ± 0.2	1.2 ± 0.3	0.69
UCP3 per mitochondrion	1.3 ± 0.2	1.1 ± 0.2	0.44

Data are means ± SE from 10 type 2 diabetic patients and 8 obese control subjects. Citrate synthase and HAD activity was measured as units · mg protein^{-1} · min^{-1}. UCP3 was determined as arbitrary units per miligram protein. UCP3 per mitochondrion was determined as arbitrary units/10^3 per citrate synthase activity.

Muscle biopsies and preparation of mitochondria. Muscle biopsies were taken from the vastus lateralis muscle using a modified Bergström needle with suction under local anesthesia (5 ml lidocaine 2%). A small part of the biopsy (~50–100 mg) was rapidly frozen in liquid nitrogen and stored at −80°C for later determination of oxidative enzyme activities, uncoupling protein (UCP)3 protein content, fiber type distribution, and oxidative stress (HNE). The major part (~100–150 mg) of the biopsy was used for isolation of mitochondria as previously described (24). Using this method, both the subsarcolemmal and intramyofibrillar mitochondrial fractions are isolated. Due to transportation (from Odense University Hospital to University of Southern Denmark, Odense, Denmark), muscle samples were on average stored for 45 min (all <1 h) in ice-cold isolation buffer before initiation of the isolation procedure. The major part of the isolated mitochondria was used for assessment of respiratory function. The remaining part was frozen in liquid nitrogen and stored at −80°C for later assessment of citrate synthase activity and maximal respiration through ETC.

Mitochondrial respiratory activity. Mitochondrial oxygen consumption was measured polarographically using a Clark-type electrode (DW1 oxygraph; Hansatech Instruments, Norfolk, U.K.). The electrode was surrounded by a temperature-controlled water-jacketed glass chamber maintaining the temperature at 25°C. The oxygraph was equipped with magnetic stirring and a gas-tight plunger ensuring a minimum of oxygen diffusion between the chamber solution and the surroundings. For data collection, the electrode was connected to a computer. The measurement was carried out in an oxygraph medium containing 225 mmol/l mannitol, 75 mmol/l sucrose, 10 mmol/l Tris, 10 mmol/l KCl, 10 mmol/l K_2HPO_4, 0.1 mmol/l EDTA, 0.8 mmol/l $MgCl_2 \cdot (6H_2O)$, and pH 7.0. A total of 5 mmol/l pyruvate + 2 mmol/l L-malate and 10 μmol/l palmitoyl-L-carnitine + 2 mmol/l L-malate was used as substrate combinations. Mitochondrial respiration was initiated by the addition of mitochondrial-rich suspension medium. After reaching a stable rate, maximal respiration was initiated by adding K-ADP (final concentration 0.3 mmol/l). After determination of the maximal ADP-initiated respiration (termed V_{max} or state 3) and respiration without ADP (termed noncoupled respiration or state 4), the oxygen tension was regained and the submaximal respiration initiated by low-rate ADP infusion. This was accomplished by the use of a microdialysis pump (CMA/102; CMA/Microdialysis, Solna, Sweden) with a pumping rate ranging from 0.1 to 20 μl/min. At ~50% of state 3 respiration, a sample was withdrawn from the respiratory chamber for calculation of ADP sensitivity ($[ADP]_{½V_{max}}$) as described previously (25).

Maximal respiration through ETC was determined in permeabilized mitochondria. Permeabilization was accomplished by addition of alamethicin to the respiratory medium (final concentration 6 μg/ml) (18). Maximal respiration was determined in the presence of 2 μmol/l cytochrome c and initiated by addition of 450 μmol/l NADH. The measurement of respiration through ETC in mitochondria from one control subject was excluded from further analysis due to signs of mitochondrial degeneration. This was confirmed by reevaluation of citrate synthase activity, which was only 25% of the expected value in the particular sample.

All respiratory measurements were related to citrate synthase activity in mitochondrial suspension (26). Respiratory control index (RCI = state 3/state 4), P/O ratio (mitochondrial efficiency), and P/O ratio during submaximal respiration (33% of state 3) were calculated. RCI describes the coupled state of the mitochondrion, and P/O ratio describes the efficiency of the mitochondrion, calculated as the amount of ADP consumed per oxygen being reduced. Both values were used to evaluate the success of the isolation procedure and the proton leak across the inner membrane. The P/O ratio at submaximal respiration was calculated to evaluate the mitochondrial efficiency, while sustaining a membrane potential (27).

Enzyme activity, fiber type distribution, and UCP3 content. The activity of 3-hydroxy-Acyl-CoA-dehydrogenase (HAD) and citrate synthase was spectrophotometrically determined at 25°C as described (28,29). Citrate synthase and HAD activity was determined as unit per miligram muscle protein per minute (BCA Protein Assay Kit; Pierce, Rockford, IL). Fiber type distribution was determined by electrophoresis as different isotypes of myosin heavy chain (type 1 fibers, type 2A fibers, and type 2X fibers) as previously described (30). Determination of percentage of type 1 fibers by either this method or the histochemical method correlates significantly ($r = 0.96$) (31). Determination of UCP3 content was analyzed in duplicate as previously described in detail (32).

Oxidative stress. HNE is a major product of endogenous lipid peroxidation that reacts with several functional groups in muscle proteins to form thioesters and Michael adducts. Protein modification by HNE was performed by immunoblotting as described (33). Freeze-dried muscle samples were homogenized for 5 min in cold lysing buffer in 1:70 wt/vol, extracted for 15 min at 4°C with slow mixing, and centrifuged at 1,000*g* for 10 min. Protein concentration of the supernatant was determined with a protein assay kit (BCA Pierce). Equal amounts of proteins (15 μg) were loaded and separated on SDS-PAGE (BioRad) and transferred to polyvinylidene difluouride membranes. After blocking in Tris-buffered saline with 5% nonfat milk, the membranes were incubated with primary polyclonal antibodies to the HNE adducts (Alpha Diagnostics), followed by incubation with horseradish peroxidise–conjugated secondary antibody (anti-goat; Santa Cruz Bio Technology). The membrane was incubated with chemiluminescence detection reagent enhanced chemiluminescence (Amersham). Actin protein content was determined with primary antibodies to actin (anti-rabbit; Sigma) on the same membrane after stripping the membrane in stripping buffer (Pierce). Quantification was performed with Quantity One 1-D Analyzing software (Bio-Rad).

Statistics. Data are presented as means ± SE. Differences between type 2 diabetic and control subjects were tested for statistical significance with the Student's unpaired *t* test. Correlation between variables was tested with Pearson correlation analysis. Statistical significance was accepted at $P < 0.05$.

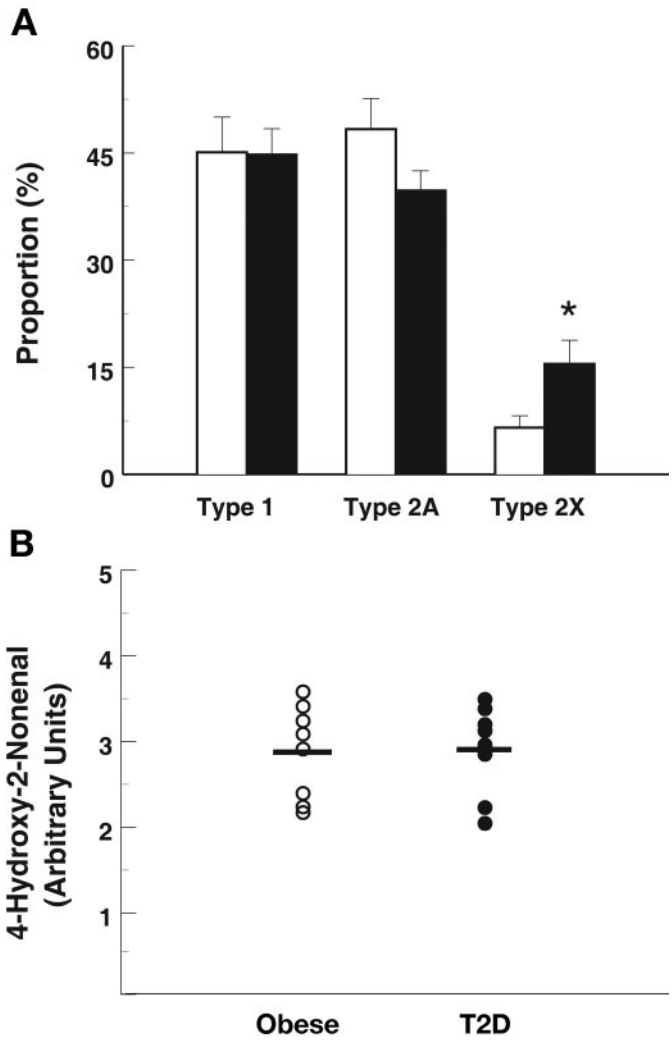

FIG. 1. Fiber type distribution and oxidative stress in human skeletal muscle from obese patients with type 2 diabetes (T2D) and obese control subjects. *A*: Proportions (%) of type 1, type 2A, and type 2X fibers in skeletal muscle from 10 diabetic (■) and 8 control (□) subjects. Data are means ± SE. *B*: Oxidative stress measured as HNE in skeletal muscle of 10 diabetic (●) and 8 control (○) subjects. The mean value for each group is indicated by a line. *$P < 0.05$ vs. control subjects.

RESULTS

Clinical, metabolic, and muscle characteristics. Type 2 diabetic patients had significantly higher concentrations of fasting plasma glucose and A1C compared with obese subjects (Table 1). There were no differences between groups in the circulating concentrations of C-peptide, insulin, FFA, total cholesterol, or triglycerides. Insulin resistance determined as HOMA-IR was significantly higher in diabetic patients compared with obese subjects.

Analyses of citrate synthase and HAD activity revealed no significant differences between type 2 diabetic patients and obese subjects (Table 1). Type 2 diabetic patients had a significant higher proportion of type 2X fibers, but no differences in type 1 or type 2A fibers were found between the groups (Fig. 1*A*). Furthermore, there were no differences in muscle oxidative stress measured as HNE (Fig. 1*B*) or in UCP3 content measured per miligram protein or per mitochondrion (citrate synthase activity) between the groups.

Mitochondrial characteristics. Maximal respiration through ETC was significantly reduced in type 2 diabetic patients compared with obese subjects (Fig. 2*A*). Maximal ADP-stimulated respiration (state 3) with pyruvate plus malate was also significantly reduced in type 2 diabetic patients compared with obese subjects, whereas state 3 respiration with palmitoyl-L-carnitine plus malate did not differ between the groups (Fig. 2*B* and *C*). In type 2 diabetic patients, RCI was significantly lower than in obese subjects but, again, only when using pyruvate plus malate as substrates (Table 2). Type 2 diabetic patients had a slightly lower P/O ratio using pyruvate plus malate, but this did not reach statistical significance. There were no significant differences in the P/O ratio during submaximal respiration or ADP sensitivity between the groups regardless of which substrates were used.

Correlation analysis. To explore potential mechanisms underlying reduced state 3 respiration and respiration through ETC, we examined the relationship between these measures of mitochondrial respiration and muscle and metabolic characteristics. In the whole study group ($n = 18$), state 3 respiration with pyruvate plus malate correlated negatively with A1C ($r = -0.51$, $P = 0.03$) (Fig. 3*A*) and tended to correlate with plasma glucose ($r = -0.46$, $P = 0.052$). Respiration through ETC was also inversely correlated with A1C ($r = -0.49$, $P = 0.048$) (Fig. 3*B*). State 3 respiration with palmitoyl-L-carnitine plus malate was positively correlated with the proportion of type 1 fibers

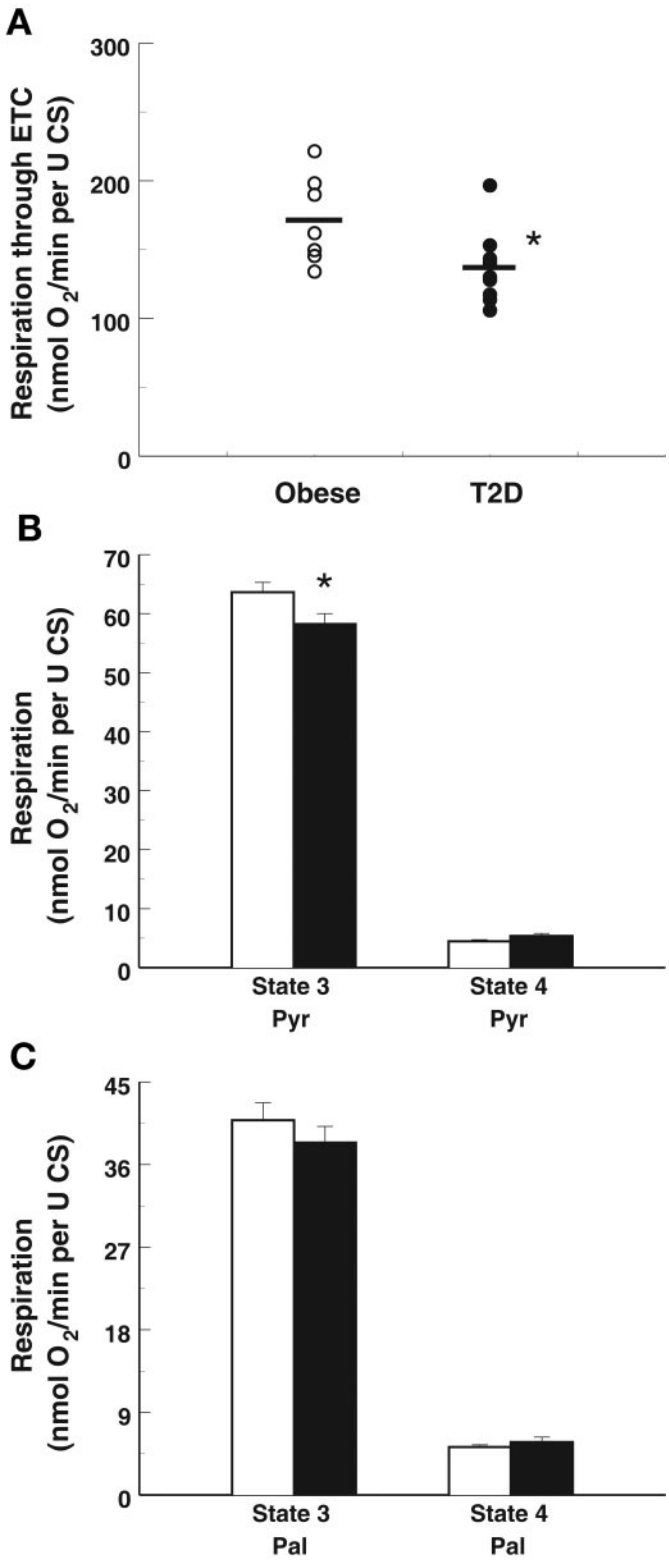

FIG. 2. Respiration in isolated mitochondria from human skeletal muscle of obese patients with type 2 diabetes (T2D) and obese control subjects. *A*: Maximal respiration through the ETC in permeabilized isolated mitochondria using NADH and cytochrome c as substrates. Data are from 10 diabetic (●) and 7 control (○) subjects. The mean value for each group is indicated by a line. States 3 and 4 respiration in isolated mitochondria with pyruvate plus malate (Pyr) (*B*) and palmitoyl-L-carnitine plus malate (Pal) (*C*) as substrate combinations. Data represent means ± SE from 10 diabetic (■) and 8 control (□) subjects. All measures of respiration were divided by citrate synthase activity (U CS) in the mitochondrial suspension to correct for mitochondrial content. *$P < 0.05$ vs. control subjects.

TABLE 2
Mitochondrial characteristics

	Type 2 diabetic subjects	Control subjects	*P*
Pyruvate + malate			
RCI	11.5 ± 1.0	14.4 ± 0.8	0.04
P/O ratio	2.47 ± 0.03	2.63 ± 0.08	0.08
P/O ratio$_{submax}$	2.24 ± 0.02	2.25 ± 0.04	0.92
[ADP]$_{Vmax}$	29.7 ± 5.7	29.6 ± 8.0	0.99
Palmitoyl-l-carnitine + malate			
RCI	7.2 ± 0.8	7.9 ± 0.6	0.47
P/O ratio	2.30 ± 0.03	2.35 ± 0.03	0.32
P/O ratio$_{submax}$	1.67 ± 0.08	1.64 ± 0.04	0.77
[ADP]$_{Vmax}$	9.8 ± 1.9	9.5 ± 1.1	0.92

Data are means ± SE from 10 type 2 diabetic patients and 8 obese control subjects. RCI is state 3 divided by state 4 respiration. P/O ratio is the amount of ADP used per oxygen atom during state 3 respiration. P/O ratio$_{submax}$ is P/O ratio determined during submaximal respiration. [ADP]$_{1/2Vmax}$ is the concentration of ADP needed to induce 50% of state 3 respiration, a measure of mitochondrial ADP sensitivity.

($r = 0.50$, $P = 0.04$) and tended to correlate with A1C ($r = -0.46$, $P = 0.06$). Neither respiration through ETC nor state 3 respiration correlated with the amount of type 2X fibers or HNE (oxidative stress).

We also studied the relationship between the amount of type 2X fibers and the other parameters measured. There was a significant positive correlation between the proportion of type 2X fibers and state 4 respiration using both pyruvate plus malate ($r = 0.55$, $P = 0.02$) and palmitoyl-L-carnitine plus malate ($r = 0.54$, $P = 0.02$) as substrates. Moreover the amount of type 2X fibers correlated positively with plasma glucose ($r = 0.48$, $P = 0.04$), FFAs ($r = 0.53$, $P = 0.02$), insulin ($r = 0.55$, $P = 0.01$), C-peptide ($r = 0.66$, $P = 0.003$), and HOMA-IR ($r = 0.76$, $P < 0.001$) (Fig. 3*C* and *D*).

DISCUSSION

Several studies have implicated a role for mitochondrial dysfunction in the pathogenesis of skeletal muscle insulin resistance (6,7,14–20). However, evidence for a difference in mitochondrial function between obese nondiabetic subjects and type 2 diabetic patients under basal conditions is scarce (19), and the extent to which this is caused by a lower mitochondrial content or an impaired functional capacity remains unknown. The most important finding of the present study was a reduced respiratory function per mitochondrion in skeletal muscle of type 2 diabetic patients compared with obese nondiabetic subjects. Moreover, in the whole study group, the measures of mitochondrial respiration were negatively associated with A1C. However, muscle oxidative stress measured as HNE was similar in type 2 diabetic patients and obese subjects, and no correlation between mitochondrial respiration and HNE content was observed. Interestingly, type 2 diabetic patients also had a twofold higher proportion of type 2X muscle fibers compared with obese subjects. In the whole study group, type 2X fiber content correlated with several markers of insulin resistance but not with state 3 respiration or respiration through ETC. These results provide evidence for a qualitative defect in the functional capacity of muscle mitochondria in type 2 diabetic patients, which together with higher type 2X fiber

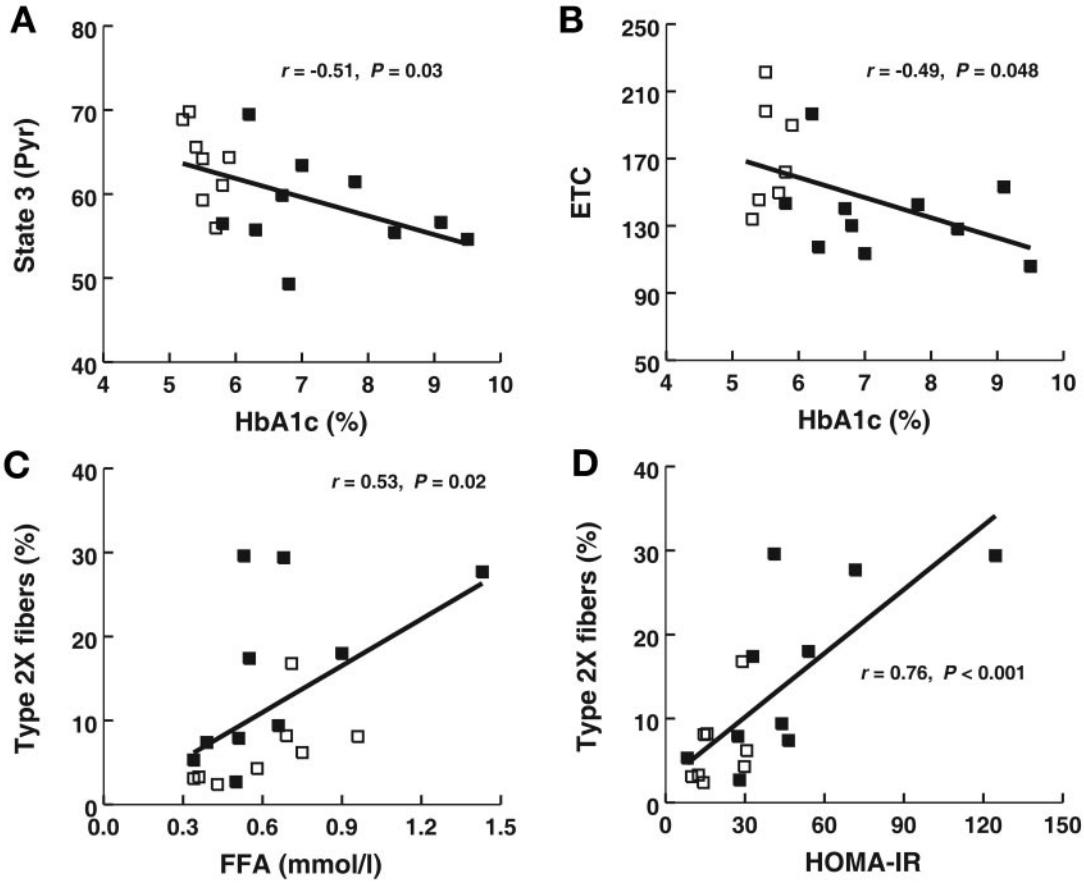

FIG. 3. Correlation analysis in the whole study group (n = 18). The levels of A1C were negatively associated with state 3 respiration with pyryvate plus malate (nmol · min^{-1} · U CS^{-1}) (*A*) and respiration through the ETC (nmol · min^{-1} · U CS^{-1}) (*B*). The proportion of type 2X fibers was positively associated with FFA levels (*C*) and HOMA-IR (*D*).

content may play a role in the muscle metabolic changes leading to type 2 diabetes in humans with obesity.

Nonhypothesis-driven approaches using DNA microarrays and proteomics have demonstrated a coordinated downregulation of nuclear-encoded genes encoding proteins of the respiratory complexes (I–V) (14–16) and a decreased protein content and phosphorylation of the ATP synthase β-subunit in skeletal muscle of type 2 diabetic patients (17). These studies have pointed to abnormalities in the respiratory complexes I–V in the inner mitochondrial membrane. Correspondingly, ETC activity measured as the activity of NADH:O_2 oxidoreductase (18) or succinate oxidase (19) has been reported to be decreased in muscle mitochondria of type 2 diabetic patients and obese subjects compared with lean subjects. Furthermore, the activity of succinate oxidase was found to be 50% lower in subsarcolemmal mitochondria in type 2 diabetic patients compared with obese subjects. However, the NADH:O_2 oxidoreductase activity corrected for mitochondrial content (citrate synthase activity) was not different between lean subjects, obese nondiabetic subjects, and type 2 diabetic patients (18). Moreover, although succinate oxidase activity related to mitochondrial DNA was reduced in type 2 diabetic patients and obese subjects compared with lean subjects, no difference in succinate oxidase activity corrected for mitochondrial content was reported between obese subjects and type 2 diabetic patients in either the total population or the subsarcolemmal population of mitochondria (19).

We report a decreased respiratory function per mitochondrion in obese type 2 diabetic patients compared with obese nondiabetic subjects. This was evident in fully coupled isolated mitochondria using pyruvate plus malate (state 3 respiration) as substrates and in permeabilized uncoupled mitochondria using NADH plus cytochrome c (maximal respiration through ETC) as substrates. A decreased respiration per mitochondrion suggests an intrinsic inhibition or damage to some or more of the involved mitochondrial pathways. When using pyruvate plus malate as substrates, the respiration in isolated mitochondria is a result of a complex interplay between tricarboxylic acid (TCA) cycle activity, ETC activity, utilization of the membrane potential, ATP synthase functionality, and the activity of adenine nucleotide translocase. Which of these factors are responsible for the reduced state 3 respiration with pyruvate plus malate in type 2 diabetic patients compared with control subjects cannot be determined from the present data. However, the concomitant finding of a significant lower respiration through ETC in type 2 diabetic patients cannot be explained by a defect in the conversion of pyruvate to acetyl-CoA. Moreover, respiration with pyruvate plus malate is not increased further by addition of substrates having the potential to increase NADH production, indicating that pyruvate dehydrogenase and TCA cycle activity are unlikely to be the limiting factors. Together with the finding of similar citrate synthase activity in the two groups, these results suggest the possibility of a defect within the respiratory complexes I–V in the inner mitochondrial membrane. The failure to observe a significant difference in respiration with palmi-

toyl-L-carnitine plus malate between obese nondiabetic and type 2 diabetic subjects could at least in part be explained by a lower respiration with palmitoyl-L-carnitine plus malate rather than with pyruvate plus malate in isolated mitochondria. Thus, with the same variations in these measurements, a larger sample size is probably required to prove significance. In vivo, however, mitochondrial oxidative metabolism is challenged by a number of substrates simultaneously, and, hence, a qualitative defect within the respiratory complexes I–V could have a more pronounced negative effect on respiration with lipids. Since only men with manifest type 2 diabetes were included, further studies are needed to determine the extent to which these findings apply to female subjects with type 2 diabetes and whether they are potential early defects in the pathogenesis of type 2 diabetes.

In the present study, A1C was negatively associated with state 3 respiration and respiration through ETC in the whole study group. This is in accordance with a previous study showing a significant inverse relationship between the content and phosphorylation of ATP synthase β-subunit and glucose levels (17). These results seem to support the hypothesis of a relationship between prolonged elevation in glucose levels and mitochondrial dysfunction caused by an increased production of reactive oxygen species (ROS) and oxidative stress (34). Mitochondria are the major source of ROS and oxidative stress (~90%), and also the primary target for oxidative damage, when ROS production exceeds the capacity of the endogenous ROS scavenging system (35). Accordingly, several protein subunits in the mitochondrial respiratory complexes (I–V) are susceptible to oxidative damage (36). However, to our knowledge, no previous studies have reported measurements of oxidative stress in skeletal muscle of insulin-resistant subjects. Surprisingly, we found no difference in muscle oxidative stress measured as HNE between type 2 diabetic patients and obese subjects. Although we did not measure other oxidative modifications such as nitration and carbonylation or local oxidative damage within mitochondria, it is likely that these measures would parallel those of HNE. Our data do not rule out a role for oxidative stress in mitochondrial dysfunction in both obesity and type 2 diabetes but indicate that the observed difference in quality of mitochondrial respiration between type 2 diabetic patients and obese subjects is not mediated by increased oxidative stress. It is therefore too early to conclude that the observed negative association between mitochondrial respiration and A1C is a causal relationship involving increased ROS production.

Numerous factors are known to influence the mitochondrial function. Some of these factors, including age, obesity, fiber type distribution, physical activity, and UCP3 protein, have also been related to insulin resistance and type 2 diabetes. Physical activity has been shown to have a positive influence on type 2 diabetes by increasing insulin sensitivity and inducing weight loss. Muscular adaptations in relation to increased physical fitness include an increased muscle oxidative capacity and increased lipid oxidative capacity, mainly caused by an increased mitochondrial volume (24). Therefore, it could be argued that a potential lower level of physical activity in type 2 diabetic patients contributed to the decrease in mitochondrial respiration. However, previous studies have shown a strong correlation between citrate synthase activity and $V\mathrm{O}_{2\mathrm{max}}$ (37), and, in our study, citrate synthase activity in type 2 diabetic patients and control subjects was in fact similar. Furthermore, several studies have shown that physical training does not affect measures of respiration per mitochondrion when using pyruvate and palmitoyl-L-carnitine plus malate as substrates (24,25,38). Finally, the values of state 3 respiration with pyruvate plus malate observed in the sedentary, obese, nondiabetic subjects in the present study were similar to those previously reported in young healthy trained and untrained subjects (38,39). For these reasons, we find it unlikely that the decreased respiration per mitochondrion in type 2 diabetic patients was due to a potential minor difference in physical activity.

In the present study, type 2 diabetic patients had a twofold higher content of type 2X fibers. This is in agreement with some (40,41), but not all, studies (42,43). Furthermore, in accordance with earlier reports of a close correlation between fiber type composition and insulin sensitivity (41,44,45), we found that type 2X fiber content was significantly associated with several markers of insulin resistance including HOMA-IR and levels of FFAs. Recent studies indicate that mitochondria from different fiber types may have different metabolic characteristics. This includes a higher UCP3 content in type 2X fibers in human skeletal muscle (46) and a lower lipid oxidative capacity per mitochondria and higher hydrogen peroxide production in type 2 fibers from rodent muscle (27,47). Correspondingly, we observed a positive association between the proportion of type 1 fibers and mitochondrial respiration with palmitoyl-L-carnitine plus malate and a correlation between type 2X fibers and noncoupled (state 4) respiration. In humans, muscle fiber type composition is more mixed than in rodents, and generally type 2X fibers have a very low oxidative capacity. Although there was no relationship between mitochondrial respiration (state 3 or through ETC) and the proportion of type 2X fibers, we cannot rule out the possibility that decreased mitochondrial respiration in type 2 diabetic patients was in part explained by an increased proportion of type 2X fibers compared with obese subjects. On the other hand, single-fiber analysis has demonstrated that reduced oxidative capacity and increased lipid content in skeletal muscle of type 2 diabetic patients are independent of the effect of fiber type (42).

Previous studies have indicated that UCP3 may play an essential role in mitochondrial dysfunction in type 2 diabetic patients (48). Despite extensive research, the main function of UCP3 is still unknown. UCP3 has been hypothesized to function as a protective mechanism against ROS production by mild uncoupling of the inner mitochondrial membrane and as a transporting protein of nonesterified fatty acids from the matrix to the intermembrane space protecting the mitochondrion against peroxidized lipids (49). At present, there is little evidence that UCP3 functions as a fatty acid transporter. However, there is some evidence supporting a role of UCP3 in the attenuation of mitochondrial ROS production (49). In the present study, we found no difference in UCP3 protein levels between type 2 diabetic patients and obese subjects. This is in contrast to a recent study showing a decreased UCP3 content in type 2 diabetic patients (50), probably due to different matching of the study subjects with respect to age, body composition, and physical activity. Our findings strongly indicate that the decrease in respiration per mitochondrion in type 2 diabetic patients compared with obese subjects is not explained by changes in UCP3 protein. On the other hand, even normal levels of

UCP3 levels may contribute to mitochondrial damage in type 2 diabetic patients in face of an increased oxidative stress (34).

In summary, we provide evidence for a qualitative defect in the respiratory function per mitochondrion in skeletal muscle of type 2 diabetic patients compared with obese nondiabetic subjects. This difference was not paralleled by increased oxidative stress in diabetic muscle. Moreover, the proportion of type 2X fibers was higher in type 2 diabetic patients and correlated with several markers of insulin resistance but not with mitochondrial respiration. These alterations may be important factors contributing to the progression from obesity to type 2 diabetes in humans. We were not able to demonstrate a role for UCP3 levels, oxidative stress, or fiber type composition in the respiratory capacity of mitochondria, but a significant negative correlation between mitochondrial respiration and A1C indicates that elevated glucose levels may contribute to mitochondrial dysfunction in obese and type 2 diabetic subjects, perhaps by other mechanisms than oxidative stress.

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