# Deficiency of Subsarcolemmal Mitochondria in Obesity and Type 2 Diabetes

**Vladimir B. Ritov,[1] Elizabeth V. Menshikova,[1] Jing He,[1] Robert E. Ferrell,[2] Bret H. Goodpaster,[1] and David E. Kelley[1]**

**The current study addresses a novel hypothesis of subcellular distribution of mitochondrial dysfunction in skeletal muscle in type 2 diabetes. Vastus lateralis muscle was obtained by percutaneous biopsy from 11 volunteers with type 2 diabetes; 12 age-, sex-, and weight-matched obese sedentary nondiabetic volunteers; and 8 lean volunteers. Subsarcolemmal and intermyofibrillar mitochondrial fractions were isolated by differential centrifugation and digestion techniques. Overall electron transport chain activity was similar in type 2 diabetic and obese subjects, but subsarcolemmal mitochondria electron transport chain activity was reduced in type 2 diabetic subjects (0.017 ± 0.003 vs. 0.034 ± 0.007 units/mU creatine kinase [CK], *P* = 0.01) and sevenfold reduced compared with lean subjects (*P* < 0.01). Electron transport chain activity in intermyofibrillar mitochondria was similar in type 2 diabetic and obese subjects, though reduced compared with lean subjects. A reduction in subsarcolemmal mitochondria was confirmed by transmission electron microscopy. Although mtDNA was lower in type 2 diabetic and obese subjects, the decrement in electron transport chain activity was proportionately greater, indicating functional impairment. Because of the potential importance of subsarcolemmal mitochondria for signal transduction and substrate transport, this deficit may contribute to the pathogenesis of muscle insulin resistance in type 2 diabetes. *Diabetes* 54:8–14, 2005**

Skeletal muscle has a high reliance on oxidative phosphorylation. Distribution of energy within myocytes is served not only by the ATP-phosphocreatine shuttle but also by subcellular compartmentalization of mitochondria in skeletal muscle (1,2). Energy generated by oxidative phosphorylation in support of muscle contraction is largely generated by mitochondria located in an intermyofibrillar location, characteristically near the Z-line of muscle fibers (3). In contrast, subsarcolemmal mitochondria generate ATP for numerous energy-requiring processes at the cell surface, including ion exchange, substrate transport, cell signaling, and protein synthesis (4). It has previously been postulated that the functional capacity of subsarcolemmal mitochondria may be crucial for fatty acid oxidation, glucose transport, and propagation of insulin signaling or other signaling cascades (4), each of importance to insulin action and thus potentially to the pathogenesis of insulin resistance in type 2 diabetes and obesity (5–7). However, the relation of subcellular distributions of mitochondria in human skeletal muscle to insulin resistance in type 2 diabetes and obesity has not been previously examined.

Prior studies have found that reduced oxidative enzyme capacity, measured in muscle homogenate, is correlated with severity of insulin resistance (8,9), a reduced capacity for lipid oxidation (10), and increased risk for obesity (11). Recently, we reported that activity of the electron transport chain is reduced and that intermyofibrillar mitochondria are smaller in type 2 diabetes (12). Both size of intermyofibrillar mitochondria and electron transport chain activity in muscle homogenate correlated with severity of insulin resistance (12). However, in that earlier study, the subpopulation of subsarcolemmal mitochondria was not specifically examined, either morphologically or with respect to electron transport chain activity. Given the importance of insulin signaling and glucose transport to insulin action, the current study was undertaken to test a novel hypothesis that a depletion of subsarcolemmal mitochondria is related to the pathogenesis of insulin resistance in muscle. To test this hypothesis, electron transport chain activity in subsarcolemmal mitochondria and intermyofibrillar mitochondrial fractions was assayed, and subsarcolemmal mitochondria were examined using a transmission electron microscope (TEM).

## RESEARCH DESIGN AND METHODS

Research volunteers were recruited by advertisement from the general community, and each potential volunteer had a screening medical examination. Three groups of volunteers were recruited: type 2 diabetic, obese nondiabetic, and nondiabetic lean subjects. The obese nondiabetic and type 2 diabetic groups were recruited to match for BMI, and these groups were sedentary. The lean nondiabetic participants were recruited to match for age, and they reported more regular physical activity, although aerobic capacity was not quantified in study participants. With regard to those with type 2 diabetes, enrollment was limited to those treated with diet and exercise alone, sulfonylureas, or metformin; the latter medications were withdrawn under medical supervision at least 4 weeks before the study. Written informed consent was obtained, and this research project was approved by the University of Pittsburgh institutional review board.

**Insulin sensitivity.** Research volunteers were admitted to the University of Pittsburgh General Clinical Research Center on the evening before these procedures, and a glucose clamp (13) was performed the next day after an

From the [1]Division of Endocrinology and Metabolism, Department of Medicine; University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania; and the [2]Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to David E. Kelley, MD, N807 Montefiore University Hospital, 3459 Fifth Ave., Pittsburgh, PA 15213. E-mail: kelley@msx.dept-med.pitt.edu.

Received for publication 1 April 2004 and accepted in revised form 22 September 2004.

CK, creatine kinase; TEM, transmission electron microscope.

© 2005 by the American Diabetes Association.

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/1/8/378934/zdb00105000008.pdf by guest on 25 January 2022

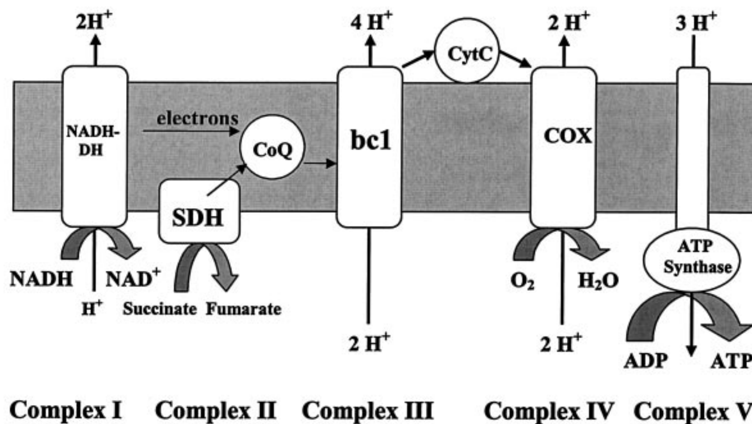


**FIG. 1. The electron-transport chain of mitochondria is depicted in this schema showing the four respiratory complexes, consisting of NADH-dehydrogenase (NADH-DH), succinate dehydrogenase (SDH), cytochrome bc1 (bc1), and cytochrome oxidase (COX), and ATP synthase (Complex V), which couples the membrane potential to phosphorylation of ADP.**

overnight fast. A percutaneous muscle biopsy of the vastus lateralis was performed (12) ~1 h preceding the start of the insulin infusion. To perform the glucose clamp, insulin was infused at 40 $mU \cdot m^{-2} \cdot min^{-1}$ for 4 h, maintaining euglycemia by infusion of dextrose. A primed (200 mg) continuous infusion (2 mg/min) of the stable isotope 6,6-$D_2$-glucose was used to measure glucose flux during the insulin infusions. The priming dose was adjusted for the severity of hyperglycemia in those with type 2 diabetes (14), with a baseline equilibriation period of 3 h. Tracer was added to 20% dextrose infusions to maintain stable plasma enrichment during insulin infusions, as previously described (15). Systemic indirect calorimetry was performed during the last 30 min of insulin infusion using an open-circuit spirometry metabolic monitor system (Delta Trac, Anaheim, CA) to estimate glucose and lipid oxidation (16). A timed overnight urine collection was obtained to measure nitrogen in order to estimate protein oxidation.

**Preparation of mitochondrial fractions.** Muscle biopsy samples of vastus lateralis muscle (~10–15 mg wet wt) were homogenized in ice-cold basic medium (in mmol/l: 100 mannitol, 80 K-gluconate, 20 K-flouride, 1 $MgCl_2$, 0.2 EGTA, 10 histidine, 10 glucose, and 10 tetraethylammonium–3-[*N*-morpholino]-2-hydroxypropanesulfonic acid, pH 7.6, at 21°C) containing 5.0 mg/ml BSA, 100 μmol/l deferoxamine mesylate, and antiprotease cocktail III, using a Polytron homogenizer according to the procedures described by Krieger et al. (17). All procedures were performed at 4°C. Soluble and particulate fractions were prepared as previously described (18,19) by centrifugation (45,000*g* for 20 min) to pellet a particulate (subsarcolemmal mitochondrial + intermyofibrillar mitochondrial) fraction containing >95% of tissue mitochondria. Aliquots (250 μl) of the particulate fraction, corresponding to ~5 mg of tissue, were suspended by Pellet Pestle for 10 s and then centrifuged (600*g* for 5 min) to separate myofibrillar fragments. Subsarcolemmal mitochondria, which remain in the supernatant, were then obtained by centrifugation (50,400*g* for 60 min). To release intermyofibrillar mitochondria, the myofibrillar fraction was incubated in 0.6 mol/l KCl/20 mmol/l pyrophosphate-$Na_4$ at 4°C under nitrogen and in the presence of antiproteases. High KCl has been shown to release sarcoplasmic reticulum vesicles from myofibrils by partially dissolving myosin (20), but without a significant effect on electron transport chain activity (21). This incubation only partially frees intermyofibrillar mitochondria, and those remaining tightly bound to cellular matrix were obtained in a centrifugation pellet (600*g* for 5 min). The supernatant, containing freed intermyofibrillar mitochondrial fraction, was centrifuged (50,400*g* for 60 min). Subsarcolemmal and the two intermyofibrillar mitochondrial fractions (free and bound) were resuspended in medium I (100 mmol/l KCl, 0.5 mmol/l EGTA, 0.1 mg/ml BSA, and 25 mmol/l $KH_2PO_4$, pH 7.1, at 21°C) and incubated with alamethicin (20 μg/ml) (12,22) to permeabilize the inner mitochondrial membrane. Alamethicin does not inhibit electron transport chain activity. Mitochondrial preparations were suspended in 500 μl of basic medium containing 0.5 mmol/l EGTA, 0.1 mg/ml BSA, and 25 mmol/l $KH_2PO_4$, pH 7.0, at 21°C and kept at −80°C until assay. The yield of mitochondrial protein was determined as earlier described (23), based on determinations of succinate dehydrogenase (19).

**Electron transport chain activity.** The electron transport chain is shown in Fig. 1. Activity of succinate oxidase (succinate: $O_2$ oxidoreductase) was measured in a reaction starting from succinate dehydrogenase and was based on assay of the accumulation of fumarate, the end product of succinate oxidation. This procedure is a modification of a previously developed assay (24). Briefly, the assay couples fumarase and malic dehydrogenase reactions to oxidize fumarate and reduce NAD, with high-performance liquid chromatography and fluorescence detection used to measure NADH (18). Succinate dehydrogenase was preactivated, as previously described, to remove inhibition by tightly bound oxalacetate (23). Activity of CK was measured as an index of muscle fiber content in biopsy samples, as previously described (12,18), and electron transport chain activity was expressed normalized to CK activity. CK activity was measured at 30°C by monitoring the generation of NADPH in a coupled enzymatic reaction (hexokinase/glucose-6P dehydrogenase) (25).

**mtDNA determinations.** mtDNA content was measured using real-time PCR, as described earlier (26). Detection of a 69-bp fragment of mtDNA (nucleotides 14918–14986) and a 77-bp fragment of β-globin, both based on markers published by Miller et al. (27), were used to determine the relative copy number of mtDNA per diploid nuclear genome. Primers and FAM-labeled Taqman TAMRA probes (no. 450025; Applied Biosystems, Foster City, CA) were designed using Primer Express software, version 1.5 (Applied Biosystems), and are listed in Table 1. DNA (mitochondrial and nuclear) was extracted from tissue samples using a QIAamp DNA mini-kit (Qiagen, Chatworth, CA). The concentration of each sample was determined using a GeneQuant spectrophotometer (Pharmacia Biotech).

**PCR conditions.** Detection of mtDNA and β-globin was performed as two separate reactions but within the same run for each sample. All samples were run in duplicate for each gene. Reactions were carried out in the presence of 1 × Taqman Universal PCR Master Mix (4304437; Applied Biosystems), 1 μmol/l each forward and reverse primer, 0.25 μmol/l FAM-labeled Taqman/TAMRA probe, and 20 ng sample DNA to a final volume of 25 μl. Amplification reactions were performed in a Prism 7700 spectrofluorometric thermal cycler (Applied Biosystems) with the following cycle conditions: 50°C for 2 min uracil-DNA N-glycosylase incubation, 95°C denaturation and enzyme activation step for 10 min followed by 40 cycles of 95°C denaturation for 15 s, and 60°C annealing and elongation for 60 s. Fluorescence spectra were recorded during the annealing phase of each PCR cycle. Sequence Detection System software (version 1.7) for the Prism 7700 was used to generate the FAM fluorescence.

**Threshold cycle calculations.** The threshold cycle number was calculated using SDS software version 1.7 (Applied Biosystems) and an automatic setting of the baseline. The baseline value was the average fluorescence of PCR cycles 3–15 plus 10 times its standard deviation. These values were used for the calculations of relative copy number calculations by expressing threshold cycle number differences of the β-globin and mtDNA PCR, as described earlier (26): $Rc = 2^{\Delta Ct}$ and $\Delta Ct = Ct_{\beta\text{-globin}} - Ct_{mtDNA}$, where Ct is the threshold cycle number and Rc is the relative copy number.

**Transmission electron microscopy.** To complement the assessment of mitochondrial distributions based on subcellular separation methods, as described above, a TEM was used to examine the thickness of the layer of

TABLE 1
Sequences of amplification primers and probes

| Primer | Sequence |
|---|---|
| mtDNA-forward | 5′–GAC GCC TCA ACC GCC TTT–3′ |
| mtDNA-reverse | 5′–GCG GAT GAT TCA GCC ATA ATT TA–3′ |
| β-Globin-forward | 5′–TGA AGG CTC ATG GCA AGA AA–3′ |
| β-Globin-reverse | 5′–AAA GGT GCC CTT GAG GTT GTC–3′ |
| **Probe** | **Sequence** |
| mtDNA probe | 5′–6FAM-CAT CAA TCG CCC ACA TCA CTC GAG AC-TAMRA–3′ |
| β-Globin probe | 5′–6FAM-CCA GGC CAT CAC TAA AGG CAC CGA-TAMRA–3′ |



Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/1/8/378934/zdb00105000008.pdf by guest on 25 January 2022

TABLE 2
Clinical characteristics of research volunteers

| | Lean | Obese | Type 2 diabetes |
|---|---|---|---|
| $n$ (F/M) | 4/4 | 3/9 | 2/9 |
| Age (years) | 43.2 ± 2.1* | 50.5 ± 3.4 | 52.6 ± 2.2 |
| Weight (kg) | 66.3 ± 5.4* | 90.9 ± 4.1 | 95.2 ± 4.8 |
| BMI (kg/m$^2$) | 22.5 ± 0.9* | 30.3 ± 1.1 | 29.4 ± 1.1 |
| Fasting glucose (mg/dl) | 82.0 ± 1.2* | 89.2 ± 2.0* | 163.8 ± 13.4 |
| $HbA_{1c}$ (%) | 5.0 ± 0.1* | 5.0 ± 0.1* | 8.0 ± 0.4 |
| Fasting insulin (μU/ml) | 5.0 ± 0.3* | 11.1 ± 0.7 | 11.4 ± 1.5 |
| $R_d$ (mg · min$^{-1}$ · kg FFM$^{-1}$) | 12.2 ± 0.7* | 6.4 ± 0.4 | 4.9 ± 0.8 |

*$P < 0.05$, lean or obese vs. type 2 diabetes. FFM, fat-free mass.

subsarcolemmal mitochondria, using a previously published method (28). Muscle samples used for the TEM were cut into small pieces (1 × 1 × 2 mm), fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon at either a longitudinal or transverse orientation. After an initial low-power screening of semithin (300 nm) sections stained with toluidine blue to optimize the plane of sectioning (29), ultrathin (60 nm) longitudinal sections were cut and mounted on copper grids and stained with lead citrate (30) and uranyl acetate (31). Sections were examined using a TEM (JEOL 100CXII) at an accelerating voltage of 80 kV. The thickness (μm) of subsarcolemmal mitochondria in the longitudinal orientations was measured using an image analysis system (National Institutes of Health image 1.61).

**Statistics.** Data are presented as the means ± SE, unless otherwise indicated. ANOVA was used to compare groups and subfractions of mitochondria, and ANCOVA was performed to adjust for the effects of age and sex. Linear regression and stepwise multiple regression was used to examine the relationships of muscle electron transport chain activity and insulin sensitivity.

## RESULTS

**Research volunteers.** The clinical characteristics of research volunteers are shown in Table 2. The obese nondiabetic and obese type 2 diabetic groups were matched for age and BMI. Insulin sensitivity tended to be lower in those with type 2 diabetes, but it was not significantly different from obese control subjects ($P = 0.17$). Fasting plasma glucose and $HbA_{1c}$ were higher in type 2 diabetic than in obese control subjects.

**Subcellular distribution of mitochondria.** Overall electron transport chain activity and that within the subsarcolemmal and intermyofibrillar mitochondrial fractions are shown in Table 3. In comparison with electron transport chain activity in muscle from lean volunteers, there were significant differences compared with obese and type 2 diabetic subjects in all subfractions of the electron transport chain, as well as in total electron transport chain activity (all $P < 0.001$ for group differences). With respect to electron transport chain activity in the subsarcolemmal mitochondrial fraction, there was an approximately sevenfold reduction in type 2 diabetes and a three- to fourfold reduction in obesity compared with lean subjects. These differences were highly significant for both type 2 diabetes and obesity ($P < 0.001$). As well, electron transport chain activity in free and bound intermyofibrillar mitochondrial fractions was reduced in type 2 diabetic and obese compared with lean subjects ($P < 0.01$), although the gradient was less than that for subsarcolemmal mitochondria, being two to threefold reduced in the intermyofibrillar mitochondrial fractions. In comparing type 2 diabetes and obesity, there was similar total electron transport chain activity in skeletal muscle. Nonetheless, in type 2 diabetes, there was a significant depression of electron transport chain activity in the subsarcolemmal mitochondrial fraction in comparison with obesity. Electron transport chain activities in the free and bound intermyofibrillar mitochondrial fractions were similar in type 2 diabetes and obesity, with a tendency toward higher electron transport chain activity in type 2 diabetes within the bound intermyofibrillar mitochondrial fraction. ANCOVA based on sex and age did not affect the significance of the comparisons.

The mitochondrial protein yield was similar across the three fractions in lean volunteers (2.1 ± 0.3, 2.1 ± 0.2, and 2.0 ± 0.2 mg/g wet wt for subsarcolemmal and free and bound intermyofibrillar mitochondrial fractions, respectively). As shown in Fig. 2, electron transport chain activity in skeletal muscle of lean volunteers was distributed evenly across the fractions, with approximately one-third of overall activity in the subsarcolemmal mitochondrial fraction, which is consistent with prior studies (32). In type 2 diabetic subjects, mitochondrial protein yield was significantly lower than in muscle from lean volunteers (0.8 ± 0.1, 1.1 ± 0.2, and 1.3 ± 0.4 mg/g wet wt; $P < 0.001$). Also, expressed as a fraction of overall electron transport chain activity, the subsarcolemmal mitochondrial fraction was smaller in type 2 diabetic subjects than in lean ($P < 0.001$) and obese ($P < 0.01$) volunteers. The distribution of muscle electron transport chain activity in obesity also revealed a deficit in the subsarcolemmal mitochondrial fraction, although this was not as severe as in type 2 diabetes.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/1/8/378934/zdb00105000008.pdf by guest on 25 January 2022

TABLE 3
Electron transport chain activity in subsarcolemmal and intermyofibrillar mitochondria fractions of human skeletal muscle

| | Lean | Obese | Type 2 diabetes |
|---|---|---|---|
| $n$ | 8 | 12 | 11 |
| Subsarcolemmal mitochondria fraction | 0.121 ± 0.022 | 0.034 ± 0.007† | 0.017 ± 0.003*† |
| Intermyofibrillar mitochondria-1 fraction | 0.130 ± 0.018 | 0.065 ± 0.014† | 0.040 ± 0.008† |
| Intermyofibrillar mitochondria-2 fraction | 0.138 ± 0.016 | 0.051 ± 0.008† | 0.068 ± 0.008† |
| Total electron transport chain activity | 0.389 ± 0.050 | 0.150 ± 0.024† | 0.125 ± 0.013† |

Succinate oxidase (units/mU CK). *$P < 0.01$, type 2 diabetes vs. obese; †$P < 0.01$, type 2 diabetes or obese vs. lean.

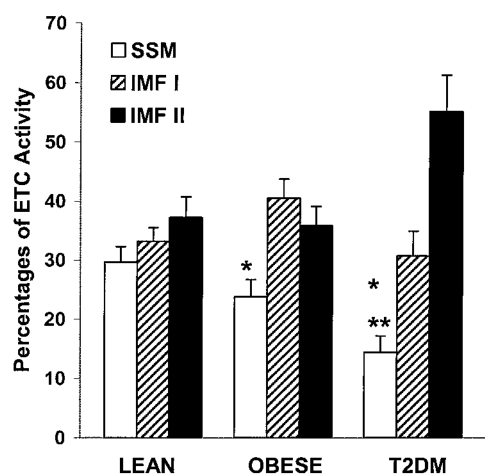


**FIG. 2. The distribution of overall succinate oxidase activity (complex II-IV) among subsarcolemmal and intermyofibrillar mitochondrial (IFM) fractions, as a percentage of overall succinate oxidase activity, is shown for skeletal muscle from lean, obese, and type 2 diabetic (T2DM) research volunteers. *Lean vs. obese or type 2 diabetic subjects, $P < 0.01$; **type 2 diabetic vs. obese subjects, $P < 0.05$. ETC, electron transport chain; SSM, subsarcolemmal mitochondria.**

Using regression analysis, we examined the potential relation of electron transport chain activity to insulin sensitivity. Consistent with our prior study (12), there was significant correlation between overall electron transport chain activity and insulin-stimulated glucose metabolism ($r = 0.67$, $P < 0.001$). The association between systemic insulin sensitivity and electron transport chain activity in subsarcolemmal and free and bound intermyofibrillar mitochondrial fractions ($r = 0.63$, 0.60, and 0.65, respectively) was similar, and each was statistically significant ($P < 0.001$).

**TEM.** A TEM was used to further and independently examine the hypothesis that there is a deficit of subsarcolemmal mitochondria in type 2 diabetes. Representative photographs of skeletal muscle from a lean volunteer and one with type 2 diabetes are shown in Fig. 3. In the muscle of lean volunteers, abundant subsarcolemmal mitochondria can be identified in the characteristic location adjacent to muscle nuclei. In contrast, in type 2 diabetes, there is a striking depletion of subsarcolemmal mitochondria. We performed quantitative measurement of the thickness of the layer of subsarcolemmal mitochondria. In type 2 diabetic subjects, the thickness of the subsarcolemmal mitochondria was reduced approximately threefold compared with that of lean volunteers (1.16 ± 0.47 vs. 2.93 ± 0.47 μm, $P < 0.01$, $n = 6$ samples from each group). Although TEM assessments support the conclusion of depleted subsarcolemmal mitochondria in type 2 diabetes, the reduction in electron transport chain activity in the subsarcolemmal mitochondrial fraction was nearly twofold greater than the reduction in mitochondria observed using a TEM.

TABLE 4
Mitochondrial DNA content in skeletal muscle in lean, obese, and type 2 diabetic research volunteers

| | Lean | Obese | Type 2 diabetes |
|---|---|---|---|
| $n$ | 6 | 12 | 5 |
| mtDNA | 2,514 ± 505 | 1,665 ± 213* | 1,850 ± 220* |
| Succinate oxidase (units/mU CK) | 0.39 ± 0.07 | 0.14 ± 0.02* | 0.11 ± 0.01* |

*$P < 0.05$ vs. values in lean.

**Mitochondrial DNA content.** To assess whether the reduction in muscle electron transport chain activity might be accounted for by reduced content of mitochondria, reduced functional capacity, or a combination of these factors, mtDNA content was determined as an index of mitochondrial content. This analysis included all volunteers from whom a sufficient sample of muscle was available, and these data are shown in Table 4. The key finding is that mtDNA content in skeletal muscle was reduced similarly in obese and type 2 diabetic subjects relative to lean volunteers. We next examined the ratio between total electron transport chain activity and mtDNA. This ratio was significantly reduced in obese and type 2 diabetic subjects relative to that found in lean volunteers. This indicates that reduced mitochondrial content, as revealed by lower mtDNA, does not fully account for the overall reduction in electron transport chain activity and that there is a reduced functional capacity of mitochondria in type 2 diabetes and obesity even after adjusting for mitochondrial content.

## DISCUSSION

Skeletal muscle is a major site for insulin-stimulated glucose utilization. Insulin resistance of this tissue is characteristically severe in type 2 diabetes (33). The pathogenesis of muscle insulin resistance in type 2 diabetes and obesity is known to involve defects of insulin

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/1/8/378934/zdb00105000008.pdf by guest on 25 January 2022

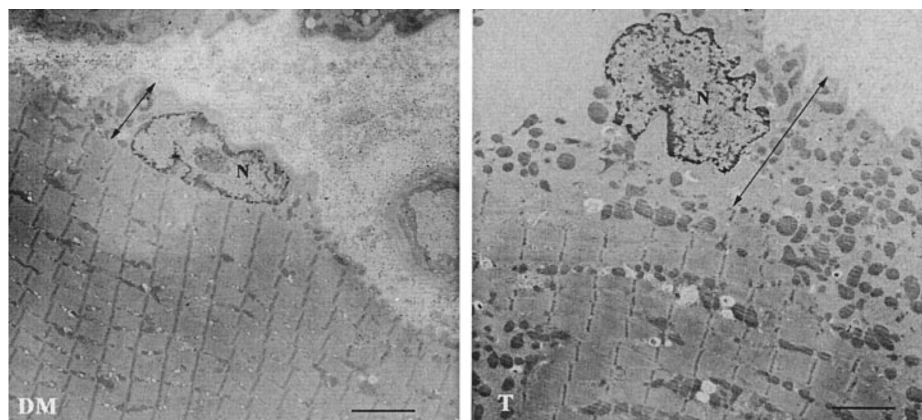


**FIG. 3. Representative transmission electron microscopy of longitudinal sections of human skeletal muscle from a lean (T) and a type 2 diabetic (DM) research volunteer are shown (bar = 2.5 μm). The thickness of the perinuclear distribution of subsarcolemmal mitochondria was measured using image analysis (National Institutes of Health image 1.61) and can be observed to be substantially depleted in type 2 diabetes.**

signaling and glucose transport (7,34–36), but the underlying pathogenesis remains uncertain. Several recent studies, including one from our laboratory, suggest that mitochondrial dysfunction might be involved in the pathogenesis of insulin resistance (12,37–39). The mechanism by which mitochondrial dysfunction causes insulin resistance remains unclear, although one attractive hypothesis is a disposition to lipid accumulation caused by impaired capacity for lipid oxidation (10,40).

The current study was undertaken to examine the subpopulation of subsarcolemmal mitochondria. Although it is recognized that mitochondria in skeletal muscle exist as a reticulum, there is compartmentalization into subsarcolemmal and intermyofibrillar mitochondrial populations (41). Subsarcolemmal mitochondria provide energy for membrane-related processes, including signal transduction, ion exchange, substrate transport, and substrate activation—steps clearly relevant to insulin action—(4), whereas the intermyofibrillar mitochondria more directly support muscle contraction. In this study, we have made the novel observation that there is a disproportionate deficiency of electron transport chain activity within the subsarcolemmal mitochondrial fraction in type 2 diabetic compared with lean active adults. This deficit of subsarcolemmal mitochondria is also evident in sedentary obese individuals, though not as severe as in type 2 diabetic individuals. This deficiency of electron transport chain activity near the cell surface in skeletal muscle in type 2 diabetes may be of importance in understanding the cell biology of insulin resistance.

In a prior study, we reported an overall reduction in electron transport chain activity in muscle in type 2 diabetes and obesity (12), but we did not address mitochondrial distribution. Also, the previous TEM examination of mitochondrial morphology, which revealed smaller mitochondria in type 2 diabetic and obese than in lean volunteers, examined only intermyofibrillar mitochondria (12). In the current study, using differential centrifugation and cell digestion procedures to separate mitochondrial fractions, a striking deficiency of subsarcolemmal mitochondria was observed in type 2 diabetes and confirmed using a TEM. In skeletal muscle of lean healthy volunteers, the distribution of electron transport chain activity across the subsarcolemmal and free and bound intermyofibrillar mitochondrial fractions was symmetrical, with approximately one-third of overall electron transport chain activity in each fraction. This observation is strongly consistent with previous findings (32). In obese and type 2 diabetic subjects, a greater proportion of overall electron transport chain activity was found in the intermyofibrillar mitochondrial fractions, although the absolute activities were reduced compared with those of lean volunteers. No significant difference between obese and type 2 diabetic subjects was observed for absolute electron transport chain activity in intermyofibrillar mitochondrial fractions. However, it was found that absolute electron transport chain activity in the subsarcolemmal mitochondrial fraction and the relative proportion of overall electron transport chain activity accounted for by this fraction were reduced in type 2 diabetic compared with both lean and obese subjects.

Two recently published studies using gene array expression analyses have reported a significant decrease in the nuclear expression of genes encoding proteins of oxidative phosphorylation and other mitochondrial proteins in type 2 diabetes (42,43). These are striking observations because this pattern of impaired gene expression was the most consistent finding in the analyses of multiple pathways. Also, Stump et al. (39) recently reported that an 8-h insulin infusion stimulates increased muscle ATP production and mitochondria protein synthesis in lean volunteers, but not in type 2 diabetic subjects. More recently, noninvasive assessments of oxidative phosphorylation of soleus muscle, performed with magnetic resonance spectroscopy, indicate mitochondrial dysfunction in insulin resistance (37,38). Taken together, the various studies support an emerging concept that insulin resistance has a crucial component related to disordered functional capacity of mitochondria. Earlier, reduced oxidative enzyme activity in skeletal muscle was identified as a risk factor for development of obesity (11,44), an association that might relate to decreased capacity for fat oxidation in skeletal muscle. Our laboratory has observed that there is impaired fat oxidation within skeletal muscle during fasting conditions in type 2 diabetes (45) and in obesity (46), and results implicated reductions in oxidative enzymes as a causative factor (10). Ex vivo studies of human muscle support this hypothesis (40). In this regard, it is important to note that one of the physiological functions postulated for subsarcolemmal mitochondria is fat oxidation (4). One mechanism by which a deficiency in subsarcolemmal mitochondria may contribute to insulin resistance might be the accumulation of intramyocellular lipids (7).

The reduction in electron transport chain activity in muscle in type 2 diabetes and obesity could be caused by reduced mitochondrial content or reduced functional capacity of mitochondria. To address this issue, we measured mtDNA content in muscle. Assessment of mtDNA is regarded as a quantitative index of mitochondrial content in tissues, including skeletal muscle (26,27). Compared with the findings in muscle from lean healthy volunteers, we did find a significant decrease in mtDNA in muscle in obesity and type 2 diabetes. This indicates that decreased mitochondrial content at least partly explains reduced electron transport chain activity. However, in type 2 diabetes, the decrement in electron transport chain activity was greater than the corresponding decrement in mtDNA content. A similar, albeit less severe, pattern was seen in obesity. These findings suggest that functional impairments in electron transport chain activity in type 2 diabetes are not fully accounted for by reduced mitochondrial content. In further support of this concept, it is noteworthy that the decrement in electron transport chain activity within subsarcolemmal mitochondria in type 2 diabetes appears to be approximately twofold greater than the decrease in the thickness of the subsarcolemmal mitochondria layer, as assessed by the TEM.

The factors that account for a disproportionate depletion of electron transport chain activity in subsarcolemmal mitochondria in type 2 diabetes are unclear and warrant further investigation. Perhaps the explanation for lower subsarcolemmal mitochondria electron transport chain activity is simply decreased physical activity. In the current study, volunteers with type 2 diabetes and those who

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/1/8/378934/zdb00105000008.pdf by guest on 25 January 2022

were obese but nondiabetic were closely matched for age, sedentary lifestyle, sex, and weight. However, a formal measure of fitness and physical activity was not performed, and potential differences might have existed. Both groups manifested a disproportionate reduction in electron transport chain activity in the subsarcolemmal mitochondrial fraction compared with lean individuals, who were more physically active. These sharp differences in subsarcolemmal mitochondria electron transport chain activity—both absolute and as a percentage of overall muscle electron transport chain activity—between sedentary type 2 diabetic and obese subjects on the one hand and the lean active volunteers on the other suggest that factors of obesity and sedentary lifestyle are potentially important determinants of the distribution of muscle electron transport chain activity. Intervention-based studies should be helpful in revealing the impact of physical activity because exercise is considered to have particular effects on the subsarcolemmal mitochondrial fraction (4).

In summary, in this study we find a disproportionately large reduction of electron transport chain activity in the subsarcolemmal mitochondrial fraction in type 2 diabetic and obese subjects compared with lean volunteers. Furthermore, despite similar overall reductions in muscle electron transport chain activity in obesity and type 2 diabetes, there is a greater deficit within the subsarcolemmal mitochondrial fraction in type 2 diabetes. The reduction in electron transport chain activity in obesity and type 2 diabetes does not appear to be fully accounted for by reduced mitochondrial content. Given the important role that subsarcolemmal mitochondria have for bioenergetic support of signal transduction, fat oxidation, and substrate transport, an impairment of electron transport chain activity in this subcellular location may have particular relevance to the pathogenesis of insulin resistance in type 2 diabetes.

## ACKNOWLEDGMENTS

This investigation was supported by funding from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (DK49200); the University of Pittsburgh General Clinical Research Center (5 M01RR00056); and the Obesity and Nutrition Research Center (P30DK462).

We gratefully acknowledge the valuable contributions of Carol Kelley, BSN, RN, the research coordinator, and the nursing staff of the University of Pittsburgh General Clinical Research Center. We also express our appreciation to the research participants.

## REFERENCES

1. Kaasik A, Veksler V, Boehm E, Novotova M, Minajeva A, Ventura-Clapier R: Energetic crosstalk between organelles. *Circ Res* 89:153–159, 2001
2. Weiss J, Korge P: The cytoplasm: no longer a well mixed bag. *Circ Res* 89:108–110, 2001
3. Cogswell A, Stevens R, Hood D: Properties of skeletal muscle mitochondria isolated from subsarcolemmal and intermyofibrillar regions. *Am J Physiol* 264:C383–C389, 1993
4. Hood D: Plasticity in skeletal, cardiac, and smooth muscle: contractile activity-induced mitochondrial biogenesis in skeletal muscle. *J Appl Physiol* 90:1137–1157, 2001
5. Saltiel A: The molecular and physiological basis of insulin resistance: emerging implications for metabolic and cardiovascular disease. *J Clin Invest* 106:163–169, 2000
6. Sheperd P, Kahn B: Glucose transporters and insulin action: implications for insulin resistance and diabetes mellitus. *N Engl J Med* 341:248–257, 1999
7. Shulman G: Cellular mechanisms of insulin resistance. *J Clin Invest* 106:171–176, 2000
8. Colberg SR, Simoneau JA, Thaete FL, Kelley DE: Skeletal muscle utilization of free fatty acids in women with visceral obesity. *J Clin Invest* 95:1846–1853, 1995
9. Simoneau JA, Kelley DE: Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. *J Appl Physiol* 83:166–171, 1997
10. Simoneau JA., Veerkamp J, Turcotte L, Kelley D: Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. *FASEB J* 13:2051–2060, 1999
11. Sun G, Ukkola O, Rankinen T, Joanisse D, Bouchard C: Skeletal muscle characteristics predict body fat gain in response to overfeeding in never-obese young men. *Metabolism* 51:451–456, 2002
12. Kelley D, He J, Menshikova E, Ritov V: Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51:2944–2950, 2002
13. DeFronzo RA, Tobin JD, Andres R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237:E214–E223, 1979
14. Finegood D, Bergman R, Vranic M: Estimation of endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps: comparison of unlabeled and labeled exogenous glucose infusates. *Diabetes* 36:914–924, 1987
15. Kelley DE, Mckolanis T, Hegazi R, Kuller L, Kalhan S: Fatty liver in type 2 diabetes mellitus: relation to regional adiposity, fatty acids, and insulin resistance. *Am J Physiol* 285:E906–E916, 2003
16. Frayn K: Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 55:628–634, 1983
17. Krieger D, Tate C, McMillin-Wood J, Booth F: Populations of rat skeletal muscle mitochondria after exercise and immobilization. *J Appl Physiol* 48:23–28, 1980
18. Ritov V, Kelley D: Hexokinase isozyme distribution in human skeletal muscle. *Diabetes* 50:1253–1262, 2001
19. Ritov V: High-performance liquid chromatography-based methods of enzymatic analysis: electron transport chain activity in mitochondria from human skeletal muscle. *Anal Biochem* 333:27–38, 2004
20. Mitchell R, Palade P, Fleisher S: Purification of morphologically intact triad structures from skeletal muscle. *J Cell Biol* 96:1008–1016, 1983
21. Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS: Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo. *Mol Cell Biochem* 184:81–100, 1998
22. Gostimskaya I, Grivennikova V, Zharova T, Bakeeva L, Vinogradov A: In situ assay of the intramitochondrial enzymes: use of alamethicin for permeabilization of mitochondria. *Anal Biochem* 313:46–52, 2003
23. Birch-Machin M, Turnbull D: Assaying mitochondrial respiratory complex activity in mitochondria isolated from human cells and tissues. *Methods Cell Biol* 6:97–117, 2001
24. Sembowich W, Knudson M, Gollnick P: Muscle metabolism and cardiac function of the myopathic hamster following training. *J Appl Physiol* 43:936–941, 1977
25. Pesce A, Kaplan L: *Methods in Clinical Chemistry.* St. Louis, C.V. Mosby, 1987
26. Szuhai K, Ouweland J, Dirks R, Lemaitre M, Truffert J, Janssen G, Tanke H, Holme E, Maassen J, Raap A: Simultaneous A8344G heteroplasmy and mitochondrial DNA copy number quantification in myoclonus epilepsy and ragged-red fibers (MERRF) syndrome by a multiplex molecular beacon based real-time fluorescence PCR. *Nucleic Acid Res* 29:E13, 2001
27. Miller F, Rosenfeldt F, Zhang C, Linnane A, Nagley P: Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age. *Nucleic Acid Res* 31:E61, 2003
28. Wu H, Kanatous S, Thurmond F, Gallardo T, Isotani E, Bassel-Duby R, Williams R: Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. *Science* 296:349–352, 2002
29. Robinson D, Ehlers U, Herken R, Herrman B, Mayer F, Schurmann F: *Methods of Preparation for Electron Microscopy. An Introduction for the Biomedical Sciences.* Tokyo, Springer-Verlag, 1987, p. 55–56
30. Reynolds E: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J Cell Biol* 17: 208, 1963
31. Haggis G: *The Electron Microscope in Molecular Biology.* London, William Glowes and Sons, Longmans, 1966, p. 77

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/1/8/378934/zdb00105000008.pdf by guest on 25 January 2022

32. Elander A, Sjostrom M, Lundgren F, Schersten T, Bylund-Fellenius, AC: Biochemical and morphometric properties of mitochondrial populations in human muscle fibers. *Clin Sci* 69:153–164, 1985
33. DeFronzo R: Lilly Lecture 1987: The trimvirate: beta cell, muscle, liver: a collusion responsible for NIDDM. *Diabetes* 37:667–687, 1988
34. Bonadonna RC, Del Prato S, Bonora E, Saccomani MP, Gulli G, Natali A, Frascerra S, Pecori N, Ferrannini E, Bier D, Cobelli C, DeFronzo RA: Roles of glucose transport and glucose phosphorylation in muscle insulin resistance of NIDDM. *Diabetes* 45:915–925, 1996
35. Kelley D, Mintun M, Watkins S, Simoneau J, Jadali F, Fredrickson A, Beattie J, Theriault R: The effect of non-insulin-dependent diabetes mellitus and obesity on glucose transport and phosphorylation in skeletal muscle. *J Clin Invest* 97:2705–2713, 1996
36. Saltiel A, Kahn C: Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414:799–806, 2001
37. Petersen K, Befoy D, Dufour S, Dziura J, Ariyan C, Rothman D, DiPietro L, Cline G, Shulman G: Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* 300:1140–1142, 2003
38. Petersen K, Dufour S, Befoy D, Garcia R, Shulman GI: Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350:664–671, 2004
39. Stump C, Short K, Bigelow M, Schimke J, Sreekumaran N: Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis and mRNA transcripts. *Proc Natl Acad Sci U S A* 100:7996–8001, 2003
40. Kim J, Hickner R, Cortright R, Dohm G, Houmard J: Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol* 279:E1039–E1044, 2000
41. Palmer J, Tandler B, Hoppel C: Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. *J Biol Chem* 252:8731–8739, 1977
42. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC: PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267–273, 2003
43. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ: Coordinated reduction in genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF-1. *Proc Natl Acad Sci U S A* 100:8466–8471, 2003
44. Zurlo F, Nemeth P, Choski R, Sesodia S, Ravussin E: Whole body energy metabolism and skeletal muscle biochemical characteristics. *Metabolism* 43:481–486, 1994
45. Kelley D, Simoneau JA: Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus. *J Clin Invest* 94:2349–2356, 1994
46. Kelley D, Goodpaster B, Wing R, Simoneau J: Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity and weight loss. *Am J Physiol* 277:E1130–E1141, 1999

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/1/8/378934/zdb00105000008.pdf by guest on 25 January 2022