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Aim: Skeletal muscle mitochondrial content is reduced in type 2 diabetes mellitus (T2DM). Whether hyperglycemia inhibits mitochondrial biogenesis and/or function is unknown. This study examined the effect of different levels of glycemia on skeletal muscle mitochondrial function in patients with T2DM.

Patients and Methods: Eleven patients with T2DM [9 males, 2 females; age, 52.8 ± 2.5 yr (mean ± se); body mass index, 30.2 ± 1.1 kg/m²] in poor glycemic control were treated with insulin aspart and NPH insulin for a median period of 46 d (range, 31–59). Mitochondrial respiration and citrate synthase activity (a marker of mitochondrial content) were measured before and after treatment. Eleven healthy subjects (age, 53.3 ± 2.7 yr; body mass index, 30.6 ± 1.1 kg/m²) were included as controls.

Results: Hemoglobin A1c (9.1 ± 0.5 to 7.5 ± 0.3%; P < 0.001) and fasting plasma glucose (12.7 ± 1.1 to 6.5 ± 0.3 mmol/liter; P < 0.001) were reduced after treatment. Mitochondrial respiration per milligram muscle was lower in T2DM compared to controls [substrates for complex I, 24% lower (P < 0.05); substrates for complex I–II, 17% lower (P < 0.05)]. Mitochondrial respiration and citrate synthase activity did not differ before and after improvements in glycemic control, but mitochondrial respiration correlated with fasting plasma glucose before (r² = 0.53; P < 0.05) but not after treatment [r² = 0.0024; not significant (NS)]. Mitochondrial respiration normalized to mitochondrial content did not differ between control subjects and patients with T2DM.

Discussion: Mitochondrial respiration and content was not improved after significant improvements in glycemic control. However, severe hyperglycemia inhibited respiration reversibly, but moderate hyperglycemia and mitochondrial function were not correlated. (J Clin Endocrinol Metab 94: 1372–1378, 2009)

Patients with type 2 diabetes are characterized by insulin resistance, which precedes and predicts the development of the disease (1). It has been suggested that decreased mitochondrial function plays a role in the pathogenesis of insulin resistance (2), and both the activity of oxidative enzymes (3–5) and the expression of genes involved in the regulation of oxidative phosphorylation (6, 7) have been shown to be lower in skeletal muscle of patients with type 2 diabetes compared with age- and body mass index (BMI)-matched control subjects. The reduction in oxidative capacity presumably leads to accumulation of intramyocellular triglycerides (IMTG) that inhibit insulin signaling (8).

It is still debated whether the reduction in oxidative capacity is due to intrinsic defects in the mitochondria (9, 10) or to a reduction in mitochondrial content (11, 12). Mitochondrial impairment has been demonstrated in a group of normoglycemic, insulin-resistant subjects with a family history of type 2 diabetes, and it has been proposed to be an early defect in the development of type 2 diabetes.

Abbreviations: BMI, Body mass index; CS, citrate synthase; FFA, free fatty acid; HbA1c, hemoglobin A1c; IMTG, intramyocellular triglycerides; NS, not significant; ROS, reactive oxygen species; VO₂max, maximum rate of O₂ consumption.
of type 2 diabetes (13). However, data from endothelial cells have shown that hyperglycemia increases oxidative stress and mitochondrial damage (14), and fasting plasma glucose and hemoglobin A1c (HbA1c) have been shown to be correlated to phosphocreatinine recovery half-time (15), rendering it possible that hyperglycemia could have adverse effects on mitochondrial biogenesis and/or function in patients with type 2 diabetes.

This study was undertaken to examine the effect of poor and good glycemic control on mitochondrial respiration and content in patients with type 2 diabetes. We improved glycemic control by intensive insulin treatment for 7 wk.

Patients and Methods

Eleven patients with type 2 diabetes (nine men) were recruited from the outpatient clinics of endocrinology at the Copenhagen University Hospitals of Hvidovre and Gentofte. Patients were eligible for the study if they were on oral antidiabetic medication (metformin and/or sulfonylurea), were weight stable with a BMI between 25 and 35 kg/m², and did not have medical conditions preventing them from participating in a bicycle maximum rate of O₂ consumption test.

After a 2-wk washout period of antidiabetic medication, patients were instructed to measure an eight-point blood sugar profile (OneTouchUltra; Lifescan, Milpitas, CA) before and after each meal, before bedtime, and at 0200 h. To optimize glycemic control, patients were then treated with multiple insulin injections (insulin aspart and NPH insulin) for a median period of 46 d (range, 31–59). The target of treatment was fasting blood glucose below 5.5 mmol/liter and postprandial blood glucose below 7.5 mmol/liter. Before and after insulin treatment, patients were admitted to our laboratory for measurements of body weight, fasting plasma glucose (Yellow Springs Instrument Model YSI 2300 STAT plus analyzer; YSI Inc., Yellow Springs, OH), fasting plasma insulin (by auto-DELPHIA automatic fluoroimmunoassay; Wallec, Inc., Turku, Finland), serum fructosamine, and HbA1c (Bayer DCA 2000+; Bayer Healthcare, Elkhart, IN). Furthermore, a muscle biopsy from musculus vastus lateralis was obtained after an overnight fast. Patients were in stable glycemic control for 2 wk before the final examination day. In the last 3 d of treatment, measurements of glucose profiles were repeated.

Eleven healthy, obese, BMI-, sex-, and age-matched controls were also included. All had fasting plasma glucose concentrations and 2-h oral glucose tolerance within the normal range. We obtained muscle biopsies and blood samples on all control subjects. Patient and control subject characteristics are shown in Table 1. The ethics committee of the municipality of Copenhagen and Frederiksberg in Denmark approved the study protocol, and oral and written consent was obtained from each patient in accordance with the Helsinki Declaration.

Preparation of muscle fibers

Muscle biopsies were taken from musculus vastus lateralis using the Bergstrom technique, and 50 mg were snap-frozen in liquid nitrogen for later biochemical analysis. A small part of the biopsy sample (5 mg) was immediately placed in ice-cold BIOPS solution containing 10 mM CaEGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM potassium 2-(n-morpholino)-enthanosulfonic acid, 0.5 mM dithiothreitol, 6.56 mM MgCl₂, 5.77 mM ATP, and 15 mM phosphocreatine (pH 7.1).

These small muscle samples were gently dissected using forceps, and fibers were incubated with 3 ml of saponin (50 g/ml) for 30 min. Muscle samples were carefully weighed before they were added to the respiration chambers. Respiration measurements were performed in a medium containing 0.5 mM EGTA, 3 mM MgCl₂, 6H₂O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, and 1 g/l-1 BSA (pH 7.1) (Mir05). The use of permeabilized fibers provides the

<table>
<thead>
<tr>
<th>TABLE 1. Subject characteristics and results of treatment</th>
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<tr>
<td>Characteristics</td>
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<tr>
<td>Age, median [range] (yr)</td>
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<tr>
<td>T2DM hyperglycemia (n = 11)</td>
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<td>54 [39–67]</td>
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<tr>
<td>T2DM normoglycemia (n = 11)</td>
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<td>54 [39–67]</td>
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<tr>
<td>P value</td>
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<td>T2DM hyperglycemia vs. normoglycemia (n = 11)</td>
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<tr>
<td>&lt;0.01</td>
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<tr>
<td>Control (n = 11)</td>
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<tr>
<td>24.1 ± 1.7</td>
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<tr>
<td>P value</td>
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<tr>
<td>T2DM vs. control</td>
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<td>&lt;0.05</td>
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<tr>
<th>Characteristics</th>
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<tr>
<td>Blood analyses</td>
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<tr>
<td>HbA1c (%) (n = 10)</td>
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<tr>
<td>9.1 ± 0.5</td>
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<tr>
<td>Fructosamine (μmol/liter)</td>
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<td>252 ± 12</td>
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<tr>
<td>Fasting plasma glucose (mmol/liter)</td>
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<tr>
<td>6.5 ± 0.3</td>
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<tr>
<td>Mean blood glucose, pre- and postprandial (mmol/liter)</td>
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<tr>
<td>7.2 ± 0.2</td>
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<tr>
<td>Fasting insulin (pmol/liter)</td>
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<tr>
<td>83 ± 32</td>
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<tr>
<td>Muscle analyses</td>
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<tr>
<td>CS (μmol/min · g) (n = 10)</td>
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<tr>
<td>71.8 ± 11</td>
</tr>
<tr>
<td>Glycogen (mmol/mg)</td>
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<tr>
<td>311 ± 26</td>
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<tr>
<td>IMTG (nmol/mg dry weight)</td>
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<td>120 ± 20</td>
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T2DM, Type 2 diabetes mellitus.

* Estimated from the maximal power output (MPO measured in Watt) with the following formula: VO₂max = 0.16 + (0.0117 × MPO) (Ref. 32).

* T2DM hyperglycemia vs. control.

* T2DM normoglycemia vs. control.

* Measured as the mean of the eight-point blood glucose profile.

* Elevated due to exogenous insulin administration.

* n = 9.
blood glucose are significant \( (P < 0.0001) \). The large error bar in the nighttime measurement is due to a limited number of measurements \( (n = 4) \).

Mitochondrial respiration protocol

Measurements of oxygen consumption were performed at 37°C using a high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). All measurements of mitochondrial respiration were performed in duplicate, and the data were averaged before further analysis. To avoid any potential oxygen limitation, all experiments were conducted after hyper oxygenation (approximately 450 nmol O2/ml). Substrates and inhibitors were added consecutively. State 2 respiration was measured after the addition of malate (2 mM), pyruvate (5 mM), and glutamate (10 mM) in saturating concentrations. State 3 respiration with electron flux through complex I was measured after the addition of ADP (5 mM), and maximal coupled respiration (state GS3), with convergent electron flux through complex I and complex II, was achieved by adding saturating concentrations of succinate (10 mM) to the muscle biopsy in the respiration chamber. The integrity of the outer mitochondrial membrane was assessed by the addition of cytochrome C (10 mM) in state GS3. If respiration did not increase more than 10% after addition of cytochrome C, the quality of the mitochondria was considered sufficient.

IMTG and glycogen measurements

Before biochemical analysis, muscle samples were freeze-dried. Using a stereomicroscope, connective tissue, visible fat, and blood were removed from the muscle samples. Muscle glycogen concentration was determined as glucose residues after hydrolysis of the muscle sample in 1 M HCl at 100°C for 2 h (16). Muscle triglyceride content was analyzed as described previously (17). In the final part of the analysis, the glycerol concentration was analyzed in triplicate on a CMA analyzer (CMA 600 microdialysis analyzer; CMA/Microdialysis AB, Stockholm, Sweden).

CS activity

CS activity was measured spectrophotometrically at 37°C by a 50-times dilution in a solution containing 100 μM acetyl-coenzyme A, 0.5 mM nicotinamide adenine dinucleotide (free acid), 1 mM sodium malate, 8 μg · ml\(^{-1}\) malate dehydrogenase (1200 U · mg\(^{-1}\); Roche Molecular Biochemicals, Indianapolis, IN), 2.5 mM EDTA, and 10 mM Tris-HCl (pH 8.0). Enzyme activities are expressed as micromoles of substrate per minute per gram dry weight muscle tissue, and CS activity was taken as a measure of mitochondrial content.

Data analysis

All comparisons were calculated using either paired or nonpaired two-tailed Student’s t test when appropriate. \( P \) values less than 0.05 were considered significant in two-tailed testing. All correlations were tested using Pearson’s method. Data are reported as means ± se.

Results

Patients were treated with insulin aspart and NPH insulin for a median period of 46 d (range, 31–59) and achieved significant improvements in glycemic control with decreases in plasma glucose, fructosamine, and HbA1c (Table 1). Figure 1 shows the mean of the blood glucose profiles on the 3 d before insulin treatment and on the last 3 d of insulin treatment. Patients received on average 74 ± 11 IU of insulin per day. Neither insulin treatment nor improved glycemic control had any effect on CS activity, which indicates unchanged muscle mitochondrial content (Table 1). CS activity correlated with maximal coupled respiration (substrates: malate, pyruvate, glutamate, succinate, and ADP) in patients with type 2 diabetes after treatment \( (r^2 = 0.84; n = 10; P < 0.01) \) and tended to correlate in control subjects \( (r^2 = 0.47; n = 9; P = 0.06) \).

Mean mitochondrial respiration per milligram of muscle was significantly lower in patients with type 2 diabetes compared with age- and weight-matched controls. Respiration was 24% lower in patients with type 2 diabetes when substrates for complex I and II were used (malate, pyruvate, glutamate, and ADP) \( (P < 0.05) \), and 17% lower when substrates for complex I and II were used (malate, pyruvate, glutamate, ADP, and succinate) \( (P < 0.05) \). Mean mitochondrial respiration did not differ before and after insulin treatment (Fig. 2). When data on mitochondrial respiration were corrected for mitochondrial content (as measured by CS activity), we found no differences between the patients with type 2 diabetes and the control subjects (Fig. 3).

There was no significant correlation between the duration of treatment and changes in mitochondrial respiration \( (r^2 = 0.10; n = 11; P > 0.2) \). Before treatment, mitochondrial respiration correlated inversely with fasting plasma glucose levels \( (r^2 = 0.53; n = 11; P < 0.05) \), but these parameters did not correlate after treatment \( (r^2 = 0.002; n = 11; NS) \) (Fig. 4). Mitochondrial respiration increased in the subjects with the most significant improvement in glycemic control during treatment. The change in fasting plasma glucose during treatment correlated to the change in mitochondrial respiration \( (r^2 = 0.63; n = 11; P < 0.01) \) (Fig. 5). Furthermore, we found a significant correlation between baseline fasting plasma glucose and the change in mitochondrial respiration during treatment \( (r^2 = 0.69; n = 11; P < 0.01) \) as well as between baseline fructosamine and change in mitochondrial respiration during treatment \( (r^2 = 0.53; n = 11; P < 0.05) \). However, the correlation between baseline HbA1c and change in mitochondrial respiration did not reach statistical significance \( (r^2 = 0.36; n = 10; P < 0.1) \).

Muscle triglyceride content and glycogen concentration did not change during insulin treatment (Table 1).

Discussion

This study indicates that significant improvement in glycemic control does not affect skeletal muscle mitochondrial respiration.
and content in type 2 diabetes. Mitochondrial respiration per milligram of muscle was approximately 20% lower in patients with type 2 diabetes compared with age- and BMI-matched control subjects, but these differences were eliminated when data were normalized to CS activity, suggesting that mitochondrial content may have contributed to the observed differences in activity. We have previously demonstrated a similar reduction in mitochondrial respiration and CS activity in a comparable group of patients with type 2 diabetes (11), and we suggest that mitochondrial content is reduced in type 2 diabetes with no intrinsic defects in mitochondrial function. This view is supported by the fact that mitochondrial respiration and CS activity are correlated in our group of patients. The reason for the decrease in mitochondrial content in type 2 diabetes is unclear.

This study is the first to measure mitochondrial respiration before and after an intensive antihyperglycemic treatment in a clinical setting. Intensive insulin therapy significantly improved glycemic control, as demonstrated by reductions in fasting plasma glucose, eight-point daily blood glucose measurements, fructosamine, and HbA1c. Mean blood glucose during the last 3 days of insulin treatment was $7.2 \pm 0.2$ mmol/liter, which included both preprandial and postprandial values. We were not able to find a correlation between the duration of treatment and change in mitochondrial function, but it is unknown whether further improvements in glycemic con-

![FIG. 2. Mitochondrial respiration with substrates for complex I (malate, pyruvate, and glutamate) and complex II (malate, pyruvate, glutamate, and succinate). Mitochondrial respiration was significantly reduced in patients with type 2 diabetes when compared with the respiration in the control group. Optimizing glycemic control had no effect on average mitochondrial respiration in the patients with type 2 diabetes. Mitochondrial respiration is measured per milligram of muscle. * , $P < 0.05$ compared with patients with type 2 diabetes. T2DM, Type 2 diabetes mellitus.](image)

![FIG. 3. Mitochondrial respiration with substrates for complex I (malate, pyruvate, and glutamate) and complex II (malate, pyruvate, glutamate, and succinate) measured as respiration per CS activity as a marker of mitochondrial content. No differences in mitochondrial respiration were observed between the groups. T2DM, Type 2 diabetes mellitus.](image)
or a longer treatment period would have an impact on mitochondrial respiration. We did, however, find a correlation between the baseline level of glycemic control, expressed as baseline levels of fasting plasma glucose and fructosamine, and mitochondrial respiration. In fact, our data showed that at high glucose levels (>15 mmol/liter) near-normalization of plasma glucose by insulin treatment improved mitochondrial function, whereas no effect was observed in patients with fasting plasma glucose levels below 15 mmol/liter. The mechanism behind this improvement in the severely hyperglycemic patients is unknown, but it could be related to the increases in plasma free fatty acid (FFA) levels associated with severe hyperglycemia. We did not measure FFA levels, but it is well established that severe hyperglycemia leads to an increase in lipolysis and an increase in plasma FFA (18), which has been shown to inhibit mitochondrial function (19).

Mitochondrial respiration and fasting plasma glucose levels correlated before, but not after, treatment. This supports our conclusion that mitochondrial function and plasma glucose levels in the normal to slightly elevated range are not directly related.

Recent animal data have suggested that very high glucose concentrations may inhibit mitochondrial function. Hyperglycemia-induced oxidative stress was recently shown to induce defects in mitochondrial biogenesis, structure, and function in high-fat, high-sucrose fed insulin-resistant mice (20). These defects in mitochondrial function have also been observed in streptozotocin-treated mice (20, 21) and were reversed by antioxidant treatment (20). Furthermore, mitochondrial dysfunction has been demonstrated in endothelial cells under hyperglycemic conditions, where the superoxide damage to mitochondria has been associated with the development of diabetic complications (14). The superoxide production is increased probably because of increased substrate oxidation, which increases the voltage gradient over the inner mitochondrial membrane and stimulates reactive oxygen species (ROS) production. Whether greater ROS production can explain the reduction in mitochondrial content in skeletal muscle in type 2 diabetes remains to be shown, but there does seem to be a link between excessive ROS production and mitochondrial dysfunction.

The question remains whether insulin resistance and decreased insulin action per se can affect mitochondrial biogenesis and function. Ten days of intensive insulin treatment had no effect on muscle mitochondrial protein synthesis and cytochrome c oxidase, and it caused only a modest increase in CS activity (22). Insulin infusion studies have confirmed that insulin up-regulates genes involved in mitochondrial biogenesis (23), and this effect has been shown to be blunted in type 2 diabetes (24). Furthermore, the increase in ATP synthesis after insulin infusion is inhibited in type 2 diabetes (25). This has led to the assumption that the impaired mitochondrial biogenesis and function in type 2 diabetes could be a result of, rather than the cause of, impaired insulin action. Our 7-wk insulin treatment did not result in greater CS activity, which is in accordance with another study applying a similar duration of insulin treatment (40 d) (26).

Defects in lipid metabolism could also have a negative effect on the mitochondria. Lipid infusion causes an immediate increase in insulin resistance (8) and a decrease in gene expression of genes involved in oxidative phosphorylation (27, 28). Furthermore, Brehm et al. (19) showed that the increase in muscular ATP production caused by hyperinsulinemic-euglycemic clamping was inhibited by concomitant lipid infusion. ATP production rate measured with magnetic

![FIG. 4.](image1) Fasting plasma glucose correlated with mitochondrial respiration (substrates: malate, glutamate, succinate, and ADP) before treatment (●), but not after treatment (○). Mitochondrial electron transport and respiration were reversibly reduced in the three subjects who were severely hyperglycemic (fasting blood glucose > 15 mmol/liter) at baseline.

![FIG. 5.](image2) The correlation between change in fasting plasma glucose and change in mitochondrial respiration during antihyperglycemic treatment in patients with T2DM. Mitochondrial respiration (substrates: malate, glutamate, succinate, and ADP) improved in patients with the poorest glycemic control at baseline but was unchanged in subjects with good to moderate glycemic control.
resonance spectroscopy increased by 60% during clamping under control conditions but was 24% lower during the lipid infusion. Chronic elevation of lipid supply has also been shown to affect mitochondrial gene expression because a high-fat diet coordinately down-regulated genes involved in oxidative phosphorylation (29). We did not find a difference in intramyocellular triglyceride levels between the patients with type 2 diabetes and obese control subjects, but we did not measure fatty acid oxidation intermediates such as diacylglycerol and ceramide, which are directly involved in the inhibition of insulin signaling (30). It has been proposed that incomplete fatty acid oxidation due to excess lipid supply to the muscle could be the link between skeletal muscle insulin resistance and mitochondrial dysfunction (31).

In conclusion, we have found that skeletal muscle mitochondrial respiration is lower in patients with type 2 diabetes compared with age- and BMI-matched control subjects. The reduction in mitochondrial respiration in type 2 diabetes is suggested to be due to a lower mitochondrial content, which is not corrected by improvements in glycemic control in subjects with baseline fasting plasma glucose below 15 mmol/l. We also demonstrate that severe hyperglycemia inhibits electron transport reversibly, possibly due to mitochondrial adaptations to increased levels of circulating FFAs or increased ROS production.

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References


