Does impaired mitochondrial function affect insulin signaling and action in cultured human skeletal muscle cells?

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Brown AE, Elstner M, Yeaman SJ, Turnbull DM, Walker M. Does impaired mitochondrial function affect insulin signaling and action in cultured human skeletal muscle cells? Am J Physiol Endocrinol Metab 294: E97–E102, 2008. First published October 23, 2007; doi:10.1152/ajpendo.00267.2007.—Insulin-resistant type 2 diabetic patients have been reported to have impaired skeletal muscle mitochondrial respiratory function. A key question is whether decreased mitochondrial respiration contributes directly to the decreased insulin action. To address this, a model of impaired cellular respiratory function was established by incubating human skeletal muscle cell cultures with the mitochondrial inhibitor sodium azide and examining the effects on insulin action. Incubation of human skeletal muscle cells with 50 and 75 μM azide resulted in 48 ± 3% and 56 ± 1% decreases, respectively, in respiration compared with untreated cells mimicking the level of impairment seen in type 2 diabetes. Under conditions of decreased respiratory chain function, insulin-independent (basal) glucose uptake was significantly increased. Basal glucose uptake was 325 ± 39 pmol/min/mg (mean ± SE) in untreated cells. This increased to 669 ± 69 and 823 ± 83 pmol/min/mg in cells treated with 50 and 75 μM azide, respectively (vs. untreated, both P < 0.0001). Azide treatment was also accompanied by an increase in basal glycogen synthesis and phosphorylation of AMP-activated protein kinase. However, there was no decrease in glucose uptake following insulin exposure, and insulin-stimulated phosphorylation of Akt was normal under these conditions. GLUT1 mRNA expression remained unchanged, whereas GLUT4 mRNA expression increased following azide treatment. In conclusion, under conditions of impaired mitochondrial respiration there was no evidence of impaired insulin signaling or glucose uptake following insulin exposure in this model system.

IT IS WELL RECOGNIZED that inherited defects of mitochondrial DNA such as the 3243 A→G tRNA mutation can lead to the development of diabetes (18, 26). Recent interest has focused on a possible role of mitochondrial dysfunction in the pathogenesis of type 2 diabetes and in particular the development of skeletal muscle insulin resistance. Kelley et al. (13) studied NADH:O2 oxidoreductase activity as an overall measure of mitochondrial electron transport chain function and found that the activity was decreased by ~40% in skeletal muscle biopsy samples from patients with type 2 diabetes compared with lean, healthy controls. Decreased mitochondrial respiration in skeletal muscle samples from type 2 diabetic patients was also recently reported by Boushel et al. (5), and this was linked to a decrease in mitochondrial content. A study of nondiabetic but insulin-resistant first-degree relatives of type 2 diabetic patients used magnetic resonance spectroscopy to measure skeletal muscle ATP synthesis (22). This was 30% lower in the relatives compared with insulin-sensitive control subjects and was associated with an increase in intramyocellular lipid content. These studies led to the intriguing question as to whether impaired mitochondrial oxidative function is a fundamental defect that contributes to the skeletal muscle insulin resistance.

Two recent studies investigated the effect of mitochondrial dysfunction on insulin action in rodent and murine muscle cell lines (17, 21). Severe depletion of mitochondrial DNA copy number led to decreased insulin-stimulated glucose uptake in both studies. However, there was no direct measurement of mitochondrial dysfunction in either of these studies, both relying on cellular ATP content as a surrogate index. As glycolytic ATP generation will continue and is likely to increase under conditions of mitochondrial dysfunction, the total cellular ATP content will underestimate the true degree of mitochondrial dysfunction. This is important because the cellular ATP content in both of these studies was decreased by ~80% under each experimental condition, suggesting extreme impairment of mitochondrial function.

The question remains whether lesser degrees of skeletal muscle mitochondrial dysfunction, comparable to those observed in type 2 diabetes, impair insulin action in human skeletal muscle. To address this specific question, we studied insulin action in primary human skeletal muscle cultures and titrated the concentration of azide, a specific inhibitor of cytochrome c oxidase (complex IV) in the mitochondrial respiratory chain (14, 15), to regulate the degree of suppression of mitochondrial respiration.

MATERIALS AND METHODS

General chemicals and reagents. Cell culture medium was obtained from Cambrex (Berkshire, UK), Opti-MEM, FBS, Trizol reagent, and the Thermoscript reverse transcription system were obtained from Invitrogen (Paisley, UK). Chick embryo extract was purchased from Sera Labs International (Sussex, UK), and penicillin-streptomycin, trypsin-EDTA, and DNase I were from Sigma (Poole, UK).

Antibodies. Anti-phospho-AMPK(Thr172), anti-phospho-Akt(Ser473), and native AMPK and Akt antibodies were purchased from New England Biolabs (Hertfordshire, UK).

Radioisotopes. 2-Deoxy-d-[2,6-3H]glucose and d-[U-14C]glucose were purchased from NEN (Boston, MA).

Subjects. Subjects recruited to this study were young, healthy, nonobese subjects with no family history of type 2 diabetes. The Newcastle and North Tyneside local research ethics committee approval was obtained before beginning the study and all subjects gave informed written consent.

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**Cell culture.** Muscle biopsies were obtained from the vastus lateralis muscle and satellite cells isolated as described previously (4). Myoblasts were cultured in Ham’s F-10 medium supplemented with 20% (vol/vol) FBS, 2% (vol/vol) chick embryo extract, 100 U/ml penicillin, and 100 µg/ml streptomycin. All experiments were carried out on cells between passages 5 and 8.

**Cell respiration measurements.** Cell respiration was measured in a high-resolution oxygraph chamber. Human myoblasts were seeded on 10-cm dishes in medium stated above. Medium was changed every 2–3 days. After reaching confluence of ~80%, medium was changed to serum-free medium with different concentrations of sodium azide. Twenty-four hours later, cells were harvested by mild trypsinization. After centrifugation, intact cells were resuspended in 100 µl of glucose-free, serum-free medium and introduced into the oxygraph chamber containing 2 ml of glucose-free, serum-free medium without added substrates at 37°C (high-resolution oxygraph chamber; Oroboros Innsbruck). After 5 min of stirring in the chamber, samples were taken for cell counts. The chamber was closed, and respiration studies were started after the signal was stabilized. Routine respiration, defined as respiration in cell culture medium without additional substrates (11), was measured for both azide-treated and untreated cells. Specificity of oxygen consumption to oxidative phosphorylation was shown by addition of 2 mM KCN at the end of measurements.

Oxygen consumption rates were calculated as the time derivative of the oxygen concentration (DATLAB Analysis Software), and traces were corrected for cell number and viability.

**ATP measurement.** ATP levels were quantified using a modified bioluminescent assay. This assay is based on the quantitative measurement of light produced from the reaction catalyzed by firefly luciferase in the presence of ATP. Cells were extracted into cold 3% perchloric acid (3% perchloric acid, 2 mM EDTA, 50 mM MOPS, 0.1% X-100). Samples were neutralized by the addition of 60 µl of oyster glycogen (240 mg/ml) and ice-cold ethanol. After centrifugation, intact cells were resuspended in 100 µl of glycogen-free, serum-free medium without additional substrates at 37°C (high-resolution oxygraph chamber; Oroboros Innsbruck). After 5 min of stirring in the chamber, samples were taken for cell counts. The chamber was closed, and respiration studies were started after the signal was stabilized. Routine respiration, defined as respiration in cell culture medium without additional substrates (11), was measured for both azide-treated and untreated cells. Specificity of oxygen consumption to oxidative phosphorylation was shown by addition of 2 mM KCN at the end of measurements.

**Glucose uptake.** Measurement of 2-deoxy-[2,6-3H]glucose uptake was performed in six-well cluster plates. Human myoblasts were serum starved with or without sodium azide for 24 h before incubation in Krebs buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO4, 1.2 mM CaCl, 20 mM HEPES, pH 7.4) with or without 100 µM insulin or cytochalasin B (10 µM) for 20 min. 2-Deoxyglucose (0.1 mM) and 0.5 µCi 2-deoxy-[2,6-3H]glucose were added to each well and incubated for a further 10 min. The reaction was terminated by washing the plate rapidly in ice-cold PBS. Cells were lysed in 0.05% SDS before scintillation counting and protein determination.

**Glycogen synthesis.** Measurement of glycogen synthesis took place in six-well cluster plates. Human myoblasts were serum starved with or without sodium azide for 24 h before incubation in serum-free medium containing 5.6 mM d-[U-14C]glucose for 1 h at 37°C with or without 100 µM insulin. The reaction was terminated by washing the plate rapidly in ice-cold PBS. Cells were lysed in 0.05% SDS before scintillation counting and protein determination.

**RESULTS**

Measurement of cellular respiratory chain function in azide-treated cells. A model of mitochondrial impairment was established by incubating human skeletal myoblasts with different concentrations of sodium azide for 24 h before measuring respiration polarographically in a high-resolution oxygraph chamber. Whole cell respiration was decreased by 35 ± 6% (mean ± SE) in cells incubated with 25 µM azide compared with untreated cells. Cells treated with 50 and 75 µM azide showed 48 ± 3 and 56 ± 1% decreases in total whole cell respiration, respectively, compared with untreated cells (Fig. 1A). ATP levels were quantified after 24 h of azide treatment. Although respiratory chain function was impaired in azide-treated cells at 24 h, ATP levels were comparable with the untreated cells (Fig. 1B). At both 50 and 75 µM azide, ATP levels were 100.8 ± 1.1 and 103.6 ± 7.7% that of the untreated control. For all further experiments, azide concentrations of 50 and 75 µM were used. Previous studies have demonstrated that, at micromolar concentrations of azide, the activity of cytochrome c oxidase (COX) is specifically reduced without affecting either mRNA or protein expression levels of COX or the activity of other mitochondrial enzymes (14, 15). Assessment of cell viability by trypan blue exclusion also demonstrated that, in this study, treatment with micromolar concen-
trations of azide did not have a detrimental effect on cell viability.

Impaired cellular respiration and glucose uptake. The effects of impaired cellular respiration in human skeletal muscle cells on insulin action were assessed by measuring rates of glucose uptake and glycogen synthesis following insulin exposure under the treatment conditions described. Human myoblasts were treated with 50 and 75 μM azide in serum-free medium for 24 h before measuring [2,6-3H]deoxyglucose uptake. Figure 2 demonstrates that treatment with azide increased basal glucose uptake at both concentrations. Untreated cells showed a basal rate of glucose uptake of 325 ± 39 compared with 669 ± 69 pmol·min⁻¹·mg⁻¹ for 50 μM azide and 823 ± 83 pmol·min⁻¹·mg⁻¹ for 75 μM azide. This increase in basal glucose uptake compared with the untreated cells was significant for both azide concentrations (***P < 0.0001). As shown in Fig. 2, glucose uptake following insulin exposure also tended to increase under all treatment conditions, indicating that glucose uptake following insulin exposure is not inhibited by azide treatment.

Impaired cellular respiration and glycogen synthesis. Glycogen synthesis following insulin exposure was also examined showed a basal rate of glycogen synthesis of 325 ± 39 compared with 669 ± 69 pmol·min⁻¹·mg⁻¹ for 50 μM azide and 823 ± 83 pmol·min⁻¹·mg⁻¹ for 75 μM azide. This increase in basal glucose uptake compared with the untreated cells was significant for both azide concentrations (both, *P < 0.0001). As shown in Fig. 2, glucose uptake following insulin exposure also tended to increase under all treatment conditions, indicating that glucose uptake following insulin exposure is not inhibited by azide treatment.
under conditions of impaired mitochondrial function. Basal levels of glycogen synthesis were increased in azide-treated vs. untreated myoblasts (Fig. 3), although this only reached statistical significance at 75 μM azide (203 ± 35 vs. 86 ± 10 pmol·min⁻¹·mg⁻¹, P = 0.005). In response to 100 nM insulin, glycogen synthesis increased from 86 ± 10 to 269 ± 28 pmol·min⁻¹·mg⁻¹ in the untreated cells (P < 0.0001). Cells treated with 50 and 75 μM azide also showed an increase in glycogen synthesis following insulin exposure. Glycogen synthesis increased from 131 ± 23 to 523 ± 37 pmol·min⁻¹·mg⁻¹ after treatment with 50 μM azide and from 203 ± 35 to 622 ± 41 pmol·min⁻¹·mg⁻¹ for 75 μM azide in response to insulin. This increase in glycogen synthesis following insulin exposure was significant under all treatment conditions (P < 0.0001).

**Impaired cellular respiration and Akt expression and activation.** To investigate whether the insulin-signaling pathway is impaired in azide-treated cells, activation of a key intermediate in the insulin-signaling pathway was examined. Western blotting using a phospho-AktSer473-specific antibody demonstrates that, in response to exposure to 100 nM insulin for 10 min, phosphorylation and activation of Akt were normal under conditions of impaired cellular respiration, while levels of the native Akt protein were also equivalent under all conditions (Fig. 4).

**Impaired cellular respiration and AMPK expression and activation.** AMPK is phosphorylated and activated in response to a reduction in the ATP-to-AMP ratio (27). Since impaired cellular respiration was associated with increased rates of basal glucose uptake, expression and phosphorylation of AMPK were evaluated after incubation with azide for 24 h. Western blotting using an antibody specific for phosphorylated AMPK on Thr172 demonstrates that the phosphorylation state of AMPK was increased in azide-treated cells compared with untreated cells (Fig. 5), whereas levels of native AMPK protein were unaltered by azide treatment.

**Impaired cellular respiration and glucose transporter expression.** Quantitative RT-PCR was used to analyze mRNA expression of GLUT1 and GLUT4 to assess whether total expression levels of these glucose transporters were altered in azide-treated cells. Human myoblast mRNA expression of GLUT1 was comparable under all treatment conditions when normalized against the reference gene β2-microglobulin (Fig. 6A). In contrast, GLUT4 expression increased following azide treatment (Fig. 6B). Compared with the untreated cells, GLUT4 mRNA expression increased after treatment with 50 and 75 μM azide, although this only reached statistical significance at the higher concentration.

**DISCUSSION**

The focus of this study was to address the specific question whether decreased mitochondrial respiration impairs insulin action in human skeletal muscle. Importantly, the dose of azide exposure was titrated to decrease mitochondrial respiration to a degree similar to that previously reported in skeletal muscle of type 2 diabetic patients (13). The key findings of this study are that impaired mitochondrial respiration results in an increase in basal glucose uptake, an increase in the absolute...
values of glucose uptake following insulin exposure, increased phosphorylation of AMPK, and an increase in the absolute values of glycogen synthesis. Insulin signaling, as assessed by phosphorylation of Akt, was unaffected by impaired mitochondrial respiration.

It is well recognized that metabolic stresses such as inhibition of oxidative phosphorylation can result in phosphorylation and subsequent activation of AMPK. This usually occurs through an increase in the cellular AMP/ATP ratio. However, in this study, ATP levels were comparable after 24-h azide treatment. Interestingly, Wedenber et al. (28) also reported normal ATP levels in mitochondrial DNA-depleted mouse muscle preparations but found decreased levels of phosphocreatine, which has been shown to activate AMPK. A time course study of inhibition of mitochondrial respiration in L6 muscle cells showed a rapid but transient decrease in ATP levels before returning toward normal (3). This shows how the cells are able to quickly respond to ATP depletion, presumably through other sources of ATP generation. This apparent discrepancy between decreased mitochondrial respiration and normal ATP levels can also be explained by the threshold effects of oxidative phosphorylation. A number of studies have demonstrated that ATP production decreases at different levels of inhibition of oxidative phosphorylation, and this in turn is dependent on the cell type (23).

The increase in basal levels of glucose uptake observed in the current study has been a consistent feature of other models of mitochondrial dysfunction (3, 8, 9, 24). There is good evidence linking the activation of AMPK to increased basal glucose uptake. AICAR (5-amino-4-imidazole carboxamide riboside) directly activates AMPK and has been shown to increase glucose uptake in rat skeletal muscle preparations (16, 28) and in cultivated human muscle cells (19). These studies provide evidence that AMPK activation directly increases glucose uptake in the absence of insulin. AMPK activation by AICAR has also been shown to increase GLUT4 mRNA and protein expression (7, 20, 29). Absolute rates of glucose uptake following insulin exposure also tended to increase following azide treatment, and importantly there was no clear inhibition of glucose uptake following insulin exposure under conditions of impaired mitochondrial respiratory chain function. Absolute values for glycogen synthesis in both the basal state and following insulin exposure were increased in azidetreated cells compared with the untreated controls. This may reflect a higher cellular free glucose concentration in the azide-treated cells. Glycogen synthase (GS) is a key enzyme in the regulation of glycogen synthesis. GS has been identified as a physiological target for AMPK, with AMPK phosphorylating (and inactivating) GS at site 2 (6). However, since the absolute values for glycogen synthesis increase under conditions where AMPK is activated, it is possible that the increased absolute levels of glucose uptake and, therefore, increased cellular glucose 6-phosphate, may override the inhibitory effects of AMPK on GS (2, 12). This has been demonstrated in vivo, where activation of AMPK by AICAR increased skeletal muscle glycogen content in the treated animals through increases in GLUT4 and hexokinase activity (10, 25).

To examine whether the insulin-signaling pathway itself was affected by impaired mitochondrial respiration, we investigated the role of Akt, which occupies a pivotal position in the insulin-signaling pathway. Both the expression and the activation of Akt were unaffected by azide treatment in this study.

In summary, although impairing mitochondrial respiration in skeletal muscle to a degree comparable to that previously reported in type 2 diabetic patients has a significant effect on basal glucose uptake, AMPK activation, and glycogen synthesis, there is no conclusive evidence of an impairment in insulin signaling and glucose uptake following insulin exposure. Further studies are required to examine the underlying mechanism behind the observations made in this study.

**GRANTS**

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**REFERENCES**


