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

Running title: Mitochondrial function and insulin action

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Abstract

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 the effects on insulin action examined. Incubation of human skeletal muscle cells with 50µM and 75µM azide resulted in a 48 ± 3% and a 56 ±1% decrease in respiration compared to 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 ± 39pmol/min/mg (mean ± SEM) in untreated cells. This increased to 669 ± 69 and 823 ± 83 pmol/min/mg in cells treated with 50 and 75µM 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 (AMPK). 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 while GLUT4 mRNA expression increased following azide treatment.

In conclusion, under conditions of impaired mitochondrial respiration, there was no evidence of impaired insulin signalling or glucose uptake following insulin exposure in this model system.
Introduction

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 and colleagues studied NADH:O₂ oxidoreductase activity as an overall measure of mitochondrial electron-transport chain function, and found that the activity was decreased by around 40% in skeletal muscle biopsy samples from patients with type 2 diabetes compared to lean, healthy controls (13). Decreased mitochondrial respiration in skeletal muscle samples from type 2 diabetic patients was also recently reported by Boushel and colleagues (5), and this was linked to a decrease in mitochondrial content. A study of non-diabetic but insulin-resistant 1st 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 to insulin sensitive control subjects, and was associated with an increase in intramyocellular lipid content. These studies lead 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 measure of mitochondrial dysfunction in either of these studies, both relying upon 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 around 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 media 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) while 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 (Herts, UK).

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

**Subjects**

Subjects recruited to this study were young healthy non-obese subjects with no family history of type 2 diabetes. Local ethics committee approval was obtained before beginning the study and all subjects gave informed written consent.

**Cell culture**

Muscle biopsies were obtained from the vastus lateralis and satellite cells isolated as described previously (4). Myoblasts were cultured in Ham’s F10 media supplemented with 20% (v/v) FBS, 2% (v/v) chick embryo extract, 100U/ml penicillin and 100µg/ml streptomycin. All experiments were carried out on cells between passage 5 and 8.

**Cell respiration measurements**

Cell respiration was measured in a high resolution oxygraph chamber. Human myoblasts were seeded on 10cm dishes in media stated above. Media was changed every 2-3 days. After reaching confluency of approximately 80%, media was changed to serum-free media with different concentrations of sodium azide. 24h later cells were harvested by mild trypsinization. After centrifugation, intact cells were resuspended in 100µl glucose-free serum-free media and introduced into the oxygraph chamber containing 2ml of glucose-free serum-free media 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 2mM 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 by using a modified bioluminescent assay. This
assay is based upon 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, 2mM EDTA, 0.5%
Triton X-100). Samples were neutralised by the addition of 60µl KOH solution
(2M KOH, 2mM EDTA, 50mM MOPS). After incubating on ice for 10mins the
samples were centrifuged for 30sec. 50µl of each sample was added to 400µl
Tris-acetate buffer (100mM Tris, 2mM EDTA, 50mM MgCl₂, ph 7.75) and
incubated at room temperature for 10min before adding 50µl ATP substrate
containing 2µg Luciferase enzyme, 25% (v/v) glycerol, 60µM luciferin, 7.5mM
DTT and 0.4mg BSA. Light emission was measured with a luminometer
(Luminometer TD20E, Promega, UK). ATP concentration in the unknown
samples was determined against ATP standards.
**RNA isolation and cDNA synthesis**

RNA was extracted from human skeletal muscle cells using Trizol, according to the manufacturer's instructions. Total RNA was DNase-treated and then reverse-transcribed using the Thermoscript reverse transcription system. Each RT reaction contained 2µg RNA, 50ng random primers and 1mM dNTPs. This mixture was heated to 65°C for 5min before adding cDNA synthesis buffer, 5mM DTT, 40U RNaseOUT and 15U Thermoscript reverse transcriptase in a final volume of 20µl. The RT reaction was performed at 50°C for 50min. The reaction was terminated by heating at 85°C for 5min.

**Quantitative real-time PCR**

Quantitative real-time PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems) using Taqman primers and probes. Reactions were performed in a final volume of 25µl with 300nM forward and reverse primers, 150nM probe, 1x universal Taqman mastermix (Applied Biosystems) and 0.3µg cDNA. The primer and probe sequences for GLUT1, GLUT4 and β2-microglobulin have been described previously (1). Results were analysed using the standard curve method from a six-point serially diluted standard curve.

**Western blotting**

Cells were lysed in extraction buffer (100mM Tris-HCl, pH 7.4, 100mM KCl, 1mM EDTA, 25mM KF, 1mM benzamidine, 0.5mM Na3VO4, 0.1% (v/v) Triton X-100, 1µg/ml pepstatin, 1µg/ml antipain and 1µg/ml leupeptin) before sonicating for 10s and snap freezing in liquid nitrogen. Protein concentrations
were determined spectrophotometrically at 595nm by a Coomassie binding method. 20µg samples were prepared in Laemmli sample buffer (0.125M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.004% (w/v) bromophenol blue) and boiled for 5min. After separation on 10% SDS-PAGE gels, proteins were transferred to PVDF membranes using a mini-Hoeffer gel transfer system. Membranes were blocked in PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk before incubating the membranes in primary antibody solution containing PBS/Tween and 1% (w/v) non-fat milk overnight at 4°C. Membranes were washed with PBS/Tween before adding the appropriate secondary antibody in PBS/Tween containing 1% (w/v) non-fat milk. Detection took place using enhanced chemiluminescence.

**Glucose uptake**

Measurement of (2,6-³H) 2-deoxy-glucose uptake took place in 6 well cluster plates. Human myoblasts were serum-starved with or without sodium azide for 24h before incubation in Krebs' buffer (136mM NaCl, 4.7mM KCl, 1.25mM MgSO₄, 1.2mM CaCl, 20mM HEPES, pH 7.4) with or without 100nM insulin or cytochalasin B (10µM) for 20min. 0.1mM 2-deoxy-glucose and 0.5µCi (2,6-³H) 2-deoxyglucose were added to each well and incubated for a further 10min. 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 6 well cluster plates. Human myoblasts were serum-starved with or without sodium azide for 24h before incubating in serum-free media containing 5.6mM D-[U-14C] glucose for 1h at 37°C with or without 100nM insulin. The reaction was terminated by washing the plate rapidly in ice cold PBS. Cells were lysed in 200µl 20% KOH, this was neutralised with an equal volume of 1M HCl and all wells were washed with 400µl H2O before heating the samples at 95°C for 10min. The samples were placed on ice before adding 50µl oyster glycogen (240mg/ml) and ice cold ethanol to precipitate the glycogen. Samples were centrifuged at 2500rpm for 10min at 4°C and the supernatants aspirated. Pellets were allowed to dry before solubilising them for scintillation counting.

**Statistical analysis**

All results are expressed as means±standard error of the mean (SEM). Comparison between treatments was by ANOVA and paired t test.
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 hours before measuring respiration polarographically in a high-resolution oxygraph chamber. Whole cell respiration was decreased by 35 ± 6% (mean±SEM) in cells incubated with 25 µM azide when compared with untreated cells. Cells treated with 50 and 75 µM azide showed a 48 ± 3% and a 56 ±1% decrease in total whole cell respiration respectively when compared with untreated cells (Fig 1a). ATP levels were quantified after 24h of azide treatment. Although respiratory chain function was impaired in azide-treated cells at 24h, 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 concentrations 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 75µM azide in serum-free media for 24h 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 ± 39pmol/min/mg compared with 669 ± 69pmol/min/mg for 50µM azide and 823 ± 83pmol/min/mg for 75µM azide. This increase in basal glucose uptake compared to the untreated cells was significant for both azide concentrations (both, p <0.0001). As shown in Figure 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 under conditions of impaired mitochondrial function. Basal levels of glycogen synthesis were increased in azide-treated versus untreated myoblasts (Figure 3), although this only reached statistical significance at 75µM azide (203 ± 35 vs 86 ± 10 pmol/min/mg, p=0.005). In response to 100nM insulin, glycogen synthesis increased from 86 ± 10 to 269 ± 28 pmol/min/mg in the untreated cells (p<0.0001). Cells treated with 50µM and 75µM azide also showed an increase in glycogen synthesis following insulin exposure. Glycogen synthesis increased from 131 ± 23 to 523 ± 37 pmol/min/mg after treatment with 50µM azide and from 203 ± 35 to 622 ± 41 pmol/min/mg for 75µM 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

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

Impaired cellular respiration and AMPK expression and activation

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

Impaired cellular respiration and glucose transporter expression

Quantitative RT-PCR was used to analyse 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 normalised against the reference gene β2-microglobulin (Figure 6A). In contrast GLUT4 expression increased following azide treatment (Figure 6B). Compared with the untreated cells, GLUT4 mRNA expression increased after treatment with 50 and 75μM 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 in order 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 signalling, as assessed by phosphorylation of Akt, was unaffected by impaired mitochondrial respiration.

It is well recognised 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 hours azide treatment. Interestingly, Wredenberg and colleagues also reported normal ATP levels in mtDNA depleted mouse muscle preparations, but found decreased levels of phospho-creatine which has been shown to activate AMPK (28). A time course study of inhibition of mitochondrial respiration in L6 muscle cells showed a rapid but transient decrease in ATP levels before returning towards 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 upon 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-imidazolecarboxamide riboside) directly activates AMPK and has been shown to increase glucose uptake in rat skeletal muscle preparations (16, 28) and in cultured 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 azide-treated cells when compared with the untreated control. This may reflect a higher cellular free glucose concentration in the azide-treated cells. Glycogen synthase is a key enzyme in the regulation of glycogen synthesis. Glycogen synthase (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 glycogen synthase (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).

In order to examine if the insulin signalling pathway itself was affected by impaired mitochondrial respiration, we investigated the role of Akt, which occupies a pivotal position in the insulin signalling pathway. Both the expression and activation of Akt were unaffected by azide treatment in this study.

In summary, while 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 signalling and glucose uptake following insulin exposure. Further studies are required to examine the underlying mechanism behind the observations made in this study.

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References


16. Lemieux K, Konrad D, Klip A, and Marette A. The AMP-activated protein kinase activator AICAR does not induce GLUT4 translocation to transverse tubules...


Figure Legends:

**Figure 1: Cellular respiration in cultured human muscle cells.** Cellular respiration was measured in human myoblasts after they had been exposed to sodium azide for 24h. Figure 1a shows the data expressed as the % respiration of the untreated cells. (n=6 from 3 separate experiments). Figure 1b shows the ATP levels measured after 24h of azide treatment expressed as a percentage of the untreated control (n=4 from 2 separate experiments).

**Figure 2: Glucose uptake following insulin exposure.** Glucose uptake was measured in human myoblasts that had been treated with 50µM and 75µM sodium azide for 24h. The rate of glucose uptake was determined over 10min in response to 100nm insulin. Figure 2 shows the absolute rates of glucose uptake. Basal glucose uptake rates were increased after azide treatment compared to untreated cells (***, p<0.0001) Results are expressed as the mean ± SEM from 5 separate experiments. Closed bars=basal conditions, and open bars = insulin treated.

**Figure 3: Glycogen synthesis following insulin exposure.** Glycogen synthesis was measured in human myoblasts that had been treated with 50µM and 75µM azide for 24h. Figure 3 shows the absolute rates of glycogen synthesis. Basal glycogen synthesis was increased after 75µM azide treatment compared to untreated cells (**, p<0.005), while insulin treatment increased glycogen synthesis under all conditions (***, p<0.0001). . Results
are expressed as the mean ± SEM from 5 separate experiments. Closed bars=basal conditions, and open bars = insulin treated.

**Figure 4: Western blot analysis of Akt activation.** Phosphorylation of Akt in response to 100nm insulin for 10min was assessed by western blot analysis. Total cell lysates from each sample (20µg) were subject to western blot analysis using phospho-specific Akt antibodies (Upstate Technology). Blots were stripped and reprobed with the native antibodies. Densitometry was determined on n=3 separate blots and the data are presented as the phospho-antibody expressed relative to the native antibody. Closed bars=basal conditions, and open bars = insulin treated.

**Figure 5: Western blot analysis of AMPK activation.** Phosphorylation of AMPK after azide treatment for 24 h was assessed by western blot analysis. Total cell lysates from each sample (20µg) were subject to western blot analysis using phospho-specific AMPK antibodies (Upstate Technology). Blots were stripped and reprobed with the native antibodies. Densitometry was determined on n=3 separate blots and the data are presented as the phospho-antibody expressed relative to the native antibody.

**Figure 6: Quantitative mRNA analysis of GLUT1 and GLUT4 expression.** Quantitation of GLUT1 and GLUT4 gene expression was assessed by real-time PCR. GLUT1 and GLUT4 expression (Figure 6A and 6B, respectively) relative to β2-microglobulin was determined from n=5 separate subjects.
Values are presented in relation to the relative expression of the untreated cells. * p=0.05 vs untreated cells.
Figure 2

![Graph showing glucose uptake (pmol/min/mg) with Azide treatment. The graph compares glucose uptake between untreated and treated samples with 50 µM and 75 µM of Azide.}

- Untreated
- 50 µM
- 75 µM

Glucose uptake (pmol/min/mg)
Figure 3

Glycogen synthesis (pmol/min/mg)

Azide treatment

- Untreated
- 50µM
- 75µM

** Stars indicate significant differences.
Figure 4

Phospho-Akt

Native Akt

Azide treatment

Relative phosphorylation

- Untreated
- 50µM
- 75µM

Azide concentration (µM)

Basal
100nM insulin
Figure 5

Phospho-AMPK

Native AMPK

(Azide conc (µM))

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