Increased mitochondrial substrate sensitivity in skeletal muscle of patients with type 2 diabetes

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Abstract

Aims/hypothesis Mitochondrial respiration has been linked to insulin resistance. We studied mitochondrial respiratory capacity and substrate sensitivity in patients with type 2 diabetes (patients), and obese and lean control participants. **Methods** Mitochondrial respiration was measured in permeabilised muscle fibres by respirometry. Protocols for respirometry included titration of substrates for complex I (glutamate), complex II (succinate) and both (octanoyl-carnitine). Myosin heavy chain (MHC) composition, antioxidant capacity (manganese superoxide dismutase [MnSOD]), citrate synthase activity and maximal oxygen uptake ($\dot{V}O_{2\max}$) were also determined. Insulin sensitivity was determined with the isoglycaemic–hyperinsulinaemic clamp technique.

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Results Insulin sensitivity was different ($p<0.05$) between the groups (patients<obese controls<lean controls). MnSOD was lower in patients than in lean controls. MHC I content was lowest in patients ($37\pm 11\%$ [mean $\pm$ SE] vs $53\pm 6\%$ and $56\pm 4\%$) vs obese controls and lean controls, respectively. $\dot{V}O_{2\max}$ was highest in lean controls (40 ± 3 ml min⁻¹ kg⁻¹ [mean $\pm$ SE]) compared with patients (25 ± 2) and obese controls (27 ± 2). Mitochondrial content (citrate synthase) was higher ($p<0.05$) in lean controls than in patients and obese controls. When normalised for mitochondrial content by citrate synthase, mitochondrial respiratory capacity was similar in all groups. However, the half maximal substrate concentration (C_{50}) for complex I was significantly lower ($p=0.03$) in patients (1.1 ± 0.2 mmol/l [mean $\pm$ SE]) than in obese (2.0 ± 0.3) and lean (1.8 ± 0.3) controls. Likewise, C_{50} for complex II was lower ($p=0.02$) in patients (3.5 ± 0.2 mmol/l [mean $\pm$ SE]) than in obese controls (4.1 ± 0.2), but did not differ from that in lean controls (3.8 ± 0.4). Substrate sensitivity for octanoyl-carnitine did not differ between groups.

Conclusions/interpretation Increased mitochondrial substrate sensitivity is seen in skeletal muscle from type 2 diabetic patients and is confined to non-lipid substrates. Respiratory capacity per mitochondrion is not decreased with insulin resistance.

Keywords Mitochondrial function · Substrate sensitivity · Type 2 diabetes

Abbreviations

C_{50}	Half maximal substrate concentration
HAD	β -Hydroxy-acyl-CoA-dehydrogenase
MHC	Myosin heavy chain
MnSOD	Manganese superoxide dismutase
mtDNA	Mitochondrial DNA

ROS	Reactive oxygen species
$\dot{V}_{\max}$	Maximal respiration
$\dot{V}O_{2\max}$	Maximal oxygen uptake

Introduction

Insulin resistance is a hallmark of type 2 diabetes and presents itself primarily in skeletal muscle and liver, both of which are highly metabolically active tissues. The mechanisms underlying insulin resistance have not yet been fully elucidated, but skeletal muscle mitochondrial dysfunction has been linked to insulin resistance and type 2 diabetes. Studies have suggested that low mitochondrial content [1, 2], decreased respiratory capacity per mitochondrion [3] and increased oxidative stress due to increased mitochondrial production of reactive oxygen species (ROS) or a reduced antioxidant defence system or both [4–6] may induce or contribute to skeletal muscle insulin resistance in patients with type 2 diabetes. However, the issue is controversial and several studies have concluded that mitochondrial respiration is not associated with insulin resistance [7–13], when respiratory capacity is expressed relative to mitochondrial content (i.e. intrinsic mitochondrial function), as estimated for example by citrate synthase activity or mitochondrial DNA (mtDNA).

Mitochondrial respiration *ex vivo* is usually measured in permeabilised fibres or in isolated mitochondria by stimulating respiration by provision of substrates in saturating concentrations to achieve maximal coupled or uncoupled respiration [7, 8]. However, this may not always be the best approach, as recent animal and cell culture studies have shown that substrate overload alters mitochondrial substrate handling and substrate oxidation in insulin-resistant models. It was proposed that this abnormality may induce or at least contribute to insulin resistance [14].

In a previous study, mitochondrial half maximal substrate concentration (C_{50}) and maximal respiration ($\dot{V}_{\max}$) were determined in biopsies from the right auricula of the heart from patients with and without type 2 diabetes. C_{50} did not differ between groups, but $\dot{V}_{\max}$ was significantly reduced in patients with type 2 diabetes [15]. However, right auricula heart tissue is probably not representative of metabolic characteristics of insulin-resistant skeletal muscle, so the generalisability of these data to diabetic muscle at large is probably limited.

With the present study, we have now determined the substrate sensitivity of the mitochondrial complexes I and II, and studied classical Michaelis–Menten kinetics in human skeletal muscle samples from patients with type 2 diabetes, and from two glucose-tolerant healthy control

groups, one obese, the other lean. Substrate sensitivity measured in permeabilised fibres integrates the entire cascade of events leading to generation of ATP in the mitochondria. It represents transport of substrate and enzyme activity in the citric cycle, which lead to generation of NADH and FADH₂, both of which are transferred to complex I and complex II in the electron transport chain for ATP production. We also determined fibre type composition in the muscle biopsies, because studies in rats have shown differences in Michaelis–Menten kinetic variables [16] and in ROS production between fibre types [17].

Thus, the aim of our study was to investigate mitochondrial substrate handling by measuring $\dot{V}_{\max}$ and the sensitivity (C_{50}) of mitochondrial respiration in skeletal muscle. This was done using three different substrates providing electrons for complex I (glutamate), complex II (succinate) and complex I+II (octanoyl-carnitine). We hypothesised that the maximal respiratory capacity would not differ between the groups, but that the sensitivity of the complexes would be decreased in patients with type 2 diabetes.

Methods

Participants We included 12 men with type 2 diabetes, ten age- and BMI-matched obese male controls and ten age-matched lean male controls in this study (Table 1). Patients with type 2 diabetes were being treated with metformin (1,000–2,000 mg; $n=8$), glimepiride (4–8 mg; $n=4$) or insulin (400 units; $n=1$), with one patient being treated by diet. Other medications included hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (simvastatin 20–40 mg; $n=8$) and ACE inhibitors (10–20 mg; $n=8$). To maintain optimal glycaemic and cardiovascular control, we deliberately chose not to discontinue any medication (i.e. induce a ‘wash-out period’). Both control groups had normal glucose tolerance (fasting glucose <6.0 mmol/l; HbA_{1c}<6.0%) and were not taking any medication. None of the participants had signs or a history of ischaemic heart disease and all were able to complete a bicycle test to measure maximal oxygen uptake ($\dot{V}O_{2\max}$) without adverse reactions. Participants were instructed to abstain from alcohol and strenuous exercise the day before each experiment. Lean control participants were characterised by lower body weight, BMI, body fat per cent and fasting plasma insulin concentrations than patients and obese control participants (Table 1). $\dot{V}O_{2\max}$ was higher in lean control participants (Table 1). Lean control participants were more physically active than the other groups, but none of them performed endurance training. Participants were examined on two separate days after an overnight (10 h) fast. At their first visit medical history was recorded and a physical examination performed, including resting and

Table 1 Participant characteristics

Variable	Type 2 diabetes	Obese controls	Lean controls
<i>n</i>	12	10	10
Age (years)	53±3	55±3	53±3
Body weight (kg)	103±3	100±5	84±3 ^a
BMI (kg/m ²)	32±1	31±1	25±1 ^a
Body fat (%)	31±1	32±1	23±2 ^a
Lean body mass (kg)	69±2	64±2	61±2
Systolic BP (mmHg)	129±5	133±5	119±3
Diastolic BP (mmHg)	76±3	82±4	70±2
$\dot{V}O_{2\max}$ (ml min ⁻¹ kg ⁻¹)	25±2	27±2	40±3 ^a
Fasting glucose (mmol/l)	8.6±0.5 ^b	5.6±0.1	5.4±0.1
HbA _{1c} (%)	7.0±0.2 ^b	5.4±0.1	5.4±0.1
Insulin (pmol/l)	64±10	50±7	25±3 ^a
NEFA (μmol/l)	482±33	548±62	501±66
Glycerol (μmol/l)	80±3	116±24	79±8
Triacylglycerol (mmol/l)	1.72±0.25	1.23±0.11	1.47±0.17
Cholesterol (mmol/l)	3.91±0.26	4.49±0.29	5.08±0.28 ^c
HDL (mmol/l)	0.89±0.06 ^b	1.29±0.08	1.18±0.11
LDL (mmol/l)	2.34±0.21	2.81±0.22	3.40±0.26 ^c

Data are means ± SE

^a $p < 0.05$ for lean control vs type 2 diabetes and obese control; ^b $p < 0.05$ for type 2 diabetes vs obese and lean controls; ^c $p < 0.05$ for lean control vs type 2 diabetes

exercise ECG. Body composition was measured with a dual-energy X-ray absorptiometry scan (Lunar Prodigy Advance; Lunar, Madison, WI, USA) and $\dot{V}O_{2\max}$ was measured. The $\dot{V}O_{2\max}$ test was performed as a graded exercise test on a bicycle ergometer (ER800; Jaeger, Würzburg, Germany), beginning at 60 W for 5 min, increasing thereafter by 35 W/min until voluntary exhaustion. Online equipment (Oxycon Pro; Jaeger) was used to measure O₂ consumption and CO₂ production. On the second visit, resting energy expenditure was measured with a ventilated hood (Oxycon Champion; Jaeger). A muscle biopsy was obtained from the vastus lateralis. Subsequently, a 120 min isoglycaemic–hyperinsulinaemic (40 mU min⁻¹ m⁻²) clamp was performed [18]. During the final 20 min of the clamp ventilated hood measurements were repeated.

The Ethics Committee of the Municipality of Copenhagen and Frederiksberg, Denmark, approved (file number H-KF-320893) the study protocol, and oral and written consent were obtained from each participant in accordance with the Helsinki Declaration.

Analytical procedures The biopsy was divided into two parts. One was prepared for respirometric measurements [7]. The other was snap-frozen in liquid nitrogen within 15 s of sampling and stored at -80°C until further analysis.

Citrate synthase and β-hydroxy-acyl-CoA-dehydrogenase (HAD) activities were measured as previously described [19], with one minor change consisting of use of a TissueLyser II (Qiagen, Hilden, Germany) to make the homogenate. The homogenates for citrate synthase and

HAD analysis were also used for Western blots. From each sample 15 μg total protein was separated by SDS-PAGE on 12.5% gels (Criterion; Bio-Rad, Hercules, CA, USA) and electrophoretically transferred to polyvinylidene difluoride membranes in a tank buffer system (transfer buffer: 25 mmol/l Tris, 192 mmol/l glycine and 20% methanol [wt/vol.]). Membranes were probed with antibodies against subunits for each of the five complexes in the mitochondrial oxidative phosphorylation system: (1) complex I (the 20 kDa subunit encoded by *NDUFB8*); (2) complex II (30 kDa subunit); (3) complex III (the 43 kDa core protein 2 subunit); (4) complex IV (22 kDa subunit II); and (5) complex V (the 55 kDa subunit α). All of the above antibodies were mixed using a kit (Human Total OXPHOS Human WB Antibody Cocktail; MitoSciences, Eugene, Oregon, USA). Antibodies against manganese superoxide dismutase (MnSOD; anti-MnSOD; Millipore, Billerica, MA, USA) were also used. Membranes were then incubated with horseradish-peroxidase-labelled secondary antibody (Dako, Glostrup, Denmark). The antigen–antibody complexes were visualised by electrochemiluminescence using a charge-coupled-device-based system (LAS-3000 Luminescent Image Analyser; Fujifilm, Tokyo, Japan) and quantified using a software package (Multi Gauge version 3.0; Fujifilm). A control homogenate was included twice on each gel. All samples were measured relative to the mean value of the control sample for each specific band. Citrate synthase and HAD homogenates were used to quantify the myosin heavy chain (MHC) composition of the muscle by SDS-PAGE [20]. Quantification of genomic and mtDNA content was performed as previously described [21].

Fig. 1 Representative traces of respiratory fluxes from the three substrate and inhibitor protocols: **(a)** Protocol-1, **(b)** Protocol-2, **(c)** Protocol-3. For further details, see the **Methods**. Black line (y -axis, left) is oxygen concentration in the chamber. Grey line (y -axis, right) is oxygen flux/mg tissue in the chamber. Addition of substrate and inhibitors as indicated; titration concentrations in parentheses above oxygen flux on trace. CytC, cytochrome *c*; Glut, glutamate; Mal, malate; Mna, malonate; Oct, octanoyl-carnitine; Rot, rotenone; Suc, succinate

Blood samples were collected in chilled tubes and immediately centrifuged for 10 min (2,500 *g*; 4°C). The plasma fraction was collected and stored at −20°C or −80°C prior to analysis. Plasma glucose concentrations during the clamp were measured on an automated glucose analyser (ABL800-flex; Radiometer, Copenhagen, Denmark). HbA_{1c} was analysed using a device (DCA2000+; Bayer Healthcare, Elkhart, IN, USA) based on the latex immunoagglutination inhibition method. Plasma insulin concentrations were determined using an ELISA kit (Dako). Plasma NEFA concentration was measured using a kit (NEFA-C test; Wako Chemical, Neuss, Germany). Concentrations of glycerol, triacylglycerol, cholesterol, HDL and LDL were measured on an automated analyser (Hitachi 912; Roche, Mannheim, Germany) using standard applications.

Mitochondrial respiration protocols Muscle samples were gently dissected on ice using sharp forceps and a magnifying glass, and permeabilised in a relaxing solution with saponin (50 µg/ml) for 30 min, followed by rinsing twice for 10 min in mitochondrial respiration medium [7]. Muscle fibres were carefully weighed and added to the respirometer.

Three substrate and inhibitor protocols (Protocol-1, -2, -3) were employed in duplicate. In Protocol-1, state 2 respiration was assessed with malate (M, 2 mmol/l), followed by state 3 respiration (M₃, 5 mmol/l, ADP). Subsequently, glutamate (GM₃, complex I substrate) was titrated in 11 steps (0.1–96.0 mmol/l), followed by cytochrome *c* (10 µmol/l) to control for outer mitochondrial membrane integrity. Finally rotenone (0.5 µmol/l) was added to inhibit

complex I. In Protocol-2, state 2 respiration was assessed with malate (M, 2 mmol/l), followed by state 3 respiration (M₃, 5 mmol/l, ADP). Glutamate (GM₃, 10 mmol/l) (complex I substrate) was added and succinate (GMS₃, complex II substrate) was titrated in ten steps (0.1–48.0 mmol/l), followed by cytochrome *c* (10 µmol/l) and malonate (5 mmol/l) in order to inhibit complex II. Finally rotenone (0.5 µmol/l) was added to inhibit complex I. Finally, in Protocol-3, state 2 respiration was assessed with malate (M, 2 mmol/l) followed by state 3 respiration (M₃, 5 mmol/l, ADP), followed by addition of octanoyl-carnitine (MO₃, providing electrons for complex I and II) titrated in ten steps (5–2,000 µmol/l). Last, cytochrome *c* (10 µmol/l) was added. Representative traces from the protocols are shown in Fig. 1.

C₅₀ was determined in isolated mitochondria from four lean control participants to study possible differences from results generated using the permeabilised fibres. Protocol-1 and -2 were used for this purpose. Isolation of mitochondria from skeletal muscle has been described in detail elsewhere [22].

Calculations Glucose clearance was calculated as the glucose infusion rate divided by glucose concentration. Standard equations were used for indirect calorimetric calculations [23]. To determine C₅₀ and $\dot{V}_{\max}$, a non-linear regression (one site saturation equation) in SigmaPlot 11.0 (Systat Software, San Jose, CA, USA) was used based on the individual raw data from the recordings of respiratory fluxes.

Statistics Data are presented as means $\pm$ SE. A *p* value of *p*<0.05 was considered significant. One- and two-way ANOVAs with repeated measures for the time factor were performed. Significant main effects or interactions were analysed by the Holm–Sidak post hoc test. If the normality test failed, data were log₁₀ transformed and reanalysed. All statistical analysis was performed in SigmaStat 3.1 (Systat Software).

Results

Plasma insulin concentrations during the clamp were similar between patients with type 2 diabetes (396 $\pm$ 46 pmol/l [means $\pm$ SE]), and obese (391 $\pm$ 20 pmol/l) and lean control participants (407 $\pm$ 11 pmol/l). Insulin sensitivity differed significantly (*p*<0.05) between the groups (patients with type 2 diabetes <obese controls<lean controls; Fig. 2). At baseline, the whole-body respiratory exchange ratio was 0.88 $\pm$ 0.01, 0.83 $\pm$ 0.02 and 0.85 $\pm$ 0.02 (means $\pm$ SE) in patients with type 2 diabetes and obese and lean control participants, respectively (*p*>0.05). With hyperinsulinaemia, the respira-

tory exchange ratio increased (*p*<0.05) in patients with type 2 diabetes, and in obese and lean controls (to 0.92 $\pm$ 0.01, 0.89 $\pm$ 0.02 and 0.92 $\pm$ 0.02 [means $\pm$ SE], respectively) with no difference between the magnitude of increase.

Skeletal muscle from patients with type 2 diabetes was characterised by less (*p*<0.05) MHC I content than in the other groups, in whom MHC I was >50% (Table 2). In accordance with the higher $\dot{V}O_{2\max}$ in lean control participants, the proportion of MHC IIX was very low in this group and significantly (*p*<0.05) lower than in patients with type 2 diabetes, with a tendency toward a lower proportion (*p*=0.069) than in obese control participants (Table 2). Citrate synthase and HAD activities were similar between patients with type 2 diabetes and obese control participants, but higher activities (*p*<0.05) were seen in lean controls. This is supported by the fact that the amount of mtDNA copies also tended (*p*=0.065) to be higher in lean control participants (Table 2), and also by the higher $\dot{V}O_{2\max}$ in lean control participants.

Protein levels of oxidative phosphorylation complexes (I–V) were similar between patients with type 2 diabetes and obese control participants, but markedly increased in lean control participants compared with the other groups (Table 2). Indicative of a decreased antioxidant capacity in patients with type 2 diabetes, MnSOD was lower than in lean control participants (*p*<0.05), with a tendency towards higher MnSOD in obese controls than in patients with type 2 diabetes (*p*=0.057; Table 2).

Mitochondrial respiration was similar between patients with type 2 diabetes and obese control participants, but higher (*p*<0.05) in lean control participants when state 3 respiration was measured with glutamate (GM₃, Protocol-1;

Fig. 2 Glucose clearance rates measured during a 120 min isoglycaemic–hyperinsulinaemic clamp in patients with type 2 diabetes (black squares), and in obese (grey circles) and lean (white triangles) control participants. Data are means (averaged for 5 min intervals) $\pm$ SE. **p*<0.05 for lean controls vs type 2 diabetes; †*p*<0.05 for lean control vs type 2 diabetes and obese control; ‡*p*<0.05 for obese control vs type 2 diabetes

Table 2 Muscle data

Variable	Type 2 diabetes	Obese controls	Lean controls
CS activity ($\mu\text{mol g}^{-1} \text{min}^{-1}$)	79 $\pm$ 5	85 $\pm$ 9	113 $\pm$ 6 ^a
HAD activity ($\mu\text{mol g}^{-1} \text{min}^{-1}$)	75 $\pm$ 4	95 $\pm$ 11	112 $\pm$ 7 ^c
mtDNA (ds copies/mg) $\times 10^6$	226 $\pm$ 15	236 $\pm$ 21	283 $\pm$ 26
MnSOD (AU)	0.64 $\pm$ 0.04	0.83 $\pm$ 0.09	0.94 $\pm$ 0.06 ^c
Complex I (AU)	0.50 $\pm$ 0.06	0.48 $\pm$ 0.07	1.07 $\pm$ 0.15 ^a
Complex II (AU)	0.59 $\pm$ 0.05	0.68 $\pm$ 0.07	1.32 $\pm$ 0.10 ^a
Complex III (AU)	0.49 $\pm$ 0.07	0.49 $\pm$ 0.09	1.20 $\pm$ 0.10 ^a
Complex IV (AU)	0.71 $\pm$ 0.07	0.62 $\pm$ 0.10	1.05 $\pm$ 0.09 ^a
Complex V (AU)	0.75 $\pm$ 0.07	0.73 $\pm$ 0.10	1.30 $\pm$ 0.10 ^a
MHC I (%)	37 $\pm$ 3 ^b	53 $\pm$ 6	56 $\pm$ 4
MHC IIA (%)	48 $\pm$ 3	35 $\pm$ 4 ^d	40 $\pm$ 4
MHC IIX (%)	15 $\pm$ 3	12 $\pm$ 4	4 $\pm$ 1 ^c

Data are means $\pm$ SE

AU, arbitrary units; CS, citrate synthase; ds, double-stranded

^a $p < 0.05$ for lean control vs type 2 diabetes and obese control; ^b $p < 0.05$ for type 2 diabetes vs lean and obese control; ^c $p < 0.05$ for lean control vs type 2 diabetes;

^d $p < 0.05$ for obese control vs type 2 diabetes

Figs. 3a, 4a), glutamate and succinate (GMS₃, Protocol-2; Figs. 3c, 5a) and octanoyl-carnitine (MO₃, Protocol-3; Fig. 3e). Inhibition of complex I with rotenone (Protocol-1) and of complex I and II with rotenone and malonic acid

(Protocol-2) always resulted in a return of respiratory rates to baseline levels, providing additional evidence of the functionality of the experimental set-up (Fig. 3a, c). When mitochondrial flux rates were expressed relative to mito-

Fig. 3 Mitochondrial respiratory rates were measured in permeabilised skeletal muscle biopsies from patients with type 2 diabetes (black squares), and obese (grey circles) and lean (white triangles) healthy control participants. The following three inhibitor and substrate protocols were used (in duplicate):

(a) Protocol-1, respiratory rate expressed per kg muscle and (b) normalised to mitochondrial content by citrate synthase (CS). Baseline, respiration in buffer; M, addition of malate (state 2); M₃, addition of ADP (state 3); GM₃, addition of glutamate (state 3); +Rot, addition of rotenone. c Protocol-2, expressed and normalised (d) as above (a, b). Protocol as Protocol-1, but with the addition of succinate (GMS₃) and malonate (+Mna), before rotenone (+Rot). e Protocol-3, expressed and normalised (f) as above (a, b). As Protocol-1, but with the addition of octanoyl-carnitine (MO₃) after state 3 with malate. Details, see the Methods. Maximal responses are shown. Data are mean $\pm$ SE. * $p < 0.05$ for lean control vs type 2 diabetes and obese control

Fig. 4 Mitochondrial respiration (O₂ flux per mg tissue) in skeletal muscle biopsies from patients with type 2 diabetes (black squares), and obese (grey circles) and lean (white triangles) control participants. Mitochondrial respiration was measured with malate, ADP and glutamate (0.1–96 mmol/l). **a** Glutamate titration, showing Michaelis–Menten kinetics. **b** Extract of the respiration curve highlighting respiration for glutamate concentration 0.1–2.5 mmol/l. Drop lines indicate C₅₀ values (x-axis) and 1/2 $\dot{V}_{\max}$ (y-axis). Data are means $\pm$ SE. * $p < 0.05$ for type 2 diabetes vs lean and obese control. T2DM, type 2 diabetes mellitus

chondrial content, e.g. citrate synthase activity (Fig. 3b, d, f) and mtDNA (data not shown), no differences between the groups were seen.

The most striking observation, however, was that mitochondrial substrate sensitivity was much higher ($p < 0.05$) in patients with type 2 diabetes than in lean and obese controls when glutamate was used as substrate (Protocol-1; Fig. 4b, Table 3). A higher sensitivity was seen in patients with type 2 diabetes than in obese control participants when succinate was used as substrate (Protocol-2; Fig. 5b, Table 3). No difference was observed in sensitivity when octanoyl-carnitine (Protocol-3; Table 3) was used as substrate. $\dot{V}_{\max}$ was similar between patients with type 2 diabetes and obese control participants, but always higher ($p < 0.05$) in lean control participants, except for under glutamate stimulation, where the difference was only significant compared with obese control participants (Table 3). No cytochrome *c* response was observed in any of the protocols (data not shown), showing that the outer mitochondrial membrane was not damaged by the

experimental procedure. Substrate control ratios (GM₃:M₃; GMS₃:GM₃; GMS₃:M₃; MO₃:M₃) and respiratory control ratios (M₃:M) were similar between groups (Electronic supplementary material [ESM] Fig. 1a–e).

In addition to the measurements in permeabilised fibres, substrate sensitivity was also determined in isolated mitochondria from a subset of the lean control group ($n = 4$). C₅₀ was significantly higher in isolated mitochondria than in permeabilised fibres, both when testing complex I (glutamate) and complex II (succinate; ESM Fig. 2).

Discussion

Main findings A major and novel finding of our study is that muscle mitochondrial substrate sensitivity was different between the experimental groups (Figs. 4b, 5b, Table 3). Patients with type 2 diabetes had a higher sensitivity than obese control participants when glutamate and succinate

Fig. 5 Mitochondrial respiration (O₂ flux per mg tissue) in skeletal muscle biopsies from patients with type 2 diabetes (black squares), and obese (grey circles) and lean (white triangles) control participants. Mitochondrial respiration was measured with malate, ADP, glutamate and succinate (0.1–48 mmol/l). **a** Succinate titration, showing Michaelis–Menten kinetics. **b** Extract of the respiration curve highlighting respiration for succinate concentration 0.1–8.0 mmol/l. Drop lines indicate C₅₀ values (x-axis) and 1/2 $\dot{V}_{\max}$ (y-axis). Data are means $\pm$ SE. * $p < 0.05$ for obese vs type 2 diabetes. T2DM, type 2 diabetes mellitus

Table 3 Mitochondrial respiratory measurements (C_{50} and $\dot{V}_{\max}$)

Variable	Type 2 diabetes		Obese controls		Lean controls	
	C_{50}	$\dot{V}_{\max}$	C_{50}	$\dot{V}_{\max}$	C_{50}	$\dot{V}_{\max}$
Glutamate	1.1±0.2 ^a	24±2	2.0±0.3	19±2 ^b	1.8±0.3	27±2
Succinate	3.5±0.2	29±3	4.1±0.2 ^c	31±4	3.8±0.4	50±4 ^d
Octanoyl-carnitine	51±12	9±1	40±7	9±1	56±7	12±1 ^d

Data are means ± SE

C_{50} is reported in millimoles per litre for glutamate and succinate, and in micromoles per litre for octanoyl-carnitine; $\dot{V}_{\max}$ is reported in picomoles per milligram per second

^a $p<0.05$ for type 2 diabetes vs lean and obese control; ^b $p<0.05$ for obese vs lean control; ^c $p<0.05$ for obese control vs type 2 diabetes; ^d $p<0.05$ for lean control vs type 2 diabetes and obese control

were used as substrates. Patients with type 2 diabetes also had a higher sensitivity than lean control participants, but only when glutamate was used as substrate. No differences were seen in mitochondrial substrate sensitivity between the three groups with octanoyl-carnitine as substrate. Substrate sensitivity was similar between obese and lean control participants with any substrate. At the same time maximal mitochondrial respiratory capacity per mitochondrion did not differ (Fig. 3b, d, f). The type 2 diabetic patients and control participants were clearly distinct in insulin sensitivity (Fig. 2), while antioxidant levels (MnSOD) and the proportion of the MHC I isoform were markedly lower in patients with type 2 diabetes.

Mitochondrial respiratory capacity The maximal coupled mitochondrial respiration per mg tissue normalised to mitochondrial content (citrate synthase activity or mtDNA) did not differ between the groups, who, in turn, had markedly different insulin sensitivity as determined by the clamp technique. Patients with type 2 diabetes and obese controls were very well matched. Moreover, even if the comparison of mitochondrial respiration (with or without correction for citrate synthase activity or mtDNA content) and insulin sensitivity is restricted to these two groups, it is evident that there is no positive relationship between mitochondrial respiration and insulin sensitivity. If the most insulin-sensitive group is brought into the comparison, i.e. the lean control group, it is also evident that per mitochondrion there is no difference in mitochondrial respiration between the three groups (type 2 diabetes, obese and lean controls). Naturally, there is a difference per amount of muscle in mitochondrial respiration in the permeabilised fibres, because the lean controls had a higher mitochondrial density, in line with a previous study in similar controls [8]. When maximal mitochondrial respiration is normalised to mitochondrial content (by citrate synthase activity), the findings in the present study are consistent with most [7–9, 24–26] but not all studies [3] using permeabilised fibres. Furthermore, calculation of

respiratory and substrate control ratios showed similar results between groups. These ratios are independent of mitochondrial content (ESM Fig. 1a–e).

The amount of mitochondria was not directly counted, but both citrate synthase and HAD activity, and mtDNA, were similar between patients with type 2 diabetes and obese controls, and were higher in lean controls, reflecting the higher level of fitness. These data are supported by measurements of protein content of the five complexes in the respiratory chain, which showed up to more than two-fold higher values in lean controls than in patients with type 2 diabetes and in obese controls (Table 2). The indices of mitochondrial density thus reflected the fact that the lean controls had a higher $\dot{V}O_{2\max}$ than the other groups.

Mitochondrial substrate sensitivity While mitochondrial respiratory capacity did not differ between groups, data on substrate sensitivity did show differences. An increased sensitivity in mitochondrial respiratory kinetics in patients with type 2 diabetes compared with obese controls was seen when glutamate and succinate were used as substrates. The increased substrate sensitivity in patients with type 2 diabetes was confined to non-lipid substrates. The lower C_{50} value found in patients with type 2 diabetes means that, for the same concentration of substrate in question, patients are closer to maximal mitochondrial respiratory capacity, i.e. they are more sensitive. The lower C_{50} value (i.e. the higher sensitivity) was not due to differences in $\dot{V}_{\max}$, since no differences were seen in $\dot{V}_{\max}$ between patients with type 2 diabetes and obese controls with any of the substrates. Mitochondrial substrate sensitivity was never different between lean and obese controls, while $\dot{V}_{\max}$ was different for all the substrates and whole-body $\dot{V}O_{2\max}$ was also different between lean and obese controls. Thus, neither $\dot{V}_{\max}$ nor $\dot{V}O_{2\max}$ is related to mitochondrial substrate sensitivity. Finally, the comparison between the two control groups shows that obesity and physical fitness are not regulating factors in substrate sensitivity for complexes I or II.

It is clear that patients with type 2 diabetes differ in substrate sensitivity for complexes I and II compared with obese controls, but not when compared with lean controls, who only differed from patients with type 2 diabetes in complex I. Thus a functional change of complex I in patients with type 2 diabetes is evident. For complex II, a change in function may also be evident in the patients with type 2 diabetes, but a firm conclusion cannot be made because C_{50} for complex II was only lower than in obese and not in lean controls. The distinction may seem trivial, but further exploration of complex II sensitivity is appropriate.

The finding of a higher sensitivity in patients with type 2 diabetes is in contrast to our initial hypothesis. We had expected that the maximal respiratory capacity per mitochondrion would be similar (shown in the present and in other previous studies), but also that the diabetic muscle would be less sensitive to substrate handling, in line with the insulin resistance concept where diabetic muscle is less sensitive to the action of insulin. The data in the present study clearly underline the need to separate the molecular events of insulin resistance from the subsequent handling of those substrates available.

Several scenarios could verify the appropriateness of an increased complex I sensitivity in patients with type 2 diabetes. First, the increased mitochondrial substrate sensitivity in patients with type 2 diabetes compared with obese controls could be an adaptive mechanism in the muscle in order to compensate for a decreased glucose flux into the cell, i.e. compensation for insulin resistance. The fact that the adaptation is confined to non-lipid substrates (C_{50} for the medium-chain fatty acid octanoyl-carnitine did not differ between groups) seems appropriate, as intracellular fatty acid availability is not in deficit in patients with type 2 diabetes [27]. Second, patients with type 2 diabetes had higher MHC II content (Table 2), which in rats has been shown to have an increased substrate sensitivity (i.e. lower C_{50} value) than type I fibres [16]. Differences in maximal mitochondrial respiration are seen between fibre types in rats [15], but with mixed muscle fibres in humans, $\dot{V}_{\max}$ in patients with type 2 diabetes and obese controls (Table 3) would be expected to be similar. Third, the increased substrate sensitivity found in patients with type 2 diabetes may serve as a regulator for ROS production, because a high mitochondrial respiratory flux rate will decrease mitochondrial membrane potential, which in turn will lead to a decrease in ROS production [28–30]. The faster the cells increase their flux rate (i.e. the more substrate-sensitive they are), the less ROS is produced. Thus a higher complex I sensitivity would be an advantage, if ROS production should need to be minimised. Moreover, the higher proportion of type II fibres found in patients with type 2 diabetes may contribute to higher ROS production

[10, 31] because type II fibres, at least in rat muscle [17], produce more ROS. This is supported by the lower antioxidant defence system in our patients with type 2 diabetes than in the other groups (Table 2). Protection from ROS can be evoked by decreasing the production of ROS or by increased antioxidant capacity, but the latter was clearly not the case in the present study (Table 2), which is consistent with other studies [4, 6]. Further experiments are needed to provide final proof of these suggestions.

It has been suggested that metformin treatment has an inhibitory effect on complex I in the electron transport chain [32, 33]. However, this inhibition was found only at concentrations above 10 mmol/l. In humans, the plasma concentration does not exceed 0.03 mmol/l (4 µg/ml). Even if metformin were to accumulate in the mitochondrial matrix by a factor 1000, as proposed in other studies [32, 33], the concentration would still be too low. Moreover, metformin has a relatively short half-life (approximate 6.5 h), which has not been taken into consideration above. These considerations fit well with our observation in the present study that patients with type 2 diabetes had similar substrate sensitivity in the metformin-treated and non-metformin-treated subgroups. They also always had higher substrate sensitivity than obese controls. Thus, physiologically, metformin treatment does not seem to play any role in mitochondrial function in human skeletal muscle.

Our additional experiment with substrate sensitivity in isolated mitochondria revealed a two- to fourfold higher sensitivity for glutamate and succinate in permeabilised fibres than in isolated mitochondria. With the higher (and not lower) sensitivity in permeabilised muscle, we have therefore provided a proof-of-concept for phenomenon, as well as providing additional arguments for using permeabilised fibres for ex vivo studies of mitochondrial respiration. The lower substrate sensitivity in isolated mitochondria is probably due to the need for an intact cytoskeleton for full effect; this intactness is destroyed in the isolation procedure [34].

Conclusion In summary, we have shown that skeletal muscle mitochondrial sensitivity for substrates for complexes I and II is markedly higher in patients with type 2 diabetes than in obese controls with normal glucose tolerance. We propose that this is an adaptive response, which serves to regulate ROS production in the face of impaired antioxidant capacity and a slightly higher content of MHC II isoforms in the muscle. We have also re-confirmed that mitochondrial respiratory capacity per mitochondrion is not linked to insulin resistance or type 2 diabetes. Finally, we have shown that obesity and physical fitness are not regulating factors in substrate sensitivity for complexes I or II.

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References

- Mootha VK, Lindgren CM, Eriksson KF et al (2003) PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267–273
- Patti ME, Butte AJ, Crunkhorn S et al (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. *Proc Natl Acad Sci USA* 100:8466–8471
- Phielix E, Schrauwen-Hinderling VB, Mensink M et al (2008) Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. *Diabetes* 57:2943–2949
- Scheede-Bergdahl C, Penkowa M, Hidalgo J et al (2005) Metallothionein-mediated antioxidant defense system and its response to exercise training are impaired in human type 2 diabetes. *Diabetes* 54:3089–3094
- Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ, Packer L (2001) The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diab Metab Res Rev* 17:189–212
- Maxwell SR, Thomason H, Sandler D et al (1997) Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus. *Eur J Clin Invest* 27:484–490
- Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsøe R, Dela F (2007) Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. *Diabetologia* 50:790–796
- Larsen S, Ara I, Rabol R et al (2009) Are substrate use during exercise and mitochondrial respiratory capacity decreased in arm and leg muscle in type 2 diabetes? *Diabetologia* 52:1400–1408
- Rabol R, Larsen S, Hojberg PM et al (2010) Regional anatomic differences in skeletal muscle mitochondrial respiration in type 2 diabetes and obesity. *J Clin Endocrinol Metab* 95:857–863
- Hey-Mogensen M, Hojlund K, Vind BF et al (2010) Effect of physical training on mitochondrial respiration and reactive oxygen species release in skeletal muscle in patients with obesity and type 2 diabetes. *Diabetologia* 53:1976–1985
- Hancock CR, Han DH, Chen M et al (2008) High-fat diets cause insulin resistance despite an increase in muscle mitochondria. *Proc Natl Acad Sci USA* 105:7815–7820
- Holloszy JO (2009) Skeletal muscle “mitochondrial deficiency” does not mediate insulin resistance. *Am J Clin Nutr* 89:463S–466S
- Kelley DE, He J, Menshikova EV, Ritov VB (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51:2944–2950
- Koves TR, Ussher JR, Noland RC et al (2008) Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* 7:45–56
- Anderson EJ, Kypson AP, Rodriguez E, Anderson CA, Lehr EJ, Neuffer PD (2009) Substrate-specific derangements in mitochondrial metabolism and redox balance in the atrium of the type 2 diabetic human heart. *J Am Coll Cardiol* 54:1891–1898
- Ponsot E, Zoll J, N’Guessan B et al (2005) Mitochondrial tissue specificity of substrates utilization in rat cardiac and skeletal muscles. *J Cell Physiol* 203:479–486
- Anderson EJ, Neuffer PD (2006) Type II skeletal myofibers possess unique properties that potentiate mitochondrial H₂O₂ generation. *Am J Physiol Cell Physiol* 290:C844–C851
- Skovbro M, Baranowski M, Skov-Jensen C et al (2008) Human skeletal muscle ceramide content is not a major factor in muscle insulin sensitivity. *Diabetologia* 51:1253–1260
- Andersen JL, Schjerling P, Andersen LL, Dela F (2003) Resistance training and insulin action in humans: effects of de-training. *J Physiol* 551:1049–1058
- Andersen JL, Aagaard P (2000) Myosin heavy chain IIX overshoot in human skeletal muscle. *Muscle Nerve* 23:1095–1104
- Kraunsøe R, Boushel RC, Hansen CN et al (2010) Mitochondrial respiration in subcutaneous and visceral adipose tissue from patients with morbid obesity. *J Physiol* 588:2023–2032
- Tonkonogi M, Sahlin K (1997) Rate of oxidative phosphorylation in isolated mitochondria from human skeletal muscle: effect of training status. *Acta Physiol Scand* 161:345–353
- Frayn KN (1983) Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 55:628–634
- Rabol R, Boushel R, Almdal T et al (2010) Opposite effects of pioglitazone and rosiglitazone on mitochondrial respiration in skeletal muscle of patients with type 2 diabetes. *Diab Obes Metab* 12:806–814
- Rabol R, Hojberg PM, Almdal T et al (2009) Improved glycaemic control decreases inner mitochondrial membrane leak in type 2 diabetes. *Diab Obes Metab* 11:355–360
- Rabol R, Hojberg PM, Almdal T et al (2009) Effect of hyperglycemia on mitochondrial respiration in type 2 diabetes. *J Clin Endocrinol Metab* 94:1372–1378
- Kelley DE, Goodpaster BH, Storlien L (2002) Muscle triglyceride and insulin resistance. *Annu Rev Nutr* 22:325–346
- Green K, Brand MD, Murphy MP (2004) Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. *Diabetes* 53(Suppl 1):S110–S118
- Iossa S, Mollica MP, Lionetti L, Crescenzo R, Tasso R, Liverini G (2004) A possible link between skeletal muscle mitochondrial efficiency and age-induced insulin resistance. *Diabetes* 53:2861–2866
- Tahara EB, Navarete FD, Kowaltowski AJ (2009) Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. *Free Radic Biol Med* 46:1283–1297
- Abdul-Ghani MA, Jani R, Chavez A, Molina-Carrion M, Tripathy D, Defronzo RA (2009) Mitochondrial reactive oxygen species generation in obese non-diabetic and type 2 diabetic participants. *Diabetologia* 52:574–582
- Brunmair B, Staniek K, Gras F et al (2004) Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? *Diabetes* 53:1052–1059
- Owen MR, Doran E, Halestrap AP (2000) Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J* 348:607–614
- Saks VA, Kuznetsov AV, Khuchua ZA et al (1995) Control of cellular respiration in vivo by mitochondrial outer membrane and by creatine kinase. A new speculative hypothesis: possible involvement of mitochondrial–cytoskeleton interactions. *J Mol Cell Cardiol* 27:625–645