CLINICAL STUDY

Early or advanced stage type 2 diabetes is not accompanied by *in vivo* skeletal muscle mitochondrial dysfunction

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Abstract

Objective: Several lines of evidence support a potential role of skeletal muscle mitochondrial dysfunction in the pathogenesis of insulin resistance and/or type 2 diabetes. However, it remains to be established whether mitochondrial dysfunction represents either cause or consequence of the disease. We examined *in vivo* skeletal muscle mitochondrial function in early and advanced stages of type 2 diabetes, with the aim to gain insight in the proposed role of mitochondrial dysfunction in the aetiology of insulin resistance and/or type 2 diabetes.

Methods: Ten long-standing, insulin-treated type 2 diabetes patients, 11 subjects with impaired fasting glucose, impaired glucose tolerance and/or recently diagnosed type 2 diabetes, and 12 healthy, normoglycaemic controls, matched for age and body composition and with low habitual physical activity levels were studied. *In vivo* mitochondrial function of the *vastus lateralis* muscle was evaluated from post-exercise phosphocreatine (PCr) recovery kinetics using ³¹P magnetic resonance spectroscopy (MRS). Intramyocellular lipid (IMCL) content was assessed in the same muscle using single-voxel ¹H MRS.

Results: IMCL content tended to be higher in the type 2 diabetes patients when compared with normoglycaemic controls ($P=0.06$). The ³¹P MRS parameters for mitochondrial function, i.e. PCr and ADP recovery time constants and maximum aerobic capacity, did not differ between groups.

Conclusions: The finding that *in vivo* skeletal muscle oxidative capacity does not differ between long-standing, insulin-treated type 2 diabetes patients, subjects with early stage type 2 diabetes and sedentary, normoglycaemic controls suggests that mitochondrial dysfunction does not necessarily represent either cause or consequence of insulin resistance and/or type 2 diabetes.

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Introduction

Insulin resistance is an early event in the pathogenesis of type 2 diabetes. However, the exact processes leading to insulin resistance remain unresolved. Previous studies have reported a strong correlation between intramyocellular lipid (IMCL) content and insulin resistance (1, 2). However, the proposed relationship between IMCL accumulation and skeletal muscle insulin resistance is not unambiguous, as it is strongly influenced by training status and/or habitual physical activity (3, 4). Nonetheless, several lines of evidence indicate that mitochondrial dysfunction, presumably associated with a reduced capacity to oxidize fatty acids, might stimulate IMCL accretion and, as such, contribute to the development of skeletal muscle insulin resistance (5).

Data to support the proposed role of skeletal muscle mitochondrial dysfunction in the development of insulin resistance and/or type 2 diabetes have been obtained with various *in vitro* methods, including

measurements of oxidative enzyme activities (6–10), mRNA and/or protein expression of oxidative phosphorylation genes (10–14) as well as mitochondrial content, morphology and respiration (8, 9, 14, 15). Furthermore, *in vivo* magnetic resonance spectroscopy (MRS) measurements of basal mitochondrial ATP synthesis rates (16–18) and post-exercise phosphocreatine (PCr) (19) and ADP (20) recovery kinetics also point towards a potential role for mitochondrial dysfunction in the aetiology of insulin resistance and/or type 2 diabetes. However, it was recently shown that *in vitro* mitochondrial respiration of permeabilized muscle fibres from biopsies of the type 2 diabetes patients and healthy controls did not differ between groups when the data were normalized for mitochondrial DNA content or citrate synthase activity (21). In other words, the type 2 diabetes patients showed normal intrinsic mitochondrial function, but an impaired oxidative capacity that was entirely attributed to a lower mitochondrial content. Environmental

factors play an important role in regulating skeletal muscle oxidative capacity, and the lower mitochondrial content in the type 2 diabetes patients might simply be the result of a reduced habitual physical activity level (21–23). Furthermore, mitochondrial dysfunction in type 2 diabetes might be secondary to impaired insulin signalling (24–26) and/or abnormal blood glucose, insulin (27, 28) and non-esterified fatty acid (NEFA) (26) levels. Therefore, the debate continues as to whether mitochondrial dysfunction represents either cause or consequence of insulin resistance and/or type 2 diabetes.

The aim of this study was to assess whether *in vivo* mitochondrial function is impaired in the early and/or overt diabetes state. Therefore, we examined *in vivo* muscle mitochondrial function in three groups of subjects, representative for different stages in the development of type 2 diabetes: long-standing, insulin-treated type 2 diabetes patients, subjects with impaired fasting glucose, impaired glucose tolerance and/or recently diagnosed type 2 diabetes, and healthy, normoglycaemic controls, all matched for age and body composition and with low habitual physical activity levels. *In vivo* mitochondrial function was determined from post-exercise PCr recovery kinetics measured with ^{31}P MRS (29–31).

Materials and methods

Subjects

Ten long-standing, insulin-treated type 2 diabetes patients (Diabetes group), 11 subjects with impaired fasting glucose, impaired glucose tolerance and/or recently diagnosed type 2 diabetes (pre-Diabetes group), and 12 healthy, normoglycaemic controls (Control group) were selected to participate in this study. All subjects were male. The diabetes patients had been diagnosed with type 2 diabetes for over 5 years, established by a fasting plasma glucose concentration larger than or equal to 7.0 mmol/l at the time of diagnosis as defined by the World Health Organization (WHO) (32). Subjects in the Control and pre-Diabetes groups had no family history of diabetes and were selected based on an oral glucose tolerance test (OGTT) according to the WHO criteria (32). Control subjects showed normal fasting glucose concentrations and normal glucose tolerance (fasting glucose <6.1 mmol/l and 2-h glucose <7.8 mmol/l). Subjects in the pre-Diabetes group had either elevated fasting plasma glucose concentrations (fasting glucose $\geq$ 6.1 and <7.0 mmol/l, and 2-h glucose <7.8 mmol/l; $n=5$), or impaired glucose tolerance (fasting glucose <7.0 mmol/l, and 2-h glucose $\geq$ 7.8 and <11.1 mmol/l; $n=2$), or recently diagnosed type 2 diabetes (<1 month; fasting glucose $\geq$ 7.0 mmol/l or 2-h glucose $\geq$ 11.1 mmol/l; $n=4$).

All diabetes patients were on exogenous insulin treatment and had been on a stable regimen of diabetes medication over the last 3 months before being recruited. Out of the ten participating diabetes patients, seven patients were treated with short (Novorapid[®], $n=6$) or rapid acting insulin (Humulin[®], $n=1$) before each meal either in combination with NPH insulin (Insulatard[®], $n=5$), premixed biphasic isophane insulin (Mixtard 30/70[®] in combination with metformin, $n=1$), or a very long-acting insulin analogue (insulin glargine, $n=1$), all administered before bedtime. Two patients were treated with premixed biphasic isophane insulin (Mixtard 30/70[®]) twice a day in combination with metformin. One patient used NPH insulin (Humulin NPH[®]) once a day before breakfast in combination with metformin and a sulphonylurea (glimepiride). Patients using thiazolidinediones were excluded from participation. None of the subjects in the pre-Diabetes group used blood glucose-lowering medication and all showed glycosylated haemoglobin (HbA_{1c}) contents below 6%. Subjects using β -blockers for less than 6 months and subjects with impaired liver function, renal failure, severe retinopathy or a history of severe cardiovascular problems were excluded from participation. Subjects with clinical signs of peripheral vascular disease (Fontaine stage II or higher) were excluded based on a thorough history considering signs of intermittent claudication, as well as a physical examination of the peripheral vascular system (no arterial bruits over the abdominal aorta, iliac artery and/or diminished pulses of femoral artery, dorsalis pedis and posterior tibial artery). The nature and the risks of the experimental procedures were explained to the subjects and all gave their written informed consent to participate in the study, which was approved by the local Medical Ethical Committee of the Máxima Medical Centre, Veldhoven, The Netherlands. This study is part of a larger project that studies mitochondrial function in chronic metabolic disease (33, 34).

Body composition

Body mass index (BMI) and waist circumference were measured using an analogue weight scale and standard measuring tape. Whole-body fat-free mass (FFM) and truncal fat mass were determined using whole-body dual energy X-ray absorptiometry (Hologic QDR-4500 Discovery A, software version 12.3.3, Hologic Inc., Bedford, MA, USA).

Habitual physical activity level

Habitual physical activity level was assessed with the Tecumseh and Minnesota occupational and leisure time activity questionnaire (35). The activity level was expressed in metabolic equivalents (MET), which is a scale of the energy cost of various physical activities in multiples of the resting metabolic rate.

Whole-body oxygen uptake capacity

Maximal whole-body oxygen uptake capacity ($VO_{2\text{peak}}$) and maximal workload capacity (W_{max}) were measured during an incremental exercise test until exhaustion, performed on a cycle ergometer (Medifit Ergometer, Medifit systems, Maarn, The Netherlands) using a ramp protocol (36). Gas exchange measurements were performed continuously (Ergostar II, PMS Professional Medical Systems, Basel, Switzerland). Maximal whole-body oxygen uptake capacity was defined as the VO_2 value remaining unchanged or increasing at <1 ml/min per kg for 30 s or more despite an increment in workload (37). Cardiac function was monitored using a 12-lead electrocardiogram with heart rate being recorded continuously (Polar Electro, Kempele, Finland). The age-predicted maximal heart rate was calculated according to the prediction model of Gellish *et al.* (38).

Blood sampling and analyses

Subjects reported at the laboratory at 0800 h after an overnight fast. After 5–10 min of supine rest, fasting samples of venous blood were collected from an antecubital vein. Subsequently, a standard OGTT was performed for all subjects, except for the diabetes patients, and blood samples were collected 2 h after ingestion of the glucose load. Blood plasma samples were collected into EDTA-containing tubes and centrifuged for 10 min at 4 °C. Aliquots of plasma were frozen immediately in liquid nitrogen and stored at -80 °C until further analyses. Plasma concentrations of glucose (Roche) and NEFA (Wako Chemicals, Neuss, Germany) were analysed with a COBAS semi-automatic analyzer (Roche). Plasma insulin was determined in duplicate by RIA (Linco, St Charles, MO, USA) for Control and pre-Diabetes groups. Cross sensitivity of exogenously administered insulin with this RIA prohibited the detection of endogenously produced insulin in the insulin-treated diabetes patients. The blood HbA_{1c} content was analysed by HPLC (Bio-Rad Diamat). For the Control and pre-Diabetes groups, the homeostasis model assessment (HOMA) index (39) was calculated.

MRS measurements

MRS measurements were performed with an 1.5 T whole-body scanner (Gyrosan S15/ACS, Philips Medical Systems, Best, The Netherlands) during two sessions. On the evening before the first MRS session, subjects received a standardized meal (mean $\pm$ s.d.: 41.0 ± 15.1 kJ per kg body weight, containing 40.7 energy% (En%) fat, 14.6 En% protein and 44.7 En% carbohydrate) after which subjects remained fasted and were allowed to drink water only. Subjects travelled by car or public transport and reported at the laboratory at 0830 h, where they received a standardized breakfast. First, ^1H MRS measurements were performed for the quantification of IMCL. After a short break, the ^{31}P MRS

protocol was carried out to familiarize the subjects to the in-magnet exercise and to determine the optimal exercise intensity for the second visit. During the second MRS session, scheduled within 1 week, ^{31}P MRS measurements were performed to assess skeletal muscle mitochondrial function. For the diabetes patients, blood glucose-lowering medication was not withdrawn before the MRS measurements.

^1H MRS

IMCL was measured in the *Musculus vastus lateralis* with image-guided single-voxel ^1H MRS using the body coil for transmission and an 8 cm diameter surface coil for signal reception. For each subject, five voxels with a size of $10 \times 10 \times 15$ mm³ were measured at different positions within the *M. vastus lateralis*. The voxels were carefully placed to avoid subcutaneous and visible interstitial fat using standard T_1 -weighted images (Fig. 1A). Spectra were recorded with a point-resolved spectroscopy (PRESS) sequence (repetition time, 1500 ms; echo time, 35 ms; spectral width, 2000 Hz; number of data points, 2048; 128 averages) using chemical-shift-selective saturation for water suppression. Unsuppressed water spectra (32 averages) were recorded from the same voxels and used as an internal reference.

^1H MRS data analysis

All spectra were fitted in the time domain by using a non-linear least-squares algorithm (AMARES) in the jMRUI software package (40) without further post-processing, except for manual phasing. The unsuppressed water spectrum was phased and fitted to a Lorentzian line shape. The zero-order phase correction from the water spectrum was applied to the corresponding water-suppressed spectrum and the total creatine (tCr) CH_3 peak was referenced to 3.02 ppm. In the water-suppressed spectrum, peaks from trimethyl ammonium and tCr CH_3 , extramyocellular lipid (EMCL) and IMCL CH_2 , and EMCL and IMCL CH_3 protons (see Fig. 1B) were fitted to the Gaussian line shapes. The positions and areas of the EMCL and IMCL CH_3 peaks were constrained with respect to the positions and areas of the EMCL and the IMCL CH_2 peaks respectively (41). To increase the reliability of the fit, the linewidth of the IMCL CH_2 peak (LW_{IMCL}) was constrained with respect to the linewidth of the water peak $LW_{\text{H}_2\text{O}}$ according to

$$LW_{\text{IMCL}} = -0.09 + 1.02 \cdot LW_{\text{H}_2\text{O}}$$

This constraint was derived from previously recorded data sets with a well-resolved IMCL peak (number of data sets = 20, $R = 0.821$, $P < 0.0001$). A soft constraint that was empirically determined from the previously recorded data sets, was applied to the linewidth of the EMCL CH_2 peak (LW_{EMCL}),

Figure 1 (A) Transversal T_1 -weighted spin-echo image of the upper leg of a type 2 diabetes patient with voxel positioning within the *M. vastus lateralis* for single-voxel ^1H MRS. The size of the voxel was $10 \times 10 \times 15 \text{ mm}^3$. The arrow points at a phantom that was located in the centre of the 8 cm diameter surface coil. (B) ^1H MR spectrum from the voxel depicted in (A). The spectrum was processed with 1 Hz line broadening. Peak annotations: tCr CH_2/CH_3 , CH_2/CH_3 protons of total creatine; TMA, CH_3 protons of trimethyl ammonium groups; EMCL CH_2/CH_3 , CH_2/CH_3 protons of extramyocellular lipid; IMCL CH_2/CH_3 , CH_2/CH_3 protons of intramyocellular lipid.

i.e. $\text{LW}_{\text{EMCL}} \leq \text{LW}_{\text{IMCL}} + 3.33 \text{ Hz}$. When the resonance frequency (δ) of the IMCL CH_2 peak could not be accurately fitted (i.e. $\delta \leq 1.25 \text{ ppm}$ or $\delta \geq 1.31 \text{ ppm}$), the position of the IMCL CH_2 peak was constrained with respect to the position of the tCr CH_3 peak (chemical shift difference = $3.02\text{--}1.28 \text{ ppm}$). IMCL was

expressed as a percentage of the water signal measured in the same voxel without water suppression. The IMCL levels determined from different voxels of one subject were averaged.

^{31}P MRS

^{31}P MRS was performed as described previously (33). In short, ^{31}P signals were collected using a 6 cm diameter surface coil placed over the *M. vastus lateralis* (spectral width, 2000 Hz; number of data points, 1024). A fully relaxed spectrum was measured at rest with a repetition time of 30 s and 24 scans. Then, spectra were acquired during a rest–exercise–recovery protocol with a repetition time of 3 s and two scans yielding a time resolution of 6 s. The first 20 spectra (2 min) were measured at rest, after which the subjects started the exercise. Subjects performed a dynamic incremental single-leg extension exercise in the supine position using a home-built MR-compatible ergometer (33). The duration of the exercise varied per subject, but never exceeded 8 min, so that at least 5 min of recovery were recorded. During the first visit, subjects performed a test run and exercised until fatigued. During the second session, the duration of the exercise was chosen to deplete PCr by about 50%, while aiming to avoid the intracellular pH dropping below 6.8.

^{31}P MRS data analysis

The ^{31}P MRS data were analysed as described previously (33). In short, spectra were fitted in the time domain by using a non-linear least-squares algorithm (AMARES) in the jMRUI software package (40). PCr, inorganic phosphate (P_i), ATP and phosphodiester (PDE) signals were fitted to the Lorentzian line shapes. Absolute concentrations of the phosphorylated metabolites were calculated after correction for partial saturation and assuming that the ATP concentration is 8.2 mM at rest (42). Intracellular pH was calculated from the chemical shift difference between the P_i and PCr resonances (43). The free cytosolic ADP concentration was calculated from the PCr concentration and pH using a creatine kinase equilibrium constant (K_{eq}) of $1.66 \times 10^9/\text{M}$ (44). Recoveries of PCr and ADP were fitted to mono-exponential functions using Matlab (version 7.30., Mathworks, Natick, MA, USA). Results are expressed as the metabolite's time constant of recovery, i.e. τ_{PCr} and τ_{ADP} , both of which are measures of the mitochondrial function (43, 45, 46). Calculation of the initial rate of PCr recovery (V_{PCr}) was based on the PCr recovery rate ($1/\tau_{\text{PCr}}$) and the difference between the resting and end-exercise PCr concentrations (47). Calculation of the maximum aerobic capacity (Q_{max}) was based on the ADP-control model (48), in which V_{PCr} has a hyperbolic dependence on the end-exercise ADP concentration according to Michaelis–Menten kinetics with a K_m of 30 μM (47).

Statistical analysis

All data are expressed as means $\pm$ s.d. Statistical analyses were performed using the SPSS 14.0 software package (SPSS Inc., Chicago, IL, USA). An ANOVA test was performed to test for overall differences between the Control, pre-Diabetes and Diabetes groups and a Bonferroni *post hoc* test was applied for pairwise comparisons between the three groups. For comparisons between two groups, differences were determined with an unpaired *t*-test. To evaluate the relationships between variables, Pearson correlation coefficients were calculated. All tests were carried out in a two-sided way and the level of significance was set at $P < 0.05$.

Results

Subjects' characteristics

The subjects' characteristics are shown in Table 1. Subjects were carefully matched for age and BMI. In accordance, FFM and truncal fat mass did not differ between the Control, pre-Diabetes and Diabetes groups ($P = 0.29$ and $P = 0.87$ respectively). $W_{\max}$ and $VO_{2\text{peak}}$ were similar for the Control and pre-Diabetes groups ($P = 1.00$), but significantly lower in the Diabetes group. The latter was paralleled by a lower maximal heart rate and a lower habitual physical activity level, but the physical activity levels were not significantly different between groups ($P = 0.12$). $W_{\max}$ and $VO_{2\text{peak}}$ correlated with both

maximal heart rate (Pearson's *R* between 0.59 and 0.68, $P < 0.01$) and percentage of the age-predicted maximal heart rate (Pearson's *R* between 0.56 and 0.62, $P < 0.01$).

The fasting plasma glucose and insulin levels were not significantly different for the Control and pre-Diabetes groups ($P = 0.39$ and $P = 0.14$ respectively), but the HOMA index was significantly higher for the pre-Diabetes group than for the Control group. Also, the glucose and insulin concentrations measured 2 h after ingestion of the glucose load during the OGTT were significantly higher for the pre-Diabetes group than for the Control group. The HbA_{1c} contents were similar for the Control and pre-Diabetes groups ($P = 1.00$) and all subjects had HbA_{1c} contents below 6%. The fasting glucose concentrations and HbA_{1c} contents were significantly higher in the Diabetes group (glucose ≥ 8 mmol/l and HbA_{1c} $\geq 6.3\%$) when compared with the Control and pre-Diabetes groups. Diabetes patients had been diagnosed with type 2 diabetes for 12 ± 7 years and had been on exogenous insulin therapy for 7 ± 8 years. Plasma NEFA concentrations did not differ between groups ($P = 0.16$).

$W_{\max}$ and $VO_{2\text{peak}}$ correlated negatively with both fasting plasma glucose (Pearson's *R* between -0.48 and -0.57 , $P < 0.01$) and HbA_{1c} (Pearson's *R* between -0.58 and -0.70 , $P < 0.01$).

IMCL content

Due to excessive EMCL contamination in the spectra, IMCL content could not be determined for two subjects

Table 1 Subjects' characteristics.

	Control	pre-Diabetes	Diabetes
<i>n</i>	12	11	10
Age (years)	56.5 $\pm$ 6.0	58.5 $\pm$ 5.0	58.8 $\pm$ 7.6
BMI (kg/m ²)	32.9 $\pm$ 4.6	32.1 $\pm$ 3.2	31.8 $\pm$ 4.0
Body weight (kg)	101.0 $\pm$ 14.7	103.9 $\pm$ 10.0	95.0 $\pm$ 14.4
FFM (kg)	70.7 $\pm$ 7.1	72.4 $\pm$ 6.9	67.2 $\pm$ 8.4
Truncal fat mass (kg)	15.5 $\pm$ 5.5	16.3 $\pm$ 2.9	15.4 $\pm$ 4.5
$W_{\max}$ (W)	247 $\pm$ 42	244 $\pm$ 30	156 $\pm$ 38* [†]
$W_{\max}$ per kg BW (W/kg)	2.48 $\pm$ 0.47	2.39 $\pm$ 0.47	1.66 $\pm$ 0.36* [†]
$VO_{2\text{peak}}$ per kg BW (ml/min per kg)	32.3 $\pm$ 5.4	33.8 $\pm$ 5.9	25.2 $\pm$ 3.5* [†]
$VO_{2\text{peak}}$ per kg FFM (ml/min per kg)	45.6 $\pm$ 6.0	48.5 $\pm$ 8.3	35.5 $\pm$ 5.2* [†]
HR _{max} (b/min)	166 $\pm$ 13	171 $\pm$ 12	140 $\pm$ 23* [†]
% predicted HR _{max}	99 $\pm$ 8	103 $\pm$ 7	84 $\pm$ 14* [†]
Activity level (MET h/day)	19.3 $\pm$ 7.4	19.5 $\pm$ 8.7	13.6 $\pm$ 4.3
Fasting glucose (mmol/l)	5.7 $\pm$ 0.2	6.6 $\pm$ 0.5	11.0 $\pm$ 2.5* [†]
Fasting insulin (μ U/ml)	16.2 $\pm$ 9.1	22.2 $\pm$ 9.6	ND
HOMA index	4.1 $\pm$ 2.2	6.5 $\pm$ 2.7 [†]	ND
2-h glucose (mmol/l)	5.7 $\pm$ 1.3	8.4 $\pm$ 2.6 [§]	ND
2-h insulin (μ U/ml)	74.8 $\pm$ 40.5	146.2 $\pm$ 64.5 [§]	ND
HbA _{1c} (%)	5.3 $\pm$ 0.3	5.5 $\pm$ 0.2	7.7 $\pm$ 1.0* [†]
NEFA (mmol/l)	0.31 $\pm$ 0.10	0.39 $\pm$ 0.13	0.46 $\pm$ 0.26
Years with type 2 diabetes	NA	NA	12 $\pm$ 7
Years of insulin therapy	NA	NA	7 $\pm$ 8

Data are expressed as means $\pm$ s.d. BMI, body mass index; FFM, fat-free mass; $W_{\max}$, maximal workload capacity; $W_{\max}$ per kg BW, $W_{\max}$ per kg body weight; $VO_{2\text{peak}}$, maximal oxygen uptake capacity per kg body weight or per kg FFM; HR_{max}, maximal heart rate during the $VO_{2\text{peak}}$ test; % predicted HR_{max}, percentage of the age-predicted HR_{max}; MET, metabolic equivalents; HOMA, homeostasis model assessment; 2 h glucose/insulin, glucose/insulin concentration 2 h after ingestion of glucose load during the oral glucose tolerance test; HbA_{1c}, glycosylated haemoglobin; NEFA, non-esterified fatty acids; NA, not applicable; ND, not determined. *Significantly different from Control (ANOVA, $P < 0.01$). [†]Significantly different from pre-Diabetes (ANOVA, $P < 0.01$). [‡]Significantly different from Control (*t*-test, $P < 0.05$). [§]Significantly different from Control (*t*-test, $P < 0.01$).

in the Control group and one subject in the Diabetes group. For all other subjects, spectra from at least two out of the five voxels were quantified. IMCL content tended to be higher for the Diabetes group when compared with the Control group (1.3 ± 0.4 ($n=10$), 1.6 ± 0.7 ($n=11$) and 2.0 ± 0.7 ($n=9$) % of the water signal in Control, pre-Diabetes and Diabetes groups respectively, $P=0.06$; Fig. 2).

Skeletal muscle mitochondrial function

Figure 3A and B show typical examples of ^{31}P MR spectra from a subject's *vastus lateralis* muscle at rest and at the end of exercise respectively. Table 2 summarizes the baseline, end-exercise and recovery ^{31}P MRS results for the Control, pre-Diabetes and Diabetes groups. At rest, PCr, P_i , ADP and PDE concentrations and intracellular pH were not significantly different for the Control, pre-Diabetes and Diabetes groups ($P=0.93$, $P=0.50$, $P=0.09$, $P=0.42$ and $P=0.10$ respectively). The end-exercise status has to be taken into account when analysing the recovery data. The end-exercise metabolite concentrations were similar for the three groups, except for the end-exercise ADP concentration, which was lower in the Diabetes group. However, for all groups, the end-exercise ADP concentration was well above the accepted K_m value of $30 \mu\text{M}$ for oxidative ATP synthesis. The average PCr depletion was 54 ± 10 , 56 ± 7 and $46 \pm 8\%$ for Control, pre-Diabetes and Diabetes groups respectively. For none of the subjects did the end-exercise pH

Figure 2 IMCL content in Control, pre-Diabetes and Diabetes groups. Bars indicate the mean values for the three groups and filled circles represent the individual data points. IMCL content tended to be higher for the Diabetes group when compared with the Control group (ANOVA, $P=0.06$).

Figure 3 Typical *M. vastus lateralis* ^{31}P MR spectra for a normoglycaemic control subject at rest (A, number of scans=60) and at the end of exercise (B, number of scans=2). Spectra were processed with 5 Hz line broadening. P_i , inorganic phosphate; PDE, phosphodiester; PCr, phosphocreatine; α , β and γ , the three phosphate groups of ATP. For this subject, the PCr depletion at the end of exercise (B) was 61% and the corresponding end-exercise pH was 6.96.

drop below 6.75. The average end-exercise pH was not different for the three groups ($P=0.72$), which is a prerequisite for comparing τ_{PCr} .

The PCr and ADP recoveries could be satisfactorily described by mono-exponential functions (average R^2 values for the mono-exponential fits were 0.96 ± 0.02 and 0.91 ± 0.06 for PCr and ADP recovery data respectively). Figure 4 illustrates both the raw data and mono-exponential fits of the PCr and ADP recoveries for one subject. Figure 5 shows the mean values and the distribution of τ_{PCr} and Q_{max} for the Control, pre-Diabetes and Diabetes groups. The ^{31}P MRS parameters for mitochondrial function, i.e. τ_{PCr} , τ_{ADP} and Q_{max} , did not differ between the Control, pre-Diabetes and Diabetes groups ($P=0.62$, $P=0.29$ and $P=0.24$ respectively; Table 2). V_{PCr} was significantly lower for the Diabetes group when compared with the

Table 2 ^{31}P MRS parameters during rest, end of exercise and recovery.

	Control	pre-Diabetes	Diabetes
Rest			
[PCr] (mM)	37.3±2.3	37.7±2.6	37.2±4.2
[P _i] (mM)	4.8±0.6	5.0±0.7	4.7±0.8
[ADP] (μM)	10.0±0.3	10.4±0.5	10.1±0.4
[PDE] (mM)	6.0±0.8	6.0±0.9	5.5±0.9
pH	7.06±0.01	7.07±0.02	7.06±0.02
End-exercise			
[PCr] (mM)	16.9±3.5	16.7±2.8	20.0±4.5
[P _i] (mM)	23.3±4.9	24.2±3.5	19.8±4.0
[ADP] (μM)	68.2±18.2	70.2±11.3	47.0±10.4*†
pH	6.91±0.08	6.92±0.07	6.89±0.12
Recovery			
τ_{PCr} (s)	44.5±10.5	41.7±6.2	46.6±16.5
τ_{ADP} (s)	19.1±3.5	16.9±5.0	20.2±6.0
V_{PCr} (mM/s)	0.47±0.09	0.51±0.08	0.39±0.09†
Q_{max} (mM/s)	0.69±0.12	0.74±0.11	0.65±0.13

Data are expressed as means ± s.d. PCr, phosphocreatine; P_i, inorganic phosphate; ADP, adenosine diphosphate; PDE, phosphodiester; pH, intracellular muscle pH; τ_{PCr} , PCr recovery time constant; τ_{ADP} , ADP recovery time constant; V_{PCr} , initial rate of PCr recovery; Q_{max} , maximum rate of oxidative ATP synthesis. *Significantly different from Control (ANOVA, $P < 0.01$). †Significantly different from pre-Diabetes (ANOVA, $P < 0.01$).

pre-Diabetes group as a result of the lower end-exercise ADP concentration.

All ^{31}P MRS parameters for mitochondrial function correlated significantly with both W_{max} (absolute Pearson's R between 0.45 and 0.60, $P < 0.01$) and $\text{VO}_{2\text{peak}}$ (absolute Pearson's R between 0.35 and 0.59, $P < 0.05$). However, τ_{PCr} , τ_{ADP} and Q_{max} did not correlate with fasting plasma glucose or $\text{HbA}_{1\text{c}}$.

Discussion

In this study, it was shown that *in vivo* skeletal muscle oxidative capacity, as determined from post-exercise PCr recovery kinetics using ^{31}P MRS, does not differ between long-standing, insulin-treated type 2 diabetes patients, subjects with early stage type 2 diabetes and healthy, normoglycaemic controls, all matched for age and body composition and with low habitual physical activity levels. Therefore, our results suggest that skeletal muscle mitochondrial dysfunction does not necessarily represent either cause or consequence of type 2 diabetes.

Maximal workload capacity and maximal whole-body oxygen uptake capacity were significantly lower in the type 2 diabetes patients and correlated negatively with both plasma glucose and blood $\text{HbA}_{1\text{c}}$ contents. The ^{31}P MRS recovery parameters, τ_{PCr} , τ_{ADP} and Q_{max} , correlated significantly with W_{max} and $\text{VO}_{2\text{peak}}$, but not with plasma glucose or blood $\text{HbA}_{1\text{c}}$ levels. Therefore, W_{max} and $\text{VO}_{2\text{peak}}$ seem to represent markers of the disease status, whereas τ_{PCr} , τ_{ADP} and Q_{max} report only on local muscle mitochondrial function. The Pearson correlation coefficient for the correlation between Q_{max} and $\text{VO}_{2\text{peak}}$ per kg FFM ($\text{VO}_{2\text{peak}}$ per kg FFM) was 0.47

Figure 4 (A) PCr and (B) ADP recovery curves from the data set that were also used in Fig. 3. Mono-exponential functions (dark lines) were fit to the actual data (filled circles) obtained every 6 s. The time constants for PCr and ADP recovery were 41.8 and 19.8 s respectively.

($R^2 = 0.22$). Therefore, only 22% of the variance in $\text{VO}_{2\text{peak}}$ per kg FFM can be explained by Q_{max} . Q_{max} was measured locally in the *M. vastus lateralis* during a dynamic single-leg extension exercise, while whole-body $\text{VO}_{2\text{peak}}$ was determined on a cycle ergometer. The remaining variance in $\text{VO}_{2\text{peak}}$ per kg FFM could be accounted for by differences in cardiovascular capacity at peak work rates that are not expected to play a significant role during the local exercise in the MR scanner. The apparent discrepancy between the results for Q_{max} and $\text{VO}_{2\text{peak}}$ per kg FFM might therefore be explained by differences in cardiac output. The latter concept seems to be supported by a significant correlation between the maximal heart rate and $\text{VO}_{2\text{peak}}$ per kg FFM (Pearson's $R = 0.64$, $P < 0.01$).

In this study, ^{31}P MRS was applied to assess *in vivo* skeletal muscle mitochondrial function from measurements during recovery from exercise. During recovery

Figure 5 (A) τ_{PCr} and (B) Q_{max} in Control, pre-Diabetes and Diabetes groups. Bars indicate the mean values for the three groups and filled circles represent the individual data points. τ_{PCr} and Q_{max} were not significantly different between Control, pre-Diabetes and Diabetes groups (ANOVA, $P=0.62$ and $P=0.24$ respectively).

from exercise, PCr is resynthesized purely as a consequence of oxidative ATP synthesis (43, 45). Therefore, τ_{PCr} provides information about mitochondrial function. Recently, Schrauwen-Hinderling *et al.* applied the same technique to study mitochondrial function in the *M. vastus lateralis* in overweight type 2 diabetes patients and BMI-matched control subjects (19). Contrary to our results, τ_{PCr} was 45% longer in the type 2 diabetes group when compared with the

control group (i.e. 39.4 ± 17.5 vs 27.0 ± 3.9 s), suggestive of impaired *in vivo* mitochondrial function in the type 2 diabetes patients. It should be noted that different exercise protocols were chosen for the ^{31}P MRS measurements in the two studies. We applied an exercise protocol that progressively increased work to deplete PCr by about 50% without severe acidification. In the previous study, exercise was performed with a single load, until a steady state was reached with an average PCr depletion of 28%. The larger PCr depletion in the current study was accompanied by a slightly larger drop in pH (change in pH was on average 0.16 in this study versus 0.04 in (19)). Several studies have shown that PCr recovery is slowed down in the presence of intracellular acidosis (30, 49, 50). However, we carefully matched the end-exercise pH for the Control, pre-Diabetes and Diabetes groups, to allow a direct comparison of τ_{PCr} between groups. As an alternative to τ_{PCr} , both τ_{ADP} and Q_{max} can also be used to assess *in vivo* mitochondrial function and these parameters have been shown to be independent of pH (30, 50–53). In accordance with the τ_{PCr} data matched for end-exercise pH, τ_{ADP} and Q_{max} also did not differ between groups.

As suggested in the literature, τ_{PCr} can be normalized for pH using a correction factor of -46 s per pH unit (49). Recalculating the values from Schrauwen-Hinderling *et al.* to a 0.12 lower end-exercise pH results in τ_{PCr} 's of about 45 and 32 s for the type 2 diabetes and control group respectively. This implies that the mitochondrial function in our Control, pre-Diabetes and Diabetes groups is similar to that of the type 2 diabetes patients in the study of Schrauwen-Hinderling *et al.*, but that the controls in the latter study show a greater mitochondrial oxidative capacity. For other studies in healthy, elderly subjects previously reported in the literature (54–56), average values for τ_{PCr} (recalculated at an end-exercise pH of 6.9 if necessary) have been shown to range between 43 and 46 s, which is well in line with the average τ_{PCr} in our Control group. As such, muscle mitochondrial function in these overweight normoglycaemic control subjects does not seem to be substantially impaired.

As an alternative to measuring muscle oxidative capacity from dynamic ^{31}P MRS experiments after exercise, Petersen *et al.* applied ^{31}P MRS saturation transfer experiments to measure mitochondrial ATP synthesis rates in resting skeletal muscle of healthy, young, lean, insulin-resistant offspring of type 2 diabetes patients and insulin-sensitive control subjects matched for age, height, weight and physical activity (17). ATP synthesis rates were $\sim 30\%$ lower in insulin-resistant subjects than in controls and it was concluded that the insulin-resistant offspring might have an inherited defect in mitochondrial phosphorylation. However, as commented by Short *et al.* (24) and Wagenmakers (25), the lower ATP synthesis rates in insulin-resistant subjects could actually be caused by

the impaired insulin signalling, jeopardizing insulin-dependent mitochondrial processes, rather than the reverse. This view is supported by studies that show that high-dose insulin infusions increase mRNA transcript levels of genes involved in mitochondrial function, mitochondrial protein synthesis and mitochondrial ATP production rates in healthy people, but not in the type 2 diabetes patients (28, 57, 58). In summary, it seems likely that the decreased basal ATP synthesis rates in insulin resistant subjects are a result of the decreased insulin sensitivity and do not necessarily reflect any intrinsic mitochondrial defect.

The diabetes patients in the current study were on exogenous insulin treatment for more than 5 years and continued their medication during the study. As blood glucose and insulin levels can affect measurements of mitochondrial function (27, 28), the higher plasma insulin levels in the Diabetes group as a result of exogenous insulin treatment might be a confounding factor in this study. Despite the insulin treatment, our diabetes patients were still hyperglycaemic (fasting plasma glucose concentration: 11.0 ± 2.5 mmol/l). Therefore, hyperglycaemia might also have affected the measurement of *in vivo* muscle mitochondrial function in the diabetes patients.

Rabøl *et al.* recently reviewed the experimental data on mitochondrial dysfunction in type 2 diabetes and concluded that evidence of an intrinsic defect in the mitochondria of type 2 diabetes patients is far from convincing (23). Considering that type 2 diabetes patients are generally physically inactive, the impairments in oxidative metabolism in type 2 diabetes patients might simply be attributed to their sedentary lifestyle. In this regard, it is important to note that in most studies physical activity has not been (strictly) controlled for. For studies in which physical activity was taken into account, the results suggest that the abnormalities in oxidative metabolism in type 2 diabetes patients can at least partly be attributed to physical inactivity (14, 28, 59, 60). In accordance, recent data from respiration measurements on permeabilized muscle fibres show that when O₂ flux is being normalized for mitochondrial DNA content or citrate synthase activity, no differences in mitochondrial respiration rate are observed between type 2 diabetes patients and healthy controls (21). These results imply that type 2 diabetes patients have normal intrinsic mitochondrial function, but an impaired oxidative capacity due to a reduced mitochondrial content, most likely secondary to lower habitual physical activity levels (21, 22). More recently, Turner *et al.* examined markers of muscle mitochondrial fatty acid oxidative capacity in rodent models of lipid-induced insulin resistance (61). Surprisingly, fatty acid oxidative capacity and protein expression of peroxisome proliferator-activated receptor- γ coactivator 1 α and mitochondrial respiratory chain subunits appeared to be upregulated. As such, the authors concluded that,

at least in these rodent models, high lipid availability does not lead to intramuscular lipid accumulation and insulin resistance by decreasing muscle mitochondrial fatty acid oxidative capacity. In accordance, in the current study we observed no differences in *in vivo* muscle mitochondrial function between long-standing type 2 diabetes patients, subjects with early stage type 2 diabetes and sedentary, normoglycaemic controls.

In conclusion, subjects with early stage type 2 diabetes as well as long-standing, insulin-treated type 2 diabetes patients do not show signs of *in vivo* skeletal muscle mitochondrial dysfunction. The latter implies that mitochondrial dysfunction does not necessarily represent either cause or consequence of insulin resistance and/or type 2 diabetes. Impairments in oxidative metabolism in type 2 diabetes patients observed in previous studies are likely to be secondary to a less active lifestyle and/or impaired insulin signalling.

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