Comparison of in vivo postexercise phosphocreatine recovery and resting ATP synthesis flux for the assessment of skeletal muscle mitochondrial function

Citation for published version (APA):

Broek, van den, N. M. A., Ciapaite, J., Nicolay, K., & Prompers, J. J. (2010). Comparison of in vivo postexercise phosphocreatine recovery and resting ATP synthesis flux for the assessment of skeletal muscle mitochondrial function. *American Journal of Physiology : Cell Physiology*, 299(5), C1136-C1143.
<https://doi.org/10.1152/ajpcell.00200.2010>

DOI:

[10.1152/ajpcell.00200.2010](https://doi.org/10.1152/ajpcell.00200.2010)

Document status and date:

Published: 01/01/2010

Document Version:

Publisher's PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.tue.nl/taverne

Take down policy

If you believe that this document breaches copyright please contact us at:

openaccess@tue.nl

providing details and we will investigate your claim.

Comparison of in vivo postexercise phosphocreatine recovery and resting ATP synthesis flux for the assessment of skeletal muscle mitochondrial function

N. M. A. van den Broek, J. Ciapaite, K. Nicolay and J. J. Prompers

Am J Physiol Cell Physiol 299:C1136-C1143, 2010. First published 28 July 2010;

doi:10.1152/ajpcell.00200.2010

You might find this additional info useful...

This article cites 67 articles, 31 of which can be accessed free at:

<http://ajpcell.physiology.org/content/299/5/C1136.full.html#ref-list-1>

This article has been cited by 1 other HighWire hosted articles

The Association of Growth Hormone Parameters with Skeletal Muscle Phosphocreatine Recovery in Adult Men

Hideo Makimura, Takara L. Stanley, Noelle Sun, Mirko I. Hrovat, David M. Systrom and Steven K. Grinspoon

JCEM, March , 2011; 96 (3): 817-823.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Updated information and services including high resolution figures, can be found at:

<http://ajpcell.physiology.org/content/299/5/C1136.full.html>

Additional material and information about *AJP - Cell Physiology* can be found at:

<http://www.the-aps.org/publications/ajpcell>

This information is current as of November 2, 2011.

Comparison of in vivo postexercise phosphocreatine recovery and resting ATP synthesis flux for the assessment of skeletal muscle mitochondrial function

N. M. A. van den Broek,¹ J. Ciapaite,^{1,2} K. Nicolay,¹ and J. J. Prompers¹

¹Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven; and ²The Netherlands Consortium for Systems Biology, Den Haag, The Netherlands

Submitted 27 May 2010; accepted in final form 23 July 2010

van den Broek NM, Ciapaite J, Nicolay K, Prompers JJ. Comparison of in vivo postexercise phosphocreatine recovery and resting ATP synthesis flux for the assessment of skeletal muscle mitochondrial function. *Am J Physiol Cell Physiol* 299: C1136–C1143, 2010. First published July 28, 2010; doi:10.1152/ajpcell.00200.2010.—³¹P magnetic resonance spectroscopy (MRS) has been used to assess skeletal muscle mitochondrial function in vivo by measuring 1) phosphocreatine (PCr) recovery after exercise or 2) resting ATP synthesis flux with saturation transfer (ST). In this study, we compared both parameters in a rat model of mitochondrial dysfunction with the aim of establishing the most appropriate method for the assessment of in vivo muscle mitochondrial function. Mitochondrial dysfunction was induced in adult Wistar rats by daily subcutaneous injections with the complex I inhibitor diphenyleneiodonium (DPI) for 2 wk. In vivo ³¹P MRS measurements were supplemented by in vitro measurements of oxygen consumption in isolated mitochondria. Two weeks of DPI treatment induced mitochondrial dysfunction, as evidenced by a 20% lower maximal ADP-stimulated oxygen consumption rate in isolated mitochondria from DPI-treated rats oxidizing pyruvate plus malate. This was paralleled by a 46% decrease in in vivo oxidative capacity, determined from postexercise PCr recovery. Interestingly, no significant difference in resting, ST-based ATP synthesis flux was observed between DPI-treated rats and controls. These results show that PCr recovery after exercise has a more direct relationship with skeletal muscle mitochondrial function than the ATP synthesis flux measured with ³¹P ST MRS in the resting state.

³¹P magnetic resonance spectroscopy; saturation transfer; high-resolution respirometry; complex I inhibition; diphenyleneiodonium

MITOCHONDRIA play a pivotal role in many cellular processes, the most important function being the production of energy in the form of ATP through a process termed oxidative phosphorylation. In the last decade, mitochondria gained interest in the field of insulin resistance (IR) and type 2 diabetes (T2D) (21, 34, 43, 49, 53, 55). Based on the in vivo observation that ATP synthesis flux in resting skeletal muscle is lower in insulin-resistant subjects and offspring of T2D patients compared with healthy controls (38, 39), it has been hypothesized that skeletal muscle mitochondrial dysfunction is a predisposing factor for IR and/or T2D. The proposed mechanism links muscle mitochondrial dysfunction to impaired fatty acid metabolism, which subsequently leads to the accumulation of intramyocellular lipids and lipid intermediates (e.g., diacylglycerol and ceramides) that interfere with the insulin signaling cascade (68).

The role of skeletal muscle mitochondrial dysfunction in the development of IR and/or T2D has been investigated using a variety of techniques (12, 16–18, 24, 31–33, 37–40, 45, 48, 52). In vitro methodologies, like the determination of gene expression levels, enzyme activities, mitochondrial content, morphology, and respiration, provide specific information on different aspects of mitochondrial energy production, but the results cannot be directly translated to in vivo mitochondrial function. ³¹P magnetic resonance spectroscopy (MRS) provides a noninvasive tool to monitor the energetic status of the cell in vivo by measuring intracellular phosphorous containing metabolites; i.e., phosphocreatine (PCr), ATP, and inorganic phosphate (P_i). ³¹P MR spectra of resting skeletal muscle are relatively constant, even in diseased states, and to assess impairments in mitochondrial energy production one needs to perturb either the chemical or the magnetic equilibrium as described below.

The resting P_i→ATP flux (V_{ATP}) can be determined by saturating the γ -ATP peak and monitoring the effect of this perturbation on the P_i magnetization in a so-called ³¹P saturation transfer (ST) experiment (3, 5). Assuming that V_{ATP} is predominantly reflecting oxidative ATP synthesis by the F₁F₀-ATP synthase in the mitochondria, V_{ATP} has been taken as a measure for mitochondrial function (39, 40). However, the interpretation of ³¹P ST data is not straightforward. The lower ATP synthesis rates in resting muscle of insulin-resistant subjects (38, 39) could actually reflect a normal regulatory response to a lower energy demand caused by impaired insulin signaling rather than an impairment of intrinsic mitochondrial function (1, 26, 51, 65). Moreover, V_{ATP} obtained from ³¹P ST measurements is composed of both mitochondrial ATP synthase flux and glycolytic exchange flux, with the latter contributing by as much as 80% at rest (3, 4, 6, 26, 27). Therefore, decreased resting V_{ATP} does not necessarily reflect a mitochondrial defect.

As an alternative to the resting state ST experiment described above, the metabolic steady state of the muscle; i.e., the chemical equilibrium, can be disturbed during exercise. During recovery from exercise, the PCr pool is replenished purely through oxidative ATP synthesis (42, 46, 58). Because the creatine kinase reaction is much faster than oxidative ATP production (64), the measurement of PCr recovery using dynamic ³¹P MRS after exercise provides an alternative method to determine the rate of oxidative ATP synthesis. The PCr recovery rate constant (k_{PCr}) thus reflects in vivo muscle mitochondrial oxidative capacity; i.e., the maximal capacity for oxidative ATP production, which is typically one order of magnitude higher than the ATP synthesis rate at rest. There-

Address for reprint requests and other correspondence: J. J. Prompers, Eindhoven Univ. of Technology, Biomedical NMR, PO Box 513, 5600 MB Eindhoven, The Netherlands (e-mail: j.j.prompers@tue.nl).

fore, the postexercise PCr recovery rate constant might be a more suitable measure for in vivo mitochondrial function compared with the resting ATP synthesis flux.

In this study, we compared in vivo ^{31}P MRS postexercise PCr recovery and resting ATP synthesis flux in a rat model of mitochondrial dysfunction with the aim of establishing the most appropriate method for the assessment of in vivo skeletal muscle mitochondrial function. Mitochondrial dysfunction was induced by daily subcutaneous injections with diphenyleneiodonium (DPI), which irreversibly inhibits complex I (NADH-ubiquinone reductase) of the respiratory chain (9, 10, 30, 44). In vivo measurements were supplemented by in vitro measurements of oxygen consumption in isolated mitochondria to confirm inhibition of complex I and to compare in vivo and in vitro measurements of mitochondrial function.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (364 ± 18 g, 14 wk old, $n = 16$, Charles River Laboratories, The Netherlands) were housed in pairs at 20°C and 50% humidity, with a 12:12-h light-dark cycle. Rats were given ad libitum access to water and normal chow [9.2% calories from fat, 67.2% calories from carbohydrates, 23.6% calories from proteins (R/M-H diet, Ssniff Spezialdiäten, Soest, Germany)]. DPI (Toronto Research Chemicals, North York, Ontario, Canada) was dissolved in a warm 5% (wt/vol) glucose solution (1.3 mg/ml) and was injected daily subcutaneously at 1 mg/kg body wt for 11 ± 2 days ($n = 8$) (10). Control animals received similar volumes of subcutaneously injected 5% (wt/vol) glucose solution ($n = 8$).

The day after in vivo MRS measurements, rats were euthanized by incising the inferior vena cava under anesthesia. Immediately thereafter, half of one tibialis anterior (TA) was freeze clamped using liquid nitrogen-cooled tongs (36) and stored at -80°C for ATP determination. The other part of the TA was frozen in liquid nitrogen and stored at -80°C for the determination of mitochondrial DNA (mtDNA) content. The second TA muscle (fresh) was used for isolation of mitochondria. All experimental procedures were reviewed and approved by the Animal Ethics Committee of Maastricht University.

^{31}P MRS. All MRS measurements were performed on a 6.3-Tesla horizontal Bruker MR system (Bruker, Ettlingen, Germany), 14 days after the start of treatment. Animals were anesthetized using isoflurane (Forene) (1–2%) with medical air (0.6 l/min), and body temperature was maintained at $35.5 \pm 0.5^\circ\text{C}$ using heating pads. Respiration was monitored using a pressure sensor registering thorax movement (Rapid Biomedical, Rimpur, Germany).

Resting V_{ATP} and postexercise k_{PCr} were measured in the TA by in vivo ^{31}P MRS. ^{31}P MRS was applied using a combination of a circular ^1H surface coil (40 mm) for making MR images and localized shimming and an ellipsoid ^{31}P MRS surface coil (10/18 mm), positioned over the TA as described previously (11). ^{31}P MR spectra were acquired applying an adiabatic excitation pulse with a flip angle of 90° . A fully relaxed ($\text{TR} = 25$ s, 32 averages) spectrum was measured at rest, followed by the ST experiment in resting TA muscle to determine V_{ATP} . Two spectra ($\text{TR} = 10.4$ s, 128 averages) were acquired for each ST experiment: a spectrum with frequency-selective saturation of the γ -ATP peak yielding the steady-state P_i magnetization in the presence of saturation (M') and a reference spectrum with saturation at a downfield frequency, equidistant from P_i , yielding the equilibrium P_i magnetization (M_0), both with a saturation pulse length of 10 s. The apparent longitudinal relaxation time of P_i (T_1) was determined by performing an eight-point inversion recovery experiment with an adiabatic full passage pulse for inversion and with γ -ATP saturation before (10 s) and during the inversion delay (inversion times = 0.01, 1, 2, 4, 6.5, 9.5, 13, and 17 s, 32 averages). The

total duration of the ST and inversion recovery experiments was about 2 h.

After the ST experiments, time series of ^{31}P MR spectra ($\text{TR} = 5$ s, 4 averages) before, during, and after muscle contractions were acquired. Muscle contractions were induced by electrical stimulation of the TA via subcutaneously implanted electrodes positioned along the distal N. Peroneus Communis (11). The stimulation protocol consisted of a series of stimulation pulses, applied every second, for a duration of 2 min. Stimulation pulse length was 100 ms, frequency was 80 Hz, and stimulation voltage varied between 2.5 and 4 V. Recovery was followed for 15 min. Three to four time series were measured for each rat.

^{31}P MR spectra were fit in the time domain by using an advanced magnetic resonance (AMARES) nonlinear least squares algorithm in the jMRUI software package (jMRUI V2.1). PCr and P_i peaks were fit to Lorentzian line shapes, whereas γ -, α -, and β -ATP signals were fit with Gaussian line shapes. Besides the cytosolic P_i signal, a second, smaller P_i peak was observed in the spectra at a frequency ~ 0.3 ppm downfield from the cytosolic P_i resonance. The two P_i signals were separately fitted, and the line widths were constrained with respect to the line width of the PCr signal. For the dynamic MRS spectra, the two P_i signals could not be distinguished, and a single P_i peak was fit to a Gaussian line shape. Concentrations of PCr and P_i were determined relative to the ATP concentration, which was determined in vitro from freeze clamped tissue as described below. ADP concentrations were calculated using the creatine kinase equilibrium (29), and intracellular pH was calculated from the chemical shift difference between PCr and the cytosolic P_i resonance (58).

The T_1 relaxation time of P_i was determined by fitting the inversion recovery data with a three-parameter monoexponential function using Origin (OriginPro 7.5 SR0, OriginLab, Northampton, MA). The V_{ATP} exchange rate constant ($k_{\text{P}_i \rightarrow \text{ATP}}$) was calculated from the T_1 of P_i and the fractional reduction of P_i magnetization upon selective saturation of γ -ATP according to: $k_{\text{P}_i \rightarrow \text{ATP}} = (1 - M'/M_0)/T_1$. V_{ATP} was then calculated by multiplying $k_{\text{P}_i \rightarrow \text{ATP}}$ with the P_i concentration at rest, as determined from the fully relaxed spectrum. The intrinsic T_1 of P_i was calculated as $T_1 = 1/(1/T_1' - k_{\text{P}_i \rightarrow \text{ATP}})$. The data of PCr recovery were fit to a monoexponential function using Matlab (version 7.04, Mathworks, Natick, MA) yielding a rate constant k_{PCr} . Results from two time series with end-stimulation pH values higher than 6.92 (63) were averaged.

TA muscle cross-sectional area in the midbelly region of the muscle was determined from the MR images using Mathematica (version 7.0, Wolfram Research, Champaign, IL).

Determination of ATP concentration. Freeze-clamped TA muscle was powdered in liquid nitrogen, mixed with 3.25 ml/g perchloric acid (6% wt/vol), and homogenized for 30 s using an Ultra-Turrax high-performance disperser (IKA Werke, Staufen, Germany). Next, the homogenate was neutralized with 5 M K_2CO_3 to pH 7.4 and centrifuged at 3,000 g for 10 min at 4°C . ATP concentration in the supernatant was determined spectrophotometrically using the hexokinase/glucose-6-phosphate dehydrogenase coupled assay as described in detail in Ref. 60.

Isolation of mitochondria. Mitochondria were isolated from one whole TA muscle through a differential centrifugation procedure as previously described (62). Briefly, TA muscle was excised, washed in ice-cold 0.9% KCl, freed of connective and adipose tissue, weighed, and minced with scissors in ice-cold medium A (5 ml for 1 g tissue) containing 150 mM sucrose, 75 mM KCl, 50 mM MOPS, 1 mM KH_2PO_4 , 5 mM MgCl_2 , 1 mM EGTA, and 0.4 mg/ml bacterial proteinase type XXIV, pH 7.4. Next, 20 ml of medium B containing 250 mM sucrose, 0.1 mM EGTA, and 20 mM MOPS, 2 mg/ml BSA, pH 7.4 were added, and the mixture was homogenized using Potter-Elvehjem homogenizer. The homogenate was centrifuged at 800 g for 10 min, 4°C . The resulting supernatant was centrifuged at 10,000 g for 10 min, 4°C . The pellet was resuspended in 15 ml of fresh ice-cold medium B and centrifuged at 10,000 g for 10 min, 4°C . Mitochondrial

pellet was resuspended in 100 μ l of medium B. Protein content was determined using BCA protein assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL).

Measurement of oxygen consumption. Oxygen consumption rate was measured at 37°C using a two-channel high-resolution Oroboros oxygraph-2 k (Oroboros, Innsbruck, Austria). Maximal ADP-stimulated (state 3) oxygen consumption rate was determined in the assay medium containing 110 mM KCl, 20 mM Tris, 2.3 mM MgCl₂, 5 mM KH₂PO₄, 50 mM creatine, 4.4 U/ml creatine phosphokinase, 1 mM ATP, and 1 mg/ml BSA, pH 7.3. All measurements were performed in 1 ml of assay medium containing 0.15 mg/ml of mitochondrial protein. To assess NADH- (through complex I) and FADH₂-driven (through complex II) oxygen consumption, 5 mM pyruvate plus 5 mM malate and 5 mM succinate plus 1 μ M rotenone were used, respectively. Oxygen consumption in resting state (state 4) was determined after addition of 1.25 μ M carboxyatractyloside. The signals from the oxygen electrode were recorded at 0.5-s intervals. Data acquisition and analysis were performed using Oxygraph-2k-DatLab software version 4.2 (Oroboros).

Determination of the relative mitochondrial copy number. Genomic DNA was isolated from a 25-mg transversal slice of mid-belly TA using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Zwijndrecht, The Netherlands). mtDNA content relative to peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) gene was measured using real-time PCR as described in Ref. 7. Primers for mtDNA were the following: forward primer, 5'-ACACAAAAGGACGAACCTG-3'; reverse primer, 5'-ATGGGGAAGAAGCCCTAGAA-3'; and for PGC-1 α : forward primer, 5'-ATGAATGCAGCGGCTCTAGC-3'; reverse primer, 5'-AA-CAATGGCAGGGTTTGTTC-3'. The relative mtDNA copy number was calculated using $\Delta\Delta$ Ct method as described in Ref. 57.

Statistical analysis. Data are presented as means $\pm$ SD. The listed n values represent the number of animals used for a particular experiment. Statistical significance of the differences was assessed using unpaired Student's t -tests in SPSS 16.0 statistical package (SPSS, Chicago, IL). Level of statistical significance was set at $P < 0.05$.

RESULTS

Animal model. Daily DPI treatment resulted in a decreased food intake (22.1 ± 1.0 and 10.8 ± 2.9 g/day for control and DPI-treated rats, respectively; $P < 0.001$) and a lower body weight (BW) at the end of the DPI treatment (375 ± 24 and 330 ± 21 g for control and DPI-treated rats, respectively; $P = 0.001$). In parallel with the 12% lower BW, TA muscle cross-sectional area was 10% smaller in DPI-treated rats compared with controls at the end of the treatment (0.43 ± 0.03 and 0.38 ± 0.03 cm² for control and DPI-treated rats, respectively; $P = 0.01$).

Mitochondrial characteristics in vitro. To assess the effect of DPI on the intrinsic in vitro mitochondrial function we determined oxygen consumption rates in mitochondria isolated from the TA muscle (results are summarized in Table 1).

Table 2. Metabolite concentrations and pH in tibialis anterior measured by in vivo ³¹P magnetic resonance spectroscopy

	Rest		End Stimulation	
	Control	DPI	Control	DPI
pH	7.19 $\pm$ 0.02	7.21 $\pm$ 0.02	7.07 $\pm$ 0.06	7.01 $\pm$ 0.06
[PCr], mM	47.5 $\pm$ 2.8	48.8 $\pm$ 2.9	22.8 $\pm$ 2.7	20.9 $\pm$ 1.2
[P _i], mM	3.0 $\pm$ 0.4	3.0 $\pm$ 0.8	28.6 $\pm$ 3.5	31.9 $\pm$ 2.8
[ADP], μ M	13.4 $\pm$ 0.6	13.9 $\pm$ 0.4	84.8 $\pm$ 10.2	89.5 $\pm$ 16.7

Data are from $n = 8$ animals for each group and are expressed as means $\pm$ SD. PCr, phosphocreatine; P_i, inorganic phosphate.

Maximal ADP-stimulated (state 3) oxygen consumption rate in the isolated mitochondria oxidizing pyruvate plus malate was 20% lower ($P < 0.001$) in the DPI-treated group compared with controls. The effect of DPI treatment on succinate oxidation was less pronounced; oxygen consumption rate in state 3 was 12% lower ($P = 0.03$) compared with controls. For both substrates oxygen consumption rates in the absence of ATP synthesis (state 4) were similar in DPI-treated and control groups.

The effect of chronic inhibition of intrinsic mitochondrial function by DPI on mitochondrial biogenesis was assessed by determining mtDNA copy number in TA muscle. The relative mtDNA copy number was not significantly different in the TA muscle of control ($2,200 \pm 323$) and DPI-treated ($2,190 \pm 369$) rats.

Muscle ATP concentration. ATP concentrations in TA muscle were 12.5 ± 3.2 mM for control rats ($n = 5$) and 11.0 ± 2.5 mM for DPI-treated rats ($n = 7$) and were not significantly different ($P = 0.4$). Because DPI treatment had no significant effect on the muscle ATP concentration, the average value for both groups combined (11.6 ± 2.8 mM) was taken as a reference for the in vivo MRS data analysis.

In vivo ³¹P MRS. Concentrations of PCr, P_i, and ADP and the intracellular pH obtained from the ³¹P MR spectra recorded in resting TA muscle are summarized in Table 2. DPI treatment had no significant effect on any of these parameters.

Figure 1 shows a representative example of a ³¹P ST spectrum measured in resting TA muscle with frequency-selective saturation of the γ -ATP peak (black) and the corresponding reference spectrum with saturation at a downfield frequency equidistant from P_i (grey). From the ³¹P ST spectra, the ratio of P_i magnetization with and without saturation of γ -ATP (M'/M_0) was determined, and this ratio was not significantly different between the groups (Table 3). The apparent T₁ (T'_1) of P_i in case of saturation of γ -ATP was determined from an eight-point inversion recovery experiment, and a representative example of P_i inversion recovery data and the corresponding

Table 1. Oxygen consumption rates in isolated tibialis anterior muscle mitochondria oxidizing different substrates in different metabolic states

	Pyruvate Plus Malate		Succinate Plus Rotenone	
	Control	DPI	Control	DPI
State 3, nmol O ₂ ·min ⁻¹ ·mg protein ⁻¹	407.6 $\pm$ 21.4	326.0 $\pm$ 38.4*	349.1 $\pm$ 27.9	307.8 $\pm$ 36.8*
State 4, nmol O ₂ ·min ⁻¹ ·mg protein ⁻¹	18.7 $\pm$ 3.2	19.8 $\pm$ 4.3	54.7 $\pm$ 11.8	52.3 $\pm$ 8.4

Data are from $n = 7$ control and $n = 8$ diphenylethionium (DPI)-treated animals and are expressed as means $\pm$ SD. State 3, maximal ADP-stimulated oxygen consumption; state 4, oxygen consumption in the absence of ATP synthesis; * $P < 0.05$ when compared with control group.

Fig. 1. Example of ^{31}P saturation transfer spectra (saturation pulse length = 10 s, TR = 10.4 s, 128 averages) with saturation of γ -ATP (black) and with saturation at a downfield frequency, equidistant from P_i (grey), measured in tibialis anterior (TA) muscle of a diphenyleiodonium (DPI)-treated rat. PCr, phosphocreatine.

mono-exponential fit is shown in Fig. 2. T_1' values of P_i were similar for control and DPI-treated animals (Table 3). The exchange rate constant $k_{\text{P}_i \rightarrow \text{ATP}}$ determined from T_1' and M'/M_0 , V_{ATP} determined from $k_{\text{P}_i \rightarrow \text{ATP}}$ and the P_i concentration, and the intrinsic T_1 of P_i were also not significantly different between control and DPI-treated animals (Table 3 and Fig. 3A).

Dynamic ^{31}P MRS measurements during and after recovery from electrical stimulation of the TA were performed to determine the rate of PCr recovery after stimulation. Concentrations of PCr, P_i , and ADP and the intracellular pH at the end of the stimulation were not significantly different between groups (Table 2). PCr concentrations during recovery were fitted with a monoexponential function (Fig. 4) yielding k_{PCr} . k_{PCr} was 46% lower in DPI-treated animals compared with control rats (Fig. 3B, $P < 0.001$). There was no relationship between V_{ATP} and k_{PCr} ($R^2 = 0.15$, $P = 0.15$).

DISCUSSION

In this study, we compared in vivo ^{31}P MRS postexercise PCr recovery and resting ATP synthesis flux in a rat model of

Table 3. Parameters determined from ^{31}P saturation transfer magnetic resonance spectroscopy

	Control	DPI
M'/M_0	0.73 ± 0.06	0.67 ± 0.08
T_1' , s	3.87 ± 0.37	3.76 ± 0.79
T_1 , s	5.38 ± 0.79	5.69 ± 1.33
$k_{\text{P}_i \rightarrow \text{ATP}}$, s^{-1}	0.07 ± 0.02	0.09 ± 0.04
V_{ATP} , $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$	8.33 ± 1.53	10.45 ± 2.82

Data are from $n = 7$ control and $n = 8$ DPI-treated animals and are expressed as means $\pm$ SD. M' , magnetization of P_i with saturation of γ -ATP; M_0 , magnetization of P_i with saturation at a downfield frequency, equidistant from P_i ; T_1' , apparent longitudinal relaxation time of P_i with saturation of γ -ATP; T_1 , intrinsic longitudinal relaxation time of P_i ; $k_{\text{P}_i \rightarrow \text{ATP}}$, $\text{P}_i \rightarrow \text{ATP}$ exchange rate constant; V_{ATP} , resting ATP synthesis flux.

Fig. 2. P_i amplitudes obtained from an inversion recovery experiment with inversion times of 0.01, 1, 2, 4, 6.5, 9.5, 13, and 17 s and with saturation of the γ -ATP peak. A mono-exponential three-parameter function (dark line) was fit to the actual data (filled circles) to determine the apparent longitudinal relaxation time (T_1'), which was 3.75 s for this example.

mitochondrial dysfunction to establish the most appropriate method for assessment of in vivo skeletal muscle mitochondrial function. Two weeks of treatment with the complex I inhibitor DPI induced mitochondrial dysfunction, as evidenced by a 20% lower oxygen consumption rate in isolated mitochondria from DPI-treated rats oxidizing pyruvate plus malate. This was paralleled by a 46% decrease in in vivo oxidative capacity, determined from postexercise PCr recovery. Interestingly, no significant difference in resting ST-based ATP synthesis flux was observed between DPI-treated rats and controls.

DPI is a nonspecific irreversible inhibitor of flavoenzymes, which causes covalent phenylation of either the flavin or the adjacent amino acid and heme groups of the proteins due to reduction of DPI to its diphenyleneiodonyl radical form during electron transport through the flavin moieties of the enzymes (35). The effects of DPI on mitochondrial function have been studied extensively in vitro (14, 20, 44). The acute inhibitory effect of DPI in submitochondrial particles and isolated mitochondria prepared from different rat tissues is primarily related to irreversible inhibition of the respiratory chain complex I, which contains flavin adenine dinucleotide as the enzyme cofactor (14, 44). It has been shown that DPI also decreases succinate state 3 oxidation in isolated rat skeletal muscle

Fig. 3. A: resting ATP synthesis flux (V_{ATP} ; data are from $n = 7$ control and $n = 8$ DPI-treated animals) measured by ^{31}P saturation transfer magnetic resonance spectroscopy (MRS); B: PCr recovery rate constant (k_{PCr} ; $n = 8$ for both groups) measured with dynamic ^{31}P MRS after muscle stimulation. Open bars, control animals; filled bars, DPI-treated rats. * $P < 0.05$ compared with controls.

Fig. 4. Representative examples of relative PCr concentrations during rest, muscle stimulation, and recovery (time resolution = 20 s) for a control animal (open symbols) and a DPI-treated rat (filled symbols). PCr concentrations are expressed as a percentage of the resting PCr concentration (left y-axis, control rat; right y-axis, DPI-treated rat). Monoexponential functions (dark lines) were fit to the recovery data, and the PCr recovery rate constants were 0.61 and 0.31 min^{-1} for the control and DPI-treated animal, respectively.

mitochondria, suggesting additional inhibition of respiratory chain complex II (15). However, the observation that this inhibition is dependent on the chloride concentration in the assay medium indicates a different potency or mechanism of action of DPI on complex II (19), which may be related to the fact that complex I and II contain a different flavin cofactor (i.e., complex II contains flavin mononucleotide). Furthermore, it has been shown that the transport of respiratory substrates into the mitochondrial matrix in isolated rat liver mitochondria is not affected by DPI (13, 20).

One may expect that the irreversible modifications of the respiratory chain complexes I and II observed *in vitro* also occur *in vivo* in animals chronically treated with DPI. Indeed, it has been shown that state 3 oxygen consumption, driven by oxidation of various NADH-delivering substrates, was significantly decreased in mitochondria isolated from gastrocnemius and soleus muscles of rats after 14 days of daily DPI treatment compared with healthy controls (9). This effect could be fully explained by specific inhibition of complex I activity. In agreement, we found that DPI treatment of similar duration as in Ref. 9 results in a significant and comparable decrease in pyruvate plus malate-driven oxygen consumption in state 3 in mitochondria isolated from TA muscle. In addition, we observed a small (12%) but significant decrease in the succinate-driven oxygen consumption in state 3. The available published data on the effects of *in vivo* DPI treatment on succinate-driven state 3 oxygen consumption in isolated skeletal muscle mitochondria are scarce and contradictory. Cooper et al. (9) found no significant impairment of succinate-driven state 3 oxygen consumption in isolated skeletal muscle mitochondria, whereas Hayes et al. (15) reported an 80% decrease in state 3 succinate oxidation in skeletal muscle mitochondria isolated from DPI-treated animals compared with controls. The different results are likely due to differences in the concentration of chloride in the assay medium, which affects the potency of DPI to inhibit succinate-driven state 3 oxygen consumption (see above) (19). Because of the low intracellular chloride concentration, the most important mode of action of DPI *in vivo* is probably inhibition of complex I.

In the present study, mtDNA copy number in TA muscle was similar for DPI-treated and control rats, indicating that

DPI treatment did not affect mitochondrial biogenesis. This is in agreement with Cooper et al. (9) who showed similar activities of citrate synthase and other TCA cycle enzymes in the muscle of DPI-treated animals compared with healthy controls. The observation that DPI treatment does not cause compensatory stimulation of mitochondrial biogenesis simplifies the interpretation of the *in vivo* determined PCr recovery rate constant k_{PCr} . It implies that the decrease in k_{PCr} in TA muscle of DPI-treated animals results from impaired intrinsic mitochondrial function rather than decreased mitochondrial number. However, the discrepancy in the magnitude of the effect of DPI observed on the *in vivo* determined k_{PCr} (46% decrease) and the *in vitro* mitochondrial function (20% decrease of state 3 oxygen consumption with pyruvate plus malate and 12% decrease of state 3 oxygen consumption with succinate plus malate) suggests that other factors than inhibition of the respiratory chain complex I and complex II may additionally affect PCr recovery *in vivo*. For example, inhibition of the creatine kinase reaction by DPI could slow down the recovery of PCr *in vivo*. However, from our ^{31}P ST data no such inhibitory effect was observed for the PCr→ATP flux (data not shown), which is in agreement with observations in a previous study (15). Alternatively, a lowered muscle tissue perfusion could be responsible for a slower PCr recovery by reducing the availability of oxygen and substrate. DPI has been shown to inhibit endothelial nitric oxide synthase (eNOS), an enzyme involved in vascular vasodilation (54). Inhibition of eNOS lowers muscle blood flow during recovery from exercise in humans (8, 47) and therefore we cannot exclude that a lower muscle perfusion in DPI-treated animals may have affected k_{PCr} .

In contrast to the oxygen consumption in isolated mitochondria and the *in vivo* PCr recovery, resting ST-based ATP synthesis flux V_{ATP} was not affected by DPI treatment. Mitochondrial ATP synthesis is a demand-driven process regulated by several error signals. Most investigated is the feedback control loop involving changes in the extramitochondrial concentrations of the ATP hydrolysis products ADP (23, 25, 66) and, to a lesser extent, P_i (66). To interpret ATP synthesis flux data in terms of mitochondrial function, it is essential to take the error signals; i.e., concentrations of ADP and P_i , into account (26). A lower ATP synthesis flux in combination with low error signals simply represents a lower ATP demand. On the other hand, a lower ATP synthesis flux in combination with normal or high error signals would indeed imply mitochondrial dysfunction or a decrease in the density of mitochondria. In our study, DPI-treated rats had both normal resting ATP synthesis flux and a normal resting P_i concentration, whereas the ADP concentration was slightly, but not significantly, elevated with respect to controls. These results imply that the resting ATP demand was not affected by treatment with DPI. The slightly increased ADP level in DPI-treated rats suggests that a higher error signal was needed to maintain a normal ATP synthesis flux. Alternatively, another, yet unidentified, error signal might have been increased in DPI-treated rats to match the normal ATP demand.

Another factor that complicates the interpretation of ^{31}P MRS ST data is that the V_{ATP} obtained from ^{31}P MRS ST measurements is composed of both mitochondrial F_1F_0 -ATP synthase flux and flux through other P_i →ATP pathways, in particular the reactions catalyzed by the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and

phosphoglycerate kinase (PGK). Although the net glycolytic contribution to the production of ATP (via GAPDH and PGK) versus that of oxidative phosphorylation is small (22), these enzymes catalyze a coupled near-equilibrium reaction, and consequently, exchange between P_i and ATP may greatly exceed the net glycolytic flux (4). Experimental proof for glycolytic exchange between P_i and ATP came from ^{31}P MRS ST experiments in yeast. $P_i \rightarrow \text{ATP}$ exchange was inhibited in yeast by iodoacetate, an irreversible inhibitor of GAPDH (6), whereas in anaerobic yeast cells overexpressing PGK, $P_i \rightarrow \text{ATP}$ exchange was increased (2). ^{31}P MRS ST experiments performed on perfused rat hearts showed that the glycolytic exchange reaction contributes significantly to the measured V_{ATP} and that only when glycolytic activity was inhibited, the measured V_{ATP} displayed a linear dependence on oxygen consumption and thus represents mitochondrial F_1F_0 -ATP synthase flux (27). For rat skeletal muscle, values for resting ATP turnover from ^{31}P MRS ST experiments are about five times higher compared with figures derived from oxygen consumption rates, whereas in human muscle the difference is even larger (reviewed in Ref. 26). This indicates that the contribution of glycolytic exchange flux to the measured V_{ATP} from ^{31}P MRS ST experiments in resting skeletal muscle can be as much as 80%. Up to this point, it has been assumed that the mitochondrial F_1F_0 -ATP synthase flux, which constitutes only a small part of the total V_{ATP} measured, is unidirectional. However, it has been shown that at low rates of respiration (e.g., resting muscle) the F_1F_0 -ATP synthase reaction is near to equilibrium and that mitochondrial exchange between P_i and ATP can make a significant contribution to the measured V_{ATP} (50). Taken together, if there would have been an effect of DPI on the rate of net resting mitochondrial ATP production, this effect might not be measurable due to the large contribution of glycolytic exchange flux and possibly also a significant contribution of mitochondrial exchange flux to the total measured V_{ATP} . To create better conditions for detecting a defect in skeletal muscle mitochondrial oxidative phosphorylation, ^{31}P MRS ST experiments would need to be performed during steady-state muscle contraction, when the F_1F_0 -ATP synthase reaction is largely unidirectional and the flux through the F_1F_0 -ATP synthase is much larger than the glycolytic exchange flux (3). However, it is very challenging to maintain steady-state muscle contractions during the lengthy ^{31}P MRS ST experiments, and this might in particular be a problem in patient groups. Moreover, in such an experiment it is crucial to achieve similar relative workloads in different subjects or animals.

Most ^{31}P MRS ST studies investigating the role of skeletal muscle mitochondrial function in the development of IR and T2D lack a proper discussion about the error signals; i.e., the concentrations of P_i and ADP. The lower V_{ATP} in IR subjects and animal models and patients with T2D (28, 38–40, 56, 67) might be explained by a lower P_i concentration (39), caused by reduced sarcolemmal P_i uptake in insulin-resistant muscle (41), rather than a decrease in the $P_i \rightarrow \text{ATP}$ exchange rate constant $k_{P_i \rightarrow \text{ATP}}$. Furthermore, it has been shown that hyperinsulinemia decreases the intracellular pH (59), resulting in a lower ADP concentration and therefore a lower error signal for oxidative ATP synthesis. In addition, all ^{31}P MRS ST studies investigating the role of skeletal muscle mitochondrial function in the development of IR and T2D were performed in resting skeletal

muscle (28, 38–40, 56, 61, 67). As was explained in the previous paragraph, in resting skeletal muscle the contribution of F_1F_0 -ATP synthesis flux to the total measured V_{ATP} will be relatively low, which further complicates the interpretation of these measurements. In a recent study, measurements of both PCr recovery and resting ATP synthesis flux were performed in T2D patients and healthy controls (61). The half-times of PCr recovery were not different between groups and also resting ATP synthesis flux was not impaired in the patients with T2D compared with controls, whereas concentrations of P_i and ADP were similar for both groups. In addition, half-times of PCr recovery were not significantly correlated with resting ATP synthesis fluxes, which is in agreement with the results of the present study. It should be noted that the animal model that we used in this study is a model of mitochondrial dysfunction but that it does not represent a model for IR of T2D. On the contrary, it has been shown that DPI can induce hypoglycemia (19, 20).

In conclusion, the rate constant of PCr recovery measured with dynamic ^{31}P MRS after exercise provides a more sensitive measure of skeletal muscle mitochondrial function than the ATP synthesis flux determined with ^{31}P ST in the resting state. The ATP synthesis flux itself represents the ATP demand of the muscle, and to interpret the data in terms of mitochondrial function it is necessary to take the error signals; i.e., the concentrations of ADP and P_i , into account. Moreover, the V_{ATP} obtained from a ^{31}P ST experiment in the resting state is dominated by glycolytic exchange flux. To detect a defect in mitochondrial oxidative phosphorylation the latter experiment would need to be done in exercising muscle.

ACKNOWLEDGMENTS

The authors thank Joep van Lier for support in fitting the ^{31}P MR spectra and Dr. Jeroen Jeneson for stimulating discussions.

GRANTS

The work of J.Ciapaite is financed by the Netherlands Consortium for Systems Biology (NCSB), which is part of the Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research. J. J. Prompers is supported by a VIDI grant from the Netherlands Organisation for Scientific Research (VIDI Grant Number 700.58.421).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. Brehm A, Krssak M, Schmid AI, Nowotny P, Waldhausl W, Roden M. Increased lipid availability impairs insulin-stimulated ATP synthesis in human skeletal muscle. *Diabetes* 55: 136–140, 2006.
2. Brindle KM. ^{31}P NMR magnetization-transfer measurements of flux between inorganic phosphate and adenosine 5'-triphosphate in yeast cells genetically modified to overproduce phosphoglycerate kinase. *Biochemistry* 27: 6187–6196, 1988.
3. Brindle KM, Blackledge MJ, Challiss RA, Radda GK. ^{31}P NMR magnetization-transfer measurements of ATP turnover during steady-state isometric muscle contraction in the rat hind limb in vivo. *Biochemistry* 28: 4887–4893, 1989.
4. Brindle KM, Radda GK. ^{31}P -NMR saturation transfer measurements of exchange between P_i and ATP in the reactions catalysed by glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase in vitro. *Biochim Biophys Acta* 928: 45–55, 1987.
5. Brown TR, Ugurbil K, Shulman RG. ^{31}P nuclear magnetic resonance measurements of ATPase kinetics in aerobic *Escherichia coli* cells. *Proc Natl Acad Sci USA* 74: 5551–5553, 1977.

6. Campbell-Burk SL, Jones KA, Shulman RG. ³¹P NMR saturation-transfer measurements in *Saccharomyces cerevisiae*: characterization of phosphate exchange reactions by iodoacetate and antimycin A inhibition. *Biochemistry* 26: 7483–7492, 1987.
7. Ciapaite J, Bakker SJ, Van Eikenhorst G, Wagner MJ, Teerlink T, Schalkwijk CG, Fodor M, Ouwens DM, Diamant M, Heine RJ, Westerhoff HV, Krab K. Functioning of oxidative phosphorylation in liver mitochondria of high-fat diet fed rats. *Biochim Biophys Acta* 1772: 307–316, 2007.
8. Clifford PS, Hellsten Y. Vasodilatory mechanisms in contracting skeletal muscle. *J Appl Physiol* 97: 393–403, 2004.
9. Cooper J, Petty R, Hayes D, Morgan-Hughes J, Clark J. Chronic administration of the oral hypoglycaemic agent diphenyleneiodonium to rats. An animal model of impaired oxidative phosphorylation (mitochondrial myopathy). *Biochem Pharmacol* 37: 687–694, 1988.
10. Cooper JM, Petty RK, Hayes DJ, Challiss RA, Brosnan MJ, Shoubridge EA, Radda GK, Morgan-Hughes JA, Clark JB. An animal model of mitochondrial myopathy: a biochemical and physiological investigation of rats treated in vivo with the NADH-CoQ reductase inhibitor, diphenyleneiodonium. *J Neurol Sci* 83: 335–347, 1988.
11. De Feyter HM, Lenaers E, Houten SM, Schrauwen P, Hesselink MK, Wanders RJ, Nicolay K, Prompers JJ. Increased intramyocellular lipid content but normal skeletal muscle mitochondrial oxidative capacity throughout the pathogenesis of type 2 diabetes. *FASEB J* 22: 3947–3955, 2008.
12. De Feyter HM, van den Broek NM, Praet SF, Nicolay K, van Loon LJ, Prompers JJ. Early or advanced stage type 2 diabetes is not accompanied by in vivo skeletal muscle mitochondrial dysfunction. *Eur J Endocrinol* 158: 643–653, 2008.
13. Gatley SJ, Sherratt HS. The effects of diphenyleneiodonium and of 2,4-dichlorodiphenyleneiodonium on mitochondrial reactions. Mechanism of the inhibition of oxygen uptake as a consequence of the catalysis of the chloride/hydroxyl-ion exchange. *Biochem J* 158: 317–326, 1976.
14. Gatley SJ, Sherratt SA. The effects of diphenyleneiodonium on mitochondrial reactions. Relation of binding of diphenylene[¹²⁵I]iodonium to mitochondria to the extent of inhibition of oxygen uptake. *Biochem J* 158: 307–315, 1976.
15. Hayes DJ, Byrne E, Shoubridge EA, Morgan-Hughes JA, Clark JB. Experimentally induced defects of mitochondrial metabolism in rat skeletal muscle. Biological effects of the NADH: coenzyme Q reductase inhibitor diphenyleneiodonium. *Biochem J* 229: 109–117, 1985.
16. He J, Watkins S, Kelley DE. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* 50: 817–823, 2001.
17. Heilbronn LK, Gan SK, Turner N, Campbell LV, Chisholm DJ. Markers of mitochondrial biogenesis and metabolism are lower in overweight and obese insulin-resistant subjects. *J Clin Endocrinol Metab* 92: 1467–1473, 2007.
18. Hojlund K, Wrzesinski K, Larsen PM, Fey SJ, Roepstorff P, Handberg A, Dela F, Vinten J, McCormack JG, Reynet C, Beck-Nielsen H. Proteome analysis reveals phosphorylation of ATP synthase beta-subunit in human skeletal muscle and proteins with potential roles in type 2 diabetes. *J Biol Chem* 278: 10436–10442, 2003.
19. Holland PC, Clark MG, Bloxham DP, Lardy HA. Mechanism of action of the hypoglycemic agent diphenyleneiodonium. *J Biol Chem* 248: 6050–6056, 1973.
20. Holland PC, Sherratt HS. Biochemical effects of the hypoglycaemic compound diphenyleneiodonium. Catalysis of anion-hydroxyl ion exchange across the inner membrane of rat liver mitochondria and effects on oxygen uptake. *Biochem J* 129: 39–54, 1972.
21. Holloszy JO. Skeletal muscle “mitochondrial deficiency” does not mediate insulin resistance. *Am J Clin Nutr* 89: 463S–466S, 2009.
22. Hood DA, Gorski J, Terjung RL. Oxygen cost of twitch and tetanic isometric contractions of rat skeletal muscle. *Am J Physiol Endocrinol Metab* 250: E449–E456, 1986.
23. Jeneson JA, Schmitz JP, van den Broek NM, van Riel NA, Hilbers PA, Nicolay K, Prompers JJ. Magnitude and control of mitochondrial sensitivity to ADP. *Am J Physiol Endocrinol Metab* 297: E774–E784, 2009.
24. Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51: 2944–2950, 2002.
25. Kemp GJ. Interactions of mitochondrial ATP synthesis and the creatine kinase equilibrium in skeletal muscle. *J Theor Biol* 170: 239–246, 1994.
26. Kemp GJ. The interpretation of abnormal ³¹P magnetic resonance saturation transfer measurements of Pi/ATP exchange in insulin-resistant skeletal muscle. *Am J Physiol Endocrinol Metab* 294: E640–E642, 2008.
27. Kingsley-Hickman PB, Sako EY, Mohanakrishnan P, Robitaille PM, From AH, Foker JE, Ugurbil K. ³¹P NMR studies of ATP synthesis and hydrolysis kinetics in the intact myocardium. *Biochemistry* 26: 7501–7510, 1987.
28. Laurent D, Yerby B, Deacon R, Gao J. Diet-induced modulation of mitochondrial activity in rat muscle. *Am J Physiol Endocrinol Metab* 293: E1169–E1177, 2007.
29. Lawson JW, Veech RL. Effects of pH and free Mg²⁺ on the K_{eq} of the creatine kinase reaction and other phosphate hydrolyses and phosphate transfer reactions. *J Biol Chem* 254: 6528–6537, 1979.
30. Majander A, Finel M, Wikstrom M. Diphenyleneiodonium inhibits reduction of iron-sulfur clusters in the mitochondrial NADH-ubiquinone oxidoreductase (Complex I). *J Biol Chem* 269: 21037–21042, 1994.
31. Mogensen M, Sahlin K, Fernstrom M, Glinborg D, Vind BF, Beck-Nielsen H, Hojlund K. Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. *Diabetes* 56: 1592–1599, 2007.
32. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC. PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34: 267–273, 2003.
33. Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, Neschen S, White MF, Bilz S, Sono S, Pypaert M, Shulman GI. Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest* 115: 3587–3593, 2005.
34. Morino K, Petersen KF, Shulman GI. Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. *Diabetes* 55, Suppl 2: S9–S15, 2006.
35. O'Donnell VB, Smith GC, Jones OT. Involvement of phenyl radicals in idonium inhibition of flavoenzymes. *Mol Pharmacol* 46: 778–785, 1994.
36. Palladino GW, Wood JJ, Proctor HJ. Modified freeze clamp technique for tissue assay. *J Surg Res* 28: 188–190, 1980.
37. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ. 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, 2003.
38. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman GI. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* 300: 1140–1142, 2003.
39. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350: 664–671, 2004.
40. Petersen KF, Dufour S, Shulman GI. Decreased insulin-stimulated ATP synthesis and phosphate transport in muscle of insulin-resistant offspring of type 2 diabetic parents. *PLoS Med* 2: e233, 2005.
41. Polgreen KE, Kemp GJ, Leighton B, Radda GK. Modulation of Pi transport in skeletal muscle by insulin and IGF-1. *Biochim Biophys Acta* 1223: 279–284, 1994.
42. Quistorff B, Johansen L, Sahlin K. Absence of phosphocreatine resynthesis in human calf muscle during ischaemic recovery. *Biochem J* 291: 681–686, 1993.
43. Rabol R, Boushel R, Dela F. Mitochondrial oxidative function and type 2 diabetes. *Appl Physiol Nutr Metab* 31: 675–683, 2006.
44. Ragan CI, Bloxham DP. Specific labelling of a constituent polypeptide of bovine heart mitochondrial reduced nicotinamide-adenine dinucleotide-ubiquinone reductase by the inhibitor diphenyleneiodonium. *Biochem J* 163: 605–615, 1977.
45. Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE. Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. *Diabetes* 54: 8–14, 2005.
46. Sahlin K. Intracellular pH and energy metabolism in skeletal muscle of man. With special reference to exercise. *Acta Physiol Scand Suppl* 455: 1–56, 1978.
47. Saltin B, Radegran G, Koskolou MD, Roach RC. Skeletal muscle blood flow in humans and its regulation during exercise. *Acta Physiol Scand* 162: 421–436, 1998.

48. Schrauwen-Hinderling VB, Kooi ME, Hesselink MK, Jeneson JA, Backes WH, van Echteld CJ, van Engelshoven JM, Mensink M, Schrauwen P. Impaired in vivo mitochondrial function but similar intramyocellular lipid content in patients with type 2 diabetes mellitus and BMI-matched control subjects. *Diabetologia* 50: 113–120, 2007.
49. Schrauwen-Hinderling VB, Roden M, Kooi ME, Hesselink MK, Schrauwen P. Muscular mitochondrial dysfunction and type 2 diabetes mellitus. *Curr Opin Clin Nutr Metab Care* 10: 698–703, 2007.
50. Sheldon JG, Williams SP, Fulton AM, Brindle KM. 31P NMR magnetization transfer study of the control of ATP turnover in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 93: 6399–6404, 1996.
51. Short KR, Nair KS, Stump CS. Impaired mitochondrial activity and insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350: 2419–2421; author reply 2419–2421, 2004.
52. Simoneau JA, Kelley DE. Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. *J Appl Physiol* 83: 166–171, 1997.
53. Sreekumar R, Nair KS. Skeletal muscle mitochondrial dysfunction & diabetes. *Indian J Med Res* 125: 399–410, 2007.
54. Stuehr DJ, Fasehun OA, Kwon NS, Gross SS, Gonzalez JA, Levi R, Nathan CF. Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyliodonium and its analogs. *FASEB J* 5: 98–103, 1991.
55. Szendroedi J, Roden M. Mitochondrial fitness and insulin sensitivity in humans. *Diabetologia* 51: 2155–2167, 2008.
56. Szendroedi J, Schmid AI, Chmelik M, Toth C, Brehm A, Krssak M, Nowotny P, Wolzt M, Waldhausl W, Roden M. Muscle mitochondrial ATP synthesis and glucose transport/phosphorylation in type 2 diabetes. *PLoS Med* 4: e154, 2007.
57. Szuhai K, Ouweland J, Dirks R, Lemaitre M, Truffert J, Janssen G, Tanke H, Holme E, Maassen J, Raap A. Simultaneous A8344G heteroplasmy and mitochondrial DNA copy number quantification in myoclonus epilepsy and ragged-red fibers (MERRF) syndrome by a multiplex molecular beacon based real-time fluorescence PCR. *Nucleic Acids Res* 29: E13, 2001.
58. Taylor DJ, Bore PJ, Styles P, Gadian DG, Radda GK. Bioenergetics of intact human muscle. A 31P nuclear magnetic resonance study. *Mol Biol Med* 1: 77–94, 1983.
59. Taylor DJ, Coppack SW, Cadoux-Hudson TA, Kemp GJ, Radda GK, Frayn KN, Ng LL. Effect of insulin on intracellular pH and phosphate metabolism in human skeletal muscle in vivo. *Clin Sci (Lond)* 81: 123–128, 1991.
60. Trautschold I, Lamprecht W, Schweitzer G. Nucleotides, Coenzymes and related compounds. In: *Methods of Enzymatic Analysis*, edited by Bergmeyer H, Wiley-VCH, 1985.
61. Trenell MI, Hollingsworth KG, Lim EL, Taylor R. Increased daily walking improves lipid oxidation without changes in mitochondrial function in Type 2 diabetes. *Diabetes Care* 31: 1644–1649, 2008.
62. van den Broek NM, Ciapaite J, De Feyter HM, Houten SM, Wanders RJ, Jeneson JA, Nicolay K, Prompers JJ. Increased mitochondrial content rescues in vivo muscle oxidative capacity in long-term high-fat-diet-fed rats. *FASEB J* 24: 1354–1364, 2010.
63. van den Broek NM, De Feyter HM, de Graaf L, Nicolay K, Prompers JJ. Intersubject differences in the effect of acidosis on phosphocreatine recovery kinetics in muscle after exercise are due to differences in proton efflux rates. *Am J Physiol Cell Physiol* 293: C228–C237, 2007.
64. Vicini P, Kushmerick MJ. Cellular energetics analysis by a mathematical model of energy balance: estimation of parameters in human skeletal muscle. *Am J Physiol Cell Physiol* 279: C213–C224, 2000.
65. Wagenmakers AJ. Insulin resistance in the offspring of parents with type 2 diabetes. *PLoS Med* 2: e289, 2005.
66. Wu F, Jeneson JA, Beard DA. Oxidative ATP synthesis in skeletal muscle is controlled by substrate feedback. *Am J Physiol Cell Physiol* 292: C115–C124, 2007.
67. Yerby B, Deacon R, Beaulieu V, Liang J, Gao J, Laurent D. Insulin-stimulated mitochondrial adenosine triphosphate synthesis is blunted in skeletal muscles of high-fat-fed rats. *Metabolism* 57: 1584–1590, 2008.
68. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 277: 50230–50236, 2002.