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

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van den Broek NM, Ciapaite J, Nicolay K, Prompers JJ. Comparison of in vivo postexercise phosphocreatine recovery and resting ATP synthesis 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 (Pi). 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 Pi−→ATP flux (VATP) can be determined by saturating the γ-ATP peak and monitoring the effect of this perturbation on the Pi magnetization in a so-called 31P saturation transfer (ST) experiment (3, 5). Assuming that VATP is predominantly reflecting oxidative ATP synthesis by the F1F0-ATP synthase in the mitochondria, VATP 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, VATP 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 VATP 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 (kPCr) 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-
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 \(^3\)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 (36 ± 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, Sniff 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 days. Control animals received similar volumes of subcutaneously injected saline.

Isolation of mitochondria. All experimental procedures were reviewed and approved by the Animal Ethics Committee of Maastricht University.

**\(^3\)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 ± 0.5°C using heating pads. Respiration was monitored using a pressure sensor registering thorax movement (Rapid Biomedical, Rimpar, Germany).

Resting V\(_{\text{ATP}}\) and postexercise k\(_{\text{PCr}}\) were measured in the TA by in vivo \(^3\)P MRS. \(^3\)P MRS was applied using a combination of a circular \(^1\)H surface coil (40 mm) for making MR images and localized shimming and an ellipsoid \(^3\)P MRS surface coil (10/18 mm), positioned over the TA as described previously (11). \(^3\)P MRS spectra were acquired applying an adiabatic excitation pulse with a flip angle of 90°. A fully relaxed (TR = 25 s, 32 averages) spectrum was measured at rest, followed by the ST experiment in resting TA muscle to determine V\(_{\text{ATP}}\). Two spectra (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 Pi magnetization (M\(_1\)) and a reference spectrum with saturation at a field level frequency, equidistant from Pi, yielding the equilibrium Pi magnetization (M\(_0\)), both with a saturation pulse length of 10 s. The apparent longitudinal relaxation time of Pi (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 \(^3\)P MR spectra (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 10 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 2 min. In three to four time series were measured for each rat.

\(^3\)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 Pi peaks were fit to Lorentzian line shapes, whereas γ-, α-, and β-ATP signals were fit with Gaussian line shapes. Besides the cytosolic Pi signal, a second, smaller Pi peak was observed in the spectra at a frequency ~0.3 ppm downfield from the cytosolic Pi resonance. The two Pi 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 Pi signals could not be distinguished, and a single Pi peak was fit to a Gaussian line shape. Concentrations of PCr and Pi 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 Pi resonance (58).

The T\(_1\) relaxation time of Pi 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\(_{\text{ATP}}\) exchange rate constant (k\(_{\text{Pi-ATP}}\)) was calculated from the T\(_1\) of Pi, and the fractional reduction of Pi magnetization upon selective saturation of γ-ATP according to: 

\[ k_{\text{Pi-ATP}} \frac{\text{Pi}_{\text{rest}}}{\text{Pi}_{\text{ATP}}} = \frac{1}{(1 + M/M_0)T_1} \]

V\(_{\text{ATP}}\) was then calculated by multiplying k\(_{\text{Pi-ATP}}\) with the Pi concentration at rest, as determined from the fully relaxed spectrum. The intrinsic T\(_1\) of Pi was calculated as T\(_1\) = 1/(1/T\(_1\) - k\(_{\text{Pi-ATP}}\)). The data of PCR recovery were fit to a monoexponential function using Matlab (version 7.04, Mathworks, Natick, MA) yielding a rate constant k\(_{\text{PCr}}\). 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\(_2\)CO\(_3\) 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\(_2\)PO\(_4\), 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-Elevejem 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
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pellet was resuspended in 100 µl 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 mM rotenone were used, respectively. Oxygen consumption in resting state (state 4) was determined after addition of 1.25 µM carboxyatractyloside. The signals from the oxygen electrode were recorded at 0.5-s intervals. Data acquisition and analysis were performed using Oroxygraph-2k-DatLab software version 4.2 (Oroboros).

Determination of the relative mitochondrial copy number. Genomic DNA was isolated from a 25-µg transversal slice of midbelly TA using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Zwijndrecht, The Netherlands). mtDNA content relative to peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) gene was measured using real-time PCR as described in Ref. 7. Primers for mtDNA were the following: forward primer, 5′-ACACCCAAAAAGGACGAACCTG-3′; reverse primer, 5′-ATGGGAAAAGCCTAGAA-3′; and for PGC-1α: forward primer, 5′-ATGATGTCGGGCTTTAGC-3′; reverse primer, 5′-CAATGGCAGGTTGTGTC-3′. The relative mtDNA copy number was calculated using ΔΔCt method as described in Ref. 57.

Statistical analysis. Data are presented as means ± 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 1. Oxygen consumption rates in isolated tibialis anterior muscle mitochondria oxidizing different substrates in different metabolic states

<table>
<thead>
<tr>
<th>Substrate</th>
<th>State 3, nmol O2·min⁻¹·mg protein⁻¹</th>
<th>State 4, nmol O2·min⁻¹·mg protein⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate Plus Malate</td>
<td>407.6 ± 21.4</td>
<td>18.7 ± 3.2</td>
</tr>
<tr>
<td>Succinate Plus Rotenone</td>
<td>326.0 ± 38.4*</td>
<td>19.8 ± 4.3</td>
</tr>
</tbody>
</table>

Data are from n = 7 control and n = 8 diphenyleneiodonium (DPI)-treated animals and are expressed as means ± 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.

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 ± 323) and DPI-treated (2,190 ± 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, P1, 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.

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

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.19 ± 0.02</td>
<td>7.21 ± 0.02</td>
</tr>
<tr>
<td>[PCr], mM</td>
<td>47.5 ± 2.8</td>
<td>48.8 ± 2.9</td>
</tr>
<tr>
<td>[P1], mM</td>
<td>3.0 ± 0.4</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>[ADP], µM</td>
<td>13.4 ± 0.6</td>
<td>13.9 ± 0.4</td>
</tr>
</tbody>
</table>

Data are from n = 8 animals for each group and are expressed as means ± SD. PCr, phosphocreatine; P1, inorganic phosphate.

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 P1 (grey). From the 31P ST spectra, the ratio of P1 magnetization with and without saturation of γ-ATP (M′/M0) was determined, and this ratio was not significantly different between the groups (Table 3). The apparent T1 (T'1) of P1 in case of saturation of γ-ATP was determined from an eight-point inversion recovery experiment, and a representative example of P1 inversion recovery data and the corresponding
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 $k_{P_i \rightarrow ATP}$ determined from $T_1'$ and M$/M_0$, $V_{ATP}$ determined from $k_{P_i \rightarrow ATP}$ 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 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.

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M'/M_0$</td>
<td>0.73 ± 0.06</td>
<td>0.67 ± 0.08</td>
</tr>
<tr>
<td>$T_1$, s</td>
<td>3.87 ± 0.37</td>
<td>3.76 ± 0.79</td>
</tr>
<tr>
<td>$k_{P_i \rightarrow ATP}$</td>
<td>5.38 ± 0.79</td>
<td>5.69 ± 1.33</td>
</tr>
<tr>
<td>$k_{PCr}$</td>
<td>0.07 ± 0.02</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>$V_{ATP}$, μmol·g$^{-1}$·min$^{-1}$</td>
<td>3.33 ± 1.53</td>
<td>10.45 ± 2.82</td>
</tr>
</tbody>
</table>

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

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.
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$\rightarrow$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 vasodilatation (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$_{1}$F$_{0}$-ATP synthase flux and flux through other P$_i$ $\rightarrow$ 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 Pi and ATP may greatly exceed the net glycolytic flux (4). Experimental proof for glycolytic exchange between Pi and ATP came from 31P MRS ST experiments in yeast. Pi→ATP exchange was inhibited in yeast by iodoacetate, an irreversible inhibitor of GAPDH (6), whereas in anaerobic yeast cells overexpressing PGK, Pi→ATP exchange was increased (2). 31P MRS ST experiments performed on perfused rat hearts showed that the glycolytic exchange reaction contributes significantly to the measured VATP and that only when glycolytic activity was inhibited, the measured VATP displayed a linear dependence on oxygen consumption and thus represents mitochondrial F1F0-ATP synthase flux (27). For rat skeletal muscle, values for resting ATP turnover from 31P 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 VATP from 31P MRS ST experiments in resting skeletal muscle can be as much as 80%. Up to this point, it has been assumed that the mitochondrial F1F0-ATP synthase flux, which constitutes only a small part of the total VATP measured, is unidirectional. However, it has been shown that at low rates of respiration (e.g., resting muscle) the F1F0-ATP synthase reaction is near to equilibrium and that mitochondrial exchange between Pi and ATP can make a significant contribution to the measured VATP (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 VATP. To create better conditions for detecting a defect in skeletal muscle mitochondrial oxidative phosphorylation, 31P MRS ST experiments would need to be performed during steady-state muscle contraction, when the F1F0-ATP synthase reaction is largely unidirectional and the flux through the F1F0-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 31P 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 31P 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 ADP and Pi, into account. Moreover, the VATP obtained from a 31P 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.

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DISCLOSURES

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REFERENCES


