Mitochondrial efficiency in rat skeletal muscle: influence of respiration rate, substrate and muscle type

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Abstract

Aim: To investigate the hypothesis that mitochondrial efficiency (i.e. P/O ratio) is higher in type I than in type II fibres during submaximal rates of respiration.

Methods: Mitochondria were isolated from rat soleus and extensor digitorum longus (EDL) muscles, representing type I and type II fibres, respectively. Mitochondrial efficiency (P/O ratio) was determined with pyruvate (Pyr) or palmitoyl-L-carnitine (PC) during submaximal (constant rate of adenosine diphosphate infusion) and maximal ($V_{\max}$, state 3) rates of respiration and fitted to monoexponential functions.

Results: There was no difference in $V_{\max}$ between PC and Pyr in soleus but in EDL $V_{\max}$ with PC was only 58% of that with Pyr. The activity of 3-hydroxyacyl-CoA dehydrogenase was threefold higher in soleus than in EDL. P/O ratio at $V_{\max}$ was 8–9% lower with PC [2.33 ± 0.02 (soleus) and 2.30 ± 0.02 (EDL)] than with Pyr [2.52 ± 0.03 (soleus) and 2.54 ± 0.03 (EDL)] but not different between the two muscles ($P > 0.05$). P/O ratio was low at low rates of respiration and increased exponentially when the rate of respiration increased. The asymptotes of the curves were similar to P/O ratio at $V_{\max}$. P/O ratio at submaximal respirations was not different between soleus and EDL neither with Pyr nor with PC.

Conclusion: Mitochondrial efficiency, as determined *in vitro*, was not significantly different in the two fibre types neither at $V_{\max}$ nor at submaximal rates of respiration. The low $V_{\max}$ for PC oxidation in EDL may relate to low activity of β -oxidation.

Keywords energetic efficiency, fatty acid oxidation, mitochondria, muscle fibre type, oxidative phosphorylation, oxygen utilization.

Several studies have demonstrated that work efficiency during cycling is related to the distribution or recruitment of type I fibres in the working muscle (Coyle *et al.* 1992, Horowitz *et al.* 1994, Hansen *et al.* 2002). Work efficiency is determined both by the efficiency of the contractile process [i.e. adenosine triphosphate (ATP) turnover performed work per] and by the efficiency of oxidative phosphorylation (OXPHOS) (i.e. ATP formation per consumed oxygen or P/O ratio). It is often claimed that the efficiency of OXPHOS is lower in type II fibres (Crow &

Kushmerick 1982, Barstow *et al.* 1996, Jackman & Willis 1996, Krstrup *et al.* 2004). Type I fibres have a lower mitochondrial proton conductance than type IIb fibres in the trout (Leary *et al.* 2003), a lower amount of uncoupling protein isotype 3 (UCP3) than type IIa and IIx fibres in humans (Russell *et al.* 2003) and a higher P/O ratio in the presence of glycerol phosphate than type IIb fibres in the rat (Jackman & Willis 1996). All of these findings support the hypothesis of a lower P/O ratio in type II fibres. However, the relevance of these intrinsic differences between mito-

chondria from type I and type II fibres for OXPHOS efficiency is unclear.

The efficiency of OXPHOS is conventionally measured in isolated mitochondria by adding a bolus of adenosine diphosphate (ADP) eliciting state 3 respiration ($V_{\max}$) and measuring the oxygen required to transfer added ADP to ATP. To our knowledge only two studies have systematically investigated if the P/O ratio is different in mitochondria from different muscle types. Pande & Blanchaer (1971) could not find any difference in P/O ratio in mitochondria isolated from different types of rat muscle when using CHO (pyruvate)- or fat (palmitoyl-carnitine)-derived substrates. In contrast Jackman & Willis (1996) showed that P/O ratio in mitochondria from type II fibres was higher (vs. type I muscles) when pyruvate and 2-oxoglutarate were used as substrates but lower when pyruvate were used in combination with glycerol-3-phosphate.

Mitochondrial efficiency is reduced (decreased coupling efficiency) by back-leakage of protons. The observed fibre-type differences in UCP3 (Russell *et al.* 2003), membrane fluidity (Leary *et al.* 2003) and proton conductance (Leary *et al.* 2003) may influence the extent of proton leak and thus the mitochondrial efficiency. However, during state 3 respiration, mitochondrial membrane potential (E_m) and proton motive force (Δp) decrease and proton leak will be reduced or abolished. Therefore, P/O ratio obtained during state 3 respiration corresponds to a theoretical peak value, which may not be achieved *in vivo*. During submaximal rates of respiration E_m will be higher than during $V_{\max}$ and the degree of uncoupling will also be higher. Potential differences in uncoupling between fibre types may therefore become manifest during submaximal respiration and reflected by differences in P/O ratio.

Measurement of P/O ratio during submaximal respiration is technically more difficult than during state 3 respiration. A steady-state submaximal rate of respiration can be obtained by maintaining a stable ADP concentration. A stable ADP concentration can be attained by constant rate of ADP infusion (Gnaiger *et al.* 2000) or by using an ATP trap (glucose + hexokinase) to recycle ADP (Willis & Jackman 1994). By using these techniques it has been shown (Willis & Jackman 1994, Gnaiger *et al.* 2000) that the P/O ratio decreases (increased degree of uncoupling) in a non-linear fashion when the respiration rate decreases. Potential fibre-type differences in P/O ratio at submaximal rates of respiration have not been investigated.

Therefore, the main purpose of the present study was to investigate the hypothesis that mitochondrial efficiency (i.e. P/O ratio) is higher in type I than in type II fibres during submaximal respirations. Mitochondria were isolated from rat soleus (mainly type I fibres) and

extensor digitorum longus (EDL; type IIa, IIx and IIb fibres) and respiration measured during submaximal rates of ADP infusions.

Materials and methods

Animal care and protocol

Fourteen male Sprague–Dawley rats, 5–8 weeks old, weighing between 120 and 200 g were used in this study. All animals were housed in box cages and maintained in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) with a 12 : 12-h light–dark cycle. The rats were provided unrestricted access to food and water. Housing and husbandry practices were in accordance with ‘Guide for the Care and Use of Laboratory Animals’ (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, Washington, 1996). Animals were killed by cervical dislocation. Mitochondria were isolated from soleus and EDL muscles, which contain 84% type I and 96% type II fibres (20% IIa, 38% IIx and 38% IIb), respectively (Delp & Duan 1996).

Isolation of muscle mitochondria

Soleus and EDL muscles were excised, trimmed free of visible connective tissue, weighed and placed in ice-cold isolation medium. Part of the muscle (5–10 mg) was frozen in liquid nitrogen and stored at -80°C for later determinations of enzyme activities. Mitochondria were isolated with a technique previously described in detail (Tonkonogi & Sahlin 1997). The muscle was finely minced and rinsed thoroughly with isolation medium [(in mmol L^{-1}) sucrose, 100; KCl, 100; Tris–HCl, 50; KH_2PO_4 , 1; EGTA, 0.1; and 0.2% bovine serum albumin (BSA), pH 7.40] incubated for 2 min with 0.2 mg mL^{-1} bacterial protease (Nagarse; EC 3.4.21.62, Type XXVII; Sigma-Aldrich, St Louis, MO, USA) and homogenized for 2 min in an ice-cooled glass homogenizer with a motor-driven (180 rpm) Teflon pestle (radial clearance 0.15 mm). The homogenate was diluted with three volumes of protease-free isolation medium and centrifuged at 750 g for 10 min. The supernatant was centrifuged at 10 000 g for 10 min and the pellet washed free from the lighter fluffy layer, suspended in the isolation medium and again centrifuged (7000 g for 3 min). The final pellet was suspended in a suspension medium (about 0.5 $\mu\text{L mg}^{-1}$ initial muscle) containing (in mmol L^{-1}): mannitol, 225; sucrose, 75; Tris, 10; EDTA, 0.1; and BSA 0.2%, pH 7.40. The isolation procedure was carried out at $0-4^\circ\text{C}$ by the same person. The order between muscles was randomized and the time for isolation was <1 h.

Mitochondrial respiratory activity

Mitochondrial oxygen consumption was measured polarographically using a Clark-type electrode (DW1 oxygraph; Hansatech Instruments, King's Lynn, Norfolk, UK) in an oxygraph at 25 °C. The electrode was connected to a computer for data collection. Respiration was measured in 485 μL oxygraph medium (in mmol L^{-1}): mannitol, 225; sucrose, 75; Tris, 10; KCl, 10; K_2HPO_4 , 10; EDTA, 0.1; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.8; and pH 7.0. Two substrate combinations was used: pyruvate (5 mmol L^{-1}) + L-malate (2 mmol L^{-1}) (Pyr) and palmitoyl-L-carnitine ($10 \mu\text{mol L}^{-1}$) + L-malate (2 mmol L^{-1}) (PC). Both Pyr and PC were dissolved in 0.5% BSA. Uncoupling of OXPHOS was evident at $20 \mu\text{M}$ PC and the concentration of PC was therefore maintained as low as possible ($10 \mu\text{M}$). After completion of state 3 and the first submaximal measurement additional PC (final concentration $6 \mu\text{M}$) was added to obtain non-limited substrate concentration.

Respiration was initiated by the addition of $12.5 \mu\text{L}$ of mitochondrial suspension. After reaching a stable rate of respiration, state 3 (V_{max}) was initiated by adding ADP dissolved in oxygraph medium (final concentration 0.3 mmol L^{-1}). The concentration of ADP was determined spectrophotometrically. After determination of V_{max} (state 3) and respiration after all added ADP had been converted to ATP (state 4) the oxygen tension in the chamber was increased (exposure of oxygraph medium to air) and ADP infused at a low rate with a microdialysis pump (CMA/102 Microdialysis Pump; CMA/Microdialysis, Solna, Sweden). Three submaximal respiration rates eliciting low, medium and high rates of respiration were performed for each measurement (Fig. 1). This corresponded to pumping rates between 0.3 and $2.0 \mu\text{L min}^{-1}$. The accuracy of the pump was tested separately by continuous weighing of delivered water.

Each oxygraph measurement was completed within 40 min and performed at oxygen concentrations ranging from 100 to $250 \mu\text{mol L}^{-1}$. The respiratory measurements from both muscles (randomized order) were performed within 2 h after the isolation procedure. Preliminary tests showed that V_{max} decreased by 5% per hour. The remaining part of the mitochondrial suspension medium and the solution in the oxygraph chamber (oxygraph solution) were placed in liquid nitrogen and stored at -80°C for later assay of citrate synthase (CS) activity. On a separate day the maximal activity of the electron transport chain (ETC) was determined in freeze-thawed mitochondria. The mitochondrial solutions were freeze-thawed three to five times and the maximal respiration was determined in the presence of NADH ($450 \mu\text{M}$) and cytochrome *c* ($2 \mu\text{M}$).

P/O ratio for V_{max} was calculated from the ratio between added ADP and consumed oxygen. P/O ratio

Figure 1 Representative oxygraphic trace of mitochondrial oxygen consumption during maximal and submaximal respiration. The example is based on mitochondria isolated from EDL with Pyr. A: Pumping rate = $0.3 \mu\text{L min}^{-1}$ ($9.2 \text{ nmol ADP min}^{-1}$). Resulting P/O ratio = 2.0. B: Pumping rate = $1.1 \mu\text{L min}^{-1}$ ($29.3 \text{ nmol ADP min}^{-1}$). Resulting P/O ratio = 2.44. C: Pumping rate = $1.4 \mu\text{L min}^{-1}$ ($36.7 \text{ nmol ADP min}^{-1}$). Resulting P/O ratio = 2.55. 'Mit' and 'ADP' denote the addition of mitochondria and ADP. 'Sample withdrawn' denotes sample taken for determination of ADP concentration. Numbers below (or to the left of) the trace denote rates of respiration ($\mu\text{mol O}_2 \text{ min}^{-1} \text{ L}^{-1}$). In order to maintain oxygen tension above 100 mmol L^{-1} the medium was at intervals exposed to air (shown as an increase in PO_2 in the trace).

for submaximal respirations was calculated from the rate of infused ADP divided by the rate of oxygen consumed after correction (determined in control experiment) for oxygen added by ADP infusion and diffusion from the ambient air. The P/O ratios were plotted against the rate of respiration and the resulting curve was fitted to a monoexponential function (Intercooled Stata 8.2; StataCorp LP, College Station, TX, USA). A best-fit analysis was also performed to confirm the use of the monoexponential function. The difference in the regression coefficient between the best-fit analysis and the fit for the monoexponential function was minimal (data not shown).

The following equation was used:

$$Y = a(1 - e^{-b(X-c)})$$

where X is the rate of respiration ($\mu\text{mol O}_2 \text{ min}^{-1} \text{ U}^{-1} \text{ CS}$), Y is the P/O ratio and a , b and c are constants. Constant a is the asymptote and describes the maximal P/O ratio, b is the gradient of the curve and c is the cut point of the x -axis.

ADP sensitivity

A sample ($50\text{--}80 \mu\text{L}$) was rapidly withdrawn from the chamber during ADP infusion eliciting a respiration of about 50% V_{max} . The sample was immediately mixed with $20 \mu\text{L}$ perchloric acid (PCA) (2.5 M) and the exact

sample volume determined from the increase in weight. The sampling was accomplished by the use of a 100 μL syringe (Hamilton, Bonaduz, Switzerland) and created a large vacuum which destroyed the mitochondria and interrupted ADP phosphorylation. This was verified by taking two samples at the same pumping rate but with different time between the sampling and the mixing with PCA (data not shown). Samples were centrifuged at 16 100 g for 5 min and the supernatant was neutralized by addition of 25 μL KHCO_3 (2.1 M) and centrifuged again at 16 100 g for 1 min. The supernatant was removed and stored at -80°C . The concentration of ADP was measured by HPLC assay according to the method used by Manfredi *et al.* (2002). In separate experiments, samples were taken at different respiratory intensities and assayed for ADP. This demonstrated that the relationship between the ADP concentration and the mitochondrial respiration followed Michaelis–Menten kinetics. The samples were withdrawn for ADP measurements when the rate of respiration was between 40% and 60% of V_{max} . Apparent K_m for ADP was calculated by assuming Michaelis–Menten kinetics.

Activity of mitochondrial enzymes

The activities of 3-hydroxyacyl-CoA dehydrogenase (HAD) and CS were determined in freeze-dried soleus and EDL muscles and in the corresponding mitochondrial-rich suspension buffer. Furthermore, the CS activity was determined in the oxygraph solutions. CS was used as a marker of mitochondrial density and as a reference base for the respiratory measurements (Wibom *et al.* 2002). Freeze-dried samples were trimmed free of blood and connective tissue, powdered and homogenized in a Triton solution (containing in mmol L^{-1} Na_2HPO_4 , 50; EDTANa_2 , 1; Triton X-100, 0.05% and pH 7.0) using a glass–glass homogenizer. Homogenized muscles or dissolved mitochondria were assayed for CS activity spectrophotometrically at 25°C according to the method used by Alp *et al.* (1976). Homogenized muscles were also assayed for HAD activity spectrophotometrically at 25°C as described by Passonneau & Lowry (1993).

Statistical methods

Values are reported as mean $\pm$ SEM. Differences in respirations, ratios and ADP sensitivity were analysed using a Student's paired *t*-test. Significance was accepted at the 5% level or at 2.5% if the same mean value was used twice (Zar 1984). Mitochondrial efficiency at submaximal respirations was fitted to monoexponential functions and statistical significance of the difference between substrates and muscles were analysed by non-

linear regression analysis. Significance was accepted at the 5% level.

Results

Based on the conservation of the CS activity the yield of isolated mitochondria was 24% (range 11–30%) for the soleus and 26% (range 19–45%) for the EDL muscle. The use of protease during mitochondrial isolation means that both subsarcolemmal and intra-myofibrillar mitochondria were harvested. Mitochondrial respiration was normalized to mitochondrial density by using CS activity as a reference base. V_{max} was not different for the two substrates in soleus, but in EDL, respiration with PC was only 58% of that with Pyr (Table 1). V_{max} (normalized to CS activity) was higher in soleus than in EDL with PC but lower with Pyr ($P < 0.001$ vs. EDL). State 4 respiration was not different between muscles but slightly higher with PC than with Pyr (soleus: $P < 0.01$, EDL: $P = 0.09$). The activity of the ETC, determined in freeze-thawed mitochondria using NADH as substrate, was similar in the two muscles (Table 1) and two- to threefold higher than mitochondrial respiration with Pyr. The respiratory control index (RCI: state 3/state 4 respirations), often used as an index of the quality of the mitochondrial preparation, was high (Table 1) and indicate that the isolated mitochondria were well coupled and of high integrity.

P/O ratio during state 3 was 8–9% higher for Pyr than for PC ($P < 0.001$, Table 1) but was not different between soleus and EDL. P/O ratio decreased when the rate of respiration decreased (Fig. 2) and at a respiration rate of 20 $\text{nmol O}_2 \text{ min}^{-1} \text{ U}^{-1}$ CS (about 20% of V_{max} with Pyr and 27% of V_{max} with PC) P/O ratio was reduced by 23% (Pyr) and 29% (PC). The relation between P/O ratio and respiration rate was well described by monoexponential functions and the asymptotes of the curves were similar to the P/O ratio obtained during V_{max} . The individual curves for soleus and EDL were defined by the three constants in the equation. Non-linear regression analysis of the monoexponential functions could not demonstrate significant differences between muscles (Table 2). This is also evident in Figure 2 where the material is presented as a common curve for the two muscles.

Apparent K_m for ADP was not significantly different between muscles but was lower for PC than for Pyr in EDL (Table 1). The activity of CS was 45% higher in soleus than in EDL muscle (165 ± 6 vs. $113 \pm 16 \mu\text{mol g dw}^{-1} \text{ min}^{-1}$; $P = 0.09$, $n = 4$) and the activity of HAD was threefold higher in soleus than in EDL muscles (158.2 ± 4.0 vs. $54.8 \pm 10.4 \mu\text{mol g dw}^{-1} \text{ min}^{-1}$; $P < 0.01$).

Table 1 Respiratory parameters

	Substrate	Soleus	EDL	Statistical significance (soleus vs. EDL)
State 3	Pyr	94.5 ± 2.6	112.6 ± 3.8	$P < 0.001$
	PC	89.4 ± 3.4	65.1 ± 2.1*	$P < 0.001$
State 4	Pyr	5.9 ± 0.4	6.1 ± 0.4	ns
	PC	6.9 ± 0.5*	7.2 ± 0.6	ns
RCI ratio	Pyr	16.2 ± 1.2	19.5 ± 1.1	$P < 0.01$
	PC	13.1 ± 0.6*	9.5 ± 0.5*	$P < 0.01$
P/O ratio	Pyr	2.52 ± 0.03	2.54 ± 0.03	ns
	PC	2.33 ± 0.02*	2.30 ± 0.02*	ns
Apparent K_m (ADP)	Pyr ($n = 5$)	11.8 ± 1.2	17.0 ± 1.4	ns
	PC ($n = 5$)	15.5 ± 3.8	8.5 ± 1.1*	ns
NADH respiration	NADH + cyt <i>c</i> ($n = 4$)	286 ± 28	273 ± 19	ns

State 3 ($V_{\max}$) is the maximal ADP-stimulated respiration and state 4 is the mitochondrial respiration when all ADP has been converted to ATP. P/O ratio is the amount of ATP formed per oxygen atom and refers to that at state 3. RCI is the ratio between state 3 and state 4 respiration. NADH respiration was measured in freeze-thawed mitochondria with NADH and cytochrome *c* as substrates. State 3, state 4 and NADH respiration are expressed as nmol O₂ min⁻¹ U⁻¹ CS. Apparent K_m for ADP is the concentration of ADP (μM) which elicits 50% of $V_{\max}$. Values are given as mean ± SEM.

* $P < 0.025$ PC vs. Pyr.

Discussion

The present study demonstrates that the efficiency of OXPHOS (P/O ratio) decreases during submaximal respiration and that the relation between respiration rate and P/O ratio is similar in soleus and EDL.

Mitochondrial efficiency at submaximal respiration

In accordance with previous studies (Willis & Jackman 1994, Gnaiger *et al.* 2000) we have shown that P/O ratio increases exponentially when respiration rate increases. The data was well described by monoexponential functions and the asymptotes of the curves were close to the P/O ratio measured during $V_{\max}$. The reduction in P/O ratio at submaximal respiration rates shows that mitochondrial oxygen consumption becomes loosely coupled to ADP phosphorylation. The mechanism for the increased uncoupling may either relate to an increased back-leakage of protons through the mitochondrial membrane or to a proton slip in the ETC (reduced H⁺/e⁻ flux). Based on the relation between mitochondrial respiration and protonmotive force (Δp) in the presence of chemical uncoupler, Brand *et al.* (1994) concluded that proton leak could account for all of the uncoupled respiration, whereas other studies suggest that a proton slip can occur (Rigoulet *et al.* 1998, Kadenbach 2003). The reduction in E_m and Δp at high rates of respiration will reduce both the leak and slip of protons and explains the increased P/O ratio when respiration rates increases. A potential artefact with the used protocol is that isolated mitochondria

might be contaminated with ATPase, which would manifest as increased respiration, especially at low rates, and thus give a false impression of reduced P/O ratio. We have compared respiration during state 4 prior to state 3, when ATP and ADP are absent, with that during state 4 after state 3, when the presence of ATP and thus potential ATPase activity would increase the respiration. The respirations during these two conditions were not significantly different but on average somewhat higher during state 4 prior to state 3. We therefore find it unlikely that contaminating ATPases contribute to the low P/O ratios at low respiration rates.

Fibre-type differences in the efficiency of the oxidative phosphorylation

In vivo studies have shown a relationship between work efficiency and the proportion (Coyle *et al.* 1992, Horowitz *et al.* 1994, Hansen *et al.* 2002) or the recruitment (Krustrup *et al.* 2004) of type I fibres. Can these *in vivo* findings be explained by intrinsic differences in mitochondrial efficiency? The similar P/O ratio in soleus and EDL both at submaximal and maximal respirations speaks against fibre-type differences in mitochondrial efficiency. However, previous studies have shown that P/O ratio is 22% lower in type IIb fibres (vs. type I fibres) during $V_{\max}$ in the presence of high concentrations (10 mM) of glycerol 3-phosphate (Jackman & Willis 1996). NADH/NAD⁺ is much lower in the cytosol than in mitochondria and the transport of reducing equivalents into mitochondrial matrix is

Figure 2 P/O ratio at different respiration rates with Pyr (a) and PC (b) in soleus (○) and EDL (■). P/O ratio at V_{max} (state 3) is shown as (mean $\pm$ SEM). The mean ($\pm$ SEM) state 4 respiration is shown at the x-axis when P/O ratio is zero. The data was fitted to a monoexponential curve $Y = a(1 - e^{-b(X-c)})$, where a , b and c are constants. The equations were similar for soleus and EDL (Table 2) and are shown as a common curve for the two muscles in the figure. Pyr: $Y = 2.43(1 - e^{-0.13(X-8.6)})$, $r^2 = 0.66$. PC: $Y = 2.25(1 - e^{-0.08(X-4.4)})$, $r^2 = 0.83$.

therefore related to a loss of energy. Transport of cytosolic reducing equivalents can occur with the glycerol phosphate shuttle and/or with the malate/aspartate shuttle. The glycerol 3-phosphate shuttle appears to be more important in type IIB than in

type I fibres (Willis & Jackman 1994) and explains why P/O ratio is reduced in the presence of glycerol 3-phosphate only in type IIB fibres (Willis & Jackman 1994). The impact of this finding for *in vivo* conditions is, however, unclear. The theoretical reduction in P/O ratio *in vivo* due to the glycerol phosphate shuttle is only about 6% and one would also expect that *in vivo* P/O ratio would be reduced also in type I fibres due to the malate-aspartate shuttle. These considerations suggest that the difference in mitochondrial efficiency between type I and type II fibres is small (<6%) during CHO oxidation.

Mitochondrial fuel selection is dependent on substrate mobilization, control of key enzymes and V_{max} of the pathway. V_{max} for PC oxidation was higher in mitochondria isolated from soleus than from EDL (Table 1) and when the 45% higher mitochondrial density in soleus is taken into account, V_{max} was about twofold higher in soleus for PC. The relatively low potential for PC oxidation together with a high glycolytic potential and a high metabolic rate will favour a switch to Pyr oxidation at lower work rate in contracting EDL than in soleus. Mitochondrial efficiency was 8–9% higher for CHO oxidation than for PC oxidation and when the substrate utilization is taken into account one may argue that mitochondrial efficiency *in vivo* is higher in type II fibres than in type I fibres during submaximal respiration. However, during low rates of respiration oxygen availability is abundant and the physiological advantage of a high P/O ratio is therefore limited during these conditions.

Fibre-type differences in mitochondrial substrate oxidation

An interesting observation was that V_{max} in soleus was similar for PC and Pyr, whereas in EDL PC oxidation was only 58% of Pyr oxidation. The ratio between HAD (a key enzyme in β -oxidation) and CS was also lower in EDL than in soleus (Fig. 3). It is well known that type I fibres have a higher capacity for FA oxidation than type II fibres. This is generally explained by enhanced capacity for transport of FA through

Table 2 Equation constants for the monoexponential fit of P/O vs. respiration rate

	Pyr			PC		
	Soleus	EDL	Statistical significance	Soleus	EDL	Statistical significance
a	2.51 ± 0.05	2.38 ± 0.08	ns	$2.23 \pm 0.07^*$	2.31 ± 0.31	ns
b	0.10 ± 0.01	0.14 ± 0.05	ns	0.10 ± 0.02	0.07 ± 0.04	ns
c	8.4 ± 1.0	8.3 ± 2.8	ns	6.4 ± 1.7	2.2 ± 6.2	ns
r^2	0.90	0.39		0.86	0.77	

The data was fitted to the equation $Y = a(1 - e^{-b(X+c)})$, where Y is P/O ratio and X is the absolute respiration. The relations are presented in Fig. 2). ns, No significant difference between soleus and EDL.

*Significantly different from Pyr within the same type of muscle.

Figure 3 Ratio between the activity of HAD and CS and the ratio between $V_{\max}$ with PC and Pyr as substrates. The bars represent the mean $\pm$ SEM ($n = 4$). * $P < 0.05$ vs. soleus.

sarcolemma, higher intramuscular triglyceride stores, increased mitochondrial volume and/or control by carnitine palmitoyltransferase 1 (Essen *et al.* 1975, Spriet & Watt 2003). Despite that all of these steps are by-passed when PC is used as a substrate the present study demonstrates that the relative rate of PC oxidation remains lower in mitochondria from EDL. The difference may relate to a low capacity of NADH/FADH formation by β -oxidation in type II fibres. The relatively low HAD activity in EDL (which may reflect the maximal flux of β -oxidation) and the two- to threefold higher respiration with saturating levels of NADH (permeabilized mitochondria) support the idea that PC oxidation *in vitro* is limited by the redox drive. However, it cannot be excluded that PC interferes with other steps in OXPHOS (Wojtczak & Schonfeld 1993) or that the difference in PC/Pyr oxidation between soleus and EDL reflects differences in PDH activity.

Fibre-type differences in ADP sensitivity

ADP concentration was in this study directly measured during ADP stimulated respiration. Estimated apparent K_m for ADP (9–17 μM , Table 1) was similar in soleus and EDL and is within the range shown by other studies using more indirect approaches (Tonkonogi & Sahlin 1997, 1999, Gnaiger *et al.* 2000, Dos *et al.* 2002). The apparent K_m measured in isolated mitochondria is considerably lower than that in permeabilized soleus fibres (Saks *et al.* 1994). The high apparent K_m in fibres has been explained by a restricted transport of ADP over the outer mitochondrial membrane, because of interaction with cytoskeleton proteins (Saks *et al.* 1994). This interaction is removed during the process of mitochondrial isolation and observed K_m for ADP may therefore not correspond to that *in vivo* but rather reflects intrinsic properties of OXPHOS regulation by ADP including ADP affinity of ANT.

An interesting finding was the higher ADP sensitivity (lower apparent K_m) in EDL with PC ($P < 0.025$ vs.

Pyr). A higher ADP sensitivity means that FA would be preferentially oxidized over CHO when ADP concentration is suboptimal, i.e. at low rates of respiration. This is of bioenergetic advantage as oxidation of FA at low rates of respiration when oxygen availability is abundant will spare the limited store of CHO to higher exercise intensities, when a high P/O ratio is essential. However, our results are based on a limited number of observations ($n = 5$) and further studies are required to verify the differences in ADP sensitivity between substrates. Further, as noted previously control of fuel selection is complex and is *in vivo* likely influenced by a number of additional factors such as fuel mobilization and enzyme control.

Perspectives

If the non-linear relationship between P/O ratio and intensity of the mitochondrial respiration is present *in vivo* one would expect a lower mitochondrial efficiency at rest and during low intensity exercise than during higher intensities. However, this is in contrast to what has been shown in the literature, where delta efficiency decreased with increments in work rate (Gaesser & Brooks 1975). Furthermore, Marcinek *et al.* (2004) measured P/O ratio in resting muscle *in vivo* with a method based on infrared spectroscopy and ^{31}P MRS. The P/O ratio was estimated to about 2.1 in resting mouse muscle presumably with FA as the main substrate (Marcinek *et al.* 2004). Theoretically, maximal P/O ratio during FA oxidation is 2.33 (Brand *et al.* 1994) and the results imply that the degree of uncoupling *in vivo* is low despite low rates of respiration. The present results from isolated mitochondria would predict a much lower P/O ratio at rest. OXPHOS is stimulated both by a push mechanism (increases in redox drive, i.e. NADH/NAD $^{+}$) and a pull mechanism [increases in ADP or ADP/(ATP $\times$ Pi)] (Kushmerick 1983). Proton leak and electron slip are stimulated by the redox drive (Rigoulet *et al.* 1998). Our experiments with isolated mitochondria were performed with saturated substrate concentrations and thus maximal redox drive, whereas the conditions *in vivo* may involve a lower redox drive. The difference between our data *in vitro* predicting a low P/O ratio in resting muscle and that determined *in vivo* demonstrating a high P/O ratio may therefore relate to differences in redox drive. Conditions of increased substrate mobilization (e.g. stress with augmented adrenaline levels or recovery from intense exercise) would according to this line of reasoning result in increased mitochondrial uncoupling and oxygen consumption. This hypothesis could be directly tested with the technique described by Marcinek *et al.* (2004).

Conflict of interest

There are no conflict of interests related to this project.

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