Inhibiting myosin-ATPase reveals a dynamic range of mitochondrial respiratory control in skeletal muscle

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Assessment of mitochondrial ADP-stimulated respiratory kinetics in PmFBs (permeabilised fibre bundles) is increasingly used in clinical diagnostic and basic research settings. However, estimates of the $K_m$ for ADP vary considerably ($\sim 20–300 \mu M$) and tend to overestimate respiration at rest. Noting that PmFBs spontaneously contract during respiration experiments, we systematically determined the impact of contraction, temperature and oxygenation on ADP-stimulated respiratory kinetics. BLEB (blebbistatin), a myosin II ATPase inhibitor, blocked contraction under all conditions and yielded high $K_m$ values for ADP of $>\sim 250$ and $\sim 80 \mu M$ in red and white rat PmFBs respectively. In the absence of BLEB, PmFBs contracted and the $K_m$ for ADP decreased $\sim 2–10$-fold in a temperature-dependent manner. PmFBs were sensitive to hyperoxia (increased $K_m$) in the absence of BLEB (contracted) at $30^\circ$C but not $37^\circ$C. In PmFBs from humans, contraction elicited high sensitivity to ADP ($K_m < 100 \mu M$), whereas blocking contraction (+ BLEB) and including a phosphocreatine/creatinine ratio of 2:1 to mimic the resting energetic state yielded a $K_m$ for ADP of $\sim 1560 \mu M$, consistent with estimates of in vivo resting respiratory rates of $<1\%$ maximum. These results demonstrate that the sensitivity of muscle to ADP varies over a wide range in relation to contractile state and cellular energy charge, providing evidence that enzymatic coupling of energy transfer within skeletal muscle becomes more efficient in the working state.

Key words: bioenergetics, blebbistatin, creatine kinase, myosin-ATPase, N-benzyltoluene sulfonamide, skeletal muscle contraction.

INTRODUCTION

Determining the sensitivity and responsiveness of the respiratory system to ADP is commonly performed in the diagnosis of mitochondrial diseases as well as in a variety of basic and applied research settings [1,2]. Respiratory sensitivity to ADP also influences a number of cellular processes dependent on mitochondrial membrane potential, including reactive oxygen species generation, subcellular calcium distribution and specific mitochondrial membrane transport mechanisms. However, in mitochondria isolated from rodent skeletal muscle, the apparent $K_m$ for ADP [3–5] is similar to the resting cellular concentration of free ADP [6–8], implying in vivo respiratory rates at rest are $\sim 50\%$ of maximal respiratory capacity – obviously an erroneous conclusion. The apparent $K_m$ for ADP is $\sim 10–15$-fold higher in PmFBs (permeabilised fibre bundles) than in isolated mitochondria from rodent cardiac and slow-twitch muscles ($\sim 300$ compared with $\sim 20–30 \mu M$ respectively) [3,4,9–12], suggesting that an additional level of regulation is preserved in fibre bundles. However, PmFBs from fast-twitch muscle (red and white gastrocnemius) are characterized by a high sensitivity to ADP ($K_m < 50–100 \mu M$), similar to isolated mitochondria from the same muscles [12]. In human skeletal muscle, which is a mixture of fast- and slow-twitch fibres, the $K_m$ for ADP is higher in PmFBs ($\sim 60–120 \mu M$ ADP [13,14]) than isolated mitochondria [5], but still predictive of much higher rates of respiration ($10–20\%$ of maximum) than at rest in vivo ($<1\%$ of maximum). The factors responsible for the obvious discrepancy in accurately assessing mitochondrial respiratory control by ADP have remained elusive.

During the course of respiration experiments, we have consistently observed that PmFBs spontaneously contract in a Ca$^{2+}$-independent manner (see Supplementary Movie S1 at http://www.BiochemJ.org/bj/437/bj4370215add.htm). To determine whether contractile activity alters ADP-stimulated respiratory kinetics, we compared the effect of two recently developed myosin II-specific inhibitors that block contractile activity: BTS (N-benzyltoluene sulfonamide) and BLEB (blebbistatin). BTS inhibits the Ca$^{2+}$-stimulated ATPase activity of myosin II subfragment 1, weakening myosin’s interaction with F-actin (filamentous actin) [15]. BLEB preferentially binds to the active site of subfragment 1 ATPase when ADP and phosphate are bound, stabilizing the intermediate state [16]. We report that spontaneous contraction of PmFBs increases mitochondrial respiratory sensitivity by severalfold in a fibre-type-specific manner that is both temperature- and tissue-oxygenation-dependent. We also report a $K_m$ value for ADP in resting human skeletal muscle that is severalfold higher than previously determined, consistent with an estimated in vivo rate of respiration of $<1\%$ of maximum. Importantly, this $K_m$ is only obtained by performing analyses with myosin-ATPase inhibition at $37^\circ$C with phosphocreatine and creatine concentrations reflective of resting muscle in vivo.

Abbreviations used: BLEB, blebbistatin; BTS, N-benzyltoluene sulfonamide; CK, creatine kinase; PmFB, permeabilised fibre bundle; RG, red gastrocnemius; SERCA, sarco/endoplasmic reticulum calcium ATPase; VDAC, voltage-dependent anion channel; WG, white gastrocnemius.

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**EXPERIMENTAL**

**Animals and reagents**

Male Sprague–Dawley rats were purchased from Charles River Laboratories. Rats were housed in a temperature- (22 °C) and light-controlled room and given free access to food and water. At the time of the experiments, rats were 8–10 weeks old and weighed 250–350 g. Skeletal muscle was obtained from anaesthetized animals (100 mg/kg intraperitoneal ketamine-xylazine). After surgery, animals were killed by cervical dislocation while anaesthetized. All procedures were approved by the Institutional Review Board of East Carolina University. All chemicals were purchased from Sigma–Aldrich. Both BLEB and BTS were dissolved in DMSO and added to the oxygraph chamber at a final concentration of 25 μM and 100 μM respectively.

**Human subjects and tissue biopsy**

Ten healthy but sedentary men were recruited to participate in this investigation. Their mean age, height, weight and BMI (body mass index) were 23.2 ± 1.7 years, 1.76 ± 0.05 m, 74.2 ± 4.9 kg and 23.9 ± 0.8 kg · m⁻² respectively. All participants were non-smokers. None of the subjects had any diseases or were taking any medications known to alter skeletal muscle metabolism. Subjects were given both oral and written information about the experimental procedures before giving their informed consent. These experiments were approved by the Institutional Review Board of East Carolina University and conducted in accordance with the Declaration of Helsinki.

On the day of the experiment, subjects reported to the clinical laboratory following an overnight fast (~12 h). A single skeletal muscle sample was obtained from the lateral aspect of vastus lateralis by a percutaneous needle biopsy technique with suction under local subcutaneous anaesthesia (1% lidocaine). A portion of the biopsy sample was immediately placed into ice-cold buffer X [50 mM K-MES, 7.23 mM K₂EGTA, 2.77 mM CaK₂EGTA, 20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 5.7 mM KCl, 7.1, 290 mOsm] and used to prepare PmFBs.

**Preparation of PmFBs**

The technique is partially adapted from previous methods [12,17] and has been described previously [18,19]. Briefly, small portions (~25 mg) of muscle were dissected and placed in ice-cold buffer X. The muscle was trimmed of connective tissue and fat. Four small muscle bundles (~2–7 mm, 1.0–2.5 mg of wet weight) of either red or white gastrocnemius were prepared from each rat. Each bundle was gently separated along the longitudinal axis with a pair of needle-tipped forceps under local subcutaneous anaesthesia (1% lidocaine). All procedures were approved by the Institutional Review Board of East Carolina University. All chemicals were purchased from Sigma–Aldrich. Both BLEB and BTS were dissolved in DMSO and added to the oxygraph chamber at a final concentration of 25 μM and 100 μM respectively.

**Mitochondrial respiration in PmFBs**

High-resolution O₂ consumption measurements were conducted in 2 ml of buffer Z using the OROBOROS Oxygraph-2k (Oroboros Instruments) with stirring at 750 rev./min. Buffer Z contained 20 mM creatine hydrate to saturate CK (creatinine kinase), which facilitates mitochondrial ADP transport [4,10,23–25], with the exception of specific experiments on human PmFBs which were conducted in the presence of 24 mM phosphocreatine and 12 mM creatine hydrate (described below). Pyruvate (5 mM) and malate (2 mM) were added as complex I substrates. ADP was titrated in step-wise increments and all experiments were completed before the oxygraph chamber [O₂] reached 150 μM. At the conclusion of each experiment, PmFBs were washed in double-distilled H₂O to remove salts, frozen at ~ −20 °C, and dried via freeze drying (Labconco). Polarographic oxygen measurements were acquired at 2 s intervals, with the rate of respiration derived from 40 data points, and expressed as pmol · s⁻¹ per mg of dry weight. Dry and wet bundle weights were consistently between 0.2–0.6 mg and ~1.0–2.5 mg respectively. Cytochrome c was added to test for mitochondrial membrane integrity, as partial loss of cytochrome c during sample preparation may limit active respiration. A cytochrome c response was detected in < 5% of all experiments and no response generated >10% increase in respiration. No relationship was observed between the relative cytochrome c response and Kₘ when grouping all human and rodent data (R² = 0.013, P > 0.05). Additionally, no significant relationship was observed in humans when using a paired t test to compare the Kₘ for those experiments showing 0–5% cytochrome c response with those few samples exhibiting a 5–10% cytochrome c response. Four PmFBs from each rat or human were run simultaneously in four separate oxygraph chambers. Two of the chambers contained either 100 μM BTS or 25 μM BLEB. A third chamber contained 1.25% DMSO (vehicle, + V) to match the content of DMSO added in the BTS and BLEB conditions with the remaining chamber serving as the control (− V) condition.

The Kₘ for ADP was determined through the Michaelis–Menten enzyme kinetics, fitting the model

\[ Y = \frac{V_{\text{max}}}{K_m + X} \]

where \( X \) = [free ADP; ADP₀] and \( Y = JO_2 \) at [ADP₁], using Prism (GraphPad Software). This equation was also used to calculate the fraction of maximal mitochondrial respiration in resting human skeletal muscle in vivo. This calculation was performed using the experimentally determined Kₘ values assuming resting [ADP] to be ~14.6 μM in human skeletal muscle [6].

**Microscopic imaging of PmFB conformation**

Images of PmFB conformation were captured immediately upon exposure to 25 °C or 30 °C before visible contraction occurred, with further images taken throughout the contraction process (Leica Microsystems). Separate images were taken of PmFBs before and after the addition of 2 mM ADP (with 5 mM pyruvate and 2 mM malate). Media temperature was maintained by a temperature-controlled base (heat-retaining gel pack and metal block).

**Statistics**

Data are presented as means ± S.E.M. Statistical analyses were performed using paired t tests, one-way or two-way ANOVA (as appropriate) with Student–Newman–Keuls method for analysis.
Muscle contraction increases mitochondrial respiratory sensitivity to ADP

of significance using Statistica software (StatSoft). DMSO (1.25%; +V) did not affect respiratory responses under any of the experimental conditions tested. Statistical analyses were performed including both −V and 1.25% DMSO (+V), but only +V data are presented for clarity. The alpha level of significance was set at \( P < 0.05 \).

RESULTS AND DISCUSSION

Inhibiting spontaneous contraction yields a higher \( K_m \) for ADP-stimulated respiration

We first tested the efficacy of inhibiting myosin-ATPase to prevent PmFB spontaneous contraction (Supplementary Movie S1). Visually, both BTS (results not shown) and BLEB (Figure 1A) substantially reduced the spontaneous contraction occurring in PmFBs from both red and white rat skeletal muscle in the absence of substrates, or during routine pyruvate/malate-supported ADP-stimulated respiration experiments (Figure 1B). Preventing contraction in rat PmFBs with BLEB increased the \( K_m \) value for ADP from \( \sim 75 \) to \( > 150 \mu M \) in red muscle, and from \( \sim 15 \) to \( > 100 \mu M \) in white muscle (Figures 2A–2D). In general, BTS was less effective than BLEB in red muscle, but tended to exert similar changes in \( K_m \) in white muscle (\( P = 0.059–0.087 \) compared with −V/+/V). Combining BLEB and BTS had no additional effect on \( K_m \) or \( V_{\text{max}} \) in either muscle (results not shown). These findings demonstrate that PmFBs spontaneously contract and that the inclusion of contraction inhibitors, particularly BLEB, lowers the respiratory sensitivity to ADP, yielding higher \( K_m \) values that are more reflective of rates of oxygen consumption at rest in vivo. Contraction of PmFBs therefore probably accounts for the higher sensitivities to ADP reported previously [9,12,26].

Temperature- and oxygen-dependent effects of spontaneous contraction on mitochondrial respiration in rat PmFBs

PmFBs remain relaxed and maintain respiratory function for as long as \( \sim 6 \) h when kept at 4°C in substrate-free buffer [26], but will begin to contract when transferred to the same buffer at 30°C (Figure 1A). A number of previous studies assessing respiratory kinetics have been performed at 25°C [9,12,26], where spontaneous contraction is not as evident in the absence of ADP but occurs readily upon ADP addition (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/437/bj4370215add.htm). To determine the influence of temperature on ADP-stimulated respiratory kinetics, rat PmFBs from both red and white muscle were subjected to ADP-titrations during complex I (pyruvate/malate)-supported respiration in the absence or presence of BLEB at 25°C or 37°C (see Supplementary Figures S2A–S2D at http://www.BiochemJ.org/bj/437/bj4370215add.htm), and compared with data obtained at 30°C. All experiments were conducted under air-saturated conditions (\( \sim 150–220 \mu M \) O₂) except in specific cases noted later. In RG (red gastrocnemius) PmFBs in the absence of BLEB (i.e. vehicle), the apparent \( K_m \) for ADP decreased markedly from \( > 200 \mu M \) at 25°C to \( \sim 75 \mu M \) at 30°C, and to \( < 25 \mu M \) at 37°C (Figure 3A). In WG (white gastrocnemius) PmFBs, \( K_m \) dropped from \( \sim 50 \mu M \) at 25°C to \( \sim 15 \mu M \) at 30 and 37°C (Figure 3C). By contrast, when contraction was inhibited by BLEB, the apparent \( K_m \) for ADP was \( > 200 \mu M \) in RG and >100 \μM in WG PmFBs at all three temperatures. In general, higher reaction temperatures did not affect the \( V_{\text{max}} \) for ADP-stimulated respiration in RG or WG, except in the presence of BLEB at 37°C in RG (Figures 3B and 3D). These findings demonstrate that, in the absence of a contraction inhibitor, the apparent \( K_m \) for ADP is markedly lower at higher temperatures. The effect is not mediated by temperature itself, but by an apparent acceleration of fibre contraction. Thus the more accurate \( K_m \) for ADP in resting muscle is obtained in contraction-inhibited PmFBs at 37°C. Importantly, however, contraction clearly increases the sensitivity of the respiratory system to ADP (up to \( \sim 10\)-fold) [24], indicating that the \( K_m \) for ADP in vivo is fluid depending on the level of contractile activity.

Temperature coefficients have been applied to respiratory flux data at 25°C and 30°C to convert into predicted respiration at 37°C (2.30 and 1.62 respectively, assuming a Q10 effect whereby flux doubles for a 10°C increase [27]). Using data from Figures 3(A)–3(D), the calculated ratios for 25–37°C conversion are \( \sim 1.1–1.3 \) in a contracted state and \( \sim 1.3–1.5 \) during relaxation (BLEB), with similar ratios for 30–37°C conversion (\( \sim 1.2–1.3 \) and \( \sim 1.0–1.4 \) during contraction and relaxation respectively, results not shown). The reason for the disparity from expected ratios is unclear. However, these results raise the possibility that factors present in PmFBs limit the effect of increased temperature on respiratory flux, and that the Q10 principle, although relevant to in vitro analyses of enzymatic activity, may not be applicable for converting respiratory flux in PmFBs, where the intracellular organization of metabolic and respiratory systems is preserved.

Oxygen concentrations as low as 20 \μM do not pose a limitation to respiration in isolated mitochondria; however, in

![Figure 1](http://www.BiochemJ.org/bj/437/bj4370215add.htm)  
**Figure 1**  Effect of spontaneous contraction on rat PmFB conformation

PmFB conformation in RG and WG following exposure to 30°C for 30 min is prevented by the myosin inhibitors BLEB and BTS (A, RG) alone (1× amplification) and (B) following ~30–45 min of ADP-stimulated respiration (0.6× amplification). +V, 1.25% DMSO.
Figure 2 Effect of contraction on ADP-stimulated mitochondrial respiratory kinetics in rat skeletal muscle

Effect of contraction on ADP-stimulated mitochondrial respiratory kinetics at 30°C in RG (A and B) and WG (C and D) PmFBs under air-saturated media (∼150–220 μM O₂). Results represent means ± S.E.M.; n = 4–5; ∗P < 0.05 compared with all other treatments at the same temperature.

Figure 3 Respiratory sensitivity to ADP and Vₘₐₓ is influenced by contraction in a temperature-dependent manner in rat skeletal muscle

Respiratory sensitivity is influenced by contraction in a temperature-dependent manner in rat RG (A and B) and WG (C and D) PmFBs under air-saturated media (∼150–220 μM O₂). Results represent means ± S.E.M.; n = 4–5; ∗P < 0.05 compared with all other treatments at same temperature; †P < 0.05, significant differences between all temperatures within the same treatment; ‡P < 0.05 compared with 30°C; or ††P < 0.05 compared with 37°C within same treatment.

PmFBs, the sensitivity to low oxygen supply is reportedly increased ∼100-fold owing to diffusion restrictions limiting oxygen supply to the core of the fibre bundle [28]. To offset this limitation, hyperoxygenation has been employed to maintain oxygen concentrations above air saturation (∼500–200 μM). To determine whether hyperoxia affects ADP respiratory kinetics when contraction is prevented, PmFBs from red and white muscle were studied in air-saturated (∼150–200 μM) and hyperoxic (∼275–450 μM) conditions in the absence or presence of BLEB at 30°C and 37°C. Interestingly, at 30°C, hyperoxia increased the apparent Kₘ for ADP 2–3-fold in both RG and WG (Figures 4A and 4C). In RG, the higher apparent Kₘ for ADP was evident regardless of whether BLEB was present. This sharp decrease in the sensitivity to ADP suggests that hyperoxia may alter the molecular components of oxidative phosphorylation, perhaps via oxidation of redox-sensitive proteins within the respiratory system [29]. Hyperoxia also increased Vₘₐₓ for ADP-stimulated respiration in RG in the absence of BLEB (Figure 4B), consistent with the notion of oxygen diffusion limitations in PmFBs under room air-saturated conditions (<200 μM O₂) described previously [28]. However, the effect of hyperoxia on Vₘₐₓ was not seen in RG when contraction was prevented or in WG in the absence or presence of the contraction inhibitor (Figure 4D).

In contrast with PmFBs at 30°C, hyperoxia at 37°C had no effect on the apparent Kₘ or Vₘₐₓ for ADP-stimulated respiration in RG or WG (Figures 4E–4H). As in earlier experiments conducted at 37°C (Figures 3A and 3C), preventing contraction with BLEB generated a markedly higher apparent Kₘ for ADP compared with PmFBs that were allowed to contract, but no difference was seen between air-saturated and hyperoxic conditions (Figures 4E and 4G). It is important to note that in RG in the absence of BLEB, hyperoxia at 30°C raised the apparent Kₘ for ADP to >250 μM (Figure 4A), whereas at 37°C the Kₘ remained below 40 μM regardless of whether hyperoxia was employed (Figures 4E and 4G). Moreover, the apparent Kₘ only increased to >250 μM when contraction was inhibited (+ BLEB; Figures 4E and 4G). These findings indicate that a 37°C reaction temperature is sufficient to prevent any effect of hyperoxia on ADP-stimulated respiratory kinetics, regardless of whether a contraction inhibitor is employed. Methodologically, this is important as initial hyperoxygenation of the respiratory buffer prevents oxygen from becoming rate-limiting during experiments with longer protocols. These findings further emphasize the importance of properly defining experimental conditions (i.e. contraction inhibitors, reaction temperature and oxygen concentrations) relative to the experimental questions being addressed when assessing respiratory function.

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Muscle contraction increases mitochondrial respiratory sensitivity to ADP

Figure 4 Respiratory sensitivity to ADP and $V_{\text{max}}$ are influenced by hyperoxic conditions in rat skeletal muscle

Hyperoxia (∼275–450 μM O₂) was compared with air-saturated media (AIR, ∼150–220 μM O₂) in a temperature-dependent manner in rat RG and WG PmFBs (A–H). Results represent means ± S.E.M.; n = 4–5; *P < 0.05 compared with other treatments at the same temperature; δP < 0.05 compared with AIR within the same treatment.

Temperature-dependent effects of spontaneous contraction on mitochondrial respiration in human PmFBs

To determine whether contraction and/or temperature influence respiratory kinetics similarly in human skeletal muscle, ADP titrations were performed on PmFBs from vastus lateralis muscle in the absence or presence of BTS or BLEB at 30 or 37°C under air-saturated conditions (Figures 5B and 5C). At 37°C, human PmFBs consistently broke apart in the absence of contraction inhibitors, making it impossible to express mitochondrial oxygen consumption to PmFB dry weight. In the presence of BTS or BLEB, however, PmFBs remained intact (Figure 5A). At 30°C, PmFBs remained intact independent of whether the contraction inhibitor was included. Compared with rodent muscle, human fibre bundles contain less connective tissue which may account for their fragility at 37°C, but this is only speculation. Regardless, oxygen consumption data at 37°C was normalized to PmFB wet weight to permit comparisons across all treatments (Figures 5C and 6C). Similar to observations in rats, preventing contraction with BLEB in human PmFBs increased the apparent $K_m$ for ADP from ∼80 μM (vehicle) to ∼175 μM at 30°C and ∼240 μM at 37°C (Figure 6A). BTS had a similar but less pronounced effect. $V_{\text{max}}$ was not different between treatments at 30°C and only slightly elevated in BLEB-treated fibres at 37°C (Figures 6B and 6C).

The contribution of phosphocreatine/creatine to ADP-stimulated mitochondrial respiratory kinetics

All of the experiments in PmFBs from rats and humans described above were performed with 20 mM creatine included in the respiration buffer. Creatine has been shown to markedly decrease the apparent $K_m$ for ADP in PmFBs of rats and humans to <50–100 μM. The effect is evident only in slow (or cardiac) fibres and has been interpreted to reflect the probable organization of intracellular energetic units utilizing creatine and phosphocreatine via CK to facilitate energy transfer between mitochondria and intracellular ATPases [30]. However, although a higher sensitivity to ADP might appear advantageous for the myofibre, as mentioned earlier this is difficult to reconcile with the fact that a $K_m$ of <50–100 μM in resting muscle predicts an in vivo respiration rate of ∼10–20% of $V_{\text{max}}$ (see the Experimental section for the calculation). The ratio of phosphocreatine/creatine...
predicts resting rates of ADP-stimulated respiration from experiments conducted at 25°C using a phosphocreatine/creatine ratio present in resting muscle yields kinetic data that is consistent with predicted rates of respiration in myofibres at rest.

**Perspectives and conclusions**

Collectively, the results from the present study demonstrate that the sensitivity of the mitochondrial respiratory system to ADP varies over a wide range and is directly related to the contractile state of the fibre, thereby optimizing the ability of skeletal muscle to respond efficiently and appropriately to large perturbations in energy demand. Why would skeletal muscle have evolved a system where the sensitivity of the mitochondrial respiratory system to ADP varies over such a wide range? In resting muscle, the lower sensitivity to ADP presumably reduces mitochondrial efficiency [i.e. the rate of ATP regeneration per rate of oxygen consumption (P/O ratio)] when energy demand is low which, together with proton leak, will tend to favour heat production. Conversely, when energy demand is elevated as a consequence of contractile activity, the increase in ADP sensitivity increases mitochondrial efficiency (P/O ratio), reflecting the priority given to energy regeneration during exercise.

The mechanism by which contraction modulates mitochondrial ADP sensitivity is unknown, but may be due to regulatory influences of force transmission from the cytoskeleton to the mitochondria itself [4,12]. ADP/ATP flux in and out of the mitochondria is mediated primarily by VDAC (voltage-dependent anion channel) in the outer membrane and ANT (adenine nucleotide translocator) in the inner mitochondrial membrane. The cytoskeletal protein tubulin has been shown previously to bind to and induce voltage-sensitive closure of VDAC in reconstituted systems [31]. When added to isolated mitochondria, tubulin partially restores the apparent high Km for ADP evident in PmFBs, presumably by restoring a limitation on VDAC-mediated permeability of the mitochondrial outer membrane. Further work will be required to establish whether reversible closure of VDAC by tubulin is responsible for regulating mitochondrial ADP sensitivity under different contractile/metabolic states. If true, this mechanism may explain how mitochondria are able to rapidly sense changes in energy demand, particularly at the onset of contraction before excessive energy deficits are created.

A number of factors that could be responsible for triggering the spontaneous contraction in PmFBs were tested, including the presence of residual ADP, redox-mediated control of SERCA (sarco/endoplasmic reticulum calcium ATPase) and cytosolic

**Figure 5** Effects of spontaneous contraction on human PmFB respiratory kinetics

(A) Conformation of PmFBs from human male vastus lateralis following ADP-stimulated respiration at 30°C without (+V) or with myosin inhibitors BLEB and BTS (0.6× amplification). (B and C) Effect of contraction on ADP-stimulated respiratory kinetics at 30°C and 37°C under air-saturated media (~150–220 μM O2). Results represent means ± S.E.M.; n = 5; *P < 0.05 compared with all other treatments at same temperature (Vmax).

in vivo fluctuates according to the energy turnover rate. One report has shown that a higher Km (~300 μM) is obtained with a ratio of phosphocreatine/creatine more typical of resting muscle (2:1, 24 mM phosphocreatine/12 mM creatine) during experiments conducted at 25°C [25]. To determine whether a phosphocreatine/creatine ratio typical of resting muscle affects ADP respiratory kinetics in the presence of contraction inhibitors at 37°C, we repeated experiments in human PmFBs in the presence of 24 mM phosphocreatine and 12 mM creatine under air-saturated conditions (Figure 7A). In the absence of contraction inhibitors (1.25% DMSO, + V), the apparent Km values were similar to previous reports (~350 μM) [25]. However, in the presence of BTS or BLEB, the apparent Km for ADP increased to ~820 μM and ~1560 μM respectively (Figure 7B). This value predicts resting rates of ADP-stimulated respiration in vivo to be <1% of Vmax, nearly 50-fold lower than previous estimates from isolated human mitochondria [5] and ~5-fold lower than previous estimates from human PmFBs [25]. Thus assessing respiratory function in the presence of a contraction inhibitor at 37°C using a phosphocreatine/creatine ratio present in resting muscle yields kinetic data that is consistent with predicted rates of respiration in myofibres at rest.

**Figure 6** Temperature-dependent effects of contraction on respiratory sensitivity to ADP and Vmax in human PmFBs

Experiments were performed under air-saturated media (~150–220 μM O2). Results represent means ± S.E.M.; n = 4; *P < 0.05 compared with all other treatments at the same temperature; †P < 0.05, significant differences between both temperatures within the same treatment.

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Figure 7 Effect of phosphocreatine/creatine balance on human PmFB respiratory kinetics

ADP-stimulated respiratory kinetics in the presence of 24 mM phosphocreatine and 12 mM creatine at 37°C, representative of ‘resting’ in vivo conditions. Results represent means ± S.E.M.; n = 4; *P < 0.05 compared with all other treatments at same temperature; #P < 0.05 compared with the same treatment with 20 mM creatine at 37°C (Figure 6). Experiments were conducted under air-saturated media (~150–220 μM O2).

Ca2+ accumulation. However, neither inhibition of CK by iodoacetamide to prevent possible residual ADP/ATP cycling between CK and myosin-ATPase, inclusion of the superoxide scavenger Tempol or TBQ [2,5-di(t-butyl)-1,4-hydroquinone] to prevent redox-triggered calcium flux through SERCA [2], nor preventing endogenous Ca2+ accumulation by EGTA (up to 5 mM) prevented spontaneous contraction (results not shown). Thus, the mechanism triggering spontaneous contraction in PmFBs remains unclear but does appear to be independent of CK-mediated cycling of endogenous adenine nucleotides or Ca2+. To conclude, the present paper reports the novel observation that spontaneous contraction occurs in PmFBs, which impart mediated cycling of endogenous adenine nucleotides or Ca2+. To conclude, the present paper reports the novel observation that spontaneous contraction occurs in PmFBs, which impart significant regulation on ADP-stimulated respiratory kinetics in a temperature-dependent manner. We report the highest significant regulation on ADP-stimulated respiratory kinetics that spontaneous contraction occurs in PmFBs, which impart controlled regulation of respiration by ADP in skeletal muscle, providing a more efficient coupling of ATP-producing to ATP-consuming systems at a time when energy turnover can rapidly and dramatically increase. From a methodological standpoint, these findings also highlight the importance of properly defining the objectives, analytical protocols and interpretive boundaries within these different parameters when designing clinical diagnostic tests and basic research experiments. Such considerations are critical in view of the rapidly expanding interests in mitochondrial processes regulating basic cell function and disease aetiology.

AUTHOR CONTRIBUTION

Christopher Perry, Daniel Kane, Ethan Anderson and Darrell Neuffer contributed to study design and conception. Christopher Perry, Daniel Kane, Chien-Te Lin and Brook Callery conducted rodent experiments. Christopher Perry, Rachel Kozy, Brook Callery, Daniel Lark, Constance Kane, Patricia Brophy and Timothy Gavin conducted human tissue experiments. Christopher Perry, Daniel Kane and Darrell Neuffer were the primary writers of the manuscript and all authors participated in manuscript preparation. Darrell Neuffer directed the project.

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Inhibiting myosin-ATPase reveals a dynamic range of mitochondrial respiratory control in skeletal muscle

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SUPPLEMENTARY ONLINE DATA

Figure S1 Effect of ADP on rat PmFB conformation

Sprague–Dawley rat RG PmFB contraction following exposure to 2 mM ADP in the presence of 5 mM pyruvate and 2 mM malate at 25 °C in buffer Z. The addition of ADP can be seen at 0 min.

Figure S2 Effect of PmFB contraction on respiratory kinetics in rat PmFB

Effect of contraction on ADP-stimulated mitochondrial respiratory kinetics at 25°C and 37°C in RG (A and B) and WG (C and D) PmFBs under air-saturated media (∼150–220 μM O2). Results represent means ± S.E.M.; n = 4–5; *P < 0.05 compared with all other treatments at the same temperature.