In vivo ATP production during free-flow and ischaemic muscle contractions in humans

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The aim of this study was to determine how ATP synthesis and contractility *in vivo* are altered by ischaemia in working human skeletal muscle. The hypotheses were: (1) glycolytic flux would be higher during ischaemic (ISC) compared to free-flow (FF) muscle contractions, in compensation for reduced oxidative ATP synthesis, and (2) ischaemic muscle fatigue would be related to the accumulation of inhibitory metabolic by-products rather than to the phosphorylation potential ([ATP]/[ADP][P_i]) of the muscle. Twelve healthy adults (6 men, 6 women) performed six intermittent maximal isometric contractions of the ankle dorsiflexors (12 s contract, 12 s relax), once with intact blood flow and once with local ischaemia by thigh cuff inflation to 220 Torr. Intracellular phosphorous metabolites and pH were measured non-invasively with magnetic resonance spectroscopy, and rates of ATP synthesis through oxidative phosphorylation, anaerobic glycolysis, and the creatine kinase reaction were determined. The force–time integral declined more during ISC (66 ± 3% initial) than FF (75 ± 2% initial, $P = 0.002$), indicating greater fatigue in ISC. [ATP] was preserved in both protocols, indicating matching of ATP production and use under both conditions. Glycolytic flux (mM s^{-1}) was similar during FF and ISC ($P = 0.16$). Total ATP synthesis rate was lower during ISC, despite adjustment for the greater muscle fatigue in this condition ($P < 0.001$). Fatigue was linearly associated with diprotonated inorganic phosphate (FF $r = 0.94 \pm 0.01$, ISC $r = 0.92 \pm 0.02$), but not phosphorylation potential. These data provide novel evidence that ATP supply and demand *in vivo* are balanced in human skeletal muscle during ischaemic work, not through higher glycolytic flux, but rather through increased metabolic economy and decreased rates of ATP consumption as fatigue ensues.

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Chemical energy in the form of adenosine triphosphate (ATP) is essential to maintain function in contracting skeletal muscle cells. Despite the approximately 100-fold increase in ATP consumption from rest to maximal-intensity contractions, the energetic demands of muscle are usually satisfied without depleting intracellular ATP (Dawson *et al.* 1977). Under most conditions, the demand for ATP is matched by ATP production from mitochondrial oxidative phosphorylation and anaerobic glycolysis, with temporal buffering from the creatine kinase reaction.

During steady-state muscle activity, where the supply of oxygen and other substrates to the mitochondria are not limited, the rate of oxidative phosphorylation increases in proportion to increases in muscle work (i.e. ATPase activity) (Chance *et al.* 1985). During ischaemia, however, the contribution of oxidative phosphorylation to the ATP pool will diminish as intracellular oxygen tension (P_{O_2}) falls below the level required to support mitochondrial ATP synthesis (Connett *et al.* 1990). It has been suggested that, under these conditions, the balance between ATP supply and demand can be accomplished by increasing reliance on ATP synthesis via substrate-level phosphorylation, which assumes that the glycolytic rate is well below the capacity for this pathway *in vivo*. While there is some evidence that muscle ATP supply is maintained during ischaemia by a compensatory increase in glycolytic flux (King *et al.* 1987; Kemp *et al.* 1994; Timmons *et al.* 1996), this finding is not universal (Conley *et al.* 1998; Hogan *et al.* 1998) and appears to depend on muscle fibre type (Greenhaff *et al.* 1993), contraction intensity (Linnarsson *et al.* 1974), and the precise manner in which oxygen delivery to the working muscle is limited (King *et al.* 1987; Gutierrez *et al.* 1988; Stainsby *et al.* 1990).

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In contrast to this concept of an energetic adjustment to ischaemia, several investigators have suggested that the balance between ATP supply and demand in ischaemic muscle is accomplished by reducing force development, and thus decreasing the demand by the ATPases (Hultman & Sjoholm, 1983; Van der Laarse *et al.* 1989; Hogan *et al.* 1996; Meyer & Foley, 1996). Indeed, many investigators have reported greater muscle fatigue, defined as an exercise-induced reduction in muscle force-generating capacity, during ischaemic compared to free-flow contractions (Harris *et al.* 1975; Greenhaff *et al.* 1993; Kemp *et al.* 1994; Hogan *et al.* 1999). However, greater fatigue during ischaemia may result from direct inhibition of contractile processes by the metabolic products consequent to increased glycolytic flux (Fabiato & Fabiato, 1978). Alternatively, a lower cytosolic phosphorylation potential ($[ATP]/[ADP][P_i]$), a proxy for the free energy of ATP hydrolysis (ΔG_{ATP}), may act as a thermodynamic 'braking function' on ATP turnover, thereby causing muscle force production to decline as ATP hydrolysis becomes less favourable (Arthur *et al.* 1992; Meyer & Foley, 1996). The former possibility is supported by studies *in vitro* demonstrating the direct effects of diprotonated P_i on muscle contractility (Nosek *et al.* 1987), and correlations *in vivo* between fatigue and $H_2PO_4^-$ (Miller *et al.* 1988), which accumulates in the muscle during glycolytic ATP turnover. The latter possibility is supported by reports of tight coupling between ATP supply and muscle force production (Hultman & Sjoholm, 1983; Dawson *et al.* 1978), particularly in the absence of inhibitory metabolite accumulation (Hogan *et al.* 1996).

The aim of the present study was to determine if a balance between ATP supply and demand is achieved during ischaemic muscle contractions *in vivo*, and to investigate the following potential mechanisms for this balance: (1) increased glycolytic flux in comparison to free-flow conditions, (2) decreased ATP demand (i.e. fatigue) induced by the accumulation of inhibitory metabolites, or (3) fatigue induced by the coupling of ATP demand to ATP supply, mediated by decreased ATP turnover in response to a reduced intracellular phosphorylation potential and independent of inhibitory metabolite levels. We hypothesized that (1) glycolytic flux would be higher during ischaemic compared to free-flow muscle contractions, presumably in compensation for reduced oxidative ATP synthesis, and (2) muscle fatigue would be related to accumulation of inhibitory metabolites rather than the intracellular phosphorylation potential.

Methods

Subjects

Twelve healthy, untrained subjects were enrolled in this study (6 men, 6 women, age 21–35 years). Written informed consent was obtained from all subjects, as approved by the University of Massachusetts, Amherst and the Yale University School of Medicine human subjects review boards. The study conformed with the *Declaration of Helsinki*.

Experimental design

Subjects performed two ankle dorsiflexion exercise protocols consisting of six 12 s isometric maximal voluntary contractions (MVC) with 12 s rest intervals between each MVC. The protocol was performed on one leg with intact blood flow (free-flow, FF) and again on the contralateral leg with circulatory occlusion (ischaemia, ISC). Approximately 1 h of rest was allowed between protocols, and the protocol was randomized as to which leg was tested first. Intracellular pH and phosphorous metabolites were measured continuously during rest, contractions, and a 10 min recovery period using phosphorous magnetic resonance spectroscopy (^{31}P-MRS). These data were used to determine the rates of ATP synthesis via oxidative phosphorylation (ATP_{OX}), anaerobic glycolysis (ATP_{GLY}), and PCr breakdown (ATP_{CK}).

Subjects were tested on two occasions, separated by at least 48 h. The first testing session took place in the Muscle Physiology Laboratory at the University of Massachusetts, where subjects were familiarized with the contraction protocols. We also assessed the degree of voluntary activation during baseline MVCs and at the end of the FF and ISC protocols. The second session took place in the Magnetic Resonance Research Center at Yale University, where subjects repeated both exercise protocols with simultaneous measures of force and intramuscular energy metabolism.

Preliminary testing

Subjects were positioned supine with their leg secured to a custom-built device that allowed measurements of ankle dorsiflexion force, surface electromyography (EMG), and electrical stimulation of the peroneal nerve as previously described (Russ & Kent-Braun, 2003). The foot was secured to a force transducer with a Velcro strap across the metatarso-phalangeal joint. A thermoplastic foot mould was used to provide an even dispersion of force across the foot. The lower leg was further immobilized with a strap just distal to the knee to minimize movement during exercise. A pair of 10 mm diameter stimulating electrodes was affixed over the common peroneal nerve, just distal to the fibular head. A recording EMG electrode was placed over the belly of the tibialis anterior muscle with the reference electrode over the distal tendon. Analog signals corresponding to force and EMG were acquired

and digitized at 500 Hz using customized Labview software (National Instruments, Austin, TX, USA). Prior to the ISC trials, a blood pressure cuff was placed around the proximal thigh and connected to a rapid cuff inflator (Hokanson, Inc., Belleview, WA, USA).

Subjects performed three brief MVCs (3–4 s duration) to assess strength, with 2 min of rest between trials. Peak force from the three trials was taken as baseline MVC. The ability to achieve full voluntary activation was assessed by measuring the central activation ratio (Kent-Braun & Le Blanc, 1996). Briefly, a train of supramaximal electrical stimuli (50 Hz, 500 ms) was imposed during the third baseline MVC. A central activation ratio was calculated as the ratio of peak voluntary force to the peak force produced during the superimposed tetanus. A ratio less than 1.0 indicates incomplete voluntary activation.

Following baseline MVC and central activation measures, subjects performed either the FF or ISC contraction protocol. Strong verbal encouragement was provided consistently to all subjects. Subjects also received visual feedback of their force output from a series of light-emitting diodes that were scaled to the baseline MVC. During the last 2 s of the final contraction, a stimulus train was imposed to assess central activation. The protocol was repeated on the contralateral leg with a thigh cuff inflated to 220 Torr. The cuff was inflated for 30 s prior to the start of the contraction series. Muscle fatigue during FF and ISC protocols was defined as the relative decline in the force–time integral (FTI) of each 12 s contraction: fatigue $= \mathrm{FTI}/\mathrm{FTI}_{\mathrm{initial}} \times 100$.

Metabolic testing

Subjects fasted for 4 h prior to the test and then consumed a nutritional supplement bar (22 g carbohydrate, 6 g fat, 15 g protein) approximately 30 min prior to the test. An MR probe assembly consisting of a 6 cm diameter ^{1}H surface coil and a coplanar 3 cm × 5 cm elliptical ^{31}P surface coil was secured over the tibialis anterior muscle. Subjects were positioned supine in a 4.0 tesla, 72 cm clear bore superconducting magnet (Bruker Biospin, Rheinstetten, Germany) interfaced with Paravision software. The foot was secured in a non-magnetic exercise apparatus, as described for the preliminary testing. Transverse gradient echo scout images of the lower leg were acquired to ensure correct positioning of the subject in the isocentre and to select a region of interest within the tibialis anterior muscle for localized shimming on the muscle water peak using the FASTMAP method. This shimming procedure yielded a full width at half-maximal height of the unfiltered PCr peak of 13 ± 8 Hz (mean ± S.D., all subjects).

While positioned in the magnet, but prior to acquisition of ^{31}P-MRS data, subjects performed two to three brief (3–4 s) MVCs separated by 2 min of rest to establish MVC force for that leg and to provide a standardized warm-up to allow for the potentiation of blood flow in response to muscle contractions (Wigmore *et al.* 2004). Two minutes after these baseline MVCs were completed, subjects performed the series of six 12 s MVCs with 12 s rest intervals under FF conditions, followed by a 10 min recovery period. Verbal encouragement and visual feedback were provided to subjects as described for preliminary testing. The subject was then removed from the magnet and repositioned to study the opposite leg after a short rest period (20–60 min). Proton imaging, shimming, and baseline MVCs were repeated. The series of 12 s contractions was then performed with circulatory occlusion, as described for preliminary testing.

^{31}P-MR spectra (125 μs hard pulse, nominal 60 deg flip angle, 2 s repetition time, 2048 data points, 8000 Hz spectral width) were obtained continuously during 1 min of rest before the contraction protocols and during the FF and ISC protocols and subsequent 10 min recovery periods. The free induction decays were then averaged to yield temporal resolution of 1 min at rest, 4 s during the contraction protocols, 12 s during the first 5 min of recovery, and 30 s during the final 5 min of recovery. A stacked plot of rest and exercise spectra for a typical subject is shown in Fig. 1.

Spectral analyses

Exponential multiplication corresponding to 10 Hz line broadening was applied to the free-induction decays before they were Fourier-transformed to the frequency domain. After phasing and baseline correction, the underlying

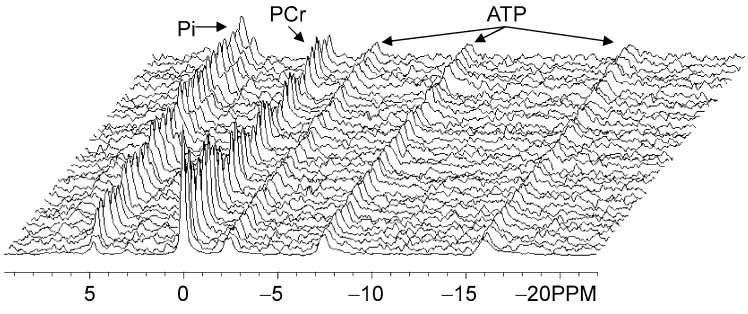

Figure 1. ^{31}P MRS spectra from the tibialis anterior muscle in a single subject
Representative stacked plot of phosphorous spectra at rest (first spectrum, 60 s average) and throughout the six 12 s MVCs with 12 s rest intervals (4 s temporal resolution). The P_i peak split into two distinct peaks during the contractions in this subject.

broad peak due to the phosphorous in bone was removed by calculating a 5th order polynomial fit of the baseline region. Peaks corresponding to PCr, phosphomonoesters (PMEs), P_i, and γ, α and β ATP were then fitted with Lorentzian-shaped curves (NUTS software, Acorn NMR, Livermore, CA, USA) to quantify the area of each peak. When two distinct P_i peaks were observed, two Lorentzian-shaped curves were used.

Metabolic calculations

Millimolar concentrations of phosphorous metabolites were calculated assuming that $[PCr] + [Cr] = 42.5$ mM and resting $[ATP] = 8.2$ mM (Harris *et al.* 1974). According to the equilibrium of the creatine kinase reaction and impermeability of the cell to P_i and free creatine, we assumed that $\Delta[P_i]$ is equivalent to $\Delta[Cr]$ during rest, exercise, and recovery(Kemp & Radda, 1994). Corrections for partial saturation of the ^{31}P-MRS peaks due to the rapid repetition time of the MR protocol were applied as previously described (Lanza *et al.* 2005). Intramuscular pH was calculated based on the chemical shift (σ) of P_i relative to PCr in parts per million:

$$\mathrm{pH} = 6.75 + \log \frac{\sigma - 3.27}{5.69 - \sigma} \quad (1)$$

When distinct P_i splitting was evident, the pH corresponding to each P_i pool was calculated separately as pH_1 and pH_2 based on the chemical shift of each peak relative to PCr. The overall muscle pH was then calculated for each spectrum as:

$$\text{Overall pH} = \mathrm{pH}_1 \frac{\text{area } P_{i_1}}{\text{total } P_i \text{ area}} + \mathrm{pH}_2 \frac{\text{area } P_{i_2}}{\text{total } P_i \text{ area}} \quad (2)$$

The concentration of the diprotonated form of inorganic phosphate ($H_2PO_4^-$) was calculated as:

$$H_2PO_4^- = \frac{[P_i]}{1 + 10^{\mathrm{pH}-6.75}} \quad (3)$$

Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) concentrations were calculated based on the equilibrium of the creatine kinase and adenylate kinase reactions, respectively, and the stoichiometry of free creatine and inorganic phosphate (Lawson & Veech, 1979):

$$[ADP] = \frac{[ATP]\,[P_i]}{K_{CK}\,[PCr]} \quad (4)$$

$$[AMP] = \frac{[ADP]^2\,K_{AK}}{[ATP]} \quad (5)$$

The equilibrium constants of the creatine kinase (K_{CK}) and adenylate kinase (K_{AK}) reactions were corrected for the effects of pH, assuming free magnesium concentration of 1 mM (Golding *et al.* 1995).

ATP synthesis from oxidative phosphorylation

The rates of mitochondrial ATP synthesis (ATP_{OX}, mM s^{-1}) were determined from the rate of PCr recovery during the 12 s rest intervals between contractions (Kemp & Radda, 1994; Jubrias *et al.* 2001). This model of oxidative phosphorylation assumes that the recovery of PCr is due entirely to oxidative ATP synthesis and that anaerobic glycolysis ceases in the absence of muscle contraction (Quistorff *et al.* 1993; Blei *et al.* 1993). However, in the present study, we observed a small amount of PCr recovery during ISC, attributable to glycolytic ATP synthesis (Crowther *et al.* 2002; Jubrias *et al.* 2003). As a result, PCr recovery between contractions in the FF protocol was adjusted for the glycolytic contribution as follows:

$$ATP_{OX} = dPCr/dt_{(total)} - dPCr/dt_{(glycolytic)} \quad (6)$$

Oxidative ATP synthesis was assumed to be negligible during ISC contractions since the oxygen trapped within the muscle during cuff occlusion would only be sufficient to generate ~4 mM ATP (Harris *et al.* 1975; Kemp *et al.* 1994), an amount that would be exhausted in ~10 s under the contraction conditions used here.

ATP synthesis from anaerobic glycolysis

Glycolytic flux (ATP_{GLY}, mM s^{-1}) was calculated during each 12 s MVC from exercise-induced changes in intramuscular pH and metabolites, after correcting for proton efflux, buffering capacity, the consumption of protons by the creatine kinase reaction, and a small contribution of oxidative metabolism to the proton pool (Kemp & Radda, 1994; Walter *et al.* 1999).

$$ATP_{GLY} = 1.5\,(-\beta(dpH/dt) + \theta\,(dPCr/dt) - mATP_{OX} - \upsilon_{eff}) \quad (7)$$

The terms dpH/dt and $dPCr/dt$ are the changes in pH and PCr for each time interval, respectively. The correction factor θ accounts for the consumption of protons when PCr is hydrolysed in the creatine kinase reaction:

$$\theta = \frac{1}{1 + 10^{\mathrm{pH}-6.75}} \quad (8)$$

This calculation of the proton stoichiometry coefficient does not account for the possible effects of intracellular Mg^{2+} and K^+ on proton stoichiometry, which may affect the magnitude of the coefficient (Kushmerick, 1997). However, this possible effect would not be expected to have a significant impact on the comparisons across conditions in the present study.

The correction factor m accounts for the production of protons during oxidative ATP synthesis:

$$m = \frac{0.16}{1 + 10^{6.1-\mathrm{pH}}} \quad (9)$$

β represents the pH buffering capacity (slykes) of the muscle, which takes into account the inherent buffering capacity of the muscle (β_{i}) and buffering due to muscle bicarbonate ($\beta_{\mathrm{CO_2}}$), inorganic phosphate ($\beta_{\mathrm{P_i}}$) and phosphomonoesters (β_{PME}), which are calculated throughout each protocol, as discussed elsewhere (Kemp & Radda, 1994; Walter *et al.* 1999):

$$\beta = \beta_{\mathrm{i}} + \beta_{\mathrm{CO_2}} + \beta_{\mathrm{P_i}} + \beta_{\mathrm{PME}} \quad (10)$$

$$\beta_{\mathrm{CO_2}} = \frac{2.3 S P_{\mathrm{CO_2}} \times 10^{\mathrm{pH}-6.1}}{(1 + 10^{\mathrm{pH}-6.1})(1 + 10^{6.1-\mathrm{pH}})} \quad (11)$$

where the solubility of CO_2 in muscle water, S, is 1.3 mM kPa^{-1} and the partial pressure of CO_2 ($P_{\mathrm{CO_2}}$) is 5 kPa.

$$\beta_{\mathrm{P_i}} = \frac{2.3\,[\mathrm{P_i}]}{(1 + 10^{\mathrm{pH}-6.75})(1 + 10^{6.75-\mathrm{pH}})} \quad (12)$$

$$\beta_{\mathrm{PME}} = \frac{2.3\,[\mathrm{PME}]}{(1 + 10^{\mathrm{pH}-6.2})(1 + 10^{6.2-\mathrm{pH}})} \quad (13)$$

Inherent buffering capacity was determined from changes in pH and PCr during the first 4 s of exercise when glycolysis and proton efflux are assumed to have a negligible effect on pH changes:

$$\beta_{\mathrm{i}} = \frac{m\mathrm{ATP_{OX}} - \theta(\mathrm{dPCr/d}t)}{\mathrm{dpH/d}t} - \beta_{\mathrm{CO_2}} - \beta_{\mathrm{P_i}} - \beta_{\mathrm{PME}} \quad (14)$$

The assumption of negligible glycolysis or proton efflux during the first 4 s of contraction stems from demonstrations that the initial pH change during contraction is entirely attributable to proton consumption or strong ion changes as the creatine kinase reaction proceeds in the forward direction (Adams *et al.* 1990). Furthermore, at least 10 s are required to achieve full activation of glycolysis during muscle contractions (Meyer *et al.* 1988). Other studies in human (Conley *et al.* 1997) and frog (Yamada & Sugi, 1987) muscle have also reported a delay in the onset of glycolysis. Proton efflux from the cell is also assumed to be initially negligible since intracellular proton accumulation is the driving force behind the Na^+/H^+ antiporter and lactate–H^+ cotransporter (Bendahan *et al.* 2003), and proton accumulation is insignificant during the first 4 s of contraction.

Proton efflux rates during exercise (υ_{eff}, mM (pH unit)$^{-1}$ min^{-1}) were estimated for each 12 s contraction, based on rates calculated during the postcontraction recovery period (Kemp & Radda, 1994; Walter *et al.* 1999) and assuming linear pH dependence of lactate–proton coefflux in muscle (Bangsbo *et al.* 1993; Newcomer *et al.* 1999). After the initial phase of acidosis early in recovery, pH increases linearly as a function of net proton efflux from the cell. A proportionality constant, λ, was determined from the slope of υ_{eff} *versus* dpH/dt during the linear portion of recovery where:

$$\upsilon_{\mathrm{eff}} = \beta(\mathrm{dpH/d}t) + m\mathrm{ATP_{OX}} + \theta\,(\mathrm{dPCr/d}t) \quad (15)$$

When these major factors contributing to the change in pH during recovery are accounted for, the remaining dpH/dt represents the rate of proton efflux. The proportionality constant, λ, was used to estimate proton efflux during the contraction protocol:

$$\upsilon_{\mathrm{eff}} = \lambda(\mathrm{pH_{rest}} - \mathrm{pH_{observed}}) \quad (16)$$

During ischaemia, we assumed negligible oxidative ATP synthesis and proton efflux (Kemp & Radda, 1994), thus simplifying the glycolytic rate calculation to:

$$\mathrm{ATP_{GLY}} = 1.5\,(-\beta\,(\mathrm{dpH/d}t) + \theta\,(\mathrm{dPCr/d}t)) \quad (17)$$

ATP synthesis from PCr breakdown

The rate of ATP synthesis from the net breakdown of PCr via the CK reaction ($\mathrm{ATP_{CK}}$, mM s^{-1}) was determined from the change in PCr during each contraction. Since the synthesis of ATP is stoichiometric with the hydrolysis of PCr in the creatine kinase reaction, the calculation of CK takes the simple form (Kemp & Radda, 1994):

$$\mathrm{ATP_{CK}} = \mathrm{dPCr/d}t \quad (18)$$

Total ATPase rate

The total ATPase rate ($\mathrm{ATP_{TOT}}$ mM s^{-1}) for each contraction was determined as (Kemp & Radda, 1994; Walter *et al.* 1999):

$$\mathrm{ATP_{TOT}} = \mathrm{ATP_{OX}} + \mathrm{ATP_{GLY}} + \mathrm{ATP_{CK}} \quad (19)$$

For each contraction, $\mathrm{ATP_{CK}}$ and $\mathrm{ATP_{GLY}}$ were also scaled to muscle force production ($\mathrm{FTI/FTI_{initial}}$), which allows comparisons across FF and ISC protocols by adjusting for any differences in muscle force due to fatigue. Metabolic economy (ME, N s (mM ATP)$^{-1}$) was determined over the course of each contraction protocol as:

$$\mathrm{ME} = \frac{\sum \mathrm{FTI}}{\sum \mathrm{ATP_{TOT}}} \quad (20)$$

Exploratory studies

To provide an estimate of the intracellular oxygen content under ischaemic conditions, exploratory studies of myoglobin desaturation were performed in one representative subject. The deoxygenated form of myoglobin (dMb) was measured during ISC using ^{1}H-MRS (Jue & Anderson, 1990). Proton spectra were acquired using a 500 μs frequency-selective Gaussian pulse, 25 ms repetition time, centred on dMb (~80 p.p.m. downfield from water). To minimize baseline artefacts due to the water signal, a water suppression pulse was applied (500 μs Gaussian pulse, centred on water, 5 ms crusher gradient). Spectra were acquired throughout the ISC protocol and during a 10 min bout of ischaemia at rest, obtained 10 min following unoccluded recovery from the ISC protocol.

Using NUTS software, dMb data were averaged to yield 12 s time resolution. Data were zero-filled, and exponential multiplication was applied using a mixed Lorentzian-Gaussian function (−300 Hz/0.1) prior to Fourier transformation. Spectra were phased (0 and 1st order) and baseline-fitted before an automated curve-fitting procedure was applied to the dMb peak for each spectrum. All dMb values were expressed relative to the peak value obtained during the 10 min ischaemic rest period, taken to reflect complete desaturation of the muscle. We used the oxygen binding curve for myoglobin to calculate intracellular P_{O_2}, assuming a P_{50} for myoglobin of 3.2 Torr, where f is the fractional desaturation of myoglobin (Richardson *et al.* 1995):

$$P_{O_2} = P_{50} \cdot \frac{1-f}{f} \quad (21)$$

On a separate day, the same subject repeated the contraction protocol following 10 min of ischaemia prior to the first contraction with ^{31}P-MRS measures obtained continuously and analysed as described above.

Statistical analyses

Student's t test for paired data was used to compare end-exercise [PCr], [P_i], [ADP], [AMP], [PME], [ATP], [$H_2PO_4^-$], pH, fatigue, and metabolic economy in FF and ISC. The non-normal distribution of CAR values necessitated non-parametric analyses using the Mann-Whitney U test. The relationships between FTI (% initial) and both $H_2PO_4^-$ and phosphorylation potential ([ATP]/[ADP][P_i]) were determined for each subject using linear regression analyses. Two-way (condition, time) repeated measures ANOVA was used to compare ATP_{GLY}, ATP_{CK} and ATP_{TOT} between FF and ISC contractions. One-way (time) repeated measures ANOVA was used to examine ATP_{OX} during the FF condition. The appropriate covariance structures were defined for each analysis. All analyses were performed using SAS software (Cary, NC, USA).

Results

Subject characteristics

Subjects were 27 ± 4 (mean ± s.d.) years of age. Height and mass were 171 ± 10 cm and 71 ± 17 kg, respectively. Systolic brachial and ankle blood pressures were 112 ± 11 Torr and 129 ± 14 Torr, respectively.

Force and central activation

All subjects were able to fully activate the ankle dorsiflexor muscles as evidenced by CAR values of ~1.0 in the unfatigued state (Table 1). Baseline MVC was ~5% higher prior to ISC compared to FF (Table 1). During the FF and ISC contraction protocols, the force–time integral progressively decreased (Fig. 2), with greater fatigue occurring during ISC compared to FF (Table 1). Although some central activation failure was evident at the end of both contraction protocols (Table 1), central fatigue appeared to play a minor role in the development of muscle fatigue, as evidenced by mean CAR = 0.92 during the final contraction in both protocols.

Phosphorous metabolites and pH

Resting [PCr], [P_i] and pH were similar prior to the two protocols (Table 1). Phosphocreatine decreased during each 12 s MVC in FF and ISC (Fig. 3*A*). Subsequently, PCr increased during the rest intervals between contractions, although the magnitude of this recovery was blunted during ISC compared to FF (Fig. 3*A* inset). At the end of the contraction protocol, PCr was depleted more in ISC compared to FF (Table 1). As expected, changes in P_i during the two protocols mirrored the changes in PCr (data not shown), and at the end of the contraction protocol P_i was higher in ISC compared to FF (Table 1). The end-exercise concentrations of ATP were similar across contraction protocols (Table 1) and did not change significantly from the assumed resting value of 8.2 mM. At the onset of FF and ISC, pH initially increased (Fig. 3*B*), and thereafter declined similarly during FF and ISC, although there was a trend for greater acidosis in ISC during the final contraction (Fig. 3*B*, Table 1). Phosphomonoesters (PMEs), consisting primarily of glucose-6-phosphate (Bloch *et al.* 1993), increased to a greater degree in ISC compared to FF, as did ADP and AMP (Table 1). Diprotonated P_i was similar at rest but increased to a greater extent in ISC compared to FF (Table 1).

Table 1. Selected variables from FF and ISC protocols

	Free flow	Ischaemia	*P*	95% CI
Force				
MVC_{pre} (N)	323 ± 27	335 ± 27	0.04	−24.7, −0.4
Fatigue ($FTI_{post}/FTI_{pre} \times 100$)	74.2 ± 2.3	64.3 ± 2.9	<0.001	6.0, 13.8
CAR_{pre}	0.99 ± 0.005	1.0 ± 0.001	0.26	−0.002, 0.004
CAR_{end}	0.92 ± 0.002	0.97 ± 0.001	0.11	−0.001, 0.01
Metabolites at rest				
[PCr] (mM)	38.2 ± 0.02	38.0 ± 0.03	0.54	−0.56, 1.02
[P_i] (mM)	4.3 ± 0.2	4.5 ± 0.3	0.71	−0.95, 0.66
pH	7.00 ± 0.01	7.01 ± 0.01	0.25	−0.04, 0.01
$H_2PO_4^-$ (mM)	1.5 ± 0.1	1.6 ± 0.1	0.89	−0.26, 0.23
PME (mM)	3.0 ± 0.4	2.7 ± 0.3	0.75	−1.1, 1.4
ADP (mM)	0.008 ± 0.0	0.008 ± 0.0	0.50	−0.002, 0.001
AMP (μM)	0.007 ± 0.0	0.008 ± 0.0	0.40	−0.004, 0.002
Metabolites at end-exercise				
[PCr] (mM)	9.9 ± 0.9	5.1 ± 0.8	<0.001	2.7, 6.8
[P_i] (mM)	32.6 ± 0.9	37.4 ± 0.8	<0.001	−6.8, −2.7
pH	6.70 ± 0.03	6.60 ± 0.05	0.10	−0.02, 0.22
$H_2PO_4^-$ (mM)	17.1 ± 0.9	21.4 ± 1.1	0.004	−6.9, −1.6
PME (mM)	8.4 ± 0.6	11.4 ± 1.3	0.07	−6.44, 0.29
ADP (mM)	0.15 ± 0.03	0.39 ± 0.09	0.02	−0.40, −0.04
AMP (μM)	2.7 ± 0.9	23.6 ± 8.9	0.04	−40.2, −1.4
[ATP] (mM)	8.5 ± 0.5	8.2 ± 0.6	0.67	−1.1, 1.7

Values are means ± S.E.M. 95% confidence intervals are presented for the difference between FF and ISC.

Pathways of ATP synthesis

Figure 4 shows absolute rates (mM s^{-1}) of ATP synthesis through ATP_{OX}, ATP_{GLY} and ATP_{CK} for each contraction. We also examined ATP synthesis rates after scaling to FTI during each contraction, to account for changes in the level of muscle force production as fatigue developed. For ease of presentation, we show only the absolute ATP synthesis rates, as scaling ATP flux to muscle fatigue produced qualitatively similar results.

The rate of oxidative ATP synthesis was constant throughout the FF contraction protocol (Fig. 4*A*, $P_{time} = 0.12$). Glycolytic flux was similar during FF and ISC ($P_{condition} = 0.22$, Fig. 4*B*). In both conditions, ATP_{GLY} increased over the course of the six contractions to a similar extent ($P_{time} < 0.001$, $P_{condition \times time} = 0.75$).

The rates of ATP generated by net PCr hydrolysis were higher during FF compared to ISC contractions (Fig. 4*C*, $P_{condition} < 0.001$), presumably due to the greater resynthesis of PCr during FF rest intervals. In both conditions, the contribution from ATP_{CK} decreased as the contractions progressed ($P_{time} < 0.001$), with the magnitude of this decline greater during ISC ($P_{condition \times time} < 0.001$).

The total ATPase rate was higher in FF compared to ISC (Fig. 4*D*, $P_{condition} < 0.001$). Total ATPase rate declined in the ischaemic condition, but not to an extent that was different from the FF ($P_{time} = 0.02$, $P_{condition \times time} = 0.19$). Metabolic economy was higher during ISC (166 ± 17 N s (mM ATP)$^{-1}$) compared to FF (126 ± 14 N s (mM ATP)$^{-1}$, $P = 0.03$).

Relationships between metabolites and fatigue

There was a strong linear relationship between FTI and intracellular $H_2PO_4^-$ during FF (Fig. 5*A*, $r = 0.94 \pm 0.01$) and ISC ($r = 0.92 \pm 0.02$). In contrast, the relationship

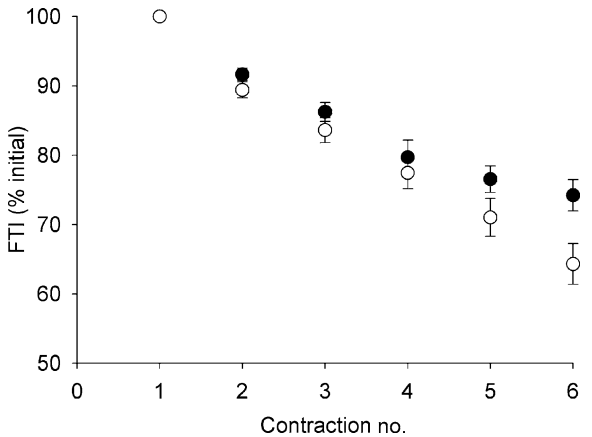

Figure 2. Muscle fatigue of the ankle dorsiflexors
The force–time integral during the 12 s MVCs, expressed relative to the first contraction, with (ISC, ○) and without (FF, ●) local circulatory occlusion. There was more fatigue during ISC compared to FF ($P < 0.001$). Values are means ± S.E.M.

between fatigue and [ATP]/[ADP][P_i] was curvilinear and shifted to the left with ischaemia, such that lower cytosolic phosphorylation potentials were observed at similar levels of fatigue in ISC compared to FF (Fig. 5*B*). The dissociation between phosphorylation potential and fatigue is also evidenced by the continued decrease in FTI despite a stable phosphorylation potential.

Exploratory studies of tissue oxygenation and energetics

Figure 6 shows the changes in myoglobin deoxygenation during the ischaemic contraction protocol in one subject. Rapid desaturation was observed at the onset of the contractions, which reached a plateau at ∼75% of the peak desaturation observed during resting ischaemia, corresponding to an intracellular P_{O_2} of 1.07 Torr.

Figure 7 shows the changes in PCr during 10 min of ischaemia at rest, followed by the ISC protocol in one subject. Phosphocreatine showed a slight downward drift during the ischaemic rest period. Thereafter, PCr decreased rapidly during the six ischaemic contractions. Notably, we observed some recovery of PCr during the ischaemic rest intervals between contractions (Fig. 7, inset). This recovery was less evident during the final stages of the contraction protocol, and no PCr recovery was observed following the final contraction until blood flow was restored.

Discussion

Our results indicate that, during ischaemic contractions when oxidative phosphorylation is no longer a viable source of ATP, the balance between ATP supply and demand is met *in vivo* not by increasing glycolytic flux, but by decreasing ATP demand. Furthermore, the present results suggest that the reduced demand for ATP during ISC is accomplished by a combination

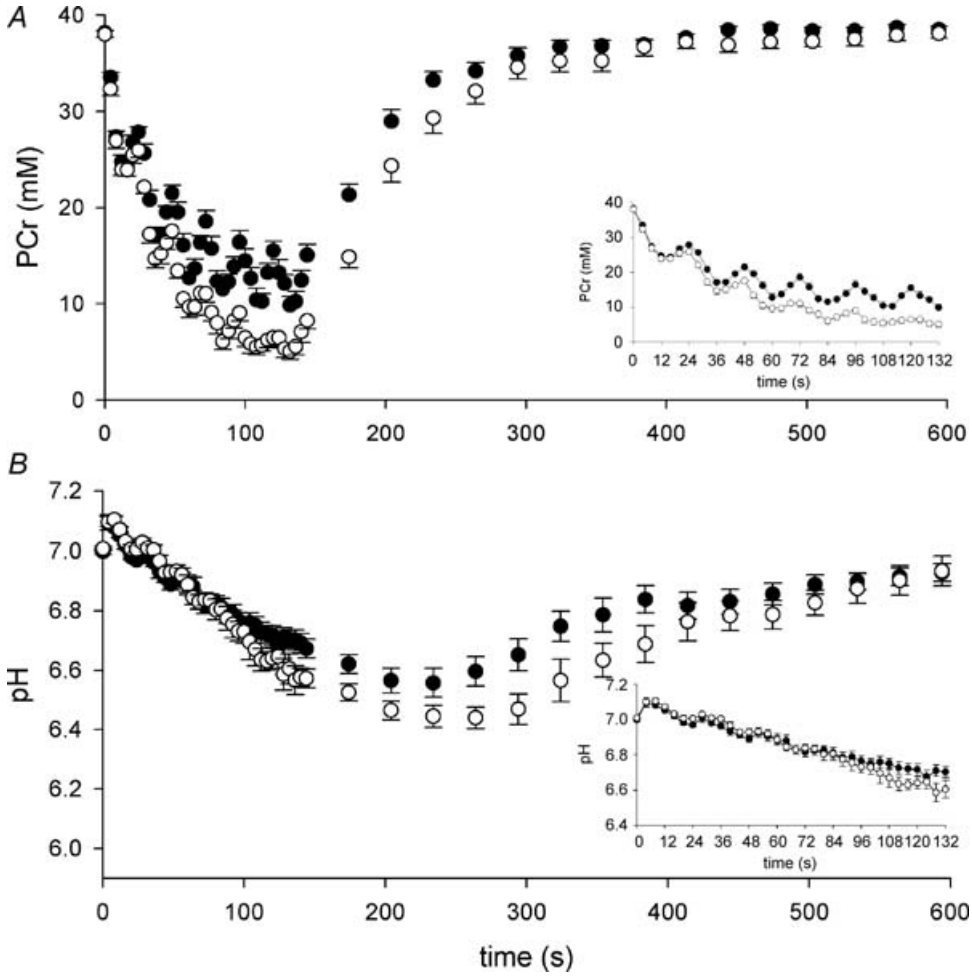

Figure 3. Phosphocreatine and pH during contractions and recovery
Phosphocreatine (*A*) decreased more during ISC (○) compared to FF (●) ($P < 0.001$). The inset shows the contraction period, illustrating transient PCr depletion and recovery during the contraction–relaxation cycle. Intracellular pH (*B*) decreased during FF and ISC, with a trend ($P = 0.10$) for greater acidosis during ISC. Values are means ± S.E.M.

of increased metabolic economy and decreased muscle force production as fatigue develops. Under both FF and ISC conditions, fatigue was related to intracellular $H_2PO_4^-$ rather than decreased ATP turnover mediated by reductions in the phosphorylation potential of the muscle.

Force and central activation

The force–time integral decreased to a greater extent during ischaemic compared to free-flow contractions. These data support the work of other investigators who have shown that skeletal muscle fatigues more during circulatory occlusion than under free-flow conditions (Harris *et al.* 1975; Greenhaff *et al.* 1993; Kemp *et al.* 1994; Russ & Kent-Braun, 2003). Further, we found that central activation was unimpaired at baseline and maintained equally well (CAR = 0.92) during both 132 s contraction protocols, indicating that the volume of active muscle was largely conserved throughout both protocols. These data support the assumption that the observed metabolic changes were representative of the entire muscle.

Anaerobic glycolysis

The magnitude of the glycolytic rates measured in the present study (0.40–1.05 mM s^{-1}) is similar to reports from other investigators who have used ^{31}P-MRS to quantify glycolytic flux *in vivo* (0.1–3.0 mM s^{-1}) (Kemp *et al.* 1994; Walter *et al.* 1999). Contrary to our hypothesis, when blood flow to the muscle was eliminated, glycolytic ATP production was unchanged compared to free-flow

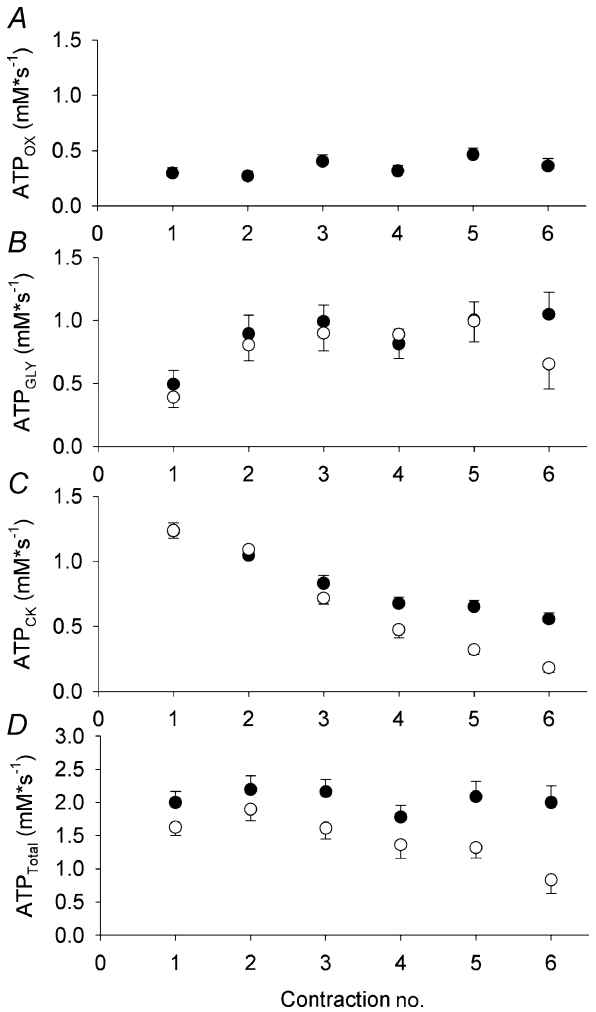

Figure 4. Rates of ATP synthesis during muscle contractions
ATP production from oxidative phosphorylation (ATP_{OX}, *A*) was similar across the 6 contractions throughout FF ($P = 0.12$), and assumed to be negligible during ISC. Glycolytic flux (ATP_{GLY}, *B*) was similar during FF (●) and ISC (○) ($P = 0.22$). ATP production from net PCr breakdown in the creatine kinase reaction (ATP_{CK}, *C*) was greater during FF than ISC ($P < 0.001$). Total ATP turnover (ATP_{TOT}, *D*), calculated as the sum of ATP_{OX}, ATP_{GLY}, and ATP_{CK}, was greater during FF compared to ISC ($P < 0.001$). Values are means ± S.E.M.

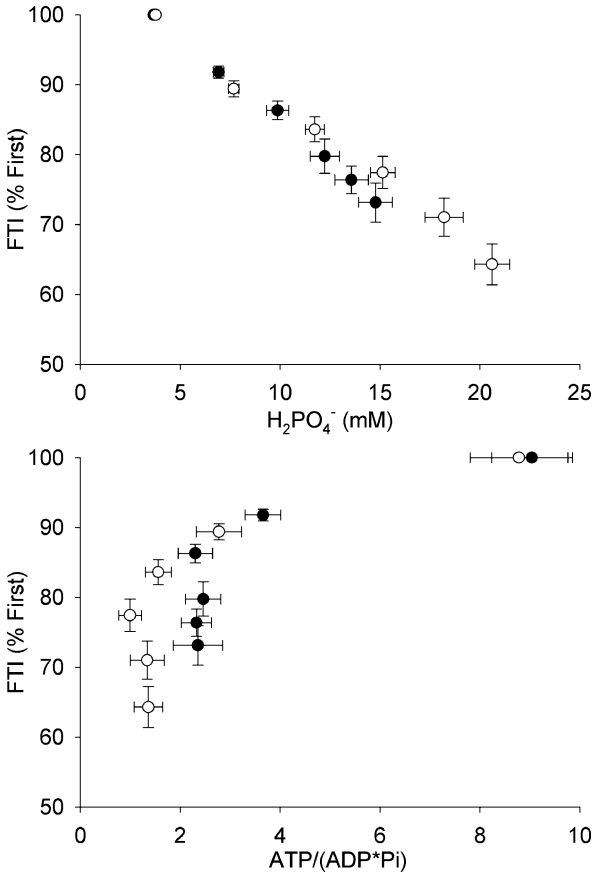

Figure 5. Relationships between muscle fatigue, pH and phosphorylation potential
Strong linear associations between fatigue and $H_2PO_4^-$ were observed during FF (●) and ISC (○) (top panel); FF $r = 0.93 \pm 0.01$, ISC $r = 0.91 \pm 0.03$. Curvilinear relationships were evident between fatigue and intracellular phosphorylation potential during FF and ISC (bottom panel). These relationships were left-shifted during ISC compared to FF. Values are means ± S.E.M.

contractions. This result conflicts with previous reports of increased glycolytic flux when oxygen delivery is limited (Linnarsson *et al.* 1974; King *et al.* 1987; Greenhaff *et al.* 1993; Kemp *et al.* 1994; Timmons *et al.* 1996; Greiner *et al.* 2005). One explanation for these disparate results may be the intensity of the muscle contractions during which glycolytic flux was measured. Since some previous studies employed submaximal contractions (Kemp *et al.* 1994; Greiner *et al.* 2005), it is possible that the ischaemia-induced increase in glycolytic flux in those studies was related to altered recruitment during ischaemia (Takarada *et al.* 2000). That is, glycolytic, Type II fibres may have been recruited more during ischaemia, which could produce higher overall glycolytic rates as a result. Consistent with this notion, muscle lactate concentration has been shown to be related to oxygen availability at submaximal but not maximal workloads (Linnarsson *et al.* 1974). The present study used maximal intensity muscle contractions, which recruit the entire population of muscle fibres, and presumably would not be affected by changes in muscle fibre recruitment between the FF and ISC protocols.

Greenhaff *et al.* (1993) overcame the issue of muscle recruitment effects by examining muscle energy metabolism in response to maximal electrical stimulation of human quadriceps muscles. Interestingly, these investigators reported similar glycogenolytic rates in type II fibres during contractions with and without ischaemia, assessed through biopsies before and immediately after stimulation. However, type I fibres exhibited substantial increases in glycolytic flux during ISC compared to FF. The authors suggested that type II fibres are functioning close to V_{max} for glycogenolysis during maximal contractions with intact circulation, unlike type I fibres, which may increase glycolytic flux when cellular concentrations of known activators of glycogenolysis are increased during ischaemia. It is somewhat surprising, then, that the present study of the tibialis anterior muscle (~76% type I fibres; Jakobsson *et al.* 1988) failed to demonstrate a similar result. While we cannot explain the discrepancy between our report and that of Greenhaff *et al.* (1993) our observations are consistent with a previous study in the same muscle group. Conley *et al.* (1998) performed maximal stimulation of the tibialis anterior muscle while using ^{31}P-MRS to assess glycolytic flux in a similar manner to that of the present study (Conley *et al.* 1998). Thus, it appears possible that the conflicting results of Greenhaff *et al.* (1993) may be related to differences in the muscle group studied, the manner in which the muscle was activated (voluntary MVC, supramaximal nerve stimulation, maximal motor point stimulation) or the methods used to quantify glycolytic flux.

The metabolic response of ischaemic muscle also appears to be sensitive to the manner in which oxygen supply is limited. Gutierrez *et al.* (1988) reported that ischaemia and hypoxia affected skeletal muscle bioenergetics in different ways. Hypoxia of rabbit hindlimb was accomplished by decreasing the P_{O_2} of arterial blood to ~5 Torr, while ischaemia was accomplished by eliminating blood flow by arterial occlusion. Blood lactate and intracellular pH changes

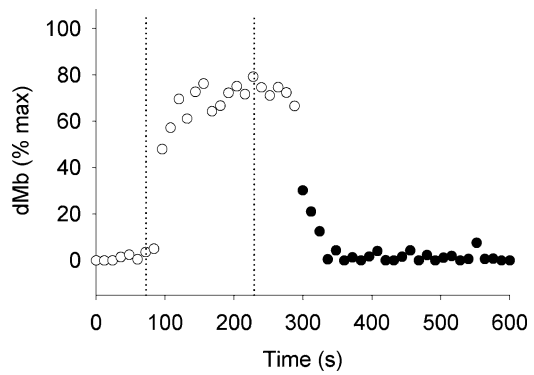

Figure 6. ^{1}H-MRS measures of deoxy-myoglobin during ischaemic contractions in one subject
The deoxy-myoglobin signal was measured during 1 min of resting cuff occlusion (○, ischaemia), ISC protocol (contraction period defined by the dotted vertical lines), cuff occlusion for 1 min following the end of the contractions, and 10 min of free-flow recovery (●, release of cuff occlusion). Ischaemic muscle contractions resulted in myoglobin desaturation to ~75% of the desaturation observed during 10 min of resting ischaemia.

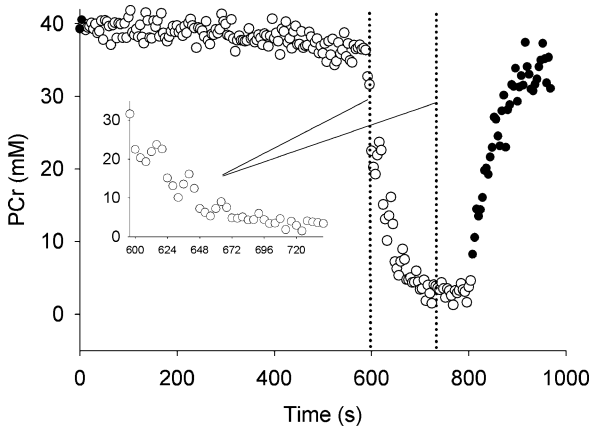

Figure 7. ^{31}P-MRS measures of phosphocreatine during prolonged ischaemia followed by ischaemic contractions in one subject
PCr decreased slightly after ~8 min of resting ischaemia (○, ischaemia). Muscle contractions were initiated after 10 min of ischaemia, resulting in a marked decrease in PCr (the start and end of the contraction periods are indicated by the dotted lines). Inset shows PCr during 6 MVC/rest intervals. PCr recovery was observed following the first 3 contractions. No recovery of PCr was evident when ischaemia was maintained for 1 min after the final MVC, but PCr recovered quickly when blood flow was restored (●, release of cuff occlusion).

were similar in control and ischaemic conditions. However, hypoxia resulted in a much greater change in blood lactate and pH from resting values compared to ischaemia. The authors highlight the possibility that, unlike complete ischaemia, hypoxia allows delivery of substrate and efflux of metabolic by-products from the muscle that may otherwise inhibit glycolytic flux. This possibility was confirmed in contracting canine gastrocnemius muscle during partial ischaemia (~30% reduction in blood flow) and free-flow hypoxia (11% fraction of inspired O_2) (Stainsby *et al.* 1990). When ischaemia was induced at the onset of muscle contractions, the net lactic acid output from the muscle did not increase compared to controls. When free-flow hypoxia was introduced at the onset of contractions, net lactic acid output was greater than controls. The results suggest that anaerobic glycolysis was able to compensate for decreased $\dot{V}_{O_2}$ during hypoxia, but not ischaemia, which likely explains why mechanical performance declined more during ischaemia compared to hypoxia (Stainsby *et al.* 1990). These studies provide an attractive explanation for the discordance between the results of the present study and those of others who have limited muscle oxygenation by free-flow hypoxia or partial ischaemia (King *et al.* 1987; Gutierrez *et al.* 1988; Stainsby *et al.* 1990; Timmons *et al.* 1996).

Our results demonstrate that during maximal voluntary contractions, glycolytic flux measured *in vivo* is similar during free-flow and ischaemia. We anticipated that glycolytic flux would increase during ISC compared to FF due to the suppression of oxidative ATP synthesis and continued high demand for ATP. It is reasonable to expect accumulation of pyruvate and NADH when mitochondrial oxygen availability is limited. Since NADH and pyruvate are both substrates for the lactate dehydrogenase reaction, lactate production could increase because of mass action effects. Glycolytic flux may also be higher during ischaemia due to greater accumulation of P_i, which is believed to be a potent activator of glycogen phosphorylase (Chasiotis *et al.* 1982). Other putative glycolytic regulators that may be relatively higher during ischaemic compared with free-flow contractions include AMP, which regulates the conversion of phosphorylase *b* to *a* through the combined effects of calcium on the activity of phosphorylase kinase (Greenhaff *et al.* 1993). Indeed, we found that P_i, AMP and ADP were higher during ISC compared to FF (Table 1). The similarity of glycolytic flux under both conditions despite elevated levels of these metabolites suggests that glycolytic flux was already near V_{max} during FF contractions, as suggested by Greenhaff *et al.* (1991, 1993) in type II fibres.

The high demand for ATP during the maximal intensity muscle contractions employed in the present study necessitated substantial glycolytic flux to supply ample ATP, even during FF contractions. Furthermore, voluntary contractions of this muscle group above 60% MVC have recently been shown to generate sufficient intramuscular pressure to compress the vasculature and fully occlude muscle perfusion (Wigmore *et al.* 2004). Therefore, it is likely that glycolytic flux increased in FF during the 12 s MVCs due to transient limitations in muscle oxygenation. Alternatively, glycolytic flux during ISC may have been limited, not by an upper limit to glycolytic flux, but by inhibition of rate-limiting steps in glycolytic reactions during ISC. Intracellular proton accumulation will eventually inhibit phosphofructokinase, thus limiting carbon flux through the distal reactions in glycolysis (Chasiotis *et al.* 1982). We observed a trend for greater acidosis during ISC compared to FF, suggesting that proton accumulation may have begun to limit glycolytic flux more during ISC than FF. We also observed a trend toward greater accumulation of phosphomonoesters, consisting principally of glucose-6-phosphate, during ISC compared to FF. This result suggests that proton accumulation during ISC may have limited glycolytic flux through inhibition of phosphofructokinase, with subsequent accumulation of glucose-6-phosphate.

Oxidative phosphorylation

The rates of oxidative ATP synthesis determined from the initial postcontraction PCr recovery during FF (0.2–0.5 mM s^{-1}) were similar to rates reported during voluntary contractions in previous studies (0.1–0.7 mM s^{-1}) (Conley *et al.* 1998; Kemp *et al.* 1994; Lanza *et al.* 2005). Interestingly, oxidative flux remained constant with each successive contraction, despite increases in several purported activators of mitochondrial respiration. The rates of oxidative ATP synthesis may have been limited by oxygen availability, even during the FF protocol. As mentioned earlier, ankle dorsiflexion above 60% MVC results in vascular occlusion due to high intramuscular pressure (Wigmore *et al.* 2004), which could have imposed an upper limit on the increase in oxidative phosphorylation during this intermittent MVC protocol.

The model of PCr recovery for estimates of oxidative phosphorylation is well-established (Kemp & Radda, 1994; Jubrias *et al.* 2001). However, we observed a small amount of PCr resynthesis during each 12 s rest interval during ischaemia following the first 3–4 contractions (Fig. 3*A*). Given that the cuff pressure (220 Torr) was significantly higher than systolic blood pressure measured at rest in the lower leg of these subjects, and mean arterial pressure increases by ~30 Torr from rest to intense contractions of the leg musculature (Andersen & Saltin, 1985), it is unlikely that this small amount of PCr recovery was due to oxygen leaking into the muscle. Further support for this notion comes from the observation that the end-exercise PCr level does not recover until blood flow is restored as

demonstrated in the present study (Fig. 7) and by others (Quistorff *et al.* 1993; Blei *et al.* 1993).

Alternatively, it is possible that a sufficient reservoir of oxygen bound to trapped haemoglobin and myoglobin could sustain oxidative ATP synthesis at low rates during blood flow occlusion. To address this possibility, we measured deoxy-myoglobin during the ischaemic contraction protocol in a representative subject. Myoglobin desaturated by ∼75%, corresponding to an intracellular P_{O_2} of 1.07 Torr, which appears to be above the P_{O_2} at which oxidative function will be limited in tissue at > 50% maximal ATP flux (Connett *et al.* 1990). An earlier study using near-infrared spectroscopy also showed that muscle deoxygenated less during a period of ischaemia with interspersed contractions than during an equivalent period of ischaemic rest (De Blasi *et al.* 1992). The incomplete desaturation of myoglobin observed during ischaemic contractions may be the result of the inhibition of oxidative ATP production by the metabolic by-products of anaerobic glycolysis (Conley *et al.* 2001) or the competition of oxygen and nitric oxide for binding sites on cytochrome oxidase in the mitochondria (Cleeter *et al.* 1994). Despite incomplete desaturation of intracellular oxygen, oxidative ATP synthesis was unlikely to be responsible for the recovery of PCr between contractions during ISC, particularly since trapped oxygen within the ischaemic muscle would only be sufficient to generate ∼4 mM of ATP through oxidative phosphorylation (Harris *et al.* 1975; Kemp *et al.* 1994).

To further confirm that PCr recovery during ISC could not be due to residual oxygen trapped within the ischaemic muscle, one representative subject performed the six intermittent contractions after a 10 min period of ischaemia at rest, which was used to completely deoxygenate the muscle. We observed a similar pattern of PCr resynthesis between ischaemic contractions as observed when only 30 s of ischaemia preceded the contraction protocol (Fig. 7). These data make it difficult to ignore the possibility that the PCr recovery observed during ISC was unrelated to oxidative ATP production.

Our demonstration of PCr recovery during ischaemic rest intervals is in contrast to several previous reports that PCr is not resynthesized during ischaemic recovery (Harris *et al.* 1975; Blei *et al.* 1993; Quistorff *et al.* 1993). These investigators have concluded that despite high levels of P_i and AMP, which are activators of phosphofructokinase and glycogen phosphorylase, anaerobic glycolysis ceases in the absence of muscle contraction, presumably due to the absence of calcium release into the cytosol by the sarcoplasmic reticulum (Connett & Sahlin, 1996). In contrast, others have reported that glycolytic flux continues for several seconds following the cessation of muscle activity (Crowther *et al.* 2002), suggesting that a small amount of PCr resynthesis between contractions during ISC could be provided by glycolysis. In the present study, the signal-to-noise associated with the 4.0 telsa field strength allowed us to examine PCr changes with a 4 s time resolution, permitting observation of changes in PCr during the first few seconds of recovery. Our PCr data during ISC provide new evidence of glycolytic ATP synthesis in resting muscle *in vivo*. Further study is necessary to clarify the factors regulating glycolytic flux in the absence of muscle contraction.

Phosphocreatine hydrolysis

The ATP derived from the net breakdown of PCr was greatest during the early phases of both contraction protocols. As the contractions progressed, the contribution of this pathway to ATP synthesis decreased, in agreement with previous studies in the ankle dorsiflexors (Lanza *et al.* 2005) and plantarflexors (Walter *et al.* 1999). This decrease was more pronounced during ISC such that FF contractions derived relatively more ATP from net PCr breakdown. This finding is undoubtedly a result of greater PCr resynthesis during rest intervals in FF compared to ISC, leading to higher PCr concentrations at the onset of subsequent contractions (Fig. 3*A*).

Total ATPase rate

Overall ATPase rates were lower during ISC compared to FF (Fig. 4*D*). Since the rate of ATP consumption depends on muscle force production, the lower ATPase rate during ISC could reflect the greater decline in muscle force during the ischaemic contractions. However, the lower ATPase rate during ISC compared to FF persisted even after we accounted for differences in muscle force production by normalizing to the degree of muscle fatigue, suggesting that the declines in muscle contractility during ISC could not entirely explain the decline in ATPase rates. Indeed, metabolic economy was higher during ISC compared to FF, in accordance with work of other investigators who have observed greater metabolic efficiency during anaerobic than aerobic ATP synthesis (Krustrup *et al.* 2003). These investigators report that heat production in exercising muscle is 60% lower when blood flow is occluded in comparison to free-flow exercise at the same workload, in accordance with early experiments *in vitro* (Curtin & Woledge, 1978). Intracellular calcium transport into the mitochondria has been implicated as a mechanism for this reduced efficiency, by causing uncoupling of oxidation and phosphorylation (Halestrap *et al.* 1993). The increased metabolic economy observed during ISC is clearly a mechanism by which the balance between ATP supply and demand is balanced in ischaemic muscle.

Relationships between metabolites and fatigue

To address the causes of greater fatigue during ISC compared to FF, we examined the relationships between fatigue, intracellular $H_2PO_4^-$, and the phosphorylation potential of the muscle. The linear association between fatigue and intracellular $H_2PO_4^-$ (Fig. 5) suggests that fatigue was related to the synergistic inhibitory effects of protons and P_i produced during glycolytic ATP turnover, perhaps leading to greater fatigability of ischaemic muscle. While glycolytic flux was similar during FF and ISC and would be expected to elicit a similar degree of acidosis under both conditions, proton efflux was presumably prevented during ISC, which could explain the trend for greater acidosis, and therefore greater accumulation of $H_2PO_4^-$ and fatigue during ISC compared to FF.

To test the hypothesis that the energetic state of the muscle will regulate force production, we examined the relationship between fatigue and intracellular phosphorylation potential, which is purported to modify the demand of the ATPases (Arthur *et al.* 1992). The phosphorylation potential of the muscle, determined as $[ATP]/[ADP][P_i]$, decreases when oxidative and glycolytic pathways cannot satisfy the demand for ATP. We observed a non-linear relationship between force and energy state: a plateau in the phosphorylation potential was seen as muscle force production continued to decrease. Further, there was a leftward shift in this relationship with ISC such that the phosphorylation potential was lower during ISC than FF at the same degree of muscle fatigue. Together, these analyses suggest that fatigue was primarily the result of inhibitory by-products of glycolytic ATP turnover, rather than lower ATP turnover due to a distinct effect of decreased phosphorylation potential in the muscle.

In summary, we show that glycolytic flux in maximally contracting human skeletal muscle *in vivo* is similar under free-flow and ischaemic conditions. We observed no net breakdown of muscle ATP during FF or ISC, suggesting that the balance of ATP supply and demand during several minutes of muscular work was maintained even during ischaemia, where oxidative ATP synthesis is negligible. This energetic balance was maintained through lower demand for ATP during ISC, accomplished by a combination of increased metabolic economy and decreased muscle force production as muscle fatigue developed.

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