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Skeletal muscle ATP turnover by ^{31}P magnetic resonance spectroscopy during moderate and heavy bilateral knee extension

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Key points

- Heavy-intensity exercise causes a progressive increase in energy demand that contributes to exercise limitation.
- This inefficiency arises within the locomotor muscles and is thought to be due to an increase in the ATP cost of power production; however, the responsible mechanism is unresolved.
- We measured whole-body O_2 uptake and skeletal muscle ATP turnover by combined pulmonary gas exchange and magnetic resonance spectroscopy during moderate and heavy exercise in humans.
- Muscle ATP synthesis rate increased throughout constant-power heavy exercise, but this increase was unrelated to the progression of whole-body inefficiency.
- Our data indicate that the increased ATP requirement is not the sole cause of inefficiency during heavy exercise, and other mechanisms, such as increased O_2 cost of ATP resynthesis, may contribute.

Abstract During constant-power high-intensity exercise, the expected increase in oxygen uptake ($\dot{V}_{O_2}$) is supplemented by a $\dot{V}_{O_2}$ slow component ($\dot{V}_{O_2sc}$), reflecting reduced work efficiency, predominantly within the locomotor muscles. The intracellular source of inefficiency is postulated to be an increase in the ATP cost of power production (an increase in P/W). To test this hypothesis, we measured intramuscular ATP turnover with ^{31}P magnetic resonance spectroscopy (MRS) and whole-body $\dot{V}_{O_2}$ during moderate (MOD) and heavy (HVY) bilateral knee-extension exercise in healthy participants ($n = 14$). Unlocalized ^{31}P spectra were collected from the quadriceps throughout using a dual-tuned (^{1}H and ^{31}P) surface coil with a simple pulse-and-acquire sequence. Total ATP turnover rate (ATP_{tot}) was estimated at exercise cessation from direct measurements of the dynamics of phosphocreatine (PCr) and proton handling. Between 3 and 8 min during MOD, there was no discernable $\dot{V}_{O_2sc}$ (mean ± SD, 0.06 ± 0.12 l min^{-1}) or change in [PCr] (30 ± 8 *vs.* 32 ± 7 mM) or ATP_{tot} (24 ± 14 *vs.* 17 ± 14 mM min^{-1}; each P = n.s.). During HVY, the $\dot{V}_{O_2sc}$ was 0.37 ± 0.16 l min^{-1} (22 ± 8%), [PCr] decreased (19 ± 7 *vs.* 18 ± 7 mM, or 12 ± 15%; $P < 0.05$) and ATP_{tot} increased (38 ± 16 *vs.* 44 ± 14 mM min^{-1}, or 26 ± 30%; $P < 0.05$) between 3 and 8 min. However, the increase in ATP_{tot} (ΔATP_{tot}) was not correlated with the $\dot{V}_{O_2sc}$ during HVY ($r^2 = 0.06$; P = n.s.). This lack of relationship between ΔATP_{tot} and $\dot{V}_{O_2sc}$, together

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with a steepening of the [PCr]–$\dot{V}_{O_2}$ relationship in HVY, suggests that reduced work efficiency during heavy exercise arises from both contractile (P/W) and mitochondrial sources (the O_2 cost of ATP resynthesis; P/O).

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Abbreviations *A*, amplitude; ATP_{tot}, total ATP turnover rate; *D*, ATP production from phosphocreatine breakdown; *k*, rate constant; *L*, ATP production from glycogenolysis; LT, lactate threshold; MRS, magnetic resonance spectroscopy; PCr, phosphocreatine; PCr_{sc}, phosphocreatine slow component; pH_i, intramuscular pH; P_i, inorganic phosphate; P/O, ATP yield per $O \rightarrow H_2O$; P/W, ATP cost per unit power output; *Q*, ATP production from oxidative phosphorylation; RI, ramp incremental; S_{pO_2}, arterial oxygenation; τ, time constant; $V_{i[PCr]}$, initial rate of phosphocreatine resynthesis; $\dot{V}_{O_2}$, rate of whole-body O_2 uptake; $\dot{V}_{O_2peak}$, peak rate of O_2 uptake; $\dot{V}_{O_2sc}$, slow component of O_2 uptake.

Introduction

During constant-power exercise below the lactate threshold (LT; moderate intensity), the rate of pulmonary oxygen uptake ($\dot{V}_{O_2}$) increases exponentially, reaching a steady state within 2–3 min. A steady-state $\dot{V}_{O_2}$ indicates that the exercise-related energy transfer is accounted for by oxidative phosphorylation. However, above the LT (heavy intensity), the dynamics of $\dot{V}_{O_2}$ become complicated by an additional, slow component ($\dot{V}_{O_2sc}$; Poole *et al.* 1994). This becomes especially important at power outputs above critical power, where the $\dot{V}_{O_2sc}$ will draw $\dot{V}_{O_2}$ inexorably towards its physiological maximum. In this intensity domain, the limit of tolerance is reached rapidly, and the exercise cannot continue unless the power output is reduced below critical power (Coats *et al.* 2003). Although the $\dot{V}_{O_2sc}$ is intimately related to exercise intolerance (Murgatroyd *et al.* 2011), the aetiology of the $\dot{V}_{O_2sc}$ remains poorly understood.

The $\dot{V}_{O_2sc}$ represents an impairment of exercise economy and is predominantly (~85%) due to increased O_2 consumption in the muscles engaged in the locomotor work (Poole *et al.* 1991; Rossiter *et al.* 2002; Krustrup *et al.* 2009). However, the intracellular source of this inefficiency is uncertain. It has been postulated that the $\dot{V}_{O_2sc}$ is related to an increased phosphate cost of force or power production; that is, an increase in the rate of ATP consumption per unit power output (or P/W) is met instantaneously by phosphocreatine (PCr; via the Lohmann reaction), the breakdown of which signals an increase in the rate of oxidative phosphorylation (Rossiter *et al.* 2002). However, distinguishing between this and the alternative hypothesis, that supra-LT exercise is associated with reductions in mitochondrial coupling (Krustrup *et al.* 2003), i.e. the ratio of the ATP resynthesized per oxygen converted to water (P/O), is technically challenging in humans.

To test these two hypotheses requires knowledge of dynamic changes in total ATP turnover rate (ATP_{tot}) in concert with power output and $\dot{V}_{O_2}$. Specifically, were the intramuscular source of the $\dot{V}_{O_2sc}$ to be caused by an increase in P/W (in line with current views; Rossiter, 2011; Poole & Jones, 2012), then the magnitude of the $\dot{V}_{O_2sc}$ during heavy exercise would be strongly related to the magnitude of the change in ATP_{tot}. Alternatively, if no proportionality between the $\dot{V}_{O_2sc}$ and the change in ATP_{tot} were evident, then changes in P/W could not be the sole source of the $\dot{V}_{O_2sc}$.

The technical challenge thus becomes, how best to establish ATP_{tot} during heavy-intensity exercise that elicits a $\dot{V}_{O_2sc}$? One approach uses ^{31}P magnetic resonance spectroscopy (MRS; Kemp *et al.* 2001; Layec *et al.* 2009*a*) to partition ATP delivery from oxidative phosphorylation, PCr breakdown and glycogenolysis. ^{31}P MRS provides direct measurement of [PCr] and allows the glycogenolytic rate (a relatively minor component of ATP_{tot} in exercise of this kind) to be estimated using reasonable assumptions about muscle H^+ buffering (Kemp *et al.* 2001, 2014). Several methods have been proposed to calculate oxidative ATP yield using ^{31}P MRS, but these show poor agreement (Layec *et al.* 2011). Previous studies to estimate ATP_{tot} during supra-LT exercise have assumed a linear $\dot{V}_{O_2}$–[PCr] relationship and a fixed time constant (τ) of PCr breakdown and resynthesis (Meyer, 1988; Walter *et al.* 1999; Lanza *et al.* 2005; Faraut *et al.* 2007) or first-order [ADP]–$\dot{V}_{O_2}$ relationship in order to transform [PCr] into a rate of oxidative ATP turnover (Layec *et al.* 2009*a*). However, it is clear that the $\dot{V}_{O_2}$–[PCr] relationship is not linear through the intensity domains (Kemp, 2008; Wüst *et al.* 2011; Kemp *et al.* 2014), and accordingly, τPCr is not invariant across exercise intensities (Yoshida & Watari, 1993, 1994; Rossiter *et al.* 2002; Jones *et al.* 2008), making this an unreliable assumption on which to base estimation of ATP_{tot}. Assuming τPCr to be invariant is equivalent to assuming that any change in [PCr] is directly proportional to change in ATP_{tot}; when τPCr changes across exercise intensity and/or duration, this proportionality is lost (Kemp *et al.* 2014). These new findings mean that the

close coherence between [PCr] and $\dot{V}_{O_2}$ during the slow component phase (Rossiter *et al.* 2002; Layec *et al.* 2009*a*) is no longer sufficient evidence to imply that an increase in P/W alone is the responsible mechanism. Consequently, a direct measurement of oxidative ATP yield during supra-LT exercise that does not rely on these assumptions is required to distinguish whether change in P/W is the dominant mechanism for the $\dot{V}_{O_2sc}$.

Oxidative ATP turnover (the dominant proportion of ATP_{tot}) at exercise cessation may be assessed directly from the initial rate of postexercise PCr resynthesis ($V_{i[PCr]}$), easily measured by ^{31}P MRS; the only assumptions required (the evidence for which is reviewed elsewhere; Kemp *et al.* 2014) are that PCr recovery is driven overwhelmingly by oxidative ATP synthesis and that any basal component of ATP turnover (i.e. ATP production not available for use by myosin ATPase, sarco(endo)plasmic reticulum Ca^{2+}-ATPase or Na^+–K^+-ATPase during exercise or PCr resynthesis during recovery) is small and reasonably constant. Therefore, temporal characterization of oxidative energy yield during dynamic exercise can be made simply by halting the exercise and measuring $V_{i[PCr]}$. Although this method has inherently poor temporal resolution (it is valid only at the instant of exercise cessation), it provides the accuracy necessary to isolate the intracellular source of inefficiency during high-intensity exercise. The other, much smaller, components of ATP_{tot} can be estimated at the end of exercise by ^{31}P MRS in ways that are relatively robust against uncertainty or changes in the underpinning assumptions.

The purpose of this study, therefore, was to characterize the rate of ATP turnover during sub- and supra-LT exercise in human quadriceps during bilateral, prone, knee-extension exercise using ^{31}P MRS. The rate of pulmonary oxygen uptake was measured in the same conditions to quantify the $\dot{V}_{O_2sc}$. We hypothesized that the close association between the dynamics of the [PCr] and $\dot{V}_{O_2}$ slow components during supra-LT exercise would be reflected in the dynamics of ATP_{tot} (measured independently), thereby confirming the hypothesis that increased P/W during heavy-intensity exercise is the predominant mechanism of the $\dot{V}_{O_2sc}$.

Methods

Ethical approval

The Biological Sciences Faculty Research Ethics Committee, University of Leeds, and the University of Liverpool Committee on Research Ethics approved this study, and all procedures complied with the latest revision of the *Declaration of Helsinki*. Written informed consent was obtained from all volunteers prior to their participation in the study.

Participants

Fourteen healthy volunteers (one female, 13 male) agreed to participate in this study [mean ± SD: age 27 ± 8 years; height 177 ± 8 cm; mass 75 ± 12 kg; bilateral knee-extension peak $\dot{V}_{O_2}$ ($\dot{V}_{O_2peak}$) 2.0 ± 0.5 l min^{-1}]. All participants were undertaking a regular exercise regimen, ranging from recreational fitness to amateur competitive sport. Volunteers were screened for cardiovascular disease risk with a resting ECG and a health history questionnaire.

Exercise protocols

All exercise tests were undertaken on an MR-compatible computer-controlled electromagnetically braked knee-extension ergometer (MRI Ergometer Up/Down, Lode BV, Groningen, The Netherlands) customized for use at 3 T by the addition of extended carbon-fibre lever arms. The participants lay prone, with their feet strapped into moulded plastic stirrups, which were attached to carbon-fibre/aluminium arms, linking to the ergometer crank arms. The participants' hips were secured to the patient bed with nylon and Velcro straps in order to isolate power production to the quadriceps and minimize movement from hip flexion/extension. Knee movements were constrained by the scanner bore, allowing for ∼35 deg of bilateral knee extension (Whipp *et al.* 1999; Cannon *et al.* 2013). No resistance was applied during knee flexion, other than the constant work required to lift the mass of the lower leg.

The testing protocol began with a rigorous familiarization phase that took place in a temperature-controlled laboratory with pulmonary gas exchange measurements. Ramp incremental (RI) and constant-power protocols were completed until reproducible physiological measurements were obtained across two consecutive visits for each condition. The second phase of the study took place within the bore of an MR scanner for measurement of muscle phosphates. The same MRI ergometer was used for both phases of the protocol.

Initially, participants completed an RI exercise test to the limit of tolerance. For this, participants lay at rest for ∼3–4 min, followed by a low-power exercise (5 W) for ∼2–4 min. The power was then increased as a function of time at 2–5 W min^{-1} (the rate of increase was dependent on the volunteer's size and strength) until the limit of tolerance was reached. Ramp rates were adjusted using 'trial and error' to determine a ramp rate that resulted in a ramp duration of ∼10–12 min. The frequency of knee extension was constrained at 90 min^{-1} with the use of a metronome. This cadence was chosen to allow synchronization with the MR scanner acquisitions (one pulse per two knee extensions) and also acted to ensure that the ergometer flywheel was maintained above its minimal operating speed. The RI was terminated upon

the participant being unable to maintain the required cadence, despite strong verbal encouragement. The results of the RI were used to determine the $\dot{V}_{O_2peak}$ and to calculate power for subsequent tests. There is a substantial learning effect with the exercise model (large gains in peak power were achieved with consecutive tests), and therefore typically, more than three RI tests were completed by each participant until reproducible performances were achieved.

A series of constant-power exercise tests were then undertaken. These consisted of an 8 min moderate-intensity bout, followed by a 6 min rest and an 8 min heavy-intensity exercise bout. During moderate-intensity exercise, the target power was 80% of estimated LT (LT was ∼60–70% $\dot{V}_{O_2peak}$), and during heavy-intensity bouts the target power was halfway between estimated LT and $\dot{V}_{O_2peak}$. These intensity domains were confirmed *post hoc* from the profile of $\dot{V}_{O_2}$ during constant-power bouts (Whipp, 1996). If necessary, power was adjusted in subsequent familiarization tests to ensure the absence (moderate) or presence (heavy) of the $\dot{V}_{O_2sc}$. Once familiarized, participants repeated this protocol three times on separate days to combine respired gas exchange data and improve the signal-to-noise ratio.

During the second phase of experiments, participants completed constant-power bouts within the bore of the superconducting magnet for ^{31}P MRS. Two trials of constant-power tests were completed in a random order consisting of the following sequences: (i) 4 min of rest, followed by 3 min of moderate exercise, 6 min of rest and 3 min of heavy exercise; and (ii) 4 min of rest, followed by 8 min of moderate exercise, 6 min of rest and 8 min of heavy exercise. Each protocol was preceded by ∼10 min of magnet shimming to optimize the MRS signal, and separated by at least 30 min outside of the MR scanner. Therefore, ∼60–90 min elapsed between the two exercise trials.

Pulmonary gas exchange

Participants breathed through a low-resistance (<0.1 kPa l^{-1} s^{-1} up to 15 l s^{-1}), low-dead-space (90 ml) mouthpiece for the measurement of respired gases. Flow rates and volumes were measured with an infrared turbine flow sensor (Interface Associates, Laguna Niguel, CA, USA), while a quadrupole mass spectrometer was used to measure respired gas concentrations after sampling air at 0.5 ml s^{-1} from the mouthpiece (MSX; nSpire Health Ltd, Hertford, UK). Gas concentration signals were time aligned with the flow sensor signal using proprietary software for the calculation of breath-by-breath gas exchange. These algorithms identified the end of each breath with the flow sensor and time aligned the changes in respired gases.

Prior to each experiment, the flow sensor and gas analysers were calibrated according to the manufacturers' instructions. The turbine volume transducers were calibrated with a 3 l syringe (Hans Rudolph Inc., Shawnee, KS, USA). The calibration was completed with flow rates ranging from 0.2 to 6 l s^{-1}, mimicking flow rates expected for humans at rest and during exercise. After the completion of the flow sensor calibration, the flow volumes were verified over 10 syringe strokes of varying flow rates and accepted when the means were within ±0.01 l, with an SD and coefficient of variation of 0.02 l and 1%, respectively. Additionally, the mass spectrometer was calibrated with atmospheric air and precision-verified gases with concentrations of O_2, CO_2 and N_2 spanning the physiological range. Following each experiment, mass spectrometer calibration factor drift was verified as negligible by sampling the calibration gases.

Data analyses for pulmonary measures. Breath-by-breath $\dot{V}_{O_2}$ was filtered for errant breaths (i.e. values resulting after sighs, swallows, coughs etc., defined as residing outside of 99% prediction limits; Lamarra *et al.* 1987). Responses from like transitions were combined to improve the signal-to-noise ratio using an averaging technique that preserves the breath-by-breath density measured during the exercise transition. This method requires time aligning and sorting of all $\dot{V}_{O_2}$ data from exercise transitions in the time domain. Time and $\dot{V}_{O_2}$ are then averaged into bins of n breaths, where n is the number of exercise transitions completed (Murgatroyd *et al.* 2011). The magnitude of the $\dot{V}_{O_2sc}$ was expressed as the difference in $\dot{V}_{O_2}$ between 3 and 8 min of exercise.

Power output and flywheel speed from the ergometer were sampled continuously and digitized by a data-recording system and stored on a PC (PowerLab 8/30 with LabChart Pro; ADInstruments Pty Ltd, Bella Vista, NSW, Australia).

^{31}P Magnetic resonance spectroscopy

Muscle phosphorus-containing metabolites were measured with a 3 T superconducting magnet (Magnetom Trio; Siemens AG, Erlangen, Germany). A one-pulse MRS acquisition was employed using a dual-tuned (^{1}H and ^{31}P) 15- and 18-cm-diameter surface RF coil (RAPID Biomedical GmbH, Rimpar, Germany), which was placed under the knee extensors, halfway between the hip and knee. The concave RF coil was stabilized with sandbags and was secured to the table once the participants' hips were strapped to the scanner table. A series of axial, sagittal and coronal gradient-recalled echo images of the thigh were acquired to confirm the placement of the RF coil relative to the knee-extensor muscles and to prescribe the volume over which shimming was achieved.

Subsequently, a standard ^{1}H shimming protocol was used to optimize the homogeneity of the magnetic field (β_0). A fully relaxed spectrum (repetition time of 10 s; number of scans = 4) was initially obtained to provide a high-resolution unsaturated resting spectrum along with a 32 scan spectrum with a repetition time of 2 s. Following this, free induction decays for ^{31}P spectra were collected every 2 s with a spectral width of 3200 Hz and 1024 data points throughout the rest-to-exercise-to-rest transitions. The ^{31}P data were averaged over four acquisitions, yielding a datum every 8 s.

Kinetic analysis of ^{31}P MRS data

Signal intensities, frequencies and line widths of inorganic phosphate (P_i), PCr, γ-ATP, α-ATP and β-ATP were determined using Java-based Magnetic Resonance User Interface (jMRUI; Naressi *et al.* 2001) in order to transform the raw data into a time series for each of the phosphates of interest. Intramuscular pH (pH_i) was estimated from the chemical shift of P_i (Moon & Richards, 1973), as follows:

$$pH_i = 6.75 + \log\left(\delta - 3.27/5.69 - \delta\right) \quad (1)$$

where δ is the chemical shift of the P_i peak, relative to PCr.

Phosphocreatine kinetics were modelled using non-linear least-squares regression (OriginPro 7.5; OriginLab Corp., Northampton, MA, USA). The ^{31}P MRS data were filtered for errant values resulting from artefacts (Rossiter *et al.* 2000) prior to characterization with the following function:

$$[PCr]_{(t)} = [PCr]_0 + A(1 - e^{-t/\tau}) \quad (2)$$

where τ is a time constant and $[PCr]_{(t)}$, $[PCr]_0$, and A are the time-variant form, baseline and fundamental amplitude, respectively. The fitting window was determined from an iterative process (Rossiter *et al.* 2001) to ensure the exclusion of phase III (steady state or slow component, depending on the intensity domain). The magnitude of the PCr slow component ($[PCr]_{sc}$) was expressed as the difference in [PCr] between the third and eighth minute of exercise.

The ATP_{tot} was estimated from the contributions from oxidative phosphorylation (Q), PCr breakdown (D) and glycogenolysis (L), which were determined from the ^{31}P MRS data acquired during exercise and recovery, using methods described elsewhere (Kemp *et al.* 2001 , 2007, 2014; Layec *et al.* 2011) and outlined below.

Production of ATP from PCr breakdown (*D*). The rate of PCr breakdown by creatine kinase (D) yields one component of ATP production (millimolar per minute) and was determined over 32 s (four spectra) immediately prior to exercise cessation, according to the following equation:

$$D = d[PCr]/dt \quad (3)$$

In the present experiments, where [PCr] is either close to steady state or changing only slowly by the end of exercise, D is a very small component of end-exercise ATP_{tot}.

Production of ATP from oxidative phosphorylation (*Q*). The rate of oxidative ATP yield (Q) is reflected in the rate of [PCr] recovery at the instant of exercise cessation ($V_{i[PCr]}$) and was calculated (millimolar per minute) as follows:

$$V_{i[PCr]} = kA \quad (4)$$

where A is the amplitude of [PCr] change (millimolar). The rate constant (k) was estimated by fitting the PCr recovery kinetics with the following function:

$$[PCr]_{(t)} = [PCr]_{end} - A(1 - e^{-kt}) \quad (5)$$

where $[PCr]_{(t)}$ is the time-dependent variant of [PCr], and $[PCr]_{end}$ is the concentration of PCr measured at the end of exercise. We make the well-evidenced assumption (Kemp *et al.* 2014) that the rate of suprabasal oxidative synthesis at the start of recovery [$V_{i[PCr]}$ from eqn (4)] is a good estimate of the suprabasal rate of oxidative synthesis at the end of exercise (Q_{end}).

Production of ATP from anaerobic glycolysis (*L*). During exercise, glycogenolysis and the resulting lactate and H^+ production cause disturbances in pH_i. These changes in pH_i are readily measured by ^{31}P MRS data and can therefore be used to estimate ATP production from glycogenolysis; 1 mol of H^+ resulting in 1.5 mol of ATP. This requires estimation of the flux rates as follows: H^+ production accompanying changes in PCr concentration via the creatine kinase reaction (H^+_{CK}, which is positive, i.e. H^+ 'consumption', when [PCr] is falling in exercise, and negative, i.e. H^+ generation, when [PCr] is rising in recovery); by the buffers of the muscle cytosol (H^+_β , which is positive, i.e. H^+ 'buffering' when pH_i is falling in exercise and negative, i.e. H^+ 'unbuffering' when pH_i is rising in recovery); and proton efflux from the cells (H^+_{efflux}). Together, these sum to the total proton yield (P) during exercise:

$$P = H^+_{CK} + H^+_\beta + H^+_{efflux} \quad (6)$$

From which:

$$L = 1.5 \times P \quad (7)$$

The number of protons consumed at the creatine kinase reaction was calculated from the time-dependent changes

in [PCr] using the proton stoichiometric coefficient, γ (Kushmerick, 1997), as follows:

$$H^+_{CK} = -\gamma D \quad (8)$$

Protons buffered (H^+_β, millimolar per minute) was calculated from the apparent buffering capacity, β_{total} (in millimoles of acid added per unit change in pH_i) and from the (smoothed) rate of pH change during exercise, as follows:

$$H^+_\beta = -\beta_{total}\left(dpH_i/dt\right) \quad (9)$$

where

$$\beta_{total} = \beta_{non\text{-}bicarbonate\text{-}non\text{-}P_i} + \beta_{P_i} \quad (10)$$

The intrinsic cytosolic buffering capacity ($\beta_{non\text{-}bicarbonate\text{-}non\text{-}P_i}$) is calculated from initial-exercise data:

$$\beta_{non\text{-}bicarbonate\text{-}non\text{-}P_i} = \beta_a - \beta_{P_i} \quad (11)$$

where the apparent $\beta(\beta_a)$ is obtained from the initial rate of change in [PCr] (ΔPCr_i) and alkalinization of pH (ΔpH_i):

$$\beta_a = \gamma \Delta PCr_i / \Delta pH_i \quad (12)$$

The value of β_{P_i} was calculated as follows:

$$\beta_X = \left(2.303 \times H^+ \times K \times [P_i]\right)/\left(K + H^+\right)^2 \quad (13)$$

where $K = 1.77 \times 10^{-7}$ (Conley *et al.* 1998). The $\beta_{bicarbonate}$ was neglected, which assumes that muscle is a closed system during short-duration exercise *in vivo* (Kemp *et al.* 1993). Proton efflux (H^+_{efflux}, millimolar per minute) was estimated for each time point of exercise assuming a linear pH-dependence constant, λ, as follows:

$$H^+_{efflux} = -\lambda \Delta pH_i \quad (14)$$

This proportionality constant, λ (millimolar per minute per pH unit) was estimated from initial recovery after exercise cessation, as follows:

$$\lambda = -V_{eff}/\Delta pH_i \quad (15)$$

At the cessation of exercise, the PCr resynthesized in the creatine kinase reaction is essentially a consequence solely of oxidative ATP production (Kemp *et al.* 2014). Therefore, H^+_{efflux} can be calculated from the rate of proton production from creatine kinase (H^+_{CK}) and the rate of pH change on the other side, as follows:

$$V_{eff} = \beta_{total}\left(dpH_i/dt\right) = \gamma V_{i,PCr} \quad (16)$$

Where ΔpH_i is very low, eqn (14) becomes unreliable, and the end-exercise rate of H^+_{efflux} is simply assumed to be equal to H^+_{efflux} calculated in from the initial recovery data by eqn (16).

In the present experiments, where pH_i is close to steady state or changing only slowly by the end of exercise, L is a very small component of ATP_{tot}.

Statistical analyses

Relationships between variables were assessed with a Pearson correlation coefficient, where appropriate. The differences between ^{31}P measures at discrete time points and across exercise intensities were compared with a two-factor (time × intensity domain) repeated-measures ANOVA. Bonferroni-corrected Student's paired t tests were used *post hoc* to identify simple effects in the case of a significant main effect. For all tests, $\alpha = 0.05$. Analyses were completed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA).

Results

During RI exercise, participants attained a peak power output of 47 ± 11 W and a $\dot{V}_{O_2peak}$ of 2.00 ± 0.48 l min^{-1}. Based on peak power output and estimated LT (~60–70% $\dot{V}_{O_2peak}$), moderate (sub-LT; 19 ± 4 W) and heavy (supra-LT; 46 ± 11 W) constant-power exercise bouts were assigned. The dynamics of $\dot{V}_{O_2}$ were examined *post hoc* to confirm the appropriate intensity assignment (Whipp, 1996; Rossiter, 2011).

During moderate exercise, there was no discernable pulmonary $\dot{V}_{O_2sc}$ (0.06 ± 0.12 l min^{-1}). However, during heavy exercise the $\dot{V}_{O_2sc}$ was 0.37 ± 0.16 l min^{-1} (Fig. 1*A*), or a 22 ± 8% increase. The [PCr] did not change between 3 and 8 min of moderate-intensity exercise (30 ± 8 *vs.* 32 ± 7 mM; n.s.). Conversely, during heavy exercise [PCr] fell from 3 to 8 min (19 ± 7 *vs.* 18 ± 7 mM or 12 ± 15% fall; $P < 0.05$; Fig. 1*B*).

The ATP yield during moderate and heavy exercise from oxidative phosphorylation (Q), PCr hydrolysis (D), lactate production (L) and, consequently, ATP_{tot} are presented in Table 1. The $V_{i[PCr]}$, calculated as described in eqn (4) is shown, along with the rate constant of PCr resynthesis (k) and the amplitude of PCr recovery (A), in Figs 2 (moderate) and 3 (heavy).

Comparisons of ATP_{tot} revealed a significant interaction (time × intensity domain; $F(1,13) = 17.2$; $P < 0.01$; $\eta^2 = 0.57$). The ATP_{tot} was not different between 3 and 8 min of moderate exercise (n.s.; Fig. 2 and Table 1), but ATP_{tot} increased (ΔATP_{tot}) during heavy exercise from 3 to 8 min (CI_{95} of the difference; $CI_{Difference}$ 1.9, 12.6 mM min^{-1}; $P < 0.05$; Fig. 3 and Table 1), equating to a 26 ± 30% increase in ATP_{tot} from 3 to 8 min (Fig. 4*A*). This percentage change in ATP_{tot} was not different to that measured in both the $[PCr]_{sc}$ and the $\dot{V}_{O_2sc}$ ($F(2,26) = 2.4$; n.s.; $\eta^2 = 0.16$; Fig. 4*A*). However, among participants the individual values of ΔATP_{tot} during heavy exercise were

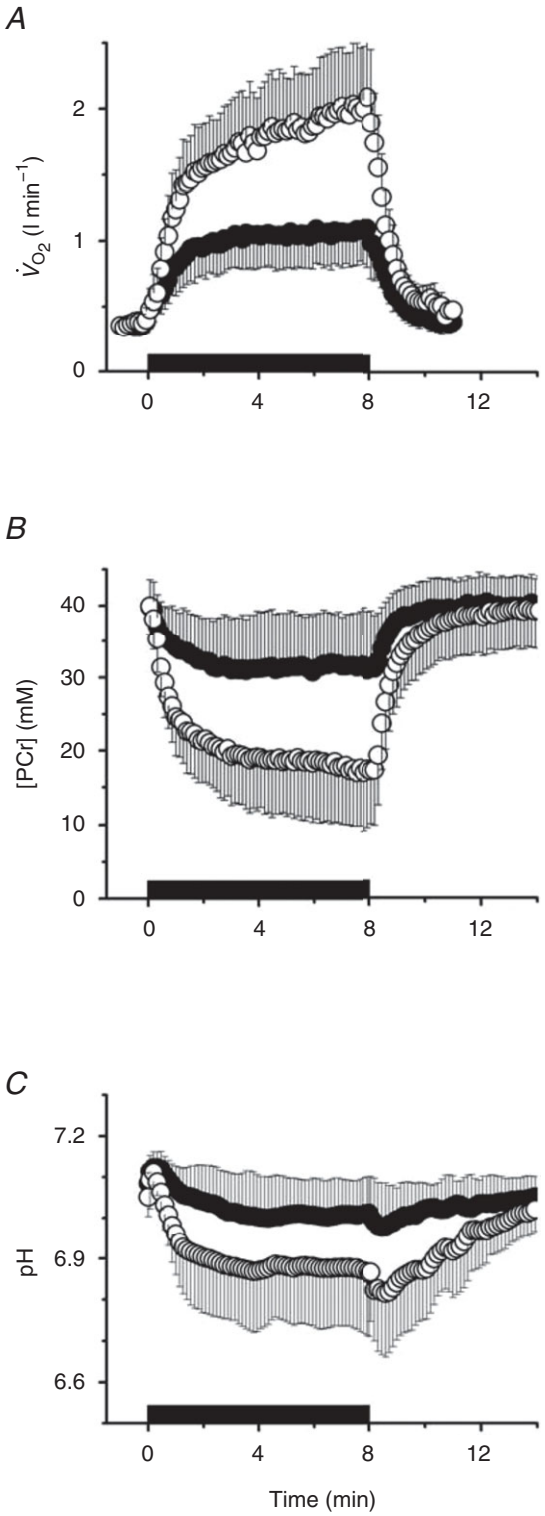

Figure 1. Rate of whole-body O_2 uptake ($\dot{V}_{O_2}$; *A*), phosphocreatine concentration ([PCr]; *B*) and pH (*C*) plotted as a function of time for moderate-intensity (filled circles) and heavy-intensity prone bilateral knee-extension exercise (open circles)
Black bar denotes exercise bout from time 0 to 8 min. Data points are 8 s means, with error bars representing SD.

Table 1. Rates of ATP turnover from oxidative phosphorylation (*Q*), phosphocreatine hydrolysis (*D*), lactate production (*L*) and the sum (ATP_{tot}) during moderate and heavy constant-power exercise at two time points

	Moderate exercise		Heavy exercise	
Parameter	3 min	8 min	3 min	8 min
Q (mM min^{-1})†	23 (14)	17 (13)	35 (17)	42 (13)*
D (mM min^{-1})	0.6 (1.2)	0.2 (1.0)	1.1 (2.6)	0.7 (0.9)
L (mM min^{-1})	1.0 (1.3)	0.3 (0.6)	1.5 (1.3)	1.3 (1.7)
ATP_{tot} (mM min^{-1})‡	24 (14)	17 (14)	38 (16)	44 (14)*

Values are presented in millimolar per minute as means (SD). *Different from 3 min; $P < 0.05$. †Time × intensity interaction; $P < 0.01$; $F(1,13) = 17.2$; $\eta^2 = 0.57$. ‡Time × intensity interaction; $P < 0.01$; $F(1,13) = 17.2$; $\eta^2 = 0.57$

not significantly correlated with the magnitude of the $\dot{V}_{O_2sc}$ (Fig. 4*B*).

To examine the relationship between $\dot{V}_{O_2}$ and [PCr], a correction for the transit delay from muscle to lung was applied. The $\dot{V}_{O_2}$ data were time corrected using 12 s difference with respect to ^{31}P measures (Rossiter *et al.* 1999; Krustrup *et al.* 2009). The relationship between $\dot{V}_{O_2}$ and [PCr] was linear during moderate exercise and the first 3 min of heavy exercise ($r^2 = 0.94$; Fig. 5). However, the slope of the [PCr]–$\dot{V}_{O_2}$ relationship was significantly steeper when data from 8 min of heavy exercise were included (-67 ± 25 *vs.* -61 ± 25 ml min mM^{-1}; $P < 0.05$).

Discussion

The [PCr] slow component ($[PCr]_{sc}$), like the $\dot{V}_{O_2sc}$, is present only during exercise above the LT. The finding that the $[PCr]_{sc}$ and $\dot{V}_{O_2sc}$ are of similar magnitude (Rossiter *et al.* 2002) led to the argument that the $\dot{V}_{O_2sc}$ is caused by an increased phosphate cost of power production (P/W) during heavy-intensity exercise. However, this is at odds with the observed dissociation between the $[PCr]_{sc}$ and $\dot{V}_{O_2sc}$ in endurance-trained individuals (Layec *et al.* 2009*b*, 2012), and both observations relied upon equivocal assumptions about the dynamic relationships between [ADP] and $\dot{V}_{O_2}$ or $\dot{V}_{O_2}$ and [PCr] (Yoshida & Watari, 1993, 1994; Rossiter *et al.* 2002; Jones *et al.* 2008; Kemp, 2008; Wüst *et al.* 2011). Our present data agree with previous reports that mean $[PCr]_{sc}$ and $\dot{V}_{O_2sc}$ magnitudes were not statistically different. Crucially, however, the data add that, among individuals, the increase in the $\dot{V}_{O_2sc}$ during heavy-intensity exercise (averaging ~22%) is not correlated with the increase in the phosphate cost of power production, ATP_{tot} (average ~26%). Thus, while the exercising limb is likely to remain the major source of the $\dot{V}_{O_2sc}$ (Poole *et al.* 1991; Rossiter *et al.* 2002; Bailey *et al.* 2010; Dimenna *et al.* 2010), the observed dissociation

between $\dot{V}_{O_2sc}$ and ΔATP_{tot} (Fig. 4*B*) strongly suggests that the progressive increase in $\dot{V}_{O_2}$ during heavy exercise is not solely due to contractile inefficiency (P/W). Thus, other explanations, such as a reduction in mitochondrial efficiency (P/O), should also be considered.

ATP turnover during moderate and heavy constant-power exercise

The primary aim of this investigation was to estimate the ATP turnover rate for exercise below and above the LT and over time without assumptions about the [ADP]–$\dot{V}_{O_2}$ or $\dot{V}_{O_2}$–[PCr] relationships. By using the most robust estimations of ATP_{tot} (Kemp *et al.* 1995; Walter *et al.* 1999; Lanza *et al.* 2005; Faraut *et al.* 2007), we provided ^{31}P MRS-derived estimates of ATP yield from oxidative phosphorylation, lactate production and PCr hydrolysis at 3 and 8 min of exercise that were unencumbered by the recently challenged assumptions about the [ADP]–$\dot{V}_{O_2}$ relationship (Kemp, 2008; Wüst *et al.* 2011; Glancy & Balaban, 2012; Kemp *et al.* 2014).

Unsurprisingly, there were no changes in ATP_{tot} during exercise below the lactate threshold, where negligible muscle fatigue is expected (Sargeant & Dolan, 1987; Yano *et al.* 2001), reflecting steady-state conditions. Conversely, during heavy exercise in which the $\dot{V}_{O_2sc}$ and $[PCr]_{sc}$ were present, ATP_{tot} was increased between 3 and 8 min of exercise. This is consistent with the suggestions that the $\dot{V}_{O_2sc}$ is consequent to increased P/W in the large locomotor muscles during supra-LT exercise (Rossiter *et al.* 2002), perhaps associated with muscle fatigue and a reduction in contractile efficiency. However, the lack of relationship between ΔATP_{tot} and $\dot{V}_{O_2sc}$ is in contrast to this postulate and challenges the current understanding of the aetiology of $\dot{V}_{O_2sc}$ (Rossiter, 2011; Poole & Jones, 2012).

Dissociation of the $\dot{V}_{O_2sc}$ and changes in the phosphate cost of exercise may have a few explanations. It may indicate an increase in $\dot{V}_{O_2}$ originating from regions within the knee extensors that are not interrogated by the surface coil. While we can only speculate on this, a similar finding has been reported where the $\dot{V}_{O_2}$ and [PCr] slow components were dissociated in endurance-trained participants but not in sedentary control subjects, despite increasing EMG activity in both participant groups during the $\dot{V}_{O_2sc}$ (Layec *et al.* 2009*b*, 2012). It was hypothesized that the exercise-trained volunteers may be better able to optimize motor unit recruitment patterns to maintain high-intensity exercise (e.g. compared with active but

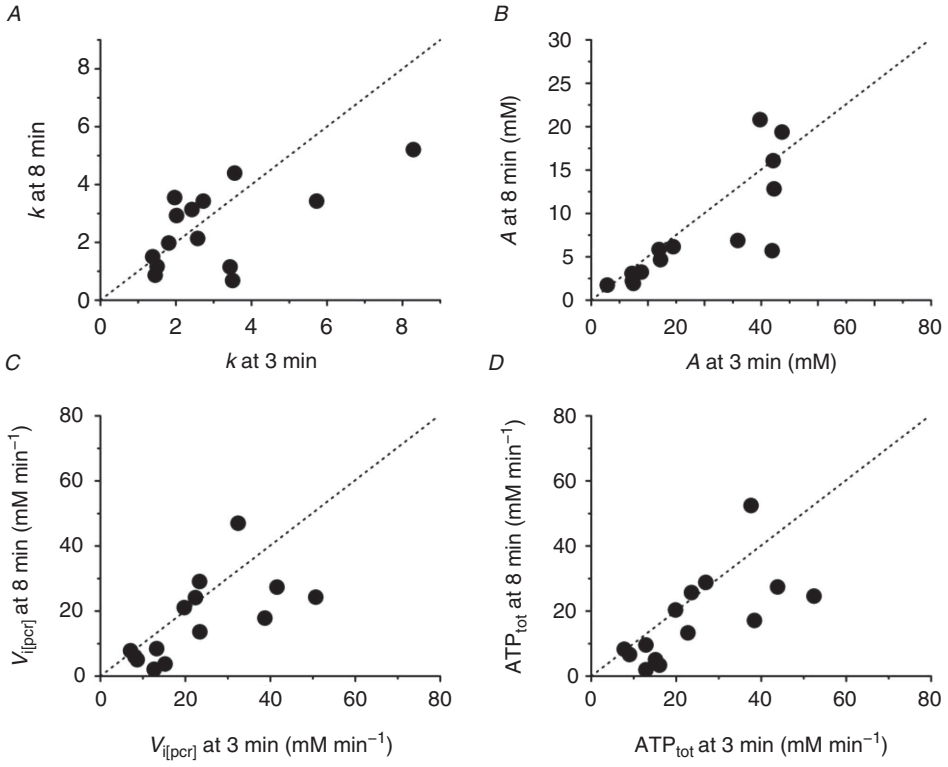

Figure 2. Moderate-intensity exercise recovery rate constant (*k*; *A*), amplitude of PCr resynthesis (termed '*A*'; *B*), initial rate of PCr resynthesis ($V_{i[PCr]}$; *C*) and total ATP turnover rate (ATP_{tot}; *D*) at 8 min of exercise, plotted as a function of 3 min of exercise
Dashed line is $y = x$.

untrained subjects; Rossiter *et al.* 2002), thereby recruiting motor unit pools that reside outside of the muscle volume being interrogated by MRS. It should be noted, however, that our surface coil interrogated a large muscle volume (~300 g) compared with alternative techniques, e.g. biopsy (~200 mg). Additionally, controversy exists about whether progressive recruitment itself is even responsible for the slow component (Zoladz *et al.* 2008; Cannon *et al.* 2011; Vanhatalo *et al.* 2011), in which case recruitment of muscle outside the surface coil view would seem to be an unlikely explanation if the recruitment pattern is stable.

The source of the $\dot{V}_{O_2sc}$ may even reside outside of the locomotor muscles. Progressive increases in respiratory (Wasserman *et al.* 1995; Żołądź & Korzeniewski, 2001) or cardiac work, or even work from non-power-producing musculature, such as stabilizing effort during cycling (Billat *et al.* 1998), may contribute to a reduction in exercise efficiency during the slow component. It is unlikely that the stabilizing effort would contribute to prone knee extension, where the work of stabilizing the torso is minimized by the body position, the ergometer and the heavy strapping used to isolate quadriceps activity. Nonetheless, the work of ventilation during prone knee extension may still contribute a meaningful proportion, particularly as the locomotor muscle mass in our study is relatively small in comparison to cycling or running.

Finally, dissociation of the $\dot{V}_{O_2sc}$ and ΔATP_{tot} could result from mitochondrial uncoupling (reduced P/O; Fig. 5). In this scenario, an increased O_2 cost of ATP resynthesis may contribute to driving the increase in $\dot{V}_{O_2}$ during heavy exercise, rather than it coming exclusively from an increased ATP cost of muscle power generation.

The $\dot{V}_{O_2}$–[PCr] relationship and mitochondrial coupling during heavy-intensity exercise

Without an invasive measure of $\dot{V}_{O_2}$ across the volume of tissue interrogated by MRS, the relationship between whole-body $\dot{V}_{O_2}$ and localized [PCr] is the next best estimate for coupling of O_2 uptake and ATP turnover. Our data show that the mean $\dot{V}_{O_2}$–[PCr] relationship was linear over the moderate intensity and during the first minutes of heavy exercise ($r^2 = 0.94$; Fig. 5). Importantly, this relationship became steeper ($P < 0.05$) with the inclusion of data from the final minutes of heavy exercise. With some important assumptions, these data suggest a reduced P/O between 3 and 8 min of heavy exercise, implicating mitochondrial uncoupling as an additional mechanism of the $\dot{V}_{O_2sc}$.

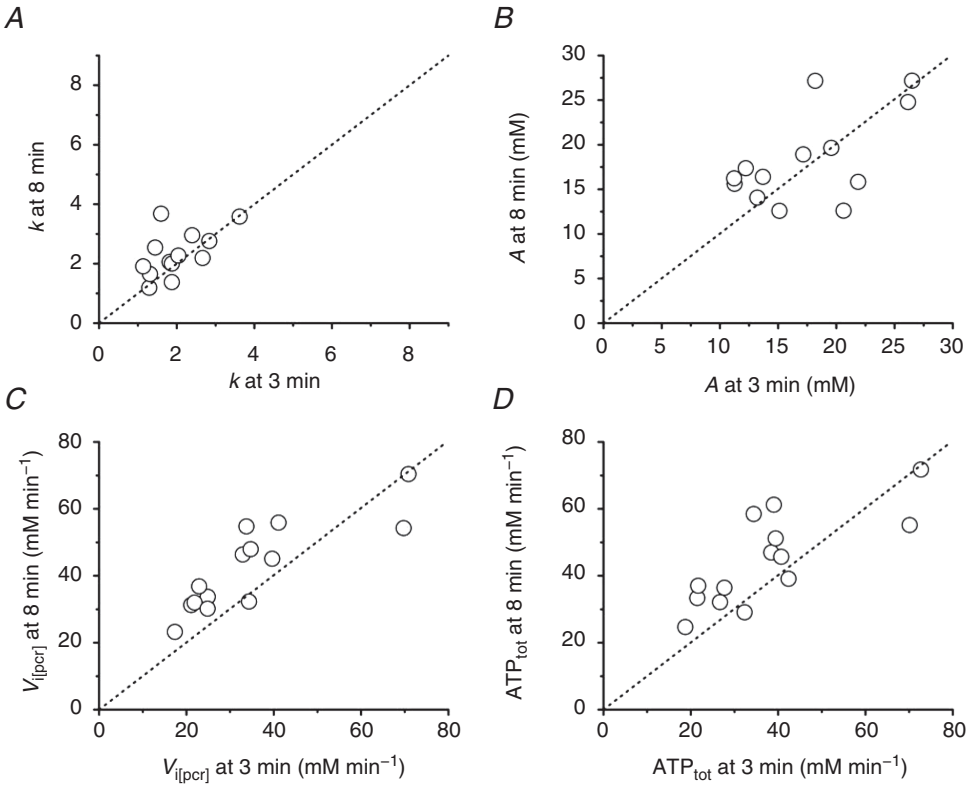

Figure 3. Heavy-intensity exercise recovery rate constant (*k*; *A*), amplitude of PCr resynthesis (termed '*A*'; *B*), $V_{i[PCr]}$ (*C*) and ATP_{tot} (*D*) at 8 min of exercise, plotted as a function of 3 min of exercise
Dashed line is $y = x$.

It is important to recognize that the slope of the $\dot{V}_{O_2}$–[PCr] relationship reflects the combined influence of mitochondrial density, the rate constant (k) of [PCr] breakdown relative to k of $\dot{V}_{O_2}$, the total [creatine] and the P/O (Meyer, 1988; Kemp *et al.* 2014). Mitochondrial density and total [creatine] are constant during acute exercise, and therefore any divergence in $\dot{V}_{O_2}$–[PCr] slope would result from changes in k[PCr] and/or P/O over the exercise intensities. While the k[PCr] was not different between 3 and 8 min of heavy-intensity exercise (p = n.s.), there was variance among individuals (Fig. 3*A*). Therefore, while we base our interpretation on the group mean, we cannot rule out the influence of variance in the individual changes in k[PCr] in interpreting the $\dot{V}_{O_2}$–[PCr] slope. In addition, we used a fixed transit delay to phase align the $\dot{V}_{O_2}$ and [PCr] measurements in the time domain. This correction provided the best fit to the kinetics that we could make, but it is a limitation for interpreting the $\dot{V}_{O_2}$–[PCr] relationship. Specifically, small errors in transit delay adjustment result in non-linear distortion when plotting single participant data, although this influence is greater during the early kinetics (first 2 min) than between 3 and 8 min of exercise, when the kinetics are slower. Finally, the progressive intramuscular acidification during exercise would be expected to dissociate the dynamics of $\dot{V}_{O_2}$ and [PCr], speeding the former and slowing the latter (Iotti *et al.* 1993; Gerbino *et al.* 1996; Layec *et al.* 2013). Therefore, while substantial assumptions necessarily underlie the interpretation of the $\dot{V}_{O_2}$–[PCr] relationship, it is currently the only way to examine change in P/O as a potential mechanism explaining the lack of relationship between the magnitude of the $\dot{V}_{O_2sc}$ and ΔATP_{tot}. These data suggest that P/O is stable during moderate-intensity exercise and the first 3 min of heavy-intensity exercise, in agreement with the other ^{31}P MRS studies (e.g. where the $\dot{V}_{O_2}$–[PCr] relationship is strikingly linear throughout the metabolic rate range; Bailey *et al.* 2010), but that sustained heavy-intensity exercise beyond 3 min may be accompanied by a reduction in P/O. Consequently, contrary to the prevailing hypothesis (Rossiter *et al.* 2002), the $\dot{V}_{O_2sc}$ may be, at least in part, a result of mitochondrial uncoupling in the active muscle during acidifying exercise.

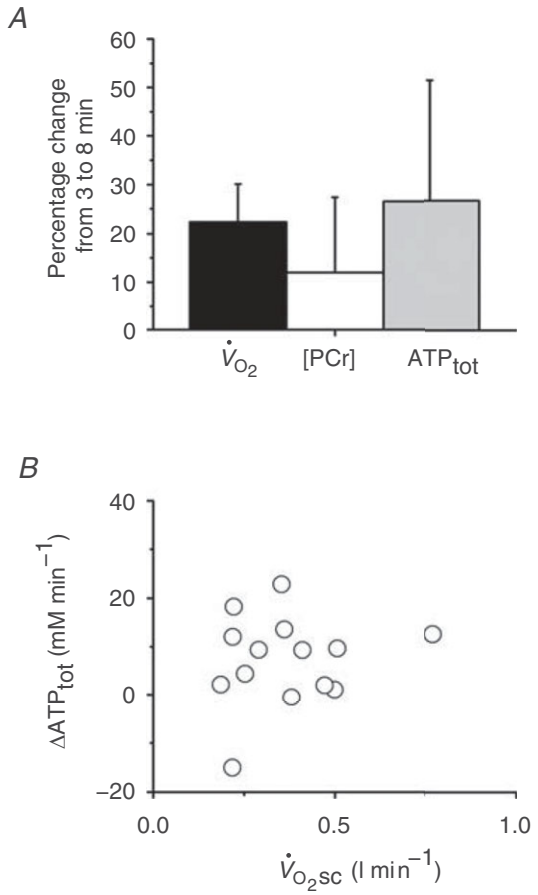

Figure 4. Magnitudes and relationship between $\dot{V}_{O_2}$ slow component and muscle [PCr] and ATP_{tot} slow components
$\dot{V}_{O_2sc}$ and $[PCr]_{sc}$ and ΔATP_{tot} from minute 3 to 8 of heavy exercise expressed as a percentage change (*A*). *B* shows ΔATP_{tot} during heavy exercise plotted as a function of the $\dot{V}_{O_2sc}$.

Potential mechanisms of mitochondrial uncoupling

There are various mechanisms that might cause the mitochondrial transmembrane proton gradient to dissipate during exercise. This proton 'leak' is regulated by uncoupling proteins and contributes to setting the resting P/O. If this process is augmented during exercise,

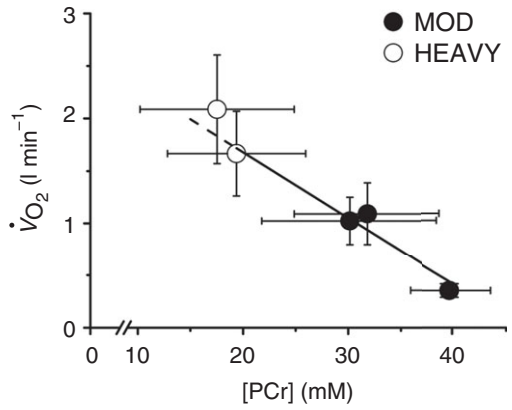

Figure 5. Relationship between pulmonary $\dot{V}_{O_2}$ and [PCr] during moderate (filled circles) and heavy exercise (open circles)
The regression shown (continuous line) was fitted to data from moderate exercise and from the first 3 min of heavy exercise and extrapolated (dashed line) to 8 min of heavy exercise. Error bars represent SD. The $\dot{V}_{O_2}$ data were phase aligned with respect to [PCr] measurements.

the ATP yield per atomic oxygen consumed would fall. Others have shown upregulation of uncoupling proteins 2 and 3 (both expressed in skeletal muscle) with an acute bout of exercise, and these can induce mitochondrial uncoupling, which is likely to minimize production of, and damage from, reactive oxygen species (Brand *et al.* 2004; Bo *et al.* 2008; Jiang *et al.* 2009). This effect may be akin to the chronic uncoupling reported with ageing, posited as a protective mechanism against damage from reactive oxygen species (Brand *et al.* 2004; Amara *et al.* 2007), particularly as leak respiration comprises a large proportion of resting $\dot{V}_{O_2}$. However, the kinetics of upregulation of uncoupling proteins are relatively slow in comparison with the duration of exercise in our study; upregulation of uncoupling proteins is typically present ∼45–90 min after acute exercise. Additionally, investigations into mitochondrial uncoupling have relied on relatively long bouts of exercise (>30 min), and evidence from human muscle suggests that acute exercise may not be sufficient to elicit the same effect size for upregulation as seen in the rat (Fernström *et al.* 2004). Therefore, upregulation of uncoupling proteins seems less likely to explain fully the lack of relationship between $\dot{V}_{O_2sc}$ and ΔATP_{tot} during heavy exercise.

Alternatively, dissociation of the $\dot{V}_{O_2sc}$ and ΔATP_{tot} may result from high $[H^+]$ or $[P_i]$ during exercise (Walsh *et al.* 2002). Low pH can reduce [ADP] from a shift in the creatine kinase equilibrium (Conley *et al.* 2001) and also serve to dissociate creatine kinase from the mitochondrial membrane, leading to a disruption in oxidative phosphorylation (Walsh *et al.* 2002). While evidence for a direct effect of acidosis is certainly not conclusive (Suleymanlar *et al.* 1992; Kemp *et al.* 2014), numerous studies show disturbances to oxidative phosphorylation through the inhibition of respiratory enzymes or reductions in the proton motive force (Hillered *et al.* 1984; Harkema & Meyer, 1997; Jubrias *et al.* 2003), but fail to result in change to P/O alone (Tonkonogi & Sahlin, 1999). Nevertheless, the variable relationships between the magnitude of the $\dot{V}_{O_2sc}$ and ΔATP_{tot}, together with a steepened $\dot{V}_{O_2}$–[PCr] relationship, suggest P/O change as a possible scenario during heavy exercise.

Technical considerations and study limitations

While limitations accompany the estimations, our study design provides an advantage over previous reports of ATP turnover rate in the literature. Prior estimations have relied on extrapolation of $V_{i[PCr]}$, which is assumed to be affected only by the [PCr] recovery amplitude. This model constrains P/O with a linear $\dot{V}_{O_2}$–[PCr] relationship, by definition (Layec *et al.* 2009*a*, which is in contrast with recent findings (Kemp, 2008; Wüst *et al.* 2011; Glancy & Balaban, 2012) and the observations in this study (Fig. 5). Conversely, [PCr] recovery dynamics may be plastic during supra-LT exercise where intracellular acidification (Yoshida & Watari, 1993, 1994), fatigue-related metabolite accumulation (Jones *et al.* 2008) and muscle fatigue (Yano *et al.* 2001; Cannon *et al.* 2011) have been reported. While the group mean for k[PCr] resynthesis (or time constant, $\tau = 1/k$) is not different following sub- and supra-LT exercise in this study and others (Rossiter *et al.* 2002), our data suggest that k[PCr] is not constant within an individual. Therefore, in our study, $V_{i[PCr]}$ (and, thus, Q and ATP_{tot}) were not constrained to increase in response only to changes in [PCr]. In other words, the augmented amplitude of [PCr] during the slow component did not result in an obligatorily faster initial rate of change following the cessation of exercise; our measurement was dependent on the recovery dynamics characterized and specific to that moment in time. Consequently, the estimations provided for oxidative ATP yield in our study are devoid of the assumptions about the $\dot{V}_{O_2}$–[ADP] and $\dot{V}_{O_2}$–[PCr] relationships.

ATP_{tot} is most heavily weighted on changes in $V_{i[PCr]}$, a measure that is sensitive to noise in the MRS signal (e.g. Fig. 7 of Rossiter *et al.* 2000); this initial rate is derived from characterization of the kinetics of [PCr] recovery. The influence of noise in [PCr] recovery kinetics, particularly in the early transient, is likely to be the largest source of variability to resolve ATP_{tot}. Conversely, the confidence in characterizing [PCr] off-kinetics is substantially greater than for pulmonary $\dot{V}_{O_2}$ or even [PCr] during the on-transient. Any improvement in the characterization of ^{31}P dynamics will take a considerable leap in signal-to-noise ratio and more rapid acquisition of spectra.

The heterogeneous nature of skeletal muscle metabolism (Koga *et al.* 2007; Damon *et al.* 2008; Saitoh *et al.* 2009; Cannon *et al.* 2013) may have obscured the characterization of [PCr] dynamics, and therefore ATP_{tot}. Using ^{31}P MRS, we measured a volume of tissue (∼300 g) that may not be representative of the entire knee extensor group responsible for the power output or the diversity of metabolic strain within this group. Finally, the unmeasured work of knee flexion is not accounted for with our ergometer. Therefore, the work of knee flexion (to lift the leg) is assumed to be constant in our experiments, but does contribute to the pulmonary $\dot{V}_{O_2}$ signal.

Conclusions

Similar to previous studies, the mean magnitudes of the $\dot{V}_{O_2}$ and [PCr] slow components were not different during heavy exercise, consistent with the prevailing hypothesis for the intramuscular source of the $\dot{V}_{O_2sc}$, i.e. an increase in the phosphate cost of force production. Although the magnitude of the $\dot{V}_{O_2sc}$ (∼22%) was similar to the increase in ATP_{tot} (∼26%) from 3 to 8 min during heavy exercise,

there was no relationship detected between these measures among individuals. Therefore, our data suggest that the pulmonary $\dot{V}_{O_2sc}$ does not originate solely from increases in the phosphate cost of power production (increased P/W). Other mechanisms, such as an increased O_2 cost of ATP resynthesis (reduced P/O) during acidifying exercise, may also contribute to generating the $\dot{V}_{O_2sc}$.

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Additional information

Competing interests

None declared.

Author contributions

D.T.C., G.J.K. and H.B.R. conceived and designed experiments and analysed data. All authors performed experiments and interpreted data. D.T.C. prepared the figures. D.T.C. and H.B.R. wrote the manuscript. All authors critically reviewed and approved the final version of the manuscript.

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