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# MRS evidence of adequate $O_2$ supply in human skeletal muscle at the onset of exercise

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## Abstract

**Purpose—**At exercise onset, intramuscular oxidative energy production responds relatively slowly in comparison to the change in ATP demand. To determine if the slow kinetics of oxidative ATP production is due to inadequate $O_2$ supply or metabolic inertia we studied the kinetics of intramyocellular deoxygenation (deoxy-myoglobin, Mb) and metabolism (phosphocreatine, PCr), using proton ($^1H$) and phosphorus ($^{31}P$) magnetic resonance spectroscopy (MRS) in 6 healthy subjects (33 ± 5 yrs).

**Methods—**Specifically, utilizing dynamic plantar flexion exercise, rest to exercise and recovery was assessed at both 60% of maximum work rate (WRmax) (moderate intensity) and 80% of WRmax (heavy intensity).

**Results—**At exercise onset [PCr] fell without delay and with a similar time constant (τ) at both exercise intensities (~33 s). In contrast, the increase in deoxy-Mb was delayed at exercise onset by 5–7 s, after which it increased with kinetics (moderate τ = 37 ± 9 s, and heavy τ = 29 ± 6 s) that were not different from τPCr ($p > 0.05$). At cessation, deoxy-Mb recovered without a time delay and more rapidly (τ ~20 s) than PCr (τ ~33 s) ($p < 0.05$).

**Conclusion—**using a unique combination of *in vivo* MRS techniques with high time-resolution, this study revealed a delay in intramuscular de-oxygenation at the onset of exercise, and rapid re-

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oxygenation kinetics upon cessation. Together these data imply that intramuscular substrate-enzyme interactions, and not $O_2$ availability, determine the exercise onset kinetics of oxidative metabolism in healthy human skeletal muscle.

**Keywords**

Myoglobin; $PO_2$; phosphocreatine; skeletal muscle; magnetic resonance spectroscopy; $VO_2$ kinetics; metabolic inertia

## Introduction

At the onset of exercise, muscle $O_2$consumption ($VO_2$) does not respond instantaneously to match metabolic demand. During this kinetic phase the substantial increase in ATP demand is met by a diminishing contribution from substrate level phosphorylation. Specifically, glycogenolysis resulting in lactate accumulation, and the rapid breakdown of phosphocreatine (PCr) via the creatine kinase reaction. Considering the key role that is played by the $VO_2$ kinetics in the ability to sustain muscular work, the mechanism (s) responsible for determining the rate of oxidative phosphorylation at the onset of exercise has been a topic of intense research over the past two decades. Indeed, whether $VO_2$ kinetics are restricted by a delay in the flux through the oxidative ATP generating pathways (i.e. "metabolic inertia"), or is a consequence of limited $O_2$ availability to the mitochondria at the onset of exercise in humans remains to be directly assessed (29).

Interestingly, there is now substantial evidence from the animal literature indicating that under normal or even conditions of enhanced convective $O_2$ delivery or peripheral $O_2$ diffusion, the fall in skeletal muscle $PO_2$ (17, 40), or rise in $VO_2$ (5, 7) is delayed at the onset of contractile activity, suggesting that intracellular factors, rather than $O_2$ delivery, limits muscle $VO_2$ during the rest to exercise transition. While providing important mechanistic insight into the determinants of $VO_2$ kinetics, it is difficult to extrapolate these results to contracting human skeletal muscles which are characterized by heterogeneous blood flow distribution, fiber type, and metabolic properties. In humans, while blood flow kinetics are faster than $VO_2$ kinetics during both moderate and severe intensity knee-extension or cycling exercise, these arterio-venous measurements may not reflect intramyocellular conditions (21), 8). Over the years, a conceptual framework describing a tipping point between an $O_2$ delivery-dependent and –independent zone (29), that varies depending on the exercise modality, intensity, and cardio-vascular fitness has received considerable attention (37). However, such investigations were limited by the use of indirect methods to assess muscle oxygenation and metabolism (near-infrared spectroscopy and pulmonary gas exchange).

The fact that myoglobin (Mb), an $O_2$-binding protein found exclusively in muscle tissue desaturates in relation to changes in intracellular partial pressure of $O_2$ ($iPO_2$) and deoxy-Mb can be non-invasively measured by MRS (31) offers a unique opportunity to discern, *in vivo*, whether $O_2$ utilization or $O_2$ transport limit the metabolic response to exercise onset in humans. Using this technique, were $iPO_2$ within active muscle to remain unchanged this would suggest that metabolic inertia was the main determinant of $VO_2$ kinetics in healthy

human muscle. This would imply adequate $O_2$ supply to support the highest rate of oxidative phosphorylation possible for the current metabolic demand. In contrast, were $iPO_2$ to fall instantaneously at exercise onset this would support the notion that $VO_2$ kinetics could be sped up if more $O_2$ were available. In addition, given the direct proportionality between the kinetics of $VO_2$ and PCr consumption (24, 34), the monitoring of [PCr] with phosphorus ($^{31}P$) MRS affords the opportunity to simultaneously determine the kinetics of metabolism within skeletal muscle at the onset of exercise.

Consequently, we aimed to determine whether $O_2$ supply or $O_2$ utilization limits metabolism at the onset of exercise in humans, using an interleaved MRS sequence. Specifically, we assessed deoxy-Mb and [PCr] during rest to dynamic plantar flexion exercise performed at both moderate (60% of maximal aerobic power, WRmax) and heavy (80% WRmax) intensities. We hypothesized that a limitation due to intrinsic metabolic inertia would manifest as a delay in the fall of $iPO_2$ and an immediate decline in [PCr] at both exercise intensities.

## Method

### Subjects

The protocol received unrestricted approval from the Pitié-Salpêtrière University Hospital Ethics Committee (Paris, France) and prior to participation in the study the 6 volunteers gave written informed consent. Subjects were not recruited with regard to exercise habits and were therefore of varying activity levels, although none of them were involved in a regular exercise training program. Mean age, height, and body weight were 33 ± 5 yrs, 174 ± 3 cm, and 71 ± 4 kg, respectively.

### Experimental protocol and set-up

Initially, each subject was familiarized with the experimental set-up and performed a graded plantar flexion exercise test to WRmax on an amagnetic pneumatic ergometer in a 4 Tesla, 46 cm internal bore, superconducting magnet (Magnex 4/60) interfaced to a Bruker Biospec MR spectrometer. The rest of experimental protocol focused upon the metabolic and oxygenation kinetics in the muscles of the lower leg during both moderate and heavy-intensity (60 and 80% of WRmax, respectively) dynamic plantar flexion exercise. The ergometer was interfaced to a PC, which allowed workload programming and the instant reading of power output. The calf of the subject's dominant leg was carefully positioned inside a 17 cm inner diameter transversal electromagnetic (TEM) $^{1}H$ transmit and receive volume coil, and an 8 cm diameter custom-built $^{31}P$ surface coil was positioned underneath the gastrocnemius, as previously described (3). 15 min after the termination of the exercise, an air-cuff was wrapped above the knee of this leg and inflated to 240 mmHg for 10 minutes to achieve complete vascular occlusion and determine the maximum deoxy-Mb signal in the lower leg.

The interleaved multi-parametric functional MR acquisition of metabolic responses ($^{31}P$-MRS) and oxygenation ($^{1}H$-MRS) was performed continuously over a five minute rest period, 10 minutes of plantar flexion exercise, and 15 minutes of recovery. Subjects

performed the plantar flexions at a constant rate of 0.67 Hz, guided by a gradient pulse generating an audible signal that was inserted into the MR sequence. The $B_0$ field homogeneity was optimized with Fastmap, an automatic localized 1st and 2nd order shim procedure. Other adjustments and the acquisition of reference images and spectra were performed in resting conditions for ~20 min. Heart rate and arterial blood $O_2$ saturation were monitored continuously using an MR-compatible patient-monitoring device (MAGLIFE, Bruker, Wissembourg, France).

## Multi-parametric MR Spectroscopy

Calf muscle intracellular [deoxy-Mb] and [PCr] were studied simultaneously by rapidly interleaved acquisitions of $^{1}$H-MRS and $^{31}$P-MRS (3). This interleaved acquisition scheme was driven by the Multi Scan Control (MSC) tool developed and made commercially available by Bruker. A complete data set was generated every 1.5 s, and comprised of the following acquisitions:

**A single $^{1}$H–NMR spectrum of deoxymyoglobin—**The n–δ proton of the proximal histidine F8 of myoglobin in the deoxygenated state was selectively excited by a 0.8 ms Gaussian pulse (64 accumulations, 256 complex points, acquisition time 6 ms, total acquisition time per spectra 384 ms). Each $^{1}$H–NMR scan acquisition was followed by a $^{31}$P-NMR scan for an effective time resolution of 1.5s (1 scan per spectrum) and 6s (4 scans per spectrum) for myoglobin and PCr respectively.

**A single $^{31}$P-NMR spectrum—**Phosphates were excited by a single 0.5 ms square pulse, and the FID 2048 complex data points were collected in 128 ms.

## NMRS data processing

The MSC tool automatically distributed the raw interleaved data into distinct $^{1}$H and $^{31}$P spectroscopy, files, which were immediately ready for processing with standard ParaVision and XWIN MRS software.

## $^{1}$H deoxymyoglobin spectra

After a 100-Hz line-broadening exponential multiplication and Fourier transformation, zero and 1rst order phases of the Mb spectrum were adjusted manually on an end-exercise acquisition. All free induction decays (FIDs) of the series were processed using these same parameter settings. After automatic baseline correction (+20/−20 ppm), the Mb peak of each spectrum was quantified by integration over 10 ppm (3, 31).

## $^{31}$P phosphate spectra

The $^{31}$P FIDs were averaged over 6 s (4 scans per spectrum) and processed in a similar fashion as the $^{1}$H spectra, except for an 8 Hz line-broadening exponential multiplication. Inorganic phosphate (Pi) and phosphocreatine (PCr) integration limits were set to 5.6–3.5 ppm and (−)1.5-(+)1.5 ppm, respectively. Intracellular pH was calculated from the chemical shift difference between the Pi and PCr signals.

### Calculation of $iPO_2$

Deoxy-Mb reached a plateau between the 8th and 10th minutes of cuff occlusion (240 mmHg), and were assumed to represent the complete deoxygenation of Mb. The fractional deoxy-Mb ($^f$deoxy-Mb) was determined by normalizing the signal areas to the average signal obtained during the last minutes of cuff ischemia. The conversion from $^f$deoxy-Mb to $PO_2$ values was calculated from the oxygen-binding curve for Mb at 37°C:

$$PO_2 = {}^f MbO_2 * MbP_{50} / {}^f dexoy - Mb \quad (1)$$

Where $^fMbO_2$ is the fraction of Mb that is oxygenated and $P_{50}$ is the $O_2$ pressure where 50% of the Mb binding sites are bound with $O_2$. The temperature-dependent Mb half saturation ($P_{50}$) of 3.2 mmHg was used (33).

Pilot assessments of the cross sectional area of the gastrocnemius-soleus muscles as a ratio of all muscles within the TEM coil revealed that although the ratio tended to increase across proximal to distal slices within the coil, the image at the center of the coil provided a robust index of this ratio. Thus, the ratio of gastrocnemius-soleus/other muscles calculated from this image was used to normalize the complete deoxy-Mb signal to only the active muscle during exercise.

### Kinetic analyses

Changes in deoxy-Mb onset and PCr offset were fit with the general exponential function:

$$\mathrm{Y(t)} = \mathrm{Y_0} + \mathrm{Y_1}(1 - \mathrm{e}^{-(\mathrm{t}-\mathrm{TD})/\tau}) \quad (2)$$

where $Y_0$ is the baseline value, $Y_1$ is the increase in each variable between baseline and steady-state, t is time, TD is the time delay, and is the time constant. The same process was used to describe the exponential recovery of deoxy-Mb and PCr onset:

$$\mathrm{Y(t)} = \mathrm{Y_0} - \mathrm{Y_1}(1 - \mathrm{e}^{-(\mathrm{t}-\mathrm{TD})/\tau}) \quad (3)$$

where $Y_0$ is the baseline value, $Y_1$ is the decrease in each variable between baseline and steady-state, t is time, TD is the time delay, and is the time constant.

Model variables were determined by non-linear least-squares regression using Origin 8.5 (OriginLab Corp, Northampton, MA), and an iterative process designed to optimize the exponential fit.

### Statistical analyses

Data were analyzed using parametric statistics, following mathematical confirmation of normal distribution using a Shapiro-Wilk test. Specifically, comparisons between PCr and deoxy-Mb kinetics, both as a function of exercise intensity, and at exercise onset and offset, were performed with repeated measures ANOVA or paired t-tests, where appropriate (Instat, San Diego, CA). Linear regression analyses were also applied to determine the association between variables. Statistical significance was accepted at $p \leq 0.05$. Data are presented as mean SE.

## Results

### Deoxy-Mb, PCr and Pi/PCr at rest and during exercise

As anticipated, compared to resting conditions during exercise there was a significant depletion of PCr, a rise in the Pi/PCr ratio, and a rise in the deoxy-Mb signal. Additionally, each of these variables revealed exercise intensity dependence (figure 1).

### Deoxy-Mb and PCr exercise onset and offset kinetics

At the onset of exercise PCr breakdown occurred immediately, whereas the deoxy-Mb increase was consistently delayed compared to the onset of exercise (table 1, figure 2 and 3). A consistent time delay (TD) could not be identified in PCr breakdown (the delay often projected into pre-exercise), and therefore the PCr fit was constrained to begin at the start of exercise (table 1). For deoxy-Mb, however, the TD was consistent, and quantitatively similar, for both moderate and heavy intensity exercise (table 1). PCr and deoxy-Mb were not different at either exercise intensity (figure 2 and table 1). The mean response time (MRT = + TD) tended to be longer for deoxy-Mb, but was not significantly different between PCr and deoxy-Mb. At exercise cessation there was no discernible delay in the re-oxygenation of Mb or PCr resynthesis at either intensity. After exercise, the kinetics of Mb re-oxygenation was significantly faster than de-oxygenation kinetics at the onset of exercise ($p < 0.05$). In addition, Mb re-oxygenation was significantly more rapid than the post exercise PCr resynthesis ($p < 0.05$), which was not different between exercise onset and recovery (table 1). For both deoxy-Mb and PCr the exercise offset kinetics were not different between exercise intensities (table 1).

### Deoxy-Mb ensemble-averaging trials

In order to further confirm the accuracy of the fitting parameters obtained from the $^{1}$H-MRS measurements of deoxy-Mb kinetics at high temporal resolution, 3 subjects performed 6 repeated trials of the moderate intensity exercise to allow ensemble averaging of the deoxy-Mb (i.e. averaging 6 data points instead of 1 data point every 1.5 seconds), utilizing a similar approach to that recommended for pulmonary $VO_2$ and PCr kinetics. Despite an improved signal-to-noise using this approach, the essential fitting parameters were unchanged and there was no qualitative or quantitative effect on the deoxy-Mb TD at the onset of exercise.

### Intracellular $PO_2$ at rest and during exercise

As Mb is almost fully oxygenated at rest (31), the deoxy-Mb signal was indistinguishable from the noise at rest with a resolution of 1.5 s, the dexy-Mb signal acquisition rate. However, based upon our previously published assessment of resting deoxy-Mb, also attained over 30 minutes (31), this baseline was assigned to 9 % deoxy-Mb, equivalent to an $iPO_2$ of 32 mmHg (figures 1 and 4). With this corrected baseline, and the finding that approximately 60–80% of the active muscle is recruited during such intensities of submaximal exercise (30), it was estimated that Mb was ~24 % deoxygenated (representing a $PO_2$ of ~11 mmHg) at moderate exercise (60% WRmax), and ~35 % deoxygenated (or a $PO_2$ of ~6 mmHg) at heavy intensity exercise (80% WRmax) (figure 4).

### Intracellular pH

At rest intracellular pH was 7.01 ± 0.01. At the end of 10 minutes of moderate intensity exercise the intracellular pH reached 6.95 ± 0.03 ($p < 0.05$ vs. rest), while at the end of the heavy-intensity exercise intracellular pH was 6.84 ± 0.02 ($p < 0.05$ vs. rest and moderate).

## Discussion

At the onset of exercise, oxidative ATP production increases relatively slowly in relation to the new energy level required to perform the work. Utilizing state-of-the-art interleaved $^{1}H$ and $^{31}P$ MRS this study sought to examine whether the kinetics of oxidative ATP production are slow because of inadequate $O_2$ supply, or because of the intrinsic properties of mitochondrial oxidative phosphorylation (i.e. metabolic inertia). At the onset of exercise, PCr began to fall immediately, as frequently documented with MRS, but there was a significant delay (~6 s) before intracellular oxygenation also fell. This observation was consistent at the onset of both moderate and heavy intensity exercise, strongly implying that $O_2$ availability within skeletal muscle is adequate to sustain the increase in oxidative phosphorylation in the first few seconds of exercise. Thus, in agreement with our initial hypothesis, these data indicate that metabolic inertia, and not $O_2$ supply, is a major determinant of $VO_2$ kinetics at the onset of exercise across a range of submaximal exercise intensities. In addition, the current study presents a second novel observation that, after an initial delay, the rate of deoxygenation at the start of exercise was similar to the PCr onset and offset kinetics. The implications and potential mechanisms responsible for these observations in terms of the metabolic control and the interplay between $O_2$ supply and utilization in response to exercise are discussed in more detail below.

### $O_2$ supply versus $O_2$ demand limitation at the onset of exercise

The issue of whether $O_2$ supply or $O_2$ demand limits the metabolic response at the onset of exercise has been debated for many years (29), with a relatively large number of studies attempting to resolve the contention. Many approaches have been adopted to address this issue in humans, including the assessment of immediate $O_2$ extraction and $VO_2$ at the onset of exercise with the direct Fick method (8, 21, 23), comparisons of cardiac output/blood flow and $VO_2$ kinetics (27), and attempts to manipulate both the rate of $O_2$ supply and $O_2$ demand (20). However, none of these approaches could assess $iPO_2$, and thus were not able to provide a definitive conclusion. With this in mind, a novel and major finding of our approach to assess intracellular oxygenation is the unveiling of a substantial delay (~6 s) in the fall of $iPO_2$ using $^{1}H$ MRS to assess myoglobin deoxygenation kinetics directly, which interestingly, to some extent, can also be considered an index of intracellular $O_2$ extraction. This delay in muscle deoxygenation was concomitant to an immediate decline in PCr at the onset of exercise. This finding supports the concept of a metabolic inertia at the onset of exercise that limits the kinetics of oxidative phosphosphorylation.

The classic study of Grassi et al. (8) interpreted a transient increase in venous $O_2$ content during the first 15 seconds of exercise (and the subsequent minimal change in leg $VO_2$) as evidence that $O_2$ delivery exceeded $O_2$ demand during the early transient. A concern that has been raised related to this study by Grassi et al. (8), was the transit time delay between

the blood actually leaving the leg and the time of sampling in the femoral vein (2). However, later, Hughson et al., (20) calculated, from arm exercise, that up to a 10 s transit delay had no effect, suggesting that this was probably not an issue in the work of Grassi et al. (8). Hughson et al. (20) also provided insight, suggesting that arm exercise below the level of the heart yielded similar results to the work of Grassi et al. (8), such that there was virtually no increase in $O_2$ extraction in the first 10–15 s of exercise. However, when exercise was performed with the arm above heart level, preventing a muscle pump effect and decreasing perfusion, $O_2$ extraction was elevated within the first 10 s of exercise onset (20) implying that $O_2$ delivery may limit $VO_2$ in this condition. In addition to this observation, there is evidence in the intact human that in certain scenarios, such as lower body negative pressure applied during supine cycling (19) and leg occlusion added to arm exercise (28), that increased $O_2$ delivery may speed $VO_2$ kinetics.

The substantial delay (~6 s) in the fall of $iPO_2$ of human skeletal muscle also confirms previous results obtained in human and animal models using indirect methods to assess muscle oxygenation and metabolism (NIRS-derived deoxy-Mb/Hemoglobin signal, microvascular $PO_2$, and the direct Fick method (29)). When comparing the present assessment of Mb with these previous measurements performed in the vascular compartment, one should not neglect the resistance to $O_2$ diffusion between red blood cells and the myocyte and the lag in the response between different compartments. With this in mind, the absolute $PO_2$ values and kinetics measured in the vasculature by these previous methods can significantly differ from that measured in the intracellular space by Mb. In fact, this is clearly illustrated by the finding in the present study that $iPO_2$ falls after only a ~6s delay, which is much shorter than previous values reported with others methods ~10–15 s such as arterio-veinous sampling (8, 20) or NIRS. In addition, unlike Mb, which is specific to muscle tissue and quantitative, the NIRS signal reflects the oxygenation status of both hemoglobin and myoglobin. The contribution of both compounds to the overall signal is controversial, likely yielding a measurement that reflects a weighted average of intracellular and extracellular compartments. It is also noteworthy that the signal from Hb reflects the contribution from arterioles, capillaries, and venules. Also, the $O_2$ phosphorescence quenching method is based on the rate of decay of porphyrin-based molecules injected intravascularly. Thus, as for arterio-veinous sampling method, this technique assesses the oxygenation state within the vasculature, not within the myocyte. Therefore, although quantitative, this method is also dependent on the lag in the response between different compartments (intracellular versus vascular). In addition, the $O_2$ phosphorescence quenching technique has been used in the frog preparation in the absence of myoglobin (22), which may affect the regulation of oxidative phosphorylation during metabolic transitions in this model. Together, these points illustrate that the assessment of intracellular oxygenation by Mb in humans generates unique and novel findings regarding the factors limiting the rate of oxidative ATP production at the onset of exercise.

In agreement with the present investigation, there is also convincing evidence supporting the hypothesis that the primary controllers of $VO_2$ are intrinsic to muscle metabolism (e.g. substrate availability, latency of mitochondrial enzymes, and damping of respiratory control transduction by CK). In this regard, it has been suggested that an enhanced pyruvate dehydrogenase (PDH) activity, a key substrate regulator for the tricarboxilic acid cycle

(TCA), following both a priming bout of exercise and pharmacologic activation by dichloroacetate (DCA) resulted in higher level of substrate available to be oxidized, a clear reduction in both PCr depletion and intramuscular lactate concentration (38), and, ultimately, a faster $VO_2$ kinetics at the onset of exercise (12). However, subsequent studies in human and dog muscle failed to confirm any difference in $VO_2$ or PCr kinetics with DCA infusion (6, 35). In addition, the time delay of the $iPO_2$ kinetics during contractile activity in intact single muscle fibers from frog was not different between control and DCA treated fibers (18). Using a similar single fiber preparation Gandra et al. (4), recently observed that the dynamics of mitochondrial NADH were also delayed at the onset of contractions, suggesting that factors intrinsic to the electron transport chain may account for the slow oxidative phosphorylation response at the onset of exercise.

Alternatively, the activation of a latent mitochondrial enzyme and transporter pool (16), perhaps through calcium signaling, has been proposed as the primary means by which ATP turnover is determined and could explain the apparently inadequate changes in substrates such as ADP. This suggestion is supported by several pieces of evidence indicating that mitochondrial calcium accumulation can result in allosteric activation of enzymatic activities (25), mitochondrial complexes such as ATP synthase (1), and can also modulate mitochondrial sensitivity to ADP via the stimulation of actomyosin ATPase (10). In accordance with these findings, allosteric features in the relationship between [ADP] and $VO_2$ measured in a canine preparation have been observed, where the apparent maximal respiration rate was time and/or ATP turnover dependent (39). It has also been suggested that the time course of calcium dependent activation of mitochondrial respiration would correspond with the biphasic feature of $VO_2$ at the onset of exercise(40), which is also consistent with our findings of an initial delay before deoxy-Mb increases mono-exponentially (figure 3). With such a scheme (parallel activation by $Ca^{2+}$), redox and phosphorylation potential would likely only serve to fine-tune mitochondrial respiration rate (16).

## Oxygenation onset kinetics and the role of creatine kinase reaction

At the risk of complicating this data set and what may otherwise seem like strong evidence for inertia in calcium dependent activation of mitochondrial respiration at the onset of exercise, it is interesting to consider the potential role of PCr in these findings. Indeed, this study did not set out to determine the role of PCr in rest-to-exercise transitions, but rather to use it as a marker of the induction of exercise metabolism that is highly correlated with $VO_2$ (34). However, in light of recent *ex vivo* and *in situ* studies (9), it is possible that PCr plays a more significant role in the current observations.

The PCr reaction is catalyzed by creatine kinase (CK) and facilitates the transfer of phosphate between creatine (Cr) and ADP. In skeletal muscle, CK is most abundant in the cytosol (although it is found also in the mitochondria) and is functionally coupled to the sites of ATP usage (e.g. myofibril ATPases, sarcoplasmic reticulum $Ca^{2+}$ ATPases, and sarcolemmal $Na^{+}$-$K^{+}$ ATPases). With the recognition that the maximal activity of CK is several fold higher than that of the ATPases, it is thought that CK rapidly rephosphorylates ADP in the vicinity of the ATPases, therefore maintaining an appropriate Gibbs free energy

(ΔG) for ATP hydrolysis. Additionally, the functional coupling of nucleotide translocase on the inner mitochondrial membrane creates an environment favorable to PCr formation and, linked by the PCr shuttle (24), facilitates the rapid transport of PCr from mitochondria to the cytosol and Cr in the reverse direction without necessitating large changes in free [ADP]. Consequently, PCr is thought to play an important role as a temporal and spatial buffer, by maintaining sufficient energy for contraction at the onset of exercise. By acting as a high capacitance energy buffer (26), PCr breakdown attenuates the increase in [ADP] during a rapid increase in ATP demand at the onset of exercise, thereby potentially slowing $VO_2$ onset kinetics. Therefore, in the context of the current study, an alternative interpretation of the delayed fall in $iPO_2$ at the onset of exercise could be a consequence of PCr damping the increase in metabolism rather than simply a metabolic latency.

In agreement with a role of the PCr-Cr shuttle in regulating of $VO_2$ kinetics, several studies utilizing CK deleted mice found that the knockout animals revealed evidence of more rapid $VO_2$ kinetics in both heart (13) and skeletal muscle (32). Similarly, pharmacological inhibition of creatine kinase in isolated single myocytes from the frog (22) and in the dog hindlimb (9) resulted in faster $VO_2$ kinetics. Of note, the exact effect of CK inhibition on the activation of oxidative phosphorylation in these studies is, however, unclear. Specifically, Kindig et al. (22) observed that the delay between the start of contractions and the fall in intracellular $PO_2$ (~8 s) was no longer apparent when CK activity was inhibited in frog while, in contrast, the time delay before $VO_2$ increased in the gastrocnemius of dog was unaffected by CK inhibition (9). The reasons for this discrepancy between these studies is uncertain, but the use of repeated contractile bouts in the same order in the frog experiments (22) might have primed the activity of the complexes in the electron transport chain (4) for the latter conditions with the CK inhibition. In addition, the absence of myoglobin, an $O_2$ buffer, in the frog preparation may play a role in the regulation of oxidative phosphorylation during metabolic transitions in this model.

### Evidence of a first order system

This study affords the interesting opportunity to go beyond the major goal of exposing the limiting factor ($O_2$ supply versus $O_2$ utilization) at the onset of exercise and examine both the on and off kinetics of both PCr and deoxy-Mb during exercise at two different intensities. Muscle oxidative metabolism is often cited as a first order system (26) because, in isolated or permeabilized mitochondria, respiratory control is well described by a first-order reaction with [ADP] (11), predicting exponential $VO_2$ response kinetics. This concept has been bolstered by the similar course of metabolic change at the beginning and cessation of exercise (26) and the close match between onset $VO_2$ and PCr dynamics (24, 34). However, other studies assessing both the onset and offset of exercise in different intensity domains have revealed an asymmetry in the dynamics of $VO_2$, suggesting more complex control (e.g. ATP/ADP or phosphorylation potential)(40).

The current findings corroborate the concept of metabolism being a first order system after an initial activation phase, with τ deoxy-Mb (37 s and 29 s during moderate and heavy exercise, respectively) not different to τ PCr (34 s and 33 s during moderate and heavy exercise, respectively). This invariant PCr time constant at the onset and offset of exercise

across both moderate and intense exercise is inconsistent with some prior findings of an asymmetry between the onset and offset of pulmonary $VO_2$ during exercise in different intensity domains (36). An explanation for this disparity might be the different exercise modes used to assess muscle metabolism (bi-lateral knee-extension vs single leg plantar flexion) and relative intensities.

Indeed, unlike the on-transient phase (8, 34), a dissociation between the kinetics of pulmonary and muscle $VO_2$ during the recovery period has recently been documented in humans (23). Such findings therefore suggest that inferences about muscle metabolic control using pulmonary $VO_2$ during the recovery period may not be appropriate. In addition, the use of a small muscle mass (single-leg plantar flexion) in the current study might have limited the influence of $O_2$ availability on PCr onset (14) and offset kinetics (15). However, in comparison to previous human studies on this topic, the current experimental conditions likely better isolated the features of muscle metabolic control, and minimized the influence of $O_2$ availability.

Finally, it is intriguing that deoxy-Mb offset kinetics were considerably faster than the onset kinetics and also faster than both the PCr onset and offset kinetics. This is likely explained by the very high affinity of Mb for $O_2$ ($P_{50}$ of myoglobin is extremely low, <5 mmHg) which makes Mb a sensitive marker for changes in mitochondrial $O_2$ consumption at the onset of exercise when Mb is almost fully saturated. However, in contrast, upon cessation of exercise, where oxidative phosphorylation rate decreases rapidly, and $O_2$ supply and demand are not necessarily tightly coupled (15), the high affinity of Mb for $O_2$ may dissociate the dynamics of mitochondrial respiration from deoxy-Mb kinetics.

### Experimental consideration: The role of $iPO_2$ heterogeneity

The present MRS technique (single pulse-acquisition sequence) combined with the use of a TEM coil provides a signal that is the weighted average of the $iPO_2$ within the sampling volume of the coil. Therefore, there is the potential for the estimated $iPO_2$ to be overestimated in the present study owing to the contribution to the deoxy-Mb signal from non-active regions of muscle. However, we attempted to minimize the influence of $iPO_2$ heterogeneity by normalizing the deoxy-Mb signal to the active muscle mass based upon the anatomical MR images of the leg at the center of the coil and the previous documentation that 60–80% of the muscle fibers are recruited during submaximal exercise of a similar intensity (30). Overall, while the approximation in the method employed here may affect the quantitative estimation of $iPO_2$, this would not affect our main conclusion, i.e. there was a significant delay (~6 s) before the fall in intracellular oxygenation. Indeed, using a similar approach (average signal of multiple fibers) we were able to detect an immediate drop in PCr at the onset of exercise. This latter finding further confirms that this kind of approach provides enough sensitivivity to detect subtle changes in muscle metabolism, and to the same extent oxygenation.

## Conclusion

This study has revealed, a significant delay (~6 s) in the fall in intracellular oxygenation at the onset of exercise from rest to both moderate and heavy intensity exercise. In addition,

after this initial delay, the rate of Mb deoxygenation was similar to PCr onset (and offset) kinetics. Together, these findings provide evidence in favor of the hypothesis that metabolic inertia, and not $O_2$ supply, is the major limitation to $VO_2$ kinetics at the onset of exercise and that, after an initial activation phase, human skeletal muscle oxidative metabolism exhibits the features of a first order system.

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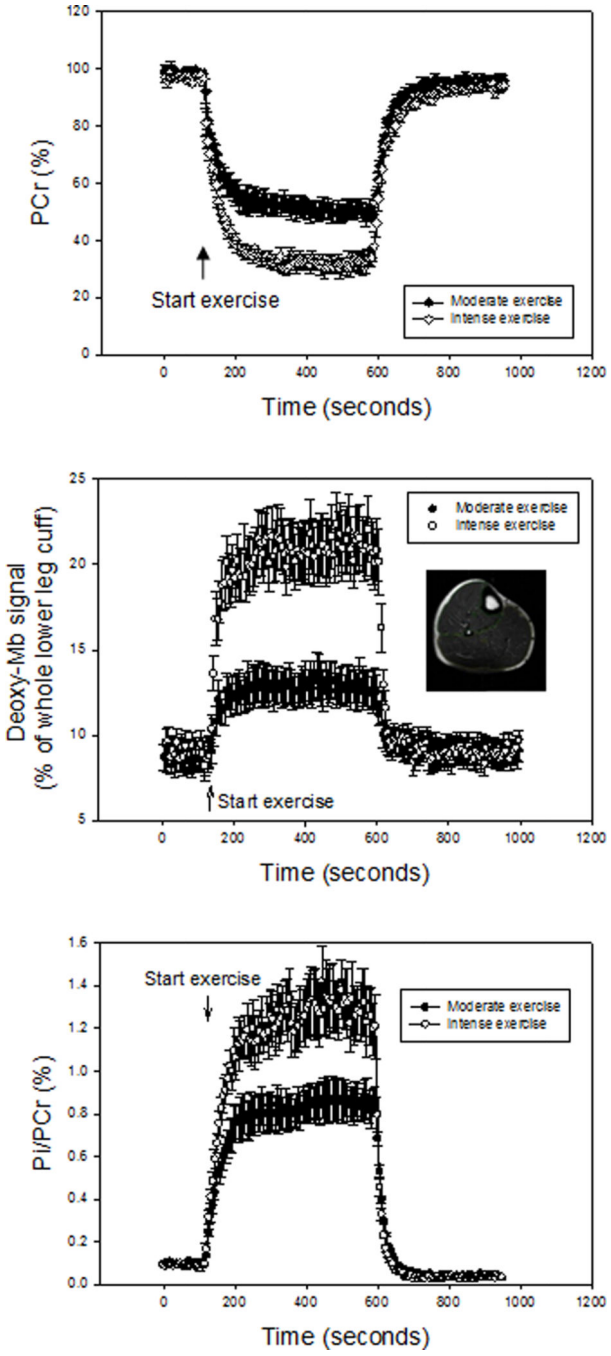


**Figure 1.**

The average phosphocreatine (PCr) depletion, deoxygenated myoglobin (deoxy-Mb) signal, and inorganic phosphate (Pi) /PCr ratio across the complete rest-exercise-rest protocol for both moderate and intense exercise. Note, because the deoxy-Mb signal was collected from all the muscle within the coil (i.e. image inlay), the signal is presented as a % of the whole lower leg and therefore, without a correction for active muscle mass, cannot be converted to intracellular $PO_2$ (see figure 4).

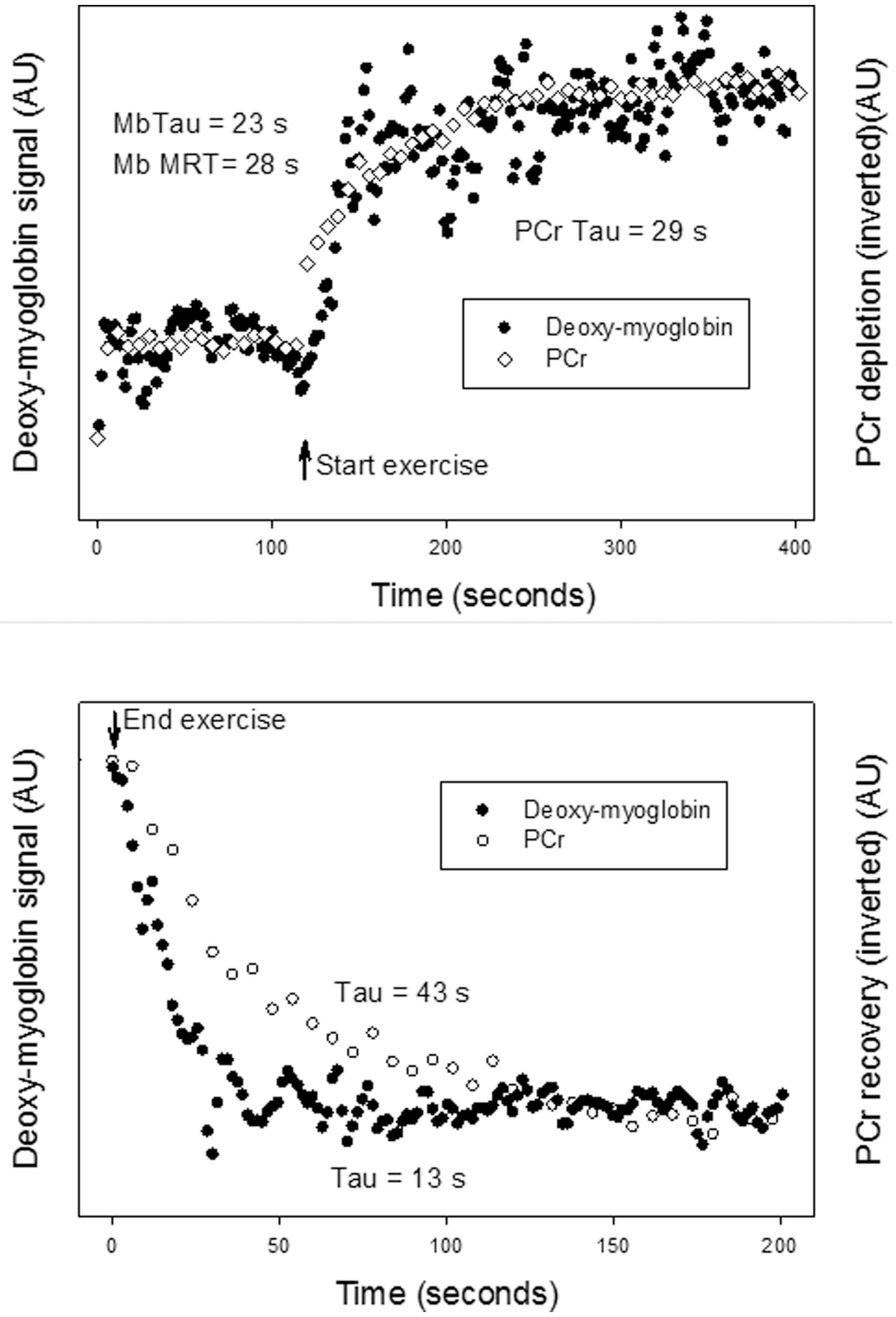


**Figure 2.**

Individual examples of the exercise onset (upper panel) and offset (lower panel) phosphocreatine (PCr) and deoxygenated myoglobin (deoxy-Mb) data. Note, in both cases the PCr change has been inverted to facilitate the comparison with the deoxy-Mb signal.

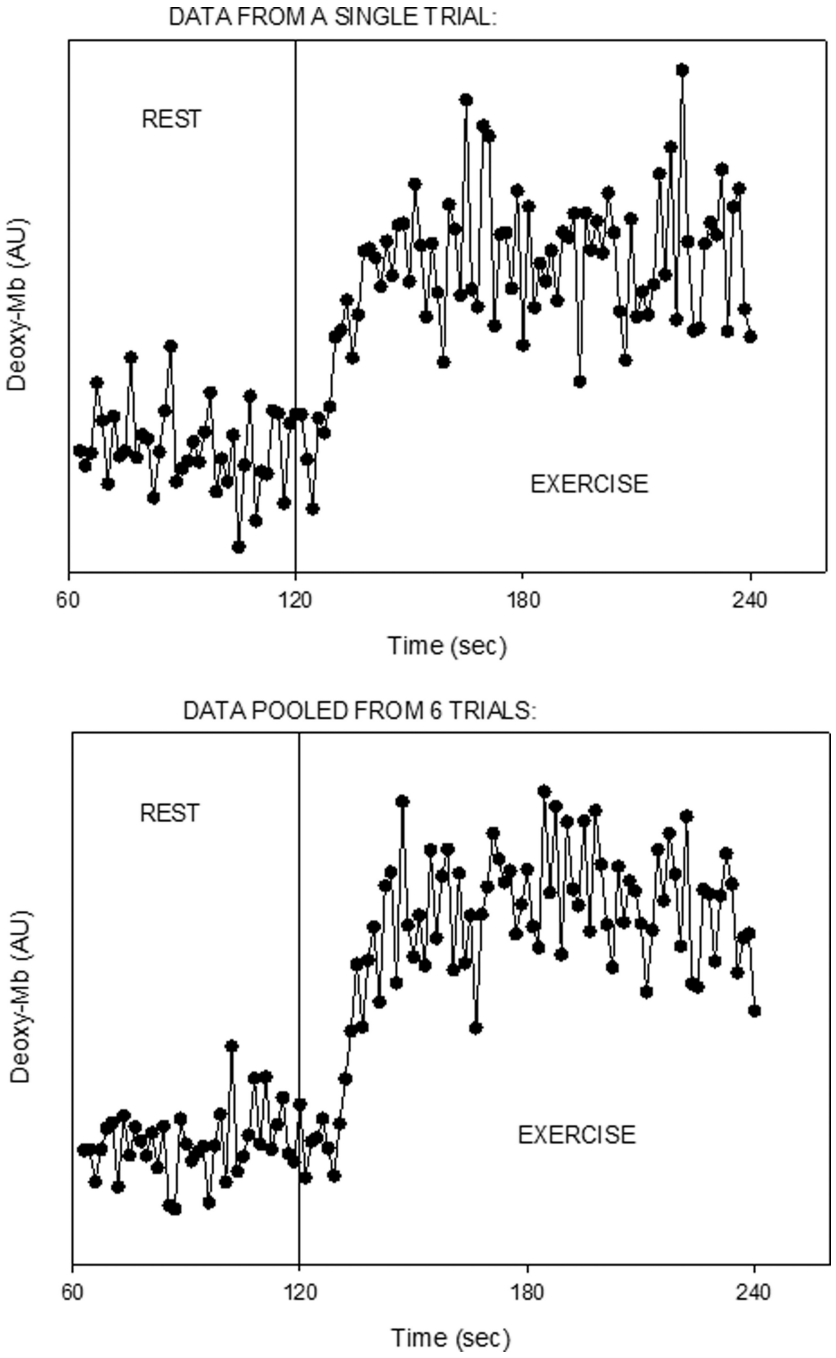


**Figure 3.**

Individual tracing of the deoxygenated myoglobin (deoxy-Mb) signal at the onset of moderate-intensity exercise (upper panel), and deoxy-Mb kinetics in the same subject, but with data compiled from six consecutive exercise bouts to improve signal-to-noise (lower panel). Note, that in both cases there is a significant delay in the rise of the deoxy-Mb signal.

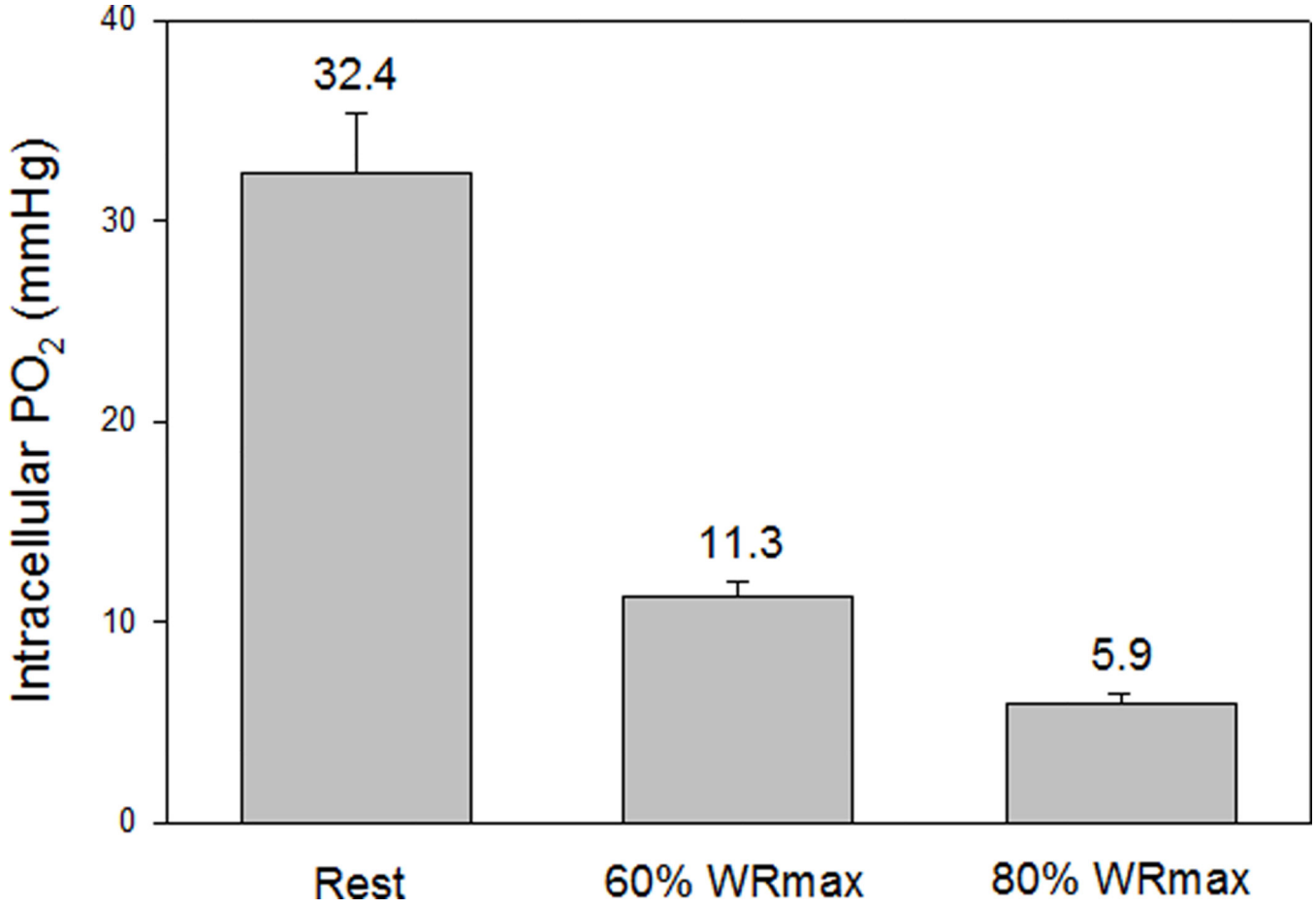


**Figure 4.**

Calculated of intracellular $PO_2$ at rest, 60% of work rate maximum (WRmax), and 80% of WRmax. Note, these calculated values utilize an assumed Mb P50 value of 3.2 mmHg and the estimated recruitment of 60% of the total muscle mass within the volume coil.

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**Table 1**

Kinetic analyses for phosphcreatine (PCr), and deoxy-myoglobin (deoxy-Mb) at the onset and offset of both moderate and intense plantar flexion exercise.

| | **Moderate Intensity Exercise (60% $WR_{max}$)** | | | | **Intense Exercise (80% $WR_{max}$)** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Onset** | | **Offset** | | **Onset** | | **Offset** | |
| | **PCr** | **deoxy-Mb** | **PCr** | **deoxy-Mb** | **PCr** | **deoxy-Mb** | **PCr** | **deoxy-Mb** |
| Delay (sec) | --- | 5 ± 2* | --- | --- | --- | 7 ± 1* | --- | --- |
| Tau (sec) | 34 ± 4 | 37 ± 9 | 35 ± 4 | 21 ± 5*# | 33 ± 4 | 29 ± 6 | 30 ± 5 | 20 ± 4*# |
| MRT (sec) | 34 ± 4 | 43 ± 9 | 35 ± 4 | 21 ± 5*# | 33 ± 4 | 39 ± 9 | 30 ± 5 | 20 ± 4*# |

Mean response time, MRT = delay + tau;

*significantly different from PCr;

#significantly different from PCr onset at the same exercise intensity, $p \leq 0.05$.