Intersubject differences in the effect of acidosis on phosphocreatine recovery kinetics in muscle after exercise are due to differences in proton efflux rates

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van den Broek NM, De Feyter HM, de Graaf L, Nicolay K, Prompers JJ. Intersubject differences in the effect of acidosis on phosphocreatine recovery kinetics in muscle after exercise are due to differences in proton efflux rates. Am J Physiol Cell Physiol 293: C228–C237, 2007. First published March 28, 2007; doi:10.1152/ajpcell.00023.2007.—31P magnetic resonance spectroscopy provides the possibility of obtaining bioenergetic data during skeletal muscle exercise and recovery. The time constant of phosphocreatine (PCr) recovery (τPCr) has been used as a measure of mitochondrial function. However, cytosolic pH has a strong influence on the kinetics of PCr recovery, and it has been suggested that τPCr should be normalized for end-exercise pH. A general correction can only be applied if there are no intersubject differences in the pH dependence of τPCr. We investigated the pH dependence of τPCr on a subject-by-subject basis. Furthermore, we determined the kinetics of proton efflux at the start of recovery. Intracellular acidosis slowed PCr recovery, and the pH dependence of τPCr differed among subjects, ranging from 33.0 to 75.3 s/pH unit. The slope of the relation between τPCr and exercise pH was positively correlated with both the proton efflux rate and the apparent proton efflux rate constant, indicating that subjects with a smaller pH dependence of τPCr have a higher proton efflux rate. Our study implies that simply correcting τPCr for end-exercise pH is not adequate, in particular when comparing patients and control subjects, because certain disorders are characterized by altered proton efflux from muscle fibers.

31P magnetic resonance spectroscopy; skeletal muscle; oxidative capacity; mitochondrial function; intracellular pH

31P MAGNETIC RESONANCE SPECTROSCOPY (MRS) provides the possibility of obtaining bioenergetic data during skeletal muscle exercise and recovery in a noninvasive manner and with a time resolution of a few seconds. This has made a major contribution to our understanding of energy metabolism, its control, and the way in which it can be affected in disease (2, 3, 11, 25, 28, 33, 40). During recovery from exercise phosphocreatine (PCr) is resynthesized purely as a consequence of oxidative ATP synthesis (39, 45, 50), and therefore measurement of the time constant of PCr recovery (τPCr) provides information about mitochondrial function. This technology was very recently applied to study in vivo mitochondrial function in patients with Type 2 diabetes (46).

Several studies have shown that cytosolic pH has a strong influence on the kinetics of PCr recovery (1, 2, 5, 8, 21, 31, 34, 44, 49, 51, 56). The slower PCr recovery in the presence of intracellular acidosis could reflect a decreased mitochondrial respiration at low pH. However, there are conflicting data about the effects of low pH on respiratory rates, ranging from inhibition (17, 22, 54) to a very small or no significant effect (10, 36, 38, 52, 56, 57) to even an increased effectiveness (15, 16). PCr recovery in the presence of intracellular acidosis could also be slowed down because of factors downstream of oxidative phosphorylation, i.e., increased ATP consumption by cellular ion pumps (4, 7, 29, 44) and/or a pH-dependent shift in the creatine kinase (CK) equilibrium (19, 30).

As an alternative to τPCr, the time constant of ADP recovery (τADP) can be used to assess oxidative capacity. The concentration of ADP is the principal error signal in the feedback loop controlling mitochondrial oxidation, and therefore ADP recovery is one of the most sensitive MRS indices of mitochondrial function (23). Finally, the maximum aerobic capacity (Qmax), which can be calculated from the 31P MRS recovery data, provides a parameter for mitochondrial function. Both τADP (1–3, 8, 31) and Qmax (31, 44, 56) have been shown to be independent of end-exercise pH. A drawback of the use of these parameters compared with τPCr is that they are indirectly derived from the PCr recovery data, with a number of assumptions.

It has been suggested that τPCr can be normalized for end-exercise pH (5, 31, 44). However, a general correction for pH can only be applied if there are no intersubject differences in the pH dependence of PCr recovery kinetics. In previous studies, data of different subjects have been grouped to investigate the effect of pH on PCr recovery (1, 2, 5, 8, 21, 31, 34, 44, 49, 51, 56). We investigated the effect of acidosis on τPCr in the vastus lateralis muscle on a subject-by-subject basis. To this end, each subject performed 10–13 exercise protocols of different intensity to reach different levels of acidification. Furthermore, we studied the pH dependence of τADP and Qmax and we determined the kinetics of proton efflux at the start of recovery to obtain a measure of the rate of pH recovery.

MATERIALS AND METHODS

Subjects. Four male and two female healthy subjects participated in this study. The nature and the risks of the experimental procedures were explained to the subjects, and all gave their written informed consent to participate in the study, which was approved by the local Medical Ethical Committee of the Maxima Medical Center, Veldhoven, The Netherlands. Subjects varied in age [mean age: 31 (SD 12) yr; 5 subjects in the range of 20–33 yr and 1 subject of 53 yr], body mass index (BMI) [mean BMI: 21.1 (SD 1.8) kg/m2; range: 17.9–22.6 kg/m2], and daily activity level, i.e., level of activities during daily living, work, and leisure time (e.g., sports). However, none of the subjects was highly trained.

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**31P magnetic resonance spectroscopy.** 31P MRS of the vastus lateralis was performed by using a 1.5-T whole body scanner (Gyroscan S15/ACS, Philips Medical Systems, Best, The Netherlands). Subjects were measured in a supine position. After transversal and sagittal scout images were collected, the magnetic field homogeneity was optimized by localized shimming on the proton signal with the body coil. The 31P signals were collected with a 6-cm-diameter surface coil placed over the vastus lateralis. From the dimension of the coil and the size and geometry of a typical upper leg, it was estimated that the majority of the signal in the unlocalized 31P MRS measurements originated from the vastus lateralis, with minimal contaminations from the adjacent rectus femoris and underlying vastus intermedius. Data were acquired after a 90° adiabatic excitation pulse with a sweep width of 2 kHz and 1,024 data points. Spectra were acquired with a repetition time of 3 s during a rest-exercise-recovery protocol (2 scans/spectrum yielding a time resolution of 6 s; total of 150 spectra/15 min). The first 20 spectra (2 min) were measured at rest, after which the subjects started the exercise (see below). The duration of the exercise varied per subject but never exceeded 9 min, so that at least 4 min of recovery was recorded.

**Exercise protocol inside magnet.** All subjects performed a single leg extension exercise that has been shown to be limited to the four muscles of the quadriceps (41) in the supine position inside the magnet. The exercise was conducted by rhythmically lifting a lever (resting on the lower leg, proximal of the foot) connected to an ergometer. The upper leg was supported with the hip joint in a 30° anteflexed position and immobilized with two 3-cm-wide Velcro straps. One contraction was performed every 1.5 s, acoustically guided by a digital metronome. The initial workload varied per subject and ranged between 7.5 and 12.5 W. This level was maintained for the first minute, and the workload was then increased by 5 W each minute. To achieve different levels of metabolic activation, and hence different degrees of cytosolic acidification, subjects performed exercises of different durations. Each subject performed 10–13 different protocols during 4–9 different sessions in a randomized order, with at least 15-min rest between different protocols within 1 session. The position of the 31P surface coil was marked on the leg during the first session, and the coil was placed at the same location during the next sessions.

The reproducibility of the 31P MRS measurements was determined in one subject. This subject performed the same protocol 10 times during 5 different sessions. In one other subject, we tested whether the position of the 31P surface coil on the vastus lateralis, when varied in the proximal and distal direction, influenced the 31P MRS measurements. For this purpose, the subject performed the same protocol 5 times, with a maximal difference of 15 cm of the position of the 31P surface coil in the proximo-distal direction.

**Data analysis.** Spectra were fitted in the time domain by using a nonlinear least-squares algorithm (AMARES) in the jMRUI software package (53). PCR, Pn, and ATP signals were fitted to Lorentzian line shapes. The three ATP peaks were fitted as two doublets and one triplet, with equal amplitudes and line widths and prior knowledge for the J-coupling constant (17 Hz). For the time series, the PCr line width triplets, with equal amplitudes and line widths and prior knowledge for shapes. The three ATP peaks were fitted as two doublets and one

The free cytosolic ADP concentration ([ADP]) was calculated from pH and [PCR] with a CK equilibrium constant (Keq) of 1.66 × 10^9 M⁻¹ (30), assuming that 15% of the total creatine is unphosphorylated at rest (7), with the equation

\[
[\text{ADP}] = \frac{[\text{ATP}][\text{Cr}]}{[\text{PCR}][\text{H}^+] K_{eq}} \tag{2}
\]

Recoveries of PCR and ADP were fitted to monoeponential functions with Matlab (version 6.1; Mathworks, Natick, MA). Results are expressed as the metabolite’s time constant of recovery, i.e., τPCR and τADP.

Calculation of the initial rate of PCr recovery (VPCR) was based on the PCr recovery rate (1/τPCR) and the difference between the resting and end-exercise [PCR] (ΔPCRr) (26):

\[
V_{\text{PCR}} = \frac{1}{\tau_{\text{PCR}}} \cdot \Delta\text{PCR} \tag{3}
\]

The calculation of the maximum aerobic capacity (Qmax) was based either on the ADP-control model (12), in which VPCR has a hyperbolic dependence on the end-exercise [ADP] ([ADP]end) according to Michaelis-Menten kinetics with a Kmax of 30 µM (26):

\[
Q_{\text{max-ADP}} = V_{\text{PCR}} \cdot \left(1 + \frac{K_m}{[\text{ADP}]_{\text{end}}} \right) \tag{4}
\]

or on a linear approximation of the ADP-control model

\[
Q_{\text{max-lin}} = \frac{1}{\tau_{\text{PCR}}} \cdot [\text{PCR}]_{\text{rest}} \tag{5}
\]

which is equivalent to the nonequilibrium thermodynamic control model (23, 35). However, this approximation is only valid when pH changes are small.

The proton efflux rate at the start of recovery was calculated as described by Kemp et al. (24, 27) from the changes in [PCR] and pH during the first 12 s of recovery according to

\[
E = (\varphi + m) \frac{\delta[\text{PCR}]}{\delta t} + \beta \frac{\delta \text{pH}}{\delta t} \tag{6}
\]

where \(\varphi\) is the amount of protons consumed per mole of PCR hydrolysis (\(\varphi = 1/[1 + 10^{pH-6.75}]\); Ref. 58), \(m\) is the number of protons produced per mole of oxidative ATP synthesis (\(m = 0.16/ [1 + 10^{pH-6.1}]\); Ref. 32), and \(\beta\) is the cytosolic buffering capacity [20 skylies, i.e., mmol·l⁻¹·PH⁻¹, plus the calculated contribution of Pi, which is given by 2.3[Pi]/(1 - \(\varphi\)) (42)]. The apparent proton efflux rate constant was calculated as \(E = k(pH - \text{pH}_{\text{rest}})\), where \(\Delta pH\) is defined as pH - pH_{rest} (23). Both the proton efflux rate \(E\) and the apparent proton efflux rate constant \(k\) at the start of recovery depend on the end-exercise pH (pH_{end}) (27), and therefore only the data sets with a pH_{end} between 6.6 and 6.8 were used to calculate an average value for \(E\) and \(k\) for each subject. These data sets with rather low pH_{end} values were chosen for two reasons: 1) they had a greater PCR depletion and therefore a larger PCr resynthesis rate and proton production rate at the start of recovery, and 2) they had a greater increase in Pi and therefore a more visible Pi peak at the start of recovery, which is important for an accurate and precise pH determination.

**Statistics.** Data are expressed as means (SD). Reproducibility is reported as the coefficient of variation [CV = (SD/mean) × 100]. Linear regression analyses were performed with the SPSS 14.0 software package (SPSS, Chicago, IL). The level of statistical significance was set at \(P < 0.05\).

**RESULTS**

**Reproducibility.** Figure 1 shows typical examples of 31P MR spectra from a subject’s vastus lateralis muscle at rest, at the end of exercise, and at two time points during recovery. For the
same data set, [PCr] and [ADP] are plotted as a function of
time in Fig. 2. At the end of recovery, both [PCr] and [ADP]
are identical to those in the resting condition. Figure 2 also
illustrates the monoexponential fits of the PCr and ADP recov-
eries. The reproducibility of the determination of 31P MRS
parameters for mitochondrial function, i.e., $\tau_{\text{PCr}}$, $\tau_{\text{ADP}}$, $V_{\text{PCr}}$
and $Q_{\max}$ calculated according to the ADP-control model
($Q_{\max}\text{-ADP}$) and calculated by a linear approximation of the
ADP-control model ($Q_{\max}\text{-lin}$), was determined in one subject,
and the results are shown in Table 1. When all 10 measure-

Fig. 1. Typical vastus lateralis muscle 31P
magnetic resonance (MR) spectra for 1 sub-
ject at rest (A; no. of scans = 60), at the end
of exercise (B; no. of scans = 2), and at 15
and 93 s of recovery (C and D, respectively;
no. of scans = 2). Spectra were processed
with 5-Hz line broadening. For this subject
the phosphocreatine (PCr) depletion at the
end of exercise (B) was 65% and the corre-
sponding end-exercise pH (pHEnd) was 6.93.
Note that the Pi signal is not discernible in the
spectrum in D. PDE, phosphodiesters; $\alpha$, $\beta$, and $\gamma$, 3 phosphate groups of ATP.

Fig. 2. PCr ([PCr]; A) and ADP ([ADP]; B)
concentrations during rest, exercise, and re-
covery obtained from the data set that was
also used in Fig. 1 with a time resolution of
6 s. The recoveries of PCr and ADP (starting
at $t = 0$) were fitted to monoexponential
functions (solid lines). The time constants for
PCr and ADP recovery ($\tau_{\text{PCr}}, \tau_{\text{ADP}}$) were 26.9
and 11.8 s, respectively.
ments were included, the CV ranged from 6.7% for $\tau_{\text{ADP}}$ to 11.3% for $\tau_{\text{PCr}}$. However, even though the exercise protocol was identical for all 10 measurements, there was still some variation in $\text{pH}_{\text{end}}$. For 7 of the 10 data sets $\text{pH}_{\text{end}}$ ranged between 6.91 and 6.96 [mean $\text{pH}_{\text{end}}$ 6.93 (SD 0.02)], while for 3 data sets $\text{pH}_{\text{end}}$ was lower than 6.9, i.e., 6.87, 6.84 and 6.81. When only the seven data sets with $\text{pH}_{\text{end}}$ above 6.9 were considered, the CV for $\tau_{\text{PCr}}$ became 6.9%, which is comparable to other studies (29, 56). No systematic differences were observed for the two measurements performed during one session.

In one other subject, we tested whether the position of the $^{31}$P surface coil on the vastus lateralis, when varied in the proximal and distal direction, influenced the $^{31}$P MRS measurements. For the five measurements with a maximal difference of 15 cm of the position of the $^{31}$P surface coil in the proximo-distal direction, the CVs for $\tau_{\text{PCr}}$ and $\tau_{\text{ADP}}$ were 4.5% and 3.0%, respectively. Therefore, it can be concluded that within a certain range the exact positioning of the $^{31}$P surface coil does not affect the parameters for mitochondrial function and that regional variations in fiber type composition in the proximo-distal direction of the vastus lateralis are probably small.

End-exercise status. To achieve different levels of metabolic activation, and hence different degrees of cytosolic acidification, subjects performed 10–13 exercises of different durations. For all measurements, homeostasis of ATP was maintained throughout the exercise protocol. None of the subjects showed a split $P_i$ peak during exercise or recovery, and therefore acidosis was not extremely heterogeneous in the measured muscle tissue. The ranges of $\text{pH}_{\text{end}}$, $\Delta\text{PCr}$, and [ADP]$_{\text{end}}$ reached for each subject are summarized in Table 2. The smallest range in $\text{pH}_{\text{end}}$ values was obtained for subject 4 and covered 0.3 pH units, while the largest range was obtained for subject 2 and covered 0.6 pH units. For subject 5, one protocol resulted in a rather low [ADP]$_{\text{end}}$ of 25 $\mu$M. For all other measurements, [ADP]$_{\text{end}}$ was well above the accepted $K_m$ value of 30 $\mu$M for oxidative ATP synthesis. In the top two rows of Fig. 3, $\Delta\text{PCr}$ and [ADP]$_{\text{end}}$ are plotted as a function of $\text{pH}_{\text{end}}$, from which it can be seen that $\Delta\text{PCr}$ was negatively correlated with $\text{pH}_{\text{end}}$ for all three subjects, whereas [ADP]$_{\text{end}}$ was not significantly correlated with $\text{pH}_{\text{end}}$.

Recovery. Recoveries of PCr and ADP could be satisfactorily described by monoeponential functions, also at low $\text{pH}_{\text{end}}$ values [average $R^2$ values for the monoeponential fits were 0.971 (SD 0.025) and 0.915 (SD 0.063) for PCr and ADP recovery data, respectively]. Table 3 lists the average values for $\tau_{\text{PCr}}$, $\tau_{\text{ADP}}$, $V_{\text{PCr}}$, $Q_{\text{max-ADP}}$, and $Q_{\text{max-lin}}$ for all subjects. For each subject, there was a strong negative linear relationship between $\tau_{\text{PCr}}$ and $\text{pH}_{\text{end}}$. The third row of Fig. 3 shows the correlation between $\tau_{\text{PCr}}$ and $\text{pH}_{\text{end}}$ for three of the subjects. Around $\text{pH}_{\text{end}}$ 7, $\tau_{\text{PCr}}$ was very similar for these three subjects, but at lower $\text{pH}_{\text{end}}$ values $\tau_{\text{PCr}}$ differed. Therefore, the pH dependence of $\tau_{\text{PCr}}$ differed, with subject 1 showing the weakest pH dependence and subject 3 showing the strongest pH dependence. The results of the linear regression analyses for all subjects are shown in Table 4. The slope of the relation between $\tau_{\text{PCr}}$ and $\text{pH}_{\text{end}}$ ranged from −33.0 to −75.3 s/pH unit. For five of the six subjects, $\tau_{\text{PCr}}$ at $\text{pH}_{\text{end}}$ 7 calculated from the linear relation between $\tau_{\text{PCr}}$ at $\text{pH}_{\text{end}}$ was very similar. Only the older subject, subject 6, had a longer $\tau_{\text{PCr}}$ at $\text{pH}_{\text{end}}$ 7.

The postexercise ADP recovery was faster than the PCr recovery (Table 3). For subjects 1–5, $\tau_{\text{ADP}}$ was again very similar, while subject 6 had a longer $\tau_{\text{ADP}}$ (Table 3). In the fourth row of Fig. 3, $\tau_{\text{ADP}}$ is plotted against $\text{pH}_{\text{end}}$ for three of the subjects. For subjects 2, 3, and 5, $\tau_{\text{ADP}}$ did not depend on $\text{pH}_{\text{end}}$ (Table 4). However, for subjects 1, 4, and 6, $\tau_{\text{ADP}}$ was significantly positively correlated with $\text{pH}_{\text{end}}$ (Table 4).

The average values of $Q_{\text{max-ADP}}$ were smaller than the average values of $Q_{\text{max-lin}}$ (Table 3). In accordance with the longer $\tau_{\text{PCr}}$ (at pH 7) and $\tau_{\text{ADP}}$, subject 6 also showed smaller values for $Q_{\text{max-ADP}}$ and $Q_{\text{max-lin}}$ compared with the other subjects (Table 3). $Q_{\text{max-ADP}}$ showed significant positive correlations with $\text{pH}_{\text{end}}$ for three of the six subjects (Fig. 3, Table 4). These are the subjects with the largest correlation coefficients for $V_{\text{PCr}}$ vs. $\text{pH}_{\text{end}}$. For each subject, there was a strong
Fig. 3. ΔPCr, end-exercise [ADP] ([ADP]_{end}), τ_{PCr} and τ_{ADP}, initial rate of PCr recovery (V_{PCr}), and maximum aerobic capacity calculated according to the ADP-control model and by a linear approximation of the ADP-control model (Q_{max-ADP} and Q_{max-lin}) plotted as a function of pH_{end} for 3 subjects. Results of the linear regression analyses (solid lines) are shown for significant correlations (P < 0.05).
positive linear relationship between $Q_{\text{max-lin}}$ and $pH_{\text{end}}$ (Fig. 3, Table 4).

For subject 5, the proton efflux rate $E$ and the apparent proton efflux rate constant $\lambda$ could not be determined. For the other five subjects, the mean total cytosolic buffering capacity $\beta$ at the start of recovery amounted to 35 (SD 1) s,lites, the mean proton efflux rate $E$ was 16 (SD 3) mM/min, and the mean apparent proton efflux rate constant $\lambda$ was 38 (SD 8) mM·min$^{-1}$·pH unit$^{-1}$. In Fig. 4, $A$ and $B$, the slope of the relation between $\tau_{\text{PCR}}$ and $pH_{\text{end}}$ is plotted against $E$ and $\lambda$, respectively. The slope of the relation between $\tau_{\text{PCR}}$ and $pH_{\text{end}}$ was positively correlated with both $E$ ($R = 0.91, P = 0.03$) and $\lambda$ ($R = 0.96, P = 0.01$).

**DISCUSSION**

Several studies have shown that cytosolic pH has a strong influence on the kinetics of PCR recovery (1, 2, 5, 8, 21, 31, 34, 44, 49, 51, 56). To establish a relationship between, e.g., $\tau_{\text{PCR}}$ and $pH_{\text{end}}$, one or a few data points of different subjects have generally been grouped. However, this procedure will not reveal intersubject differences in the pH dependence of $\tau_{\text{PCR}}$ and $pH_{\text{end}}$. In Fig. 4, $A$ and $B$, the slope of the relation between $\tau_{\text{PCR}}$ and $pH_{\text{end}}$ is plotted against $E$ and $\lambda$, respectively. The slope of the relation between $\tau_{\text{PCR}}$ and $pH_{\text{end}}$ was positively correlated with both $E$ ($R = 0.91, P = 0.03$) and $\lambda$ ($R = 0.96, P = 0.01$).

**Table 3. Averages of $^{31}$P MRS recovery parameters for different exercise protocols**

<table>
<thead>
<tr>
<th>Subject</th>
<th>$\tau_{\text{PCR}}$, s</th>
<th>$\tau_{\text{ADP}}$, s</th>
<th>$V_{\text{PCR}}$, mM/s</th>
<th>$Q_{\text{max-ADP}}$, mM/s</th>
<th>$Q_{\text{max-lin}}$, mM/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.7 (6.3)</td>
<td>11.1 (2.5)</td>
<td>0.71 (0.12)</td>
<td>0.96 (0.08)</td>
<td>1.10 (0.22)</td>
</tr>
<tr>
<td>2</td>
<td>38.4 (10.8)</td>
<td>10.4 (1.3)</td>
<td>0.70 (0.09)</td>
<td>0.97 (0.14)</td>
<td>1.06 (0.29)</td>
</tr>
<tr>
<td>3</td>
<td>44.4 (12.0)</td>
<td>12.3 (1.4)</td>
<td>0.66 (0.11)</td>
<td>0.83 (0.16)</td>
<td>0.94 (0.31)</td>
</tr>
<tr>
<td>4</td>
<td>37.9 (6.0)</td>
<td>12.6 (1.7)</td>
<td>0.66 (0.07)</td>
<td>0.90 (0.07)</td>
<td>1.00 (0.15)</td>
</tr>
<tr>
<td>5</td>
<td>35.0 (4.9)</td>
<td>13.0 (2.1)</td>
<td>0.68 (0.15)</td>
<td>0.97 (0.13)</td>
<td>1.12 (0.18)</td>
</tr>
<tr>
<td>6</td>
<td>51.1 (9.6)</td>
<td>16.1 (2.5)</td>
<td>0.56 (0.05)</td>
<td>0.76 (0.07)</td>
<td>0.86 (0.18)</td>
</tr>
</tbody>
</table>

Data are presented as means (SD).

The slower PCR recovery in the presence of intracellular acidosis could reflect a decreased mitochondrial respiration at low pH. The mechanisms by which protons affect oxidative phosphorylation include (1) a direct effect on the mitochondria, i.e., a decreased oxidative capacity at low pH, or (2) an indirect effect, because a low pH decreases the ADP concentration through the constraints set by the CK equilibrium resulting in a lower signal for mitochondrial ATP supply. There are conflicting data about the effects of low pH on respiratory rates. Hypercapnic acidosis has been found to reduce the aerobic capacity of perfused cat soleus muscle by a factor of 3 (17). However, it is not clear whether this change was caused by acidosis per se or by some other effect of hypercapnic perfusion. Moreover, it has been shown that in skinned fibers from rat soleus muscle the rate of respiration is impaired by lactic acidosis and elevated $[P_i]$ (54). Jabrubas et al. (22) showed that intracellular acidosis inhibits oxidative phosphorylation in vivo in hand and limb muscle, and their results suggest that pH has a direct effect on mitochondrial function, because oxidative flux did not increase during exercise that generated acidosis despite a significant rise in $[ADP]$. In contrast, in vitro studies isolated mitochondria suggest that the effect of acidosis on oxidative phosphorylation is very small (10, 36, 52, 57) and not significant in the range pH 6.5–7.5 (10). Likewise, an in vivo study of electrically stimulated rabbit muscle showed that CO$_2$-induced acidosis (to pH 6.7) did not decrease the maximum aerobic capacity (38). Moreover, in human medial gastrocnemius muscle aerobic ATP synthesis rates were not lowered by acidosis (56). Other reports have suggested that mitochondrial respiration is even more effective at low pH (15, 16).

**Table 4. Correlation of $^{31}$P MRS recovery parameters with $pH_{\text{end}}$**

<table>
<thead>
<tr>
<th>Subject 1 (n = 10)</th>
<th>Subject 2 (n = 12)</th>
<th>Subject 3 (n = 13)</th>
<th>Subject 4 (n = 12)</th>
<th>Subject 5 (n = 13)</th>
<th>Subject 6 (n = 11)</th>
<th>Grouped Data (n = 71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\tau_{\text{PCR}}$</td>
<td>$R$</td>
<td>-0.99*</td>
<td>-0.98*</td>
<td>-0.94*</td>
<td>-0.87*</td>
<td>-0.85*</td>
</tr>
<tr>
<td>Slope, s/U</td>
<td>-42.9±2.1</td>
<td>-57.9±3.5</td>
<td>-75.3±8.1</td>
<td>-56.2±9.9</td>
<td>-33.0±6.3</td>
<td>-62.9±7.7</td>
</tr>
<tr>
<td>$\tau_{\text{ADP}}$</td>
<td>s at pH 7, s</td>
<td>25.3</td>
<td>22.8</td>
<td>26.4</td>
<td>26.8</td>
<td>28.7</td>
</tr>
<tr>
<td>$R$</td>
<td>0.86*</td>
<td>0.40</td>
<td>0.53</td>
<td>0.78*</td>
<td>0.13</td>
<td>0.89*</td>
</tr>
<tr>
<td>Slope, s/U</td>
<td>14.8±3.1</td>
<td>2.9±2.1</td>
<td>4.9±2.4</td>
<td>14.2±3.6</td>
<td>2.3±5.4</td>
<td>15.8±2.6</td>
</tr>
<tr>
<td>$V_{\text{PCR}}$</td>
<td>R</td>
<td>0.13</td>
<td>0.74*</td>
<td>0.55</td>
<td>-0.26</td>
<td>0.06</td>
</tr>
<tr>
<td>Slope, mM·s$^{-1}$·U$^{-1}$</td>
<td>0.11±0.29</td>
<td>0.36±0.10</td>
<td>0.41±0.19</td>
<td>-0.20±0.24</td>
<td>0.07±0.37</td>
<td>0.16±0.10</td>
</tr>
<tr>
<td>$Q_{\text{max-ADP}}$</td>
<td>R</td>
<td>0.52</td>
<td>0.88*</td>
<td>0.83*</td>
<td>0.40</td>
<td>0.14</td>
</tr>
<tr>
<td>Slope, mM·s$^{-1}$·U$^{-1}$</td>
<td>0.30±0.17</td>
<td>0.67±0.12</td>
<td>0.86±0.18</td>
<td>0.31±0.23</td>
<td>0.14±0.31</td>
<td>0.37±0.10</td>
</tr>
<tr>
<td>$Q_{\text{max-lin}}$</td>
<td>R</td>
<td>0.96*</td>
<td>0.97*</td>
<td>0.92*</td>
<td>0.85*</td>
<td>0.85*</td>
</tr>
<tr>
<td>Slope, mM·s$^{-1}$·U$^{-1}$</td>
<td>1.45±0.16</td>
<td>1.56±0.13</td>
<td>1.88±0.25</td>
<td>1.41±0.27</td>
<td>1.24±0.23</td>
<td>1.18±0.13</td>
</tr>
</tbody>
</table>

$R$, correlation coefficient determined from linear regression analysis; slope values (± SE) represent steepness of correlation. $\tau_{\text{PCR}}$ at pH 7 was obtained from the linear relation between $\tau_{\text{PCR}}$ and $pH_{\text{end}}$, U, pH unit. *$P < 0.01$. 

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PCr recovery in the presence of intracellular acidosis could also be slowed down because of factors downstream of oxidative phosphorylation, consistent with the observation that the recovery of oxyhemoglobin saturation measured by near-infrared spectroscopy is not affected by acidosis (34). Ion pumping reactions also require ATP, and therefore not all the ATP that is synthesized oxidatively during recovery is available for the CK reaction. At low pH the amount of ATP that is shuttled to cellular ion pumps might be increased (29, 44) in order to reestablish pH homeostasis. It has been reported that ion pumping reactions can consume ~43% of the total ATP produced (4, 7). The slow PCr recovery at low pH has also been attributed to a pH-dependent shift in the CK equilibrium. The CK $K_{eq}$ depends on proton and metal ion concentrations (30). Iotti et al. (19) showed that at the end of muscular exercise $K_{eq}$ can increase even more than threefold compared with rest, because of a decrease in pH and an increase in the free Mg$^{2+}$ concentration. Therefore, net PCr resynthesis throughout recovery behaves as a function of both intracellular pH and net ATP flux (2, 56). This was confirmed by a model for ATP production (according to the ADP-control model; see Eq. 4) and pH recovery, which reproduced the main features of recovery from exercise, including the feature that PCr recovery is slowed when the pH is low (48). Finally, with the incremental exercise protocol that we used, part of the pH dependence of PCr recovery could originate from selective fiber type recruitment, i.e., recruitment of mainly oxidative type I fibers (with short $\tau_{PCr}$) during the low exercise intensities with a high pH$_{end}$ and recruitment of relatively more type II fibers (with long $\tau_{PCr}$) during the higher exercise intensities with a low pH$_{end}$.

The observed intersubject differences in the pH dependence of $\tau_{PCr}$ are likely to reflect differences in the rate of pH recovery. Unfortunately, the recovery of pH could not be investigated, because the P$_i$ peak consistently disappeared within the noise after ~1 min of recovery (Fig. 1D) and for the exercises at higher intensities was not fully recovered by the end of the time series. This phenomenon has been reported before in the literature (1, 8, 21, 29, 43, 50) and has been attributed to sequestering of P$_i$ inside the mitochondria, where it becomes “NMR invisible” (2, 20), or trapping of P$_i$ into the glycolgenolytic pathway during exercise leading to phospho-monoester production (6). The recovery of pH is much slower than PCr recovery (1, 2, 55, 56), and therefore it was attempted to increase the signal-to-noise ratio of the P$_i$ peak by the summation of spectra during the recovery phase. However, even when four spectra were added, yielding a time resolution of 24 s, the position of the P$_i$ peak could not be accurately determined.

The recovery of cytosolic pH to the resting value is a function of net proton efflux (50). Several mechanisms are responsible for proton efflux, such as sodium/proton exchange, sodium-dependent chloride/bicarbonate exchange, efflux of undissociated lactic acid, and outward proton-lactate cotransport. The change in proton concentration in the cell can be calculated from the change in pH multiplied by the cytosolic buffer capacity and equals the proton efflux rate minus the rate of proton generation by PCr resynthesis and aerobic ATP production (24). We calculated proton efflux rates $E$ and apparent proton efflux rate constants $\lambda$ at the start of recovery. Both $E$ and $\lambda$ are pH dependent (27), and therefore only the data sets with a pH$_{end}$ between 6.6 and 6.8 were used to calculate an average value for $E$ and $\lambda$ for each subject. The mean values that we found for the total cytosolic buffering capacity $\beta$, $E$, and $\lambda$ correspond with values reported by Kemp et al. (27), calculated for a similar pH$_{end}$ range with exactly the same formulas. The slope of the relation between $\tau_{PCr}$ and pH$_{end}$ was positively correlated with both $E$ ($R = 0.91$, $P = 0.03$) and $\lambda$ ($R = 0.96$, $P = 0.01$), indicating that subjects with a smaller pH dependence of $\tau_{PCr}$ have a higher proton efflux rate, most likely as a result of a better blood flow due to, e.g., an increased capillary density possibly related to the subject’s fiber type composition. Higher proton efflux rates will lead to faster pH recovery, and therefore the observed correlations support our hypothesis that the intersubject differences in the pH dependence of $\tau_{PCr}$ are caused by differences in the rate of pH recovery.

To overcome the problems of pH determination during recovery associated with the transient loss of P$_i$ signal, Chen et al. (14) modeled the pH recovery based on the CK equilibrium by considering the transition from exercise to recovery as a step function input. The entire pH recovery was characterized by calculating the time required for pH recovery ($t_{pHrec}$), and a strong linear correlation was observed between $t_{pHrec}$ and the half-time of PCr recovery in normal subjects (average pH$_{end}$ ~6.7). This strong correlation corroborates the link between the PCr recovery rate and the overall pH recovery rate. Moreover, the large variation in $t_{pHrec}$ within normal subjects ($t_{pHrec}$ ranged from ~2 to 18 min) implies that differences in the pH dependence of $\tau_{PCr}$ can be significant, as we demonstrated in the present study. Certain disorders, e.g., hypertension and mitochondrial myopathy (24) and dermatomyositis and polymyositis (9), are associated with altered proton efflux from muscle fibers, which will affect the rate of pH recovery.
Therefore, when comparing PCr recovery measurements between patients and control subjects, differences in proton efflux rates or rates of pH recovery should be considered, as these might lead to systematic changes of τPCr, in particular when pHend is low.

As an alternative to τPCr, the kinetics of ADP recovery can be used to assess oxidative capacity. Because [ADP] is the principal error signal in the feedback loop controlling mitochondrial oxidation, ADP recovery is one of the most sensitive MRS indices of mitochondrial function (23). It has been shown that, in contrast to τPCr, τADP is independent of pHend (1–3, 8, 31), which was confirmed by a theoretical model (48). We investigated the pH dependence of τADP for each subject. For three subjects τADP was not significantly correlated with pHend, while for the other three subjects τADP was positively correlated with pHend, i.e., ADP recovery became faster at low pH. This phenomenon was also observed by Larson-Meyer et al. (29), but we doubt that it has any physiological meaning. It has been reported that the recovery of ADP is not always monoeXponential and that [ADP] can decrease below the resting level (1).

We also observed an ADP undershoot in some of our data sets. However, it was difficult to quantify this effect, because the undershoot occurs in the period during which the PI peak becomes invisible, resulting in a less reliable PH estimation. When the ADP recovery data with an undershoot are fitted with a monoeXponential function, the time constant will be underestimated (13), and this could explain the positive correlation between τADP and pHend. Furthermore, it was assumed that the CK $K_{eq}$ was not affected by the different metabolic conditions present after exercise, and therefore changes in pH and the free Mg$^{2+}$ concentration (18), in particular for the exercises at higher intensities, are sources of error for the [ADP] calculation that might lead to deviations in τADP.

$V_{PCr}$ is a measure of the actual mitochondrial ATP synthesis rate and therefore does not represent an absolute measure of oxidative capacity. Still, a number of studies have reported that $V_{PCr}$ is independent of pH (31, 44, 56). This is a consequence of [ADP]end being either similar for different degrees of acidification (44) or well above the accepted $K_{eq}$ value of 30 μM for oxidative ATP synthesis (31, 56). The latter was also the case for most of our measurements, and for five of the subjects $V_{PCr}$ was independent of pHend. $V_{PCr}$ can be calculated from the product of $1/\tau_{PCr}$ and ΔPCr (Eq. 3) (26, 31, 44), as in our study, or $V_{PCr}$ can be measured directly from the first data points (typically 10–14 s) (8, 29, 56). Boska et al. (8) applied both methods and found good correlations between calculated and measured $V_{PCr}$ in control subjects ($R = 0.753$) and patients with peripheral vascular occlusive disease ($R = 0.646$). However, Walter et al. (56) observed that at low pH (pHend 6.45) the calculated $V_{PCr}$ was about two times smaller than the measured $V_{PCr}$. Under conditions in which intracellular pH is decreased, any model that relies on $\tau_{PCr}$ is no longer valid and $V_{PCr}$ should be measured directly from the initial phase of recovery. However, this method is extremely sensitive to sampling rate and signal-to-noise values (56). Moreover, it has been shown that $V_{PCr}$ measured from the first 10 s of recovery is underestimated by up to 56% and that a 1- to 2-s time window is needed for the determination of $V_{PCr}$ requiring very high-time-resolution 31P MRS data (37). The fact that $V_{PCr}$ calculated from $\tau_{PCr}$ was still independent of pHend in five of our subjects results from the fact that $1/\tau_{PCr}$ and ΔPCr vary in opposite directions as a function of pHend (Fig. 3). For the sixth subject changes in these two quantities apparently did not compensate each other completely.

In the literature, $Q_{max-ADP}$ has been found to be independent of pH (31, 44), although in the study of Walter et al. (56) only $Q_{max-ADP}$ based on the measured $V_{PCr}$ was pH independent. In our study, $Q_{max-ADP}$ was based on the calculated $V_{PCr}$ and [ADP]end (Eq. 4). Although $V_{PCr}$ and [ADP]end were not significantly correlated with pHend (except for 1 subject), these parameters tend to vary in opposite directions as a function of pHend (Fig. 3), resulting in significant positive correlations between $Q_{max-ADP}$ and pHend for three of six subjects. For each subject, there was a strong positive linear relationship between $Q_{max-ADP}$ and $\tau_{PCr}$, showing an equally strong relationship with pHend. The slopes of the relation between $Q_{max-ADP}$ and pHend were much smaller than for $Q_{max-ADP}$ and thus at low pH the error is smaller for $Q_{max-ADP}$. However, in accordance with Walter et al. (56), our data show that none of the models that rely on $\tau_{PCr}$ is reliable to predict $Q_{max}$ in the presence of intracellular acidosis (56).

In conclusion, intracellular acidosis slowed PCr recovery, and the pH dependence of τPCr differed among subjects, ranging from -33.0 to -75.3 s/pH unit. The effect of acidosis on PCr recovery kinetics after exercise correlated with the kinetics of proton efflux at the start of recovery, strongly indicating that the intersubject differences in the pH dependence of τPCr reflect differences in the rate of pH recovery. Our study implies that simply correcting τPCr for pHend with a general formula is not adequate, in particular when comparing patients and controls, because certain disorders are characterized by altered proton efflux from muscle fibers. Also, matching for pHend is not sufficient when subject groups systematically differ in proton efflux kinetics. Therefore, τPCr can only be used as a measure of mitochondrial function when pHend is close to resting values. Avoiding a decrease in intracellular pH along with a sufficient drop in PCr to model PCr recovery may, however, be difficult in untrained subjects or patients (56). An exercise protocol that progressively increases work, like that used in the present study, has been reported to be successful in decreasing PCr without severe acidification as opposed to sustained-load exercise (22, 56). Indeed, we obtained data sets with pHend close to 7 with a drop in PCr of roughly 50%. Alternatively, one could use an exercise protocol of short duration (9 s) with very rapid contractions, which has the advantage that it is believed to simultaneously recruit all fibers (56), or a gated protocol in which the acquisition is gated to contractions of short duration without significant muscle acidification that are repeated in a steady state for as many times as necessary to obtain the desired signal-to-noise ratio (47). The kinetics of ADP recovery is independent of pHend. Disadvantages of using τADP as a measure of mitochondrial function are the complex time-dependent undershoot of ADP during recovery and the assumptions that have to be made to calculate [ADP] (1). $Q_{max}$ can only be used when based on $V_{PCr}$ directly.
measured from the initial recovery data points. However, the reproducibility of the latter parameter is much lower than for $\tau_{PC}$ and $\tau_{ADP}$ (56).

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