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ORIGINAL PAPER

Phosphocreatine recovery overshoot after high intensity exercise in human skeletal muscle is associated with extensive muscle acidification and a significant decrease in phosphorylation potential

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Abstract The phosphocreatine (PCr) recovery overshoot in skeletal muscle is a transient increase of PCr concentration above the resting level after termination of exercise. In the present study [PCr], [ATP], [P_i] and pH were measured in calf muscle during rest, during plantar flexion exercise until exhaustion and recovery, using the ^{31}P NMR spectroscopy. A significantly greater acidification of muscle cells and significantly lower phosphorylation potential (ΔG_{ATP}) at the end of exercise was encountered in the group of subjects that evidenced the [PCr] overshoot as well as [ADP] and [P_i] undershoots than in the group that did not. We postulate that the role of the PCr overshoot-related transiently elevated [ATP]/[ADP_{free}] ratio is to activate different processes (including protein synthesis) that participate in repairing numerous damages of the muscle cells caused by intensive exercise-induced stressing factors, such as extensive muscle acidification, a significant decrease in ΔG_{ATP}, an elevated level of reactive oxygen species or mechanical disturbances.

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Introduction

At the onset of physical exercise of human skeletal muscles, the concentration of phosphocreatine (PCr) decreases significantly because of a transient mismatch between the rate of ATP consumption and production. At the end of fatiguing exercise of maximal intensity in predominantly glycolytic human skeletal muscles, [PCr] is almost completely depleted (see, e.g., [1–5]). The phosphocreatine concentration recovers rapidly during muscle recovery, reaching the pre-exercise level within a few minutes after the termination of exercise (see, e.g., [3, 6–8]). In some studies it has been shown that after the termination of exercise, the muscle PCr concentration rises transiently even above its pre-exercise level (the so-called PCr overshoot) [7, 9–16]. This phenomenon may be attributed to either oxidative muscles/muscle fibers after long-term submaximal exercise [13–15] or to glycolytic muscles/muscle fibers after short-term, very intensive exercise of maximal intensity [7, 16]. In some cases this effect is still present even 90 min after the termination of exercise [10]. The selective PCr recovery overshoot manifestation in oxidative skeletal muscle may be promoted by a higher level of parallel activation of oxidative phosphorylation than in glycolytic skeletal muscle, while the appearance of this overshoot in glycolytic skeletal muscle may be caused by a presence of stressing factors, such as an extensive muscle acidification (see [17, 18]). The [PCr] recovery overshoot is accompanied by the [ADP_{free}] undershoot and [P_i] undershoot (and, consequently, ΔG_{ATP} overshoot)

[9, 13]. On the other hand, the [ADP_{free}] recovery undershoot may in some cases appear without the [PCr] overshoot [19–21]. This observation indicates that these undershoots/overshoots are not strictly related to each other and that it is the [ADP_{free}] undershoot that conditions the PCr overshoot, but not inversely.

The mechanisms underlying the phenomenon of [PCr] overshoot remain unclear. It was proposed recently by Korzeniewski [17] that a slow decay, after the termination of exercise of the parallel activation of oxidative phosphorylation, which is turned on during skeletal muscle work [22, 23], is responsible for the phenomenon of the [PCr] recovery overshoot. This idea was further developed and quantitatively elaborated by Korzeniewski and Zoladz [18]. In particular, it was postulated that the [ADP_{free}] undershoot is not accompanied by the [PCr] overshoot if the decay of extensive muscle acidification after the termination of exercise lasts longer than the decay of [ADP_{free}] undershoot [18]. All these theoretical studies were performed using the computer dynamic model of oxidative phosphorylation developed by Korzeniewski and Zoladz [24]. It has been proposed that the role of the [PCr] overshoot/[ADP_{free}] undershoot during muscle recovery is the stimulation of ATP-using processes activated by a high [ATP]/[ADP_{free}] ratio, for instance, protein synthesis [25], participating in muscle cell repair after stress-generating exercise [18]. The relevant stressing factors may comprise extensive muscle cell acidification, elevated level of reactive oxygen species, significant decrease in the phosphorylation potential or mechanical damage.

In this study we try to support the above proposal and hypothesize that the PCr recovery overshoot is associated with one of the intensive-exercise-related stressing factors, namely extensive muscle acidification (and also a great decrease in the phosphorylation potential) during and immediately after exercise. Our additional aim is to validate the supposition that the PCr recovery overshoot is generated by some mitochondrial mechanism (causing the ADP and P_i recovery undershoots) and not by the creatine kinase mechanism (shift in the CK equilibrium caused by changes in [H^+] and [Mg^{2+}]). At the same time, we attempt to show that the creatine kinase mechanism is important for the manifestation, size and duration of the PCr overshoot.

Materials and methods

Subjects

Twenty healthy young men aged (mean ± SEM) 23.2 ± 0.8 years old, with height 184.2 ± 1.6 cm, body mass 74.0 ± 1.6 kg and BMI 21.8 ± 0.3 kg m^{-2}, volunteered for this study. The subjects reported to the laboratory in the morning hours after a light breakfast. All procedures were approved by the local ethics committee and performed according to the Declaration of Helsinki. Subjects gave informed written consent and were aware of the aims of the study.

Exercise protocol

The exercise protocol started with 5 min seating in rest, followed by performing one leg (right) plantar flexion exercise with a frequency of 60 cycles per minute—until fatigue. The resisting force of the pedal amounted to about 50% of the maximal voluntary contraction force of this muscle group, determined for each subject. The exercise was stopped when the subject declared fatigue. Subsequently, after the finishing exercise, the subjects remained seated with the fatigued leg positioned in the magnet.

^{31}P NMR spectroscopy

A ^{31}P MR signal was acquired on an MRI research system equipped with a 4.7-T/310-mm horizontal bore superconducting magnet (Bruker) and a digital MARAN DRX console (Resonance Instruments). A dedicated, home built $^{31}P/^{1}H$ (81.05/200 MHz) double-tuned MR probe head with a two-turn 50-mm ID surface coil for muscle study was used. The probehead was placed in the magnet in such a position that the surface coil was located 10 cm distal of the fossa poplitea under the calf to acquire MR signal from the gastrocnemius and soleus muscles.

MR signals were acquired with a pulse-acquisition sequence using a single square RF pulse with repetition time of 5 s. Then 16k data points of ^{31}P MR signal (FID) were recorded with the sampling rate of 10 μs. The power and duration of the RF pulse were calibrated using phantoms to obtain the ^{1}H and ^{31}P signals from the region of interest i.e. 15 mm under the surface of the calf. The static magnetic field over the volume of interest was corrected manually (so-called "shimming") to achieve homogeneity of about 0.1–0.2 ppm using proton signal from muscle water. During the experimental protocol (pre-exercise rest, exercise, post-exercise rest/recovery), the MR signals were acquired continuously and written as a single file.

Signal analysis

The signals were extracted and accumulated as a postprocessing to obtain the desired temporal resolution of 20 s. Pre-exercise signals were accumulated (10 min) and processed independently to obtain good quality pre-exercise

rest spectra. According to the SVD fitting model, the PCr signal was prepared. The model was used for homonuclear reference deconvolution. It was performed to convert line shapes to the Lorentzian type and therefore to improve the overall quality of the signal analysis.

After the preprocessing, MR data sets were analyzed in the time domain using the "JMRUI v.3.0" software package [26]. The signals were filtered to reduce spectral bandwidth using the "ER-Filter" procedure. AMARES method (JMRUI) requires no more than 2,048 time domain data points. The ER-Filter reduces the bandwidth of the spectrum and number of time domain data points at the same time [27]. It cuts out a selected region of the spectrum and performs inverse Fourier transform yielding time domain signal from the selected region. In our case the acquired MR signal was over-sampled (16k time domain data points/100 kHz spectrum width). After the ER-Filter procedure had been carried out, the signal consisted of 1,024 time domain data points with a bandwidth of 6360 Hz.

Then the signals were fitted in the time domain using the "AMARES" algorithm [28] employing prior knowledge for ATP signal components. α and γ ATPs were fitted as doublets with an amplitude ratio of 1:1 and β ATP as a triplet with amplitude ratios of 0.5:1:0.5 [28] with line splitting of 17 Hz and equal spectral linewidth and phase. The amplitudes were T_1 corrected. In this study, as in the most of the experiments with different types of model exercises, the T_{1s} were assumed constant [29–32].

Relative concentrations of $[P_i]$/[ATP] and [PCr]/[ATP] as well as chemical shifts of every spectral component were assessed.

Typical ^{31}P spectra for single subjects belonging to group A (with no PCr recovery overshoot) and group B (with significant PCr recovery overshoot; see below) recorded at rest, during exercise and in the recovery period after exercise are presented in Fig. 1a, b.

Calculations of the derivative parameters

Intracellular pH, $[Mg^{2+}]$, $[ADP_{free}]$ and $[\Delta G_{ATP}]$ were calculated using the equations and constants as presented below (see Eqs. 1–10). Absolute concentrations of PCr and P_i were obtained by assuming that the concentration of ATP in calf muscle at rest is 8.2 mM (see [2, 33–35]) and of [TCr] 42 mM (see [2, 34]). $[K^+]$ was assumed to be a constant value of 0.11 M (see [35]).

Intracellular pH was calculated applying the Henderson-Hasselbalch equation with pK = 6.75, $\delta_1 = 3.27$, $\delta_2 = 5.69$ (see, [33]), which utilizes dependence of pH on the P_i chemical shift δ_{Pi} relative to PCr chemical shift δ_0 (see Eq. 1).

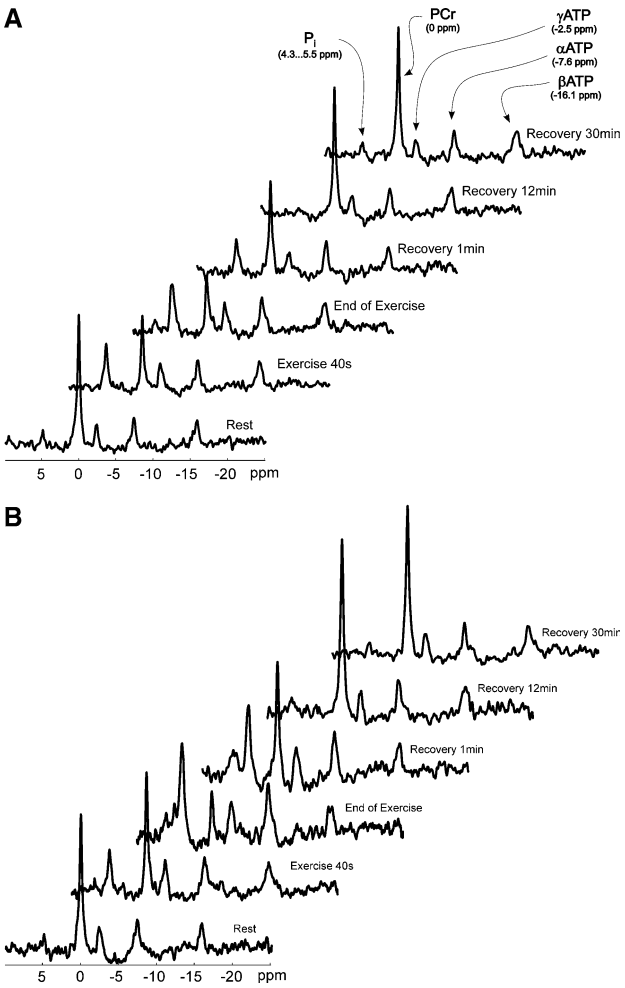

Fig. 1 Typical ^{31}P spectra recorded at rest, during exercise and in the recovery period after exercise for group A (individuals without PCr recovery overshoot) (**a**) and group B (individuals with PCr recovery overshoot) (**b**)

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

where δ is an experimental value $\delta = \delta_{Pi} - \delta_0$ and is expressed in ppm.

pMg and hence the concentration of free cytosolic magnesium ($[Mg^{2+}]$) was calculated using Eq. 2 and calibration constants provided by Iotti et al. [36]. It involves the dependence of free magnesium concentration on chemical shift of βATP.

$$\mathrm{pMg} = y_0 + \frac{c_1}{c_2 - \exp\left(\frac{\delta_\beta - \delta_0}{d}\right)} + a_1\left(\delta_\beta - \delta_0\right) + a_2\left(\delta_\beta - \delta_0\right)^2 + b([\mathrm{PCr}] - 27) \quad (2)$$

where $d = D_1 + D_2(\mathrm{pH} - 7)^2$, $a_1 = A_1 + A_2(\mathrm{pH} - 7)$ with calibration constants: $y_0 = 10.43$, $A_1 = -0.20$ ppm^{-1}, $A_2 = -0.29$ ppm^{-1}, $D_1 = 0.105$ ppm^{-1}, $D_2 = 0.22$ ppm^{-1}, $a_2 = 0.128$ ppm^{-2}, $b = -0.005$ mM^{-1}, $c_1 = -25.66$,

$c_2 = 3.728$ and with experimental values pH, [PCr] and δ_β, δ_0 (chemical shifts of βATP and PCr respectively).

$[ADP_{free}]$ was calculated using Eqs. 3–7 and constants according to Kemp et al. [35] (see also [37]).

$$[ADP_{free}] = [ATP]\left(\frac{[TCr]}{[PCr]} - 1\right)\frac{1}{K_{CK}^{app}}\frac{1}{[H^+]} \quad (3)$$

$$K_{CK}^{app} = K_{CK}^{true}\frac{f_{ADP}f_{PCr}}{f_{ATP}} \quad (4)$$

$$\frac{1}{f_{ATP}} = 1 + \frac{[Mg^{2+}]}{K_{Mg\,ATP}} + \frac{[K^+]}{K_{K\,ATP}} + \frac{[H^+]}{K_{H\,ATP}}\left\{1 + \frac{[Mg^{2+}]}{K_{Mg\,HATP}} + \frac{[H^+]}{K_{H_2\,ATP}}\right\} \quad (5)$$

$$\frac{1}{f_{ADP}} = 1 + \frac{[Mg^{2+}]}{K_{Mg\,ADP}} + \frac{[K^+]}{K_{K\,ADP}} + \frac{[H^+]}{K_{H\,ADP}}\left\{1 + \frac{[Mg^{2+}]}{K_{Mg\,HADP}} + \frac{[H^+]}{K_{H_2\,ADP}}\right\} \quad (6)$$

$$\frac{1}{f_{PCr}} = 1 + \frac{[Mg^{2+}]}{K_{Mg\,PCr}} + \frac{[K^+]}{K_{K\,HPCr}} + \frac{[K^+]}{K_{H\,PCr}}\left\{1 + \frac{[K^+]}{K_{KH_2\,PCr}} + \frac{[H^+]}{K_{H_2\,PCr}}\right\} \quad (7)$$

ΔG_{ATP} was calculated by using Eqs. 8–10 and constants according to Kemp et al. [35] (see also [37]).

$$\Delta G_{ATP} = \Delta G_{ATP}^0 + R\,T\,\ln\frac{[ADP_{free}][P_i]}{[ATP]} \quad (8)$$

$$-\Delta G_{ATP}^0 = R\,T\left[\ln\left(K_{ATP}^{true}\right) + \ln\left(\frac{f_{ATP}}{f_{ADP}f_{P_i}}\frac{1}{[H^+]}\right)\right] \quad (9)$$

$$\frac{1}{f_{P_i}} = 1 + \frac{[Mg^{2+}]}{K_{Mg\,Pi}} + \frac{[K^+]}{K_{K\,P_i}} + \frac{[H^+]}{K_{H\,P_i}}\left\{1 + \frac{[H^+]}{K_{H_2\,P_i}} + \frac{[K^+]}{K_{KH\,P_i}}\right\} \quad (10)$$

where R is a gas constant = 8.3145 J K^{-1} M^{-1}, and T is an absolute temperature in Kelvin = 310 K.

Statistics

Data are presented as mean (± SEM). For comparison of the studied variables in groups A ($n = 15$) and B ($n = 5$) as well as for comparison of the data obtained at rest, during exercise as well as during the recovery period, a non-parametric Wilcoxon-Mann-Whitney test with a non-asymptotic, exact, two-sided p value was used, using StatXact software, version 6.1.

Results

Muscle [PCr], [ATP], [P_i] and pH were measured in the calf muscle in 20 subjects during rest, maximal exercise and 30 min of recovery. Because the studied subjects finished the exercise due to exhaustion after varied periods of time (about 3–6 min), it was impossible to present average values of the measured variables during exercise, and therefore in the figures below only the variable values at rest, at the end of exercise and during muscle recovery are presented. The duration of exercise was not particularly significant for our experiment, because intensive exercise served only for production of some stressing factors, especially the end-exercise muscle cell acidification.

Based on the [PCr] recovery pattern two main groups of subjects were distinguished: group A (*the subjects without [PCr] recovery overshoot, $n = 15$*) and group B (*the subjects with [PCr] overshoot equal to at least 5% above the pre-exercise [PCr], $n = 5$*). This is shown in Fig. 2 where the time courses of [PCr] for the same subjects as in Fig. 1 are presented. It should be mentioned that the mean (±SEM) time of work to fatigue in both groups was almost identical, equal to 4.87 ± 1.13 min in group A and 4.86 ± 1.14 min in group B. Anyway, as mentioned above, we were more interested in a pronounced decrease in pH at the end of exercise in relation to its resting value than in the duration time of the exercise that caused this final acidification.

The resting [PCr] tended to be lower ($p = 0.15$) in group B (overshoot group) than in group A (the "no overshoot group") (31.76 ± 2.17 mM vs. 35.10 ± 0.88 mM), while the resting [P_i] was higher (5.91 ± 1.17 mM vs. 3.96 ± 0.17 mM) ($p < 0.02$) (see Fig. 3). [PCr] decreased during exercise to 17.16 ± 1.51 mM (i.e., about 50% of the pre-exercise concentration) in group A and to 10.80 ± 2.20 mM (i.e., about 30% of the pre-exercise concentration) in group B (A vs. B, $p = 0.08$). During recovery, [PCr] returned to its resting value in the former case ($p = 0.11$, rest vs. recovery 6–22 min), while in the latter case an average [PCr] overshoot of over 2 mM ($p < 0.0625$, rest vs. recovery 6–22 min) (about 8% above the pre-exercise [PCr]) that lasted at least 30 min was observed. It is noteworthy that the kinetics of the PCr resynthesis was significantly faster in group A than in group B. In group A [P_i] increased during work to 18.23 ± 1.17 mM and returned to its resting value during recovery. In group B the exercise-induced increase in [P_i] was greater than in group A (to 24.53 ± 1.22 mM) ($p < 0.02$), and a P_i recovery undershoot of over 2 mM took place and lasted at least 30 min ($p = 0.06$). The rate of the decrease in [P_i] during recovery was slightly higher in group A than in group B. [ATP], assumed to be 8.2 mM at rest, remained essentially constant during work and recovery in group A. In group B it dropped slightly during exercise and at the end of exercise, and during recovery was approximately constant at 7.81 ± 0.33 mM (Fig. 3).

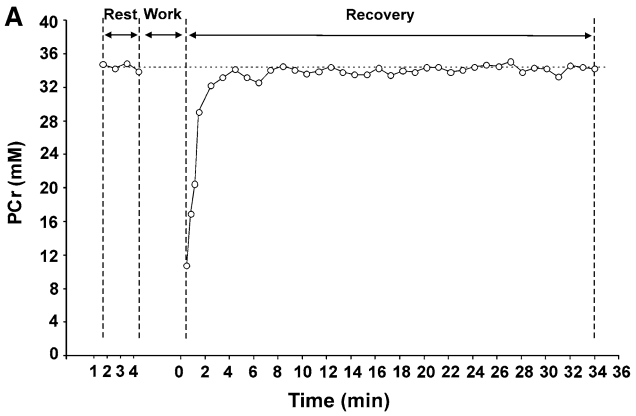

Fig. 2 Changes in [PCr] concentration in the calf muscle at rest and during the recovery period after fatiguing exercise in typical single subjects (the same as in Fig. 1) from group A (no PCr recovery overshoot) (**a**) and B (PCr recovery overshoot present) (**b**). The *dashed horizontal lines* represent the pre-exercise concentrations of PCr

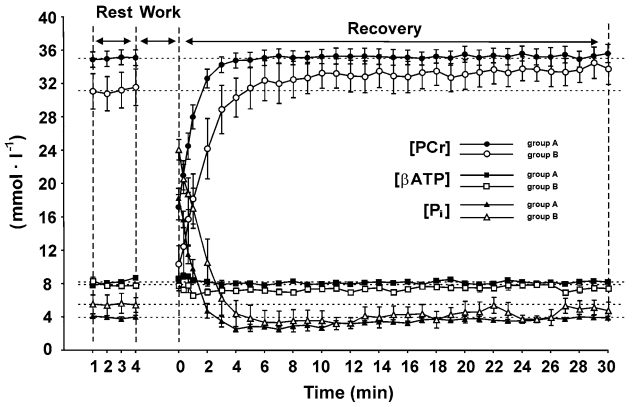

Fig. 3 Mean (± SEM) values of [PCr], [P_i] and [ATP] concentration in the calf muscles at rest, at the end of work and during the recovery period after fatiguing exercise in the subjects from group A (no PCr overshoot) and B (PCr overshoot present). The *dashed horizontal lines* represent the pre-exercise concentrations of [PCr], [P_i] and [ATP]

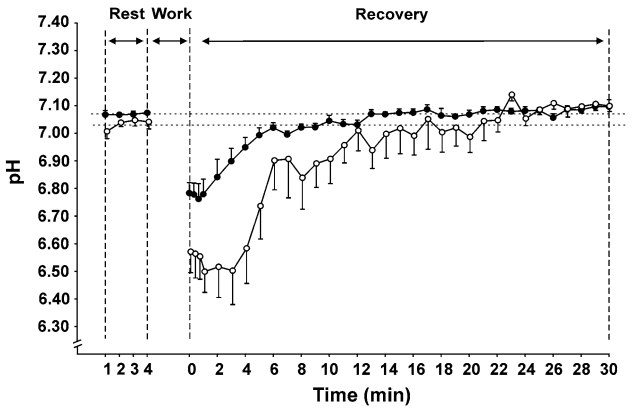

Fig. 4 Mean (±SEM) values of pH in the calf muscles at rest, at the end of work and during the recovery period after fatiguing exercise in the subjects from group A (no PCr overshoot) and B (PCr overshoot present). The *dashed horizontal lines* represent the pre-exercise muscle pH

The resting pH was similar in group A (7.07 ± 0.01) and group B (7.04 ± 0.02) (Fig. 4). pH decreased much more at the end of exercise in group B (to 6.57 ± 0.08, by 0.46 pH units) than in group A (to 6.78 ± 0.04, by 0.29 pH units). The end-exercise pH in group B was significantly lower ($p < 0.05$) than in group A. During recovery, in group A pH started to increase quickly after an initial lag phase (exhibiting a small undershoot related to CK reaction) lasting about 1 min and returned near the resting value after about 6 min. The lag phase at the onset of recovery (exhibiting a bigger CK reaction-related undershoot) was significantly longer in group B (about 4 min), and after a quick increase terminating about 6 min after the onset of recovery, the pH value was still lower by about 0.15 pH units than the resting pH value. It subsequently took about 6 min for the pH to reach the resting value (Fig. 4).

The calculated free cytosolic ADP [ADP_{free}] at rest tended to be higher in group B than in group A (24.92 ± 6.85 μM vs. 15.93 ± 2.21 μM) (Fig. 5), but this difference was not statistically significant ($p = 0.20$). During exercise [ADP_{free}] increased to 87.95 ± 14.12 μM (by 73.82 μM) in group A and to 95.01 ± 22.54 μM (by 70.09 μM) in group B. The end-exercise ADP_{free} concentrations were not statistically different. In group A ($n = 15$) a small [ADP_{free}] recovery undershoot of about 3 μM took place ($p = 0.013$, rest vs. recovery 3–22 min) and lasted about 10 min, while in group B ($n = 5$) a significant [ADP_{free}] undershoot of about 12 μM lasting at least 30 min was observed ($p = 0.0625$, rest vs. recovery 3-22 min). Interestingly, [ADP_{free}] during recovery was essentially identical in both groups. The rate of the decrease in [ADP_{free}] was faster in group A than in group B (Fig. 5).

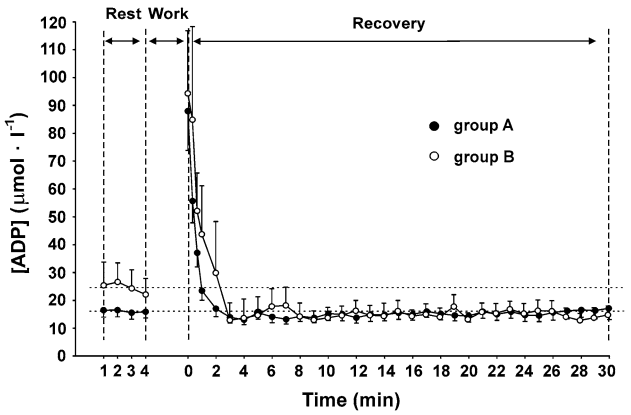

Fig. 5 Mean (± SEM) values of [ADP_{free}] in the calf muscles at rest, at the end of work and during the recovery period after fatiguing exercise in the subjects from group A (no PCr overshoot) and B (PCr overshoot present). The *dashed horizontal lines* represent the pre-exercise muscle [ADP_{free}] concentration

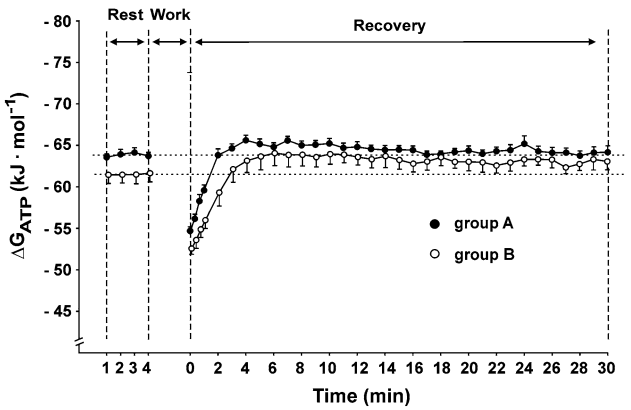

Fig. 6 Mean (± SEM) values of ΔG_{ATP} in the calf muscles at rest, at the end of work and during the recovery period after fatiguing exercise in the subjects from group A (no PCr overshoot) and B (PCr overshoot present). Note the ΔG_{ATP} overshoot in the recovery period. The *dashed horizontal lines* present the pre-exercise muscle ΔG_{ATP}

The (absolute value of the) phosphorylation potential at rest was higher (more negative) in group A than in group B (-63.85 ± 0.45 vs. -61.51 ± 1.09 kJ mol^{-1}) ($p < 0.02$). It decreased during exercise to -54.70 ± 0.49 kJ mol^{-1} in group A and to -52.47 ± 0.70 kJ mol^{-1} in group B. This difference was statistically significant ($p < 0.05$). In both groups there was a ΔG_{ATP} overshoot during recovery: in group A it equalled 2 kJ mol^{-1} and lasted about 15 min, while in group B it equalled about 3 kJ mol^{-1} and lasted at least 30 min (Fig. 6). The increase in the phosphorylation potential at the beginning of recovery was faster in group A than in group B.

In this study the pre-exercise concentration of magnesium ions [Mg^{2+}] in group A was equal to 0.70 ± 0.06 mM. At the end of exercise, it decreased to 0.37 ± 0.09 mM, which constituted a statistically significant difference in relation to rest ($p = 0.00006$). However, after the termination of exercise [Mg^{2+}] quickly increased, and after 3 min of recovery equalled 0.65 ± 0.09 mM and was not significantly different from the resting value ($p = 0.45$). [Mg^{2+}] was essentially constant during the rest of recovery. In group B the pre-exercise concentration of [Mg^{2+}] was equal to 0.67 ± 0.11 mM. At the end of exercise it increased to 1.02 ± 0.46 mM, but this increase was not statistically significant ($p = 0.63$). After the termination of exercise [Mg^{2+}] quickly (within about 3 min) returned near the initial level and remained essentially constant during the rest of recovery. [Mg^{2+}] after 3 min of recovery equalled 0.87 ± 0.26 mM and was not significantly different ($p = 0.63$) from the baseline. Therefore, the changes in [Mg^{2+}] did not have any significant influence on the appearance of the [PCr] recovery overshoot that started after 4 min of recovery. Of course, these changes in [Mg^{2+}] were taken into account when [ADP_{free}] was calculated from the creatine kinase equilibrium.

Discussion

The main finding of this study is that the [PCr] recovery overshoot after intensive calf muscle exercise until exhaustion in humans is associated with an extensive muscle acidification and enhanced drop in the phosphorylation potential (ΔG_{ATP}) at the end of exercise (see Figs 4, 6). At the same time, all subjects studied exhibited [ADP_{free}] recovery undershoot, [P_i] undershoot and ΔG_{ATP} overshoot. Therefore, these undershoots/overshoots are not necessarily correlated with the PCr overshoot, in agreement with previous studies [19–21]. On the other hand, a pronounced [ADP_{free}] undershoot is a necessary prerequisite for the appearance of the [PCr] overshoot (see below).

Two main potential (groups of) mechanisms determining the [PCr] kinetics during skeletal muscle recovery after exercise can be considered. The mitochondria-related mechanism(s) determines the relationship between the rate of ATP production and [ADP] (and [P_i]) (assuming that the ATP concentration is essentially constant), while the creatine-kinase-related mechanism(s) influence the apparent equilibrium between the [ATP]/[ADP_{free}] ratio and [PCr]/[Cr] ratio. The mitochondria-related mechanisms involve the kinetic properties of oxidative phosphorylation: (1) "basic" properties in the absence of the direct external activation and (2) the degree of the (postulated) direct parallel activation of oxidative phosphorylation complexes at a given moment of recovery. The CK-related mechanisms involve changes in [H^+] and [Mg^{2+}] during recovery. Creatine kinase catalyzes the quick reversible reaction:

$$PCr^{2-} + MgADP^{-} + H^{+} \leftrightarrow Cr + MgATP^{2-}$$

At a given pH and $[Mg^{2+}]$, there is a constant relationship between the [ATP]/$[ADP_{free}]$ ratio and the [PCr]/[Cr] ratio. High levels of both $[H^{+}]$ and $[Mg^{2+}]$ (in particular: in relation to rest) decrease the [PCr]/[Cr] ratio at a given [ATP]/$[ADP_{free}]$ ratio and therefore tend to counteract the appearance of the PCr recovery overshoot.

The present data suggest that mitochondrial mechanisms (e.g., a slow decay of parallel activation during recovery) constitute the crucial factor that is necessary for the appearance of the [PCr] recovery overshoot, as proposed previously [17, 18]. This happens through generation of the $[ADP_{free}]$ recovery undershoot. However, this undershoot, especially if it is small and short lasting, can be 'masked' by creatine kinase mechanisms and thus prevent the appearance of the [PCr] overshoot. In particular, the elevated level of $[H^{+}]$ (and, to a lesser extent, $[Mg^{2+}]$) after the termination of exercise shifts the equilibrium of creatine kinase and thus lowers the [PCr]/[Cr] ratio at a given [ATP]/$[ADP_{free}]$ ratio. In order to 'hide' completely the $[ADP_{free}]$ undershoot, the acidification must be (1) large enough (in relation to the size of the $[ADP_{free}]$ undershoot) and (2) long enough (again, in relation to the duration of the $[ADP_{free}]$ undershoot). In group A the $[ADP_{free}]$ undershoot is very tiny and ends after about 10 min of recovery, and therefore can be masked by moderate initial acidification that vanishes completely after about 12 min of recovery. On the contrary, in group B the $[ADP_{free}]$ undershoot is more pronounced and lasts for at least 30 min. Therefore, it can be potentially masked only during the first 4–5 min of recovery when an extensive acidification (in relation to rest) takes place, and after this period the [PCr] overshoot appears. Anyway, [PCr] needs some time to return from its end-exercise level near its initial (resting) level during recovery.

$[ADP_{free}]$ (and, to a smaller extent, $[P_i]$) is the main feedback regulator of the muscle energetic system in general, and of oxidative phosphorylation in particular, while the [PCr]/[Cr] pair only buffers the [ATP]/$[ADP_{free}]$ ratio and helps to overcome possible diffusion limitations for $[ADP_{free}]$ [the creatine kinase (CK) shuttle, [38]]. Therefore, in a steady-state (or quasi-steadystate), the [PCr]/[Cr] ratio will reflect (through CK equilibrium) the [ATP]/[ADP] ratio, and not inversely: this statement does not necessarily concern transient states, because absolute (in mM) changes in $[ADP_{free}]$ are much smaller than absolute changes in [Cr] and [PCr]. For this reason the combined influence of the [ATP]/$[ADP_{free}]$ ratio and apparent CK equilibrium will decide about the appearance and size of the PCr recovery overshoot (the plateau phase of the overshoot constitutes a quasi-steadystate). It has been proposed previously [18] that the size and time of the decay of the parallel activation of oxidative phosphorylation during recovery determine the extent and duration of the $[ADP_{free}]$ undershoot ([ATP]/$[ADP_{free}]$ overshoot). On the other hand, the concentration of H^{+} (and Mg^{2+}) determines the apparent equilibrium constant of creatine kinase. Therefore, whereas a mitochondrial mechanism—parallel activation—is a necessary condition of the presence of the [PCr] recovery overshoot, both mitochondrial and CK mechanisms determine the size and duration of this phenomenon.

The strong initial acidification during muscle recovery and the related [PCr] overshoot seem to be associated with a slower kinetics at the onset of recovery of all variables measured (or calculated): [PCr], $[P_i]$, $[ADP_{free}]$ and phosphorylation potential. This seems to be related to a slowed down rate of ATP resynthesis by oxidative phosphorylation in mitochondria, because this process is the main source of ATP during muscle recovery [39]. Several potential explanations of this relationship exist. First, the [PCr] overshoot may be related in our experimental system to glycolytic type II muscle fibers that contain less mitochondria and therefore have less active oxidative phosphorylation than oxidative type I muscle fibers. Second, some experimental data suggest that oxidative phosphorylation may be inhibited by high proton concentration [40]. However, the acidification that was supposed to block oxidative phosphorylation in that study (pH 6.88) was much smaller than in group B in our study. Additionally, low pH does not inhibit oxidative phosphorylation in isolated mitochondria in the physiological pH range [41]. Third, because of the pronounced initial acidification in group B, the ADP_{free} concentration is smaller in the transient state after termination of exercise at a given [PCr]/[Cr] ratio than in group A (because of a shifted CK equilibrium). This would slow down the [PCr] recovery that is stimulated by $[ADP_{free}]$. On the other hand, in the quasi-steadystate where the overshoots appear, it is the [PCr]/[Cr] ratio that reflects the [ATP]/$[ADP_{free}]$ ratio, and not inversely, as discussed above. Generally, the reason for the difference between group A and group B in the dynamics of metabolite concentrations at the onset of recovery is not quite clear.

pH at the beginning of recovery is more acidic (by about 0.2 pH units), has a longer "lag phase" (4 vs. 1 min) and exhibits a greater undershoot in group B than in group A (Fig. 4). The lower pH is most probably related to a greater intensity of anaerobic glycolysis. On the other hand, the longer and more pronounced undershoot results from the lower level of [PCr] at the end of exercise and thus to a greater amount of [PCr] that must be resynthesized by CK. This is associated with greater proton production. Also other parameters of the system, such as buffering capacity for protons or the intensity (rate constant) of the proton

efflux, may influence the degree of muscle acidification during exercise in different (groups of) individuals.

In the present study no statistically significant differences in [Mg^{2+}] between rest and 3–30 min of recovery were measured in group A, although [Mg^{2+}] transiently decreased in the initial stage of recovery (0–3 min). In group B [Mg^{2+}] transiently increased during exercise and at the beginning of recovery, but this increase was not statistically significant. Anyway, such a transient increase could only lead, through a shift in the apparent CK equilibrium, to a decrease of the [PCr]/[Cr] ratio at a given [ATP]/[ADP_{free}] ratio. Therefore, this phenomenon could not be responsible for the appearance of the [PCr] recovery overshoot. Additionally, after 3 min of recovery (before [PCr] overshoot started) [Mg^{2+}] returned in group B to the resting value (no statistically significant difference). There was also essentially no decrease in [ATP] in group A, while in group B a small decrease in [ATP] was observed during exercise and at the beginning of recovery (see Fig. 3). This could be caused by AMP deamination, the main source of the increase of magnesium ion concentration in the cytosol during skeletal muscle exercise (see, e.g., [42]).

Generally, the [PCr] overshoot can be regarded as an epiphenomenon of the [ADP_{free}] recovery undershoot and the extent of muscle cell acidification during recovery, both determining the [PCr]/[Cr] ratio through the creatine kinase (CK) equilibrium. The muscle acidification during exercise is of course brought about by anaerobic glycolysis, while the return of pH to the initial (resting) value depends on the proton efflux from cytosol to blood [43]. These processes are relatively well understood. Generally, the rate of changes in pH is inversely proportional to the proton buffering capacity, provided that all other factors are unchanged. Therefore, in order to explain the biochemical background of the [PCr] recovery overshoot phenomenon, one must explain the basis of the [ADP_{free}] undershoot (and the related [P_i] undershoot and ΔG_{ATP} overshoot).

It has been postulated recently by Korzeniewski [17] and further developed by Korzeniewski and Zoladz [18] that [PCr] overshoot (and, first of all, [ADP_{free}] undershoot) is caused by a slow decay after the termination of exercise of the direct activation of oxidative phosphorylation complexes taking place during skeletal muscle contraction (due to the so-called parallel-activation or each-step-activation mechanism of the regulation of oxidative phosphorylation postulated by Korzeniewski [22, 23]). It has been postulated that this mechanism plays an important role in the training-induced improvement of muscle metabolic stability and acceleration of the VO_2 on kinetics at the onset of exercise [44, 45]. The factors that can increase the characteristic decay time of the activation of oxidative phosphorylation after the termination of exercise $t_{1/2}$ can cause the appearance/enlargement of the [PCr] recovery overshoot, [P_i] undershoot, [ADP_{free}] undershoot and phosphorylation potential overshoot [17, 18, 23]. One such factor may be muscle stress during exercise, for instance caused by a pronounced decrease in the phosphorylation potential (ΔG_{ATP}), increase in muscle temperature, reactive oxygen species production, mechanical stress and/or very low muscle pH at the end of exercise in muscle cells.

In this study a more pronounced (in size and duration) [ADP_{free}] undershoot (group B) is clearly associated with a higher muscle cell acidification and ΔG_{ATP} decrease during exercise. This fact confirms the conclusion that there is a tight connection between the intensity of stress-generating factors and the [ADP_{free}] undershoot.

Muscle cell stress may originate in various types of muscle fibers, depending upon the kind of exercise and its intensity, and can lead to temporary disturbances in muscle cell functioning as well as to damage of many proteins and other compounds. For this reason, it is necessary to resynthesize these components of the cell quickly, and therefore to activate RNA synthesis, protein synthesis and other relevant processes. It was demonstrated that RNA synthesis and protein synthesis are very sensitive (much more than, e.g., sodium/potassium and calcium ion circulation) to the [ATP]/[ADP_{free}] ratio (and/or phosphorylation potential) [25]. Therefore, as proposed in [18], the function of the [PCr] overshoot and especially the related [ADP_{free}] undershoot could be to activate recovery of the exercise-induced disturbances in muscle cell functioning as well as to accelerate the repair of the damages of muscle cells occurring during stressing exercise. In this context, the [ADP_{free}] undershoot would be the phenomenon that really matters ([ATP] is essentially constant in skeletal muscle under most conditions). Unfortunately, we have no direct evidence that group B recovered from damages faster than group A. However, if the damages in group B are actually greater, even the same time of recovery from damages would mean that the repair is more intensive in this group.

Group A and B had different numerical force (it was not able to predict in advance how many subjects would fall into each group). However, the statistical analysis performed by us proves that the differences between the groups were statistically significant.

A possible alternative explanation of the [ADP_{free}] recovery undershoot and [PCr] recovery overshoot could be a turning off of all (or most) 'basic' ATP-using processes that keep the cell alive (e.g., protein synthesis, [Na^+]/[K^+] circulation) in the first stage of muscle recovery. However, first, the size of the [ADP_{free}] undershoot/[PCr] overshoot generated in this way would be small (see [18]). Second, it is difficult to imagine how the cell could survive the switching off for at least 30 min of all or most of the ATP-using processes necessary for its basic functioning.

In the spectra presented in Fig. 1, at the end of exercise and in the initial stage of recovery, some signal can be seen (in the phosphomonoesters-PME-spectral region) that comes most probably from sugar phosphates. This suggests accumulation of some glycolytic metabolites (probably mostly glucose-6-phosphate, see, e.g., [46]). The extent and duration of sugar phosphate accumulation seem to be significantly greater in group B than in group A. However, while this phenomenon can contribute to the observed P_i undershoot [47], it is not able to directly account for the PCr overshoot. First, in group B the signal from sugar phosphates evidently gradually vanishes during 30 min of recovery (see Fig. 1b), while the PCr overshoot is still present (this can be seen in Fig. 2 where time courses of [PCr] for the same subjects as in Fig. 1 are presented). Second, the P_i concentration has no direct influence on the CK equilibrium. Third, a decreased concentration of P_i that is an activator (although less important than ADP) of oxidative phosphorylation would be compensated by an appropriate increase in [ADP]. This would mean a decrease in the ATP/ADP ratio, decrease in the PCr/Cr ratio and consequently decrease in [PCr]. Therefore, any accumulation of phosphate groups in glycolytic metabolites would counteract the appearance of the PCr overshoot. Fourth, as is demonstrated in [47], the accumulation of sugar phosphates and the related significant P_i undershoot can appear without any PCr overshoot. Therefore, the accumulation of phosphate groups in glycolytic metabolites by itself cannot be the reason for the PCr recovery overshoot.

However, like the significant decrease in pH in group B observed in our experiment, the greater accumulation of sugar phosphates can be an additional indicator of a stronger direct activation of glycogenolysis during exercise. It was recently observed in [48] that adrenaline activates glycogenolysis in resting anaerobic frog skeletal muscle, which is accompanied by an increase in sugar phosphates and PCr as well as by muscle acidification. Direct activation of glycolysis during muscle work that persists during recovery could potentially contribute to the PCr recovery overshoot through additional ATP production and pyruvate supply to oxidative phosphorylation. However, it has been demonstrated that anaerobic glycolysis essentially does not contribute significantly to PCr resynthesis during muscle recovery in humans, at least under anoxic conditions [39]. Additionally, it must be emphasized that such an effect would be an element of the parallel activation mechanism postulated by us. Glycolysis is a part of the substrate dehydrogenation and, more generally, ATP supply system. The direct parallel activation of glycolysis by some cytosolic factor during skeletal muscle contraction was proposed in [49]. Anyway, both cytosol acidification and sugar phosphate accumulation vanish long before 30 min of recovery when PCr overshoot is still present. For all these reasons, it is unlikely that the discussed mechanism contributes significantly to the PCr overshoot.

The appearance of the [PCr] overshoot in the skeletal muscle may also have some beneficial effect on its maximal power-generating capabilities (for an overview of this point see, e.g., [4, 5, 50–52]). This temporarily increased pool of [PCr] in skeletal muscle may significantly increase the amount of [ATP] resynthesized via creatine kinase reaction during the initial stage of exercise and thus allow maintaining a high power output for a longer period of time while performing maximal sprinting exercise.

In summary, the main finding of this study is that the appearance of the [PCr] recovery overshoot is associated with extensive muscle acidification during recovery (and also with a stronger exercise-related decrease in the phosphorylation potential). Our investigations indicate that the necessary condition of the appearance of the [PCr] overshoot is a significant $[ADP_{free}]$ recovery undershoot ($[ATP]/[ADP_{free}]$ overshoot) caused by some mitochondria-related mechanism, while the size and duration of this phenomenon are co-determined by a creatine-kinase-related mechanism, namely the shift in the apparent equilibrium of CK caused by changes in $[H^+]$ (and $[Mg^{2+}]$). It is suggested, along with the earlier proposal [17, 18], that the $[ADP_{free}]$ undershoot is caused by a slow decay during muscle recovery of the parallel activation of oxidative phosphorylation complexes [22, 23], which is turned on during muscle work. A possible role of this undershoot is an activation, through the elevated $[ATP]/[ADP_{free}]$ ratio the recovery of the exercise-induced disturbances in muscle cell functioning, as well as an activation of different processes in the cell, including recovery of the exercise-induced disturbances in muscle cell functioning as well as RNA and protein synthesis, participating in repairing muscle cells after damages caused by such stress-generating factors as low pH, low ΔG_{ATP}, elevated muscle temperature, free radical production and mechanical stress.

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