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Inorganic Phosphate Is Transported into Mitochondria in the Absence of ATP Biosynthesis: An *in Vivo* ^{31}P NMR Study in the Human Skeletal Muscle

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We used 31-phosphorus magnetic resonance spectroscopy to study *in vivo* the transport of inorganic phosphate (Pi) from cytosol into mitochondria as assessed by the kinetics of Pi recovery during ischemia after aerobic exercise in human skeletal muscle. In all subjects during the first 30 s of ischemia inorganic phosphate showed a marked decrease from the value measured at the end of exercise, whilst phosphocreatine maintained the same value reached at the end of exercise. Our results show that Pi transport from cytosol into mitochondria is active in the absence of ATP biosynthesis and lasts 30 s possibly as a consequence of a decreased pH gradient, due to symport of Pi and H^+ associated with an inactive electron transport chain during ischemia.

Pi is present inside the cell in at least two separate pools: cytosolic and mitochondrial. Pi is transported from cytosol into mitochondria by two different carriers: a specific phosphate carrier (1) and the dicarboxylate carrier (2), the former accounting for about 90% of the over-all transport activity (3). Early experiments by Coty & Pedersen (3) on rat liver mitochondria suggested that the driving force of this process was a pH gradient across the mitochondrial membrane, and showed that inhibitors of respiration and ATP hydrolysis had no effect on Pi transport. Wohlrab & Flowers later showed (1), with experiments on liposomes incorporating the carrier protein, that Pi transport is in fact pH gradient-dependent.

To study Pi transport from cytosol into mitochondria *in vivo,* inorganic phosphate must first be accumulated in the cytosol, a condition easily obtained in the skeletal muscle just by exercising. During work, phosphocreatine (PCr) decreases and Pi accumulates stoichiometrically, both being quantitatively correlated with the intensity of the work performed. During recovery from exercise, [Pi] decreases and [PCr] increases back to their respective resting levels, Pi being transported into mitochondria to allow the biosynthesis of ATP, and PCr being regenerated by the creatine kinase reaction (4).

Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) is the choice method to study Pi transport *in vivo* as, in the experimental conditions of *in vivo* measurements, it reveals only the free or rapidly-exchanging metabolites in the cytosol, while mitochondrial phosphorus-containing metabolites are MR-invisible (5). Therefore the kinetics of Pi recovery from muscular exercise is a reliable *in vivo* index of Pi transport from cytosol into mitochondria (6).

We report the results of an *in vivo* study performed in experimental conditions where ATP biosynthesis was inhibited to define whether ATP biosynthesis is a requirement for Pi transport from cytosol into mitochondria in human skeletal muscle.

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MATERIALS AND METHODS

^{31}P-MR spectroscopy. Spectroscopic measurements were performed according to the quantification and quality assessment protocols defined by the EEC Concerted Research Project on ''Tissue Characterisation by MRS and MRI'', COMAC-BME II.1.3 (7). MR spectra were acquired by a G.E. 1.5 T Signa System and a surface coil supplied by G.E. The stimulation-response sequence was repeated every 5 s. All studies were performed on gastrocnemius muscle by placing the surface coil directly on the skin. After optimising magnetic field homogeneity (FWMH 0.25–0.35 ppm) 60 transients were accumulated during rest (5 min). During exercise, data were collected for one min (12 FIDs) for each level of work. During ischemic recovery 2-FID data blocks (10 s) were recorded for 120 s. After the cuff was released, during aerobic recovery 2-FID data blocks were recorded for 60 s, while longer time blocks were collected during the following 4 min. The accumulated spectra were transferred to the data station and processed using a 4 Hz line broadening and manual phasing. The limits of all the peaks were marked manually on each spectrum after phasing. The computer was used to integrate the areas between the limits as previously reported (8). Intracellular pH was calculated from the chemical shift of Pi from PCr (9). The chemical shift was carefully determined from the centroid of the PCr peak to the centroid of the Pi peak.

Subjects, exercise, and recovery protocols. Ten healthy individuals (all males) aged 17–38 volunteered for this study. All subjects performed in-magnet isokinetic exercise by rhythmically pressing a pedal connected with a pneumatic ergometer (8). The pressure in the ergometer was increased every minute by small increments according to the ramp protocol exercise (10). As soon as the last minute of exercise was completed, a cuff, previously positioned around the thigh, was quickly inflated by compressed air to a pressure well above the systolic pressure to ensure ischemic conditions. Contractions were continued for another 10 s to allow the residual oxygen present in the tissue to be used up, then one 2-FID data block was recorded to be considered ''zero time'' of the ischemic recovery and exercise stopped. Ischemia was maintained for other 2 min, then pressure was released to allow aerobic recovery.

RESULT

All subjects were asked to stop exercise at different degrees of metabolic activation, to accumulate different amounts of Pi in the cytosol. Figure 1 reports two 2-FID spectra recorded from the gastrocnemius muscle of subject # 6 collected at the end of exercise (upper spectrum) and from 20 to 30 s of ischemic recovery (lower spectrum). The Pi signal was remarkably decreased after 30 s of ischemic recovery, following aerobic exercise, while the intensity of PCr and ATP signals were substantially unchanged. Figure 1 also reports the patterns of Pi, PCr, ATP and the sum of Pi+PME (phosphomonoesters) during ischemic recovery recorded from the same individual. The PME peak under these experimental conditions mainly represents phosphorylated monosaccharide intermediates of the glycolytic pathway.

All subjects showed the same behaviour, i.e. a major decrease of Pi during the first 30 s of ischemic recovery (Table 1) paralleled by unchanged ATP and PCr signals. Cytosolic pH at the end of exercise varied from 6.56 to 6.88 in the different subjects according to the degree of metabolic activation and did not show any substantial changes during ischemic recovery. Pi recovery in all cases was best fitted by a monoexponential function and the rate of Pi recovery was assessed by the time constant of the fitting equation. Pi decrease was calculated by the difference between the Pi value measured at zero time of ischemic recovery and its asymptotic value defined by the monoexponential equation. The mean value of Pi decrease from zero time was 22.8% $\pm$ 9.2 (SD), and the degree of Pi decrease in each individual was independent of the degree of metabolic activation, as assessed by PCr depletion at the end of exercise, while the rate of Pi recovery was inversely correlated to the cytosolic pH reached during ischemia.

The sum of Pi plus PME also showed a major decrease in all subjects with a pattern well described by the same monoexponential function describing Pi recovery (Figure 1). However, it was not possible to quantitate the exact degree of Pi+PME decrease in all subjects because of the low signal-to-noise ratio of the PME peak.

DISCUSSION

We designed our experimental protocol to reproduce *in vivo* the experimental conditions showing that Pi transport from cytosol into mitochondria *in vitro* is driven by a pH gradient

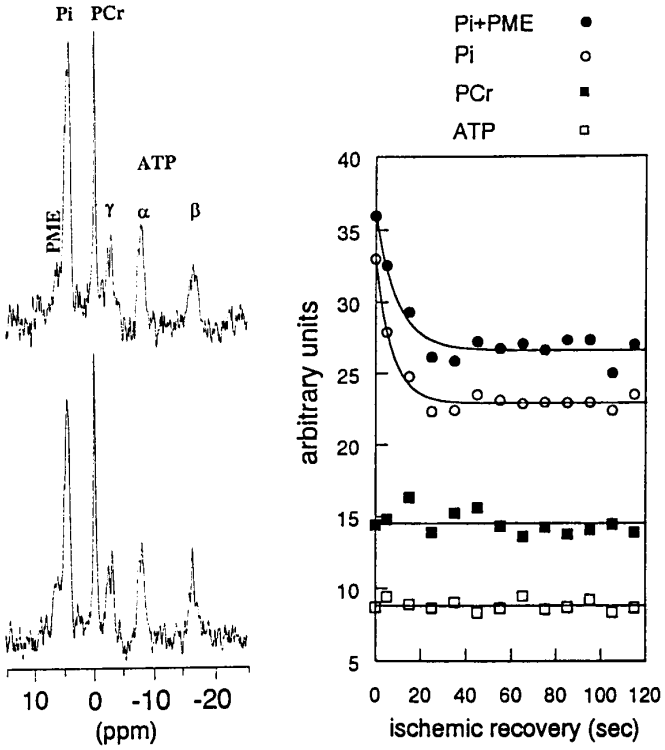

FIG. 1. Two-FID (10-sec) ^{31}P MRS spectra from gastrocnemius muscle of a healthy male volunteer (case # 6) collected at the end of exercise (upper spectrum) and at 20–30 s of ischemic recovery (lower spectrum). On the righthand side is reported the pattern of ischemic recovery of ATP, PCr, Pi, and the sum of Pi+PME following aerobic exercise from case # 6. Pi decrease was 25% of Pi value measured at zero time.

between the two compartments (1) and is independent of mitochondrial respiration (3). We reasoned that: aerobic exercise generates a pH gradient between mitochondrial matrix and cytosol *in vivo,* and the absence of oxygen supply during ischemia blocks ATP biosynthesis. Our results show that Pi transport from cytosol into mitochondria, in the human skeletal muscle *in vivo,* is active during ischemic recovery following aerobic exercise in the absence of ATP biosynthesis, possibly at the expense of the pre-existing pH gradient. The rate of Pi transport during ischemia was also directly dependent on cytosolic pH, as previously found during aerobic recovery (6).

It has been shown that in the human skeletal muscle in the absence of oxygen there is total absence of PCr and Pi recovery (11, 12), both being constant during ischemic recovery, a result that binds Pi transport from cytosol into mitochondria to PCr biosynthesis. We also

TABLE 1

Pi Recovery during Ischemia after Aerobic Exercise in the Calf Muscle of Ten Healthy males. Pi Decrease was Calculated by the Difference between the Pi Value at Zero Time of Ischemic Recovery and its Asymptotic Value Defined by the Equation Best Fitting the Experimental Points and Reported as Percentage of Pi at Zero Time (ΔPi%)

Case #	1	2	3	4	5	6	7	8	9	10
Age	17	19	20	21	26	29	31	32	33	38
ΔPi%	−15	−41	−11	−30	−12	−25	−25	−21	−19	−29

failed to find any Pi changes during ischemic recovery in the same experimental conditions (data not shown). However, in these studies ischemic recovery was performed after ischemic exercise, thus precluding a correct pH gradient between cytosol and mitochondria driving Pi transport. Moreover, had Pi recovery occurred at the beginning of ischemia, it would have been masked by simultaneous Pi accumulation from ATP breakdown during exercise.

It has been suggested that during recovery Pi is trapped in the glycogenolytic pathway by a direct exchange between Pi and phosphomonoesters (PME) (13). Under our experimental conditions, the monoexponential pattern of Pi+PME decrease argues against this interpretation, although we cannot exclude that some Pi is used by an active glycogenolytic pathway.

Pi transport during ischemic recovery lasted approximately 30 s in all individuals. We cannot offer any straightforward explanation for this. Our interpretation is that the pH gradient is reduced after a short time due to symport of Pi and H^+ (14) and/or the mitochondrial Pi pool, is already replenished after approximately 30 s.

Our results also define a new experimental condition that allows the assessment of Pi post-exercise recovery independently of PCr recovery. This protocol will be extremely useful to study specific muscle diseases in which Pi transport is impaired due to an altered cellular ionic traffic as in Becker patients (15) and asymptomatic carriers of Duchenne and Becker muscular dystrophy (10, 15).

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