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Levels of Energy-Related Metabolites in Intact and Isolated Perfused-Superfused Rat Skeletal Muscles

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Summary

Adenosine 5'-triphosphate (ATP), phosphocreatine (PCr), creatine (Cr), inorganic phosphate (Pi), lactate (LAC), pyruvate (PYR) and glycogen as glucose (GLU) were determined and free adenosine 5'-diphosphate (ADP) was calculated from ATP:creatine phosphokinase (CPK) reaction in the gracilis muscle of cold-acclimated rats *in vivo*, and in completely isolated muscles under medium perfusion and superfusion *in vitro*, using the freeze-clamping method. The mean *in vivo* levels (μmol/g w.w.) were: ATP 4.8, PCr 12.0, Cr 7.8, Pi 1.6, LAC 1.6, PYR 0.09, GLU 22.9, ADP 0.62×10^{-3}. Isolation of the muscle (about 11 min of anoxia followed by perfusion in the air with a high pO_2 medium) decreased macroergic phosphate levels (ATP 3.0 , PCr 8.3). In isolated muscles perfused with a high pO_2 medium (99 kPa O_2, perfusion rate 70 μl/min) and simultaneously superfused with a low pO_2 medium (6.2 kPa O_2, 2.3 ml/min) at 28 °C *in vitro* the levels of metabolites were (μmol/g w.w.): ATP 3.1, PCr 8.5, Cr 5.6, Pi 0.9, LAC 2.1, PYR 0.19, GLU 6.6, ADP 0.44×10^{-3}. The mean steady oxygen uptake of the isolated muscle was 97 nmol $O_2 \times min^{-1} \times g^{-1}$ w.w. Thus, the levels of macroergic phosphates and their derivatives are lower after isolation and perfusion of the muscle, but the creatine charge [PCr]/([PCr]+[Cr]] remains stable (0.61 *in vivo* versus 0.60 in the isolated muscle). This indicates that the steady-state and high energy status of the isolated perfused-superfused gracilis muscle is maintained.

Key words

Skeletal muscle – Energetics – Metabolites – Flow-dependent respiration

Introduction

In the isolated perfused-superfused, relaxed gracilis muscle from cold-acclimated rats, the oxygen consumption and heat production rates increase in a flow-rate dependent manner as the medium flow rate increases. However, the comparison of indirect and direct calorimetry revealed a discrepancy between both energy fluxes, i.e. oxygen consumption versus heat production, the former being higher in the range of perfusion rates from 50 to 150 $\mu l \times min^{-1}$ (Chinet and Mejsnar 1989).

Basically, this phenomenon can have two alternative explanations. Either the flow-rate effect on metabolism means a storage recuperation of an exhausted muscle which is out of the steady state, or it represents a flow-dependent component of the steady-state energy metabolism.

If the latter alternative is valid, according to the non-linear thermodynamic model (Prigogine 1967), the increase in the perfusion rate increases the total affinity of oxidative phosphorylation reactions. A propagation of the chemical potential of reactants along the sequence of reactions would result in the elevated concentration of PCr and ATP with increasing perfusion rate (Mejsnar 1991). The simultaneous increase of PCr and ATP measured by ^{31}P-nuclear magnetic resonance spectroscopy (Mejsnar *et al.* 1992) is in favour of this.

To verify one of the alternatives considered above, the metabolic states of the gracilis muscle under investigation must be characterized at various phases of its preparation. The aim of this study was to determine the effects of isolation, perfusion in the air, and simultaneous perfusion and superfusion *in vitro* on the

levels of some selected metabolites of the gracilis muscle from cold-acclimated rats. The intact, freeze-clamped muscles from anaesthetized animals served as controls.

Methods

Male rats of the Wistar strain weighing 380 to 500 g were held individually in cages for at least 3 weeks in a cold room (7 °C) under artificial light conditions (12 hours of light, 12 hours of dark). They were fed a standard rat diet and water *ad libitum*.

An incision of the skin on the ventromedial part of left thigh was made under general anaesthesia, muscles were exposed and a substantial part of *m. gracilis cranialis* was freeze-clamped with tongs precooled in liquid nitrogen. The rest of the tissue was cut off and the frozen muscle was stored in liquid nitrogen until analyzed (usually the same day). The rat was then sacrificed under anaesthesia. Mean time from the skin incision to the freeze-clamping of the muscle was 5.5 s (4.5 to 6.5 s). This protocol represents a preparation of the first (control) group of muscles *in vivo*.

The second experimental group of six animals was used to clarify the changes in metabolite levels associated with the process of muscle isolation. The procedure was essentially the same as in the third group (see below) with the exception of the final perfusion-superfusion period in the chamber. The muscle was isolated, rinsed through with heparin, perfused in the air with a high-pO_2 medium, excised, mounted in a frame and freeze-clamped after slight drying and analyzed for the metabolites.

In the third experimental group (8 animals) the muscle was vascularly isolated, cannulated, rinsed through with heparin, and perfused with Krebs' bicarbonate buffer without phosphate that was oxygenated with an oxygen-rich gas mixture (94.8 % O_2, 5.2 % CO_2; high-pO_2 medium). Glucose (5 mM final concentration) was added as an external substrate. A period of anoxia was an inevitable part of the vascular isolation (lasting 11 min on the average). The muscle was then excised and mounted in a metal frame at physiological length. This period when the muscle was perfused in the air lasted for about 21 min. Finally, the muscle was placed in a small glass chamber at a stable temperature of 28 °C. Simultaneously, the muscle was perfused with the high-pO_2 medium (mean pO_2 99 kPa) at the rate 70 μl x min^{-1} and superfused with the low-pO_2 medium (pO_2 6.2 kPa, flow rate 2.3 ml x min^{-1}). This is necessary to ensure an open capillary bed of the muscle and low perfusion pressure and thus to avoid swelling of the muscle (Chinet and Mejsnar 1989). The perfusion pressure was monitored (blood pressure sensor and analyzer, Tesla, Czech Republic) and oxygen consumption was measured continuously using an oxygen polarographic electrode and analyzer (Radiometer Copenhagen, Denmark). The muscle was perfused and superfused at constant rates for about one hour (63 min on the average). Then the chamber was opened, the muscle slightly dried with a piece of cellulose tissue and freeze-clamped with precooled metal tongs. The mean time from opening to freezing was 17 s.

Analyses of metabolites

A sample of frozen muscle tissue was quickly weighed and pulverized in a mortar under liquid nitrogen in a cold room (3 °C). The powder was quantitatively transferred to a small test tube filled with cold perchloric acid and ethanol (2 % $HClO_4$, 20 % ethanol). The exact amount of the sample was estimated from the difference of its weight before and after addition of the tissue into the test tube. The ratio $HClO_4$: tissue was adjusted to 5 μl : 1 mg. The thawing sample was homogenized and centrifuged (10 000 x *g*, 10 min, cold). The supernatant was divided into two parts for analyses of adenosine 5'-triphosphate (ATP), phosphocreatine (PCr), lactate (LAC), pyruvate (PYR), and inorganic phosphate (Pi) in one sample, and creatine (Cr) in the other. The pH in the first sample was adjusted by a saturated solution of potassium carbonate to pH 5–6, the Cr analysis was performed at pH 7 (samples neutralized with a potassium carbonate triethylammonium buffer). After neutralization and centrifugation, the aliquot parts of the supernatant (from 40 to 300 μl) were used for analyses of the metabolites. Standard optic enzymatic tests (Bergmeyer 1984 and 1985) measuring changes in the absorbancy of nicotinamideadenine dinucleotides at 366 or 334 nm wavelength (photometer Eppendorf with a line recorder) were used for analyses of ATP, PCr, Cr, LAC and PYR. Pi was measured by the phosphomolybdenate method. Glycogen was extracted from a separate sample of the frozen muscle tissue according to Good *et al.* (1933). After acid hydrolysis (5 N H_2SO_4 at 105 °C for 2 hours) glycogen was analyzed as glucose using the glucose-oxidase commercial kit (Oxochrom-Glucose, Bio-La-Test). The tissue level of free adenosine 5'-diphosphate (ADP) was calculated from the ATP:creatine phosphokinase (CPK) reaction, using the apparent equilibrium constant $K'_{eq} = 5 \times 10^{-10}$ M and assuming cellular pH equals 7 (McGilvery and Murray 1974, Kushmerick 1983):

$$[ADP] = \frac{K'_{eq} \times [ATP] \times [Cr]}{[PCr] \times [H]^{0.8}}$$

All enzymes, substrates and chemicals (except of the Oxochrom-Glucose test) were from Böhringer, Germany.

Results of all analyses were compared by the unpaired Student t-test at the significance level 0.05.

Results

The tissue levels of metabolites from the three experimental groups are summarized in Table 1. A comparison of the intact control muscles with isolated muscles perfused-superfused at a constant rate (70 μl/min and 2.3 ml/min respectively) for 63 minutes at 28 °C revealed a significant decrease in the content of ATP, PCr, Cr, Pi, and glycogen. Changes in lactate and pyruvate as well as in free ADP concentrations were not significant. It is obvious from Table 1 and Fig. 1 that the fall in the levels of some metabolites is a consequence of muscle isolation and subsequent manipulation. A comparison of the second and third group shows no significant difference between the two groups except that glycogen decreases significantly during *in vitro* perfusion-superfusion period.

Table 1
Levels of metabolites in muscles of the three experimental groups

Group	ATP	PCr	Cr	Pi	LAC	PYR	ADP x10^{-3}	GLU
Controls *in vivo*	4.8	12.0	7.8	1.6	1.6	0.09	0.62	22.9
± S.E.M. (n = 7)	0.33	0.77	0.33	0.14	0.11	0.03	0.06	2.1
Isolated, perfused in the air	3.0**	8.3**	6.1	1.2	2.3	0.07	0.44	26.6
± S.E.M. (n = 6)	0.22	0.41	0.73	0.12	0.36	0.01	0.07	4.2
Isolated, perfused and superfused	3.1**	8.5*	5.6*	0.9**	2.1	0.19	0.44	6.6**
± S.E.M. (n = 8)	0.22	0.99	0.70	0.14	0.71 (n=3)	0.15 (n=3)	0.07	2.9

*The results are expressed as means ± S.E.M. in µmol/g wet weight. Significant differences from the controls: * = $p<0.05$; ** = $p<0.01$*

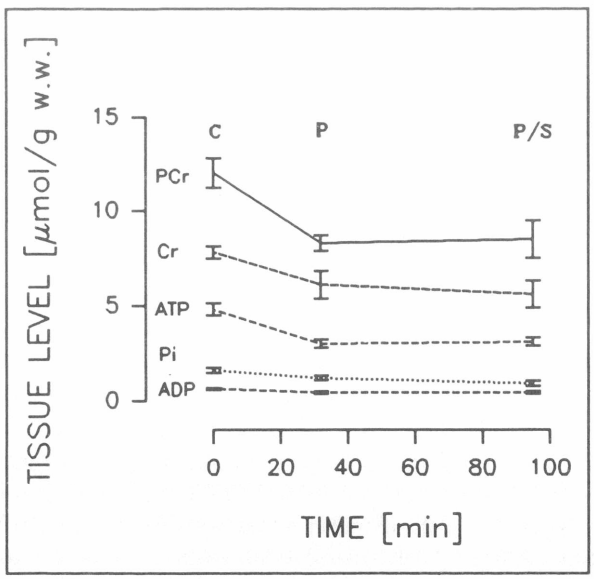

Fig. 1
Changes in metabolites characterizing ATP hydrolysis and the CPK reaction in skeletal muscles of the three experimental groups. C – controls *in vivo*, n = 7; P – isolated muscles perfused in the air, n = 6; P/S – isolated muscles perfused-superfused *in vitro*, n = 8. The results are plotted in the time scale of the experiments.

A ratio of [ATP]/[ADP] and a creatine charge [PCr]/([PCr]+[Cr]) that can characterize the cellular system of macroergic phosphates in the muscles are summarized in Table 2. No significant difference among the groups was found in the ATP/ADP ratio or in the creatine charge.

Table 2
The creatine charge and the ATP/ADP ratio in muscles of the three experimental groups

Group	Creatine charge [PCr]/([PCr]+[Cr])	Ratio [ATP]/[ADP]	
Controls *in vivo*	0.61	7.8	x10^3
± S.E.M. (n = 7)	0.01	0.4	
Isolated, perfused in the air	0.58	7.2	x10^3
± S.E.M. (n = 6)	0.02	0.6	
Isolated, perfused and superfused	0.60	8.9	x10^3
± S.E.M. (n = 8)	0.04	2.0	

The perfusion pressure and oxygen consumption of the muscle *in vitro* indicated the vitality of the preparation. The average perfusion pressure during perfusion in the air was 19.2±2.3 kPa but, after exposing the muscle to the low-pO_2 superfusion medium in the chamber, the perfusion pressure fell in 5 min to 15 kPa. In the next 15 to 20 min it further decreased and then became stabilized (9.1±1.4 kPa). Simultaneously, the oxygen consumption reached a stable level. The mean oxygen consumption in the muscles at the end of perfusion-superfusion period was 97±12 nmol $O_2 \times min^{-1} \times g^{-1}$ w.w. The average weight of muscles of this group was 405±22 mg. As the mean oxygen delivery was 82.5±3.6 nmol O_2/min to the muscles and 64.9±8.5 nmol O_2/min to the metabolic chamber, and the actual oxygen uptake by the muscles was 38.4±5.5 nmol O_2/min, we can state that the oxygen supply to the system was sufficient.

Discussion

Considerable variability exists in the reported data on the levels of cellular metabolites in muscles of different animal species and even in different muscles of the same species (e.g. Marsh *et al.* 1992, Van der Meulen *et al.* 1992). The results apparently depend on the muscle and the prevailing type of muscle fibres in different muscles.

Our values of ATP and PCr measured *in vivo* (4.8 and 12.0 μmol/g w.w., respectively) are close to those that have been found in the perfused-superfused muscle *in vitro* by ^{31}P-NMR spectroscopy (4.5 and 14.3 μmol/g w.w., respectively) (Mejsnar *et al.* 1992). There were, however, small differences in the procedure the same muscle was manipulated (shorter adaptation time of animals, different perfusion/ superfusion medium and the time schedule during the muscle isolation). This might be responsible for the slight differences in the levels of ATP and PCr in the perfused-superfused muscle reported here in comparison to values found by Mejsnar *et al.* (1992). Another possibility is an artifactual PCr hydrolysis (or ATP hydrolysis) during freezing. This explanation was offered by Kushmerick and Meyer (1985) who found a similar discrepancy between NMR spectroscopy and chemical analysis in PCr and Pi in rat skeletal muscles.

We consider, on the basis of the close similarity of our methods and results with those of Marsh *et al.* (1992), that our values are correct. The possible differences in the content of glycogen or other metabolites could be due to the acclimation to cold of our experimental animals. The presence of non-shivering thermogenesis in skeletal muscles of cold-acclimated rats (Janský and Hart 1963, Mejsnar and Mejsnarová 1971, Grubb and Folk 1976) should be consistent with a more effective oxidative metabolism of substrates in the resting muscle. The increased demand for oxidative pathways may change the proportion of fast-glycolytic : slow-oxidative fibres, with a consequent decrease of the PCr/Cr buffering energetic system and glycogen.

A comparison of the control group with isolated muscles clearly shows a decrease of macroergic phosphates levels. This is certainly no surprise as the 11 min anoxia is quite a long period to induce such changes. On the other hand, one would expect a proportional increase in Cr (and Pi or ADP as well). We have however found a decrease in Cr and Pi and no significant change in ADP. Furthermore, neither LAC nor PYR were changed significantly. Clearly one has to suggest that during vascular occlusion and subsequent anoxia the muscle is restricted to glycolysis and lactate levels should increase. This would lead to the lowering of pH. As creatine is unstable at lower pH, a non-enzymatic conversion to creatinine may occur, which could lead to the observed decrease in the total creatine content. Increased level of AMP (the product of adenylate kinase reaction maintaining a low level of cellular ADP and a high phosphorylation potential) would result in its increased deamination (with a possible release of inosine 5'-monophosphate out of the cell), which may help in buffering of cellular pH (Herniö and Saris

1967). This would improve pH, and in the following perfusion period, lactate but also creatinine would be washed out of the muscle. As a result, the final change of lactate in the tissue is nonsignificant and the total Cr decrease occurs. The content of lactate in the medium released by the muscle was not monitored, but the release of lactate was documented in the vascularly isolated and artificially perfused *gracilis muscle* by Kolář (1977). Because we did not measure the total levels of ADP and AMP, it is possible that their concentrations were changed as a consequence of the isolation of the muscle.

Another cause of the simultaneous decrease in the concentrations of metabolites after isolation of the muscle could be the swelling of the tissue during perfusion with an artificial medium. This cannot be excluded as we did not use albumin to avoid scumming of the medium.

The most surprising fact was that there was no change in the glycogen content between the controls and isolated muscles perfused in the air. There is no satisfactory explanation of this finding at present, except that there might be a difference in the glycogen content between the two groups at the beginning, perhaps due to differences in body weight or age of the animals.

The content of glycogen in muscles is subjected to variations. In the rat *gastrocnemius-plantaris* muscles, the glycogen content (resting, control value) was 29.5 μmol/g wet weight (Hespel and Richter 1992). Our control values are slightly lower. A substantial increase in the glycogen content in muscles was induced by a higher glucose supply in the food by these authors, whereas a significant decrease was found in animals with restricted food intake and forced to exhaustive muscular work a day before the analysis. This indicates that muscle glycogen levels are liable to changes.

The fall of the glycogen content in muscles perfused-superfused *in vitro* (group 3) seems to be a logical consequence of the recovery from anoxia and the subsequent maintenance of the stable metabolic rate. We used exogenous glucose as a possible substrate. Nevertheless, inner stores of chemical energy may be more preferable. In this connection, possible functional heterogeneity of the muscle with respect to the delivery of substrates and oxygen to muscle fibres may also be important (Chinet 1990).

It is not possible to make a quantitative energy balance analysis of the preparation on the basis of available limited data but we can evaluate the energy status of the muscle. The energy status of the macroergic phosphates can be expressed in different ways, e.g. the ratio of ATP to free ADP, the muscle phosphorylation potential [ATP]/[ADP][Pi], the energy charge ([ATP]+0.5[ADP])/([ATP]+[ADP]+[AMP]), or the creatine charge [PCr]/([PCr]+[Cr]). As the analysis of freeze-clamped muscles gives total cellular concentrations of ADP (and AMP), whereas only free ADP is important for characterizing the energy of splitting of ATP in cells, we calculated the free levels of ADP from the CPK reaction as presented in the Methods. It is assumed that the CPK reaction is near to equilibrium in the skeletal muscle and PCr and Cr are unbound (Kushmerick 1983); therefore the free levels of ADP may be calculated.

From the data of Marsh *et al.* (1992) the ratio of ATP/ADP can be calculated, giving values from 7.04 to 10.94 in different muscles. Our results are three orders of magnitude higher and lie between the ratios 3.66×10^2 calculated from Schulte *et al.* (1992) and 2.5×10^4 calculated from Kushmerick (1983).

Unfortunately, in our case, the muscle phosphorylation potential, the energy charge, or the ratio ATP/ADP were of little significance in deciding the stability of the energetic metabolism of the muscle. This is because the free cytosolic ADP was not measured independently of ATP, PCr, and Cr, but was derived from the assumed equilibrium of the CPK reaction. But as PCr and Cr were analysed separately, the ratio of PCr/total creatine (creatine charge) gives some insight into the cellular status of macroergic phosphates.

Using the creatine charge we did not find any significant differences between the three groups of muscles. The result is close to the value reported by Schulte *et al.* (1992) for resting trout white muscle (0.49).

The stability of the creatine charge in isolated perfused-superfused muscles *in vitro* indicates that after the isolation, despite lowering of the total capacity of adenylate nucleotides and the PCr/Cr system, the muscle is (concerning energetics) in good condition with a high energy status. The stable oxygen uptake and perfusion pressure support this conclusion.

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Reprint Requests

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