Preservation of Mitochondrial Functions in Human Skeletal Muscle during Storage in High Energy Preservation Solution (HEPS)

Daniela Skladal[a], Wolfgang Sperl[a], Reinhard Schranzhofer[a], Martin Krismer[b], Erich Gnaiger[c], Raimund Margreiter[c], Frank N. Gellerich[c]

[a]Department of Pediatrics, [b]Orthopaedic Clinic, [c]Department of Transplant Surgery, Clinical and Interdisciplinary Bioenergetics; University Hospital of Innsbruck, A-6020 Innsbruck, Austria

Introduction

Several enzyme systems have to be examined for the diagnosis of mitochondrial myopathies, including the complexes of respiratory chain, the pyruvate dehydrogenase complex and enzymes of the Krebs cycle [1-4]. These investigations are carried out on frozen biopsy specimens [3,4]. For functional characterization of mitochondria in human tissues measurements have to be performed immediately after obtaining the biopsy specimens. Large amounts of tissue are required (>500-1000 mg) for investigation of isolated mitochondria [1-4]. This imposes a serious limitation for ethical reasons particularly in pediatric patients. In addition, there is the danger of artifacts since mitochondria separated from their cellular environment are fairly unstable.

Recent investigations of saponin-skinned fibers from human heart and skeletal muscle introduced an alternative approach to characterize mitochondrial functions in biopsies [5-8]. Permeabilized fibers have the advantage that the mitochondria remain in a more physiological surrounding. It is even possible to characterize the mitochondrial function in as little as 30 mg muscle tissue obtained by needle biopsy when high resolution respirometry [9] is applied in combination with multiple substrate-inhibitor titrations [8]. With the aid of these techniques mitochondrial defects have been functionally defined in several patients [6-8].

The stability must be defined of mitochondrial functions during storage of muscle biopsies for several reasons. *(1)* A respirometric experiment lasts for 30-60 min. Although two replicate measurements can be carried out simultaneously in the two-channel *Oxygraph*, serial replicates can only be interpreted appropriately if the samples are sufficiently stable for hours. *(2)* More detailed analyses can be specifically designed and performed on the same biopsy, on the basis of initial respirometric information. *(3)* Long-term stability for >24 h would decisively increase the accessibility of this diagnostic method. It would be possible to send biopsy samples to clinical bioenergetics centers for investigation. *(4)* Information on long-term storage in skeletal muscle can provide insights into the mechanisms of mitochondrial damage during cold hypoxic preservation and thus lead to an improved storage method for hearts used for transplantation.

A medium with good preservation properties for the storage and permeabilization of muscle fibers has been described [5]. In particular, this medium contains 5 mM ATP and 15 mM creatine phosphate and may be appropriately called High Energy Preservation Solution, HEPS. In the present study we characterize the stability of mitochondrial function in skinned fibers from human skeletal muscle up to 30 hours.

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METHODS

Biopsy: Muscle specimens were obtained from *m. quadriceps* (*m. vastus lateralis*) by open biopsy of patients (40-82 years) who underwent routine orthopaedic surgery under general anaesthesia.

Isolation, cold storage and permeabilization of muscle fibers: Muscle fibers were transported to the laboratory in 0.9 % NaCl at 4 °C. For dissection with small needles the fibers were put into a droplet of HEPS (10 mM EGTA-CaEGTA buffer, free Ca^{2+} concentration 0.1 μM, 9.5 mM $MgCl_2$, 3 mM KH_2PO_4, 20 mM taurine, 5 mM ATP, 15 mM creatine phosphate, 49 mM K^+-MES, 29 mM imidazole-HCl, pH 7.1). The fibers were stored for up to 30 h in this medium on ice. For subsequent permeabilization fibers were put into 1 ml HEPS plus 50 μg saponin and gently stirred for 30 min at 4 °C. The fibers were washed two times in incubation medium to remove the saponin and the adenine nucleotides.

Respirometric measurements: Fiber bundles were weighed after removing the adherent liquid by cellulose. Fibers of about 5 to 15 mg were measured in chamber volumes set at 1.5 ml, using an OROBOROS® *Oxygraph* (Anton Paar, Graz, Austria). The respiratory measurements were performed at 30 °C in an incubation medium consisting of 75 mM mannitol, 225 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 0.5 mM Na_2EDTA, 5 mM $MgCl_2$, 1 mg/ml BSA, 20 mM Tris-HCl, pH 7.4. Glutamate (10 mM) plus malate (2 mM) or pyruvate (10 mM) plus malate (2 mM) were used as mitochondrial substrates. The oxygen concentration at air saturation of the medium was considered to be 200 nmol O_2/ml at 95 kPa barometric pressure. Weight-specific oxygen flux [nmol O_2/(s·mg wet weight)] was calculated as the time derivative of oxygen concentration (DATGRAF 2.2 Analysis Software, OROBOROS®).

RESULTS AND DISCUSSION

The mitochondria of muscle fibers remain functionally intact after 6.5 and 29 hours of cold storage, as judged by the criteria shown in Fig. 1 (compare left and right panel). Without substrates the respiratory rate of the permeabilized fibers is negligibly low. This indicates the lack of endogenous substrates for oxidative phosphorylation. After addition of substrates (Glu) the oxygen flux increased. This endogenous rate of respiration might be due to different mechanisms which can be discriminated by further titrations. *(1)* The presence of adenine nucleotides (AdN) which are highly concentrated in the HEPS medium and were not completely washed out after permeabilization. *(2)* ATP splitting activities in the extramitochondrial space of the fibers. *(3)* Uncoupled respiration, which is ruled out by the observation of a low state 4 respiration in the presence of atractylate (Atr), when AdN transport into the mitochondria is inhibited. Oxygen flux was high after adding 1 mM ADP (state 3), and after uncoupling by FCCP.

Respiration rates are plotted as a function of storage time in Fig. 2. Only minor (insignificant) changes of mitochondrial function occurred during cold storage up to 30 h in any state of mitochondrial activity. Similar results were obtained with pyruvate plus malate as substrates (data not shown). These results indicate that mitochondrial functions are well preserved in HEPS medium.

In some experiments (Fig. 1, left panel) non-stationary (decreasing) rates of respiration were observed under endogenous and state 3 conditions. The linear decline of oxygen flux (second time derivative) at high ADP does not change sig-

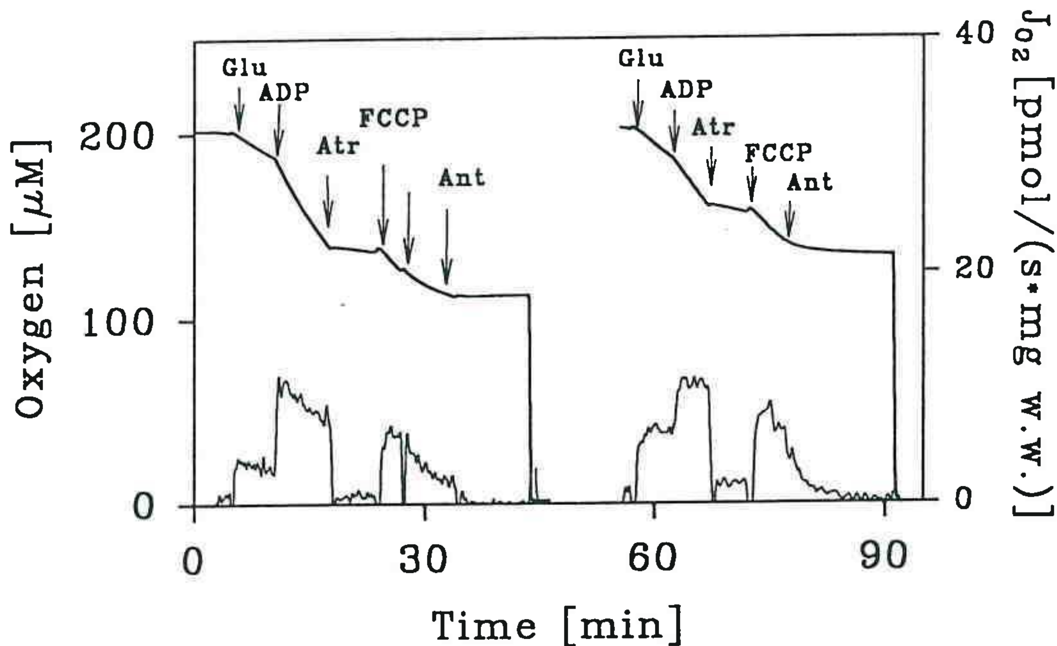

Fig. 1. Typical oxygraph traces of permeabilized muscle fibers (*m. vastus lateralis*) after different storage times in HEPS, showing oxygen concentration [μM O_2], upper traces, and oxygen flux [pmol/(s·mg w.w.)], lower traces. Left panel: measurement after 6.5 h storage, 8.6 mg w.w. Right panel: measurement after 29 hour storage; 13 mg w.w. Additions: Glu, 10 mM glutamate plus 2 mM malate; ADP, 1 mM ADP; Atr, 50 μM atractylate; FCCP 10 μM FCCP; Ant, 10 μM antimycin A; Dit, a few mg dithionite for zero calibration.

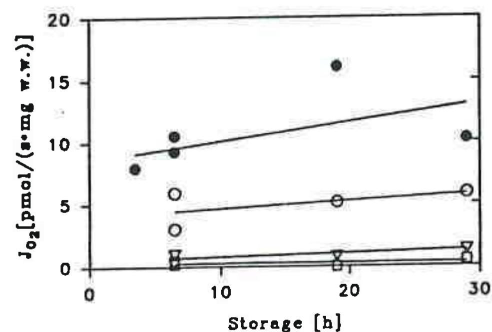

Fig. 2. Rates of respiration in permeabilized muscle fibers using glutamate plus malate as substrates as a function of time of storage in HEPS, showing a representative experiment and various states (cf. Fig. 1). Endogenous (○); state 3 (●); state 4 (▿); antimycin A (□). Lines are the linear regressions. Slopes are not significantly different from zero.

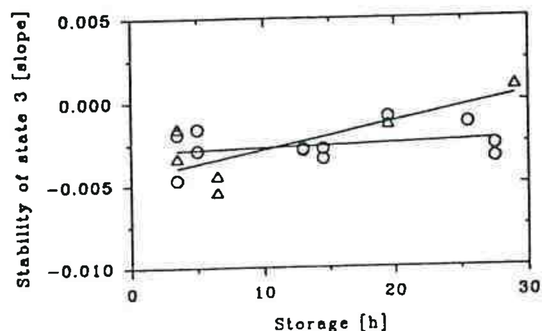

Fig. 3. Stability of state 3 respiration (slope of oxygen flux versus time) as a function of cold storage time in HEPS. Data from a typical experiment, with glutamate plus malate (△), and pyruvate plus malate (○). The difference in the regression lines is not significant at a probability level of 0.05.

nificantly during the time of cold storage (Fig. 3). The reason for the non-stationary rates of respiration in some permeabilized fibers remains unclear. Product inhibition might occur either by ATP or Krebs cycle products which accumulate in the mitochondrial microcompartment.

CONCLUSION

Absolute levels and stability of oxygen flux were used as indicators for mitochondrial integrity. The main mitochondrial functions remained preserved in HEPS-medium during 30 h storage of dissected muscle fibers. Such a period of time is sufficient for sending biopsy specimens to diagnostic centers. This increases extensively the accessibility of the respirometric method for the diagnosis of mitochondrial diseases and injury.

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