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Cryopreservation of mitochondria and mitochondrial function in cardiac and skeletal muscle fibers

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Abstract

Long-term preservation of muscle mitochondria for consequent functional analysis is an important and still unresolved challenge in the clinical study of metabolic diseases and in the basic research of mitochondrial physiology. We here present a method for cryopreservation of mitochondria in various muscle types including human biopsies. Mitochondrial function was analyzed after freeze–thawing permeabilized muscle fibers using glycerol and dimethyl sulfoxide as cryoprotectant. Using optimal freeze–thawing conditions, high rates of adenosine 5′-diphosphate-stimulated respiration and high respiratory control were observed, showing intactness of mitochondrial respiratory function after cryopreservation. Measurement of adenosine 5′-triphosphate (ATP) formation showed normal rates of ATP synthesis and ATP/O ratios. Intactness of the outer mitochondrial membrane and functional coupling between mitochondrial creatine kinase and oxidative phosphorylation were verified by respiratory cytochrome *c* and creatine tests. Simultaneous confocal imaging of mitochondrial flavoproteins and nicotinamide adenine dinucleotide revealed normal intracellular arrangement and metabolic responses of mitochondria after freeze–thawing. The method therefore permits, after freezing and long-term storage of muscle samples, mitochondrial function to be estimated and energy metabolism to be monitored in situ. This will significantly expand the scope for screening and exchange of human biopsy samples between research centers, thus providing a new basis for functional analysis of mitochondrial defects in various diseases.
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Mitochondria play a central role in hereditary mitochondrial diseases [1], ischemia reperfusion injury [2], and apoptosis [3,4]. Thus, the analysis of mitochondrial oxidative phosphorylation is fundamental in the diagnosis of many diseases and in the basic research of mitochondrial physiology. Mitochondrial defects are usually analyzed in frozen biopsy samples using histochemical, enzymatic, or molecular biology methods. However, direct assessment of mitochondrial function by measuring coupled respiration [5,6] and ATP synthesis [7] provides more full information, and the study of oxidative phosphorylation in skeletal muscles remains an important initial screening procedure for the potential presence of mitochondrial diseases.

Estimation of mitochondrial function is conventionally performed after isolation of mitochondria from tissue [8]. This, however, requires relatively large amounts of muscle tissue and therefore is not suitable for clinical tests of small human biopsies. Application of chemically permeabilized fibers and cells permits mitochondria to be studied in situ directly in biopsy samples and without their isolation [6,9–11]. This provides a

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basis for the analysis of mitochondrial function and dysfunction in very small tissue samples including human biopsies without isolation artifacts [12–14]. Specifically designed substrate/inhibitor titration protocols [6] and flux control analysis [12,15] allow putative mitochondrial defects to be localized. The confocal imaging technique affords the unique possibility to investigate mitochondrial changes in distinct mitochondrial subpopulations within the cell [16], thus offering a topological assay of mitochondrial injury.

On the other hand, the major limitation of investigating mitochondrial function is that it has to be performed immediately after biopsy to guarantee mitochondrial intactness. Many approaches of mitochondrial investigation, however, require serial and relatively time-consuming experiments that may lead to progressive, storage-dependent deterioration of mitochondrial function [17]. These time limitations reduce the possibilities to perform basic mitochondrial studies (muscle bioenergetics) and to analyze mitochondrial defects in various diseases. Therefore, recent advances in mitochondrial research have made the long-term preservation of biopsies for consequent functional analysis increasingly important. Moreover, since only a limited number of centers perform functional analysis of mitochondria, it is also of considerable importance to be able to ship frozen samples to a distant center for additional assays and thus eliminate the necessity for the patient to travel to the center to provide a second fresh biopsy.

Previous studies demonstrated preservation of mitochondria in frozen sperm, in some cells, and at least partially in DMSO[1] freeze–thawed isolated liver mitochondria [18–20]. However, muscle mitochondria are not easy to cryopreserve in a fully functionally intact state, and simple freeze–thawing of muscles is associated with severe damage of mitochondria. As a result, up to now it was impossible to investigate the functional characteristics of mitochondria (e.g., coupled respiration and ATP synthesis) in frozen biopsy specimens, which demonstrates the need for cryopreservation methods.

To overcome these limitations we tested several procedures for cryopreservation of permeabilized fibers isolated from different muscle types, including human biopsies. Various concentrations of glycerol and DMSO were applied to find an optimum, and the freezing procedure was optimized for the best preservation of mitochondrial functional integrity. We showed that in the optimally cryopreserved preparations oxidative phosphorylation was comparable to that of fresh, unfrozen muscles. After such cryopreservation, mitochondrial respiratory parameters, such as resting- and active-state respiration and respiratory control ratios with various substrates, were not significantly different from control values. Rates of ATP synthesis, ATP/O ratios, and the intracellular arrangement of mitochondria revealed by confocal imaging were similar to those of corresponding control fibers. Furthermore, cytochrome *c* and creatine tests demonstrated the intactness of the outer mitochondrial membrane.

[1] *Abbreviations used:* DMSO, dimethyl sulfoxide; miCK, mitochondrial creatine kinase; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid.

Materials and methods

Patients, human biopsies

Human biopsies (M. vastus lateralis) were taken from otherwise healthy orthopedic patients and immediately placed into ice-cold isotonic NaCl solution.

Animals

Adult Wistar rats (male, 250–300 g) were used in all animal experiments.

Preparation of permeabilized muscle fibers

Muscle fibers were prepared from human biopsy samples (M. vastus lateralis) and from rat M. soleus and rat myocardium (left ventricle) by careful dissection in ice-cold relaxing solution, containing 10 mM Ca–EGTA buffer, free concentration of calcium 0.1 μM, 20 mM imidazole, 20 mM taurine, 49 mM K-MES, 3 mM K_2HPO_4, 9.5 mM $MgCl_2$, 5 mM ATP, 15 mM phosphocreatine, pH 7.1. Dissection of each sample was performed using two pairs of extrasharp antimagnetic forceps. Optical inspection during tissue preparation was aided by using petri dishes with black bottoms. For permeabilization, fibers were incubated with 50 μg/ml saponin for 30 min as previously described [13–15].

In cryopreservation experiments, carefully dissected fibers were immersed in 100 μl of relaxing solution (using 1.8 ml NUNC cryotubes) additionally containing various glycerol or DMSO concentrations and 10 mg/ml fatty acid-free BSA and equilibrated with cryopreservation solution for 5 s. These fibers were then immediately frozen in liquid nitrogen at uniform freezing rates and generally kept in liquid nitrogen. In some cases and, especially for shipping, they were also stored on dry ice. For further analysis, tubes were placed in a water bath at 37 °C. When the cryopreservation medium was completely thawed, fibers were immediately transferred and washed in the medium for respiration measurement containing 2 mg/ml BSA (for composition see below) to minimize time of contact with glycerol or DMSO. Fibers were then permeabilized as described above and used for respiration or ATP synthesis analysis. They can be stored on ice in the same respiration medium for several hours without changes in mitochondrial function.

Cryopreservation of nonpermeabilized dissected fibers is preferred, but there is evidence that freezing of already permeabilized fibers is similarly effective. Human M. vastus lateralis fibers from some biopsy samples ($N = 7$) were also frozen in liquid nitrogen, but then sent on dry ice (−70 °C) from Magdeburg to Bordeaux and again kept in liquid nitrogen to simulate conditions of sample shipping (sharing).

Respiration measurements

Respiration measurements were performed at 25 °C using a high-resolution Oroboros oxygraph (Anton Paar, Graz) or Hansatech oxygraph in a medium consisting of 110 mM mannitol, 60 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM Na_2EDTA, and 60 mM Tris–HCl (pH 7.4). In some experiments using parallel measurements of oxygen consumption and ATP synthesis to inhibit adenylate kinase as a possible source for ATP formation, di(adenosine-5′) penta-phosphate was added in a final concentration of 50 μM.

Cytochrome c and creatine tests

Cytochrome *c* test was used to assess the integrity of the outer mitochondrial membrane. For this test, 10 μM cytochrome *c* was added during state 3 respiration (+1 mM ADP) with succinate. The functional activity of mitochondrial creatine kinase (miCK) was evaluated respirometrically in the presence of 10 mM glutamate + 5 mM malate and 0.1 mM ADP on the basis of the percentage increase in the respiration rate after addition of 30 mM creatine (VCr %).

Measurement of ATP synthesis

The rate of mitochondrial ATP production in permeabilized muscle fibers was measured by bioluminescence assay using a Lumat LB 9507 luminometer (“Berthold Ortec”) as described [7] and luciferin/luciferase reaction, providing a light signal which in the range 10^{-7}–10^{-6} M is proportional to the ATP concentration in the solution. Rate of ATP synthesis was measured in parallel with the rate of mitochondrial respiration. For this purpose, five or six 10-μl aliquots were taken from the oxygraph chamber every 30 s at different times before and after addition of 1 mM ADP. Samples were quenched in 100 μl DMSO to stop the reaction and frozen at −80 °C until ATP determination. Before measurement, samples were diluted by adding 4.9 ml of ice-cold distilled water and kept on ice during measurement. For each set of measurements, calibration curves (five points in a range 0–200 nM ATP) were used to quantitatively determine the ATP concentration in oxygraph samples. Atractyloside (an inhibitor of ATP–ADP translocase) was added in a final concentration of 100 μM at the end of the measurements to completely inhibit ATP synthesis. The decline in ATP concentration was subsequently measured and in each experiment the rates of ATP synthesis were corrected for the corresponding rates of ATP hydrolysis.

Confocal imaging of mitochondria

Isolated saponin-permeabilized fiber bundles were fixed between a Heraeus flexiperm chamber (Hanau, Germany) and a microscope glass slide. Then 200 μl of respiration medium was immediately added to the chamber. Fully oxidized state of mitochondrial flavoproteins was achieved by substrate deprivation and equilibration of the medium with air at room temperature. Flavoprotein autofluorescence was imaged using a confocal microscope (LSM-510NLO; Zeiss) with a 40X water immersion lens (NA 1.2). The use of such water immersion prevented geometrical aberrations when observing living cells. Autofluorescence of flavoproteins was excited with the 488-nm line of an argon laser, and the laser output power was set to an average power of 8 mW. The fluorescence signals were collected using a multiline beam splitter with maximum reflections at 488 ± 10 nm (for rejection of the 488-nm line) and at 543 nm (for rejection of 543-nm line). The flavoprotein signal was then passed through a 505- to 550-nm band-pass filter before being collected through a pinhole (one Airy disk unit).

In simultaneous imaging of flavoproteins and NADH, the fluorescence of NADH was excited by two-photon absorption using a femtosecond-pulsed infrared laser (Tsunami+MilleniaVIII; SpectraPhysics). Pulse frequency was set at 100 MHz with a pulse width of 100 fs. The infrared line was tuned to 720 nm, giving a maximum two-photon absorption of NADH. The laser output power was set to an average power of 400 mW. The fluorescence signals were collected through a multiline beam splitter with maximum reflections at 488 nm and above 700 nm (for rejection of infrared excitation). A second 490-nm beam splitter was used to discriminate the NADH signal from the flavoprotein signal. The flavoprotein signal passed through a 500- to 550-nm band-pass filter with an additional infrared rejection filter before being collected through a pinhole. The NADH signal was redirected to a 390- to 465-nm band-pass filter with an additional infrared rejection filter.

Materials

Saponin, MES, taurine, creatine phosphate, cytochrome *c*, ADP, ATP, and bovine serum albumin (essentially fatty acid free) were purchased from Sigma (Deisenhofen, Germany). Double-distilled glycerol was obtained from Serva (Heidelberg, Germany). Dimethyl

sulfoxide (p.a.) was purchased from Riedel-de Haen (Hannover, Germany).

Results and discussion

Some assays of oxidative phosphorylation important for the study and diagnosis of mitochondrial pathologies can be performed only on fresh muscle mitochondria. These include the analysis of coupled respiration and ATP synthesis, which are the most reliable means of assaying for putative complex V (ATP synthase) defects. Although mitochondrial function can be significantly stabilized by fixing the mitochondria in permeabilized cells or fibers or by applying specially designed preservation solutions with added protective agents (substrates, antioxidants, protease inhibitors; [17]), such preservation is still limited to several hours of cold storage. On the other hand, simple freezing of muscles causes significant damage of mitochondrial functional integrity through mechanisms which are not fully understood. Application of cryoprotective permeating or nonpermeating agents or their combinations, which minimizes the detrimental effects of increased solute concentration and ice crystal formation, protects mitochondria during freeze–thawing. Although mitochondrial cryopreservation is established for sperm, some cells, and isolated liver mitochondria [18–20], cryopreservation of muscle mitochondria (for batch storage, shipping, etc.), with few exceptions [15], has not been used for respiration measurement or other studies of energy metabolism in heart or skeletal muscles.

Testing of various cryoprotective agents and optimal conditions for cryopreservation

Several additives were screened in previous studies as cryoprotective agents including sugars, serum proteins, and solvents. Glycerol and DMSO have been shown to be most effective for cryopreservation of mitochondria [18,19]. Our study tested various concentrations of glycerol and DMSO to determine the optimal conditions for preservation of mitochondrial function. Fig. 1 shows the respiratory capacity of mitochondria in permeabilized human muscle fibers with glutamate + malate or succinate + cytochrome *c* as substrates, after freezing in glycerol (A)- or DMSO (B)-based media. When freezing in glycerol medium the best preservation is seen at 20% glycerol, which gives fibers having nearly control rates of respiration when using succinate in the presence of ADP and cytochrome *c* (Fig. 1A, filled squares; cf. [15]). Likewise, a similar glycerol concentration has been used for cryopreservation of skinned fibers in mechanical experiments of muscle tension [21]. Although some studies have shown glycerol to be less toxic than DMSO [22], another study demonstrates a markedly higher

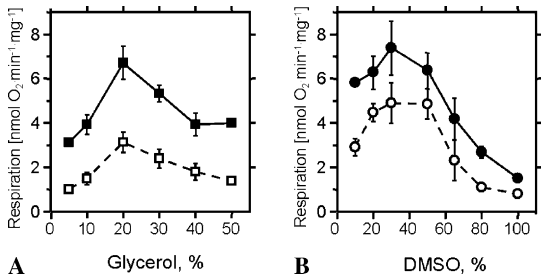

Fig. 1. Respiratory capacities of mitochondria in permeabilized human skeletal muscles after freezing in cryopreservation solutions with 10 mg/ml BSA and various concentrations of glycerol (A) or DMSO (B). Respiration was measured in the presence of 1 mM ADP, at 25 °C, with 10 mM glutamate + 5 mM malate (open symbols, dashed lines) or with 10 mM succinate + 10 μM cytochrome *c* (closed symbols, full lines) as mitochondrial substrates and expressed per milligram of dry weight. Values are means ± SD (two to six determinations for each concentration). When not visible, SD is smaller than symbol size.

cryoprotection potential of DMSO [23]. In our studies of mitochondrial preservation DMSO was clearly more effective than glycerol (Fig. 1). Indeed, for ADP-stimulated, NAD-linked (glutamate + malate) respiration, glycerol-preserved fibers showed less than 50% of respiratory activity in relation to unfrozen permeabilized muscle fibers. On the contrary, respiratory capacities with succinate + cytochrome *c* after freezing at optimal glycerol or DMSO concentrations were not significantly different from those of corresponding controls. A much better preservation of glutamate + malate-supported respiration was observed with DMSO at concentrations between 20 and 50%, with a steep decline in respiratory capacity at concentrations higher than 50% (Fig. 1B). Respiration of glycerol freeze–thawed fibers with succinate (even under optimal glycerol concentration) can be significantly stimulated by cytochrome *c* (data not shown). This clearly indicates a depletion of a substantial part of endogenous cytochrome *c* and a rupture of the outer mitochondrial membrane during freeze–thawing with glycerol. Therefore, in all further freeze–thaw experiments and tests of mitochondrial integrity in situ in permeabilized muscle fibers we used 30% DMSO + 10 mg/ml BSA (taking it as a standard cryopreservation solution). Interestingly, as a more penetrating reagent DMSO may cause greater dehydration and hence minimize freezing injury. Since a cryoprotective agent (DMSO) may have a toxic side effect on mitochondria, a standard equilibration period (time between mixing fibers with cryopreservation medium and freezing) of 5 s was used. Rapid and uniform freezing was critical for cryopreservation of mitochondria and was achieved by freezing in liquid nitrogen fibers immersed in rather small (0.1 ml) volumes of medium. For better preservation, fibers were transferred to a respiration medium immediately on thawing to minimize exposure to DMSO.

Table 1
Effect of cryopreservation on respiratory capacities of mitochondria in permeabilized fibers of various muscle types[a]

Muscle type	Control			Freeze–thawed		
	Pyr/Mal	Glu/Mal	Succ	Pyr/Mal	Glu/Mal	Succ
Rat ventricle	24.9 ± 2.6 ($N = 13$)	25.1 ± 3.7 ($N = 11$)	30.6 ± 4.1 ($N = 13$)	24.0 ± 1.7 ($N = 4$)	21.1 ± 5.7 ($N = 6$)	27.6 ± 3.3 ($N = 4$)
Rat M. soleus	15.5 ± 1.6 ($N = 5$)	15.4 ± 1.9 ($N = 3$)	20.3 ± 1.2 ($N = 5$)	14.2 ± 2.8 ($N = 5$)	15.7 ± 1.5 ($N = 3$)	$18.1 \pm 1.2^*$ ($N = 4$)
Human M. vastus lateralis	9.2 ± 1.5 ($N = 3$)	8.7 ± 1.6 ($N = 12$)	10.9 ± 2.5 ($N = 12$)	8.0 ± 1.3 ($N = 3$)	7.5 ± 1.2 ($N = 6$)	9.0 ± 1.3 ($N = 4$)

[a] Rates of respiration (V_{O_2}) were measured after addition of 1 mM ADP at 25 °C as described under Materials and methods and expressed in nmol O_2 min^{-1} mg^{-1} dry weight. Experiments were performed with 10 mM pyruvate + 5 mM malate (Pyr/Mal), 10 mM glutamate + 5 mM malate (Glu/Mal), or 10 mM succinate (Succ).

* Significantly different from corresponding control ($p < 0.05$).

Effect of DMSO freeze–thawing on spatial organization and function of mitochondria in fibers isolated from various muscle types

Table 1 summarizes the effect of cryopreservation on respiratory capacity of mitochondria of various muscles, demonstrating that in almost all cases the coupled respiration of cryopreserved fibers (in the presence of saturating ADP concentration) is either identical or not less than 90% that of corresponding control values. Moreover, rates of resting-state respiration (before addition of ADP) and respiratory control ratios (the ratio of active-state respiration, after addition of 1 mM ADP, and resting-state respiration) after freeze–thawing were not significantly different from those of controls (data not shown). Only in one case of rat soleus fibers and with succinate as the mitochondrial substrate was the rate of respiration of frozen fibers significantly lower than that of control fibers ($p < 0.05$; 89% of control; Table 1). Typical oxygen consumption traces of permeabilized control and cryopreserved fibers isolated from human vastus lateralis muscles are shown in Fig. 2 with particular emphasis on the stimulatory effect of cytochrome *c*. It is seen that the rates of ADP-stimulated respiration of permeabilized fresh fibers with pyruvate + malate and after subsequent succinate addition were similar to those for DMSO freeze–thawed muscle fibers and in both cases were not increased by externally added cytochrome *c* (Fig. 2). These experiments show that, in contrast to glycerol-cryopreserved muscles, the optimal freeze–thaw procedure with DMSO completely protects the integrity of the outer mitochondrial membrane and ensures complete cytochrome *c* conservation.

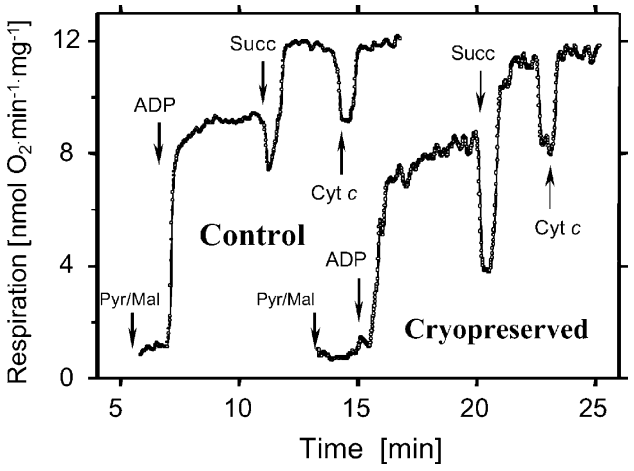

Fig. 2. Representative oxygraph traces of permeabilized control and cryopreserved skeletal muscle fibers obtained from human biopsies. Cytochrome *c* test. Additions: Pyr/Mal, 10 mM pyruvate + 5 mM malate; 1 mM ADP; Succ, 10 mM succinate; Cyt *c*, 10 μM cytochrome *c*. Respiration was measured at 25 °C as described under Materials and methods and calculated per milligram of dry weight.

Mitochondrial creatine kinase (isoenzyme of creatine kinase) is localized at the outer side of the inner mitochondrial membrane in the vicinity of adeninenucleotide translocase (ATP–ADP carrier). Activation of miCK by its substrate creatine results in rapid utilization of mitochondrially produced ATP for synthesis of phosphocreatine and local regeneration of ADP, which is transported by ATP–ADP translocase back into mitochondria [24,25]. As a result, mitochondrial respiration is preferentially regulated by ADP produced via miCK. Addition of creatine, therefore, significantly activates respiration at a submaximal concentration of exogenous ADP, and the degree of this activation indicates the extent of functional coupling between mitochondrial creatine kinase reaction and oxidative phosphorylation. Thus, it can be used as a very sensitive test for functional coupling as an important step in intracellular energy transfer [24,25]. Activation of respiration after addition of 30 mM creatine (VCr %) in the presence of submaximal ADP (0.1 mM) was $55 \pm 10\%$ ($N = 5$) in control and $55 \pm 15\%$ ($N = 4$) in freeze–thawed myocardial fibers. These identical stimulatory effects of creatine demonstrated complete preservation of miCK coupling and efficiency in freeze–thawed fibers isolated from rat heart.

Fig. 3 shows the spatial organization and function of mitochondria within permeabilized rat myocardial fibers

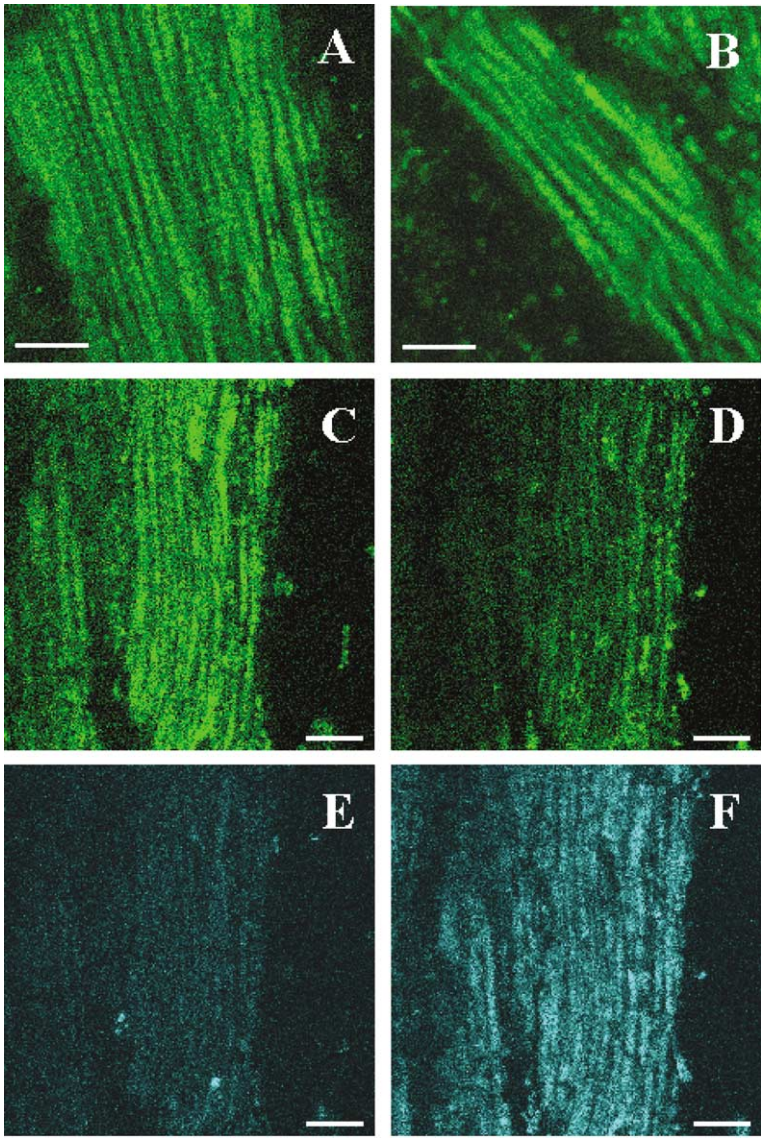

Fig. 3. Confocal fluorescence images of mitochondria in permeabilized myocardial fibers. (A, B) Autofluorescence of mitochondrial flavoproteins. Fully oxidized state of mitochondrial flavoproteins was achieved by substrate deprivation and equilibration of the medium with air at room temperature. Regular mitochondrial arrangement can be seen in control fibers (A) and in freeze–thawed fibers (B) using cryopreservation medium with 30% DMSO + 10 mg/ml BSA. Scale bar, 10 μm. (C, E) Simultaneous confocal imaging of the autofluorescence of mitochondrial flavoproteins (C) and NADH (E) in cryopreserved myocardial fibers in the oxidized state. (D, F) Corresponding flavoproteins and NADH autofluorescence images in the partially reduced state after addition of 5 mM glutamate. The fluorescence signal of flavoproteins diminishes with reduction of the respiratory chain after addition of glutamate (D), and the NADH fluorescence signal increases (F). Scale bar, 10 μm.

as determined by fluorescent confocal microscopy before and after freeze–thawing with 30% DMSO + 10 mg/ml BSA. Confocal imaging of the autofluorescence of mitochondrial flavoproteins in their oxidized state (Figs. 3A and B) demonstrates that the regular mitochondrial arrangement was not changed in cryopreserved fibers as compared to control. The two-photon excitation techniques also permitted detection (imaging) of mitochondrial NADH fluorescence across freeze–thawed myocardial fibers simultaneously with flavoprotein imaging (Figs. 3C–F). In the oxidized state of the mitochondrial respiratory chain, achieved by substrate deprivation in the aerated open chamber, the intensive fluorescence of flavoproteins, but not of NADH, can be seen (Figs. 3C and E). Addition of respiratory substrate glutamate (5 mM) reduces the flavoproteins, decreasing their fluorescence intensity and, at the same time, strongly increases the NADH fluorescence signal (Figs. 3D and F). These fluorescence changes demonstrate normal metabolic responses of mitochondria in freeze–thawed fibers to the addition of mitochondrial substrate, similar to our observations of fresh unfrozen rat myocardial fibers (data not shown) or previously reported data on mice skeletal muscle fibers [16], indicating

complete preservation of all enzyme systems involved in substrate transformation and mitochondrial redox change. Thus, neither the distribution nor the functional response of mitochondria was altered in cryopreserved myocardial fibers.

Direct measurement of the rate of ATP synthesis provides information on the efficiency of oxidative phosphorylation and, with parallel measurement of respiration, permits the ATP/O ratio in situ to be calculated as an important mitochondrial index [7]. This parameter can be significantly lowered in various pathologies including ischemia–reperfusion injury, Luft syndrome, etc. [26]. The results of simultaneous measurement of mitochondrial respiration and ATP synthesis in freeze–thawed permeabilized fibers of various muscle types are summarized in Table 2, which shows that ATP production and ATP/O ratios do not differ from corresponding control values. In the presence of a high concentration of ADP (in our experiments, 1 mM), ATP can also be produced by the adenylate kinase system without involving oxidative phosphorylation. To exclude this possibility and correctly assess the rate of mitochondrial ATP synthesis, possible ATP production from ADP by adenylate kinase reaction was minimized by adding a specific inhibitor, diadenosine pentaphosphate (50 μM). Since ATP synthesis and hydrolysis contribute equally to the steady state ATP concentration, ATP hydrolysis was measured after full inhibition of oxidative phosphorylation by atractyloside (specific inhibitor of ATP–ADP translocase), to calculate the actual rate of mitochondrial ATP production. The rate of ATP hydrolysis never exceeded 15% of the corresponding net ATP synthesis rate before addition of atractyloside, and in each experimental run it was used for the correction. When calculating ATP/O ratios, to obtain the phosphorylation-related rate of respiration, the rate with atractyloside (7 to 11% of the respiration rate before addition of the inhibitor, depending on muscle type) was always subtracted from the net rate of oxygen consumption in the presence of ADP. Importantly, ATP/O ratio data as presented in Table 2 are within the previously published range for intact unfrozen cells [7]. In line with previous findings, ATP/O ratio with complex II substrate succinate was found to be 1.6 ± 0.4 and with artificial substrate for complex IV *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride + ascorbate it was 0.7 ± 0.1 (in both cases $N = 3$). It has been shown that DMSO may have an uncoupling effect on liver mitochondria [18], demonstrating that this agent by itself affects mitochondrial function. In contrast to this finding, no uncoupling of muscle mitochondria by DMSO was found in our study (Table 2). Most probably, this is due to a minimal time of mitochondrial exposure to this cryoprotective agent due to the application of permeabilized muscle fibers, which allows a very fast transfer of samples from the cryopreservation medium to the medium without DMSO and eliminates the need for any centrifugation.

Taken together, freeze–thawed fibers of various types of muscle demonstrated good preservation of mitochondrial functional integrity, normal ATP production (oxidative phosphorylation), and energy transfer through effectively coupled mitochondrial creatine kinase. They can therefore be used as an attractive alternative to the investigation of freshly isolated muscle preparations. Muscle samples may be prepared in single-use portions and batch-stored for further analysis. This will be particularly important for integrating various approaches in mitochondrial research by sharing biopsies between various scientific centers using diverse or, on the contrary, highly standardized samples. Importantly, cryopreservation of functional mitochondria ensures long-term stability, but does not correct any mitochondrial defects already present in muscles. Additionally, this technique allows long-term storage of muscle fibers to serve as controls in diagnostic investigations.

Table 2
Mitochondrial respiration and ATP synthesis in permeabilized fibers of various muscle types before and after cryopreservation[a]

Muscle type	Control			Freeze–thawed		
	V_{ATP}[b]	V_{O_2}	ATP/O[c]	V_{ATP}[b]	V_{O_2}	ATP/O[c]
Rat ventricle	119.6 ± 33.3 ($N = 8$)	25.6 ± 3.8 ($N = 8$)	2.5 ± 0.3 ($N = 8$)	106.3 ± 19.6 ($N = 3$)	24.2 ± 1.2 ($N = 3$)	2.4 ± 0.3 ($N = 3$)
Rat M. soleus	67.2 ± 15.2 ($N = 3$)	15.4 ± 1.9 ($N = 3$)	2.5 ± 0.1 ($N = 3$)	59.4 ± 6.3 ($N = 3$)	15.7 ± 1.5 ($N = 3$)	2.1 ± 0.1 ($N = 3$)
Human M. vastus lateralis	n.d.	8.9 ± 1.7 ($N = 7$)	n.d.	34.4 ± 8.4 ($N = 7$)	7.4 ± 1.1 ($N = 7$)	2.6 ± 0.3 ($N = 7$)

n.d., not determined.

[a] Rates of ATP synthesis (V_{ATP}) and respiration (V_{O_2}) were measured after addition of 1 mM ADP at 25 °C and expressed in nmol ATP or O_2 per min and per mg dry weight. Experiments were performed with 10 mM glutamate + 5 mM malate.

[b] To find the actual rate of ATP production, V_{ATP} was corrected for the rate of ATP hydrolysis (rate added) measured after inhibition of oxidative phosphorylation by the addition of 100 μM of atractyloside.

[c] For the calculation of ATP/O ratio, rate of respiration V_{O_2} was corrected for the rate in the presence of 100 μM of atractyloside (6–8% of noninhibited rate). This rate was subtracted to obtain the phosphorylation-related rate of respiration. ATP/O ratio was always calculated using V_{ATP} and V_{O_2} values from the same experiment (respirometric run).

Summarizing, we demonstrate that this method is suitable for routine studies in mitochondrial physiology and clinical applications. Minimization of sample size is especially important for studies of mitochondrial oxidative phosphorylation in human biopsies, where the amount of available tissue is limited. Cryopreservation of muscle mitochondria in permeabilized fibers not only may significantly expand the scope for analysis of each muscle sample, but also may permit creation of banks of samples with well-defined pathologies accessible to all modern technologies for mitochondrial research. In the future, this technique will allow better diagnosis and much broader screening of mitochondrial injuries in patients with various pathologies.

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