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Role of mitochondria–cytoskeleton interactions in respiration regulation and mitochondrial organization in striated muscles

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ABSTRACT

The aim of this work was to study the regulation of respiration and energy fluxes in permeabilized oxidative and glycolytic skeletal muscle fibers, focusing also on the role of cytoskeletal protein tubulin βII isotype in mitochondrial metabolism and organization. By analyzing accessibility of mitochondrial ADP, using respirometry and pyruvate kinase–phosphoenolpyruvate trapping system for ADP, we show that the apparent affinity of respiration for ADP can be directly linked to the permeability of the mitochondrial outer membrane (MOM). Previous studies have shown that MOM permeability in cardiomyocytes can be regulated by VDAC interaction with cytoskeletal protein, βII tubulin. We found that in oxidative soleus skeletal muscle the high apparent K_m for ADP is associated with low MOM permeability and high expression of non-polymerized βII tubulin. Very low expression of non-polymerized form of βII tubulin in glycolytic muscles is associated with high MOM permeability for adenine nucleotides (low apparent K_m for ADP).

1. Introduction

Striated muscles such as cardiac and skeletal muscles have a common contractile unit named sarcomere and similar mechanism of contraction based on the conversion of free energy of ATP hydrolysis in ATPase reaction into mechanical energy for contraction. In cardiac cells, structural and functional organization of metabolism allowing connection of ATP-consuming sites such as sarcomere, sarcoplasmic reticulum and subsarcolemmal ion pumps with ATP-synthesizing systems was named intracellular energetic units (ICEUs) [1–3]. In mitochondria the energy transfer is carried out by mitochondrial interactosome (MI) supercomplex [1,4]. This complex is situated at the contact sites of the outer and inner mitochondrial membranes (MIM) and is composed of ATP synthasome (including ATP synthase, coupled to the respiratory chain complexes, adenine nucleotide translocase (ANT) and inorganic phosphate carrier), mitochondrial creatine kinase (MtCK) and voltage dependent anion channel (VDAC), interacting with cytoskeletal protein βII tubulin and possibly with some other cytoskeletal proteins [1,4–6]. The restriction of adenine-nucleotides diffusion at the level of MOM creates a basis for the compartmentalization of energy transfer within ICEUs [7,8]. The intracellular energy flux within ICEUs is supported by phosphocreatine/creatine kinase (PCr/CK) pathway and the transfer of phosphoryl groups mainly occurs via the system of various specifically localized isoenzymes of CK and other phosphoryl-transferring kinases [2,7–9].

These mechanisms have been shown mostly for cardiac cells, but the information regarding the regulation of respiration and control of energy fluxes in various skeletal muscles is still limited. According to the myofibrillar ATPase activity, enzyme pattern and mitochondrial content, muscle fibers can be divided into three main groups: 'slow twitch oxidative fiber' (type I), 'fast twitch oxidative' (type IIA) and 'fast twitch glycolytic' fibers (types IIB, IIX) [10]. Slow twitch oxidative muscles such as *m. soleus* (consisting of about 84% type I and 7% type IIA fibers) display relatively low ATPase activity and large capacity for oxidative phosphorylation with high mitochondrial content (still significantly lower than in the heart) [11]. They are able to sustain low intensity workloads for long periods of time. Fast glycolytic muscles, i.e. white portion of rats *m. gastrocnemius* (GW) (consisting of about 92% type IIB fibers) display three- to fivefold higher ATPase activity than oxidative muscles and are

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able to support high intensity workloads for short periods of time [11,12]. As a general rule, skeletal muscles consist of mixture of oxidative and glycolytic muscle fibers. For example, red portion of gastrocnemius muscle (GR) is formed of 51% type I and 35% type IIA fibers [11]. Relative to the cell volume mitochondria occupy about 35% in cardiac myocytes, about 6–10% in oxidative and only 1% in glycolytic skeletal muscle cells [13–15]. It has been shown that isolated mitochondria from oxidative and glycolytic muscles display similar characteristics. For instance, there are similar maximal rates of ADP-stimulated respiration per mg of mitochondrial protein and similar activities of isolated respiratory chain complexes [16,17]. Proteomic analysis of isolated mitochondria has revealed only few differences of protein contents between them [17]. However, several experimental studies using cell permeabilization have indicated distinct patterns of mitochondrial regulation in oxidative and glycolytic muscle fibers. Major differences were found in the apparent affinity of oxidative phosphorylation for ADP. In particular, the apparent K_m for ADP in the heart and *m. soleus* has been shown to be an order of magnitude higher than that of glycolytic muscles [12,18–20]. Several recent studies suggested that it can be associated with different permeability of MOM for ADP regulated by the binding of heterodimeric αβ tubulin to VDAC [1,21–25]. Our recent immunochemical studies of the distribution of β tubulin isoforms in cardiomyocytes linked this phenomenon to the presence of mitochondria-specific isoform of βII tubulin [26,27].

In the present work, we studied: i) the relationship between the apparent K_m for ADP and MOM permeability in skeletal muscle fibers by estimating respirometrically accessibility of mitochondrial ADP in the presence of excess of PK–PEP trapping system for external ADP, ii) flux control that different MI complexes exert on the total energy flux in oxidative and glycolytic permeabilized skeletal muscle fibers and iii) the dependence of MOM permeability on βII tubulin distribution, considering polymerization–depolymerization equilibrium of tubulin and mitochondrial arrangement. We hypothesized that the differences in mitochondrial affinity for ADP between oxidative and glycolytic muscles might be explained by different distribution pattern and/or by different free protein content of βII tubulin which may participate in feedback regulation of mitochondrial metabolism.

2. Material and methods

2.1. Laboratory animals and chemicals

Male Wistar rats weighing 150–200 g were used in the experiments. The animals were housed at constant temperature (22 °C) in environmental facilities with a 12:12 h light–dark cycle. Animal procedures were approved by "Comité d'éthique pour l'expérimentation animale" of Grenoble (33_LBFA-VS-01) and National Committee for Ethics in Animal Experimentation (Estonian Ministry of Agriculture).

2.2. Preparation of permeabilized fibers

Rats were anaesthetized with sodium pentobarbital (40–50 mg kg^{-1}) intraperitoneal injection, decapitated and, the muscles of interest were placed into a plastic Petri dish containing ice-cold isolation solution A of the following composition: 10 mM Ca-EGTA buffer (2.77 mM of CaK_2EGTA + 7.23 mM K_2EGTA) 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 $MgCl_2$, 5.7 mM ATP, 15 mM PCr, pH 7.1. Muscle-fiber bundles were separated from each other using extra-sharp antimagnetic forceps under a microscope of a cold light source. To study the regulation of mitochondrial respiration of muscle, fibers were permeabilized by saponin treatment (50 μg/mL) keeping the mitochondrial membranes intact [20,28]. The permeabilization procedure was followed by triple wash in ice-cold Mitomed solution containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 3 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES, 110 mM sucrose, 0.5 mM dithiothreitol, 2 mg mL^{-1} fatty acid free BSA, pH 7.1. The aim is to wash out saponin and other metabolites, especially traces of ADP or ATP, and proteases released for damaged lysosomes due to the saponin effect. To protect fibers of the proteolytic effect of lysosomal enzymes during experiments Mitomed is supplemented with 2 mg mL^{-1} BSA and leupeptin 1 μM [29]. The studied muscles are as follows: soleus; red portion of gastrocnemius muscle (GR), white portion of gastrocnemius muscle (GW), extensor digitorum longus (EDL), and left ventricle muscle (LV).

Heart mitochondria were isolated as described previously in [30] using trypsin.

2.3. Measurements of oxygen consumption

The rates of oxygen uptake were determined with a high-resolution respirometer (oxygraph-2 K, OROBOROS Instruments, Austria) in Mitomed solution supplemented with 5 mM glutamate and 2 mM malate. These measurements were carried out at 25 °C and taken the solubility of oxygen as 240 nmol mL^{-1} [31]. The respiration rates of permeabilized cardiomyocytes were expressed in nmol of oxygen consumed per minute per nmol of cytochrome aa3. The content of mitochondrial cytochrome aa3 was measured spectrophotometrically according to the method described previously [4]. Measurements of cytochrome aa3 content in skeletal muscles were limited by the necessity to increase the amount of the samples because of their lower mitochondrial content. As a result, decreased optical density compromised the quality of cytochrome aa3 measurements in spectrophotometry. The respiration rates of permeabilized muscle fibers were expressed in $nmolO_2$ min^{-1} mg^{-1} dry weight fibers. Wet fibers were dried at 100 °C for 24 h. Respiration rates were not compared between different muscles, but inside each muscle fiber-type between ADP- and Cr-stimulated respirations.

One of the most reliable quality tests of the intactness of membrane structures for permeabilized fibers is the cytochrome c test used to check the integrity of MOM [28]. Measurement of cytochrome c release from mitochondria in permeabilized cells can be studied qualitatively by Western blot and quantitatively by spectrophotometry. Western blot analysis is highly specific for cytochrome c, but it's time-consuming and requires separate labeling of isolated mitochondrial and cytosolic fractions. Isolation of mitochondria embedded into muscle fibers cytoskeleton gives two fractions: light or damaged mitochondria with increased MOM permeability and cytochrome c release and intact mitochondria. Time is also a very important factor because the aim of the cytochrome c release study is to select permeabilized fibers with intact mitochondria for the measurements of oxygen consumption. Permeabilized fibers or cells were used for respirometry studies during the first 3 h after permeabilization. Appaix et al. (2000) developing method of spectrophotometric measurement of cytochrome c in permeabilized cells showed that their results were equal to those of oxygraphic determination of cytochrome c-dependent respiration of permeabilized cardiomyocytes [32]. This experiment is carried out in KCl-solution (125 mM KCl, 20 mM HEPES, 5 mM KH2PO4, 3 mM Mg acetate, 0.4 mM EGTA, 0.3 mM DTT) supplemented with respiratory substrates (glutamate and malate) and 2 mM of ADP to get the maximal rate of respiration. Cytochrome c is a highly soluble hemoprotein of the respiratory chain that transfers electrons and is loosely associated with the outer side of the inner mitochondrial membrane. If MOM is disrupted, cytochrome c leaves mitochondria decreasing maximal respiration rate and consequently, in this situation its addition in presence of ADP will increase respiration rate. Fig. 1A shows high maximal rates of ADP-stimulated respiration and high respiration control ratio (RCR) which is estimated by the ratio between maximal ADP-stimulated and basal respiration rates (V_{maxADP}/V_0), and indicates preserved flux through the electron transport chain after saponin permeabilization. Subsequently, the addition of carboxyatractyloside (CAT) gives us information about the integrity of mitochondrial inner membrane (MIM). CAT inhibits in irreversible way ANT interrupting ATP/ADP exchange