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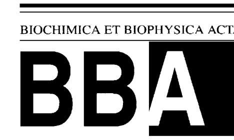

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Mitochondrial subpopulations and heterogeneity revealed by confocal imaging: Possible physiological role?

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Abstract

Heterogeneity of mitochondria has been reported for a number of various cell types. Distinct mitochondrial subpopulations may be present in the cell and may be differently involved in physiological and pathological processes. However, the origin and physiological roles of mitochondrial heterogeneity are still unknown. In mice skeletal muscle, a much higher oxidized state of subsarcolemmal mitochondria as compared with intermyofibrillar mitochondria has been demonstrated. Using confocal imaging technique, we present similar phenomenon for rat soleus and gastrocnemius muscles, where higher oxidative state of mitochondrial flavoproteins correlates also with elevated mitochondrial calcium. Moreover, subsarcolemmal mitochondria demonstrate distinct arrangement and organization. In HL-1 cardiomyocytes, long thread mitochondria and small grain mitochondria are observed irrespective of a particular cellular region, showing also heterogeneous membrane potential and ROS production. Possible physiological roles of intracellular mitochondrial heterogeneity and specializations are discussed.
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1. Introduction

Confocal imaging of mitochondria in situ in permeabilized non-fixed preparations of tissue or in living cells revealed that mitochondria are either clustered or arranged in highly organized, tissue specific manner. Moreover, mitochondria in the cell may be very heterogeneous in many structural and functional aspects under normal [1,2] and pathological [3,4] conditions. There is growing evidence that different mitochondrial subpopulations localized in different cell regions may be present in the tissue, but the data showing mitochondrial heterogeneity with respect to their biochemical properties respiratory or enzymatic activities, calcium, membrane potential, coupled state, etc. are rather divergent, probably due to methodological difficulties of the isolation and separation of individual mitochondrial populations from the tissue [5]. Functional heterogeneity of mitochondria was apparent in several ways. Using imaging techniques, heterogeneity of mitochondrial redox potentials has been shown in mice skeletal muscle [6] and cardiomyocytes [2,7]. Heterogeneity of mitochondrial calcium has been studied in cardiac cells under pathological conditions [4,8]. Functional static and dynamic heterogeneity of mitochondria has been reported for a number of various cells including hepatocytes, HUVEC, astrocytes, and various human carcinoma cells [1,2]. However, metabolic consequences or causes of mitochondrial heterogeneity as well as its physiological and pathophysiological significance are still not clear and require further investigation. In our study, a hypothesis considering highly specialized mitochondria within one cell was tested. Applying a confocal imaging approach, possible functional differences of mitochondrial subpopulations were studied in situ in permeabilized, non-fixed muscle fibers of rat soleus and gastrocnemius skeletal muscles and in vivo in HL-1 and adult rat cardiomyocytes. Our findings suggest high degree of metabolic and possible functional heterogeneity in various cell types.

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2. Material and methods

2.1. Confocal imaging of mitochondria, ROS and mitochondrial calcium in living cells

To detect the mitochondrial functional state at the level of the single mitochondrion, images were acquired and analyzed by fluorescent confocal microscopy. Muscle fibers were permeabilized with saponin (50 μg/ml) as described in [6] and fixed at both ends in a flexiperm chamber (Hanau, Germany) with microscopic glass slide. The fully oxidized state of mitochondrial flavoproteins (maximal fluorescence intensity) was achieved by substrate deprivation and equilibration of the medium with air. HL-1 cells were cultured using fibronectin-gelatin substrate and a specially formulated growth medium. Cells were placed in Lab-Tek® chambered coverglasses (Nalge Nunc, Rochester, NY) and incubated in the same growth medium at least 15 h before analysis, chamber volume 0.3 ml, $10–20\times10^3$ cells per chamber. Flavoprotein autofluorescence was imaged using a confocal microscope (LSM510 NLO, Zeiss) with a 40× water immersion lens (NA 1.2). The autofluorescence of flavoproteins was excited with the 488 nm line of an argon laser. The fluorescence was collected through a 510-nm dichroic beam-splitter and a 505–550-nm band pass filter. To analyze mitochondrial inner membrane potential, cells were incubated for 30 min at room temperature with 50 nM tetramethylrodamine methyl ester (TMRM, Sigma), a fluorescent dye that accumulates in mitochondria on the basis of their membrane potential added directly to the cell culture medium. In control experiments, dissipation of membrane potential was observed after addition of 5 μM antimycin A, 4 μM FCCP, and 0.5 μM rotenone (data not shown). Production of ROS was examined using 2′,7′-dichlorodihydrofluorescin diacetate, (DCF-DA, Sigma), a dye that forms a fluorescent product upon oxidation. For this cells were incubated with DCF-DA (20 μM) for 30 min in the dark. ROS-induced green fluorescence of DCF-DA was imaged similar to flavoprotein fluorescence, except the laser power was set to 2–3%. Importantly, this low power laser setting allows differentiating green DCF-DA fluorescence from low intensity signal originated from mitochondrial flavoproteins. To analyze the level of mitochondrial matrix calcium $[Ca^{2+}]_m$, permeabilized muscle fibers were preloaded with fluorescent Ca^{2+} specific probe Rhod-2 AM (5 μM, Molecular Probes, Eugene, OR, USA) for 60 min at room temperature. Cationic dye Rhod-2 has a net positive charge allowing, under our experimental conditions, its specific accumulation in muscle mitochondria. The digital images of TMRM, DCF-DA and Rhod-2 fluorescence were acquired with inverted confocal microscope (Leica DM IRE2) with a 63× water immersion objective or with a microlens-enhanced Nipkow disk-based confocal system UltraVIEW RS (Perkin Elmer, Wellesey MA, USA) mounted on an Olympus IX-70 inverse microscope with 40× water immersion objective (Olympus, Nagano, Japan). Images were acquired using the UltraVIEW RS software. The DCF fluorescence was excited with the 488 nm line of argon laser for excitation and 510 to 550 nm for emission. TMRM and Rhod-2 fluorescence was measured using 543 nm for excitation (Helium-Neon laser) and greater than 580 nm for emission.

3. Results and discussion

In our study, imaging of mitochondrial organization and mitochondrial functional states were performed in intact cells and permeabilized muscle fibers. Mitochondrial clustering has been reported as one of specific ways of mitochondrial organization in various cell types, that may be associated with the specific cellular demands [9,10]. Cardiac mitochondria are organized in a “lattice” of parallel rows surrounding the contractile myofilaments, forming structural and functional complexes with sarcoplasmic reticulum and sarcomere [11–13]. Fig. 1 shows also clusters of mitochondria surrounding the nuclei in adult rat cardiomyocytes. It can be seen that some of these mitochondria demonstrate higher signal of autofluorescence of mitochondrial flavoproteins than that of intermyofibrillar mitochondria (Fig. 1A) possibly due to distinct intrinsic properties of these perinuclear subsets. These clustered mitochondria are more densely packed in the perinuclear region, visible also from confocal images of autofluorescence of mitochondrial NADH (Fig. 1B). Such mitochondrial clustering may serve to drive mitochondrial metabolism to generate ATP close to the nucleus as has been shown recently for parotid acinar cells [9] and in a line with the concept of the integrated phosphotransfer network and energetic channeling between mitochondria and nucleus suggested by Dzeja et al. [10]. Taken together, these findings strongly suggest that clusters of perinuclear mitochondrial subpopulation may play an important physiological role in the mechanisms for nuclear import, as well as for regulating a variety of other nuclear functions.

Other subpopulations of mitochondria with cluster organization are subsarcolemmal mitochondria in skeletal muscle. Measurement of autofluorescence of mitochondrial flavoproteins which are fluorescent in the oxidized, but not reduced state has been widely used to examine the metabolic status of cells. The redox state of flavoproteins is in equilibrium with the redox state of mitochondrial NADH pool. The intensity of flavoprotein

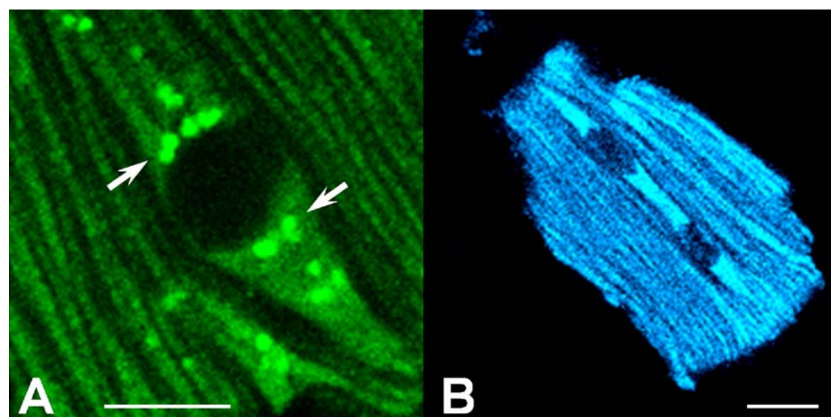

Fig. 1. Perinuclear mitochondrial clustering in rat cardiomyocytes revealed by confocal fluorescent microscopy. (A) Confocal imaging of autofluorescence of mitochondrial flavoproteins shows significantly higher intensity and therefore higher oxidized state of some perinuclear mitochondria (arrows). (B) Two photon confocal imaging of autofluorescence of NADH in perinuclear clusters of mitochondria. Scale bars, 10 μm.

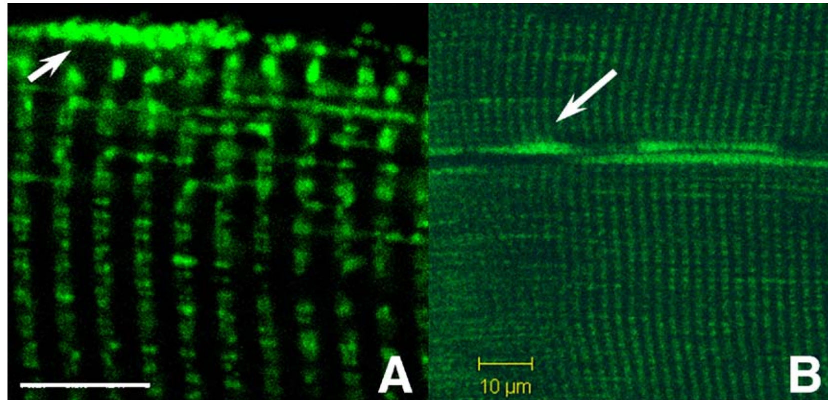

Fig. 2. Subsarcolemmal mitochondria in rat skeletal muscles. (A) Confocal imaging of autofluorescence of mitochondrial flavoproteins in rat soleus. Mitochondria located near the outer cell membrane demonstrate interconnections. (B) Confocal imaging of autofluorescence of mitochondrial flavoproteins in rat gastrocnemius. In both cases of rat soleus and rat gastrocnemius, significantly higher autofluorescence of mitochondrial flavoproteins can be seen in subsarcolemmal mitochondrial subpopulation (arrows). Scale bars, 10 μm.

autofluorescence signal reflects electron transport through respiratory chain and is related therefore to mitochondrial metabolic state and activity. Monitoring of flavoprotein autofluorescence both by confocal fluorescent imaging of intact and permeabilized cells or by FACs analysis indicated that mitochondria may be highly heterogeneous with respect to their oxidative state. Such heterogeneity of mitochondrial oxidative state has been documented for rat cardiomyocytes [7] and skeletal muscles [6]. In mice skeletal muscle, subsarcolemmal mitochondria demonstrate more then 4 times higher signal for flavoprotein autofluorescence, pointing thus to significantly different biochemical properties of distinct mitochondrial subpopulations [6]. In our work, using confocal imaging of permeabilized, non-fixed muscle fibers in physiological salt solution, functional differences between subsarcolemmal and intermyofibrillar mitochondrial were further tested in other muscles such as rat soleus and rat gastrocnemius. Fig. 2 shows that in rat skeletal muscles (soleus and gastrocnemius) subsarcolemmal mitochondrial subsets have significantly higher intensity of flavoprotein autofluorescence, indicating more oxidized state of these mitochondria. Importantly, this high oxidative state of subsarcolemmal mitochondria correlates well with significantly higher Ca^{2+} levels in this subpopulation revealed from imaging of specific mitochondrial calcium probe Rhod-2 (Fig. 3). These higher calcium levels in subsarcolemmal mitochondria can also be responsible for higher mitochondrial respiration via activation of certain mitochondrial dehydrogenases [14–16], or due to recently proposed alternative way of calcium participation in the regulation of mitochondrial function through changes in the affinity of mitochondria to their main regulator ADP [17]. Furthermore, clusters of subsarcolemmal mitochondria are densely packed (Figs. 2 and 3). In addition, these mitochondria show less regular arrangement as intermyofibrillar mitochondria or cardiac mitochondria [18]. Interestingly, in rat soleus, confocal imaging of mitochondria located near the outer cell membrane demonstrates numerous contacts and interconnections, showing clear mitochondrial network in this muscle type (Figs. 2A and 4).

Several years ago, an interesting concept of mitochondrial clusters as intracellular power-transmitting cables has been

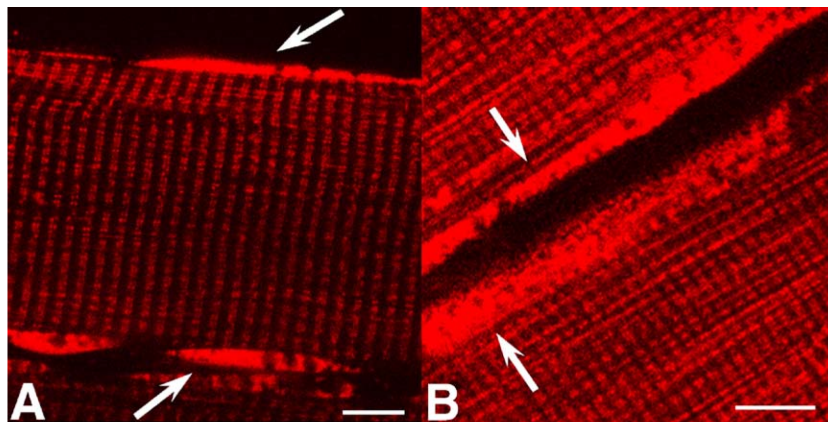

Fig. 3. Confocal imaging of mitochondrial calcium in subsarcolemmal mitochondria in rat skeletal muscles visualized by mitochondrial calcium specific probe Rhod-2. Specificity for mitochondrial calcium in permeabilized muscles has been demonstrated previously by us, showing the colocalization of Rhod-2 with mitochondrial flavoproteins [28] or MitoTracker [18]. Significantly higher fluorescence of Rhod-2 can be seen in subsarcolemmal mitochondrial subpopulation (arrows). Scale bars, 10 μm.

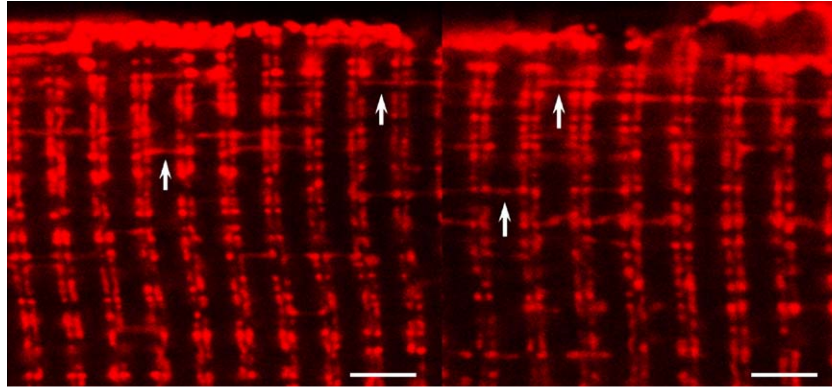

Fig. 4. Organization of subsarcolemmal mitochondrial clusters in rat soleus. Clusters of subsarcolemmal mitochondria are densely packed. As in Fig. 2A, mitochondria located near the outer cell membrane demonstrate interconnections and mitochondrial network (arrows). Soleus mitochondria were labeled with Rhod-2 as in Fig. 3 and described in Materials and methods. Scale bars, 5 μm.

proposed by Skulachev [19,20]. This theory suggests that mitochondria may exist in two interconverting forms: - small isolated particles and extended filaments, networks, or clusters connected by intermitochondrial junctions. It is assumed that, in the diaphragm muscle, oxygen and mitochondrial substrates from a capillary are first consumed by a subsarcolemmal mitochondrial cluster. H^+ gradients created by these mitochondria may be then transferred to intermyofibrillar mitochondria through connecting mitochondrial filaments (a cable-like energy transporting system). Our data, demonstrating the existence of subsarcolemmal mitochondria in muscles in more oxidized and active state, as well as our findings of mitochondrial interconnections in the vicinity of the cell membrane are consistent with this theory of Skulachev. Furthermore, these mitochondrial interconnections may be important for synchronization of mitochondrial functional activities in muscle cells during contraction cycle, in addition to the metabolic feedback regulation of respiration via energy transfer networks [10–13,17,18]. In addition, our data support the concept of possible region specific specializations of mitochondria in living cells, although additional experimental evidence is required to examine this hypothesis. Moreover, we cannot exclude that mitochondrial functional heterogeneity we observed might reflect "metabolic" heterogeneity caused by subcellular localization or specific microenvironment of distinct mitochondrial subsets.

The metabolic differences between subsarcolemmal and intermyofibrillar mitochondria may have important functional and physiological consequences. Indeed, subsarcolemmal mitochondria are located close to the cell periphery and therefore exposed to higher oxygen levels than other mitochondria inside the cell. Such localization close to source of

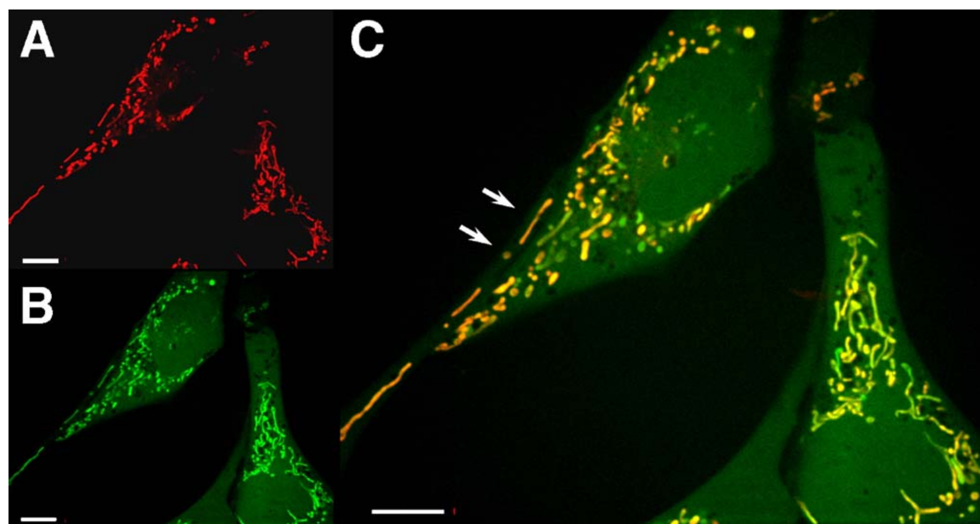

Fig. 5. Confocal fluorescent imaging of mitochondria in cultured HL-1 cardiomyocytes demonstrates heterogeneity of mitochondrial morphology and membrane potential. (A) Simultaneous confocal images of red fluorescence of mitochondrial membrane potential sensitive probe TMRM and (B) green fluorescence of ROS sensitive probe DCF. (C) Fluorescence of TMRM and DCF is shown as a merge image. Mitochondria in living HL-1 cells show clear different morphology: long thread and small isolated grain mitochondria (arrows). The homogeneous yellow-brown color of mitochondria is seen as a result of spatial colocalization of TMRM and DCF (C). However, some mitochondria with only green fluorescence indicate partially depolarized mitochondria with lower TMRM signal, demonstrating heterogeneity in mitochondrial membrane potentials. HL-1 cells were cultured as described in [29]. Scale bars, 10 μm.

oxygen may explain more oxidized state of this mitochondrial subset, potentially leading to more active mitochondrial respiration. According to Skulachev hypothesis [20], subsarcolemmal mitochondria may serve as a 'protection barrier' maintaining permissive levels of oxygen in the cell. Our data support this hypothesis. Indeed, this clustered, densely packed (Figs. 3 and 4), highly oxidized (Fig. 2) and probably actively respiring subsarcolemmal population of mitochondria may defend intracellular structures against high oxygen concentration outside the cell, providing thus important shielding mechanism against oxidative stress inside the cell. Functional features of subsarcolemmal mitochondria described above are in a good accordance with the findings demonstrating significantly higher expression of uncoupling protein-3 (UCP-3) in subsarcolemmal mitochondria as compared with intermyofibrillar subpopulation [21]. There is now increasing evidence that mitochondrial uncoupling proteins play a central role in the regulation of ROS production by the mitochondrial respiratory chain complexes [22]. Higher content of UCP-3 may, in turn, lead to "mild" uncoupling of mitochondria suppressing ROS overproduction due to high oxygen level in this region of the cell. This hypothesis is consistent also with our confocal imaging observation demonstrating that, despite existence of oxygen gradients, mitochondrial ROS levels in muscle fibers detected by either DCF-DA fluorescence or specific mitochondrial superoxide probe MitoSOX were similar in different mitochondrial subpopulations (data not shown). Therefore, both more active respiration and a "mild" uncoupling may compensate possible increase of ROS generation due to higher oxygen concentration near the cell membrane.

Interestingly, in HL-1 non-contracting cardiomyocytes, long thread mitochondria form a dynamic reticulum as in many other cell types (Fig. 5). However, it can be seen that a number of small isolated grain mitochondria can also be present in these cells, demonstrating thus very different mitochondrial morphology irrespective of a particular cellular region (e.g., periphery versus perinuclear region). Moreover, it can be seen that HL-1 cells do not exhibit the strictly regular ("crystal-like") mitochondrial distribution typical for rat cardiac cells [18]. It is known that proper regulation of mitochondrial dynamics is crucial for the cell. Mitochondrial morphology is regulated by fission and fusion processes, which may be modulated by organelle-associated proteins [23] or by energy substrates [24]. Thread–grain transitions might be transient and may occur under various stressful conditions, representing also an early event in apoptosis [25], however the metabolic consequences of these transitions as well as the presence of different mitochondrial morphologies in the cell remain largely unknown. Interestingly, cells with targeted null mutations in Mfn1 or Mfn2 and lacking ability for mitochondrial fusion show high degree of mitochondrial functional heterogeneity [26].

In contrast to skeletal muscles, HL-1 cells show clear heterogeneity of the fluorescence of the mitochondrial membrane potential sensitive probe TMRM and ROS sensitive probe DCF visible in green in the merge image combining TMRM and DCF fluorescence, which normally should result in uniform brownish color (Fig. 5C). Similar heterogeneity of ROS and large variations in mitochondrial membrane potentials among cells of the same type, and within one cell have also been shown in human carcinoma and some other cells [2,27]. The origin and biological implications of such morphological and functional heterogeneity of mitochondria are not clear and require further investigation using confocal imaging approach as a tool to address these questions. In this respect, our data show that HL-1 cells can be considered as a useful model to study highly dynamic mitochondrial transitions, fragmentation and heterogeneity under normal and pathological conditions (e.g., hypoxia–reoxygenation, oxidative stress and apoptosis).

Taken together, our data suggest an important physiological role of heterogeneity of mitochondria and mitochondrial subpopulations, as well as their possible cellular region-specific specializations.

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