Molecular and Cellular Biochemistry **256/257:** 359–365, 2004.

Subcellular heterogeneity of mitochondrial function and dysfunction: Evidence obtained by confocal imaging

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Abstract

Beyond their fundamental role in energy metabolism, mitochondria perform a great variety of other important functions (e.g. in Ca^{2+} homeostasis, apoptosis, thermogenesis, etc.), thus suggesting their region-specific specializations and intracellular heterogeneity. Although mitochondrial functional heterogeneity has been demonstrated for several cell types, its origin and role under physiological and, in particular, pathophysiological conditions, where the extent of heterogeneity may significantly increase, remain to be elucidated. The present work thus investigated the static and dynamic heterogeneity of mitochondria and mitochondrial function in various cell types in which mitochondria may cope with specific functions including cardiomyocytes, hepatocytes and some cultured carcinoma cells. Modern confocal and two-photon fluorescent microscopy was used for the investigation and direct imaging of region-specific mitochondrial function and heterogeneity. Analysis of the autofluorescence of mitochondrial flavoproteins in hepatocytes and carcinoma cells permitted significant intracellular heterogeneity of mitochondrial redox state to be demonstrated. Comparative homogeneity and clear colocalization of mitochondrial flavoproteins, membrane potential and calcium-sensitive probes were observed in both isolated cardiomyocytes and permeabilized myocardial fibers. After ischemia reperfusion, however, or under conditions of substrate deprivation, significant heterogeneity of all these parameters was detected. Some methodological issues, mechanistic aspects, possible metabolic consequences of mitochondrial functional heterogeneity and its impact under pathological conditions are discussed. (Mol Cell Biochem **256/257**: 359–365, 2004)

Key words: confocal imaging, heterogeneity, mitochondria, mitochondrial function

Abbreviations: BODIPY®FL-C_{11}-PC – 1,2-bis-(4,4-difluoro-5,7dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-sn-glycero-3-phosphocholine; CIR – cold ischemia reperfusion; DAF-2DA – 4,5-diaminofluorescein diacetate; DASPMI – 2-(p-dimethylaminostyryl) pyridyl methyl iodide; DCF – 2,7-dichlorodihydro-fluorescein diacetate; DHA – dihydroxyacetone; MPT – mitochondrial permeability transition; ROS – reactive oxygen species; TMRE – tetramethylrodamine ethyl ester

Introduction

Mitochondria are the main source of energy in the form of ATP in many cells and also perform a great variety of important functions. They synthesize amino acids, pyrimidines, lipids, heme, hormones and other metabolites. Mitochondria play key roles in maintaining cellular redox potential, ionic regulation (Ca^{2+} homeostasis), thermogenesis, etc. However, the interplay of these roles of mitochondria under both normal and pathological conditions is poorly understood. Moreo-

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ver, mitochondria are directly involved in pathophysiological mechanisms of ischemia reperfusion injury, oxidative stress, inherited diseases, toxicological injury, and side-effects of pharmacological treatments [1, 2]. Damaged mitochondria cause organ injury via several mechanisms including diminished cellular energy status, production of reactive oxygen species, disturbance of ionic balance, cytochrome *c* release and induction of apoptosis [1, 3–5]. A dysbalance between energy production and energy demand and a disturbance in energy transfer networks play important roles in many pathologies, and this complex problem is currently the subject of intensive research in several metabolic subspecialties of physiology. It has been shown that mitochondria localized in various cell regions may have different morphological and biochemical properties, demonstrating clear intrinsic heterogeneity [6–8]. Various mitochondrial subpopulations such as intermyofibrillar, subsarcolemmal and perinuclear mitochondria, are present in muscles, and may be differently involved in physiological [9] and pathological processes [10]. In addition, mitochondria subsets may show a different efficiency of ATP production in different regions of a cell. Therefore, valuable information regarding mitochondrial physiology and pathophysiology may be lost by analyzing 'averaged' mitochondria, for example in respirometric studies of isolated mitochondria, permeabilized cells or muscle fibers, enzyme analysis of tissue homogenates, etc. Laser-excited fluorescence measurements performed with perfused tissues, cell or mitochondrial suspensions can be used to assess mitochondrial function and dysfunction [11]. These studies, however, cannot discriminate the fluorescence of mitochondria in different intracellular regions. Alternatively, confocal fluorescence imaging distinguishes the signals from various mitochondrial subpopulations [9], small clusters or even from single mitochondrion, providing quantitative measurement of their regional dynamics. We here report that functional heterogeneity of mitochondria is a feature of many cell types and was observed in several ways with respect to their energetic status, redox potential and calcium content. Importantly, the extent of mitochondrial functional heterogeneity can significantly increase under pathological conditions, such as ischemia reperfusion injury.

Results and discussion

A confocal imaging study of mitochondria can be performed with intact and permeabilized cells and may include simultaneous imaging of the autofluorescence of flavoproteins and NADH (redox potentials), imaging of the mitochondrial inner membrane electrical potential using potentiometric mitochondria-specific dyes: TMRE, JC-1, DASPMI, and calcium with Rhod-2 and in various metabolic states. Table 1 summarizes possible approaches that can be used for direct imaging of various important mitochondrial parameters, as well as the functional heterogeneity of mitochondria between the cells or within a cell. Simultaneous measurement of flavoproteins (Fp) and NADH affords a unique opportunity for ratio imaging (Fp/NADH). Because of Fp and NADH, opposite fluorescence behavior, this Fp/NADH ratio can be used as a sensitive indicator of change in mitochondrial function [9, 22]. Thus, a digital imaging approach enables quantitative measurement of regional dynamics of mitochondria within a cell, providing valuable information on the complex functioning of mitochondrial systems for oxidative phosphorylation. Moreover, mitochondrial imaging can be topologically colocalized with imaging of region-specific production (cellular distribution) of ROS and NO, providing a link between mitochondrial local permeability transition, local changes in ROS and calcium. Importantly, confocal imaging of mitochondria can be easily combined with immunofluorescent methods in order to identify cytoskeletal components when studying the specific structural organization of the mitochondrial functional complexes and other cellular structures (intracellular energetic units) as a basis for effective energy metabolism [13, 23]. Thus, the important advantage is that this method directly visualizes the heterogeneity of mitochondria and complex mitochondrial functions and interactions within living or permeabilized cells [9, 15]. Furthermore, mitochondrial imaging permits regional intracellular defects to be detected in topological assays in clinical studies. This is of special importance for understanding the pathophysiological basis of several diseases involving compromised mitochondrial function.

Several mitochondrial subpopulations can be present in the tissue. In muscles, intermyofibrillar, subsarcolemmal and perinuclear mitochondrial subsets are clearly distinguishable by their intracellular location (Fig. 1). These subpopulations may be differently involved in intracellular metabolic pathways. Intermyofibrillar mitochondria might be mainly responsible for synthesizing ATP used for muscle contraction, and the subsarcolemmal subpopulation can also actively participate in energy production for ion pumping, whereas the role of mitochondria clustered around the nucleus is to sustain nuclear import [24]. Figure 1 clearly shows a regular mitochondrial arrangement typical for cardiomyocytes, and relatively homogeneous fluorescence of TMRE, a potential-sensitive probe, shows the homogeneity of mitochondrial membrane potential ($\Delta\Psi_m$), probably except for the perinuclear regions. The perinuclear mitochondrial clusters are also easily seen from their flavoproteins or NADH autofluorescence (not shown) and have a significantly higher signal than do intermyofibrillar mitochondria. This may reflect a compact packing or distinct intrinsic properties of these mitochondrial subsets. Subsarcolemmal and interfibrillar mitochondrial subpopulations with various functional (biochemical) properties were obtained from skeletal and cardiac

Table 1. Approaches for the investigation of mitochondrial function and heterogeneity by fluorescent confocal imaging

Parameter	Fluorescent Probe/marker/color	Localization	Reference
Mitochondrial redox state (flavoproteins)	Autofluorescence, green	Mitochondrial inner membrane	[9, 12]
Mitochondrial redox state (NADH)	Autofluorescence, blue	Mitochondrial matrix,cytoplasm	[13, 14]
Mitochondrial membrane potential ($\Delta\Psi_m$)	TMRE, red JC-1, red/green DASPMI/green	Mitochondrial inner membrane	[3, 9, 12, 15]
Mitochondrial calcium	Rhod-2, red	Mitochondrial matrix,	[16, 17]
Reactive oxygen species (ROS) production	DCF, green	Mitochondria, cytoplasm	[18, 19]
Nitric oxide (NO) production	DAF-2DA, green	Mitochondria, cytoplasm	[20]
Phospholipases (phospholipase A_2) activity	BODIPY®FL-C_{11}-PC, green	Cellular membranes	[21]

muscles using selective isolation procedures [6, 7]. In the heart, these two spatially separated mitochondrial subpopulations may be differently involved in physiological and pathological processes [25]. For example, the selective decline of oxidative phosphorylation after global warm ischemia in a perfused rabbit heart model was demonstrated solely in subsarcolemmal mitochondria [10]. Using an imaging technique, heterogeneity of mitochondrial redox potentials (autofluorescence of mitochondrial flavoproteins) was established in skeletal muscle, with a difference of more than four times in the signal observed between subsarcolemmal and intermyofibrillar mitochondrial subpopulations but with similar functional behavior [9]. In isolated cardiomyocytes heterogeneity of the mitochondrial redox state was visible as a metabolic wave propagation under conditions of substrate deprivation [12].

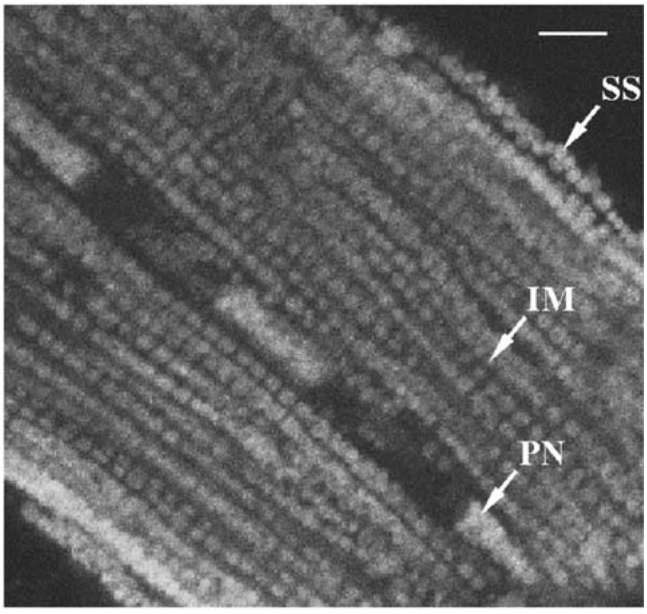

Fig. 1. Several subpopulations of mitochondria in a single rat myocardial fiber. The arrows show intermyofibrillar (IM), subsarcolemmal (SS) and clustered perinuclear (PN) mitochondria. Mitochondria were visualized using a fluorescent membrane potential-sensitive probe TMRE (50 nM). Scale bar, 5 μm.

Some clear examples of mitochondrial heterogeneity revealed in our study by fluorescent confocal microscopy are shown in Fig. 2. As mentioned above, our experiments with intact cardiomyocytes demonstrated rather homogeneous flavoprotein fluorescence and hence homogeneous distribution of the redox state of intermyofibrillar mitochondria. In line with previous finding [12] significant heterogeneity was observed only under conditions of substrate deprivation, where local regions with a high signal (probably due to the mitochondria's fully oxidized state) can be seen (Fig. 2A). However, our experiments showed no visible wave propagation of redox potential. In contrast to cardiac cells, mitochondrial clusters with a high flavoprotein signal were also observed in liver cells (hepatocytes) under normal conditions (Fig. 2B), despite the rather homogeneous distribution of fluorescence of another redox marker NADH (Fig. 2D). Strong heterogeneity of mitochondrial membrane potential was observed by monitoring TMRE fluorescence in cultured human MS57-8T carcinoma cells preloaded with 50 nM TMRE. Figure 2C shows a merge image combining flavoprotein (green) and TMRE (red) fluorescence. The intracellular regions with only green fluorescence (indicated by arrow) demonstrate deenergized mitochondria (collapsed membrane potential) with very low TMRE accumulation together with the normal mitochondria within the same cell. An interesting example of mitochondrial heterogeneity between cells is shown in Fig. 3, where simultaneous measurement of the fluorescence of mitochondrial flavoproteins, calcium (Rhod-2) and NADH was performed. Despite equivalent surrounding conditions, two MCF-7 carcinoma cells show very low flavoprotein fluorescence, which is indicative of highly reduced mitochondria (Fig. 3C; arrows), whereas almost all the mitochondria of two other cells were in a highly oxidized state. The difference between these two cell groups is also clearly seen in a merge image (Fig. 3D). In contrast to flavo-

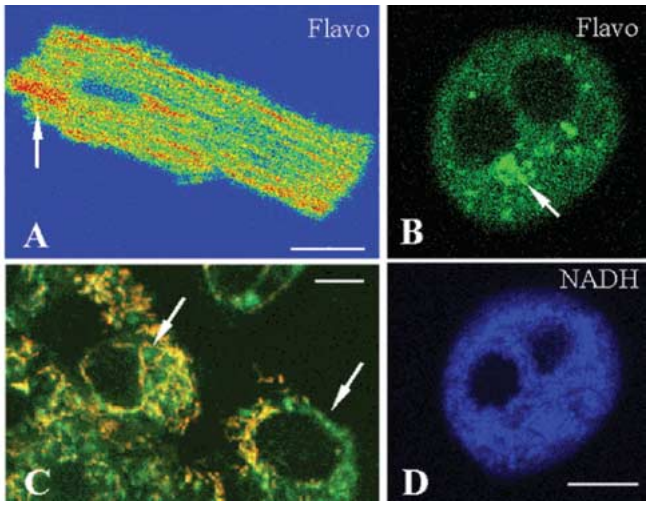

Fig. 2. Intracellular heterogeneity of the mitochondrial redox state and membrane potential in various cells. (A) Static heterogeneity of mitochondrial flavoprotein fluorescence in intact cardiomyocyte with substrate (glucose) deprivation. For better visualization of the changes in the fluorescence signal, an artificial color (rainbow) method was used. (B, D) Simultaneous imaging of flavoproteins and NADH fluorescence in intact liver cell (hepatocyte). In contrast to the homogeneous intracellular distribution of NADH fluorescence (D), individual clusters of mitochondria with high flavoprotein fluorescence (indicated by arrow) are seen (B). NADH fluorescence was monitored using two-photon laser excitation. C: Merge image combining fluorescence of mitochondrial flavoproteins (green) and TMRE (red) in MS57-8T carcinoma cultured cells. The intracellular regions with only green fluorescence (arrows) demonstrate deenergized mitochondria and a heterogeneity of mitochondrial membrane potential within the same cell. In all cases (A–D): scale bar, 10 μm.

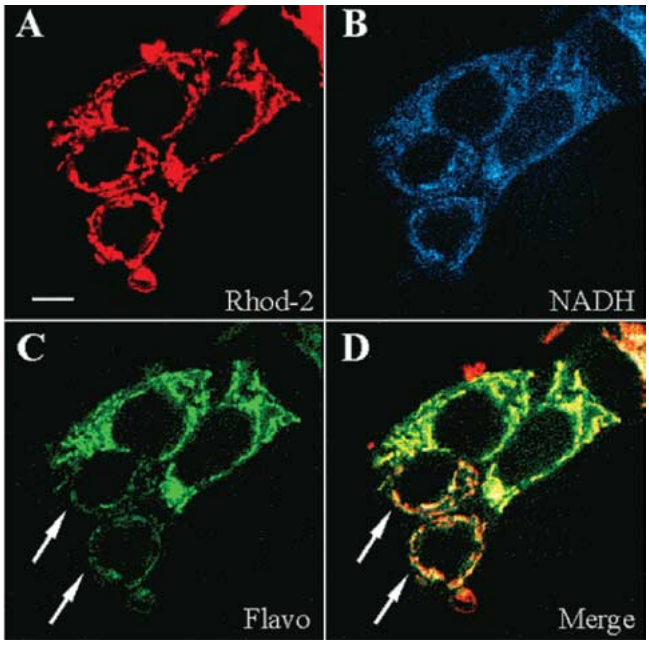

Fig. 3. Mitochondrial heterogeneity between cells under the same surrounding conditions. Simultaneous confocal images of fluorescence of mitochondrial calcium probe Rhod-2 (A), mitochondrial NADH autofluorescence (B), and mitochondrial flavoprotein autofluorescence (C) in MCF-7 carcinoma cells preloaded with 5 μM Rhod-2. (D) Merge image combining all three fluorescence images. NADH fluorescence was excited by two-photon infrared laser. Scale bar, 10 μm.

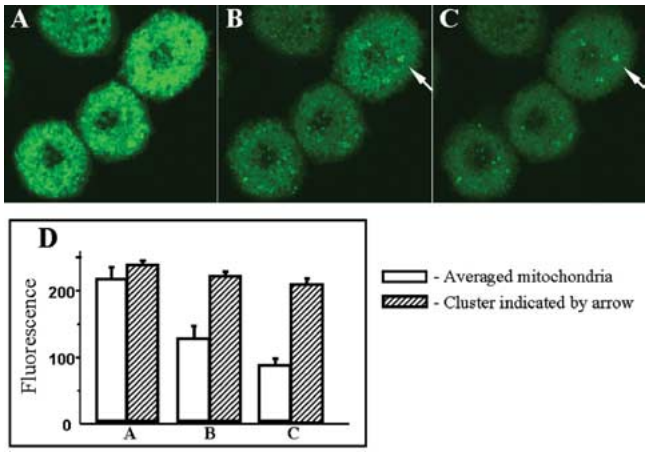

Fig. 4. Heterogeneity of mitochondrial dynamics in intact hepatocytes in response to substrate addition. Intensive fluorescence of mitochondrial flavoproteins (A) remarkably decreases in almost all mitochondria after addition of 10 mM dihydroxyacetone (B) and further decreases after 5 min of incubation (C). (D) Quantitative measure of change (arbitrary units) in fluorescence of small 'non-responsive' clusters (indicated by arrow) and in averaged mitochondrial fluorescence outside these clusters. Error bars show S.D.

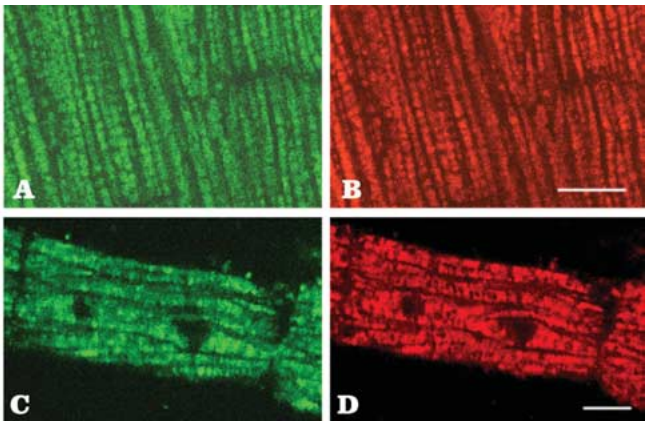

Fig. 5. Heterogeneity of mitochondrial flavoprotein redox state and calcium after cardiac ischemia reperfusion injury. Simultaneous confocal images of the fluorescence of mitochondrial flavoproteins (A) and the calcium-specific probe Rhod-2 (B) within permeabilized myocardial fibers isolated from control rat hearts. Permeabilized myocardial fibers preloaded with 5 μM Rhod-2. (C, D) Corresponding images after ischemia and *in vivo* reperfusion show irregularities in both flavoproteins and Rhod-2 fluorescence. Scale bar, 10 μm.

protein fluorescence, the fluorescence signal of NADH and Rhod-2 (mitochondrial calcium) was identical in all four cells (Figs 3A and 3B).

Importantly, static heterogeneity of mitochondria (distinct morphology and biochemical properties of mitochondrial subsets) can be distinguished from dynamic heterogeneity where individual time behavior and distinct time responses of mitochondria are observed. The dynamic and static heterogeneity of mitochondrial flavoproteins were observed in experiments with intact hepatocytes (Fig. 4). Mitochondrial flavoproteins are fluorescent in their oxidized state and show some minor initial heterogeneity of fluorescence (Fig. 4A). Flavoproteins were then partially reduced by adding substrate, 10 mM dihydroxyacetone (DHA), which resulted in a strong decline in fluorescence in almost all mitochondria (Figs 4B and 4C). However, some small mitochondrial clusters (indicated by arrow) or single mitochondrion maintained at almost the initial fluorescence signal (Fig. 4D), very clearly demonstrating their different response to DHA. Obviously, this effect can not be explained by diffusion or other restrictions on substrate transport since 'responding' and 'non-responding' mitochondria are distributed equally within cells (Figs 4B and 4C). Functional static and dynamic heterogeneity of mitochondria were recently reported for a number of cells including HUVEC, astrocytes, HeLa cells, with kinetic differences between perinuclear and peripheral mitochondria [15]. The authors of this elegant study suggested that different mitochondrial subpopulations, clusters or even single mitochondrion may carry out diverse processes in distinct regions of a cell. However, many important aspects were not addressed, particularly concerning metabolic consequences of mitochondrial heterogeneity and physiological and pathophysiological impacts of this very interesting phenomenon. Previous findings and our results indicate that the structural organization of a cell, as well as local energy and other cellular demands, may determine features of mitochondrial region-specific functional behavior, leading to functional specialization of mitochondria. This may very well be related to the general problem of regulation of energy metabolism.

The phenomenon of subcellular compartmentalization, the existence of microdomains with restricted diffusion, functional enzyme coupling and direct channeling result in strong metabolic heterogeneity [23, 26–28]. Accordingly, the environment of each single mitochondrion can significantly differ from that of other mitochondria, potentially causing region-specific alterations in mitochondrial properties and function in addition to the intrinsic mitochondrial heterogeneity. Therefore, an important and still unresolved question is how the heterogeneity of mitochondrial function and regional specializations of mitochondria are genetically defined and to what extent this heterogeneity may be dependent on environmental aspects.

Heterogeneous mitochondrial damage is suggested in various pathologies including myopathies, ischemia reperfusion and apoptosis, where heterogeneous release of mitochondrial cytochrome *c* has been directly demonstrated [29, 30]. Mosaic distribution of genetic mitochondrial defects (e.g. COX deficiency) is well established in numerous diseases. Elevated production of ROS and NO (oxidative stress) occurring in various pathological processes results in modification of mitochondrial proteins and phospholipids and may cause cyclosporin A-sensitive pores to open in the inner mitochondrial membrane (permeability transition) [3, 31, 32]. Thus, the original heterogeneity of mitochondria can be supplemented by the appearance of mitochondrial subsets with a different severity or pattern of injury. This second heterogeneity may be a consequence of non-equal oxygen, pH, calcium and ROS distribution in the cell. Since mitochondrial damage inhibits oxidative phosphorylation and increases ROS, heterogeneity of injury would cause spatial heterogeneity of ATP and ROS production. Both local energy deficit and elevated ROS generation are damaging for mitochondria in this particular region, and in turn, cause a significant increase in the extent of heterogeneity and thus an amplification of damage. In pathological cells, in various metabolic diseases, or when substrates deprivation is given, this increase in the extent of heterogeneity may correlate with the degree of injury, which would have a critical impact on energy metabolism and cell viability. Moreover, different mitochondrial subsets may be differently involved in the pathophysiological process, have diverse sensitivities to injury, or cause dissimilar metabolic consequences. In apoptosis, heterogeneity of mitochondrial function and cytochrome *c* release may play a crucial role as determinants of apoptotic pathways [4, 5, 30]. In these pathways, one mitochondrial subpopulation with dissipated membrane potential and suppressed oxidative phosphorylation supplies cytochrome *c* for activation of caspases and induction of apoptosis, while another mitochondrial subpopulation with normal membrane potential, cytochrome *c* content and respiration provides the ATP production necessary for execution of the apoptotic program [33].

Studies of ischemic canine heart revealed that the distribution and extent of the ultrastructural alterations were not uniform in the ischemic zone, showing a striking heterogeneity in the extent of damage [34]. This suggests that, at least in the early stages, ischemic damage may advance at greatly varying rates in different regions. It was demonstrated that not all structures were equally affected. While in the one fiber the myofilaments were well preserved and mitochondria were severely injured with ruptured membranes and low-density matrix, the neighboring fiber may show intact mitochondria and sarcolemma and, at the same time, completely disorganized myofibrils. Importantly, ischemic injury in the heart does not evolve in a uniform manner, and regional differences in metabolism and energy requirements exist in ischemic myocardium. Myocardium injury and tissue necrosis usually

originate in the endocardium and, with time, migrate toward to the epicardial surface as a 'wave front of cell death' [35]. Furthermore, earlier studies using NADH fluorescence imaging of perfused rat heart surface revealed extremely steep oxygen gradients in ischemia and, therefore, heterogeneous anoxic regions of myocardium surrounded by sharp areas with an almost normal oxygen supply [36]. Direct, non-invasive indicators, NADH videofluorimetry and microvascular *p*O2 measurement using the Pd-porphyrin phosphorescence technique have been applied to assess the balance between oxygen supply and consumption [37]. These indicators have shown a relatively homogeneous distribution under physiological conditions, supporting the idea of regional matching of oxygen supply with oxygen consumption. NADH videofluorimetry, however, has demonstrated large increases in heterogeneity for this matching and the existence of steep oxygen gradients in compromised (ischemic, hypoxic) hearts.

The increase in mitochondrial heterogeneity after ischemia reperfusion injury of the heart was apparent when analyzing two important mitochondrial parameters, redox state and calcium (Fig. 5). Permeabilized myocardial fibers isolated from control rat hearts showed the regular mitochondrial arrangement usual for cardiac tissue, and rather homogeneous fluorescence intensity of flavoproteins and Rhod-2, a specific probe for mitochondrial calcium, showed homogeneity of mitochondrial redox state and calcium (Figs 5A and 5B). However, after 10 h cold storage in University of Wisconsin (ViaSpan) solution, rat heart transplantation (1 h second ischemia), and subsequent *in vivo* reperfusion myocardial fibers showed functional mitochondrial heterogeneity with respect to their redox state and matrix calcium, visible as irregular distribution of intensity of flavoprotein and Rhod-2 fluorescence (Figs 5C and 5D). These findings suggest that discrete subpopulations of mitochondria with various degrees of mitochondrial damage may co-exist upon ischemia reperfusion. Interestingly, heterogeneity of mitochondrial calcium has been demonstrated in the model of simulated (chemical) ischemia, in NaCN-treated (poisoned) cardiac cells [38]. These cells showed groups of mitochondria that expelled calcium from the matrix, probably due to depressed membrane potential at onset of permeability transition. Thus, ischemia reperfusion of the heart causes heterogeneous mitochondrial function, most probably as a result of heterogeneous mitochondrial damage owing to local production of reactive oxygen species and region-specific mitochondrial permeability transitions. Such a mitochondrial imaging approach may therefore create a basis for topological estimation of mitochondrial injury in many pathologies.

In summary, the problem of mitochondrial heterogeneous damage is central in studies of mitochondrial diseases, in studies of mechanisms of apoptosis and ischemia reperfusion injury. However, numerous questions regarding mitochondrial specialization and integration in various pathways or cell functions remain unanswered, just as the origin and role of heterogeneity of mitochondria under physiological and, in particular, pathophysiological conditions remain to be elucidated. We believe that the study of heterogeneous mitochondrial function represents a new area in the general concept of 'bio-complexity' and will potentially lead to the integration of mitochondrial bioenergetics and cell physiology with various physiological and pathophysiological implications. New information on the role of heterogeneous function and damage of mitochondria will be important if we are to understand the basic mechanisms of mitochondrial regulation in normal cells and its impairment in various pathologies. This, in turn, will open a promising avenue for the development of new strategies in the diagnosis and therapy of many metabolic diseases.

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