- BRIEF COMMUNICATIONS

Functional heterogeneity of mitochondria after cardiac cold ischemia and reperfusion revealed by confocal imaging

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1. Abstract

Background.

Mitochondria play a critical role in ischemia-reperfusion injury of the heart. The purpose of the present study was to analyze the intracellular region-specific functional state of mitochondria after cold ischemia-reperfusion in a rat heart transplant model.

Methods.

Imaging of the mitochondrial functional state in situ in nonfixed myocardial fibers was performed by confocal microscopy of mitochondrial flavoprotein autofluorescence as redox state indicator; fluorescence of Rhod-2, a specific probe for mitochondrial calcium; and of tetramethylrhodamine ethyl ester fluorescence to monitor the mitochondrial membrane potential.

Results.

This imaging demonstrated that, in contrast to control fibers, 10-hr heart cold storage, heterotopic cardiac transplantation, and 24-hr reperfusion result in a highly heterogeneous mitochondrial functional state (mitochondrial calcium content, redox state, and inner membrane potential), thus suggesting local permeability transitions and heterogeneous mitochondrial damage.

Conclusions.

Imaging of in situ mitochondria allows topologic assessment of mitochondrial defects and heterogeneity, consequently providing new insights into the mechanisms of cardiac ischemia-reperfusion injury.

Mitochondrial damage plays a central role in ischemia-reperfusion injury ([1]). Intracellular functional heterogeneity of mitochondria has been demonstrated for several cell types, suggesting that various mitochondrial subpopulations may be differently involved in physiologic and pathologic processes ([2]). Mitochondrial subpopulations may be differently involved in physiologic and pathologic processes including cardiomyopathy, apoptosis, and normothermic ischemia-reperfusion. Using an imaging technique, the heterogeneity of the mitochondrial redox state has been established in skeletal muscle ([3]) and isolated cardiomyocytes ([4]). Heterogeneity of mitochondrial calcium has been shown in the model of simulated (chemical) ischemia of cardiac cells after cyanide treatment, with single or small groups of mitochondria that have expelled their matrix calcium ([5]). Heterogeneous mitochondrial damage is suggested in various abnormalities including ischemia-reperfusion injury and apoptosis, where heterogeneous release of mitochondrial cytochrome *c* has been demonstrated ([6]). In the context of organ preservation and transplantation, the present work investigated mitochondrial heterogeneity in situ in nonfixed myocardial fibers after cold ischemia, heterotopic cardiac transplantation, and subsequent reperfusion using a rat heart model. Long-term normothermic reperfusion was used to mimic the clinical situation in heart transplantation. Confocal fluorescent microscopy was applied for the investigation and direct imaging of the heterogeneity of the mitochondrial functional state with simultaneous recording of several mitochondrial parameters, including mitochondrial calcium, inner membrane potential, permeability changes, and redox state.

Adult male Lewis rats were used in a syngeneic heart transplantation model. All animals were housed under standard conditions with free access to diet and water according to local guidelines and the Austrian Animal Care Law. All experiments were performed with approval of the National Animal Welfare Committee and followed the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 85-23, revised in 1985). Heterotopic cardiac transplantation was performed according to the technique described by Ono and Lindsey ([7]). After intravenous injection of heparin into the donor, hearts were flushed in a retrograde fashion through the ascending aorta with ice-cold histidine-tryptophan-ketoglutarate preservation solution and subsequently stored on ice for 10 hr at 0°C in the same preservation solution. Before transplantation, grafts were again flushed through an intra-aortic cannula. The donor aorta and pulmonary artery were anastomosed to the recipient abdominal aorta and inferior vena cava. Vascular clamps were removed and hearts were reperfused after exactly 30 min of anastomosis (second ischemia). After reperfusion for 24 hr, relaparotomy was performed under terminal anesthesia. Permeabilized myocardial fibers were isolated from the left ventricle and prepared by gentle agitation in the relaxing solution supplemented with saponin as described ([3]). Hearts without cold storage and transplantation served as controls. Permeabilized myocardial fibers were fixed at both ends in a Heraeus Flexiperm chamber (Hanau Instruments, Hanau, Germany). The fully oxidized state of mitochondrial flavoproteins (maximal fluorescence intensity) was achieved by substrate deprivation and equilibration of the medium with air. To detect the mitochondrial functional state at the level of the single mitochondrion, fluorescent images were acquired and analyzed using a confocal microscope (LSM510-NLO; Carl Zeiss, Jena, Germany) with a 40× water immersion lens (objective, NA 1.2). The use of such a water immersion prevented geometric aberrations. Flavoprotein fluorescence was measured using 488 nm for excitation and 505 to 550 nm for emission. Rhod-2 and tetramethylrhodamine ethyl ester (TMRE) fluorescence was measured using 543 nm for excitation and greater than 580 nm for emission. These fluorescent images of exclusively mitochondrial origin were compared in permeabilized control fibers and after 10 hr of heart cold storage, heterotopic cardiac transplantation, and 24 hr of reperfusion, conditions that produce diminished contractile performance and a complex pattern of multiple mitochondrial damage, including damage of complex I, mitochondrial uncoupling, and cytochrome *c* release.

Cardiac mitochondria are organized in a “lattice” of parallel rows surrounding the contractile myofilaments. In controls, the regular banded mitochondrial arrangement typical for cardiomyocytes was clearly seen from the fluorescence pattern of mitochondrial flavoproteins, whereby the relatively homogeneous signal showed the homogeneity of the mitochondrial redox state (Fig. 1A). Simultaneous red fluorescence imaging of a specific mitochondrial calcium probe (4 μM) showed the relative homogeneity of the mitochondrial Ca^{2+} content in individual fibers (Fig. 1C). Moreover, clear spatial co-localization of Rhod-2 (red) and flavoprotein (green) fluorescence can be seen from the uniform brown color of mitochondria in the merge image (Fig. 1E). Similarly, imaging of myocardial fibers preloaded with

mitochondrial inner membrane potential sensitive-probe TMRE (50 nM) demonstrated a typical regular mitochondrial distribution and homogeneous pattern of mitochondrial membrane potential ($\Delta\Psi_m$) (not shown). After cold ischemia-reperfusion (CIR), however, myocardial fibers showed irregularities in the fluorescence of mitochondrial flavoproteins caused by regional differences in their redox state (Figs. 1B and 2A). Simultaneous imaging of Rhod-2 fluorescence in these fibers showed numerous discrete "black holes" (Fig. 1D), indicating mitochondria that lost Ca^{2+}, also visible as green spots in the merge image combining Rhod-2 and flavoprotein fluorescence (Fig. 1F).

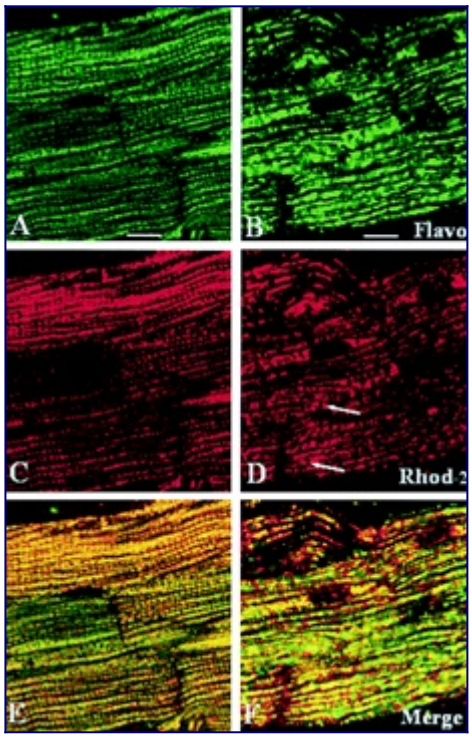

Figure 1: *Heterogeneity of mitochondrial redox state and calcium in permeabilized myocardial fibers after cold ischemia and reperfusion. (A) Simultaneous confocal images of green mitochondrial flavoprotein autofluorescence and (C) red fluorescence of mitochondrial calcium probe Rhod-2 (4 μM) in permeabilized myocardial fibers isolated from control rat hearts. (B and D) Corresponding images after cold ischemia-reperfusion show mitochondrial irregularities and heterogeneity distributed equally within fibers. (D, arrows) Mitochondria with low Rhod-2 fluorescence, indicating loss of mitochondrial Ca^{2+}. (E and F) Fluorescence of mitochondrial flavoproteins and Rhod-2 is shown as a merge image. (E) In control fibers, the homogeneous brown color of mitochondria is seen as a result of exact spatial co-localization of flavoproteins and Rhod-2. (F) In contrast, widespread numbers of mitochondria with only flavoprotein (green) or only Rhod-2 (red) fluorescence are seen after CIR (bar=10 μm).*

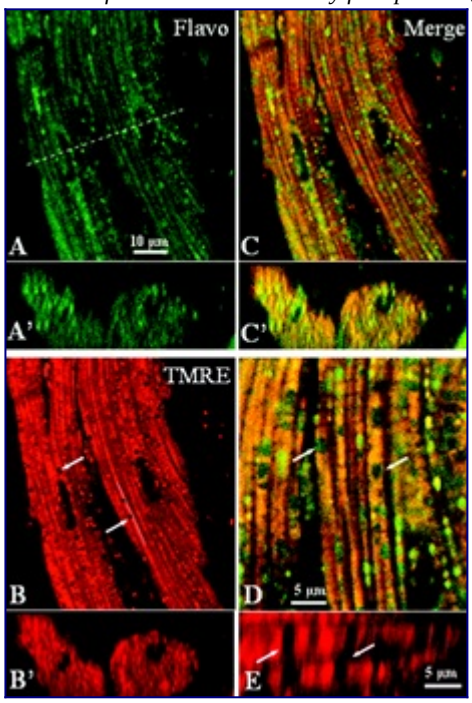

Figure 2: *Heterogeneity of mitochondrial membrane potential and local MPT after CIR detected by TMRE. (A) Simultaneous confocal images of green mitochondrial flavoprotein autofluorscence and (B) red fluorescence of $\Delta\Psi_m$ probe TMRE (50 nM) in permeabilized myocardial fibers from hearts after CIR. (B, arrows) Depolarized mitochondria lacking TMRE fluorescence. (C) Fluorescence of mitochondrial flavoproteins and TMRE is shown as a merge image. As in Figure 1 (F), numerous mitochondria with only flavoprotein (green) fluorescence are seen (bar=10 μm). (D) Merge image acquired with higher magnification. As in B, depolarized mitochondria as a result of local MPT are shown (arrows) (bar=5 μm). (A'–C') Corresponding Z cross-sections (A, dashed line). (E) Z section through B longitudinal to the fiber (B, dotted line), showing overlying-underlying planes of mitochondria. (arrows) Pairs of de-energized mitochondria with low TMRE fluorescence (black holes), demonstrating possible synchronization of permeability transition between two mitochondrial pairs along the z-axis and thus reflecting possible communication between adjacent mitochondria (electrical coupling) within a single sarcomere as described in Zorov et al ([8]) (bar=5 μm).*

In CIR-injured hearts, similar black holes in TMRE fluorescence (Fig. 2B and E) and spots with only green flavoprotein fluorescence in merge images (Fig. 2C) show individual de-energized mitochondria, lacking TMRE sequestration because of collapse of $\Delta\Psi_m$. These depolarized mitochondria are more clearly seen at higher magnification (Fig. 2D), indicating the CIR-induced local mitochondrial permeability transition (MPT) in some mitochondria as a possible cause of the loss of mitochondrial calcium shown in Figure 1(D). Recently, similar local MPT has been demonstrated as a result of photoactivation in isolated adult cardiomyocytes together with increased production of reactive oxygen species (ROS) at sites of spontaneously de-energized mitochondria ([8]). A large body of evidence points to MPT and the subsequent cellular energy deprivation as a fundamental mechanism for ischemia-reperfusion cell injury and death ([1]). Subsarcolemmal and intermyofibrillar mitochondrial subpopulations have been described in muscles ([9]). They are clearly distinguishable by their intracellular location and may be differently involved in physiologic and pathologic processes. Moreover, different functional behavior of these subpopulations has been reported in situ ([3]). However, CIR-induced mitochondrial heterogeneity was observed over the entire fibers (Figs. 1 and 2) and therefore was not related to the differences between intermyofibrillar and subsarcolemmal mitochondria.

Thus, our results show that CIR causes abnormal intracellular distribution of mitochondrial redox-electrical potentials and calcium, most likely because of heterogeneous mitochondrial damage as a determinant of ischemia-reperfusion injury of the heart. This heterogeneous damage can be strongly related to mitochondrial ([8]) or nonmitochondrial ([10]) local ROS production, local MPT gating, and heterogeneous cytochrome *c* release. However, additional experimental evidence is required to examine this hypothesis. In the future, confocal imaging of the mitochondrial functional state together with region-specific ROS production should be used to analyze human myocardial biopsy specimens for local mitochondrial abnormalities induced by ischemia-reperfusion. Direct imaging of various functional parameters of mitochondria allows topologic assessment of mitochondrial defects, thus providing a greater understanding of the mechanisms of ischemia-reperfusion injury and potentially leading to new diagnostic approaches and additional improvements in clinical care.

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