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Original article

Mitochondrial instability during regional ischemia–reperfusion underlies arrhythmias in monolayers of cardiomyocytes

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ABSTRACT

Regional depolarization of the mitochondrial network can alter cellular electrical excitability and increase the propensity for reentry, in part, through the opening of sarcolemmal K_{ATP} channels. Mitochondrial inner membrane potential ($\Delta\Psi_m$) instability or oscillation can be induced in myocytes by exposure to reactive oxygen species (ROS), laser excitation, or glutathione depletion, and is thought to be a major factor in arrhythmogenesis during ischemia–reperfusion. Nevertheless, the correlation between $\Delta\Psi_m$ recovery kinetics and reperfusion-induced arrhythmias has been difficult to demonstrate experimentally. Here, we investigate the relationship between subcellular changes in $\Delta\Psi_m$, cellular glutathione redox potential, electrical excitability, and wave propagation during coverslip-induced ischemia–reperfusion (IR) in neonatal rat ventricular myocyte (NRVM) monolayers. Ischemia led to decreased action potential amplitude and duration followed by electrical inexcitability after ~15 min of ischemia. $\Delta\Psi_m$ depolarization occurred in two phases during ischemia: in phase 1 (<30 min ischemia), mitochondrial clusters within individual NRVMs were depolarized, while phase 2 $\Delta\Psi_m$ depolarization (30–60 min) was characterized by global functional collapse of the mitochondrial network across the whole ischemic region of the monolayer, typically involving a propagating metabolic wave. Oxidation of the glutathione (GSSG:GSH) redox potential occurred during ischemia, followed by recovery upon reperfusion (i.e., lifting the coverslip). $\Delta\Psi_m$ recovered in the mitochondria of individual myocytes quite rapidly upon reperfusion (<5 min), but was highly unstable, characterized by subcellular oscillations or flickering of clusters of mitochondria in NRVMs across the reperfused region. Electrical excitability also recovered in a heterogeneous manner, providing an arrhythmogenic substrate which led to formation of sustained reentry. Treatment with 4′-chlorodiazepam, a peripheral benzodiazepine receptor ligand, prevented $\Delta\Psi_m$ oscillation, improved GSH recovery rate, and prevented reentry during reperfusion, indicating that stabilization of mitochondrial network dynamics is an important component of preventing post-ischemic arrhythmias. This article is part of a Special Issue entitled 'Mitochondria'.

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1. Introduction

Although the relative rate of mortality from cardiovascular disease has declined, coronary heart disease remains a leading cause of death, responsible for ~1 of every 6 deaths in the United States [1]. Ischemia caused by coronary occlusion, thrombosis, or spontaneous spasm leads to a variety of changes in ion homeostasis, energetics and oxidative processes that contribute to mitochondrial and cellular injury. Reperfusion of the ischemic area is the first line therapy to decrease ischemic damage and prevent cell death; however, reperfusion itself can lead to a more severe damage, a phenomenon known as reperfusion injury [2–4]. Reperfusion is also associated with an increased risk of potentially fatal arrhythmias [5–7].

Several arrhythmogenic mechanisms have been proposed to be involved in ischemia–reperfusion (IR)-induced arrhythmias. In pathological situations such as IR, abnormal automaticity and triggered activity [8] arising from ectopic pacemakers in the myocardium, particularly near the border zone between well-perfused and ischemic or infarcted tissue, can contribute to increased risk of arrhythmias [9]. Heterogeneous alterations in gap junctional conductance, resting membrane potential, excitability, and action potential duration (APD) together promote reentrant arrhythmias by increasing dispersion of refractoriness, slowing conduction velocity (CV), and/or creating regional excitation block (for a detailed review see ref. [10]).

The ability of the mitochondrial network of the cardiomyocyte to restore energy production, control reactive oxygen species (ROS), restore proton and ion gradients, and limit necrotic and apoptotic cell

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death is a key determinant of survival after IR. Mitochondrial failure can scale to affect cardiac cell function, and ultimately to the organ level, causing electrical and contractile dysfunction [11]. Dynamic instabilities of mitochondrial inner membrane potential ($\Delta\Psi_m$) are known to occur as the level of oxidative stress on the mitochondria increases. If a number of mitochondria in the network approach a threshold of redox stress, a state called mitochondrial criticality is reached, rendering the mitochondria hypersensitive to small perturbations. In this state, depolarization of only a few mitochondria can lead to a propagated wave of depolarization, complete collapse, or oscillation of $\Delta\Psi_m$ in the whole network of a cardiac myocyte [12–14]. Coupling between mitochondria occurs through the autocatalytic mechanism called ROS-induced ROS-release (RIRR) [15–17]. RIRR was originally demonstrated by increased ROS generation via photodynamically-induced mitochondrial depolarization [15]. However, an increase in mitochondrial ROS generation due to inhibition of scavenging systems can also lead to a similar critical state [18]. In its initial description, activation of the permeability transition pore (PTP) was suggested as the main mechanism of RIRR [15], as evidenced by inhibition with the adenine nucleotide translocase inhibitor bongkrekic acid and by an increase in inner membrane calcein permeability; however, cyclosporine A (CsA) could not prevent RIRR induced by laser excitation [15]. Our previous studies revealed that RIRR also underlies self-sustained cell-wide mitochondrial oscillations in adult cardiomyocytes triggered either by highly localized laser excitation [19], or glutathione depletion [18]. These oscillations were also insensitive to CsA and independent of $[Ca^{2+}]_c$, but do not involve a large change in membrane permeability. Ligands of the mitochondrial benzodiazepine receptor, such as 4′-chlorodiazepam (4′-Cl-DZP) or PK-11195, were shown to stabilize the stressed mitochondria and prevent depolarization and oscillation of $\Delta\Psi_m$ [19]. These effects were interpreted to suggest that activation of the ROS-sensitive energy-dissipating inner membrane anion channel (IMAC) [20,21] was the main mechanism involved in RIRR and the mitochondrial instability resulting from oxidative stress [19].

With regard to the effects of $\Delta\Psi_m$ loss or oscillation on cellular electrophysiology, decreasing cellular ATP/ADP ratio during $\Delta\Psi_m$ depolarization can activate sarcolemmal K_{ATP} channels and profoundly alter cellular electrical excitability and APD [22,23]. In isolated cardiomyocytes subjected to metabolic stress, $I_{K,ATP}$ oscillated in synchrony with $\Delta\Psi_m$ depolarization, and induced APD oscillations [24]. We proposed that the resulting heterogeneous spatiotemporal instability of $\Delta\Psi_m$ and action potentials (AP) would increase the dispersion of repolarization in the myocardium, increasing the vulnerability to cardiac arrhythmias. This was supported by experiments in intact perfused hearts subjected to antioxidant depletion or IR, whereby 4′-Cl-DZP suppressed arrhythmias and preserved cardiac contractile function [11,25–27]. However, cellular oscillations in $\Delta\Psi_m$ following IR were not demonstrated in the intact heart, in part due to technical limitations.

Recently, using computational and experimental methods, we explored the impact of regional mitochondrial depolarization, i.e., the formation of a metabolic current sink, on electrical propagation and arrhythmogenesis in monolayers of cardiomyocytes [28]. Induction of metabolic sinks, through regional chemical uncoupling of mitochondria in NRVM monolayers, decreased AP amplitude (APA) and caused slowing of conduction velocity (CV), shortening of APD and wavelength, and finally, inexcitability of the sink area. Using glibenclamide to inhibit K_{ATP} channels, we provided evidence that the opening of K_{ATP} channels was a major factor in producing these effects, as well as in causing reentry. Since IR induces much more complex changes in ion balance, ROS, and energetics than simple chemical uncoupling of oxidative phosphorylation, it remains to be determined if similar arrhythmogenic mechanisms are involved in the response to IR. To explore this, here we utilize a coverslip-induced IR model [29,30] to study the electrophysiological effects, cytosolic GSH/GSSG redox and $\Delta\Psi_m$ responses to IR in NRVM monolayers. The findings reveal complex patterns of $\Delta\Psi_m$ loss and recovery during ischemia and reperfusion, which strongly influence the occurrence of post-ischemic reentrant arrhythmias.

2. Materials and methods

2.1. NRVM monolayers

Ventricles of 2-day old neonatal Sprague–Dawley rats (Harlan Laboratories) were excised, chopped into small pieces, washed with HBSS (Invitrogen) and then trypsinized overnight at 4 °C. All animal protocols conformed to the guidelines of the National Institutes of Health [31]. The next day, cardiomyocytes were isolated using collagenase and two rounds of preplating were done to reduce the fibroblast concentration. 10^6 cells were plated on each fibronectin-coated (25 μg/ml) circular cover glass (D = 22 mm) and cultured in Medium 199 (Invitrogen) containing 10% heat-inactivated bovine serum (Invitrogen). The medium was changed daily. Experiments were performed on beating and confluent monolayers on the 3rd to 5th days of culture.

2.2. Inducing ischemia and reperfusion

Experiments started by equilibration of the monolayers with a modified Tyrode's solution consisting of (in mmol/l): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 0.33 NaH_2PO_4, 5 HEPES, and 5 glucose. The monolayer was paced by application of voltage pulses at 1 Hz at the edge of the monolayer using bipolar point electrodes. A 15 mm circular glass coverslip (#1, Fisher Scientific) was placed on the center of the monolayer to reduce availability of nutrients and O_2 and create a restricted extracellular space, hence inducing ischemic conditions. Reperfusion was performed by removing the coverslip after 1 h of ischemia.

2.3. Measuring $\Delta\Psi_m$

To study the effect of IR on mitochondrial function, the fluorescent potentiometric indicator tetramethylrhodamine methyl ester (TMRM) was used to record $\Delta\Psi_m$. Monolayers were loaded with 50 or 100 nmol/l TMRM for 1 h in the 37 °C incubator. They were then washed and put in normal Tyrode's solution in the heated (37 °C) chamber of an inverted microscope (Eclipse TE2000-E, Nikon). Excitation light of 545 ± 12 nm was used and the emitted fluorescence (605 ± 35 nm) was recorded with an EMCCD camera (Cascade II, Photometrics, Tucson, AZ) using Micro-Manager (Vale Lab, UCSF, CA) or custom software developed in LabVIEW (Texas Instruments, Dallas, TX). Mitochondrial depolarization leads to loss of TMRM from the matrix and into the cytoplasm, which causes a decrease in the spatial dispersion of TMRM fluorescence. Spatial dispersion was quantified by calculating the coefficient of variation of the image fluorescence intensity, defined as the ratio of standard deviation to the mean (SD/mean), a dimensionless measure that provides a superior indicator for mitochondrial polarization than fluorescence intensity alone [32,33], avoiding artifacts related to changes in dye load, illumination, bleaching, etc. (see Supplemental Fig. 1). The data was analyzed using ImageJ, MATLAB (MathWorks, Natick, MA), and Origin (OriginLab, Northampton, MA).

2.4. Measuring oxidative stress

Cyto-Grx1-roGFP2 [34] adenovirus was added to the media on the second day of culture. Experiments were done on the 4th–5th days of culture. Images were taken using a spinning disk confocal microscope (Andor Revolution XD, Olympus) at 10× magnification and were analyzed using ImageJ and MATLAB. For each data point, two images were taken using laser excitation at 405 nm and 488 nm and recording the emission at 525 ± 25 nm. The excitation ratio (405 nm/488 nm)

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