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Optical imaging of mitochondrial function uncovers actively propagating waves of mitochondrial membrane potential collapse across intact heart

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Abstract

Polarization of the mitochondrial membrane potential ($\Delta\Psi_m$) is critical for normal mitochondrial function and cellular energetics. Mitochondrial dysfunction, manifesting as disrupted $\Delta\Psi_m$ polarization (i.e. *depolarization or hyperpolarization*), underlies several important and highly prevalent diseases, including a variety of cardiac and neurological disorders. As such, $\Delta\Psi_m$ instability might form a unifying mechanism for a class of metabolic disorders affecting excitable tissues. Here, we measured the spatiotemporal kinetics of $\Delta\Psi_m$ changes across the intact heart using high-resolution optical $\Delta\Psi_m$ imaging and uncovered surprisingly complex spatial patterns and dynamically fluctuating changes in $\Delta\Psi_m$ that developed into actively propagating waves of mitochondrial depolarization during global ischemia. Our data further indicated that the recovery of $\Delta\Psi_m$ upon reperfusion is dictated by the duration of the preceding ischemic insult. Post-ischemic electrical and functional recovery was dependent on early $\Delta\Psi_m$ recovery but independent of overall cellular injury measured using a standard assay of lactate dehydrogenase release. These findings reveal a novel mechanism by which instabilities in cellular energetic properties that are independent of irreversible cellular injury can scale to the level of the intact organ via an organized process of active conduction involving the multi-cellular network. This highlights the importance of investigating cellular metabolic properties in the context of the intact organ.

Keywords

Mitochondria; Oxidative Stress; Mitochondrial membrane potential; Energetics

INTRODUCTION

Polarization of the mitochondrial membrane potential ($\Delta\Psi_m$) is critical for normal mitochondrial function and cellular energetics, providing the electron-motive force

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DISCLOSURES None

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necessary for efficient shuttling of electrons down the electron transport chain and the generation of adenine triphosphate (ATP) through a highly regulated process of oxidative phosphorylation. Mitochondrial dysfunction, manifesting as disrupted $\Delta\Psi_m$ polarization (i.e. *depolarization or hyperpolarization*), underlies several important and highly prevalent diseases, including a variety of cardiac and neurological disorders [1-3]. For example, several neurodegenerative diseases have been mechanistically linked to $\Delta\Psi_m$ collapse (depolarization), secondary to oxidative stress associated with aging and/or genetic mutations. As such, $\Delta\Psi_m$ instability might form a unifying mechanism for a class of metabolic disorders affecting excitable tissues.

In the heart, metabolic insults, including acute ischemia, ischemia-reperfusion injury, and inflammation, are characterized by oxidative stress, manifested as increased production of reactive oxygen species (ROS), and ensuing mitochondrial dysfunction [2,4]. Excessive generation and/or reduced scavenging of ROS in isolated cardiomyocytes results in $\Delta\Psi_m$ depolarization, with subsequent loss of ATP synthesis, and cell death through activation of necrotic or apoptotic pathways [5].

As mitochondrial dysfunction represents a common pathway for these pathophysiological processes, intensive study in isolated cells and organelles has led to the development of novel therapeutic strategies to stabilize mitochondrial function in diseased tissues in order to ameliorate injury [4,6]. Despite recent advances in our understanding of mitochondrial biochemistry at the subcellular/molecular levels, the pathophysiological consequences of mitochondrial dysfunction at the level of the intact mammalian organ remain unknown.

The development of fluorescent $\Delta\Psi_m$ reporter dyes has facilitated the investigation of mitochondrial integrity at the cellular and tissue levels [7-9]. Since $\Delta\Psi_m$ depolarization is a fundamental early event that regulates mitochondrial energetics within individual cells, the goal of the present study was to investigate the spatio-temporal patterns of $\Delta\Psi_m$ changes in an intact tissue subjected to acute ischemia-reperfusion (I-R) injury. Using a semi-quantitative technique of $\Delta\Psi_m$ imaging in the intact perfused heart, we report the existence of complex spatiotemporal gradients of $\Delta\Psi_m$ during metabolic stress produced by ischemic injury. Our findings reveal for the first time the presence of adjacent zones of polarized and depolarized $\Delta\Psi_m$ that coexist during the first minute of myocardial ischemia. Importantly, we uncover the existence of waves of $\Delta\Psi_m$ depolarization which actively propagate across the epicardial surface with an average velocity of $\sim 20 \mu\text{m/sec}$. Spatially discordant and rapidly fluctuating (~ 10 sec time constant) changes (ie ripples) in the polarity of the $\Delta\Psi_m$ slope precede $\Delta\Psi_m$ depolarization, suggesting a mechanism linking unstable $\Delta\Psi_m$ kinetics to organized mitochondrial dysfunction across hundreds of cells within the intact ischemic heart. Finally, early recovery of $\Delta\Psi_m$ and not the extent of irreversible cellular injury reported by lactate dehydrogenase release, correlated with functional (electrical and contractile) recovery of the heart upon reperfusion. Our present findings highlight the importance of investigating cellular metabolic properties in the context of the intact organ.

METHODS

High-resolution optical $\Delta\Psi_m$ imaging in ex vivo perfused rat hearts

Rats (N=38) were anesthetized with Pentobarbital Sodium (30mg/kg, IP). Their hearts were rapidly excised, washed with ice cold cardioplegic solution, transferred to a Langendorff apparatus, and retrogradely perfused through the aorta with oxygenated (95% O_2 -5% CO_2) Tyrodes solution containing (in mM): NaCl 121.7, NaHCO_3 25, KCl 4.81, MgSO_4 2.74, CaCl_2 2.5 and dextrose 5.0, pH=7.40, temperature = $37 \pm 0.5^\circ\text{C}$. Perfusion pressure was maintained at $\sim 60\text{mmHg}$ by adjusting perfusion flow. Hearts were suspended in the buffer filled, temperature controlled chamber which was tightly regulated at 37°C throughout the

entire protocol. Volume conducted electrocardiograms were recorded for rhythm analysis using non-contact silver electrodes (Grass Instruments) placed within the chamber. ECG signals were recorded continuously throughout the entire *ex vivo* perfusion protocol (Figure 1A, red trace). These signals were amplified (ECG100-MP150 Amplifier, Biopac Systems, CA, USA) and displayed in real time using the AcqKnowledge 3.9 software package (Biopac Systems). Hearts were positioned such that the mapping field was centered over a 4×4mm region of left ventricular epicardium, midway between apex and base. These preparations remained stable for over 4 hours of perfusion. The *ex vivo* experimental protocol was typically completed in less than 2 hours. In a subset of experiments (N=6), a buffer filled latex balloon (Harvard apparatus) for measuring LV cavity pressure (Figure 1A, blue trace) was carefully inserted through the mitral valve into the LV cavity as previously reported [10].

We developed a semi-quantitative imaging technique of optical $\Delta\Psi_m$ mapping in *ex vivo* perfused guinea pig [11] and rat [12,13] hearts using the $\Delta\Psi_m$ -sensitive dye tetramethylrhodamine methylester (TMRM). This method allows the assessment of mitochondrial function at a 50 μ m resolution within the intact organ. Briefly, following cannulation, hearts were allowed to stabilize for ten minutes at physiological temperature ($37 \pm 0.5^\circ\text{C}$). Hearts were then stained with TMRM (250nM; Molecular Probes Inc.) mixed in a 500 mL volume of Tyrodes solution (dye loading phase) for 20 minutes. This was followed by a 20-30 minute dye washout phase during which perfusion was switched back to dye free Tyrodes solution. TMRM background fluorescence intensity was measured periodically (in 1 minute intervals) throughout the dye staining and washout phases using a 6400 pixel CCD based optical imaging approach that allowed the measurement of normalized $\Delta\Psi_m$ with high spatial resolution (50 μ m) over a 4×4mm window of the epicardial surface. To measure TMRM background fluorescence, hearts were excited with filtered light ($525 \pm 20\text{nm}$) emitted from a 200W quartz tungsten halogen lamp (Newport Corporation, CT, USA). Emitted fluorescence was filtered ($585 \pm 20\text{nm}$ for TMRM) and focused onto the high resolution CCD camera (Scimeasure, GA, USA). Background fluorescence intensity was measured as the amplitude difference before and after excitation.

During the dye loading procedure, a marked rise (over >50 fold) in background fluorescence was verified for multiple pixels in order to confirm adequate dye staining of the heart. During dye washout, the stability of TMRM background fluorescence was evaluated from multiple pixels in real time, as this baseline pre-ischemic level served for normalization purposes during the I-R protocol. The staining procedure did not affect LV pressure, perfusion pressure, or the ECG. In all experiments, the dye washout phase was associated with stable signal intensity, which was required to normalize subsequent changes in TMRM fluorescence caused by I-R injury. In preliminary experiments, we ensured the stability of background fluorescence for over 3 hours of steady state perfusion.

High-throughput analysis of optical signals was performed. Peak emitted TMRM fluorescence signal from each of 6400 pixels was measured before and after excitation achieved by a computer automated filter shutter switch. TMRM background fluorescence was baseline corrected by subtracting fluorescence levels before dye staining (<0.1%) for each pixel. Background corrected TMRM fluorescence ($\Delta\Psi_m$) during the I-R protocol was then normalized to the value of steady-state TMRM fluorescence achieved during the dye washout phase for each of the 6400 individual pixels.

Normalized $\Delta\Psi_m$ measurements during I-R injury across the imaged 4×4mm region of the heart which were reported as a percentage of the steady-state pre-ischemic value on a pixel-by-pixel basis were plotted as contour maps using Delta Graph 5.6 (Red Rock Software). These maps served to illustrate the dynamic changes in the spatial distribution of $\Delta\Psi_m$.

Ex Vivo Models of Ischemia-Reperfusion Mediated Myocardial Stunning

Ischemia-reperfusion (I-R) injury promotes mitochondrial dysfunction and oxidative stress. In the present study we used two distinct protocols of I-R injury which do not cause myocardial infarction but result in functionally divergent outcomes in terms of electrical stability following reperfusion. Specifically, 7.5 and 15 minutes of ex vivo global no-flow ischemia, followed by reperfusion in normal rat hearts were used to modulate mitochondrial function by oxidative stress without causing major LV infarction. These protocols, which result in reversible myocardial stunning [14] were specifically selected on the basis of post-ischemic functional (electrical and contractile) recovery, with the majority (50-75%) of 15 min but not 7.5 min (<10%) ischemic hearts exhibiting arrhythmias within the first 5 minutes of reperfusion. In a subset of experiments, regional I-R injury was induced by transient (7.5 or 15 min) occlusion of the left anterior descending coronary artery (LAD).

In addition to ischemia, controlled interventions known to increase oxidative burden such as perfusion with deoxygenated solution to produce hypoxia (Supplemental Figure 1) or the mitochondrial uncoupling agent FCCP (Supplemental Figure 2) produced marked depolarization of $\Delta\Psi_m$, serving as positive controls.

Measurement of lactate dehydrogenase (LDH) release and TTC Staining—LDH release as an index of cytotoxicity and cellular injury was measured in the perfusate of hearts during baseline perfusion and reperfusion following 7.5 and 15 min ischemic episodes. Three 1mL samples of perfusate were collected from each of 8 hearts during baseline perfusion. Multiple (n=20) 1mL samples of perfusate were collected during 20 min of reperfusion from hearts subjected to 7.5 min (N=4) and 15 min (N=4) of ischemia. Samples were incubated with the reaction mixture using the *in vitro* toxicology lactate dehydrogenase assay kit (TOX-7, Sigma). LDH release was measured by a colorimetric assay (490nm detection) as described previously [15]. Average LDH release during reperfusion following 7.5 min and 15 min of ischemia were compared to baseline perfusion and to each other.

Triphenyltetrazolium chloride (TTC) staining was performed in a subset of experiments to confirm that the arrhythmias which we readily observed following 15 minutes of ischemia were not caused by significant myocardial infarction in this model of myocardial stunning [14]. TTC staining in a subset of hearts was completed after 2 hours of reperfusion following 15 minutes of ischemia to allow for complete dehydrogenase washout, as previously reported in detail [16]. Hearts were perfused with 1% TTC solution (10 min, 37°C), incubated in 1% TTC solution (10 min, 37°C), and then fixed in formalin for sectioning and imaging.

RESULTS

TMRM loading of perfused rat hearts for 20 minutes resulted in a robust increase in fluorescence intensity (Figure 1B). Following dye washout, background fluorescence was stable (Figure 1B) exhibiting less than 3.5% variation for at least 4 hours in continuously perfused, non-ischemic control hearts. Changes in $\Delta\Psi_m$ during the course of ischemia could be quantitatively measured as a background subtracted and normalized value relative to the maximum pre-ischemia TMRM fluorescence for each optical pixel (Figures 1B and 1C).

Spatio-temporal changes in $\Delta\Psi_m$ during Ischemia

On average, global no-flow ischemia in rat myocardium decreased normalized TMRM fluorescence by 38% after 3 minutes and 56% after 7 minutes, consistent with progressive depolarization of $\Delta\Psi_m$ (Figure 1C). Remarkably, changes in $\Delta\Psi_m$ during the course of

ischemia were both spatially and temporally heterogeneous. Shown in Figure 2A is the distribution of $\Delta\Psi_m$ across a 4×4mm region of epicardium at 10, 60, 180, and 360 seconds of ischemia. As evident, well defined zones of polarized (yellow-black) and depolarized (red) $\Delta\Psi_m$ emerged early (within 10 seconds of ischemia) across the heart.

Zones of rapid $\Delta\Psi_m$ depolarization during the early phase of ischemia consistently formed the originating focus of ‘waves’ of $\Delta\Psi_m$ depolarization (Figure 2B, traces A-J), which actively propagated across the epicardial surface with a mean velocity of $19.6\pm3.9\ \mu\text{m}/\text{sec}$. As shown in Figure 2A, the expanding zone of $\Delta\Psi_m$ depolarization (red) was sharply demarcated from the polarized zone ahead of the wavefront producing a large spatial gradient (red to black transition) of $\Delta\Psi_m$. Finally, once $\Delta\Psi_m$ at a given region was depolarized, it remained stable throughout the subsequent ischemic period (Figure 2B). In contrast, areas of polarized $\Delta\Psi_m$ during early ischemia exhibited low amplitude $\Delta\Psi_m$ fluctuations (Figure 2B, insert) before undergoing rapid and final depolarization by the propagating wavefront.

Detailed analysis of the spatial distribution of $\Delta\Psi_m$ (Figure 3A) and its temporal first derivative ($\delta\Delta\Psi_m/\delta t$, Figure 3B) across the 4×4 mm² mapping field revealed the existence of neighboring regions with alternating positive and negative deflections in their $\Delta\Psi_m$ slope (Figure 3B, *). Spatially, these regions appeared as ripples that extended across several millimeters of tissue ahead of the depolarization wavefront (Figures 3A and 3B), with peak-to-peak distances ranging from 500 to 1000 μm . Each ripple therefore spanned several adjacent cardiomyocytes (mean size $\sim 150\times 35\mu\text{m}$ [17]). These ripples initially reflected out of phase changes in the kinetics of $\Delta\Psi_m$ between adjacent regions of ischemic tissue (Figure 3B).

Next, we investigated the temporal evolution of these metabolic ripples which accompanied the wave of $\Delta\Psi_m$ depolarization (Figure 4). Remarkably, these spatially alternating regions in the polarity of the $\Delta\Psi_m$ slope also exhibited a temporal oscillation as they fluctuated in time between positive and negative deflections with a frequency of $\sim 0.1\text{Hz}$ (Figure 4, bottom row: ischemia 60-180 sec). Finally, these spatially discordant regions of $\Delta\Psi_m$ kinetics were eventually abolished by the main depolarization wavefront (Figure 4, ischemia 190 sec). Interestingly, the leading edge of the $\Delta\Psi_m$ depolarization wavefront (Figure 4 top row) was also characterized by an alternating polarity in its slope (Figure 4, bottom row 150-180 seconds) having a similar time constant as the spatially discordant ripples that had extended several millimeters ahead of it (Figure 4).

Reperfusion mediated changes in $\Delta\Psi_m$

We then investigated the dynamic changes in $\Delta\Psi_m$ upon reperfusion of the heart following ischemic injury. Shown in Figure 5 are contour maps of normalized $\Delta\Psi_m$ at various time points of reperfusion that followed either 15min (Figure 5A) or 7.5min (Figure 5B) episodes of global no-flow ischemia. Interestingly, reperfusion following 15 minutes of ischemia was not associated with sustained recovery of $\Delta\Psi_m$ but rather caused further $\Delta\Psi_m$ depolarization (Figures 5 A, C). In stark contrast, although the average level of $\Delta\Psi_m$ depolarization following 7.5 and 15 minutes of ischemia were comparable (Figure 5D, end ischemia), reperfusion led to stark differences in the $\Delta\Psi_m$ response. Specifically, full recovery of $\Delta\Psi_m$ after ~ 2 minutes of reperfusion (Figure 5B, C, and D) occurred following 7.5- but not 15-minute episodes of ischemia. On average, $\Delta\Psi_m$ was significantly ($p=0.0119$) more depolarized during the early course of reperfusion (120 to 300 seconds) following 15 min compared to 7.5 min episodes of ischemia (Figure 5D).

To further explore the dependence of reperfusion mediated $\Delta\Psi_m$ recovery upon the duration of the preceding ischemic insult, we investigated changes in $\Delta\Psi_m$ in an *ex vivo* model of

regional ischemia produced by proximal LAD occlusion for 15 (Figure 6A) and 7.5 (Figure 6B) minutes, respectively. As shown in Figure 6A, regional ischemia caused a heterogeneous response, with depolarization of $\Delta\Psi_m$ (red) being confined to the ischemic zone. In fact, perfused regions of the heart proximal to the occlusion site (blue area in Evans stained preparation) exhibited polarized $\Delta\Psi_m$. Again, reperfusion following 15 minute episodes of regional ischemia failed to recover $\Delta\Psi_m$ in the previously ischemic zone. In sharp contrast, regional ischemia for 7.5 minutes failed to depolarize $\Delta\Psi_m$ (Figure 6B). Surprisingly however, early reperfusion led to a heterogeneous response characterized by a transient depolarization of $\Delta\Psi_m$ that was limited to the previously ischemic zone (Figure 6B 10seconds, traces F-L). $\Delta\Psi_m$ was fully recovered following 2 minutes of reperfusion (Figure 6B).

Early $\Delta\Psi_m$ recovery but not LDH release predicts post-ischemic electrical and functional recovery

Since we found that post-ischemic $\Delta\Psi_m$ recovery was dependent on the duration of the preceding ischemic episode, we proceeded to measure the extent of cellular injury indexed by LDH release during reperfusion of hearts following 7.5 and 15 min of ischemia, respectively (Figure 7). In sharp contrast to $\Delta\Psi_m$ recovery, LDH release during reperfusion was comparable ($p = \text{NS}$) in the 7.5 and 15 min ischemia groups. Reperfusion in both groups was associated with a marked ($> 3\text{-fold}$, $p < 0.0001$) increase in LDH activity compared to baseline, pre-ischemic levels (Figure 7). Interestingly, this model of *ex vivo* I-R injury was not associated with significant myocardial necrosis, as TTC staining following 15 minutes of ischemia failed to demonstrate areas of non-viable myocardium (Figure 7).

Finally, measurement of LV cavity pressure and ECG tracings during the course of I-R injury in both groups revealed successful electrical and contractile recovery in all hearts undergoing 7.5 min of ischemia. In sharp contrast, $>65\%$ of hearts undergoing 15 minutes of ischemia exhibited reperfusion mediated VF within the first 5 minutes of reperfusion. Interestingly, the subset of ischemic hearts that electrically recovered following 15 minutes of ischemia exhibited delayed and incomplete contractile recovery compared to the 7.5 min group. As such, $\Delta\Psi_m$ recovery but not the extent of cellular injury assayed by LDH release or TTC staining, was predictive of post-ischemic functional and electrical recovery.

DISCUSSION

The main contributions of the present study are as follows: 1) we identified waves of $\Delta\Psi_m$ depolarization that propagate across the heart with a mean velocity of $\sim 20 \mu\text{m/sec}$, several orders of magnitude slower than myocardial action potential propagation; 2) we elucidated complex spatio-temporal metabolic instabilities that precede and accompany the formation of these organized waves; 3) we established the dependence of sustained $\Delta\Psi_m$ recovery during reperfusion on the duration of the preceding ischemic insult; and 4) we revealed a dependence of functional recovery of hearts following I-R injury on early $\Delta\Psi_m$ recovery and not upon irreversible cellular injury measured by LDH release.

To our knowledge, this is the first report that details the complex spatiotemporal changes in $\Delta\Psi_m$ in the heart under conditions of relatively mild (7.5 and 15 min) I-R injuries which cause myocardial stunning but not irreversible tissue injury and infarction [14]. A major strength of this report is the elucidation of functionally important changes in $\Delta\Psi_m$ that are independent of myocyte loss. We describe for the first time the emergence of regional patterns of polarized and depolarized $\Delta\Psi_m$ within 10 seconds of ischemia. Furthermore, we define zones of early $\Delta\Psi_m$ depolarization as sources of active inter-cellular propagation of metabolic collapse across the heart. Finally, we identify persistent $\Delta\Psi_m$ recovery as an important metabolic index for predicting functional outcome following I-R injury.

Ischemia induced depolarization of $\Delta\Psi_m$

$\Delta\Psi_m$ depolarization is triggered by opening of mitochondrial ion channels [18,19]. Specifically, during metabolic insults, increased mitochondrial ROS production triggers the opening of the inner mitochondrial membrane anion channel (IMAC) and/or the permeability transition pore (PTP) [18]. This results in ROS release from mitochondria and $\Delta\Psi_m$ depolarization. In isolated cardiomyocytes, ROS diffusion within the cytosol triggers further ROS release from neighboring mitochondria, initiating a feedback cycle of ROS-induced ROS release (RIRR) and $\Delta\Psi_m$ depolarization [20-22]. In the present study, we identified at the tissue level the presence of $\Delta\Psi_m$ ripples prior to mitochondrial collapse (Figures 3 and 4). This suggests patterns of wave behavior spreading across the myocardium ahead of the main wave of $\Delta\Psi_m$ depolarization, with propagation reflecting the direct interaction between adjacent cells within the intact ischemic tissue. Although we did not directly image ROS levels, it is conceivable that ROS diffusion at the interface between depolarized (acting as ROS sources) and polarized (ROS sinks) regions can drive the propagation of $\Delta\Psi_m$ collapse, in a manner that extends the notion of RIRR from a subcellular to a multi-cellular phenomenon. Our findings indicate that mitochondria within ischemic myocardium respond initially through low amplitude high frequency oscillations of $\Delta\Psi_m$ before undergoing $\Delta\Psi_m$ collapse. As such, in the present study we extend the phenomenon of mitochondrial oscillations in isolated cardiomyocytes *in vitro* to the level of the intact heart [22,23].

Since mitochondrial function of individual cells is highly influenced by network properties it is critical to investigate mitochondrial function within the milieu of the intact heart. Specifically, our findings indicate that the heterogeneous metabolic substrate of the heart during the early onset of ischemia, determines the ensuing patterns of $\Delta\Psi_m$ propagation during the remaining course of ischemia, which may ultimately dictate myocardial excitability and contribute to the formation of zones of conduction block by heterogeneous activation of surface ATP sensitive potassium channels [24,25]. Finally, our findings reveal a novel mechanism by which $\Delta\Psi_m$ within a given zone can be modulated by the mitochondrial function of adjacent zones. The amplification and propagation of $\Delta\Psi_m$ depolarization across the electrically coupled syncytium presents novel opportunities to limit injury by potentially targeting areas of early $\Delta\Psi_m$ collapse that form the origin of the organized propagating wavefront of mitochondrial dysfunction.

Reperfusion mediated changes in $\Delta\Psi_m$

Prompt restoration of oxygenated blood flow to ischemic myocardium (i.e. reperfusion) is required for preventing irreversible cell damage and death. Unfortunately, restoration of blood flow, in itself, results in additional cardiac damage, known as reperfusion injury. Such oxidative damage, which is mediated by bursts of ROS, is more severe when reperfusion therapy is delayed. Effective strategies to limit or prevent reperfusion injury have proven elusive. Despite an improved understanding of the pathophysiology of this process, the vast majority of clinical trials aimed at preventing reperfusion injury have been quite disappointing. A major finding of the present report is the demonstration that the successful recovery of $\Delta\Psi_m$ upon reperfusion is indeed highly dependent on the duration of the preceding ischemic episode. It is important to note that we specifically investigated two distinct episodes of ischemia (7.5 and 15 min) because of their differential outcomes in terms of electrical and functional recovery following reperfusion. Both durations of ischemic insult are considered mild in terms of expected infarction [14] and our TTC stains confirmed that. Therefore, a major strength of our approach is the assessment of metabolic function in two I-R injury protocols that produce highly distinct functional outcomes without causing permanent cell death or infarction. Despite a comparable degree of $\Delta\Psi_m$ depolarization following 7.5 and 15 minutes of global no-flow ischemia, reperfusion led to

markedly divergent responses. Interestingly, sustained $\Delta\Psi_m$ recovery but not LDH release during reperfusion is predictive of post-ischemic functional and electrical recovery. Our data also suggest that comparably increased levels of LDH release after 7.5 and 15 min of ischemia occur independent of irreversible tissue injury. Hence, our findings indicate that LDH release may be highly influenced by cytotoxicity associated with non-cardiomyocyte cell types in the heart, such as endothelial cells and fibroblasts, which have been shown to exhibit a lower threshold for apoptosis [26,27]. As such, LDH release may not be a good predictor of post-ischemic functional recovery.

Our present data also highlight the notion that reperfusion is a highly complex phenomenon which could either reverse or exacerbate ischemia mediated changes in $\Delta\Psi_m$. In fact our findings regarding additional $\Delta\Psi_m$ depolarization upon reperfusion following a long episode of ischemia are consistent with ROS induced damage during this phase. This is further supported by the transient $\Delta\Psi_m$ depolarization upon reperfusion following a 7.5 min episode of regional ischemia, which itself failed to depolarize $\Delta\Psi_m$.

Finally, it is well known that mitochondria serve as final mediators of cardiomyocyte life and death [1,28]. In addition to their critical role in generating ATP, mitochondria can trigger apoptosis and necrosis through formation of the mitochondrial permeability transition during reperfusion [18]. Our study was focused on a model of myocardial stunning which does not result in irreversible myocardial injury and LV infarction. However, our findings that $\Delta\Psi_m$ recovery during reperfusion is critically dependent on the duration of the ischemic insult are consistent with findings of increased rates of apoptosis (known to be triggered by irreversible $\Delta\Psi_m$ depolarization) upon reperfusion following long episodes of ischemia. A better understanding of the biophysical properties of the complex spatio-temporal $\Delta\Psi_m$ dynamics under conditions of oxidative stress as well as the influence of cardioprotective agents and interventions known to limit oxidative stress offers the potential for designing more effective strategies to modulate mitochondrial function and limit damage caused by ischemia-reperfusion injury [29].

Limitations

Ischemic injury in humans typically results in a transmural gradient of blood flow and tissue perfusion, which are expected to produce highly complex alterations in local metabolic properties across the ventricular wall. Our measurements in the present study were limited to the epicardial layer (<250um in depth [30]). Therefore, future investigations of transmural heterogeneities in metabolic properties in response to I-R injury are warranted. These measurements can be achieved by applying the optical technique, which we describe here to the transmural surface of arterially perfused wedge preparations, as we and other have reported in the past [31,32].

The experimental approach described in this manuscript assumes a regionally homogeneous distribution of $\Delta\Psi_m$ under baseline conditions of normal perfusion. Similar assumptions have been made with regards to NADH and ROS levels by other investigators using similar techniques in the past [33,34]. Because this is not a ratiometric imaging technique, we can only report relative (not absolute) changes in $\Delta\Psi_m$ during the course of an experiment.

Finally, in this study we exclusively focus on the early phase of reperfusion because we had found that this time window represents a vulnerable period for post-ischemic arrhythmias that are dependent on mitochondrial function [25].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Gustafsson AB, Gottlieb RA. Heart mitochondria: gates of life and death. *Cardiovasc Res*. 2008 Jan 15; 77(2):334–43. [PubMed: 18006487]
2. Honda HM, Korge P, Weiss JN. Mitochondria and ischemia/reperfusion injury. *Ann N Y Acad Sci*. 2005 Jun; 1047:248–58. [PubMed: 16093501]
3. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*. 2006 Oct 19; 443(7113):787–95. [PubMed: 17051205]
4. Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev*. 2008 Apr; 88(2):581–609. [PubMed: 18391174]
5. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell*. 2004 Jan 23; 116(2):205–19. [PubMed: 14744432]
6. Fatokun AA, Stone TW, Smith RA. Oxidative stress in neurodegeneration and available means of protection. *Front Biosci*. 2008; 13:3288–311. [PubMed: 18508433]
7. Scaduto RC Jr, Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophysical journal*. 1999 Jan; 76(1 Pt 1):469–77. [PubMed: 9876159]
8. Zorov DB, Koberinsky E, Juhaszova M, Sollott SJ. Examining intracellular organelle function using fluorescent probes: from animalcules to quantum dots. *Circ Res*. 2004 Aug 6; 95(3):239–52. [PubMed: 15297386]
9. Matsumoto-Ida M, Akao M, Takeda T, Kato M, Kita T. Real-time 2-photon imaging of mitochondrial function in perfused rat hearts subjected to ischemia/reperfusion. *Circulation*. 2006 Oct 3; 114(14):1497–503. [PubMed: 17000908]
10. Brown DA, Aon MA, Akar FG, Liu T, Sorrairain N, O'Rourke B. Effects of 4'-chlorodiazepam on cellular excitation-contraction coupling and ischaemia-reperfusion injury in rabbit heart. *Cardiovasc Res*. 2008 Jul 1; 79(1):141–9. [PubMed: 18304929]
11. Akar FG, Aon MA, Tomaselli GF, O'Rourke B. Spatial heterogeneity of the mitochondrial membrane potential underlies reperfusion related arrhythmias. *Circulation*. 2006; 114(18):269.
12. Jin HWCE, Lyon A, Stocks J, Akar FG. Spatial Heterogeneity Of The Mitochondrial Membrane Potential Underlying Arrhythmias In Pressure Overload Hypertrophy. *Circulation*. 2008; 118(18):S531.
13. Lyon AR, P J, Liang LF, Hajjar RJ, Akar FG. Gene Transfer Of The Peripheral Type Benzodiazepine Receptor Alters The Spatio-temporal Dynamics Of The Mitochondrial Membrane Potential ($\Delta\psi(M)$) Across The Intact Heart. *Circulation*. 2008; 118(18):S528.
14. Kusuoka H, Marban E. Cellular mechanisms of myocardial stunning. *Annual review of physiology*. 1992; 54:243–56.
15. Inserte J, Garcia-Dorado D, Ruiz-Meana M, Solares J, Soler J. The role of Na^+-H^+ exchange occurring during hypoxia in the genesis of reoxygenation-induced myocardial oedema. *J Mol Cell Cardiol*. 1997 Apr; 29(4):1167–75. [PubMed: 9160868]
16. Schneider S, Chen W, Hou J, Steenbergen C, Murphy E. Inhibition of p38 MAPK α/β reduces ischemic injury and does not block protective effects of preconditioning. *American journal of physiology*. 2001 Feb; 280(2):H499–508. [PubMed: 11158945]
17. Severs NJ. The cardiac muscle cell. *Bioessays*. 2000 Feb; 22(2):188–99. [PubMed: 10655038]
18. Weiss JN, Korge P, Honda HM, Ping P. Role of the mitochondrial permeability transition in myocardial disease. *Circ Res*. 2003 Aug 22; 93(4):292–301. [PubMed: 12933700]
19. O'Rourke B. Mitochondrial ion channels. *Annual review of physiology*. 2007; 69:19–49.

20. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J Exp Med*. 2000 Oct 2; 192(7):1001–14. [PubMed: 11015441]
21. Brady NR, Elmore SP, van Beek JJ, Krab K, Courtoy PJ, Hue L, et al. Coordinated behavior of mitochondria in both space and time: a reactive oxygen species-activated wave of mitochondrial depolarization. *Biophysical journal*. 2004 Sep; 87(3):2022–34. [PubMed: 15345578]
22. Aon MA, Cortassa S, Marban E, O'Rourke B. Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. *J Biol Chem*. 2003 Nov 7; 278(45):44735–44. [PubMed: 12930841]
23. Aon MA, Cortassa S, O'Rourke B. The fundamental organization of cardiac mitochondria as a network of coupled oscillators. *Biophysical journal*. 2006 Dec 1; 91(11):4317–27. [PubMed: 16980364]
24. Weiss JN, Venkatesh N. Metabolic regulation of cardiac ATP-sensitive K⁺ channels. *Cardiovasc Drugs Ther*. 1993 Aug; 7(Suppl 3):499–505. [PubMed: 8251419]
25. Akar FG, Aon MA, Tomaselli GF, O'Rourke B. The mitochondrial origin of postischemic arrhythmias. *J Clin Invest*. 2005 Dec; 115(12):3527–35. [PubMed: 16284648]
26. Park M, Shen YT, Gaussin V, Heyndrickx GR, Bartunek J, Resuello RR, et al. Apoptosis predominates in nonmyocytes in heart failure. *American journal of physiology*. 2009 Aug; 297(2):H785–91. [PubMed: 19465551]
27. Scarabelli T, Stephanou A, Rayment N, Pasini E, Comini L, Curello S, et al. Apoptosis of endothelial cells precedes myocyte cell apoptosis in ischemia/reperfusion injury. *Circulation*. 2001 Jul 17; 104(3):253–6. [PubMed: 11457740]
28. Aon MA, Cortassa S, Akar FG, O'Rourke B. Mitochondrial criticality: a new concept at the turning point of life or death. *Biochim Biophys Acta*. 2006 Feb; 1762(2):232–40. [PubMed: 16242921]
29. Weiss JN, Yang L, Qu Z. Systems biology approaches to metabolic and cardiovascular disorders: network perspectives of cardiovascular metabolism. *J Lipid Res*. 2006 Nov; 47(11):2355–66. [PubMed: 16946414]
30. Girouard SD, Laurita KR, Rosenbaum DS. Unique properties of cardiac action potentials recorded with voltage-sensitive dyes. *J Cardiovasc Electrophysiol*. 1996 Nov; 7(11):1024–38. [PubMed: 8930734]
31. Akar FG, Rosenbaum DS. Transmural electrophysiological heterogeneities underlying arrhythmogenesis in heart failure. *Circ Res*. 2003 Oct 3; 93(7):638–45. [PubMed: 12933704]
32. Akar FG, Yan GX, Antzelevitch C, Rosenbaum DS. Unique topographical distribution of M cells underlies reentrant mechanism of torsade de pointes in the long-QT syndrome. *Circulation*. 2002 Mar 12; 105(10):1247–53. [PubMed: 11889021]
33. Salama G, Lombardi R, Elson J. Maps of optical action potentials and NADH fluorescence in intact working hearts. *Am J Physiol*. 1987 Feb; 252(2 Pt 2):H384–94. [PubMed: 3812752]
34. Lu LS, Liu YB, Sun CW, Lin LC, Su MJ, Wu CC. Optical mapping of myocardial reactive oxygen species production throughout the reperfusion of global ischemia. *J Biomed Opt*. 2006 Mar-Apr. 11(2):021012. [PubMed: 16674187]

Figure 1. $\Delta\Psi_m$ mapping in the intact perfused heart

A. Raw traces of the volume conducted ECG (red) and LV cavity pressure (blue) during the course of I-R injury in a representative heart.

B. Background fluorescence ($585\pm 20\text{nm}$) upon excitation with filtered ($525\pm 20\text{nm}$) light during loading with TMRM (250nM) for 20 min followed by dye washout (20 min).

C. Normalized and baseline corrected TMRM fluorescence ($\Delta\Psi_m$) during global ischemia (black trace) and non-ischemic control (purple trace) hearts.

Figure 2. Ischemia-induced $\Delta\Psi_m$ depolarization waves in the intact heart

A. Contour maps measured at 10, 60, 180 and 360 seconds of global ischemia indicating active propagation of $\Delta\Psi_m$ collapse. Relative changes in $\Delta\Psi_m$ are mapped to a color scheme: baseline $\Delta\Psi_m$ (black), depolarized $\Delta\Psi_m$ (red), hyperpolarized $\Delta\Psi_m$ (yellow). Scale bar = 1 mm.

B. Temporal $\Delta\Psi_m$ changes during global ischemia measured at equidistant sites (A-J) along the main axis of propagation of the $\Delta\Psi_m$ depolarization wavefront. Data points are fitted to non-linear regression curves (Boltzmann). Insert shows evidence of simultaneous regions of $\Delta\Psi_m$ hyperpolarization and depolarization during early ischemia.

Figure 3. Ripples of fluctuating $\Delta\Psi_m$ kinetics precede the main depolarization wave in the intact heart

A. Contour map of $\Delta\Psi_m$ measured at 20 seconds of ischemia (left). Representative trace indicating the dynamic temporal changes in $\Delta\Psi_m$ at a select site before and after the onset of ischemia (right).

Color scale: baseline (black), depolarization (red), hyperpolarization (yellow).

B. Contour map of the first temporal derivative of $\Delta\Psi_m$ ($\delta\Delta\Psi_m/\delta t$) measured following 20 seconds of ischemia. Representative temporal trace of the first derivative of $\Delta\Psi_m$ from the same site shown in **A**.

Figure 4. Spatio-temporal fluctuations of $\Delta\Psi_m$ during global ischemia

Successive contour maps of $\Delta\Psi_m$ (above) and its first derivative (below) acquired at 10 second intervals during global no-flow ischemia in a representative rat heart. These data illustrate the presence of spatially and temporally discordant kinetics of $\Delta\Psi_m$ that exist proximal to the main depolarization wave of $\Delta\Psi_m$ collapse.

Color scale: a) $\Delta\Psi_m$ contour maps: baseline (black), depolarization (red), hyperpolarization (yellow); b) $\delta\Delta\Psi_m/\delta t$ contour maps: baseline (black), positive slopes (turquoise), negative slopes (purple).

Figure 5. Reperfusion following global ischemia induces further $\Delta\Psi_m$ depolarization
 $\Delta\Psi_m$ contour maps at end ischemia and during reperfusion in hearts subjected to global ischemia. Spatio-temporal $\Delta\Psi_m$ changes associated with reperfusion following representative long (15 min, Panel **A**) and short (7.5 min, Panel **B**) episodes of global ischemia. **C**. Representative temporal changes in $\Delta\Psi_m$ associated with a short (red trace) and long (black trace) episodes of I-R injury. **D**. Average data showing differences in the $\Delta\Psi_m$ response to reperfusion following short (red, n=6) and long (blue, n=3) episodes of global no-flow ischemia. Two way ANOVA, $p < 0.001$.

Figure 6. Spatio-temporal $\Delta\Psi_m$ changes caused by regional I-R injury

$\Delta\Psi_m$ contour maps during regional ischemia–reperfusion injury caused by LAD occlusion. **A.** Representative contour maps measured at the end of a long (15 min) episode of regional ischemia followed by reperfusion for 10 minutes. Temporal changes in $\Delta\Psi_m$ at reperfusion recorded from discrete locations within in the non-ischemic zone (A-B), borderzone (C-D) and ischemic zone (E-F) after 15 minutes of regional ischemia. **B** Representative contour maps measured at the end of a short (7.5 min) episode of regional ischemia followed by reperfusion. Fifteen minutes of regional ischemia induces regional $\Delta\Psi_m$ depolarization that is limited to the ischemic zone. Reperfusion fails to reverse $\Delta\Psi_m$ depolarization. Brief (7.5 minutes) regional ischemia-reperfusion induced the collapse of $\Delta\Psi_m$ in the ischemic zone. Transient $\Delta\Psi_m$ depolarization was followed by full recovery following reperfusion. Temporal changes in $\Delta\Psi_m$ at reperfusion from pixels in the non-ischemic zone (A-C), borderzone (D-G) and ischemic zone (H-L) after brief (7.5 minutes) regional ischemia demonstrating rapid collapse and recovery of $\Delta\Psi_m$ upon reperfusion. Image of a representative heart subjected to regional I-R injury demonstrating presence of ischemic and non-ischemic zones after perfusion with Evan's blue stain (bottom).

Figure 7. Functional and electrical recovery following 7.5 but not 15 minutes of ischemia
Representative traces of volume conducted ECG (red) and LV cavity pressure (blue) measured during early reperfusion after 7.5 min (left) and 15 min (right) of ischemia (top, left). LV cavity pressure traces recorded during entire I-R injury protocol from hearts that electrically recovered following reperfusion. 7.5 min pre-ischemic heart is associated with more rapid and complete functional recovery (bottom, left). Cross sections of triphenyltetrazolium chloride (TTC, 1%) stained heart that underwent 15 minute I-R injury protocol reveals absence of necrotic tissue (top, right). LDH release measured during reperfusion following 7.5 and 15 min ischemic episodes reveals a comparable increase in both groups (bottom, right) compared to baseline perfusion.