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Uncoupling of increased cellular oxidative stress and myocardial ischemia reperfusion injury by directed sarcolemma stabilization

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Abstract

Myocardial ischemia/reperfusion (I/R) injury is a major clinical problem leading to cardiac dysfunction and myocyte death. It is widely held that I/R causes damage to membrane phospholipids, and is a significant mechanism of cardiac I/R injury. Molecular dissection of sarcolemmal damage in I/R, however, has been difficult to address experimentally. We studied here cardiac I/R injury under conditions targeting gain- or loss-of sarcolemma integrity. To implement gain-in-sarcolemma integrity during I/R, synthetic copolymer-based sarcolemmal stabilizers (CSS), including Poloxamer 188 (P188), were used as a tool to directly stabilize the sarcolemma. Consistent with the hypothesis of sarcolemmal stabilization, cellular markers of necrosis and apoptosis evident in untreated myocytes were fully blocked in sarcolemma stabilized myocytes. Unexpectedly, sarcolemmal stabilization of adult cardiac myocytes did not affect the status of myocyte-generated oxidants or lipid peroxidation in two independent assays. We also investigated the loss of sarcolemmal integrity using two independent genetic mouse models, dystrophin-deficient *mdx* or dysferlin knockout (Dysf KO) mice. Both models of sarcolemmal loss-of-function were severely affected by I/R injury *ex vivo*, and this was lessened by CSS. In vivo studies also showed that infarct size was significantly reduced in CSS-treated hearts. Mechanistically, these findings support a model whereby I/R-mediated increased myocyte oxidative stress is uncoupled from myocyte injury. Because the sarcolemma stabilizers used here do not transit across the myocyte membrane this is evidence that intracellular targets of oxidants are not sufficiently altered to affect cell death when sarcolemma integrity is preserved by synthetic stabilizers. These findings, in turn, suggest that sarcolemma destabilization, and consequent Ca^{2+} mishandling, as a focal initiating mechanism underlying myocardial I/R injury.

Introduction

Coronary heart disease (CHD) is the most common form of heart disease accounting for 20% of all deaths in the U.S. [1]. CHD most often presents as a myocardial infarction (MI), during which cardiac tissue becomes ischemic due to blockage of the coronary artery.

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Conflict of interest: J.M. Metzger is on the scientific advisory board of and holds shares in Phrixus Pharmaceuticals Inc., a company developing novel therapeutics for heart failure.

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Restoration of blood flow by thrombolytics or angioplasty is necessary to limit the amount of tissue death; however, reperfusion itself also induces significant injury[2]. This phenomenon, referred to as myocardial ischemia reperfusion (I/R) injury, can also occur in numerous other clinical settings, such as angina, cardiac surgery, transplantation, or cardiac arrest and resuscitation[3–7]. Cardiac I/R causes tissue damage, cardiac dysfunction and can eventually lead to heart failure.

Myocardial I/R injury is a complex multi-factorial process and the precise mechanism underlying injury is challenging to dissect. Numerous components of cardiac I/R injury include ATP depletion, contracture, cell swelling and membrane destabilization[8–12]; ionic imbalances, especially intracellular Ca^{2+} dysregulation; Ca^{2+} activated proteases calpain[13] and caspase[14]; mitochondrial membrane depolarization[15–17]; and marked disturbances in redox state. Of these, altered redox state is widely considered a major early initiator of cellular damage in cardiac I/R injury [2, 5, 18–21]. Proposed targets of increased oxidants include sarcoplasmic reticulum Ca^{2+} ATPase, sarcomeric proteins and membrane phospholipids [20, 22, 23]. The mitochondrial membrane is differentially damaged by lipid peroxidation compared to the sarcolemma[24–26]. Mechanistic dissection of these redox-dependent pathways and I/R injury has proven difficult to elucidate and important to understand. Further, conflicting results of anti-oxidant therapies, including negative and adverse effects, raise questions about the precise role of oxidants in I/R injury[27, 28].

Whereas it is established that membrane integrity is important in I/R cardiac injury[29], the dissection of the roles of other factors in membrane stabilized myocytes has not been addressed. Therefore, we investigated the status of mitochondria, Ca^{2+} , and oxidants in myocytes with and without membrane stabilizers. To probe mechanism, we implemented models of gain- and loss-of- sarcolemma integrity during I/R. Copolymer sarcolemmal stabilization (CSS) using poloxamers, including poloxamer 188 (P188), provides a unique gain-of-function tool [30–32] as these absorb onto the membrane surface, serving as cell surface interacting “stabilizers”, but do not transit across the phospholipid outer membrane barrier [33, 34]. Copolymers such as P188 have been investigated in the treatment of myocardial I/R; however, the proposed mechanisms largely centered on improved blood flow by rheological effects. Some pre-clinical studies examining these mechanisms were promising, but large clinical trials failed to show an effect. In this study, we show for the first time the cardiac myocyte membrane stabilizing effect by CSS in I/R. Primary markers of I/R-mediated cellular damage, including intracellular Ca^{2+} dysregulation, mitochondrial membrane depolarization, membrane leak, and myocyte necrosis and apoptosis, were all significantly blocked in sarcolemma stabilized compared to control cardiac myocytes. Surprisingly, myocyte oxidative stress and subsequent lipid peroxidation were significantly increased in I/R, with both the timing and magnitude of I/R-mediated peroxidation the same in control and sarcolemma stabilized myocytes, which is in conflict with the prevailing oxidant-induced myocyte damage hypothesis in I/R.

We also utilized two independent genetic models of loss-of-membrane integrity where sarcolemma stability and repair were disabled. Dystrophin-deficient *mdx* hearts are an established model of myocytes with impaired sarcolemmal integrity [32, 35]. Loss of fully intact dystrophin has also been reported in I/R injury[36–42], but the effect of dystrophin deficiency has not been investigated. Membrane repair pathways have also gained attention in cardioprotection from I/R injury[43]. The membrane repair protein dysferlin has recently gained attention as contributing to cardiac injury[44], but has not been investigated in I/R injury. Consistent with our hypothesis, both models of sarcolemmal loss-of-function exhibited significantly amplified cardiac I/R injury compared to controls, a result not shown previously. In addition, application of membrane stabilizers enabled cardiac function in both sarcolemmal loss-of-function models, even though dystrophin-deficient muscles are known

to have increased oxidative stress[45, 46]. We also examined infarct size after 24 hours, and found that CSS nearly completely blocked infarct. This finding is consistent with the effects of myocyte extrinsic oxidants also being blocked by CSS. Taken together, these results inform a model of I/R injury wherein myocyte generated oxidants alone are insufficient as primary mediators of cellular damage in reperfusion. The data support a model in which sarcolemma integrity plays a primary role in I/R injury and establishes direct sarcolemma stabilization as a candidate therapeutic target to protect the myocardium in I/R.

Materials & Methods

Animals

All animals were used and cared for according to principles outlined in the *NIH Guide for the care and use of laboratory animals* (NIH publication no. 85-23, Revised 1996) and protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota. C57BL/10, mdx, 129, and Dysferlin knockout mice were obtained from Jackson Labs (Bar Harbor, ME). Sprague Dawley rats were obtained from Harlan.

Cell culture and chemicals

Adult rat ventricular cardiac myocytes were isolated by collagenase digestion, as previously described[47]. Myocytes were plated on laminin at a density of 2×10^4 cells/well on 6-well plates in M199 for 24 hours, unless otherwise stated. Tri-block copolymers, including P188 (8400 Da mol. wt.; hydrophobic/ hydrophilic ratio 0.16) and P338 (13,400 Da mol. wt., hydrophobic/hydrophilic ratio 0.16) were obtained from BASF Corp. PEG8000 was obtained from Sigma. A schematic diagram showing relative molecular weights and hydrophobicity/hydrophobicity is shown in Supplemental Figure 1A. Fluorescent probes were from Molecular Probes, unless otherwise stated.

In vitro ischemia/Reoxygenation (sI/R)

Myocytes were exposed to simulated ischemia (sI) in the following buffer[48, 49]: (lactic acid 20mM, 2-deoxyglucose 10mM, NaCl 118mM, HEPES 10mM, NaHCO_3 24mM, NaH_2PO_4 1mM, CaCl_2 2.5mM, MgCl_2 1.2 mM, KCl 16mM, pH 6.4) at 0.2% O_2 with 5% CO_2 using an OxyCycler (Biospherix, Lacona, NY). Myocytes were reoxygenated (R) in M199 for 1 hour. Effective doses for sarcolemmal stabilization are shown in Supplemental Figure 1B.

Permeability Assays

For LDH assays, media was collected after 1 hour reoxygenation. Samples were incubated in 100mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 120uM NADH, 2.3mM pyruvate, and 0.033% bovine serum albumin at 37 degrees, and the conversion of NADH to NAD^+ was determined by reading absorbance values at 340nm every 1 minute for 30 minutes[50]. Myocyte permeability was also assessed using a cTnI ELISA kit (Life Diagnostics, West Chester, PA) according to the manufacturer's instructions. Cellular permeability was assessed by incubating the myocytes with fluorescently labeled dextrans (Dextran-FITC) of various sizes. In intact cells, dextran is usually excluded. Sarcolemmal permeability causes internalization of the dextrans. After washing extracellular dextran away, cells were lysed in RIPA buffer and internalized fluorescent dextran-FITC was measured by exciting at 488nm, and emission read at 520nm[51].

Fluorescence Assays for viability

7×10^3 adult cardiac myocytes were plated on laminin coated 96-well glass bottom Optilux plates (BD Falcon). The following day myocytes were pre-loaded with the appropriate fluorescent indicator to ensure equal loading and then exposed to simulated ischemia as described above. Myocytes were reoxygenated in Tyrode's buffer, and fluorescence measured using a Molecular Devices Gemini plate reader. To measure mitochondrial membrane potential, myocytes were loaded with 1 μ M Mitotracker Green (MTG) and 200nM tetramethylrhodamine methyl ester (TMRE, from Anaspec) for 30 minutes at 37 degrees. Mitotracker green accumulates in mitochondria and covalently binds to the inner membrane. TMRE is a cationic dye that accumulates in polarized mitochondria, and is released if mitochondria depolarize. In polarized mitochondria MTG fluorescence is quenched by FRET to TMRE, but loss of TMRE by depolarized mitochondria leads to increased MTG fluorescence. Thus, mitochondrial depolarization was measured by exciting at 488nm, and measuring emission at 520nm[52]. To assess cell viability, cells were loaded with 2 μ M calcein-AM for 30 minutes at 37 degrees. Calcein-AM is cell permeable, but not fluorescent until endogenous ATP-dependent esterases hydrolyze the AM moiety. Fluorescence was measured by exciting at 488nm, and emission read at 520nm[53].

Fluorescence Assay for lipid peroxidation

Myocytes were plated on Optilux plates as described above, and were equilibrated with 10 μ M Bodipy C11(581/591) in 5% fetal calf serum in M199 for 12 h at 37 deg C prior to simulated ischemia. Bodipy C11(581/591) fluoresces at 595nm, but upon oxidation shifts to 520nm. Accordingly, we measured the ratio of 595/520 fluorescence during reoxygenation to assess lipid peroxidation[54, 55].

ROS assay

Myocytes were plated on Optilux plates as described above, and were equilibrated with 5 μ M H₂DCFDA for 30 min at 37 deg C prior to simulated ischemia. DCF fluorescence was measured during reoxygenation at Ex 488nm, Em 520nm[56].

Ca²⁺ measurements

Resting cellular Ca²⁺ content was determined by loading cells with the Ca²⁺ indicator Fluo-4, 2 μ M at room temperature for 30 minutes prior to simulated ischemia. Fluorescence was measured during reoxygenation by exciting at 488nm, and emission read at 520nm[57]. Ca²⁺ transients were measured by loading blebbistatin treated cells with the ratiometric Ca²⁺ indicator Fura-2 AM for 15 minutes, followed by a 15-minute de-esterification in Tyrode's. Ca²⁺ transients were recorded using an Ionoptix system[58]. Blebbistatin treatment is commonly used in optical mapping experiments to measure Ca²⁺. It inhibits contraction, but doesn't appear to affect electrophysiological properties[59]. We found that blebbistatin treated myocytes still released LDH after sI/R, but retained Ca²⁺ transients (data not shown). Accordingly, myocytes were selected randomly to measure Ca²⁺ transients, independently of contracture or contraction.

Caspase-3 Assay

Myocytes were lysed in Caspase lysis buffer (50mM HEPES, 0.1% CHAPS, 1mM DTT, 0.1mM EDTA). Lysates were diluted in Caspase Assay buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 10mM DTT, 1mM EDTA, 10% Glycerol, 10 μ M Ac-DEVD-AMC), and fluorescence was measured by exciting at 380nm, and emission read at 460nm. Protein concentration was determined using a BCA Protein Determination kit (Pierce), and caspase activity was normalized to RFU/ μ g. Recombinant Caspase-3 (R&D Systems) standard dilutions were used to ensure linearity of the assay.

Ex vivo Ischemia/Reperfusion

Hearts cannulated via the aorta and perfused with Krebs-Henseleit solution bubbled with 95% O₂ and 5% CO₂, as described with minor modifications[60]. Hearts were equilibrated for 20 minutes while stimulated at 7Hz, exposed to global no-flow ischemia for 15 minutes, and then reperused for 60 minutes. We exposed hearts to 15 minutes of ischemia as mdx and dysferlin-deficient hearts are much more susceptible to I/R than the C57BL/10 and 129S controls, which themselves are more susceptible to I/R injury than other strains[61]. Hearts were excluded if initial baseline LV developed pressure was less than 70mmHg, or if flow was less than 1 ml/min or greater than 5 ml/min.

In vivo Ischemia/Reperfusion

Mice were anesthetized with isoflurane, and an incision was made between the ribs to expose the heart. A 8-0 nylon suture with a 3.5mm tapered needle was introduced into the left ventricular free wall, and tied around a piece of PE-60 tubing to occlude the coronary artery for 30 minutes. Subsequently, the PE-60 tubing was removed to allow reperfusion, the incision was closed, and the animal was allowed to recover for 24 hours. Infusions of saline or P188 (460 mg/kg[32]) were performed by intravenous injection into the subclavian vein. The procedure was similar for rats, however, we used 7-0 suture and PE-150 tubing for the occlusion.

Assessment of serum cTnI and infarct size

Mice and rats were anesthetized with sodium pentobarbital, and blood was collected from the vena cava in heparinized syringes. Hearts were cannulated and retrogradely perfused with 1% TTC for 5 minutes. The original occlusion was tied, and the heart was perfused with 1% Evans blue. Hearts were sliced in 2mm sections, and incubated at 37 degrees for 15 minutes to allow TTC stain development. Stained sections were then imaged on a Zeiss stereoscope. Area at risk was determined by assessing Evans blue negative staining, and infarct size determined by negative TTC staining. Quantitation of infarct size/area at risk was performed by thresholding signals using Image J. Serum cTnI levels were assessed by ELISA according to manufacturer's directions (Life Diagnostics).

Statistics

Statistical analysis was done with Prism 5 software (GraphPad). One-way ANOVA, Newman Keuls post-hoc tests were used for statistical testing.

Results

Cardiac I/R in vitro

To investigate the role of sarcolemma integrity in I/R injury we tested cardiac myocytes with and without membrane stabilizers. To this end, we adapted a well validated simulated ischemia/reperfusion (sI/R) protocol [48, 49] that causes significant damage and death to adult rat cardiac myocytes (Figure 1). Adult rat cardiac myocytes exposed to simulated ischemia/reperfusion exhibited a robust release of LDH, as expected (Figure 1A). To implement as a direct tool for gain-of-sarcolemma integrity, we used several tri-block copolymer-based membrane sealants (Supplemental Figure 1A) which we have used in previous studies in dystrophic myocardium. Of these, poloxamer 188 (P188) is the best biologically characterized of the synthetic membrane stabilizers[32, 35]. Copolymer based sarcolemmal stabilization (CSS) fully blocked sI/R-induced LDH release (Figure 1A and Supplemental Figure 1B). As a control, the polymer polyethylene glycol of comparable molecular weight (PEG-8000) did not prevent LDH release. Interestingly another copolymer of similar hydrophilicity/hydrophobicity composition to P188 but larger molecular weight

(CSS-extended) was able to completely prevent LDH release at 10-fold lower concentrations (Supplemental Figure 1B). CSS was most effective to block membrane leak when present throughout the sI/R protocol (Supplemental Figures 1C & D). In addition, the release of the clinically relevant cardiac specific biomarker cardiac troponin I (cTnI) was also significantly increased in I/R and this was fully blocked by membrane stabilization (Figure 1B). We next assessed the uptake of FITC-labeled dextran (40kDa), a commonly used assay to quantify cell wounding which is normally excluded from myocytes. Conjugated dextran uptake was significantly increased by sI/R and uptake prevented by CSS (Figure 1C). As dysregulated intracellular Ca^{2+} is a feature of I/R, we next assessed intracellular Ca^{2+} content and transient kinetics in sI/R. In myocytes pre-loaded with the fluorescent Ca^{2+} indicator Fluo-4 prior to sI, intracellular diastolic Ca^{2+} was significantly increased during reoxygenation, consistent with I/R-induced sarcolemmal permeability to Ca^{2+} , and this was significantly blocked by membrane stabilization (Figure 1D). Additionally, recordings of Ca^{2+} transients using the ratiometric Ca^{2+} indicator Fura-2 showed impaired Ca^{2+} decay kinetics in sI/R which were prevented by gain-of-sarcolemma integrity (Figures 1E & F).

We next determined whether gain-of-sarcolemma integrity would impact myocyte viability in sI/R. Calcein-AM fluorescence requires ATP-dependent esterase activity and is a well accepted indicator of cell viability in I/R. Myocytes pre-loaded with calcein prior to sI showed a significant decrease in calcein fluorescence during reoxygenation which was fully prevented by sarcolemma stabilization (Figures 2A&B). Apoptosis is also implicated in oxidative stress and I/R injury, so we assessed the activity of caspase-3 in lysates of myocytes. Here, sI/R induced significant caspase-3 activity, an effector of apoptosis, and this was also fully blocked by sarcolemma stabilization (Figure 2C). Thus markers of necrotic and apoptotic-mediated cell death were both fully blocked by membrane stabilization.

Mitochondrial membrane depolarization is implicated in myocyte death in I/R, so we determined mitochondrial membrane potential by using a dynamic FRET assay between Mitotracker Green (MTG) and the mitochondrial membrane potential-dependent indicator TMRE (Figure 3A). In this assay, green fluorescence by MTG is quenched by TMRE in healthy cells, whereas depolarized mitochondrial membranes lose TMRE causing MTG fluorescence to increase. In myocytes pre-loaded with MTG and TMRE prior to sI, MTG fluorescence during reoxygenation was significantly increased, indicating loss of FRET between MTG and TMRE and thus mitochondrial membrane depolarization, and this was significantly blocked by sarcolemma stabilization (Figures 3B & C). Qualitatively similar results were found using TMRE only (data not shown).

We next assessed myocyte redox state using two independent methods during myocyte-generated oxidant production in sI/R. As a probe of cellular oxidative stress we first pre-loaded myocytes with H_2DCFDA (dihydrodichlorofluorescein) that is oxidized to fluorescent DCF (2',7'-dichlorofluorescein). Here, in sI/R using isolated adult cardiac myocytes, DCF fluorescence during reoxygenation was significantly increased (Figures 4A & B). Because of the importance to use other independent cellular biomarkers to track cell redox status[62–64], we further assessed lipid peroxidation as a second measure of altered redox state using the lipid analog C11-Bodipy (581/591) which exhibits a fluorescent spectral shift upon oxidation[54, 55]. Consistent with the DCF findings, cardiac myocytes pre-loaded with C11-Bodipy then exposed to sI/R exhibited a significant fluorescent shift in C11-Bodipy during reoxygenation indicating marked lipid peroxidation in cardiac myocytes (Figures 4C & D). Interestingly, neither the onset nor the magnitude of sI/R-mediated increases in DCF fluorescence and C11-Bodipy spectral shift were altered in the copolymer-based sarcolemma stabilized myocytes (Figures 4A–D). As a positive control to markedly perturb cardiac myocyte redox state, we next used peroxide and found that H_2O_2 -

induced a marked increase in DCF fluorescence and this also was not significantly altered by direct sarcolemma stabilization (Supplemental Figures 1C & D). Peroxide-induced lipid peroxidation measured by C11-Bodipy spectra analysis was also not affected by membrane stabilization (Supplemental Figures 1E & F). The results of these independent assessments of oxidative stress are evidence that the I/R-induced oxidant production and lipid peroxidation in myocytes are not altered by sarcolemma stabilization and support the conclusion that copolymers do not serve as an antioxidant [30].

Ex vivo isolated heart studies

To determine whether gain-of-sarcolemma integrity would translate to the organ level, isolated hearts were exposed to global no-flow ischemia and then reperfusion *ex vivo*. I/R caused a significant increase in LDH release into the perfusate (Figure 5A), as well as an increase in fluorescent dextran uptake into cardiac tissue (Figure 5B), both of which were significantly blocked in sarcolemma stabilized hearts. There was also a significantly improved LV pressure recovery in I/R in sarcolemma stabilized hearts *ex vivo* (Figure 5E). Consistent with the *in vitro* findings in Figure 1, sarcolemma stabilization *ex vivo* was most effective when present throughout the I/R injury protocol (Supplemental Figure 2D). Interestingly, an extended CSS (CSS-ext) was also able improve functional recovery from I/R in isolated hearts at 10-fold lower concentrations (Supplemental Figure 3). To investigate organ level I/R in loss-of-sarcolemma integrity models we used mice with pre-existing genetic defects in sarcolemmal integrity and repair. Dystrophin deficiency is the cause of Duchenne muscular dystrophy (DMD) and cardiomyopathy by increased sarcolemmal fragility is implicated in the pathogenesis of the disease. Dystrophin-deficient *mdx* mouse hearts exposed to 20 minutes of global ischemia were severely damaged with negligible functional recovery (Supplemental Figure 3A); accordingly, we shortened the ischemic duration to 15 minutes. Here, *mdx* hearts showed significantly decreased LV pressure and elevated LV end-diastolic pressures during I/R compared to C57BL/10 controls (Figure 6; Table 1). Thus, loss-of-membrane integrity precipitated greater I/R injury. The application of gain-of-sarcolemma integrity in the DMD membrane defect model significantly mitigated functional deficits (Figures 6C,D and Table 1).

Recently, membrane repair pathways have been shown to play a role in protection from stress-induced cardiac damage[43, 44]. For example, MG53 knockout mice are highly susceptible to I/R injury[43] and dysferlin deficiency results in Limb Girdle Muscular Dystrophy Type 2B showing stress-induced cardiomyopathy[44]. Dysferlin-deficient (Dysf KO) cardiac myocytes have been shown to have compromised membrane repair[44]. The specific role of dysferlin has not been investigated in I/R injury. We used dysferlin knockout (Dysf KO) mice as a loss-of-function model to determine whether sarcolemmal repair is integral to maintain functional recovery from I/R. Dysf KO mouse hearts exposed to 20 minutes ischemia had, like *mdx* hearts, poor functional recovery (Supplemental Figure 3B); accordingly, we shortened the ischemic duration to 15 minutes. Dysf KO hearts showed significantly decreased function compared to 129 controls in I/R (Figure 6E; Table 2; Supplemental Figure 3B). Other models of dysferlin deficiency, eg. A/J and SJL, are also significantly more susceptible to I/R injury than controls (Supplemental Figure 3C). In these models of defective membrane repair, sarcolemma stabilization significantly blocked the LV functional deficits (Figure 6G,H and Table 2).

In vivo studies

To examine I/R *in vivo*, we occluded coronary arteries in adult rodents for 30 minutes followed by 24 hours reperfusion. Here, release of cTnI into the serum was significantly blocked by sarcolemma stabilization in both mice (Figure 7A) and rats (Figure 7B). Gain-in-sarcolemma integrity implemented only in reperfusion did not affect the increased serum

cTnI levels (Supplemental Figure 4). Infarct size was assessed by staining with Evans blue and TTC. In all groups there was similar Evans blue staining, indicating similar areas at risk. Sarcolemma stabilization produced a marked increase in TTC staining, indicative of increased myocardial viability in I/R (Figures 7C & D).

Discussion

Myocardial ischemia reperfusion injury exacts a tremendous toll on morbidity and mortality in humans world-wide[65]. The mechanistic roads to cardiac I/R injury are complex and critical to elucidate. Myocyte oxidant production and membrane destabilization are key in I/R yet it has been challenging to dissect their specific roles. It is widely considered, nevertheless, the perturbed myocyte redox state is a dominant mechanism underlying cardiac damage in I/R [2, 5, 18, 19, 21]. The main new finding here, in contrast to the prevailing model, is evidence of an uncoupling between the myocyte-derived altered redox state and cellular damage and death in I/R. We used here in gain- and loss-of-sarcolemma integrity models, sarcolemma interfacing molecules as a direct tool to dissect the roles of membrane integrity and oxidative stress in I/R. Results show that the I/R-mediated altered redox state, as measured by two independent assays[64], is alone insufficient to cause cell injury and death in the context of preserved membrane integrity by sarcolemma stabilizers. Thus, we find that in cardiac I/R, elevated oxidants cause significant lipid peroxidation but this is insufficient in itself to precipitate cardiac I/R injury and death when sarcolemma integrity is maintained. We speculate the uncoupling of oxidative stress as a sole agent initiating cell damage could account for the previously unexplained negative clinical trial results using antioxidant therapies in I/R[27, 28]. Our results, instead, point to sarcolemma integrity as a focal mechanism in the myocardial I/R injury pathway.

Mechanistically, our findings can be discussed in terms of a model that incorporates the characteristic timing and magnitude of myocyte generated oxidant production in I/R and yet, with targeted gain-of-sarcolemma integrity, cellular function and myocyte viability are preserved (Figure 8). Here, in the absence of sarcolemma stabilization, reperfusion leads to cell swelling and membrane instability causing elevated intracellular Ca^{2+} , mitochondrial membrane depolarization in a feed-forward degenerative pathway. In contrast, directed outer membrane stabilization interrupts this cycle despite marked I/R-mediated disturbances in redox state. Our results also call into question the role of other proposed membrane and non-membrane targets of oxidants, including intracellular proteins, lipids and DNA[13, 22] in terms of their contribution to acute I/R injury. This model is further supported by our findings in genetic models of defective membrane integrity (dystrophin deficiency) and repair (dysferlin deficiency) where injury is increased and membrane destabilization more dramatic compared to controls. Collectively, these results shed new light on the mechanism of cardiac I/R injury by supporting a direct primary role of membrane integrity in I/R. Presently there is no effective therapy protecting the myocardium in I/R. Based on these results we propose that strategies to enable membrane stabilization may offer a new approach to improve myocardial viability and function in I/R.

It is well accepted that oxidative stress is an early component of reperfusion(67) and it is hypothesized that the oxidation of phospholipids is a central causative factor in cellular damage. Among the myocyte phospholipid membranes, the mitochondrial membrane is thought to be the most susceptible to lipid peroxidation and key to mitochondrial membrane depolarization and dysfunction[26]. In this context, it is revealing that the synthetic membrane stabilizers used here are restricted to engaging the sarcolemma surface and, by virtue of their amphiphilic structure, absorb into the outer membrane surface but do not gain access into the cell[30]. Thus the effects demonstrated here are not explained by copolymers stabilizing the putative intracellular phospholipid targets of oxidants. For example, oxidative

stress is thought to directly alter the sarcoplasmic-reticulum Ca^{2+} ATPase to cause myocyte Ca^{2+} dysregulation[23]. In our experiments both the diastolic Ca^{2+} and Ca^{2+} transient decay rate were significantly preserved by CSS in I/R even though myocyte redox state was markedly disrupted. Thus sarcolemma stabilization is sufficient to protect key cell functions even under conditions that favor increased lipid peroxidation. This model is further supported by the recent demonstration that copolymers themselves do not serve as free radical scavengers[33]. This point is in keeping with our finding here that even non-physiological extensive H_2O_2 -mediated lipid peroxidation is not altered by copolymers. Because the copolymers used here are restricted from cell entry any proposed effect of membrane stabilizers to protect the sarcolemma from oxidant-mediated damage would have to result in myocyte-generated oxidants targeting the outer membrane of the sarcolemma phospholipid bilayer. In this case, myocyte function would be preserved by copolymers affecting membrane lipid packing and/or fluctuations to suppresses free radical diffusion into the phospholipid bilayer[33].

Reduced membrane integrity has been proposed to play a role in I/R injury and consequent myocardial necrosis; however, the precise mechanism and understanding whether this a direct effect or downstream secondary event has not been established[29]. Altered sarcolemma integrity in I/R is inferred by the detection of cardiac myocyte specific contractile proteins in plasma where detection of troponins in the blood is in wide use as definitive clinical evidence of ischemic injury[66, 67]. Among the myriad of alterations in I/R is severe intracellular Ca^{2+} dysregulation caused in part by direct membrane permeability to Ca^{2+} [2]. Unregulated Ca^{2+} entry causes marked mechanical damage to myocytes leading to irreversible hypercontracture [8–11, 68–70]. Intracellular Ca^{2+} dysregulation also initiates mitochondrial depolarization which is known to play a significant role in I/R-induced myocyte death[16, 71, 72]. Our findings directly support the hypothesis that the decisive and irreversible event leading to myocyte death is when the sarcolemma becomes compromised and can no longer maintain a barrier between intra- and extra-cellular components[29]. Although the majority of I/R-induced myocyte loss is widely believed to be necrotic death, apoptosis also plays a significant role[29]. ATP content is thought to control the fate between necrotic and apoptotic death. In isolated hearts, we did not see a difference in contracture during ischemia, providing evidence that membrane stabilization does not affect ATP content. However, membrane stabilization significantly improved functional recovery, perhaps due to the improved ability to regenerate and maintain ATP and thus reduce necrosis. It could therefore be hypothesized that an injured cell with sufficient ATP could then undergo apoptosis. Membrane stabilization also inhibited activation of Caspase-3, an end effector of apoptosis; however, Caspase-3 does not distinguish between extrinsic or intrinsic pathways. Sarcolemmal disruption may cause activation of the receptors in the extrinsic apoptotic pathways, and stabilization may reduce their activity. Membrane stabilization may also reduce Ca^{2+} overload which can activate the intrinsic apoptotic pathways initiated by mitochondria or calpain activation[73].

The concept of membrane damage in I/R injury has been hypothesized since at least the 1950's[74, 75]. Indeed, this remains the basis for utilizing intracellular components as biomarkers for clinical diagnosis of myocardial infarct[66, 67]. Ultrastructural studies of myocardium exposed to I/R show that membrane damage does indeed occur in I/R[76–80]. In this context, it is surprising that relatively little focus has been placed on determining the possible direct role of sarcolemma integrity in I/R injury. In one previous study, myosin antibodies conjugated to liposomes prevented death in H9C2 cardiac-like cells during hypoxic stress in vitro, presumably through a plug and seal membrane stabilization process[81]. In follow up studies, these immunoliposomes were shown to confer cardiac protection in I/R; however, immunoglobulin alone was also shown to be protective, presumably through an oncotic mechanism[82, 83], thus leaving uncertain the specific role

of membrane stabilization in I/R. Due to uncertainties of the mechanism by which lipids-protein complexes could exact membrane protection, and further being mindful of the paradoxical cardiotoxicity effects of these compounds and their extremely limited bioavailability due to their rapid clearance[83], we used protein-free, chemically inert, non-metabolized and fully synthetic molecules to test the membrane stabilization hypothesis in I/R. Synthetic block copolymers used here consist of tandem linear block arrays of polyethylene oxide(PEO)/propylene oxide(PPO) moieties[84]. To gain insight into the mechanism of copolymer-based membrane stabilization, we used a formulation of PEO-PPO-PEO shown to be beneficial in the context of membrane stabilization in progressive cardiomyopathy owing to dystrophin deficiency[32, 35]. Along with blocking dilated cardiomyopathy in dystrophic canines the long-term administration of sealants had no detected effects on kidney or liver [35]. In the present study, this same copolymer was protective during I/R in normal hearts. To address the role of hydrophobicity and the PPO core domain in membrane stabilizing function, which we and others hypothesize to target membrane lipid bilayer deformations during stress[85], we tested exclusive hydrophilic polymer compounds of comparable mass (ie, PEG 8000). These hydrophobic moiety-deficient synthetic polymers were ineffective in I/R. In complement, extended copolymer structures at fixed PPO/PEO ratio, which yield highly potent sealing function, shows that total molecular mass of the copolymer is critical in conferring membrane stabilization. Collectively, these findings suggest an optimal feature of alternating hydrophobic/hydrophilic polymer arrays in conferring membrane interaction and sealing function in I/R.

Membrane fragility is also implicated in several forms of dystrophic cardiomyopathy. In this light, loss of dystrophin has been observed in I/R and this has been postulated to have a role in I/R injury due to loss of membrane stability[36–40, 42]. Preconditioning prevents the loss of dystrophin, leading to the hypothesis that dystrophin is important in preconditioning[37, 38]. The loss of other dystrophin glycoprotein complex components, such as sarcoglycans, has also been observed[42]. Although these previous studies show stress-induced loss of dystrophin, the causal effect of dystrophin loss on I/R injury was not established. It is possible that the loss of dystrophin in I/R is a secondary effect, but this could potentially lead to a feed-forward cycle of additional damage. However, the consequences of dystrophin deficiency in I/R injury have not been previously investigated. Evidence is provided here that dystrophin deficiency causes increased susceptibility to I/R and sealant-based membrane stabilization limits injury in this model, even though previous studies have shown that dystrophin deficient muscles have increased oxidative stress[46]. In addition, recent work suggests that components of the endogenous membrane repair pathway are important for cardioprotection[43, 44]. For example, deficiency in the membrane repair protein MG53 results in increased susceptibility to I/R-induced cell death[43] in keeping with our results here on dysferlin deficient hearts. Recombinant MG53 has been shown to improve pathology in dystrophic skeletal muscle suggesting a new muscle repair approach to protect striated muscle in disease[86]. More recently it has been shown that MG53 has diverse functions outside of muscle repair and can cause insulin resistance and metabolic syndrome[87]. These significant off-target effects, however, raise concerns about implementing MG53 in therapeutic application for membrane repair *in vivo*. Our results show for the first time that dystrophin and dysferlin have potentially unappreciated roles in I/R injury. Future studies could be useful to determine the role of dystrophin and dysferlin and related proteins in I/R injury *in vivo*. For example, it would be important to determine whether membrane stabilization can rescue the I/R-induced loss of dystrophin. Further, the membrane repair pathway has not been investigated in I/R injury *in vivo*. Understanding the role of the membrane repair pathways may be beneficial in developing therapeutics for treating ischemic heart disease.

Previously, synthetic copolymers were proposed as rheological agents to increase blood flow during thrombolytic therapy after myocardial infarction[88–90]. In the CORE clinical trial of RheothRx (chemically identical to P188) the copolymer was administered four hours after the initiation of reperfusion[88]. The use of RheothRx in this context had no clinical benefit. Our results show for the first time that an alternative mechanism and use of copolymers are effective in protection from I/R. In previous studies using poloxamer as a rheological agent after I/R, membrane damage and downstream effectors of Ca^{2+} dysregulation and mitochondrial dysfunction had already occurred in the myocytes prior to sealant delivery. Our findings indicate that membrane damage in I/R is an early precipitating event and requires timely measures to be an effective treatment modality. Membrane stabilization of cardiomyocytes must be in place early in ischemia/reperfusion to be effective. However, pre-treatment of myocytes with stabilizers in vitro was not effective in preventing LDH release, nor did pretreatment of isolated hearts or animals with occlusions in vivo provide protection from I/R. These data suggest that membrane stabilization may wash out immediately at reperfusion, and therefore also needs to be provided during reperfusion to be effective.

In conclusion, our findings support a model of cardiac I/R injury in which intracellular oxidant production alone is insufficient to precipitate myocyte death. By targeted gain-of-sarcolemma integrity key markers of cell injury and death are prevented despite I/R-mediated increased oxidative stress. This supports the hypothesis that sarcolemmal disruption is a direct effector of I/R-induced myocyte injury and death and not a secondary downstream event. In addition, impaired sarcolemmal integrity by loss of dystrophin or associated proteins, or membrane repair pathways by dysferlin, may have underappreciated roles in ischemic heart disease[39, 41]. Taken together, these findings point to the sarcolemma as an attractive target to improve cardiac viability and function in I/R. It can be questioned if membrane instability is an early event in I/R how practical then is sealant administration in the clinical setting. We propose that direct pre-emptive sarcolemmal stabilization by copolymer sealants could be beneficial in a wide range of clinical settings, including in recurring ischemic episodes, perinatal asphyxia [4], unstable angina, acute coronary syndrome, angioplasty[91], coronary artery bypass [91], heart transplantation, or resuscitation after cardiac arrest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CSS	copolymer-based sarcolemmal stabilization
cTnI	cardiac troponin I
DYSF KO	dysferlin knockout mice
H₂DCFDA	dihydrodichlorofluorescein diacetate
I/R	ischemia/reperfusion

LDH	lactate dehydrogenase
mdx	dystrophin-deficient
MTG	Mitotracker Green
P188	poloxamer 188
P338	poloxamer 338
PEO	polyethylene oxide
PEG	polyethylene glycol 8000
PPO	polypropylene oxide
sI/R	simulated ischemia/reperfusion
TMRE	tetramethyl rhodamine ester

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Highlights

- Evidence of sarcolemma instability as a primary upstream effector in I/R injury.
- Membrane sealants increase cell viability despite increased oxidative stress.
- Sealants limit Ca^{2+} and mitochondrial dysfunction in I/R.
- Membrane sealants limit cardiac injury during I/R in vivo.

Figure 1.

Copolymer-based sarcolemmal stabilization (CSS) prevents sarcolemmal permeability during sI/R *in vitro*. A) Myocytes exposed to sI/R exhibited LDH release into culture media, which was completely inhibited by CSS. B) Myocyte release of the cardiac-specific biomarker cTnI was also increased by sI/R, and was prevented by membrane stabilization. C) Myocytes exposed to sI/R in the presence of extracellular Dextran-FITC (40 kDa), a marker of membrane permeability, showed an increase in Dextran-FITC uptake, which was blocked by membrane stabilization. Figures A–C are representatives of at least 3 experiments with similar results. D) Diastolic Ca²⁺ assessed by Fluo-4 fluorescence shows increased diastolic Ca²⁺ following sI/R, which was prevented by CSS (n=4). E) Representative individual Ca²⁺ transient traces in blebbistatin-treated myocytes loaded with the ratiometric dye Fura-2 in sI/R. F) Summary of Ca²⁺ transient reuptake kinetics in sI/R show that CSS normalizes sI/R induced Ca²⁺ kinetics (n=9–13 myocytes per group). Data are presented as mean ± SEM; *P<0.05 versus control, #P<0.05 versus sI/R.

Figure 2.

Membrane stabilization improves viability after sI/R. A) Myocytes loaded with Calcein-AM, an indicator of cell viability, exhibit decreased fluorescence after sI/R and was prevented by copolymer-based sarcolemmal stabilization (CSS) (n=4 replicates). B) Quantification of calcein fluorescence at 30 min reoxygenation. C) Lysates from myocytes exposed to sI/R have significantly increased Caspase-3 activity and is blocked by sarcolemmal stabilization. Results are compiled from 3 independent experiments, expressed as fold over control for each experiment. Data are presented as mean $\pm$ SEM. *P<0.05 compared to control, #P<0.05 compared to sI/R.

Figure 3. Membrane stabilization prevents si/R-induced loss of mitochondrial polarization in vitro. A) Diagram of MTG + TMRE FRET assay to determine mitochondrial polarization. B) Myocytes exposed to si/R show increasing MTG fluorescence, indicating loss of quenching by TMRE and thus mitochondrial depolarization. Sarcolemmal stabilization prevents loss of TMRE and thus decreased MTG fluorescence. Representative experiment shown of 3 independent experiments with similar results. C) Quantification of MTG fluorescence at 60 min reoxygenation. Results are compiled from 3 independent experiments, expressed as fold over control for each experiment. Data are presented as mean $\pm$ SEM. * $P < 0.05$ compared to control, # $P < 0.05$ compared to si/R.

Figure 4.

Membrane stabilization does not affect sI/R-induced oxidant production. A) Fluorescence of the oxidative stress indicator DCF increases after exposing myocytes to sI/R, which is not affected by copolymer-based sarcolemma stabilization (CSS). B) Summary data of DCF fluorescence at 60min reoxygenation. C) Myocytes pre-loaded with the lipid peroxidation sensor C11-Bodipy^{581/591} prior to sI show increased green/red fluorescence ratio during reoxygenation, indicative of oxidized phospholipids, which is not affected by membrane stabilization. D) Summary data of sI/R-induced lipid peroxidation at 60min reoxygenation. Data are presented as mean $\pm$ SEM. Data shown are representative of 3 experiments with similar results. * $P < 0.05$ compared to control.

Figure 5.

Membrane stabilization prevents I/R-induced sarcolemmal permeability and functional deficits in isolated hearts *ex vivo*. A) Isolated mouse hearts exhibit increased release of LDH during reperfusion and this was significantly reduced by sarcolemmal stabilization. B) I/R caused a significant uptake of Dextran-FITC during reperfusion which was inhibited by sarcolemmal stabilization (n=3–6 hearts per group). C–D) Representative LV pressure traces of hearts exposed to I/R: C) C57BL/10 control hearts, D) sarcolemmal stabilized C57BL/10 control hearts. E) C57BL/10 control hearts exposed to I/R show decreased functional recovery which was mitigated by sarcolemmal stabilization. F) LV End Diastolic Pressures in C57BL/10 control hearts were not significantly changed by P188 treatment (n=6–14 hearts per group). Data are presented as mean $\pm$ SEM. *P<0.05 compared to control, #P<0.05 compared to I/R.

Figure 6.

I/R-induced functional deficits in Dystrophin-deficient *mdx* hearts and Dysferlin-deficient (Dysf KO) hearts ex vivo are blocked by sarcolemmal stabilization. A–B) Representative traces of *mdx* hearts exposed to I/R: A) Dystrophin-deficient *mdx* hearts, B) sarcolemmal stabilized *mdx* hearts. C) *mdx* hearts exposed to I/R show severe LV pressure deficits following I/R and lessened by sarcolemmal stabilization. D) *mdx* hearts exposed to I/R have significantly elevated left ventricular end diastolic pressures (LVEDP) and is blocked by sarcolemmal stabilization (n=6–14 hearts per group). E–F). Representative LV pressure traces of Dysf KO hearts exposed to I/R: E) Dysf KO hearts, F) Sarcolemma stabilized Dysf KO. F) Dysf KO hearts exposed to I/R exhibit significant functional deficits and are blocked by by sarcolemmal stabilization. G) Dysf KO hearts have increased LVEDP after I/R and this is lessened by sarcolemmal stabilization (n=11–15 hearts per group). Data are presented as mean $\pm$ SEM. *P<0.05 compared to controls.

Figure 7.

I/R-induced myocardial injury is significantly blocked by sarcolemmal stabilization *in vivo*. cTnI release during I/R injury *in vivo* is significantly blocked by sarcolemmal stabilization in mice (A) and in rats (B). Serum cTnI was below detection levels (B.D.) in sham treated animals. C) Infarct size in rats was measured by Evans blue and TTC staining. Representative staining is shown in (C). D) Myocardial infarct (MI) size relative to area at risk (AAR) in rat hearts was quantified using Image J. Data are presented as mean $\pm$ SEM. n=20–23 mouse hearts per group, and 4–5 rat hearts per group. Two-tailed t-test was used for statistical testing. *P<0.05 compared to I/R.

Figure 8.

Model of sarcolemma stabilization in I/R. A) In the absence of sarcolemma stabilization, I/R causes sarcolemma instability leading to increases in intracellular Ca^{2+} . Dysregulated Ca^{2+} causes necrosis by initiating mitochondrial depolarization and/or activating caspase to cause apoptosis. I/R disrupts the redox state which can damage cellular components such as membranes. B) Copolymer-based sarcolemma stabilization prevents I/R-induced sarcolemmal damage and significantly blocks Ca^{2+} dysregulation and mitochondrial depolarization, even in the presence of significant oxidative stress and altered myocyte redox state, leading to blocking necrotic and apoptotic cell death pathways.

Table 1

Summary of isolated heart Langendorff I/R data in C57BL/10 and mdx mice.

	C57BL/10	C57BL/10 +P188	mdx	mdx + P188
n=	14	12	10	6
baseline LV developed pressure (mmHg)	91.2 ± 6.4	98.6 ± 3.1	97.0 ± 4.9	92.8 ± 4.2
LV end-diastolic pressure, 15' ischemia (mmHg)	64.0 ± 5.3	72.1 ± 3.6	65.7 ± 2.0	52.0 ± 5.5
LV end-diastolic pressure, 60' reperfusion (mmHg)	30.1 ± 4.4	23.1 ± 4.0	47.7 ± 3.3 [*]	33.0 ± 5.4
LV developed pressure, 60' reperfusion (mmHg)	25.2 ± 4.1	41.5 ± 3.4 [*]	13.2 ± 2.4 [*]	32.3 ± 8.2

Data are presented as mean ± SEM.

* P<0.05 compared to C57BL/10 controls.

Table 2

Summary of isolated heart Langendorff I/R data in 129 and Dysf KO mice.

	129	129 + P188	Dysf KO	Dysf KO +P188
n=	8	10	13	13
baseline LV developed pressure (mmHg)	95.8 ± 5.6	90.1 ± 6.0	90.8 ± 3.7	86.7 ± 4.9
LV end-diastolic pressure, 15' ischemia (mmHg)	70.4 ± 8.5	64.6 ± 5.3	73.0 ± 2.9	52.83 ± 6.4
LV end-diastolic pressure, 60' reperfusion (mmHg)	45.5 ± 3.5	31.0 ± 4.3*	46.6 ± 4.1	30.3 ± 3.9*
LV developed pressure, 60' reperfusion (mmHg)	29.8 ± 3.3	37.0 ± 2.9*	21.1 ± 2.3*	35.4 ± 2.7

Data are presented as mean ± SEM.

* P<0.05 compared to 129 controls.