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Functional Importance of Oxidative Post-Translational Modifications of Ryanodine Receptor in Cardiac Sarcoplasmic Reticulum Ca2+ Handling During Oxidative Stress

Stefan R. Mazurek
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LOYOLA UNIVERSITY CHICAGO

FUNCTIONAL IMPORTANCE OF OXIDATIVE POST-TRANSLATIONAL MODIFICATIONS OF RYANODINE RECEPTOR IN CARDIAC SARCOPLASMIC RETICULUM Ca^{2+} HANDLING DURING OXIDATIVE STRESS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CELLULAR AND MOLECULAR PHYSIOLOGY

BY

STEFAN R. MAZUREK

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DECEMBER 2014
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<td>Action Potential</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular</td>
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<td>AMI</td>
<td>Acute Myocardial Infarction</td>
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<tr>
<td>β-AR</td>
<td>β-Adrenergic</td>
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<tr>
<td>Calcium</td>
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<td>[Ca²⁺]ᵢ</td>
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<td>DTDP</td>
<td>Dithiodipyridine</td>
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<td>ECC</td>
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<td>ECG</td>
<td>Electrocardiogram</td>
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<td>FFR</td>
<td>Force Frequency Response</td>
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<td>LTCC</td>
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<td>mBB</td>
<td>Monobromobimane</td>
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<td>NCX</td>
<td>Na⁺/Ca²⁺ exchanger</td>
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<tr>
<td>NEM</td>
<td>N-Ethylmaleimide</td>
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<tr>
<td>PTM</td>
<td>Post-Translational Modification</td>
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<td>RyR</td>
<td>Ryanodine Receptor</td>
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<td>SA</td>
<td>Sinoatrial</td>
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<tr>
<td>SERCA</td>
<td>Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase</td>
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<td>SCD</td>
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<td>Spontaneous Ca²⁺ Wave</td>
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<td>SNAP</td>
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<td>T-tubule</td>
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CHAPTER I: INTRODUCTION

Free ionic calcium (Ca\textsuperscript{2+}) plays an essential role as a second messenger that initiates muscle contraction in the heart (Bers, 2002). In the event that intracellular Ca\textsuperscript{2+} regulation is compromised, both the contractility and electrical excitability of the heart can be altered (Rubart & Zipes, 2005; Morgan, 1991). As a consequence, heart function may not be able to maintain the necessary cardiac output to meet the metabolic demand of the body.

During a cardiac cycle, the movement of Ca\textsuperscript{2+} is regulated by proteins that function as channels and pumps, which localize on the sarcolemma and the sarcoplasmic reticulum (SR) (Bers, 2002). The SR is a specialized organelle in muscle that acts as a Ca\textsuperscript{2+} store for the purpose of generating a robust rise in cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) during systole. This cytosolic Ca\textsuperscript{2+} release event is known as a global (non-localized) Ca\textsuperscript{2+} transient. During diastole, Ca\textsuperscript{2+} is actively sequestered back into the SR, which allows for relaxation and priming for the next cycle (Periasamy & Huke, 2001). The molecular machinery that is responsible for Ca\textsuperscript{2+} release from the SR is the ryanodine receptor (RyR; type2 isoform) complex (Bers, 2004).

The intrinsic automaticity of the sinoatrial (SA) node dictates the rate of contraction of the heart. When an action potential (AP) is elicited, it will propagate
through the atria, atrioventricular (AV) node, His-Purkinje system, and ventricles from endocardial to epicardial eliciting cardiac muscle contraction in an order that is necessary for proper pump function of the heart (Anderson et al., 2009). This order of excitation is essential for creating pressure gradients that promote the movement of blood into and out of each chamber. The result is efficient blood flow from the venous return, through the respiratory circulation, and out to the systemic circulation. Therefore, any change in the order or overall excitation of the myocardium due to arrhythmogenesis can lead to sudden cardiac death (SCD).

Cardiac arrhythmias that originate from the ventricle, as opposed to the atria, can severely compromise the pump function of the heart and are therefore potentially life-threatening. These events can override the normal excitation of the sinus node, which can be distinguished clinically on the electrocardiogram (ECG). If ventricular tachycardia is sustained, it can devolve into fibrillation and SCD. These lethal arrhythmias can occur as the result of an ischemic episode, myocardial infarction, or in the failing heart (Pye & Cobbe, 1992; Gheeraert et al., 2006). The underlying arrhythmogenic activity is frequently attributed to spontaneous action potentials due to triggered activity (Xing & Martins, 2004), which also has the potential of initiating reentrant tachyarrhythmias (Nattel et al., 2008; Heijman et al., 2013). Delayed afterdepolarization (DAD), a mechanism responsible for triggered activity, is a spontaneous depolarization of the sarcolemma that can elicit action potentials in a zone
of the myocardium that normally has no pacemaker activity. Consequently, they can gain automaticity and persist as an ectopic pacemaker. It has been determined that the underlying mechanism of DADs is spontaneous $\text{Ca}^{2+}$ release from the SR during diastole (Fozzard, 1992). As a result of SR $\text{Ca}^{2+}$ mishandling, $\text{Ca}^{2+}$ can be extruded from the cell via the electrogenic $\text{Na}^+$/\text{Ca}^{2+} exchanger (NCX) causing depolarization of the sarcolemma (Spencer & Sham, 2003). If this depolarization event reaches threshold of voltage gated $\text{Na}^+$ channels, a spontaneous action potential can be elicited and can propagate to neighboring myocardium. Therefore, understanding the molecular mechanisms that promote spontaneous SR $\text{Ca}^{2+}$ release in heart disease is imperative for designing a therapeutic strategy against arrhythmogenesis.
CHAPTER II: LITERATURE REVIEW

Cardiac Excitation-Contraction Coupling

Excitation-Contraction Coupling (ECC) is the cellular mechanism that connects the electrical stimulus to the contraction of the heart. During ECC, there is a global rise in [Ca\textsuperscript{2+}] that triggers contraction with the binding of Ca\textsuperscript{2+} to the myofilament protein troponin C. This induces an allosteric change in the troponin-tropomyosin complex allowing for myosin heads to form a cross bridge interaction with actin (Solaro & Rarick, 1998). Once this Ca-dependent interaction takes place, mechanical force is generated with the hydrolysis of ATP by the acto-myosin ATPase that facilitates cross-bridge cycling. These cycles will persist as long as [Ca\textsuperscript{2+}] is present at a sufficient concentration or until ATP is no longer available (Goldman, 1987). In the mammalian myocardium, the average [Ca\textsuperscript{2+}] threshold for contraction is between 0.1 µM - 0.5 µM and the half maximal force is generated at approximately 5 µM (Endo M & Kitazawa T, 1977).

The transport systems involved in the movement of Ca\textsuperscript{2+} into and out of the cytosolic milieu contribute directly to the activation and relaxation of the myofilaments (Bassani et al., 1992). Furthermore, they are responsible for steady state Ca\textsuperscript{2+} within the myocyte. This means that for a given heart rate (cardiac demand remains constant), Ca\textsuperscript{2+} influx into the cytosol (from extracellular) during systole must be balanced by Ca\textsuperscript{2+}...
extrusion (intracellular to extracellular) during diastole so that there is no net change in SR Ca\(^{2+}\) content from each cardiac cycle (conservation of mass). This is also set by SR Ca\(^{2+}\) reuptake by the SR Ca-ATPase (SERCA) and SR Ca\(^{2+}\) leak during diastole that is primarily mediated by the RyR2 (Bers, 1985). Thus, the relative activity of RyR2 plays a role in setting SR Ca\(^{2+}\) content on a beat to beat basis.

**The Molecular Components of Cardiac Ca\(^{2+}\) Cycling**

Activated by an influx of Ca\(^{2+}\) from voltage gated L-type Ca\(^{2+}\) channels (LTCCs) during an AP, RyR2 mediates the release of Ca\(^{2+}\) from the SR during systole. This mechanism is known as Ca-induced Ca\(^{2+}\) release (CICR) (Fabiato, 1983). In order for membrane excitation to simultaneously activate SR Ca\(^{2+}\) release in ventricular myocytes, sarcolemmal invaginations called transverse tubules (T-tubules) descend deep into the myocyte. The SR forms a junction with the T-tubule creating a specialized subcellular microdomain called the dyadic cleft that allows for efficient activation of CICR (Soeller & Cannell, 1999). Within the dyadic cleft, RyRs and LTCCs interact in a highly organized lattice forming a critical domain termed the couplon. The cluster of RyRs within the couplon comprise the calcium release unit (CRU) (Franzini-Armstrong & Jorgensen, 1994; Scriven *et al.*, 2013). It has been estimated that RyR2 complexes form clusters of approximately 100 in size on the junctional SR membrane (Franzini-Armstrong *et al.*, 1999). However, the exact value currently remains a debated issue. Other investigators have estimated RyR cluster number to be smaller in size (Hayashi *et al.*, 2009; Baddeley
et al., 2009). These RyR2 clusters align with LTCCs in the dyadic cleft via junctophilins (Garbino et al., 2009). The global rise in $[\text{Ca}^{2+}]$, elicited by an AP is termed the $\text{Ca}^{2+}$ transient. In a ventricular myocyte, SR $\text{Ca}^{2+}$ release is the result of the spatio-temporal activation of thousands of RyR2 clusters that make up individual $\text{Ca}^{2+}$ release sites. The amplitude of the $\text{Ca}^{2+}$ transient during systole is the result of local subcellular recruitment of $\text{Ca}^{2+}$ release units (Stern, 1992). SR $\text{Ca}^{2+}$ release allows for a robust rise in global $[\text{Ca}^{2+}]_i$, which is important for fast allosteric activation of troponin C and myofilament contraction. During diastole, there are two major $\text{Ca}^{2+}$ transport systems that compete for cytosolic Ca; SERCA and the sarcolemmal NCX. The sarcolemmal Ca-ATPase and the mitochondrial $\text{Ca}^{2+}$ uniporter compete as well, but are considered to be minor components (Bassani et al., 1992). Immediately after the global rise in cytosolic $\text{Ca}^{2+}$ as a result of CICR, the majority of $\text{Ca}^{2+}$ is sequestered back into the SR by SERCA and to a lesser extent is extruded from the cell by NCX. The contribution of SERCA and NCX to decreasing $[\text{Ca}^{2+}]_i$ during diastole is variable among species. It has been estimated that NCX contributes only 7% to $\text{Ca}^{2+}$ removal in mouse and rat myocardium (Bassani et al., 1994; Sham et al., 1995). Whereas, in the human and rabbit myocardium, SERCA and NCX contribute approximately 70% and 30% to cardiac relaxation, respectively (Bers & Bridge, 1989). There are two major contributors to the $\text{Ca}^{2+}$ transient in the heart; Inward $\text{Ca}^{2+}$ current ($I_{\text{Ca}}$) and SR $\text{Ca}^{2+}$ release. While $I_{\text{Ca}}$ from the LTCC contributes approximately 25-30% to the $\text{Ca}^{2+}$ transient in the rabbit ventricle, the
majority of Ca\(^{2+}\) comes from SR Ca\(^{2+}\) release by RyR2 (Bassani et al., 1993). At steady state, \(I_{Ca}\) and SR Ca\(^{2+}\) release must be balanced by Ca\(^{2+}\) extrusion and SR Ca\(^{2+}\) uptake. Therefore, any changes in \(I_{Ca}\), SR Ca\(^{2+}\) release, SR Ca\(^{2+}\) reuptake, or sarcolemmal Ca\(^{2+}\) extrusion can have a profound effect on Ca-dependent inotropy (force) and lusitropy (relaxation).

**Sarcoplasmic Reticulum Ca\(^{2+}\) Release Channel**

The SR is an intracellular organelle network that functions as a Ca\(^{2+}\) source and sink during the systolic and diastolic phase of the cardiac cycle, respectively. Separated into two morphologically distinct but contiguous sections, the longitudinal (non-junctional) SR spans the length of the sarcomere and the terminal cisternae (junctional) SR form junctions in parallel with the T-tubule (Winegrad, 1965). The junctional SR is functionally and structurally specialized to participate in CICR (Block et al., 1988).

Concentrated here, across from sarcolemmal LTCCs, are RyR2 clusters that make up the SR Ca\(^{2+}\) release sites (Lai et al., 1988). This junction forms the dyadic cleft. In addition to the RyR2 clusters, it has been proposed that isolated or “rogue” RyRs can localize in longitudinal or free SR (Sobie et al., 2006).

RyR2 has a relatively low selectivity given that it’s permeable to many different divalent and monovalent cations. Furthermore, the channel has a very high conductance of approximately 100 pS for divalent cations (Gillespie & Fill, 2008). Its characteristically low selectivity for Ca\(^{2+}\) is suggested to be fundamental to its
physiological role to produce a fast and large Ca\textsuperscript{2+} release event. While Ca\textsuperscript{2+} needs to compete with other cations for occupancy of the channel pore, it has been proposed that RyR2 has surface or vestibule charges that may enhance the permeation of Ca (Mead-Savery et al., 2009; Gillespie, 2008).

**Ryanodine Receptor Complex**

Predominantly expressed in cardiac muscle, the type 2 isoform of RyR2 is a tetrameric channel with a total molecular weight of approximately 564 kDa (Takeshima et al., 1989). Activated by Ca\textsuperscript{2+} during an AP, RyR2 acts as the primary SR Ca\textsuperscript{2+} release channel (Fill & Copello, 2002). Although Ca\textsuperscript{2+} is the central physiological activator of RyR, other free ions and small molecules can alter its activity including Mg\textsuperscript{2+} and ATP (Eager & Dulhunty, 1998; Masumiya et al., 2001). There are a number of proteins that interact with RyR2 as well, each of which can modulate the channel’s activity. Proteins that interact on the cytosolic side of RyR2 include: calmodulin, FK-506 binding proteins (FKBP), sorcin, and Homer-1 (Balshaw et al., 2001; Bers, 2004). The two known kinases that are scaffolded on RyR, protein kinase A (PKA) and calcium/calmodulin dependent kinase (CaMKII), have been shown to phosphorylate RyR2 at Ser-2809 and Ser-2815, respectively (Marx et al., 2000; Wehrens et al., 2004). Also, there are three known associated protein phosphatases that play a role in regulating RyR2 phosphorylation including PP1, PP2A, and PP2B. Spanning the SR membrane, but also associated with RyR2, are the auxiliary proteins junctin and triadin. Their function is thought to be
important for RyR’s ability to sense luminal $\text{Ca}^{2+}$ ([Ca$^{2+}]_{\text{SR}}$) via interactions with the SR Ca$^{2+}$ binding protein calsequestrin (Bers, 2004). All of the aforementioned proteins that make up the RyR2 complex are necessary for proper function of RyR2 channel activity.

While oxidative stress can directly affect RyR2, it is important to acknowledge that oxidative stress can indirectly affect RyR2 channel activity as the result of a change in function of associated regulatory proteins (Zissimopoulos et al., 2007; Erickson et al., 2008; Humphries et al., 2007). For the purpose of feasibility, the scope of this work will only examine the effect of oxidative post-translational modifications (PTMs) of the RyR.

**Ca-induced $\text{Ca}^{2+}$ release of RyR2**

Unlike ECC in skeletal muscle, local Ca$^{2+}$ entry from LTCC is an absolute requirement for SR Ca$^{2+}$ release in cardiac muscle. Fabiato and Fabiato were the first to characterize cardiac CICR by using skinned cardiac myocytes and directly controlling Ica (Ca$^{2+}$ current) at the dyadic cleft (Fabiato & Fabiato, 1975). Further work by Fabiato showed that SR Ca$^{2+}$ release by CICR was graded, having a dependence on both time (duration of the Ca$^{2+}$ pulse) and trigger ([Ca$^{2+}$] amplitude). Moreover, it was shown that the introduction of high [Ca$^{2+}$] immediately after the Ca$^{2+}$ pulse trigger would cause a decrease in SR Ca$^{2+}$ release. It was concluded that this characteristic was due to a Ca-dependent inactivation site on the cytosolic side of RyR2 (Fabiato, 1985). While the activation characteristics of RyR2 have since been confirmed, inactivation of RyR2 or termination of CICR still remains controversial. A more recent study was unable to show
that high [Ca\(^{2+}\)] promotes inactivation of CICR. Unlike Fabiato’s work, this study carried out pulsatile Ca2+ uncaging in an intact cellular environment (Nabauer & Morad, 1990). Their results suggest the existence of other CICR termination mechanisms. In vitro and in vivo studies have shown that CICR termination, RyR2 channel activity, and channel restitution are indeed dependent on luminal (SR) [Ca\(^{2+}\)] (Gyorke et al., 2004; Stevens et al., 2009; Terentyev et al., 2002; Gyorke et al., 2002; Qin et al., 2008). Furthermore, it has been demonstrated that SR Ca\(^{2+}\) release terminates at a critical level of [Ca\(^{2+}\)]\(_{SR}\), which is dependent on RyR2 channel gating (Zima et al., 2008a). The fractional release of SR Ca\(^{2+}\) for a given [Ca\(^{2+}\)]\(_{SR}\) during CICR is therefore indicative of RyR2 channel activity (Bassani et al., 1993; Bassani et al., 1995; Shannon et al., 2000a). For example, RyR2 sensitization to cytosolic Ca\(^{2+}\) by the presence of 250uM caffeine results in a lower SR termination level, increased SR Ca\(^{2+}\) fractional release, and increased cytosolic Ca\(^{2+}\) transient (Domeier et al., 2009). These characteristics of increased RyR2 activity are suggested to have pathological significance in the event of arrhythmogenic spontaneous SR Ca\(^{2+}\) waves during SR Ca\(^{2+}\) overload and promoting abnormally low SR Ca\(^{2+}\) content seen in the failing heart (Zima et al., 2014).

**Local Control, Luminal Regulation, and Inter-RyR2 Regulation**

The mechanism of CICR can be logically thought of as a self-regenerative process since Ca\(^{2+}\) release from the SR would diffuse throughout the dyadic cleft and activate neighboring RyR2 clusters. If CICR was unopposed, SR Ca\(^{2+}\) release would continue until
[Ca\(^{2+}\)]_{SR} was fully exhausted. However, during an AP, SR Ca\(^{2+}\) release is graded with Ica and CICR is halted before [Ca\(^{2+}\)]_{SR} is entirely depleted (Zima et al., 2008a; Shannon et al., 2003; Stern & Cheng, 2004). This suggests that the positive feedback mechanism of CICR within the dyadic cleft is countered by channel inactivation or a termination process. There is a general consensus that the control of CICR is a local phenomenon that is in part due the spatial organization of RyR2 clusters within the dyadic cleft. The local control theory, which was first described by Stern, states that CICR between RyR2 clusters is limited by physical separation as well as the high [Ca\(^{2+}\)] necessary to activate a RyR2 cluster (Stern, 1992). Small increases in [Ca\(^{2+}\)]\(_i\), however, have also been shown to significantly increase SR Ca\(^{2+}\) leak in resting permeabilized myocytes (Bovo et al., 2011). These results suggest that high local [Ca\(^{2+}\)]\(_i\) is not a limiting factor for RyR2 channel activation. Furthermore, even though [Ca\(^{2+}\)]\(_i\) is changing dynamically throughout each cardiac cycle, SR Ca\(^{2+}\) leak (stochastic events immediately after an elicited Ca\(^{2+}\) transient) remains relatively constant due to its dependence on [Ca\(^{2+}\)]\(_{SR}\). The ability for RyR2 to maintain a constant leak rate with contrasting [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_{SR}\) during a cardiac cycle is proposed to be an essential mechanism for setting and maintaining an appropriate SR Ca\(^{2+}\) load (Zima et al., 2010).

While Fabiato was the first to suggest an inactivation mechanism for RyR2 ([Ca\(^{2+}\)]\(_i\) dependent), a number of other mechanisms have since been proposed that implicate luminal regulation ([Ca\(^{2+}\)]\(_{SR}\) dependent) of RyR2 in the termination of CICR.
Without changes in RyR2 channel activity, SR Ca\(^{2+}\) release terminates at a critical level that remains constant on a beat to beat basis (Domeier et al., 2009). Moreover, the termination level observed from a single RyR2 cluster (Ca\(^{2+}\) spark events) also remains constant (Zima et al., 2008a). This intrinsic property of RyR2 implies that there is the potential for a Ca\(^{2+}\) sensor to exist on the luminal side of the channel. While few argue the existence of a luminal Ca\(^{2+}\) binding site on RyR2 that acts as the primary [Ca\(^{2+}\)]\(_{SR}\) sensor (Chen et al., 2014), multiple studies claim that the luminal Ca\(^{2+}\) sensing mechanism is solely regulated by a complex made up of junctin, triadin, and calsequestrin (Gyorke et al., 2004; Qin et al., 2008; Beard et al., 2004; di Barletta et al., 2006). Furthermore, evidence linking the naturally occurring mutations in calsequestrin and triadin to catecholaminergic polymorphic ventricular tachycardia (CPTV) provide further support for the latter mechanism (Liu et al., 2006; Roux-Buisson et al., 2012).

While the dependence of SR Ca\(^{2+}\) release on total SR Ca\(^{2+}\) load is widely accepted as fact, the theory that RyR2 activity is solely regulated by a luminal mechanism remains highly debatable. Other mechanisms have been proposed that explain termination of SR Ca\(^{2+}\) release as a RyR2 current dependent process (Yonkunas MJ et al., 2012; Guo et al., 2012). A recently proposed theory called pernicious attrition explains the RyR2 dependence on SR Ca\(^{2+}\) load by the magnitude of the trans-SR Ca\(^{2+}\) driving force (Gillespie & Fill, 2013), simply meaning that as RyR2 Ca\(^{2+}\) current increases with increasing SR Ca\(^{2+}\) load, inter-RyR2 CICR within or between nearby clusters will facilitate
SR Ca\(^{2+}\) release. Intuitively, as SR Ca\(^{2+}\) load falls, local Ca\(^{2+}\) release will fail to sustain inter-RyR2 CICR leading to the termination of SR Ca\(^{2+}\) release. While RyR2 gating is stochastic in nature, the decreased local Ca\(^{2+}\) will no longer be sufficient to activate channels that have spontaneously closed. Therefore, any PTMs that promote the open conformation of RyR2 or enhance the channel’s sensitivity to \([\text{Ca}\(^{2+}\)]_i\) would likely hinder this termination process and therefore, allow for a greater amount of SR Ca\(^{2+}\) release.

**SR Ca\(^{2+}\) Leak**

The RyR, being the main pathway for Ca\(^{2+}\) release from the SR, is responsible for triggered (by LTCC) Ca\(^{2+}\) release during systole as well as non-triggered SR Ca\(^{2+}\) release events during diastole. Non-triggered Ca\(^{2+}\) release events are referred to as SR Ca\(^{2+}\) leak. By definition, RyR2 channel gating is stochastic in nature (Stern, 1992; Laver et al., 2013). The probability of RyR2 channel opening (\(P_o\)) is primarily dependent on local \([\text{Ca}\(^{2+}\)]_i\), but can also be affected by a number of other factors (i.e. ATP, Mg\(^{2+}\), etc.) that have the ability to regulate channel activity (Eager & Dulhunty, 1998; Masumiya et al., 2001). The open probability of RyR2 is described in single channel recordings as having a value of 0<\(P_o<1\), where \(P_o=1\) is a 100% likelihood of being open. Since Ca\(^{2+}\) is the main physiological activator of RyR, \(P_o\) nears the value 1 when local \([\text{Ca}\(^{2+}\)]_i\) is high (~100 \(\mu\)M) at the dyadic cleft (e.g. LTCC activation). As \(P_o\) approaches 1 during systole, the SR Ca\(^{2+}\) release events summate to form a global Ca\(^{2+}\) transient. During repolarization and relaxation of the myocardium, RyR2 \(P_o\) approaches 0 as \([\text{Ca}\(^{2+}\)]_i\)
declines back down toward basal diastolic levels (~100nM) (Xu et al., 1998b; Rousseau & Meissner, 1989). Tightly controlled [Ca\textsuperscript{2+}], by extrusion and reuptake machinery promotes a low P\textsubscript{o} for RyR2, which limits spontaneous Ca\textsuperscript{2+} release from the SR and ensures synchronized relaxation of the contractile apparatus.

SR Ca\textsuperscript{2+} leak mediated by RyR2 can occur as spontaneous Ca\textsuperscript{2+} sparks as well as non-spark-mediated leak which is undetectable with current microscopy techniques (Zima et al., 2010). Ca\textsuperscript{2+} sparks are elementary Ca\textsuperscript{2+} release events that arise from the activation of individual RyR2 CRUs. These release events are observed as spatially restricted rises in [Ca\textsuperscript{2+}], that predominantly occur at junctional SR adjacent to the z-lines of sarcomeres. Cheng and colleagues were the first to visualize Ca\textsuperscript{2+} sparks in 1993 (Cheng et al., 1993). Soon after, a growing body of work described the average Ca\textsuperscript{2+} spark to increase local [Ca\textsuperscript{2+}], by approximately 200-400 nM and have a spatial width of about 2\textmu m (Cannell et al., 1995; Lopez-Lopez et al., 1994; Satoh et al., 1997).

Spontaneous Ca\textsuperscript{2+} sparks can be observed during diastole of electrically paced isolated myocytes as well as myocytes at rest. Experimentally, the frequency of Ca\textsuperscript{2+} spark events can be used to determine RyR2 channel activity. While Ca\textsuperscript{2+} spark events occur at a very low frequency in a healthy denervated myocyte, a significantly increased Ca\textsuperscript{2+} spark propensity during diastole can be elicited by exposing myocytes to high levels of sympathetic stimulation using the β-Adrenergic (β-AR) agonist isoproterenol (100 nM) (Santiago et al., 2013). As the frequency of Ca\textsuperscript{2+} sparks during diastole increases over
time, Ca\textsuperscript{2+} sparks can summate causing local [Ca\textsuperscript{2+}], to rise to a level at which neighboring clusters become activated. Consequently, the summation and subsequent activation of RyR2 clusters within a junction can propagate an asynchronous global SR Ca\textsuperscript{2+} release event known as a spontaneous Ca\textsuperscript{2+} wave (SCW). The SCWs are a form of SR Ca\textsuperscript{2+} leak that is considered sufficient to induce spontaneous APs and is therefore considered the major substrate for DADs (Shiferaw et al., 2012; Xie & Weiss, 2009).

**Cardiac Arrhythmias**

Sudden cardiac death (SCD) is responsible for upwards of 50% of all deaths associated with cardiovascular disease in the United States and other developed countries (Reddy S.K. & Yusuf S., 1998). Ventricular tachyarrhythmias that devolve into fibrillation are the primary cause of most SCD cases (Zipes & Wellens, 1998). Although the electrophysiological changes are mostly understood, the molecular mechanisms that cause these changes and the onset of life threatening arrhythmias during myocardial stress still remain elusive.

**Pathophysiology of Arrhythmias**

The pathogenesis of cardiac arrhythmias is divided into three primary mechanisms: abnormal or enhanced automaticity, re-entry of excitation, and triggered activity. Although each mechanism can induce arrhythmias by itself, the likelihood of multiple mechanisms acting in concert is increased as a result of cardiovascular disease.
Abnormal automaticity is defined as an arrhythmic event in which a latent pacemaker, a region of myocardium that exhibits automaticity properties, overrides the intrinsic rhythm of the SA node. Enhanced normal automaticity refers to an abnormally accelerated AP rate produced by the normal pacemakers (SA or AV node). Although all cardiomyocytes possess intrinsic automaticity to some degree, a variety of factors can enhance or inhibit pacemaker’s or cardiac tissue’s automaticity properties. These factors include changes in intracellular pH, ionic imbalances (i.e. hypo/hyperkalemia - calcemia), or elevated catecholamines (Mangoni & Nargeot, 2008).

The mechanism of re-entry of excitation is essentially the repetitive propagation of an AP due to an abnormal circuit of conduction. There are two potential factors that can generate irregular conduction pathways, allowing re-entry to manifest. The first of these is the occurrence of an anatomical conduction block. In the case of an acute myocardial infarction (AMI), a region of myocardium that becomes ischemic or infarcted can either slow or block AP conduction. If an anatomical block only allows one pathway of normal conduction (unidirectional block), an AP wave front can “re-enter” back toward excitable tissue (relative refractory or repolarized) that lies around the anatomical block (Kleber & Rudy, 2004). The second cause of re-entry, termed functional re-entry, is dependent on heterogeneities in conduction and not anatomic obstacles (Allesie MA et al., 1976). It has been suggested that the primary cause of
these heterogeneities is abnormal channel expression/conductance as a consequence of structural remodeling (Valderrabano, 2007; Kleber & Rudy, 2004).

Although re-entry substrates can exist in the diseased heart, they normally remain quiescent unless electrical instability triggers them. Ectopic foci have been implicated as the major contributor for initiating re-entry of excitation (Nattel et al., 2008). Moreover, it is well accepted that the underlying cause for ectopic firing is both abnormal automaticity and triggered activity.

**Triggered activity**

As described previously, there are multiple mechanisms that can potentially elicit arrhythmias in disease states. Substrates for these arrhythmias include abnormal conduction and spontaneous excitability, which can result from I/R injury or occur during the progression to heart failure (Fozzard, 1992; Xing & Martins, 2004). Spontaneous APs, due to triggered activity, have been implicated in initiating and maintaining tachyarrhythmias (Kujala et al., 2012). Furthermore, there is evidence suggesting that triggered activity from DAD is the main mechanism facilitating ectopic foci in ischemic myocardium (Xing & Martins, 2004).

Triggered activity is defined as a spontaneous impulse that arises from an afterdepolarization, which can potentially occur anywhere throughout the myocardium. Although all cardiomyocytes have some degree of intrinsic automaticity, the underlying mechanisms inducing afterdepolarizations involve the enhancement or generation of
automaticity properties (Fozzard, 1992). Ultimately, this tissue region can become an ectopic focus that can initiate bouts of arrhythmias.

Afterdepolarizations, the cause of triggered activity, are fluctuations in the membrane potential or irregular depolarizations of the sarcolemma that occur following the upstroke of an AP. If a depolarization reaches the threshold of voltage gated Na\(^+\) channels, a “triggered” ectopic AP can be generated (Zipes, 2003). Afterdepolarizations are categorized into two types of mechanisms, which manifest at different phases during the AP. While DADs occur once repolarization is complete (phase 4), early afterdepolarizations (EADs) can occur during either phase 2 or 3 of the AP (Clusin, 2003). A number of abnormal conductance properties have been implicated in the pathophysiology of EADs including enhanced I\(_{\text{Na}}\) (Zaza et al., 2008), I\(_{\text{Ca,L}}\) (Yamada et al., 2008), and I\(_{\text{Cl}}\) (Verkerk et al., 2003) or decreased activity of I\(_{\text{K1}}\) (Maruyama et al., 2011). Furthermore, it has also been shown that enhanced NCX by increased Ca\(^{2+}\) release from the SR can promote EAD formation (Patterson et al., 2006). The occurrence of DADs, on the other hand, is solely associated with enhanced NCX activity due to spontaneous Ca\(^{2+}\) release from the SR. Therefore, DADs occur through a SR-dependent mechanism, while EADs can occur through both a SR- and sarcolemma-dependent mechanisms (Clusin, 2003).

Without proper AP propagation along the correct pathway of excitation, the heart as a pump is rendered insufficient. Although arrhythmogenesis can occur through
a number of different mechanisms, multiple mechanisms can act synergistically in the
generation and maintenance of arrhythmic episodes. While triggered activity has been
implicated as a major mechanism underlying ectopic foci, it has also been suggested to
initiate re-entry of excitation. Therefore, elucidating the molecular mechanism involved
in promoting triggered activity after an ischemic event is crucial in designing new
therapeutic strategies in the prevention of life-threatening arrhythmias.

**SR Ca\(^{2+}\) Cycling in Heart Disease**

The heart vitally relies on tightly controlled Ca\(^{2+}\) cycling and signaling for proper
pump function. In both ischemic and non-ischemic etiologies of heart disease, the heart
undergoes a large number of changes that contribute to the disease phenotype as well
as to the progression into heart failure. In these pathophysiological states, both the
contractile function as well as the electrical properties of the myocardium becomes
dysfunctional. Abnormal Ca\(^{2+}\) handling, which is a prevailing hallmark described in I/R
injury and the failing heart, is considered to be a major downstream effect that
ultimately promotes the disease phenotype (Talukder et al., 2009; Zima et al., 2014). In
the healthy myocardium, [Ca\(^{2+}\)] is robust and dynamic in nature allowing the molecular
machinery of the sarcomere to respond in an appropriate fashion. However, this
second messenger system is dynamic. In fact, the intrinsic ability of the myocardium to
modify the Δ[Ca\(^{2+}\)], and duration of each Ca\(^{2+}\) transient event is fundamental property of
the heart so that it can match cardiac output with the demand of the body (Lakatta,
Abnormal [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_{SR}$, due to impaired Ca$^{2+}$ cycling machinery, promotes both diastolic and systolic dysfunction of the heart (Zile & Gaasch, 2011). Furthermore, the inability of the failing heart to maintain an adequate [Ca$^{2+}$]$_{SR}$ is an important contributor to the hearts lack of capacity to meet cardiac demand (Zima et al., 2014). Another consequence of impaired Ca$^{2+}$ cycling machinery is the increased risk for arrhythmias. Here, the abrupt increase in [Ca$^{2+}$]$_{SR}$ as a result of stress (e.g. β-AR) can cause the formation of SCWs (Eisner et al., 2009).

**Ischemia-Reperfusion Injury**

An ischemic episode or AMI in the heart are most commonly a result of coronary artery disease. In the event that a coronary artery becomes obstructed as a result of atherosclerotic plaques, the downstream blood flow slows or completely ceases creating a hypoxic environment for the non-perfused myocardium (Roberts, 1972). In the ischemic environment, metabolites build up within the interstitium and intracellularly due to the energy consumption of the working myocardium and the lack of blood perfusion. Commonly associated with ischemia are complex cellular metabolic changes including a decrease in [ATP] and a subsequent increase in free [Mg$^{2+}$], [ADP] and inorganic phosphate ([Pi]), as well as a drop in intracellular pH (Carmeliet, 1999). Furthermore, increased reactive oxygen species (ROS) generation is prominent as a result of ischemic injury (Misra et al., 2009). All the aforementioned factors are known to modulate the activity of the proteins required for intracellular Ca$^{2+}$ cycling,
particularly the RyR. Consequently, enhanced RyR-mediated SR Ca\(^{2+}\) leak results in abnormally elevated diastolic [Ca\(^{2+}\)].

Depending on the degree of ischemia, infarction of the tissue can soon follow (within minutes). Current medical interventions to restore blood flow to the ischemic region have proven to be successful in reducing the progression of necrosis and drastically improving mortality rates after ischemia or AMI. However, a major complication associated with reperfusing blood to the ischemic region is the increased risk of arrhythmogenesis (Yavuz, 2008). These bouts of arrhythmias have the potential of initiating reentrant tachyarrhythmias, which can devolve into fibrillation and ultimately SCD (Bunch et al., 2007).

**Heart Failure**

Congestive heart failure (CHF) can be simply defined as the inability for cardiac output to meet the metabolic demand from the body. Today, CHF remains the number one cause of hospitalization for ages 65 and older in the United States and is ultimately the end result of cardiac remodeling due to chronic hypertension, abnormalities in cardiac valves, or coronary artery disease (Hall MJ et al., 2008). Although the clinical criteria for CHF are not standardized, individuals with systolic heart failure are normally characterized as having a left ventricle ejection fraction (LVEF) less than 40%. Individuals with diastolic HF may have an ejection fraction in the healthy range (LVEF>55%), however, their cardiac output remains inadequate due to impaired
ventricular filling (Dickstein et al., 2008). Researchers have documented different cellular changes of the myocardium that prove to be dependent on the etiology of HF (Sen et al., 2000). Cardiovascular disease risk factors can promote a variety of cellular changes in the myocardium, which ultimately affect the development of CHF. Coronary artery disease, a major risk factor for AMI, is the leading cause of ischemic HF. However, increased cardiac afterload (e.g. hypertension) is a common cause of non-ischemic HF (Cowburn et al., 1998). Due to progressive cardiac remodeling, patients who suffer from CHF have a poor prognosis as well as a low quality of life. In patients suffering from end stage CHF, death either results from pump failure or SCD (Lane et al., 2005).

The normal healthy heart has an intrinsic inotropic property that allows for an increase in force generation in response to increased heart rates. This is either termed the positive force frequency response (FFR) or the Bowditch effect. This mechanism allows the heart to increase its cardiac output so that it can meet the body’s metabolic demand during increased stress (Lakatta, 2004). In the failing heart, however, increasing pacing frequencies are associated with blunted FFR. This characteristic is prominent in the failing heart and is implicated as an underlying mechanism contributing to pump failure. While it has mostly been attributed to decreased SERCA activity and enhanced NCX activity (Hasenfuss et al., 1996), it has also been proposed that increase RyR2 activity can play a critical role (Kushnir et al., 2010).
A number of abnormal electrical properties have been characterized in the failing myocardium. One of the hallmark changes that take place in HF is a prolongation of the AP. The cellular changes that have been implicated in AP prolongation include alterations in Ca\(^{2+}\) handling, depolarizing currents, and repolarizing currents. The latter is a notable change that is commonly observed in a number of different animal models of HF (Tomaselli et al., 1994). In the past decade, researchers have defined changes in the expression as well as the activity of ion channels that are involved in the cardiac AP, particularly downregulation of outward K+ current (I\(_{to}\) and I\(_{k1}\)). As a result of diminished repolarizing currents, the failing myocardium becomes predisposed to afterdepolarizations (Janse, 2004). Moreover, AP prolongation is heterogeneous throughout the failing heart, which creates an increased transmural and interventricular dispersion of repolarization (Akar & Rosenbaum, 2003). This creates conditions favorable for the generation of torsade de pointes, a type of triggered arrhythmia (Wu et al., 2002).

Prolongation of the AP and abnormal conduction makes the failing myocardium more susceptible to arrhythmogenesis. However, abnormal Ca\(^{2+}\) homeostasis has been implicated as the underlying cause of arrhythmias in HF. In other words, an ectopic impulse due to triggered activity acts as the initiating event for other mechanisms of arrhythmogenesis (i.e. re-entry of excitation). Abnormal SR Ca\(^{2+}\) cycling, a hallmark of HF, is an important contributor to the heart’s depressed contractile function and the
increased incidence of triggered activity (Pogwizd & Bers, 2004); implicating RyR2 and SERCA dysfunction as the primary cause. In a majority of studies, HF is associated with increased SR Ca$^{2+}$ leak and decreased SR Ca$^{2+}$ reuptake mediated by RyR2 and SERCA, respectively. However, there is conflicting evidence with respect to the contribution of RyR2 to impaired SR Ca$^{2+}$ cycling. Furthermore, pathophysiological differences have been observed between ischemic and idiopathic HF with regards to SR Ca$^{2+}$ cycling dysfunction. A study by Sen et al. found that impaired SERCA activity was the primary impairment in ischemic HF, whereas SERCA activity in idiopathic HF was not significantly different when compared to healthy myocardium (Sen et al., 2000). Furthermore, impaired SR Ca$^{2+}$ release was only observed in idiopathic HF. These results suggest that the underlying mechanisms responsible for SR Ca$^{2+}$ cycling dysfunction may depend on the etiology of HF.

Independent of the etiology, HF is commonly defined as a condition of chronic oxidative stress due to compromised energetics. Moreover, the impaired cardiac metabolism is considered to play an important role in the progression of disease. As the disease progresses, oxidative stress worsens due to the increasing energy demand and workload of the failing heart, thus perpetuating a deleterious cycle (Seddon et al., 2007). Although HF is associated with a large number of complex changes, the focus of this work is directed at understanding the role oxidative stress on SR Ca$^{2+}$ release. To date, RyR2 has been characterized in HF as having an increased phosphorylation level,
an increased oxidation level, and a decreased association of auxiliary proteins. All of the aforementioned have been associated with increased channel activity. However, the current understanding of oxidative modifications of RyR2 and their contribution to defective SR Ca\(^{2+}\) cycling in HF remains unclear.

**Oxidative Stress**

Oxidative stress is a prominent feature in the onset and progression of a number of disease states, including cardiovascular disease. Although the generation of ROS has been shown to play an important role in normal cell signaling (Thannickal & Fanburg, 2000), during periods of oxidative stress excessive ROS production can have detrimental effects on normal protein function and cell viability (Stadtman & Berlett, 1998). Furthermore, increased ROS production can induce lipid peroxidation and DNA damage that can compromise the structural and genetic integrity of the cell.

In order to counteract any ROS produced, the cell has an intrinsic antioxidant system that allows for the breakdown of ROS into nontoxic molecules. Some of the major components of the cellular antioxidant system include superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx) (Slodzinski et al., 2008; Yamawaki et al., 2003), which act as selective scavenging enzymes. The nonspecific antioxidants include glutathione and thioredoxin systems. Reduced glutathione (GSH), a highly abundant low-molecular-weight thiol, is considered the largest of the antioxidant pools (~5mM) and is arguably the most important antioxidant system in the myocardium.
GSH is considered the first line of defense against ROS, because it is ubiquitous throughout all cellular compartments. Maintenance of the GSH pool by reduction of oxidized GSH (GSSG) is carried out by glutathione reductase (Schafer & Buettner, 2001).

Oxidative stress can be simply defined as increased ROS production that overwhelms the cellular antioxidant defense (Giordano, 2005). Depending on the etiology of disease, oxidative stress can manifest at different time points and from different sources. The following will review the fast onset of acute oxidative stress in IR injury as well as the slow onset of chronic oxidative stress in HF.

**Acute Oxidative Stress in Ischemia-Reperfusion Injury**

It is has been well characterized that restoring blood flow to the ischemic region drastically increases ROS production, which further increases oxidative stress. The generation of ROS, due to an increased supply of oxygen to ischemic myocardium, has been implicated as the underlying factor that promotes ischemia-reperfusion injury (Vanden Hoek et al., 1996; Zweier et al., 1987). In this condition, the electron transport chain (ETC) in the mitochondria becomes uncoupled (ETC complex I and III) and incompletely reduces $O_2$ forming superoxide radicals (Turrens, 1997). This sudden burst of ROS overwhelms the intrinsic antioxidant system. The GSH/GSSG ratio can decrease considerably during I/R (Ceconi et al., 1988; Werns et al., 1992), which can contribute to mitochondrial ROS spill over (Aon et al., 2007; Aon et al., 2010; Brown et al., 2010). Other sources of ROS, including NADPH oxidase (NOX), uncoupled nitric oxide synthase
(NOS), and xanthine oxidase (XO), are also believed to play a role in I/R injury (Becker, 2004; Zweier & Talukder, 2006; Angelos et al., 2006). A recent study implicated DADs as the major mechanism of arrhythmogenesis in a dog model of AMI. Furthermore, they went on to show that the occurrence of DADs could be prevented with IV infusion of the ROS scavenger TEMPO. The results from these studies implicate oxidative stress as a major factor in the generation of triggered activity in I/R.

During myocardial infarction, excessive β-AR stimulation manifests in the ischemic region due to elevated concentrations of catecholamines (Lameris et al., 2000). Both ex vivo and in vivo I/R studies have shown that the main source of endogenous catecholamines is in fact from nonexocytotic release at sympathetic nerve endings that innervate the myocardium (Lameris et al., 2000; Kurz et al., 1995). β-AR stimulation is considered to be an important contributor in I/R injury. Increased β-AR stimulation can further increase energy demand and intracellular ROS production (Christensen & Videbaek, 1974). Studies that block PKA activation via beta-blockers or direct inhibition of PKA has proven to be effective in reducing infarct size (Makaula et al., 2005; Spear et al., 2007). A recent study done by Nagasaka et al., showed that mitochondrial ROS production was significantly increased in the presence of PKA catalytic subunit in permeabilized myocytes (Nagasaka et al., 2007). Furthermore, the ROS production was associated with a depolarization of the mitochondrial membrane potential suggesting...
that PKA-mediated ROS production could contribute to mitochondrial dysfunction and cardiac injury.

**Chronic Oxidative Stress in Heart Failure**

The mechanisms that are responsible for the progression of heart failure are very complex and have been under intensive investigation for many years. However, one of the common features that have been implicated to play an important role in the pathophysiology of HF is chronic oxidative stress (Belch *et al.*, 1991; Hill & Singal, 1997; Mallat *et al.*, 1998). Both experimental and clinical studies have measured an increase in ROS production in HF (Mak & Newton, 2001; Giordano, 2005; Ventura-Clapier *et al.*, 2004). In a mouse model of MI induced HF, Ide et al. showed that the levels of ROS and lipid oxidation were associated with impaired mitochondrial function (Ide *et al.*, 2001). Furthermore, in a canine model of HF, they demonstrated that the E-transport chain of HF mitochondria is significantly more prone to uncoupling and subsequent ROS production (Ide *et al.*, 1999). These results also provide evidence for a positive correlation between depressed contractility and the level of ROS production. Chronic oxidative stress has also been shown to manifest in HF as an imbalance of ROS production to antioxidant defense. Furthermore, evidence has shown that antioxidant activity progressively deteriorates in the failing heart post MI (Hill & Singal, 1997). Furthermore, transgenic mice that have null mutations in mitochondrial SOD or
thioredoxin develop dilated cardiomyopathy and HF, which suggests that oxidative stress plays a primary role in the development of HF.

**Oxidative Post Translational Modifications**

Recent emphasis has been placed on the study of oxidative PTMs and their important role in the regulation of RyR2 activity (Eager & Dulhunty, 1998; Xu et al., 1998a; Marengo et al., 1998; Gonzalez et al., 2010; Sanchez et al., 2005). Elevated ROS production, which is associated with increased cardiac demand (Heinzel et al., 2006), has been suggested to play a role in the augmentation of SR Ca\(^{2+}\) release (Zima & Blatter, 2006). Therefore, oxidative PTMs of RyR2 may function as a mechanism for positive inotropy in the healthy heart. However, in the case of myocardial I/R or HF, abnormally elevated ROS level can cause irregular Ca\(^{2+}\) cycling and, therefore, cardiac arrhythmias (Belevych et al., 2009; Terentyev et al., 2008; Belevych et al., 2012).

In the presence of oxidative stress, free thiols of cysteine residues are the first subjected to oxidation. Depending on the degree of oxidative stress, protein free thiols can be oxidized by ROS to form sulfenic (R-SOH), sulfinic (R-SO\(_2\)H), or sulfonic (R-SO\(_3\)H) acid products (Giles & Jacob, 2002). GSH attenuates ROS production during oxidative stress either by directly scavenging free radicals or acting as a substrate for the major antioxidant enzyme GSHPx. Also, GSH can readily react with protein sulfenic acids forming the reversible PTM S-glutathionylation. The reversible reduction of S-glutathionylation is carried out mainly by the enzyme glutaredoxin (GRX). The
formation of sulfinic and sulfonic acids, however, are considered biologically irreversible. Thus, the formation of the protein-glutathione mixed disulfide (S-glutathionylation - PSSG) is thought to have a protective role during changes in cellular redox state (Townsend, 2007). However, it has been proposed that S-glutathionylation may play a role in promoting protein disulfide formation (PSSP) of both intra- and intermolecular species (Bass et al., 2004; Cumming et al., 2004; Brennan et al., 2004).

In all tissues, the ratio between oxidized and reduced GSH (GSSG/GSH) is an important indicator of the redox state. Changes in cellular redox environment potentially affects the activity of many proteins, including Ca\(^{2+}\) channels and pumps (Zima & Blatter, 2006). As a result, oxidative stress can potentially promote abnormally elevated [Ca\(^{2+}\)]\(_i\) in the myocardium during diastole (Kourie, 1998; Giordano, 2005). In cardiomyocytes, cytosolic glutathione is mainly reduced under normal physiological conditions. During oxidative stress, however, the GSSG/GSH ratio can increase significantly (Ceconi et al., 1988; Werns et al., 1992) as well as total protein-glutathione mixed disulfides (Tang et al., 2011). The increased formation of glutathione mixed disulfides is a common feature of oxidative stress due to the abundance of glutathione and the ready conversion of reactive thiols. Recent studies have been implicated glutathione mixed disulfides as a critical signaling mechanism that plays a causative role, rather than a protective role, in cardiovascular disease. With respect to SR Ca\(^{2+}\) cycling,
however, it is unclear if increased glutathione mixed disulfide are beneficial or detrimental.

**Cardioprotection and Nitric Oxide**

Cardiac ischemia preconditioning is a phenomenon whereby short episodes of ischemia result in cardioprotection against a severe ischemic insult. The vast body of research studying ischemic preconditioning has yielded many different molecular mechanisms (Zaugg et al., 2003). Given its complex nature, the crucial downstream targets that give a tissue the ability to resist ischemic injury make up a sizeable list that has steadily grown over the recent years. Nitric oxide (NO) signaling, an important regulator in many physiologic processes, and subsequent protein S-nitrosylation is commonly identified as an important molecular intermediate allowing for ischemic preconditioning. Several cardioprotection studies defined many downstream targets of NO, having identified the cardioprotective effect as the result of covalently linked NO with reactive protein thiols (S-nitrosylation). These downstream targets include proteins that are involved in mitochondrial metabolism, apoptosis, ROS defense, protein trafficking, myofilament contraction, and Ca$^{2+}$ handling. Overall, increased S-nitrosylation in the myocardium is anti-apoptotic and anti-inflammatory (Sun & Murphy, 2010; Lima et al., 2010). As mentioned previously, the reperfusion of blood or reintroduction of O$_2$ to the ischemic tissue stimulates oxidative phosphorylation in impaired mitochondria, which results in a burst of ROS production. Recent studies have
found that S-nitrosylation of mitochondrial protein complexes (I, IV, and V) of the electron transport chain (ETC) inhibits their activity, which limits oxidative phosphorylation (Rassaf et al., 2014; Zhang et al., 2005; Sun et al., 2007). Furthermore, S-nitrosylation of myofilament proteins decreases their sensitivity to Ca\(^{2+}\), decreasing myofilament cross-bridge formation, which subsequently reduces ATP consumption (Nogueira et al., 2009). By promoting energy conservation in the myocardium, S-nitrosylation limits ROS production from uncoupled mitochondria during IR.

The cardioprotective effects of S-nitrosylation on Ca\(^{2+}\) machinery, although independent, complements the effect seen in the mitochondria. In both IR and HF, impaired Ca\(^{2+}\) cycling commonly leads to an increase in diastolic [Ca\(^{2+}\)]\(_i\), as well as depleted [Ca\(^{2+}\)]\(_{SR}\). In a state of [Ca\(^{2+}\)] overload, the diastolic function of the heart is impaired and the likelihood of arrhythmogenesis is increased. Evidence of S-nitrosylation-dependent cardioprotection has been documented for the major components of Ca\(^{2+}\) cycling, preventing [Ca\(^{2+}\)] overload (Loyer et al., 2008). For LTCC, S-nitrosylation of the channel has been shown to reduce the channel activity. Also, SERCA activity has been reported to increase in response to S-nitrosylation. Paradoxically, RyR2 activity has been shown to increase in response to S-nitrosylation (Gonzalez et al., 2009). Other studies, however, showed that hyponitrosylation of RyR2 caused the channel to be more susceptible to oxidation by ROS, leading to increased SR Ca\(^{2+}\) leak and arrhythmogenesis (Gonzalez et al., 2010). Moreover, it has been suggested that S-
nitrosylation can potentially prevent irreversible oxidation of cysteine residues (Sun & Murphy, 2010). Thus, S-nitrosylation of RyR2 may act as a protective PTM against oxidative stress and detrimental SR Ca\(^{2+}\) leak. By maintaining SR Ca\(^{2+}\) load and preventing [Ca\(^{2+}\)], S-nitrosylation plays a very important role in cardiac function during periods of oxidative stress.

Redox Regulation of RyR2 Activity

It is well established that oxidative stress can cause ROS-induced PTMs of proteins. The RyR2 has approximately 360 cysteine residues per tetrameric channel, with an estimated 84 of those are in a reduced free thiol state (Xu et al., 1998a). Each free thiol residue can serve as a target for a number of oxidative modifications including S-nitrosylation, S-glutathionylation, or a disulfide cross-bridge. To date, a number of in vitro studies have shown that both ROS and other free radicals can induce changes in RyR2 channel activity (Zima & Blatter, 2006; Kawakami & Okabe, 1998). Bilayer studies have shown that RyR2 channel activity is increased in the presence of ROS (Boraso & Williams, 1994; Zima et al., 2004; Anzai et al., 1998). It has been proposed that oxidative modifications, specifically S-nitrosylation and S-glutathionylation, of RyR2 can prevent SR Ca\(^{2+}\) leak (Marengo et al., 1998; Bull et al., 2008; Zima et al., 2003; Boraso & Williams, 1994). Recent work from Gonzalez et al. identified that enhanced xanthine oxidase superoxide production caused a decrease in cardiac RyR2 S-nitrosylation with an overall decrease in free thiols promoting SR Ca\(^{2+}\) leak in heart failure rats (Gonzalez et al.,
2010). Moreover, an increase in overall oxidation of RyR2 with abnormal SR Ca\(^{2+}\) release has also been observed in post-MI dog hearts (Belevych et al., 2009). Oxidation of RyR2 is also thought to play a role in myocardial preconditioning before an ischemic insult. For example, tachycardia-induced preconditioning was proven to reduce the infarct size after ischemia (Domenech et al., 1998). It was later identified that tachycardia stimulated NADPH oxidase dependent S-glutathionylation of RyR, increasing RyR2 Ca\(^{2+}\) release and decreasing SR Ca\(^{2+}\) leak in SR microsomal preparations (Sanchez et al., 2005). It still remains controversial whether or not increased channel activity (single channel) or increased Ca\(^{2+}\) release rates (SR microsomes) can also coincide with decreased SR leak within a cellular milieu.

**Intersubunit Cross-linking**

The protein-protein interaction between RyR2 subunits (intersubunit) has been implicated in channel gating (Kimlicka et al., 2013; Strauss & Wagenknecht, 2013; Abramson & Salama, 1989) and therefore, likely plays a role in regulating SR Ca\(^{2+}\) release. In the past decade, there has been a great amount of progress in defining the quaternary structure of RyR. Although only a small portion of the cytosolic domain has been crystallized to date, high resolution cryo-EM studies have provided insight into conformational changes that occur as a result of channel activation. By superimposing the 3-D crystal structure of the N-terminal domain (RyR1 a.a. 1-532) within the 3-D matrix created using images from cryo-EM, it was determined that the intersubunit gap
between N-terminal domains becomes widened by ~7 angstroms in the open conformation. These results suggest that any protein-protein interactions that are taking place in the closed conformation, are likely disrupted as the result of channel opening (Van, 2014). Although the N-terminal domain is only responsible for a small portion of the intersubunit interaction, it is of particular interest because a large number of disease mutations have been found to localize within it. In fact, a majority of these mutations were found facing the intersubunit boundary. Here, the mutations all had side chain properties that would consequently disrupt normal wild-type interdomain interactions. Moreover, the mutations were all associated with a gain-of-function phenotype (increased RyR channel activity) (Kimlicka et al., 2013). These functional results are consistent with the structural evidence, supporting the claim that RyR channel activity is indeed affected by changes in the intersubunit interactions.

Recent work by Han et al. demonstrated that in the presence of an oxidant (H₂O₂), RyR1 undergoes covalent disulfide cross-linking between neighboring subunits (intersubunit cross-linking) that is reversible with the reducing agent DTT. In parallel, Cryo EM images showed that RyR1, which normally has a cytosolic structure that resembles a pinwheel, undergoes major morphological changes as a result of H₂O₂ treatment. These morphological changes, however, are reversible with the treatment of DTT (Han et al., 2006). A different cryo EM study used a non-selective crosslinking agent (glutaraldehyde) to induce intersubunit crosslinking. In these conditions, RyR1 adopted
a conformation that resembled that of the open state. In both these studies, the authors suggest that intersubunit crosslinking leads to activation of RyR1 as a result of structural changes that directly affect gating of the channel (Strauss & Wagenknecht, 2013).

Similar to other oxidative PTMs, there is functional evidence showing that inter-subunit cross-linking of RyR1 is associated with increased channel activity. The association of intersubunit cross-linking and increased channel activity was first shown in vitro using RyR1 (Aghdasi et al., 1997). This study demonstrated with single channel recordings of RyR1 that treatment with diamide increased channel P_o and the formation of cross-linked dimer in a dose dependent manner. The thiol alkylating agent NEM, however, was shown to oxidize RyR1 in a multiphasic manner, which was dependent on concentration and time. While sub-millimolar (200 µM) concentrations of NEM would only induce an inhibitory effect of RyR1 by decreasing channel activity, sequential treatment of NEM at millimolar (4-5mM) concentrations led to further oxidation of RyR1 causing an increase in channel activity. Most importantly, pretreatment with sub-millimolar NEM effectively prevented diamide induced intersubunit cross-linking. These results suggest that the first phase of sulfhydryls, which are most susceptible to oxidation, are involved in intersubunit cross-linking.

Abramson and Salama were the first to suggest that intersubunit cross-linking is involved in the gating of RyR1 (Abramson & Salama, 1989). They argue that thiol
oxidation is a necessary requirement for RyR1 channel opening. In order for this hypothesis to be correct, the transition from conducting to non-conducting states would have to coincide with the reduction of the principal disulfides regulating gating. Furthermore, because the cytosolic environment is maintained at a highly reduced state, this proposed gating mechanism assumes that dynamic disulfide formation is present without oxidative stress. Recently, a study done by Zissimopoulos et al. provides some biochemical evidence to support this hypothesis for both RyR1 and RyR2. The major limitation to this study, however, was that full length RyR was not used in the experimental approach (Zissimopoulos et al., 2013). Their work shows that N-terminal fragments of RyR2 self-assemble into oligomers similar to that of RyR1. Interestingly, unlike RyR1, N-terminal fragments of RyR2 were covalently linked by endogenous disulfide bonds in ambient conditions (absence of exogenous ROS treatment). Even though both RyR1 and RyR2 N-terminal fragments formed disulfide linked oligomers with H$_2$O$_2$ in a dose dependent manner, the authors suggest that a difference in sequence homology between isoforms may explain the disparity in disulfide bond formation. Alternatively, if the requisite cysteine residues are in fact conserved between isoforms, other oxidative PTMs (e.g. glutathionylation, nitrosylation) that resist disulfide formation may be unique to RyR1.

In summary, there is a growing body of structural and functional evidence that suggests an effect of intersubunit cross-linking on RyR2 channel gating. Although some
work has been done to examine the effects of intersubunit cross-linking on RyR2 channel activity, no studies have examined its role in SR Ca$^{2+}$ cycling within the cellular environment. Furthermore, studies of RyR intersubunit cross-linking have focused primarily on the skeletal isoform. In light of recent work, the susceptibility for intersubunit cross-linking appears to be increased for the cardiac isoform of RyR (Zissimopoulos et al., 2013). The set of experiments described in this dissertation attempts to define a potential mechanism for RyR2 cysteine oxidation and intersubunit cross-linking during increased protein-glutathione mixed disulfides resulting from oxidative stress (Fig. 1). Also, I examine the incidence of intersubunit cross-linking in models of oxidative stress in the heart. This work attempts to address the effect of RyR2 intersubunit cross-linking on SR Ca$^{2+}$ cycling in the heart during periods of oxidative stress. Defining the contribution of RyR2 intersubunit cross-linking to SR Ca$^{2+}$ leak is a necessary step in determining a mechanistic link between oxidative stress and arrhythmogenesis. Ultimately, this research has the potential to shed light on principal cysteine residues of RyR2 as therapeutic targets for potentiating cardiac arrhythmias.
Figure 1. Schematic of Oxidative Stress and Cysteine Oxidation of RyR2

**Oxidative Stress and Glutathione**

Top Panel, Schematic depicting increased mitochondrial ROS and subsequent oxidation of RyR2 free thiols. GSH acts to attenuate ROS production and react with sulfenic acid side chains. RyR2 S-glutathionylation and potentially disulfide formation are a result. **Bottom Panel**, Electron cryo-microscopy image depicting RyR2 intersubunit cross-linking promoting the open conformation of the channel.
CHAPTER III: AIMS AND HYPOTHESES

The overall goal of this project is to determine the role of ROS production in the regulation of RyR-mediated Ca\(^{2+}\) release under pathological conditions induced during I/R injury and in the failing heart. Changes in PTMs of RyR2 have been implicated in abnormal Ca\(^{2+}\) handling that promotes the onset and progression of cardiovascular disease. Due to their association with many disease phenotypes, recent emphasis has focused on the study of oxidative PTMs. Furthermore, a growing body of evidence suggests oxidative PTMs as a potent regulator RyR2 activity (Zima & Blatter, 2006; Eager & Dulhunty, 1998; Xu et al., 1998a; Marengo et al., 1998; Gonzalez et al., 2010; Sanchez et al., 2005). However, little has been done to specifically characterize oxidative PTMs of RyR2 as a result of oxidative stress within the ischemic or failing myocardium. Moreover, it is not yet known how these modifications contribute abnormal SR Ca\(^{2+}\) handling and arrhythmogenesis. This project tests the following central hypothesis:

**Selective oxidation of cysteine residues on RyR2 will enhance SR Ca\(^{2+}\) fractional release and leak in cardiomyocytes.** Oxidative PTMs of RyR2, observed as a result of experimentally induced oxidative stress, will manifest in models of acute or chronic oxidative stress (I/R and HF).
Specific aim #1: Characterize RyR2 oxidation and SR Ca\(^{2+}\) release in vivo as a result of increasing intracellular glutathione mixed-disulfides. Commonly observed in many disease phenotypes, including cardiovascular disorders, is the prominence of glutathione mixed-disulfides as the result of oxidative stress. This intrinsic ability for the cell to buffer ROS and form reversible protein mixed disulfides with glutathione (S-glutathionylation) is imperative for protection against irreversible oxidation. However, increased S-glutathionylation during oxidative stress is also thought to play a causative role in the progression of cardiovascular disease. I hypothesize that increased glutathione mixed-disulfides will promote oxidation of RyR2 favoring augmented SR Ca\(^{2+}\) fractional release and SR Ca\(^{2+}\) leak. Therefore, any elevation in SR Ca\(^{2+}\) load will in turn drastically increase SR Ca\(^{2+}\) leak, promoting arrhythmogenic Ca\(^{2+}\) waves. To test this hypothesis, changes in SR Ca\(^{2+}\) cycling parameters will be measured in intact myocytes. RyR2 oxidation will be determined by measuring relative free thiol content and western blot analysis. Prevention and reversibility of RyR2 oxidation will be determined both biochemically and functionally. In isolated myocytes, the cellular redox environment will be manipulated experimentally using two approaches: 1) Introduce GSSG within permeabilized myocytes and 2) Increase intracellular GSSG/GSH ratio using the thiol oxidant diamide.

Specific aim #2: Determine the role of RyR2 oxidation on abnormal SR Ca\(^{2+}\) release in I/R and HF. It is generally accepted that ischemic and non-ischemic
cardiomyopathy are characteristically associated with oxidative stress and abnormal Ca$^{2+}$ cycling; both being associated with an increased risk of arrhythmia and have been implicated in the progression of HF. In these studies, I will discern the redox status of RyR2 after an ischemic insult and in the failing heart as well as the functional effect on SR Ca$^{2+}$ release. I hypothesize that oxidative stress in I/R and HF promotes the oxidation of RyR2 favoring augmented SR Ca$^{2+}$ fractional release and SR Ca$^{2+}$ leak. Furthermore, the oxidation of RyR2 will decrease frequency dependent facilitation associated with the failing heart. To test this hypothesis, SR Ca$^{2+}$ fractional release and SR Ca$^{2+}$ leak will be measured in isolated myocytes from I/R, HF, and sham hearts. RyR2 oxidation will be determined both biochemically and functionally.
CHAPTER IV: METHODS

Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. All experiments were performed at room temperature (22-24°C).

Animal Models

The use of rabbit hearts to study Ca\(^{2+}\) dependent arrhythmias in both I/R and HF is important because the electrophysiology and SR Ca\(^{2+}\) regulation is similar to that of a human (Bers, 2000). New Zealand White rabbits (2-2.5 kg; Harlan Laboratories, Indianapolis, IN) received containing heparin (400U/kg) were anesthetized with sodium pentobarbital (100 mg/kg I.V.). Experimental protocols (ex-vivo and in-vivo), animal surgery, handling, and cell isolation were approved by the Institutional Animal Care and Use Committee at both Loyola University Medical Center and Rush University Medical Center.

Ex-vivo Low Flow I/R

In order to induce the effect of I/R injury globally throughout the entire heart, rabbit hearts underwent low flow ischemia and reperfusion via ex-vivo perfusion on a Langendorff apparatus (Langendorff Oskar, 1903). Afterwards, myocytes were isolated
from both left and right ventricles for experiments examining RyR2 oxidation and SR Ca\(^{2+}\) handling. After the rabbit was sacrificed, the excised heart was rinsed in 1.5 mM Ca\(^{2+}\) Tyrode containing heparin (1.5U/ml) and cannulated to the Langendorff apparatus and perfused at a constant pressure of 55 mmHg at 37°C with 1.5 mM Ca\(^{2+}\) Tyrode without heparin for 10 minutes until stabilization of the sinus rate and the pressure of the left ventricle (LVP). The hearts were allowed to pace at their intrinsic sinus rhythm of approximately 120-180 beats/min (2-3Hz). A “pseudo-electrocardiogram” (ECG) was recorded using two silver hook electrodes on each ventricle. LVP was measured with the aid of a latex balloon at the end of a polyethylene catheter inserted into the left ventricle. ECG and LVP were recorded simultaneously with an amplifier (IWorx, CB science) and LabScribe software throughout the entire duration of the experiment. Changes in sinus rate, propensity of arrhythmia, and LVP was monitored throughout. Low flow ischemia was carried out by reducing the perfusion flux to approximately 5% of baseline for 30 min. The initial flow was then restored for 15 minutes (reperfusion). In hearts that were used solely for glutathione ratio analysis, tissue biopsies were collected from ventricular myocardium before and after low flow I/R. Tissue biopsies were excised using a circular punch blade so that both endo- and epicardial regions of the ventricular wall were included.
Ex-vivo Coronary Ligation I/R

Local I/R injury was performed using coronary ligation on excised rabbit hearts via ex-vivo perfusion on a Langendorff apparatus. Afterwards, tissue was isolated from the left ventricle, flash frozen in liquid nitrogen, and later homogenized for biochemical analysis examining RyR2 oxidation. Excised hearts were rinsed in 1.5 mM Ca$^{2+}$ Tyrode containing heparin (1.5U/ml), cannulated on the Langendorff, and perfused at a constant pressure of 55 mmHg at 37°C with 1.5 mM Ca$^{2+}$ Tyrode without heparin for 10 minutes until stabilization of the sinus rate and LVP. The hearts were allowed to pace at their intrinsic sinus rhythm and monitored with ECG. ECG and LVSP were recorded as described in the low-flow I/R protocol. Local ischemia was carried out by ligating the left anterior descending coronary (LAD) using a 5-0 polypropylene suture (Ethicon, USA). The suture was tightened around the LAD and tied around a small piece of polyethylene tubing, inducing ischemia. After 30 min ischemia, the tubing was removed and the coronary ligature was released allowing for reperfusion of the ischemic myocardium for 15 min. Tissue was isolated from ischemic (downstream of coronary) and remote zones from the left ventricle.

Non-Ischemic HF

The HF myocytes used in the presented studies were isolated from a rabbit HF model that was implemented by Dr. Lothar A Blatter, from Rush University. New Zealand White rabbits (Harlan Laboratories, Indianapolis, IN) undergo two separate
surgeries to induced hypertrophy and HF (a combined insult of aortic insufficiency and constriction was used to induce left ventricular volume and pressure overload, respectively). This model has shown to produce many similar symptoms of human HF including non-reentrant ventricular arrhythmias. Aortic insufficiency was performed by puncturing the aortic valve with a beveled 5-Fr micropuncture introducer, which was introduced through the left carotid artery and guided by echocardiography. Two-dimensional echocardiography was used to confirm and determine the degree of aortic valve insufficiency. Two weeks later, aortic constriction was performed on the abdominal aorta proximal to the renal arteries. Depending on the severity of aortic insufficiency, a 4-0 silk thread was tightened around the aorta in order to reduce the diameter between 25-40%. The progression of hypertrophy and HF was monitored using echocardiography at 2 to 4 week intervals after both surgeries. All parameters were calculated relative to the baseline measurements taken prior to the surgeries. The development of hypertrophy and the subsequent onset of HF was carried out over the course of approximately 5-8 months. Once left ventricular end-diastolic diameter was increased >50% and left ventricular fractional shortening was decreased by approximately 40% (depending on morbidity), hearts were categorized as HF. Rabbits were sacrificed and single cardiomyocytes were isolated.
**Myocyte Isolation**

Ventricular myocytes were isolated from sham (control), I/R, and HF rabbit hearts. Following thoracotomy hearts were quickly excised, mounted on a Langendorff apparatus. Ex-vivo sham or I/R protocols were carried out as described previously. All hearts were then retrogradely perfused with nominally Ca$^{2+}$ free Tyrode solution for 5 min, preventing CICR and relaxing the muscle. In sequence, minimal essential medium Eagle (MEM; 9.7g/L), 20 μM Ca$^{2+}$ (CaCl$_2$), Liberase Blendzyme (45 μg/ml; Roche Applied Science, IN, USA)-containing solution at 37°C was perfused for 16 min. Atria were removed from the heart, followed by the aorta and residual connective tissue. The free wall of the left ventricle was separated from the septum and the right ventricle. Each ventricle was then lightly minced (~5 pieces), gently shaken, filtered, and washed in a MEM, 50 μM Ca$^{2+}$, and 10 mg/ml bovine serum albumin-containing solution at 37°C. Viable myocytes were settled for 10-12 minutes in conical tubes and then resuspended in MEM, 50 μM Ca$^{2+}$-containing solution at room temperature (22–24°C). Using slow capillary action, isolated myocytes were re-equilibrated to ~1-1.5 mM Ca$^{2+}$ using 2 mM Ca$^{2+}$ Tyrode solution.

**Confocal Microscopy**

Changes in [Ca$^{2+}$]$_i$ and [Ca$^{2+}$]$_{SR}$ were measured with laser scanning confocal microscopy (Radiance 2000 MP, Bio-Rad, UK) equipped with a ×40 oil-immersion objective lens (N.A.=1.3).
Measurements of $[\text{Ca}^{2+}]_i$:

To record $[\text{Ca}^{2+}]_i$ we used the high affinity $\text{Ca}^{2+}$ indicator Fluo-4 (Molecular Probes/Life Technologies, Grand Island, NY). To load the cytosol with $\text{Ca}^{2+}$ indicator, cells were incubated at room temperature with 10 µM Fluo-4/AM for 15 minutes in Tyrode solution (in mM: NaCl 140; KCl 4; CaCl$_2$ 2; MgCl$_2$ 1; glucose 10; HEPES 10; pH 7.4), followed by a 20 minute wash. Fluo-4 was excited with the 488 nm line of an argon laser and fluorescence was measured at >515 nm with a photomultiplier tube after passing through a 515-nm long-pass filter. Action potentials were induced by electrical field stimulation using a pair of platinum electrodes, which were connected to a Grass stimulator (Astro-Med. Inc., USA) set at a voltage ~50% above the threshold. Fluo-4 recordings were acquired in line-scan mode (3 ms per scan; pixel size 0.12 µm). SR $\text{Ca}^{2+}$ load was measured from the peak amplitude of the $[\text{Ca}^{2+}]_i$ transient induced by the rapid application of 10 mM caffeine.

Measurements of $[\text{Ca}^{2+}]_{\text{SR}}$ and SR $\text{Ca}^{2+}$ leak:

To record $[\text{Ca}^{2+}]_{\text{SR}}$ we used the low affinity $\text{Ca}^{2+}$ indicator Fluo-5N (Molecular Probes/Life Technologies, Grand Island, NY). To load the SR with $\text{Ca}^{2+}$ indicator, myocytes were incubated with 5 µM Fluo-5N/AM for 2.5 hours at 37°C.

For intact cell experiments, action potentials were induced by electrical field stimulation using a pair of platinum electrodes, which were connected to a Grass stimulator (Astro-Med. Inc., USA) set at a voltage ~50% above the threshold for
contraction. To avoid motion artifacts, the scan line was positioned along the short axis (transversal scan) in the central region of the cell where cell motion is minimal during contraction. Stimulation frequency was 0.5 Hz. Changes in $[\text{Ca}^{2+}]_{\text{SR}}$ were calculated by the formula (Cannell et al., 1994): $[\text{Ca}^{2+}]_{\text{SR}} = K_d \times R/(K_d/[\text{Ca}^{2+}]_{\text{SR \,\text{diast}}} - R + 1)$, where $R$ was the normalized Fluo-5N fluorescence ($R = (F - F_{\text{min}})/(F_0 - F_{\text{min}})$); $F_0$ and $F_{\text{min}}$ were the fluorescence level at rest and after depletion of the SR with caffeine (10 mM), respectively. The $K_d$ (Fluo-5N Ca$^{2+}$ dissociation constant) was 390 μM based on in situ calibrations (Zima et al., 2010), and $[\text{Ca}^{2+}]_{\text{SR \,\text{diast}}}$ (diastolic $[\text{Ca}^{2+}]_{\text{SR}}$ at 0.5 Hz) was 800 μM (Shannon et al., 2003).

Fluo-5N/AM loaded myocytes were permeabilized with saponin (0.005%) to remove cytosolic Fluo-5N. In order to avoid photobleaching, Fluo-5N was excited with a low laser energy (0.5% Argon). To improve the signal-to-noise ratio of the low intensity Fluo-5N signal, fluorescence was collected with an open pinhole and averaged over the entire cellular width of the 2-D image. The saponin-free cytosolic solution was composed of (in mM): K-aspartate 100; KCl 15; KH$_2$PO$_4$ 5; MgATP 5; EGTA 0.35; CaCl$_2$ 0.12; MgCl$_2$ 0.75; phosphocreatine 10; HEPES 10; creatine phosphokinase 5 U/ml; dextran (MW: 40,000) 8%, and pH 7.2 (KOH). Free $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$ of this solution were 150 nM and 1 mM, respectively (maxchelator.stanford.edu). SR Ca$^{2+}$ leak was calculated as a function of $[\text{Ca}^{2+}]_{\text{SR}}$ ($[\text{Ca}^{2+}]_{\text{SRT}}$) over time ($d[\text{Ca}^{2+}]_{\text{SRT}}/dt$) after inhibiting SERCA with TG. $[\text{Ca}^{2+}]_{\text{SRT}}$ was calculated as: $[\text{Ca}^{2+}]_{\text{SRT}} = B_{\text{max}}/(1 + K_d/[\text{Ca}^{2+}]_{\text{SR}}) + [\text{Ca}^{2+}]_{\text{SR}}$;
where $B_{\text{max}}$ and $K_d$ were 2700 μM and 630 μM, respectively (Shannon et al., 2000c). The rate of SR Ca$^{2+}$ leak ($d[Ca^{2+}]_{\text{SR}}/dt$) was plotted as a function of $[Ca^{2+}]_{\text{SR}}$ for each time point (15 s) during $[Ca^{2+}]_{\text{SR}}$ decline. Changes in SR Ca$^{2+}$ leak are presented as relative changes in the rate of leak ($d[Ca^{2+}]_{\text{SR}}/dt$) for a given $[Ca^{2+}]_{\text{SR}}$ bin. The complete SERCA inhibition was confirmed by measuring $[Ca^{2+}]_{\text{SR}}$ recovery after SR Ca$^{2+}$ depletion with caffeine and then $[Ca^{2+}]_{i}$ elevation to drive Ca$^{2+}$ uptake. $F_{\text{min}}$ was measured after depletion of the SR with 10 mM caffeine in the presence of 5 mM EGTA. After $F_{\text{min}}$ was determined, cells were pretreated with the myosin inhibitors (blocking myofilament cross-bridge cycling) 2,3-butanedione monoxime (10 mM) and blebbistatin (10 μM) for approximately 5 min in order to prevent irreversible myocyte contraction during application of high $[Ca^{2+}]_{i}$ during $F_{\text{max}}$. $F_{\text{max}}$ was measured following an increase of $[Ca^{2+}]_{i}$ to 10 mM in the presence of caffeine. Caffeine keeps RyRs open allowing $[Ca^{2+}]_{i}$ equilibration across the SR membrane (Shannon et al., 2003). The Fluo-5N signal was converted to $[Ca^{2+}]_{i}$ (Free Ca$^{2+}$) using the formula: $[Ca^{2+}]_{\text{SR}} = K_d(F-F_{\text{min}})/(F_{\text{max}}-F)$, where $K_d$ was 390 μM. SR Ca$^{2+}$ leak was measured as the changes of total $[Ca^{2+}]_{\text{SR}}$ ($[Ca^{2+}]_{\text{SR}}$) over time ($d[Ca^{2+}]_{\text{SR}}T/dt$) after complete SERCA inhibition with thapsigargin (TG; 10 μM).

All 2-D and line scan measurements for $[Ca^{2+}]_{i}$ and $[Ca^{2+}]_{\text{SR}}$ were analyzed with ImageJ software (NIH, USA). Spark properties from line scan measurements were analyzed using the ImageJ plugin Sparkmaster (Picht et al., 2007).
**ROS Production Measurements**

Relative changes in the cellular level of ROS was measured within intact myocytes using the ROS-sensitive fluorescent dye, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; Molecular Probes/Life Technologies, Grand Island, NY). Myocytes were incubated in 20 μM H2DCFDA, 2 mM Ca$^{2+}$ Tyrode solution for 40 min at room temperature, entrapping the dye within the cytosol. H$_2$DCFDA excitation (~450-500nm) was carried out using the 488 nm line of an argon laser and the emitted fluorescence (~510-570nm) was collected using a PMT via a long pass filter transmitting wavelengths >510nm. Live 2-D recordings were acquired at 15 s intervals. The fluorescence intensity (F) was determined by integrating the fluorescence for the entire volume of the cell. The ROS level (arbitrary units) was presented as the normalized fluorescence ($F/F_0$), where $F_0$ is the baseline fluorescence recorded during the initial 2-D scan. All experiments were performed using the lowest laser power available (0.5%) and fluorescence was collected with an open pinhole in order to ensure that photoproduction of ROS by laser illumination was minimized. The rate of ROS production was estimated by the slope of a fitted linear function to the H$_2$DCFDA signal ($d(F/F_0)/dt$). Relative differences in the rate of ROS production under different experimental conditions were determined as a percentage. To ensure specificity for cytosolic ROS, the application of ROS scavenger Tiron was used to limit endogenous ROS
production. Exogenous H$_2$O$_2$ (1mM) was applied as a positive control as well to determine a standardized rate of ROS production.

**RyR2 Single Channel Recordings**

SR vesicles isolated from rabbit ventricle were incorporated into planar lipid bilayers and RyR2 channel activity was measured. For recording of Cs$^+$ currents through RyRs, the cis- and trans-chambers contained (mM): CsCH$_3$SO$_3$ 400; CaCl$_2$ 0.1; Hepes 20; pH 7.3. The cis- and trans-chambers corresponded to the cytosolic and luminal side of the RyR2 channel, respectively. After fusion of vesicles and incorporation of RyR channels, Free [Ca$^{2+}$] in the cis-chamber was adjusted to 3 µM by adding an appropriate amount of EGTA. Single channel currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at a holding potential of -20 mV. Currents were filtered at 1 kHz and sampled at 5 kHz. The mean open probabilities ($P_o$) of the channels were used as an index of activity. $P_o$ values were calculated from the 50% threshold analysis using pClamp software (Axon Instruments).

**Measurements of RyR2 Intersubunit Cross-Linking and S-Glutathionylation**

Alteration in the electrophoretic mobility of the 560 kDa subunit (measured with 34C primary antibody; DSHB, USA) was used to detect RyR2 cross-linking between subunits (~1MDa) (Aghdasi et al., 1997). A specific antibody raised against GSH was used to determine RyR2 S-glutathionylation (1:10000; Virogen). Previous studies have proven its ability to detect RyR2 S-glutathionylation levels (Aracena-Parks et al.,
Whole tissue isolations were minced and homogenized using a glass mortar and pestle in a buffer solution containing Tris-maleate (10 mM), NaCl (0.5%), and protease inhibitor cocktail (Roche Applied Science, IN, USA) at a pH of 6.8. All myocytes used in these experiments were freshly isolated and treated in suspension. Intact ventricular myocytes were treated with diamide in Tyrode solution for 5 min. Permeabilized myocytes were treated with either GSH or GSSG (5mM) in internal solution for 5 min. Myocytes were pelleted via centrifugation and lysed in non-reducing Laemmlı buffer containing N-ethylmaleimide (5 mM) to block free sulfhydryl groups. The addition of 200 mM DTT was used to confirm reversibility of RyR2 cross-linking and to be used as a reference for total RyR. Lysate samples were incubated at 70°C for 10min, ran on 3-15% SDS-PAGE, and transferred onto nitrocellulose membrane using Tubo-transfer (Bio-Rad, USA). Immunoblot against RyR2 was carried out using the monoclonal 34C primary antibody (1:1000) (DSHB, USA) and anti-mouse HRP-conjugated secondary antibody (1:5000) (Santa Cruz, USA). Western blots were quantified using the UVP EpiChemi imaging system (BioRad, USA) and ImageJ software (NIH, USA). Relative RyR2 cross-linking was analyzed as ((Total RyR2 - Monomeric RyR) / (Total RyR)).

Functional studies examining the contribution of S-glutathionylation on SR Ca^{2+} leak (confocal microscopy), introduced GSH (5mM) and human recombinant glutaredoxin-1 (5 units/µL) (Cayman Chemical, USA).
**Free Thiol Content Measurement of RyR**

The content of free thiols in RyRs was measured using the irreversible alkylating agent monobromobimane (mBB), which becomes fluorescent once it reacts with a free thiol. Previous studies have demonstrated the efficacy mBB for detecting free thiols, including those on RyR2 (Kosower & Kosower, 1987; Terentyev et al., 2008). All myocytes used in these experiments were freshly isolated from HF, sham, and I/R and treated in suspension. To determine the relative free thiol content, maximal free thiols (entirely reduced) was determined by treating myocytes with the reducing agent dithiothreitol (DTT; 5 mM). As a baseline fluorescence (entirely oxidized), myocytes were incubated with the oxidizing agent 2,2′-dithiodipyridine (DTDP; 0.5 mM). After treatment for 5 min, myocytes were permeabilized with saponin (0.005%) for ~30 sec so that mBB (400 μM) can be introduced intracellularly. Myocytes were incubated with mBB for one hour and then washed 3 times in order to remove unbound label and GSH. Myocytes were then lysed in reducing Laemmli Buffer (containing 10% 2-mercaptoethanol) and equally loaded and ran out on 4-15% SDS-PAGE. The gel was first imaged using UV light to measure mBB emission (490 nm) with the UVP EpiChemi imaging system (BioRad, USA). Afterwards the gel was stained with Coomassie Blue in order to normalize the mBB signal to total RyR2 protein.
Glutathione Ratio Assay

The severity of oxidative stress was determined from Sham and I/R tissue biopsies isolated before and at the end of each protocol. Tissue biopsies were homogenized using a bead-beater homogenizer (Biospec Products; USA) and the resulting homogenate protein concentration was determined using Coomassie Bradford assay (Thermo; USA). The addition of 5-sulfo-salicylic acid dihydrate (5% w/v) was used to precipitate protein from homogenate samples. Samples were vortexed, incubated on ice for 10 min, and centrifuged at 14,000 rpm at 4°C for 10 minutes. The resulting supernatant was used for analysis of GSSG/GSH content. The concentration of GSH was defined using a fluorometric glutathione detection assay (Arbor Assay K006), utilizing a fluorescent label that covalently binds to GSH. The sample fluorescence was measured using a fluorometer (OLIS DM 45, USA) at an excitation/emission of 390/510 nm. A standard plot of known GSH concentrations was developed before each set of experiments. After GSH fluorescence was determined for each sample, the subsequent reduction of GSSG by glutathione reductase would yield total GSH fluorescence. Absolute concentrations were determined based on the linear fit of the standard fluorescence. Absolute GSSG concentration was quantified as ((Total GSH - Free GSH) / 2). Values presented are percent changes in GSSG/GSH.
HEK293 Ca-Induced Ca^{2+} Release Model

Using HEK293 cells grown on glass coverslips cultured in 2%FBS MEM, recombinant human RyR2 and SERCA2a were co-expressed via polyethylenimine-mediated transient transfection (PEI; 1μg/ml). Cloned cDNA encoding human GFP-RyR2wt was kindly provided by Dr. Christopher George (University of Cardiff, UK) and CFP-SERCA2a was kindly provided by Dr. Seth Robia (Loyola University Chicago). This approach was also employed so that differences in redox sensitivity of the RyR2 mutant (C3602A) and RyR2wt could be assessed. Before each experiment, HEK293 cells cultured on a glass coverslip were loaded into a chamber and washed in relaxing solution containing (in mM): potassium aspartate 150; MgCl\(_2\) 0.25; EGTA 0.1; HEPES 10; pH 7.2. Using confocal microscopy to measure [Ca\(^{2+}\)], HEK293 cells were permeabilized with internal solution containing saponin (0.005%). The saponin-free internal solution contained the high affinity Ca\(^{2+}\) indicator Rhod-2 tripotassium salt (40 μM) and included (in mM): K-aspartate 100; KCl 15; KH\(_2\)PO\(_4\) 5; MgATP 5; EGTA 0.35; CaCl\(_2\) 0.12; MgCl\(_2\) 0.75; phosphocreatine 10; HEPES 10; creatine phosphokinase 5 U/ml; dextran (MW: 40,000) 8%, and pH 7.2 (KOH). Free [Ca\(^{2+}\)] and [Mg\(^{2+}\)] of this solution were 300 nM and 1 mM, respectively. Prior to the experiments, the free [Ca\(^{2+}\)] was established by introducing increments of 50 nM free [Ca\(^{2+}\)] in a step wise manner until spontaneous Ca\(^{2+}\) waves (SCW) were induced. Only HEK293 cells co-expressing RyR2wt and SERCA2a produced SCW and did not occur in cells expressing RyR2wt without expressing SERCA2a.
(and vice versa). Recorded [Ca\(^{2+}\)]\(_i\) fluorescence was normalized to the initial baseline fluorescence (F/F\(_0\)). The mean amplitude and propensity for SCWs was calculated as a relative change during treatment compared to control steady state. Mean values calculated in control for each cell were normalized to 100%.

**Statistics**

Data are presented as mean +/- standard error of the mean (SEM) of n measurements or averaged values from each myocyte isolation. Statistical comparisons between groups were performed with the Student’s t test for paired or unpaired data sets. Differences were considered statistically significant at P<0.05. Comparisons among multiple sample groups were performed with one way analysis of variance (ANOVA). Statistical analysis and graphical representation of averaged data was carried out on OriginPro7.5 software (OriginLab, USA).
CHAPTER V: RESULTS

AIM #1: Characterize RyR2 oxidation and SR Ca\(^{2+}\) release in vivo as a result of increasing intracellular glutathione mixed-disulfides

Determining Endogenous ROS Levels in Stressed Myocytes

In order to determine a physiologically relevant concentration of \(\text{H}_2\text{O}_2\) treatment, oxidative stress was simulated in isolated ventricular myocytes. Using fluorescence microscopy, direct measurements of cytosolic ROS level was done using the irreversible fluorescent ROS indicator \(\text{H}_2\text{DCFDA (DCF)}\).

In order to induce oxidative stress, myocytes were stressed under supramaximal \(\beta\)-AR stimulation. Excessive \(\beta\)-AR stimulation, which is associated with AMI, has been implicated as an underlying factor that can promote oxidative stress. As described previously by Bovo and colleagues, supramaximal \(\beta\)-AR induces a significant increase in ROS production that coincides with the onset of SCWs in electrically paced myocytes. Moreover, the authors determined that scavenging ROS prevented the onset of SCWs (Bovo et al., 2012). These results suggest that the concentration of intracellular ROS produced during \(\beta\)-AR stress was sufficient to promote abnormal SR Ca\(^{2+}\) handling.

In paced (0.75 Hz) myocytes loaded with DCF, the rate of ROS production was measured (Fig. 2A) before and after the application of isoproterenol (0.1\(\mu\)M). Since the initial rise in ROS production is not evident until 6-8 min during \(\beta\)-AR stimulation,
A, Representative DCF fluorescence plot measuring intracellular ROS production during isoproterenol (0.1 µM) application in myocytes treated with and without MnTBPA (25 µM). B, ROS production was measured relative to baseline fluorescence as a percentage. n=4 (11 myocytes total).
DCF fluorescence was recorded for approximately 15 min of isoproterenol treatment in order to establish a relative change in ROS production. At the end of each experiment, the myocytes were then perfused with 1mM H$_2$O$_2$ in order to determine a standardized rate of change in DCF fluorescence for a given concentration of ROS. We performed analysis on the linear rate of ROS production during isoproterenol treatment relative to that of the external application of H$_2$O$_2$ (Fig. 2B). Based on the resulting percentage (~10%), overall ROS concentration during β-AR stimulation was estimated to be approximately 100µM. Here, our estimate of ROS concentration in stressed myocytes did in fact corroborate the existing literature as a physiologically relevant concentration of ROS during oxidative stress (Stone & Yang, 2006).

**Effects of Oxidants on SR Ca$^{2+}$ Fractional Release and Leak**

In order to assess the effect of oxidative stress on SR Ca$^{2+}$ handling, the first experimental approach examined RyR-mediated SR Ca$^{2+}$ release in isolated myocytes before and after oxidant treatment. Here, my aim is targeted toward defining a molecular mechanism that links oxidative stress and abnormal RyR2 activity. Using the Ca$^{2+}$ indicator Fluo-4AM, cytosolic [Ca$^{2+}$], was measured in isolated ventricular myocytes paced at 0.75 Hz using field stimulation (Fig. 3A). SR Ca$^{2+}$ load was measured as the amplitude of the Ca$^{2+}$ transient induced by caffeine (10 mM) application, whereas LTCC contribution was estimated from the amplitude of the AP-induced Ca$^{2+}$ transient during the application of caffeine. Because the SR is completely depleted of Ca$^{2+}$ in the
Figure 3. SR Ca$^{2+}$ Fractional Release from Cytosolic and SR Ca$^{2+}$ Fluorescence Measurements in Isolated Ventricular Myocyte

A, Representative plot of Fluo-4 fluorescence measuring cytosolic Ca$^{2+}$ during field stimulation (red arrows) and caffeine induced release of SR Ca$^{2+}$ content. LTCC contribution was determined by field stimulation after SR Ca$^{2+}$ depletion (caffeine; 10mM). RyR2 contribution was calculated subtracting LTCC from the Ca$^{2+}$ transient elicited before caffeine application. SR Ca$^{2+}$ fractional release was determined by dividing RyR2 contribution by total SR Ca$^{2+}$ content (caffeine).

B, Representative Fluo-5N measure of SR Ca$^{2+}$. SR Ca$^{2+}$ fractional release was calculated as the amount of Ca$^{2+}$ released from the SR relative to the total SR Ca2+ content ((diastolic [Ca$^{2+}]_{SR}$ - [Ca$^{2+}]_{SR}$ termination) / diastolic [Ca$^{2+}]_{SR}$).

C, Fluo-4 fluorescence measuring LTCC Ca$^{2+}$ transient after blocking LTCC with Nifedipine(5 µM).
presence of 10mM caffeine, the AP-induced Ca\(^{2+}\) transient during caffeine application is mainly mediated by LTCC (Bovo et al., 2013). In order to determine if reverse mode of NCX contributed to electrically induced Ca\(^{2+}\) transients after SR depletion, myocytes were treated with LTCC blocker nifedipine (5uM) in a separate experiment (Fig. 3C). No measurable Ca\(^{2+}\) transients could be elicited after 3 min of nifedipine treatment suggesting that the contribution of reverse-mode of NCX was undetectable.

With the application of 50 µM H\(_2\)O\(_2\) (Fig. 4A), there was a tendency (~50% cells) for Ca\(^{2+}\) transient amplitudes to increase within the initial minutes of treatment suggesting that H\(_2\)O\(_2\) was having a positive inotropic effect with respect to SR Ca\(^{2+}\) cycling. This effect diminished over time as both transient amplitude and SR Ca\(^{2+}\) load decreased suggesting that SR Ca\(^{2+}\) leak was prevailing over reuptake. Furthermore, basal diastolic [Ca\(^{2+}\)]

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tended to be elevated during treatment. Although SR Ca\(^{2+}\) content diminished over time, the resultant SR fractional release was significantly increased after treatment. In a parallel study, we determined changes in [Ca\(^{2+}\)]\(_{SR}\) termination (SR Ca\(^{2+}\) depletion during an AP), which is a direct measurement of RyR-mediated SR Ca\(^{2+}\) release (Fig. 4B). This was done by measuring changes in [Ca\(^{2+}\)]\(_{SR}\) during field stimulation using myocytes with Fluo-5N entrapped within the SR. With respect to steady state control (5 min before treatment), SR Ca\(^{2+}\) load (diastolic [Ca\(^{2+}\)]\(_{SR}\)) did not significantly change after 5 min of H\(_2\)O\(_2\) treatment. [Ca\(^{2+}\)]\(_{SR}\) termination, however, was significantly decreased (0.30 ± 0.05). Thus, the calculated SR Ca\(^{2+}\) fractional release was significantly
Figure 4. SR Ca^{2+} Fractional Release with H_{2}O_{2} Treatment

A, Representative traces of Ca^{2+} transients before and during H_{2}O_{2} application (50 µM; 5 min). B, Representative traces of [Ca^{2+}]_{SR} depletions in a ventricular myocyte before and during H_{2}O_{2} application. C) Average data for relative diastolic [Ca^{2+}]_{SR}, [Ca^{2+}]_{SR} termination, and SR Ca^{2+} fractional release after 5 min H_{2}O_{2} treatment. *P<0.05; n=3 (6 myocytes total)
increased (0.68 ± 0.04; Fig. 4C). The tendency for SR Ca\(^{2+}\) load to decrease over time with \(\text{H}_2\text{O}_2\) treatment (Fluo-4A; Caffeine transient) did agree with SR Ca\(^{2+}\) measurements.

\(\text{H}_2\text{O}_2\) can potentially oxidize a number of different protein residues including the two most susceptible to oxidation, cysteine and methionine. Furthermore, \(\text{H}_2\text{O}_2\) is known to readily react with phospholipids and therefore, can potentially damage the integrity of the cell membrane. Although the use of \(\text{H}_2\text{O}_2\) experimentally is physiologically relevant in the study of oxidative stress, it is highly reactive (fatty acid oxidation, methionine oxidation, etc.) and can complicate the experimental results as well as defining a molecular mechanism. Therefore, a more selective oxidant approach was used in the following experimental designs.

Diamide, a diazine carbonyl compound, is a chemical oxidant that has been shown to be selective for sulphhydryl groups (i.e. protein thiols; \(R-SH\)). In particular, diamide has been demonstrated to be a potent chemical oxidizer of the cellular GSH pool (Kosower et al., 1972) and can selectively induce oxidative PTMs on cysteine residues of proteins. In order to identify the effects of thiol oxidation on Ca\(^{2+}\) cycling in electrically paced (0.75 Hz) myocytes, cytosolic [Ca\(^{2+}\)] and SR Ca\(^{2+}\) load was measured before and after the treatment of diamide. In the presence of diamide (25 \(\mu\text{M}\)), Ca\(^{2+}\) transient amplitude remained relatively unchanged (Fig. 5A). However, SR Ca\(^{2+}\) load was decreased (by 25\%) and LTCC-mediated transient amplitude was elevated (by 16\%). The effect on [Ca\(^{2+}\)]\(_i\) by diamide fully developed 1 min after diamide application and was not
Figure 5. SR Ca\(^{2+}\) Fractional Release with Diamide Treatment

A, Representative traces of Ca\(^{2+}\) transients before and during diamide application (25 µM; 5 min). B, Representative traces of [Ca\(^{2+}\)]\(_{SR}\) depletions in a ventricular myocyte before and during diamide application. C) Average data for relative diastolic [Ca\(^{2+}\)]\(_{SR}\), [Ca\(^{2+}\)]\(_{SR}\) termination, and SR Ca\(^{2+}\) fractional release after 5 min diamide treatment. *P<0.05; n=5 (9 myocytes total)
reversible after washout. These results suggest that diamide increases fractional SR Ca\(^{2+}\) release and depletes SR Ca\(^{2+}\) load.

In order to determine if diamide affects other components of Ca\(^{2+}\) handling, [Ca\(^{2+}\)]\(_i\) decline was analyzed during the AP-induced and caffeine-induced Ca\(^{2+}\) transients under control conditions and in the presence of diamide. The decline of [Ca\(^{2+}\)]\(_i\) during the caffeine application is due to Na\(^+-\)Ca\(^{2+}\) exchange (NCX), whereas during the AP-induced Ca\(^{2+}\) transient [Ca\(^{2+}\)]\(_i\) declines as the result of the combined activities of SR Ca\(^{2+}\) -ATPase (SERCA) and NCX (Bassani & Bers, 1994). Diamide slightly accelerated the decline of the AP-induced Ca\(^{2+}\) transients (by 19±8%), but did not affect the kinetics of the caffeine-induced Ca\(^{2+}\) transient. These results suggest that diamide stimulates SERCA-mediated SR Ca\(^{2+}\) uptake, but not NCX-mediated Ca\(^{2+}\) extrusion.

Determining the contribution of RyR2-mediated Ca\(^{2+}\) release by measuring [Ca\(^{2+}\)]\(_i\) alone was not sufficient since LTCC-mediated Ca\(^{2+}\) transient amplitude did not remain constant with the application of diamide. Therefore, changes in [Ca\(^{2+}\)]\(_{SR}\) depletion were determined using field stimulated myocytes with Fluo-5N entrapped within the SR (Fig. 5B). In the presence of 25 µM diamide, diastolic [Ca\(^{2+}\)]\(_{SR}\) (0.56 ± 0.07) as well as relative [Ca\(^{2+}\)]\(_{SR}\) termination level (0.28 ± 0.04) decreased significantly, which resulted in an increased fractional SR Ca\(^{2+}\) release (0.52 ± 0.03; Fig. 5C). Therefore, these results indicate that RyR-mediated SR Ca\(^{2+}\) release is significantly increased with the application of the oxidant diamide.
Effect of Thiol Oxidation on Steady State [Ca\textsuperscript{2+}]\textsubscript{SR}

Since SR Ca\textsuperscript{2+} load is highly dependent on RyR-mediated SR Ca\textsuperscript{2+} leak, steady state diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} was measured with increasing oxidation of RyR2 by diamide. Here, our approach was to superfuse myocytes with increasing concentrations of diamide (10, 50, and 500 µM) while measuring diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} in field-stimulated myocytes (0.5 Hz). We found that increasing concentrations of diamide up to 50 µM caused a decrease in diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} (10 µM: 48.75% ± 2.2; 50 µM: 30.5% ± 3.2; 500 µM: 30.0 ± 2.8; Fig. 6C). The relative change in diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} in the presence of diamide (at steady state) was calculated as a percentage of control [Ca\textsuperscript{2+}]\textsubscript{SR} (Fig. 6D). When increasing concentrations of diamide were applied in sequence (Fig. 6C), the application of 500 µM diamide had no additional effect on [Ca\textsuperscript{2+}]\textsubscript{SR} suggesting that the effect due to oxidation is already maximal at 50 µM diamide. The alkylating agent N-ethylmaleimide (NEM; 500 µM) has been shown previously to irreversibly block reactive sulphydryls skeletal RyR2 type 1 (2). Here, myocytes were perfused with NEM (500 µM) for 1min before the application of diamide. With the application of NEM, diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} increased initially and gradually fell back toward control levels. Consequently, the application of diamide had no effect on diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} after NEM application (Fig. 7). The previous results suggested that oxidation of SERCA by diamide increases SERCA activity. Thus, thiol oxidation of RyR2 by diamide decreases diastolic [Ca\textsuperscript{2+}]\textsubscript{SR} presumably by stimulation of RyR-mediated Ca\textsuperscript{2+} leak (not compensated by SERCA).
Figure 6. RyR2 Oxidation and Diastolic $[\text{Ca}^{2+}]_{\text{SR}}$ with Diamide Treatment

A, Western immunoblot (non-reducing) against RyR2 from total lysate of isolated myocytes subjected to different concentrations of diamide (5 min). For each sample, the reducing agent DTT (200 mM) was added in order to measure total RyR. B, Relative RyR2 cross-linking in the presence of diamide (*$p<0.05$; $n=3$). No measurable cross-linking is observed in control myocytes without diamide treatment. C, Representative trace of $[\text{Ca}^{2+}]_{\text{SR}}$ measured with the sequential application of increasing diamide concentrations. D, Percent change in steady state $[\text{Ca}^{2+}]_{\text{SR}}$ with the presence of diamide in proportion to $[\text{Ca}^{2+}]_{\text{SR}}$ before diamide application. *$p<0.05$ vs. control $n=3$ (7 myocytes total)
Figure 7. RyR2 Oxidation and Diastolic $[Ca^{2+}]_{SR}$ with N-Ethylmaleimide and Diamide

A, Representative trace of $[Ca^{2+}]_{SR}$ measured in an intact ventricular myocyte with NEM application for 1 min followed with sequential application of 10 µM and 50 µM diamide. B, Percent change in steady state $[Ca^{2+}]_{SR}$ with the presence of diamide in proportion to $[Ca^{2+}]_{SR}$ before diamide application. C, Western immunoblot (non-reducing) against RyR2 from total lysate of isolated myocytes subjected to diamide (50 µM) with or without pretreatment with NEM (500 µM). n=6 (8 myocytes total)
**RyR2 Oxidative PTMs Induced by the Thiol Oxidant Diamide**

Diamide has been shown previously to induce inter-subunit disulfide cross-linking of RyR1 from skeletal muscle (Aghdasi *et al.*, 1997). Therefore, we measured relative cross-linking of RyR2 in isolated ventricular myocytes in parallel with \([\text{Ca}^{2+}]_{\text{SR}}\) measurements. Using western blot analysis with non-reducing SDS-PAGE we quantified the amount of monomeric RyR2 relative to total RyR. Total RyR2 was measured from each protein sample in the presence of the reducing agent DTT (200 mM). With increasing diamide concentrations, the level of monomeric RyR2 dissipated while cross-linked RyR2 became more pronounced (Fig. 6A). Relative cross-linking ratios were calculated as the difference in total RyR2 and monomeric RyR2 divided by total RyR ((Total RyR2 – Monomeric RyR2) / Total RyR2). While there was no measurable cross-linking without diamide, RyR2 is nearly maximally (~90%) cross-linked in the presence of 50 µM diamide (10 µM: 20.7% ± 1.3; 25 µM: 57.2% ± 5.2; 50 µM: 87.0 ± 9.1; 100 µM: 93.0 ± 4.7; Fig. 6B). Steady state diastolic \([\text{Ca}^{2+}]_{\text{SR}}\) was maximally effected by 50 µM diamide, as shown previously, suggesting that intersubunit cross-linking of RyR2 is a strong regulator of \([\text{Ca}^{2+}]_{\text{SR}}\).

**Effect of Oxidized Glutathione SR \(\text{Ca}^{2+}\) leak**

Increased protein glutathione mixed disulfides are a common result of oxidative stress. Here, permeabilized myocytes were used as a different approach to investigate how GSSG affects RyR2 and SR \(\text{Ca}^{2+}\) leak. Previous studies have used GSSG to induce protein-
Figure 8. RyR2 Oxidation and SR Ca\textsuperscript{2+} Leak with GSH and GSSG

A, Representative [Ca\textsuperscript{2+}]\textsubscript{SR} profiles; 5 mM GSH (black) or 5 mM GSSG (gray). SR Ca\textsuperscript{2+} leak was measured as the change in free [Ca\textsuperscript{2+}]\textsubscript{SR} as a function of time after the application of TG. B, Fold change of SR Ca\textsuperscript{2+} leak rates. C, Western immunoblot (non-reducing) against RyR from total lysate of permeabilized myocytes with the addition of GSH or GSSG (5 min). *P<0.05 n=3 (9, 6, 7 myocytes total; Control, GSH, GSSG respectively)
glutathione mixed disulfides in vitro and in vivo (Tang et al., 2011; Wang et al., 2005). To date, the effect of GSSG on RyR2 has only been studied using in-vitro assays with SR microsomes. Since GSH cannot permeate the cellular membrane, controlled manipulation of the cellular GSH environment was achieved by introducing GSH into the cell after rendering the cellular membrane permeable using a mild detergent (saponin). Membrane permeabilization provides several advantages in the context of our experiments: the cytosolic milieu is tightly controlled and cytosolic GSH and GSSG levels can be easily manipulated. This also allows us to control for cytoplasmic ion and ATP concentrations that can also affect RyR2 channel function. Using myocytes loaded with Fluo-5N entrapped within the SR, either GSH (5mM) or GSSG (5mM) was introduced immediately after sarcolemma permeabilization with saponin. SR Ca^{2+} leak was estimated from the rate of [Ca^{2+}]_{SR} decay after the inhibition of SERCA with TG (10 μM).

With the introduction of GSSG in permeabilized myocytes (before TG), we observe a slow decline in [Ca^{2+}]_{SR} as opposed to GSH where [Ca^{2+}]_{SR} is maintained at a steady state (Fig. 8A). With the application of TG, GSSG significantly increased SR Ca^{2+} leak approximately 3-fold relative to the basal leak rate (no glutathione), whereas GSH had no significant effect on leak (Fig. 8B). These results suggest that RyR2 is already highly reduced and therefore is unaffected by the addition of GSH in the cytosolic environment of permeabilized myocytes. SR Ca^{2+} leak in cardiomyocytes, mediated primarily through RyR, occurs in the form of Ca^{2+} sparks as well as spark-independent leak (Zima et al.,
At low diastolic \([\text{Ca}^{2+}]_{\text{SR}} < 300 \mu\text{M}\), SR \(\text{Ca}^{2+}\) leak occurs mostly in the absence of \(\text{Ca}^{2+}\) sparks. Whereas at high diastolic \([\text{Ca}^{2+}]_{\text{SR}} > 600 \mu\text{M}\), sparks became a significant contributor to SR \(\text{Ca}^{2+}\) leak. We found that GSSG increased SR \(\text{Ca}^{2+}\) leak to the same degree at all measured \([\text{Ca}^{2+}]_{\text{SR}}\) levels (Fig 4B), suggesting that GSSG stimulates both spark and spark-independent \(\text{Ca}^{2+}\) leak.

Since diamide stimulates SR \(\text{Ca}^{2+}\) release and RyR2 cross-linking (Fig 5 and 6), we investigated whether GSSG has a similar effect on the RyR2 structure in permeabilized myocytes. Using western blot analysis with non-reducing SDS-PAGE, we found that GSSG induces cross-linking of RyR2 (46.9 ± 12.5%; \(n=3\)). The RyR2 cross-linking was not observed in control permeabilized myocytes (no glutathione) or after incubation of myocytes with GSH. Moreover, pretreatment with NEM (500 \(\mu\text{M}\)) prevented cross-linking of RyR2 induced by GSSG (Fig. 8C). These results demonstrate that GSSG alone can induce RyR2 inter-subunit cross-linking.

Importantly, S-glutathionylation of RyR2 itself has been shown to be associated with increased channel activity (Bull et al., 2008). In order to determine if S-glutathionylation of RyR2 is contributing to the enhanced SR \(\text{Ca}^{2+}\) leak under these experimental conditions, permeabilized myocytes were treated with glutaredoxin (5 units/\(\mu\text{L}\)) and GSH (5 mM) as an attempt to reverse any S-glutathionylation induced by acute GSSG treatment (Aracena-Parks et al., 2006). During this treatment period, there was no significant change in the rate of decline of \([\text{Ca}^{2+}]_{\text{SR}}\) (Fig. 9A,B). Next, western blot
Figure 9. Contribution of RyR2-Glutathionylation in GSSG Induced SR Ca\textsuperscript{2+} Leak

A, Representative [Ca\textsuperscript{2+}]_{SR} profile; 5 mM GSSG (red) 5mM GSH + 5U/µL GRX (blue). B, Absolute rate of decline of [Ca\textsuperscript{2+}]_{SR}. n=3 (6 myocytes total) C, Western immunoblot (non-reducing) against RyR2 and GSH (PSSG) from total lysate of permeabilized myocytes with the addition of GSSG (5 min).
analysis using a pan anti-GSH Ab was carried out to determine the level of RyR2 S-glutathionylation. Using this technique, there was no detectable S-glutathionylation of RyR2 (cross-linked) as a result of GSSG treatment (Fig. 9C). These results suggest that irreversible oxidation in the form of inter-subunit cross-linking is the major contributor of SR Ca\(^{2+}\) leak under our conditions.

**Effect of Oxidized Glutathione on Ca\(^{2+}\) Spark Frequency**

In previous publications, it has been shown that increased spark-mediated Ca\(^{2+}\) leak occurs in the presence of oxidative stress (Yan et al., 2008; Zima et al., 2004). In order to determine the effect of glutathione on Ca\(^{2+}\) sparks, we measured spark properties in permeabilized myocytes with GSH or GSSG introduced into the cytosol after a steady state control frequency was established (Fig. 10A). We found that GSSG caused a significant increase in spark frequency 1 min after application. However, spark frequency declined back to control levels after 5 min. Although not significant, spark amplitude declined over time during the presence of GSSG (Fig. 10B). These results suggest that increased RyR-mediated SR Ca\(^{2+}\) leak leads to depleted SR Ca\(^{2+}\) load and therefore, decreased Ca\(^{2+}\) spark amplitude. Furthermore, there was no significant difference in Ca\(^{2+}\) spark frequency and amplitude between control and GSH (Fig. 11).

**Effect of Oxidized Glutathione on Single RyR2 Channel Activity**

It has been reported previously that both GSH and GSSG affects RyR2 channel open probability (P\(_o\)). We therefore investigated the effect of GSH and GSSG on RyR2
Figure 10. Ca\textsuperscript{2+} Spark Properties with GSSG

A, Representative confocal linescan images of cytosolic Ca\textsuperscript{2+} fluorescence (Rhod-2) and corresponding traces of Ca\textsuperscript{2+} sparks in control and GSSG (1 and 5 min after application). B, Absolute values of spark frequency and amplitude for both control and GSSG. Spark frequency was significantly increased with GSSG at 1 min compared to control. *P<0.05; n=4 (7 myocytes total)
Figure 11. Ca\textsuperscript{2+} Spark Properties with GSH

A, Representative confocal linescan images of cytosolic Ca\textsuperscript{2+} fluorescence (Rhod-2) and corresponding traces of Ca\textsuperscript{2+} sparks in control and GSH (1 min after application). B, Absolute values of spark frequency and amplitude for both control and GSH. n=4 (8 myocytes total)
Figure 12. RyR2 Single Channel Recordings with GSSG and GSH

RyR $P_0$ was measured using a lipid bilayer apparatus. **A**, Representative RyR single channel recordings in the presence of either GSH or GSSG. (C – closed and O – open state) **B**, RyR $P_0$ in the presence of 5 mM GSH and 5 mM GSSG. *P < 0.05; GSH, n=4; GSSG, n=5
activity by measuring single channel current in planar lipid bilayers (Fig. 12A). In these experiments, SR microsomes from rabbit hearts were incorporated into the bilayer and GSH or GSSG was introduced into the cis (cytosolic) side of the chamber. We found that GSH decreased $P_o$ of RyR2 (from 0.083±0.019 in control to 0.039±0.010 (n=4) in the presence of GSH), while GSSG significantly increased $P_o$ (from 0.092±0.015 in control to 0.193±0.033 (n=5) in the presence of GSSG; Fig. 12B). The effect of GSSG was measured within 30 seconds of application and was completely reversed with the application of DTT.

**Simplified Cardiac CICR in HEK293 Cells**

In order to determine the redox sensitivity of RyR2 independent of its auxiliary proteins (making up the cardiac RyR2 complex), an alternative approach was developed in HEK293 cells expressing recombinant RyR2 so that cardiac CICR could be mimicked *in vivo*. HEK293 cells have the ability to be easily transfected with large cDNA plasmids allowing for co-expression of both recombinant RyR2 and SERCA2a. Also, an advantage to the HEK293 cell line is that it lacks endogenous RyR2, thereby permitting the study of only recombinant RyR2. This approach generates SCWs, recorded using Rhod-2 Ca$^{2+}$ indicator within the cellular environment, that are spatially and temporally similar to those that occur in cardiomyocytes (Fig. 13A). This technique also makes it possible to characterize redox sensitive residues by comparing the propensity of SCWs of RyR2wt and RyR2 possessing cysteine mutations during oxidative stress. Ultimately,
mutagenesis of the critical cysteines involved in RyR2 intersubunit cross-linking will allow us to explore their functional relevance using the HEK293 cell CICR model. Of particular interest is the cysteine at residue 3602 of RyR2, which has been shown to be highly redox sensitive and suggested to participate in disulfide formation (Aracena-Parks et al., 2006). Generated by Dr. Bovo, the RyR2 mutant C3602A plasmid was used in these experiments in order to determine if intersubunit cross-linking or redox sensitivity is affected.

Effect of Oxidants on Spontaneous Ca\textsuperscript{2+} Release in HEK293

Our initial studies determined that Ca\textsuperscript{2+} release from the ER can only be elicited if RyR2 is coexpressed with SERCA2a in HEK293 cells. Neither the expression of RyR2 or SERCA2a alone was sufficient to induce Ca\textsuperscript{2+} release events. Therefore, all experiments were done in cells expressing both proteins. By perfusing HEK293 cells with Tyrode solution at high extracellular Ca\textsuperscript{2+} (>2mM), cytosolic SCW could be elicited with the plasma membrane intact. However, all redox studies were carried out in permeabilized cells. This was done in order to avoid variability in \([\text{Ca}^2+]_i\) as a result of changes in plasmalemma permeability during oxidative stress. Comparable to cardiomyocytes, Ca\textsuperscript{2+} release from the ER could be elicited with the application of caffeine at a high concentration (10 mM). Importantly, the observed SCWs were sensitive to the application of caffeine at a low concentration (100 μM), which is indicative of enhanced sensitivity of RyR2 to \([\text{Ca}^2+]_i\). This characteristic behavior of RyR2 not only confirms the
Figure 13. WT and C3602A RyR2 SCWs in HEK293 with Diamide Treatment

A, Only cells that co-expressed RyR2 and SERCA2a (HEK293 cell membrane marked by white line; Left) generate ER Ca release. Representative SCW in HEK293 cell and ventricular myocyte (Right) B, Conserved residues of RyR1 and RyR2 cytosolic motif; RyR1 3635 and RyR2 3602 Cysteine (yellow) C, Representative cytosolic Ca^{2+} fluorescence traces from HEK293 expressing recombinant WT and C3602A RyR2 of SCW in control and diamide (100 µM). D, Relative change in SCW propensity. *P<0.05 ; WT, n=7; C3602A, n=4
origin of the SCW, but it also allows us to determine the activity of RyR2 by measuring the propensity of SCW before and after treatment with oxidants.

After permeabilization, cytosolic solution containing Ca\(^{2+}\) was introduced into the HEK293 cells promoting the generation of SCWs. Prior to experimentation with oxidants, \([\text{Ca}^{2+}]_i\) of 250 nM was determined to be the average threshold to elicit spontaneous Ca\(^{2+}\) release by RyR, which was lower than what was measured previously in cardiomyocytes. Increasing \([\text{Ca}^{2+}]_i\) to 300 nM promoted SCWs that occurred in an oscillatory manner and therefore, could more easily be calculated as a rate. After 5 minutes post-permeabilization, the steady state (control) SCWs were recorded for 1 min before treatment. Figure 13B shows a representative recording of the average fluorescence taken from the cytosolic region of an individual HEK293 cell. With the introduction of diamide (100 μM), the relative change in the rate of SCW was significantly increased immediately after application. Furthermore, the increased SCW propensity was maintained for the duration of the experiment. The relative change in amplitude of SCWs, however, was significantly decreased over time. This change seen in Ca\(^{2+}\) wave properties is typical for increased RyR2 channel activity, as shown with low dose caffeine treatment (Zima et al., 2008b). Using the identical cytosolic solution (300 nM \([\text{Ca}^{2+}]_i\)), oscillating SCWs were elicited in permeabilized HEK293 cells expressing RyR2 C3602A. Similar to RyR2wt, the relative change in the rate of SCW was significantly increased, while the relative change in amplitude of SCWs was significantly
decreased as a result of diamide treatment (Fig. 13C, D). In all untreated HEK293 cells (no changes in oxidation), the rate and amplitude of SCWs remained unchanged throughout the experimental time window.

Next, we examined if recombinant RyR2 expressed in HEK293 cells is susceptible to intersubunit cross-linking. Qualitatively, both monomeric and cross-linked RyR2 was measured using western blot analysis with non-reducing SDS-PAGE. To ensure the cross-linking was redox dependent, RyR2 was measured from each sample in the presence of the reducing agent DTT (200 mM). As demonstrated previously in isolated myocytes, diamide treatment induced intersubunit cross-linking of RyR2 (Fig. 4).

Similarly, HEK293 cells treated in culture with diamide (100 μM) for 5 min induced intersubunit cross-linking of recombinantly expressed RyR2wt. Furthermore, C3602A RyR2 mutant presented no resistance against diamide induced intersubunit cross-linking (Fig. 14), suggesting it is not relevant in intersubunit cross-linking or increased RyR2 activity under the experimental conditions.

**Protective Effect of Nitrosyl Donor on Steady State [Ca^{2+}]_{SR}**

A growing body of evidence has implicated NO as an important molecule that is protective against oxidative stress. The covalent attachment of NO with a reactive free thiol, termed S-nitrosylation, is a ubiquitous and biologically reversible PTM that has been shown to play an important role in cardioprotection. One potential mechanism of
Figure 14. WT and C3602A RyR2 Diamide Induced Intersubunit Cross-linking in HEK293

Western immunoblot (non-reducing) against RyR2 from total lysate of HEK293 expressing recombinant WT and C3602A RyR2 with the addition of diamide (100 µM).
NO dependent cardioprotection is the ability for S-nitrosylation to prevent further thiol oxidation during periods of oxidative stress.

The pharmacological agent S-Nitroso-N-acetylpenicillamine (SNAP) is commonly used experimentally as a NO donor in intact ventricular myocytes. Furthermore, it has been shown to significantly increase total protein S-nitrosylation within minutes of incubation. In order to determine if S-nitrosylation of RyR2 is protects against thiol oxidation by diamide, steady state \([\text{Ca}^{2+}]_{\text{SR}}\) was measured with and without pretreatment with SNAP (50 μM). As with the previous experiment (Fig. 6), the steady state \([\text{Ca}^{2+}]_{\text{SR}}\) was measured during the diastolic phase in field-stimulated myocytes (0.5 Hz). The relative change in diastolic \([\text{Ca}^{2+}]_{\text{SR}}\) in the presence of SNAP, diamide, or SNAP + diamide was calculated as a percentage of control \([\text{Ca}^{2+}]_{\text{SR}}\) (Fig. 15A, B). While diastolic \([\text{Ca}^{2+}]_{\text{SR}}\) decreases during the application of diamide (10 μM), the pretreatment of SNAP (3 min) resulted in a significantly less drop of \([\text{Ca}^{2+}]_{\text{SR}}\) during the application of diamide. There was no measurable change in diastolic \([\text{Ca}^{2+}]_{\text{SR}}\) during SNAP treatment alone. Further analysis comparing both treatment groups shows that treatment with SNAP results in a significantly smaller drop in diastolic \([\text{Ca}^{2+}]_{\text{SR}}\) during diamide treatment. These results suggest that increased S-nitrosylation of RyR2 limits thiol oxidation and subsequent SR Ca\(^{2+}\) leak.

Next, relative cross-linking of RyR2 was measured from isolated ventricular myocytes treated with diamide or SNAP+diamide. Using western blot analysis with non-
Figure 15. RyR2 Intersubunit Cross-linking and Diastolic $[Ca^{2+}]_{SR}$ with SNAP and Diamide Treatment

A, Representative traces of $[Ca^{2+}]_{SR}$ measured in intact ventricular myocytes with the sequential application of SNAP and diamide vs. diamide alone. B, Percent change in steady state $[Ca^{2+}]_{SR}$ with the presence of diamide or SNAP + diamide in proportion to $[Ca^{2+}]_{SR}$ before treatment. C, Western immunoblot (non-reducing) against RyR2 from total lysate of isolated myocytes subjected to diamide (50 µM) with and without pretreatment of SNAP (50 µM) D, Relative RyR2 cross-linking (n=3). *P<0.05  n=3
reducing SDS-PAGE, relative cross-linking was determined using the quantified monomeric RyR2 and total RyR2 (with DTT). Consistent with previous experimental results, untreated myocytes presented low to undetectable cross-linked RyR2. As expected, treating myocytes with diamide (50 μM) for 5 min decreased the level of monomeric RyR2, which yielded a near maximal cross-linking ratio. By treating myocytes with SNAP (50 μM) for 5 min before introducing diamide, intersubunit cross-linking was significantly lower with respect to diamide treatment alone (Fig 15C, D).

**Effect of Nitrosyl Donor on Spontaneous Ca^{2+} Release in HEK293 cells**

Not unlike other oxidative species, NO has the potential to covalently modify proteins that play a role in the regulation of RyR2. Furthermore, previous studies have implicated S-nitrosylation of RyR2 in affecting the binding affinity of regulatory proteins making up the RyR2 complex. In order to directly examine the effect of S-nitrosylation on RyR2 activity, the simplified CICR HEK293 model was utilized. The ability to study the channel activity of recombinant RyR2 void of scaffolded regulatory proteins is the major advantage of this system. Therefore, any effect of S-nitrosylating agents on channel activity is likely due to a direct modification of RyR2.

Using the same approach, both RyR2 and SERCA2a were coexpressed in HEK293. For these experiments, both RyR2wt and RyR2 C3602A were expressed in parallel to determine any differences in NO sensitivity. Oscillating SCWs were generated once the plasma membrane was permeabilized and cytosolic solution with a [Ca^{2+}] of 300 nM
was introduced. After 5 minutes post-permeabilization, the steady state (control) SCWs were recorded for 1 min before treatment. Figure 16 shows a representative recording of the average fluorescence (cytosolic Ca\textsuperscript{2+}) within an individual HEK293 cell. Since permeabilization makes RyR2 accessible to impermeable pharmacological agents, both SNAP (50 μM) and GSNO (200 μM) could be used as nitrosyl donors. The mean amplitude and propensity for SCWs was calculated during the presence of GSNO, SNAP, and sequential treatment of diamide (100 μM). Analysis of the data is presented as a relative change in the mean values calculated from control and treated for each cell (Fig. 16A,B). With the introduction of SNAP, the relative change in propensity of SCW was decreased at the 3 min time point of treatment. Although decreased, the SCW propensity was not significantly changed in the presence of GSNO. Furthermore, the relative amplitude of SCWs was unaffected in cells treated with either SNAP or GSNO.

To test whether nitrosyl donors are capable of protecting RyR2 from the effects of oxidative stress, diamide was introduced immediately following the 3 min pretreatment period. Both the propensity and amplitude of the SCWs did not significantly change during the 5 min of diamide treatment. Further analysis of SCW parameters in the presence of diamide from cells pretreated with and without nitrosyl donors proved to be significantly different. While diamide treatment with no nitrosyl donor significantly increased SCW propensity, these results suggest that nitrosyl donors
Figure 16. RyR2 WT and C3602A RyR2 SCWs in HEK293 with SNAP, GSNO, and Diamide Treatment

A, Representative cytosolic Ca\(^{2+}\) fluorescence traces from HEK293 expressing recombinant WT and C2602A RyR2 of SCW in control, SNAP (50 µM) + diamide (100 µM), and diamide alone. B, Relative change in SCW propensity for C2602A RyR2 with diamide, GSNO (200 µM), and GSNO + diamide. C, Relative change in SCW propensity and amplitude for WT RyR2 with GSNO, SNAP, GSNO + diamide, and SNAP + diamide. *P<0.05; WT, n=7; C3602A, n=4
prevent the effects of diamide by limiting further oxidation of RyR2 (e.g. Intersubunit Cross-linking).

Since cysteine 3602 of RyR2 is a potential target for S-nitrosylation, changes in SCW propensity in cells expressing RyR2wt and RyR2 C3602A were compared. With all treatment groups, there was no difference in the relative change of SCW propensity. These results suggest that cysteine residue 3602 does not play a role in oxidation dependent changes in RyR2 activity.
Aim #2: Determine the role of RyR2 oxidation on abnormal SR Ca\(^{2+}\) release in I/R and HF

Ischemia-Reperfusion

Resulting from I/R, intracellular Ca\(^{2+}\) overload and oxidative stress are the major mechanisms that have been implicated in cardiomyocyte death, stunning, and arrhythmogenesis. It is generally accepted that these mechanisms are interdependent and have the ability to influence one another through multiple signaling pathways. In order to study isolated myocytes that have been subjected to I/R, an ex-vivo low-flow ischemia model in Langendorff perfused rabbit hearts was established in our laboratory. Coronary occlusion I/R models are notoriously difficult to isolate viable myocytes from myocardium that has undergone I/R. Low-flow I/R ensures that all myocytes isolated for biochemical and functional studies will have undergone I/R. It is imperative that low flow ischemia was sufficient to promote arrhythmogenesis during reperfusion (at increased oxidative stress). In order to determine if the heart exhibited these attributes, the left ventricular pressure (LVP) and electrical activity (ECG) was monitored during throughout the protocol. Figure 17 depicts a representative recording of the ECG and LVP at steady state, ischemia, and reperfusion. Since the ECG electrodes were placed across the ventricles, no P wave can be discerned on the recording. However, the QRS complex and T wave were clearly observed so that the rate of pacemaker automaticity and regular conduction pathway could be defined. Based on the polarity and intrinsic
frequency of the QRS complex, ventricular ectopic beats (not originating or following the normal conduction pathway) were recorded and analyzed qualitatively. Changes in LVP were calculated during the ischemia and reperfusion phases relative to the average pressure during steady state. With low-flow ischemia, the relative change in pacemaker automaticity was markedly decreased from that of the steady state frequency. With reperfusion, the relative heart rate was significantly increased and arrhythmias were observed in 100% of all hearts used for single myocyte isolation and experimentation. While the LVP was significantly decreased during ischemia, no significant difference was observed in LVP during reperfusion compared to control steady state.

**Effects of I/R on SR Ca\(^{2+}\) Fractional Release and Leak**

In order to assess the effect of I/R on SR Ca\(^{2+}\) handling, RyR-mediated SR Ca\(^{2+}\) release was measured in isolated myocytes from low flow I/R hearts and sham hearts. Using the Ca\(^{2+}\) indicator Fluo-4AM, cytosolic Ca\(^{2+}\) was measured in isolated ventricular myocytes. Similar to previous fractional release measurements, myocytes were paced at 0.75 Hz and SR Ca\(^{2+}\) load was measured as the amplitude of the Ca\(^{2+}\) transient induced by caffeine (10 mM) application. LTCC contribution was estimated from the amplitude of the AP-induced Ca\(^{2+}\) transient during the application of caffeine. The average SR fractional release in myocytes isolated from I/R hearts was significantly higher than that of sham (Fig. 18A). These results indicate that RyR-mediated SR
Representative recordings of LVP and ECG during low flow I/R A, Control full perfusion B, Low-flow ischemia C, Reperfusion; arrhythmogenesis D, Reperfusion; tachycardia E, Intrinsic heart rate before, during, and after low-flow ischemia F, Relative change in LVP before, during, and after low-flow ischemia. n=11
Ca\(^{2+}\) release is significantly increased as a result of I/R.

Next, the effect of I/R on RyR-mediated SR Ca\(^{2+}\) leak was investigated in permeabilized myocytes. Not only is SR Ca\(^{2+}\) leak used as a complementary parameter to SR fractional release, but this approach allows for a more direct measurement of RyR2 channel activity as a result of PTMs. I/R injury can result in changes in cytoplasmic ion concentrations and ATP concentration, which can have a profound effect RyR2 channel function. With this approach, membrane permeabilization allows us to control for all cytoplasmic variables that may potentially be different between I/R and sham. Using myocytes loaded with Fluo-5N entrapped within the SR, cytosolic solution containing \([\text{Ca}\(^{2+}\)]\), of 150 nM was introduced immediately after sarcolemma permeabilization with saponin. SR Ca\(^{2+}\) leak was estimated from the rate of [Ca\(^{2+}\)]\(_{\text{SR}}\) decay after the inhibition of SERCA with TG (10 μM). For each myocyte leak protocol, the [Ca\(^{2+}\)]\(_{\text{SR}}\) decay was differentiated in order to determine SR Ca\(^{2+}\) leak rate for a given [Ca\(^{2+}\)]\(_{\text{SR}}\). The resulting leak rates were compiled and averaged within 100 μM ([Ca\(^{2+}\)]) increments (Table 1). Myocytes isolated from I/R hearts had a significantly increased SR Ca\(^{2+}\) leak rate than that of sham myocytes (Fig. 18B).

**Effects of I/R on Redox Environment and the Oxidation of RyR2**

While studies have implicated oxidative stress in SR Ca\(^{2+}\) dysfunction, it is not yet clear to what extent the oxidation of RyR2 plays in abnormal SR Ca\(^{2+}\) handling in I/R. During oxidative stress, the redox potential can shift leading to an increase in GSSG. To
Figure 18. SR Ca\textsuperscript{2+} Leak and Fractional Release in Sham and I/R Myocytes

A, SR Ca\textsuperscript{2+} fractional release from Sham (CNTL) and I/R myocytes. B, SR Ca\textsuperscript{2+} leak rates plotted from table 1; SHAM (Control; black) or low flow I/R (I/R; gray). SR Ca\textsuperscript{2+} leak was measured as the change in free [Ca\textsuperscript{2+}]\textsubscript{SR} as a function of time after the application of TG.*P<0.05 Control, n=4; I/R, n=5
Table 1. SR Ca\(^{2+}\) Leak Rates of I/R Myocytes

### Control (Sham)

<table>
<thead>
<tr>
<th>[Ca(^{2+})](_{SR}) bin (μM)</th>
<th>[Ca(^{2+})](_{SR}) (Mean)</th>
<th>Leak Rate μM/s (Mean ± SEM)</th>
<th>[Ca(^{2+})](_{SR}) (Mean)</th>
<th>Leak Rate μM/s (Mean ± SEM)</th>
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<tr>
<td>0-100</td>
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<td>1.66724 ± 0.20</td>
<td>37.6</td>
<td>1.2248 ± 0.15</td>
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<td>101-200</td>
<td>153.6</td>
<td>3.40569 ± 0.24</td>
<td>151.6</td>
<td>4.93024 ± 0.75*</td>
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<tr>
<td>201-300</td>
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<td>239.9</td>
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<td>13.17702 ± 2.31*</td>
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<td>13.20784 ± 1.84</td>
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Number of rabbit myocyte isolations used for leak measurements was n=3. [Ca\(^{2+}\)]\(_{SR}\) was divided into 100 μM bins and [Ca\(^{2+}\)]\(_{SR}\) data was compiled and averaged within each bin. Leak rates were analyzed as the decrease in Ca\(^{2+}\) over time (μM/s).
Figure 19. Relative RyR2 Free Thiols in I/R

A, Representative mBB and comassie gel of RyR2 from sham (CNTL), I/R, and I/R treated with DTT (5 mM; Fmax). B, Relative RyR2 free thiols. n=3 C, GSSG/GSH ratios from fluorometric analysis. n=3 *P<0.05
exam the effect of low-flow I/R on the cellular redox environment, the levels of GSH and GSSG were measured from cardiomyocytes isolated from low flow I/R hearts. Using a fluorescent probe, GSH was measured in deproteinated lysate from ventricular tissue biopsied before and after I/R. Analysis of GSH and Total GSH fluorescence determined that the GSSG/GSH ratio was significantly elevated in tissue after I/R indicating that oxidative stress was induced during reperfusion (Fig. 19C). In order to determine if oxidation of RyR2 took place as a result of I/R, changes in relative free thiol content were measured in isolated myocytes from sham and I/R hearts. For each isolation, myocyte aliquots were either untreated, completely reduced with DTT (5 mM; $F_{\text{max}}$), or oxidized with DTDP (0.5 mM; $F_{\text{min}}$) and then incubated with the fluorescent thiol probe mBB. By resolving RyR2 using reducing SDS-PAGE, analysis of RyR2 free thiol content determined that RyR2 was significantly more oxidized in myocytes isolated from I/R hearts compared to that of sham hearts (Figure 19A,B).

Given that RyR2 was determined to be susceptible to intersubunit cross-linking, the next approach was directed toward determining if the oxidative stress induced in low flow I/R was sufficient to induce this modification. Using western blot analysis with non-reducing SDS-PAGE we measured monomeric RyR2 relative to total RyR. Total RyR2 was measured from each protein sample in the presence of the reducing agent DTT (200 mM). In myocytes isolated from I/R hearts, the cross-linked form of RyR2 was undetectable.
Intersubunit Cross-Linking of RyR2 in Coronary Occlusion Model of I/R

Since low flow I/R may not be sufficient to induce oxidative stress at a severe magnitude that promotes cross-linking, a coronary occlusion model was developed in order to localize the area of ischemia and induce 100% occlusion. Using a similar protocol to low flow I/R, hearts underwent ischemia and reperfusion for identical periods of time. Monomeric RyR2 relative to total RyR2 was measured from isolated tissue biopsies taken from the area of ischemia and remote myocardium. Tissue isolated from the ischemic region had a significantly decreased level of monomeric RyR2 and an observable cross-linked form of RyR2 (Fig. 20A). Relative cross-linking ratios were calculated as the difference in total RyR2 and monomeric RyR2 divided by total RyR. Analysis of relative cross-linking in ischemic myocardium was calculated as 0.16 ± 0.07 (Fig. 20B). These results suggest that coronary occlusion I/R is sufficient to induce intersubunit cross-linking of RyR2, which likely promotes abnormal SR Ca\(^{2+}\) handling at the area of injury.

Cardiac Function of Failing Hearts in Rabbit Model of HF

Non-ischemic HF was induced in rabbits using a combined insult of aortic insufficiency and constriction. Two-dimensional echocardiography was used to determine the progression of HF at 2 to 4 week intervals after insult. The calculated parameters for diastolic left ventricular internal dimension (LVIDd) and left ventricular fractional shortening (LVFS) was determined prior to sacrifice and myocyte isolation
Figure 20. RyR2 Intersubunit Cross-linking in Coronary Occlusion I/R

**A**

Western immunoblot (non-reducing) against RyR2 from total lysate of whole tissue biopsies from remote and I/R myocardium

**B**

Relative RyR2 cross-linking

*P<0.05; n=3
Table 2. Echocardiography Parameters of Failing Rabbit Hearts

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<th>HF Rabbit</th>
<th>LVIDd Initial (cm)</th>
<th>LVIDd Before Sac. (cm)</th>
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<th>LVFS Initial (%)</th>
<th>LVFS Before Sac. (%)</th>
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</table>

Total number of rabbits used in the combined studies was n=9. The left ventricular internal diameter during diastole (LVIDd) and left ventricular fractional shortening (LVFS) was measured prior to surgery (initial) and before myocytes isolation (Sacrifice).
Table 3. SR Ca\textsuperscript{2+} Leak Rates of HF Myocytes

<table>
<thead>
<tr>
<th>[Ca\textsuperscript{2+}]\text{SR} (µM)</th>
<th>[Ca\textsuperscript{2+}]\text{SR} (Mean)</th>
<th>Leak Rate µM/s (Mean ± SEM)</th>
<th>[Ca\textsuperscript{2+}]\text{SR} (Mean)</th>
<th>Leak Rate µM/s (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-100</td>
<td>42.8</td>
<td>1.02978 ± 0.18</td>
<td>45.2</td>
<td>1.11663 ± 0.26</td>
</tr>
<tr>
<td>101-200</td>
<td>147.6</td>
<td>1.55087 ± 0.22</td>
<td>142.8</td>
<td>2.75434 ± 0.23</td>
</tr>
<tr>
<td>201-300</td>
<td>247.3</td>
<td>2.66749 ± 0.24</td>
<td>242.5</td>
<td>5.35821 ± 0.61*</td>
</tr>
<tr>
<td>301-400</td>
<td>349.7</td>
<td>3.88337 ± 0.81</td>
<td>340.4</td>
<td>7.84119 ± 1.12*</td>
</tr>
<tr>
<td>401-500</td>
<td>456.5</td>
<td>4.39206 ± 0.82</td>
<td>449.7</td>
<td>10.08685 ± 2.93*</td>
</tr>
<tr>
<td>501-600</td>
<td>554.1</td>
<td>5.34739 ± 0.97</td>
<td>549.3</td>
<td>12.49379 ± 1.13*</td>
</tr>
<tr>
<td>601-700</td>
<td>642.1</td>
<td>7.49380 ± 1.12</td>
<td>654.1</td>
<td>18.08933 ± 1.81*</td>
</tr>
<tr>
<td>701-800</td>
<td>754</td>
<td>9.82630 ± 1.06</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>801-900</td>
<td>856.2</td>
<td>12.32001 ± 1.29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>901-1000</td>
<td>970.2</td>
<td>16.88586 ± 1.31</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Number of rabbit myocyte isolations used for leak measurements was n=3. [Ca\textsuperscript{2+}]\text{SR} was divided into 100 µM bins and [Ca\textsuperscript{2+}]\text{SR} data was compiled and averaged within each bin. Leak rates were analyzed as the decrease in Ca\textsuperscript{2+} over time (µM/s).
(Table 2). The percent change in both parameters was calculated relative to the baseline measurements taken prior to the surgeries.

**Effects of HF on SR Ca\(^{2+}\) Fractional Release and Leak**

In order to assess the effect of HF on SR Ca\(^{2+}\) handling, RyR-mediated SR Ca\(^{2+}\) release was measured in isolated myocytes from failing hearts and sham hearts. Using the Ca\(^{2+}\) indicator Fluo-4AM, cytosolic Ca\(^{2+}\) was measured in isolated ventricular myocytes. Identical to the protocol for I/R, myocytes were paced at 0.75 Hz and SR Ca\(^{2+}\) load was measured as the amplitude of the Ca\(^{2+}\) transient induced by caffeine (10 mM) application. LTCC contribution was estimated from the amplitude of the AP-induced Ca\(^{2+}\) transient during the application of caffeine. The average SR fractional release in myocytes isolated from failing hearts was significantly higher than that of sham (Fig. 21A). These results indicate that RyR-mediated SR Ca\(^{2+}\) release is significantly increased in the myocytes isolated from failing hearts.

Next, the effect of HF on RyR-mediated SR Ca\(^{2+}\) leak was investigated in permeabilized myocytes. As with I/R injury, HF can affect the cytosolic milieu and intracellular Ca\(^{+}\) buffering, which can have a profound effect RyR2 channel function. Using myocytes loaded with Fluo-5N entrapped within the SR, cytosolic solution containing [Ca\(^{2+}\)]\(_i\) of 150 nM was introduced immediately after sarcolemma permeabilization with saponin. SR Ca\(^{2+}\) leak was estimated from the rate of [Ca\(^{2+}\)]\(_{SR}\) decay after the inhibition of SERCA with TG (10 μM). For each myocyte leak protocol,
A, SR Ca\textsuperscript{2+} fractional release from Sham (CNTL) and HF myocytes. B, SR Ca\textsuperscript{2+} leak rates plotted from table 1; SHAM (Control; black) or HF (red). SR Ca\textsuperscript{2+} leak was measured as the change in free [Ca\textsuperscript{2+}]\textsubscript{SR} as a function of time after the application of TG. *P<0.05 Control, n=5; HF, n=4
the $[Ca^{2+}]_{SR}$ decay was differentiated in order to determine SR Ca$^+$ leak rate for a given $[Ca^{2+}]_{SR}$. The resulting leak rates were compiled and averaged within 100 μM ($[Ca^{2+}]$) increments (Table 3). Myocytes isolated from failing hearts had a significantly increased SR Ca$^{2+}$ leak rate than that of sham myocytes (Fig. 21B).

**Force Frequency Response in HF**

A blunted FFR is a characteristic defect that has been implicated in impaired contraction of the failing heart. This limitation has been attributed to abnormal activity of Ca$^{2+}$ cycling machinery, including RyR2. However, whether or not an increase in oxidation of RyR2 can promote a blunted FFR remains unknown. Using myocytes isolated from rabbit HF and sham, the percent FFR was investigated in myocytes loaded with Fluo-4AM. Ca$^{2+}$ transients were recorded at a pacing frequency of 0.2 Hz, followed by 0.75 Hz. Before each recording, myocytes were given 2 minutes at each pacing frequency so that Ca$^{2+}$ cycling could reach steady state. For each line scan recording, the average Ca$^{2+}$ transient amplitude was determined for each pacing frequency. Frequency facilitation from 0.2 to 0.75 Hz was calculated as the relative change in amplitude. Analysis of transient amplitudes determined frequency facilitation was significantly lower in HF compared to that of sham (Fig. 22A, B). These results indicate that FFR is indeed blunted in the myocytes isolated from failing rabbit hearts.

In order to test the hypothesis that oxidative stress can induce a blunted FFR phenotype, frequency facilitation was determined in healthy myocytes exposed to low
Figure 22. Frequency Facilitation with Diamide Treatment and HF

A, Representative Ca\(^{2+}\) transients from control and HF myocytes at 0.2 Hz and 0.75 Hz pacing frequencies. Frequency facilitation was measured as the percent increase in Ca\(^{2+}\) transient amplitude. B, Relative facilitation from 0.2 to 0.75 Hz. n=4 C, Representative Ca\(^{2+}\) transients from control myocytes at 0.2 Hz and 0.75 Hz pacing frequencies with and without the treatment of diamide (10 µM). D, Relative facilitation from 0.2 to 0.75 Hz. *P<0.05 n=3 (7 myocytes total)
concentration of the cysteine oxidant diamide (10 μM). Ca$^{2+}$ transients were recorded from each myocyte at both 0.2 and 0.75 Hz in two consecutive sets (Fig. 22C). The myocytes subjected to oxidative stress were perfused with diamide during the second set of measurements (beginning 2 minutes prior to 0.2 Hz recording). Frequency facilitation from 0.2 to 0.75 Hz was calculated as the relative change in amplitude and was determined for each set. Analysis of transient amplitudes found a significantly lower relative facilitation in the presence of diamide compared to control (Fig. 22D).

**Oxidation of RyR2 in HF**

Previous studies have showed an increase in oxidation of RyR2 in animal models of heart failure. In order to determine if RyR2 is more oxidized in our rabbit HF model, changes in relative free thiol content were measured from myocytes isolated from sham and failing hearts. For each isolation, myocyte aliquots were either untreated, completely reduced with DTT ($F_{\text{max}}$), or oxidized with DTDP ($F_{\text{min}}$) and then incubated with the fluorescent thiol probe mBB. By resolving RyR2 using reducing SDS-PAGE, analysis of RyR2 free thiol content determined that RyR2 was significantly more oxidized in myocytes isolated from failing hearts compared to that of sham (Fig. 23).

With the availability of human myocardium tissue samples from failing hearts (provided by Dr. deTombe’s laboratory), the existence of intersubunit cross-linking was investigated. Western blot analysis with non-reducing SDS-PAGE yielded evidence that intersubunit cross-linking was indeed present in failing myocardium (Fig. 24).
Figure 23. Relative RyR2 Free Thiols in HF

A, Representative mBB and comassie gel of RyR2 from sham (CNTL), HF, and HF treated with DTT (5 mM; Fmax).  

B, Relative RyR2 free thiols. n=3  *P<0.05
**Figure 24. RyR2 Intersubunit Cross-linking in Human HF**

**A**

Human DCM LV

<table>
<thead>
<tr>
<th>DTT</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>560 kDa</td>
<td>▶</td>
<td>▶</td>
</tr>
</tbody>
</table>

**B**

Human NF LV  | Human DCM LV

<table>
<thead>
<tr>
<th>DTT</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crosslinked</td>
<td>▶</td>
<td>▶</td>
<td>▶</td>
<td>▶</td>
</tr>
<tr>
<td>560 kDa</td>
<td>▶</td>
<td>▶</td>
<td>▶</td>
<td>▶</td>
</tr>
</tbody>
</table>

**A**, Western immunoblot (non-reducing) against RyR2 from total lysate of tissue biopsies taken from human HF. **B**, Western immunoblot (non-reducing) against RyR2 from total lysate of tissue biopsies taken from both human non-failing (NF) and HF.
This evidence provided the rationale to investigate whether or not this oxidative modification was present in the rabbit model of non-ischemic HF. Using the isolated myocytes from rabbit HF, monomeric RyR2 and total RyR2 was determined. However, myocytes isolated from failing rabbit hearts exhibited no detectable form of cross-linked RyR2.
CHAPTER VI: DISCUSSION

Evaluating External Oxidative Stress on SR Ca Release

Oxidative stress is a recurrent mechanism underlying the pathogenesis of many disease states. Being no exception, both ischemic and nonischemic cardiovascular disease has been associated with oxidative stress (Zweier et al., 1987; Belch et al., 1991; Hill & Singal, 1997; Mallat et al., 1998; Vanden Hoek et al., 1996). For the initial study, oxidative stress was evaluated in healthy isolated myocytes stressed with high β-AR stimulation. β-AR stress, commonly observed in I/R injury and in the failing heart, has been shown to promote oxidative stress. The aim of this study was to define a relevant concentration of ROS that can be used experimentally to mimic oxidative stress. Using fluorescence microscopy, intracellular ROS concentration was estimated to be approximately 100 µM based on an external exposure of standardized H₂O₂. Although DCF is not selective for one particular free radical, it is most sensitive to H₂O₂ and lipid peroxides (Roth G & Valet G, 1990; Hoffman et al., 2008). DCF has been evaluated as a less sensitive indicator of the hydroxyl radical, a highly reactive free radical formed via the fenton reaction, which can potentially make the calculation here an underestimated ROS concentration.
Intracellular GSH is arguably the first line of defense against ROS. In particular, GSH is a highly abundant redox buffer in the heart; as a result, increased GSSG/GSH ratio predominates in cardiovascular disease where oxidative stress is common. Oxidative stress also promotes protein-glutathione mixed disulfides, which are increased due to the abundance of the reducing equivalent GSH. It is well-established that RyR2 activity can be modulated by redox-sensitive compounds. Furthermore, it has been shown that GSSG/GSH can affect the activity of different proteins including RyR2 (Zima & Blatter, 2006). Experimentally, both physiological and chemical oxidants can promote increased GSSG/GSH as well as protein-glutathione mixed disulfides. However, the effect of the aforementioned oxidative changes on RyR2 oxidation, channel activity, and SR Ca\(^{2+}\) release in ventricular myocytes has not been systematically explored.

**SR Ca Fractional Release**

The use of external ROS to induce oxidative stress is imperative to dissect the effect of oxidative stress in abnormal SR Ca\(^{2+}\) handling due to the complex nature of cardiovascular disease. By mimicking oxidative stress in healthy ventricular myocytes with an acute application of H\(_2\)O\(_2\), the results show that cytosolic Ca\(^{2+}\) transient amplitudes increase within minutes of treatment (Fig. 4). Moreover, direct measurements of SR Ca\(^{2+}\) using fluorescent Ca\(^{2+}\) dye (Fluo-5N) entrapped within the SR determined SR Ca\(^{2+}\) fractional release to be markedly elevated with H\(_2\)O\(_2\) treatment. SR Ca\(^{2+}\) load also decreased over time suggesting that increased SR Ca\(^{2+}\) leak caused a shift
in steady state Ca\(^{2+}\) cycling that resulted in a depletion of SR Ca\(^{2+}\) content. Overall, this data suggests that oxidative stress, induced by an external physiological oxidant (H\(_2\)O\(_2\)), increases RyR-mediated Ca\(^{2+}\) release from the SR during ECC.

Although H\(_2\)O\(_2\) is a physiologically relevant oxidant, it can potentially oxidize a number of different protein residues including the two most susceptible to oxidation, cysteine and methionine. Furthermore, H\(_2\)O\(_2\) oxidizes phospholipids, which can potentially damage the integrity of the sarcolemma and sarcoplasmic reticulum. Therefore, the subsequent experimental approaches were designed to more selectively manipulate cytosolic GSSG in order to study its effect on cardiac RyR2 channel activity in both a cellular environment and in vitro.

Since GSSG is not membrane permeable, the diazine carbonyl compound diamide was used to oxidize intracellular GSH within intact myocytes. It has been shown experimentally that diamide preferentially oxidizes cysteine thiol residues, particularly GSH (forming GSSG) in comparison to other thiol containing molecules (Kosower et al., 1972). Either directly or indirectly (via GSSG formation), diamide can promote the formation of protein-glutathione mixed disulfides as well as protein-protein disulfides (Brennan et al., 2004; Hansen et al., 2009). Therefore, any change in SR Ca\(^{2+}\) release in the presence of diamide is likely the effect of oxidative modifications involving cysteine residues alone (i.e. GSSG, PSSG, or PSSP). With the acute application of diamide, our results show that cytosolic Ca\(^{2+}\) transient amplitudes are maintained despite a decrease
in SR Ca\(^{2+}\) load (Fig. 5A). As a result, fractional SR Ca\(^{2+}\) release was significantly increased in the presence of diamide. This data suggests that elevated cytosolic GSSG increases RyR-mediated Ca\(^{2+}\) release from the SR during ECC. Furthermore, AP-induced Ca\(^{2+}\) transients during caffeine application were also augmented suggesting that LTCC activity is increased in the presence of diamide (Fig. 5A). These results would then corroborate a recent study by Tang et al. showing that LTCC activity is increased as a result of S-glutathionylation (Tang et al., 2011). Because changes in LTCC complicate our measurements of RyR-mediated Ca\(^{2+}\) release using cytosolic Ca\(^{2+}\) measurements, we measured relative changes in SR Ca\(^{2+}\) depletion. Our results show that in the presence of diamide SR Ca\(^{2+}\) load as well as [Ca\(^{2+}\)]\(_{SR}\) termination level during APs is significantly decreased, therefore causing fractional SR Ca\(^{2+}\) release to be increased (Fig. 1B).

Because the sensitivity of RyR2 to CICR is reduced with decreasing [Ca\(^{2+}\)]\(_{SR}\) (Shannon et al., 2000b), these results suggest that RyR2 is more sensitive to [Ca\(^{2+}\)]\(_{SR}\) in the presence of diamide.

**Steady State SR Ca\(^{2+}\) and Leak**

The resulting measurements of steady state diastolic [Ca\(^{2+}\)]\(_{SR}\) clearly demonstrate that diamide causes a dose-dependent decrease in [Ca\(^{2+}\)]\(_{SR}\) and an increase of RyR2 inter-subunit cross-linking (Fig 6). There was no further decrease in diastolic [Ca\(^{2+}\)]\(_{SR}\) when diamide concentration was increased from 50 to 500 µM (Fig. 6C). In parallel, RyR2 subunits were almost entirely cross-linked in the presence of 50 µM diamide.
Given these results, we hypothesize that RyR2 cross-linking increases diastolic SR Ca$^{2+}$ release (i.e. Ca$^{2+}$ leak), which cannot be compensated by SERCA, resulting in a decreased steady state diastolic [Ca$^{2+}$]$_{SR}$. A decrease in diastolic [Ca$^{2+}$]$_{SR}$ could also be explained by inhibition of SERCA activity. However, we found that the decay rate of the AP-induced Ca$^{2+}$ transient was increased in the presence of diamide. Moreover, there was a tendency for steady state [Ca$^{2+}$]$_{SR}$ to increase during the application of 500 µM diamide suggesting that SERCA activity may be enhanced. This effect may be due to the ability of diamide to cross-link phospholamban which can subsequently relieve SERCA inhibition (Robia et al., 2007).

Since diamide alone has the potential of directly oxidizing cysteine residues on RyR, a more direct approach was carried out by introducing GSSG directly into the cytosol. This was achieved by using saponin-permeabilized myocytes. Although we are unable to measure changes in SR Ca$^{2+}$ fractional release (as with intact myocytes) with this technique, we can confidently detect changes in RyR2 activity induced by GSSG in a tightly controlled cytosolic environment. Consistent with the effects of diamide, our results show that GSSG significantly increases RyR-mediated SR Ca$^{2+}$ leak (Fig. 8). By analyzing Ca$^{2+}$ leak at different [Ca$^{2+}$]$_{SR}$, we found that GSSG increases two major components of SR Ca$^{2+}$ leak: spark and non-spark-mediated Ca$^{2+}$ leak (Fig. 8B). Our data also suggests that augmentation of spark-mediated SR Ca$^{2+}$ leak after acute application of GSSG causes a decrease in SR Ca$^{2+}$ load. This would explain why we
observe a decrease in Ca\(^{2+}\) spark amplitude and frequency over time (Fig. 10).

Importantly, we found that GSSG within permeabilized myocytes is sufficient to induce cross-linking of RyR2 (similar to diamide). In accordance with previous bilayer studies (Zable et al., 1997; Feng et al., 2000), our single channel recordings reveal that GSSG significantly increases \(P_o\) of RyR2 and this effect can be restored by DTT.

**Intersubunit Cross-linking of RyR2**

The results of these studies reveal a novel mechanism by which GSSG can affect RyR-mediated Ca\(^{2+}\) release in rabbit ventricular myocytes. We found that oxidation of RyR2 by GSSG is associated with formation of a disulfide bond between two subunits within the tetramer (i.e. inter-subunit cross-linking). Because the inter-subunit dynamic within the RyR2 complex has been proposed to play an important role in channel gating (Abramson & Salama, 1989; Orlova et al., 1996), we suggest that inter-subunit cross-linking increases RyR2 activity and SR Ca\(^{2+}\) leak, causing depletion of SR Ca\(^{2+}\) load.

Protein-glutathione mixed disulfides can occur through the interaction between a reactive cysteine within a protein and GSH. This modification can also occur through a thiol/disulfide exchange mechanism in which GSSG reacts directly with a free protein thiol (Tang et al., 2011; Wang et al., 2005; Schafer & Buettner, 2001). In this study, GSSG was used at high concentrations in order to promote thiol/disulfide exchange between free thiols on RyR2 and GSSG. Although the concentration of GSSG is at an extreme range, it is my belief that these findings introduce important new insights into molecular
mechanisms of redox-modification of RyR2. The results of this study reveal a novel mechanism by which oxidative stress can affect RyR2 activity and SR Ca\(^{2+}\) release in ventricular myocytes. With oxidative stress promoting the formation of GSSG, the subsequent oxidation of RyR2 induced the formation of a disulfide bond between two RyR2 subunits. In control conditions, RyR2 was detected only as a single ~560 kDa band. However, after treatment of myocytes with GSSG or diamide, RyR2 can be measured as two separate bands: as a monomer with molecular mass of ~560 kDa and as a dimer >1MDa. The monomeric structure of RyR2 can be restored by DTT, indicating that redox modification of the RyR2 was involved in this effect. Although the exact mechanism that promotes intersubunit cross-linking is unclear, it has been suggested that S-glutathionylation acts as an intermediate for protein disulfide formation (Bass et al., 2004; Cumming et al., 2004; Brennan et al., 2004). Furthermore, the data presented here suggest that mixed disulfide formation between RyR2 and GSH during oxidative stress may facilitate RyR2 inter-subunit cross-linking leading to increased RyR2 activity. It is important to note that S-glutathionylation of RyR2 itself has been shown to be associated with increased channel activity (Bull et al., 2008). The attempt to enzymatically reverse S-glutathionylation of RyR2 potentially induced by the acute application of GSSG showed no change in the rate of decline of [Ca\(^{2+}\)]\(_{\text{SR}}\) (Fig. 9). Furthermore, western blot analysis using a pan anti-GSH Ab could not detect RyR2 S-glutathionylation. It is possible that this technique is not sensitive enough to detect
small changes in S-glutathionylation of RyR2. Altogether, the results suggest that irreversible oxidation in the form of inter-subunit cross-linking is the major contributor of SR Ca\(^{2+}\) leak under our conditions.

Both intra- and inter-subunit interactions have been proposed to play an important role in RyR2 channel gating (Zissimopoulos & Lai, 2007). Therefore, intra- or inter-subunit cross-linking as a result of oxidative stress would potentially affect RyR2 channel activity. Although we cannot rule out intra-subunit cross-linking in our experiments, our results suggest that RyR2 inter-subunit cross-linking plays an important role in increasing cardiac RyR2 activity and augmentation of diastolic SR Ca\(^{2+}\) leak. It was first suggested by Abramson and Salama that disulfide formation between subunits is involved in the channel opening of skeletal RyR1 (Abramson & Salama, 1989). It has recently been shown by Zissimopoulos and colleagues that the cardiac isoform of RyR2 forms an inter-subunit disulfide between N-terminal domains under ambient conditions. However, their studies suggest that N-terminal inter-subunit cross-linking plays an important role in cardiac RyR2 tetramer assembly as well as mediating channel closure. Furthermore, they show that disrupting the inter-subunit interaction between N-terminal domains of RyR2 subunits can promote channel opening (Zissimopoulos et al., 2013). In our experiments, however, cardiac RyR2 inter-subunit cross-linking was not measurable in control conditions suggesting that it is not likely present in the healthy heart. Based on our results, we hypothesize that inter-subunit cross-linking of
RyR2 as a result of oxidative stress promotes abnormal inter-subunit interactions that facilitate channel opening and increased diastolic Ca\(^{2+}\) leak.

**Simplifying RYR2 CICR during Oxidative Stress**

A major limitation to studying the oxidation of RyR2 in cardiomyocytes is the inability to distinguish a direct effect of the channel itself as opposed to an indirect effect by regulatory proteins. The auxiliary proteins that make up the RyR2 complex are likely affected by changes in redox. In order to determine the effects of channel oxidation on SR Ca\(^{2+}\) release, RyR2 must be expressed in a non-cardiac system where any activity from regulatory proteins is considered nominal. In these studies, the HEK293 cell line was used as an expression system for both recombinant RyR2 and SERCA2a. In addition, all experiments were carried out after permeabilization of the plasma membrane so that the intracellular milieu could be tightly controlled. The results from these studies clearly show that SCWs in HEK293 are sensitive to intracellular Ca\(^{2+}\) as well as caffeine, which is highly characteristic of RyR2. Ultimately, this approach allows for a strong argument to be made for the relevance of oxidative PTMs of RyR2 on SR Ca\(^{2+}\) release.

Given the previously acquired results from isolated myocytes, RyR2 intersubunit cross-linking was investigated in the HEK293 expression system. Although recombinant RyR2 formed a functional channel in HEK293, it was not known if the cysteine residues involved in intersubunit cross-linking were accessible to one another. Here, the results
clearly show that diamide induces the same cross-linking effect in recombinantly expressed RyR2 compared to those endogenously expressed in myocytes. These results suggest that the intersubunit folding motifs and domain interactions are likely preserved in recombinantly expressed RyR2. Furthermore, the data suggest that RyR2 intersubunit cross-linking does not require any auxiliary proteins. The functional results corroborate those from ventricular myocytes in that diamide induced cross-linking of RyR2 facilitates Ca\(^{2+}\) release from the ER in HEK293. This is evident with the increased SCW propensity as well as the decreased SCW amplitude over time. Similar to the spark measurements in myocytes, spark amplitude had a tendency to decrease over time in the presence of GSSG. This suggest that the depletion of [Ca\(^{2+}\)]\(_{\text{SR}}\) due to enhanced leak, not compensated by Ca\(^{2+}\) reuptake, causes a subsequent decrease in trans-SR Ca\(^{2+}\) driving force.

The mutagenesis of the cysteine at residue 3602, which has been suggested to be involved in intersubunit cross-linking, did not prove to prevent diamide induced intersubunit cross-linking. Moreover, it did not prevent or mitigate the effect of diamide on SCWs. Even though cysteine 3602 has been shown to undergo oxidative PTMs, the results from these studies suggest its oxidation state holds no bearing on the effects seen with diamide.
Protective Implications of S-Nitrosoylation in Oxidative Stress

S-nitrosoylation has been implicated as an important oxidative PTM that promotes ischemic preconditioning. Targets of S-nitrosoylation include proteins that are involved in mitochondrial metabolism, apoptosis, ROS defense, protein trafficking, myofilament contraction, and Ca\(^{2+}\) handling. Although it has been suggested that S-nitrosoylation can prevent irreversible oxidation of cysteine residues, the ability of S-nitrosoylation to prevent intersubunit cross-linking of RyR2 or increased SR Ca\(^{2+}\) leak was not yet investigated. By pretreating myocytes with the NO donor SNAP before the application of diamide, the steady state [Ca\(^{2+}\)]\(_{\text{SR}}\) is maintained at a higher level compared to that of diamide alone. Although the SNAP concentrations used did not completely prevent a drop in [Ca\(^{2+}\)]\(_{\text{SR}}\), SNAP only partially prevented intersubunit cross-linking induced by diamide. These results suggest that S-nitrosoylation, like NEM, prevents intersubunit cross-linking and the subsequent drop in [Ca\(^{2+}\)]\(_{\text{SR}}\). A major difference between SNAP and NEM is that S-nitrosoylation is readily reversible whereas S-alkylation is irreversible. Therefore, SNAP likely promotes a weaker protective effect against further oxidation than that of NEM.

In the HEK293 studies, SNAP pretreatment proved to prevent the increase in SCW propensity as well as the decrease in SCW amplitude seen with diamide application. These results corroborate with the steady state [Ca\(^{2+}\)]\(_{\text{SR}}\) studies done in myocytes, which provides more evidence of the protective effect of S-nitrosoylation.
against detrimental Ca\(^{2+}\) leak induced by oxidative stress. Previous publications have implicated deficient S-nitrosylation in abnormal SR Ca\(^{2+}\) handling (Gonzalez et al., 2010). Therefore, S-nitrosylation of RyR2 is likely an essential PTM to protect against oxidative stress and subsequent SR Ca\(^{2+}\) leak. By maintaining SR Ca\(^{2+}\) load and preventing [Ca\(^{2+}\)]\(_i\) overload, I propose that S-nitrosylation plays a very important role in cardiac function during periods of oxidative stress.

**Oxidative Stress in Ischemia-Reperfusion Injury and Heart Failure**

The magnitude of oxidative stress in cardiovascular disease has a large bearing on the overall welfare of myocardium. It is well established that severe oxidative stress is a major stimuli that leads to necrosis (Ryter et al., 2007; Hanus et al., 2013). Furthermore, [Ca\(^{2+}\)]\(_i\) overload and subsequent mitochondrial Ca\(^{2+}\) overload will in turn activate necrotic signaling pathways as well as promote the opening of the mitochondrial permeability transition pore; the latter causing mitochondrial rupture and subsequent cell death (Halestrap et al., 2004). Since intersubunit cross-linking of RyR2 induces a severe form of SR Ca\(^{2+}\) leak, it is likely that this oxidative PTM has a role in promoting [Ca\(^{2+}\)]\(_i\) overload and necrosis. Also, intersubunit cross-linking of RyR2 likely sets the stage for triggered activity and the genesis of ectopic foci.

The presented studies provide evidence for RyR2 intersubunit cross-linking in I/R and HF. However, these results were only observable in whole tissue isolations. While RyR2 intersubunit cross-linking was undetectable in isolated myocytes from I/R or HF,
increased oxidation of RyR2 was evident. Furthermore, I/R and HF myocytes exhibited increased SR Ca\textsuperscript{2+} fractional release and leak with no detectable RyR2 intersubunit cross-linking suggesting other PTMs are likely responsible. These results suggest that isolated myocytes (intact and viable after enzymatic separation) from I/R and failing hearts have survived due to the fact that they have not undergone the degree of oxidative stress capable of inducing RyR2 intersubunit cross-linking.
CHAPTER VII: CONCLUSION

In conclusion, the data presented suggests that protein-glutathione mixed disulfides play an important role in facilitating abnormal Ca$^{2+}$ cycling during oxidative stress in the heart. In this study, we introduce a new mechanism for increased RyR-mediated Ca$^{2+}$ leak from the SR: Oxidative stress promotes an increase in overall glutathione mixed disulfides in turn facilitating intermolecular cross-linking of RyR2 subunits and a subsequent increase in channel activity that promotes SR Ca$^{2+}$ leak and fractional release. The data presented in this study implicates intersubunit cross-linking as an important mechanism for the regulation of RyR2 channel activity and steady state [Ca$^{2+}$]$_{SR}$. Preventing RyR2 intersubunit cross-linking has the potential to act as a beneficial therapy in the treatment of I/R injury and HF.
BIBLIOGRAPHY

Reference List


Heijman J, Wehrens XH, & Dobrev D (2013). Atrial arrhythmogenesis in catecholaminergic polymorphic ventricular tachycardia--is there a mechanistic link between sarcoplasmic reticulum Ca(2+) leak and re-entry? Acta Physiol (Oxf) 207, 208-211.


for in vivo oxidant stress in ventricular dilatation and progression to heart failure. *Circulation* 97, 1536-1539.


Periasamy M & Huke S (2001). SERCA pump level is a critical determinant of Ca(2+)homeostasis and cardiac contractility. *J Mol Cell Cardiol* 33, 1053-1063.


VITA

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In August of 2009, Stefan joined the Department of Cell and Molecular Physiology at Loyola University Medical Center (Maywood, IL). Shortly thereafter, he joined the laboratory of Dr. Aleksey V. Zima, where he studied mechanisms of oxidative stress, calcium regulation, and arrhythmogenesis in rabbit hearts, focusing specifically on oxidative post translational modifications of the Ryanodine Receptor.