

Electronic Thesis and Dissertation Repository

---

2-15-2017 12:00 AM

## Up-regulation of junctophilin-2 prevents ER stress and apoptosis in hypoxia/reoxygenation-stimulated H9c2 cells

Zijun Su, *The University of Western Ontario*

Supervisor: Dr.Tianqing Peng, *The University of Western Ontario*

Joint Supervisor: Dr.Stephen Sims, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology

© Zijun Su 2017

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>



Part of the [Cardiovascular Diseases Commons](#)

---

### Recommended Citation

Su, Zijun, "Up-regulation of junctophilin-2 prevents ER stress and apoptosis in hypoxia/reoxygenation-stimulated H9c2 cells" (2017). *Electronic Thesis and Dissertation Repository*. 4433.  
<https://ir.lib.uwo.ca/etd/4433>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact [wlsadmin@uwo.ca](mailto:wlsadmin@uwo.ca).

## Abstract

Ischemic heart disease is the leading cause of death, and reperfusion which can restore blood flow is the primary therapy. However, reperfusion can induce further damage to cardiomyocytes, a condition described as ischemia-reperfusion (I/R) injury. I/R is now recognized as a combination determining the final myocardial infarction size. Although the mechanisms underlying I/R-induced cardiac injury remain incompletely understood, emerging evidence suggests that intracellular  $\text{Ca}^{2+}$  mishandling during I/R plays a key role in cell death. Junctophilin-2 (JPH2) is a junctional membrane-binding structural protein. It mechanically maintains the fixed distance between the T-tubule and the sarcoplasmic reticulum (SR), thus allowing the proper  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release for stable excitation-contraction coupling. Down-regulation of JPH2 has been observed in diseased hearts and is related to cardiac dysfunction and T-tubule remodeling. In this study, we show that the protein levels of JPH2 are down-regulated in cardiomyocytes following hypoxia/reoxygenation (H/R), a condition simulating I/R. Up-regulation of JPH2 protects cardiomyocytes against H/R-induced apoptotic cell death. Furthermore, we reveal that up-regulation of JPH2 reduces ryanodine receptor-2 (RyR2)-mediated SR  $\text{Ca}^{2+}$  leak and inhibits calcium-dependent calpain activation in H/R-stimulated cardiomyocytes. Lastly, up-regulation of JPH2 prevents endoplasmic reticulum stress in response to H/R. In summary, we demonstrate for the first time that JPH2 prevents H/R-induced apoptosis by blocking  $\text{Ca}^{2+}$  leakage via RyR2 in cardiomyocytes. Thus, up-regulating JPH2 may represent a new therapeutic strategy to treat ischemic heart disease.

## Keywords

Cardiomyocytes; Junctophilin-2; RyR2; Calcium; Calpain; ER stress; JNK1/2; Apoptosis; hypoxia/reoxygenation

## Dedication

To my mom and my dad, who love me the most and give me the best.

## Acknowledgments

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Tianqing Peng, for providing me the opportunity to pursue my master's degree at Western University. I have been extremely lucky to have a supervisor who cared so much about my work. Thank you for all your important advice to apply not only within the lab but also in life. This thesis could not have been completed without your significant patience and continued guidance.

I would like to extend my sincere gratitude to my co-supervisor, Dr. Stephen Sims, who facilitated my research and helped me all the way. Your valuable suggestions, constructive criticism, and precious encouragement inspired me to get through tough times during my project.

Besides my mentors, I would like to acknowledge my advisory committee, Dr. Martin L. Duennwald and Dr. Xiufen Zheng. I always felt like you were truly interested in and cared about my work and that you also wanted me to succeed. This meant a lot, and I greatly appreciate it. I would also like to thank the rest of the examination committee, Dr. Rui Tao and Dr. Patrick Lajoie, for their insight, feedback, and recommendations.

My colleagues helped tremendously throughout my thesis. Particularly, I would like to acknowledge Dr. Dong Zheng whose expertise and support helped me in learning experiment techniques, and without whose help my work would not be done. Rui. Ni, Shuai Li, and Yifan Huang also offered me endless support and treated me like their little brother, which will never be forgotten. Special thanks are extended to Ryan Beach and Brandon Kim for their immeasurable help in the calcium experiment. Also, thank you for your friendship; I am so glad to work with you guys.

Last but not the least, I would like to acknowledge the Department of Pathology and Laboratory Medicine. Tracy and Susan, I deeply appreciate your extensive work to make the examination happen. I am indebted to you for all your patient help. Dr. Chandan Chakraborty, participating in your journal club courses not only expanded my horizon but also enlightened my thesis. Your consistent care shaped me to adapt to the graduate study better, and your strict requirements pushed me to become an above-and-beyond researcher. I

will always remember your inculcation, especially the words "Learning English should be a life-long exercise."

# Table of Contents

|   |      |
|---|------|
| Abstract .....  | i    |
| Dedication .....  | ii   |
| Acknowledgments.....  | iii  |
| Table of Contents .....   | v    |
| List of Tables .....  | viii |
| List of Figures .....   | ix   |
| Chapter 1 .....   | 1    |
| 1 Introduction .....  | 1    |
| 1.1 Myocardial ischemia/reperfusion injury .....                      | 1    |
| 1.2 Ca <sup>2+</sup> mediated cell death in ischemia-reperfusion..... | 3    |
| 1.2.1 Ca <sup>2+</sup> : a universal second messenger.....            | 3    |
| 1.2.2 Ca <sup>2+</sup> signaling in cardiomyocytes .....              | 4    |
| 1.2.3 Ca <sup>2+</sup> homeostasis in cardiomyocytes .....            | 5    |
| 1.2.4 Ca <sup>2+</sup> overload after ischemia/reperfusion .....      | 7    |
| 1.3 Junctophilin-2 .....  | 8    |
| 1.3.1 The biophysiology of JPH2 .....                                 | 8    |
| 1.3.2 JPH2 and heart diseases .....                                   | 11   |
| 1.4 ER stress and ischemia/reperfusion injury.....                    | 13   |
| 1.5 Calpain/calpastatin system in ischemic heart .....                | 16   |
| 1.6 Rationale .....   | 19   |
| 1.7 Hypothesis.....   | 20   |
| Chapter 2.....  | 21   |
| 2 Materials and Methods.....  | 21   |
| 2.1 Cell culture.....   | 21   |
| 2.2 Neonatal mouse cardiomyocyte isolation .....                      | 21   |

|           |   |    |
|-----------|---|----|
| 2.3       | Adenoviral infection .....  | 22 |
| 2.4       | Hypoxia/reoxygenation (H/R) .....   | 22 |
| 2.5       | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis .....         | 23 |
| 2.6       | Caspase-3 activity measurement.....   | 25 |
| 2.7       | Cellular DNA fragmentation.....   | 26 |
| 2.8       | Calpain activity .....  | 26 |
| 2.9       | Single cell calcium photometry and SR leak .....  | 27 |
| 2.10      | Statistical Analysis.....   | 27 |
| Chapter 3 | .....   | 28 |
| 3         | Results .....   | 28 |
| 3.1       | Establishment of an in vitro model of Hypoxia/Reoxygenation injury .....                                      | 28 |
| 3.2       | H/R down-regulated JPH2 protein expression in cardiomyocytes .....  | 31 |
| 3.3       | Adenoviral vector mediated up-regulation of JPH2 expression in heart cells.....                               | 33 |
| 3.4       | Up-regulation of JPH2 prevented apoptosis induced by H/R.....   | 35 |
| 3.5       | JPH2 regulated RyR2-mediated aberrant Ca <sup>2+</sup> release from SR in H/R-stimulated cardiomyocytes. .... | 37 |
| 3.6       | Blockage of RyR2 reduced apoptosis induced by H/R.....  | 43 |
| 3.7       | Up-regulation of JPH2 prevented calpain activation in H/R-stimulated cardiomyocytes. ....                     | 45 |
| 3.8       | Up-regulation of JPH2 attenuated ER stress in H/R-stimulated cardiomyocytes.                                  | 49 |
| Chapter 4 | .....   | 51 |
| 4         | Discussion, Limitation & Future direction .....   | 51 |
| 4.1       | Discussion.....   | 51 |
| 4.1.1     | JPH2 downregulation and its contribution to apoptosis in H/R injury .....                                     | 51 |
| 4.1.2     | Decreased JPH2 increases aberrant SR Ca <sup>2+</sup> release via RyR2, which contributes to H/R injury ..... | 52 |

|                  |  |    |
|------------------|--|----|
| 4.1.3            | Up-regulation of JPH2 prevents Calpain activation in H/R-stimulated cardiomyocytes ..... | 53 |
| 4.1.4            | Up-regulation of JPH2 attenuates the induction of ER stress.....                         | 54 |
| 4.1.5            | Concluding remarks .....   | 55 |
| 4.2              | Limitations and future direction.....  | 56 |
| Chapter 5        | .....  | 58 |
| 5                | References .....   | 58 |
| Curriculum Vitae | .....  | 70 |

## List of Tables

|   |    |
|---|----|
| Table 1:Composition of separating gel .....                                       | 23 |
| Table 2: Composition of stacking gel .....  | 24 |
| Table 3: Composition of loading buffer.....                                       | 24 |
| Table 4: O <sub>2</sub> concentration in different time points after hypoxia..... | 28 |

## List of Figures

|  |    |
|--|----|
| Figure 1: Ca <sup>2+</sup> cycling in cardiomyocyte, cited from[43] .....  | 5  |
| Figure 2: Ca <sup>2+</sup> overload after ischemia, cited from [65] .....  | 8  |
| Figure 3: Ca <sup>2+</sup> leakage through RyR2, cited from [77] .....   | 11 |
| Figure 4: Assessments of apoptosis on H9c2 cells following H/R.....  | 30 |
| Figure 5: Time course of JPH2 protein expression following H/R in H9c2 cells .....                               | 32 |
| Figure 6: JPH2 protein expression in Ad-JPH2 infected H9c2 cells .....   | 34 |
| Figure 7: Effect of JPH2 up-regulation on apoptosis in H9c2 cells after H/R .....                                | 36 |
| Figure 8: Measurement of SR Ca <sup>2+</sup> release in H9c2 cells following H/R.....                            | 39 |
| Figure 9: Effects of JPH2 up-regulation on aberrant SR Ca <sup>2+</sup> release in H9c2 cells following H/R..... | 42 |
| Figure 10: Effect of dantrolene on apoptosis in H9c2 cells following H/R.....                                    | 44 |
| Figure 11: Measurement of calpain activation in H9c2 cells following H/R .....                                   | 46 |
| Figure 12: Effects of JPH2 up-regulation on calpain activation in H9c2 cells following H/R .....                 | 48 |
| Figure 13: Effect of JPH2 over-expression on ER stress in primary neonatal cardiomyocytes following H/R .....    | 50 |
| Figure 14: Summary of the findings .....   | 56 |

## Chapter 1

### 1 Introduction

#### 1.1 Myocardial ischemia/reperfusion injury

Cardiovascular disease (CVD) is the leading cause of death globally. It kills more than 17.3 million people per year, and the number is predicted to climb to 23.6 million by 2030 [1]. In Canada, 1.6 million people are suffering from CVD, and more than 66,000 Canadians die from it each year, nearly one death every seven minutes [2]. Ischemic heart disease (IHD), also known as coronary artery disease (CAD), is the most common and deadly type of CVD, accounting for 46% of cardiovascular deaths in men and 38% in women [3].

Most IHDs are related to coronary atherosclerosis, a formation of plaque involving intense inflammatory and immunological developments on the artery wall [4]. A narrowed coronary artery cuts down the blood flow to supply myocardial oxygen and nutrients, which decreases ATP generation and causes myocardial injury [5]. The most severe manifestation is when the atherosclerotic plaque ruptures and completely occludes the coronary artery, a condition called myocardial infarction [6]. A short period after oxygen deprivation (ischemia), anaerobic glycolysis engages and substitutes oxidative phosphorylation as the significant source of ATP production to maintain the basic energy demand in the myocardium [7]. Cardiac contraction is abated to reduce energy demand in the same way as the ATP-consuming biosynthetic processes including gluconeogenesis and lipid and protein synthesis. However, if the ischemic event continues, the glycolytic pathway is unable to remedy ATP depletion and insufficient to maintain the minimal cellular metabolism [8]. The entire cardiomyocyte becomes swollen due to the influx of sodium and water caused by the failure of the ATP sodium pump, leading to cytoskeleton dispersion, a dilated endoplasmic reticulum, and the formation of "blebs" at the cell surface [9]. Even at this point, the cellular derangement is still repairable if the hypoxia can be reversed. If ischemia duration further extends, intensely disturbed intracellular ions, especially impairment of  $\text{Ca}^{2+}$  homeostasis, activates membrane-bound endogenous

phospholipase and consequently accelerates the degradation of membrane phospholipids [10]. As a result, plasma and organelle membranes are dysfunctional. Once the cells lose the permeability barrier, the cellular damage becomes irreversible, and necrosis ensues [11]. Accordingly, the crucial step to rescue the myocardium from lethal cell injury is to remove the ischemic condition when the damage remains reversible. Studies of reperfusion were initiated 40 years ago by Marko et al. [12] and Ginks et al. [13], and it has been proven that therapies to restore blood flow in ischemic myocardium salvage reversibly injured cardiomyocytes from necrosis. Since then, reperfusion strategies like thrombolysis, intervention, and bypass surgery have been widely incorporated into clinical practice during the last three decades [14]. This therapy limits the extent of infarct size, preserves the cardiac contraction function, and prevents the onset of heart failure, which all ultimately lead to a significant reduction in the acute mortality after myocardial infarction [15]. However, as a result of a decrease in mortality rate for acute myocardial infarction due to improved reperfusion therapies, there is an increase in the incidence of heart failure after myocardial infarction [16]. Post-myocardial infarction heart failure is now the most rapidly rising cardiovascular condition to affect the lives of Canadians. Over 500,000 Canadians are afflicted with heart failure, and up to 50% of them die within five years of diagnosis [17]. Thus, myocardial infarction and subsequent heart failure is a tremendous personal struggle for Canadians and a significant financial burden for our health care system [18].

In fact, studies have shown that reperfusion itself induces further disruptions and additional injuries secondary to the ischemic damage, a condition described as “ischemia/reperfusion (I/R) injury” [19]. I/R injury not only reduces the benefits of blood flow restoration but also converts a population of cardiomyocytes from reversible to irreversible injury [4, 5]. The four most recognized forms of I/R injury include arrhythmia, myocardial stunning, microvascular obstruction, and lethal myocardial injury [20]. Paradoxically, although reperfusion could damage the myocardium, which may cause heart failure later, until now, there has been no better solution than this strategy to rescue the severe acute ischemic heart from a total dysfunction. Therefore, the clinical research has shifted from solely ischemic damage to I/R injury together as the important combination contributing to the final myocardial infarct size [21].

For many years, necrosis was the predominant form of I/R-induced cell death [22]. This random, uncontrolled sudden cell death happens to those already-damaged ischemic cells in the initial minutes after reperfusion in response to the overwhelming stress [23]. Recent studies have revealed that apoptosis is not only involved in the I/R injury but also has a significant contribution to the IR injury [24]. Unlike necrosis, this programmed, energy-consuming form of cell death happens to those peripheral ischemic cells that suffer lower damage comparing to the central ischemic cells and survive the initial minutes of reperfusion [25]. These cells can be rescued since apoptotic progression requires time and can be regulated. Therefore, prevention of late apoptosis in prolonged reperfusion has attracted more attention as a therapeutic strategy to reduce IR injury after myocardial infarction [8, 25, 26]. Although the mechanisms of I/R-induced cell death (necrosis and apoptosis) have not been completely understood, ample evidence suggests that calcium overload and altered calcium handling is one of the major mediators initiating disruption and lethal cell injury.

## 1.2 $\text{Ca}^{2+}$ mediated cell death in ischemia-reperfusion

### 1.2.1 $\text{Ca}^{2+}$ : a universal second messenger

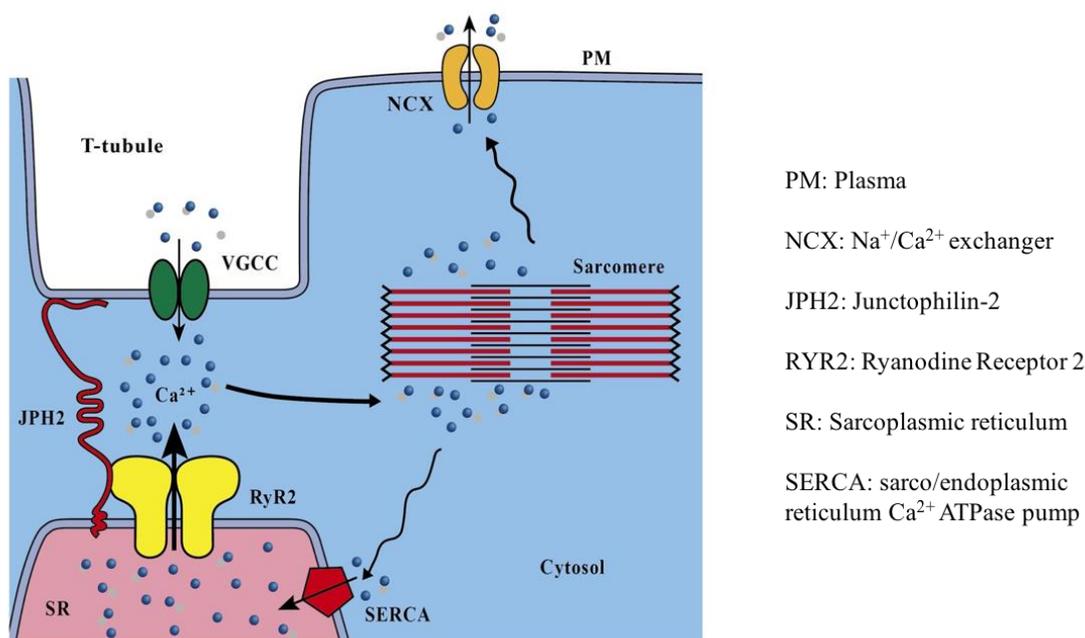
In the long-time river of evolution, the selection of  $\text{Ca}^{2+}$  as the universal carrier of signals had been the hallmark of the transition from the unicellular to the multicellular life form [27, 28]. The exchange of intercellular signals allows a higher level of functional interplay and coordination among the cells of the organisms other than just the competition of nutrients [27]. However, extracellular-secreted signal molecules are complex and mostly act as a first messenger that binds to the receptors on the target cell membrane and may not cross it. Thus, it requires the second messenger to interact and transmit the signals within the cell [29]. The  $\text{Ca}^{2+}$ , given its chemical properties, is one of the perfect molecules to accommodate the binding signals. Unlike other active-site metals that directly participate in the enzyme catalysis,  $\text{Ca}^{2+}$  is an allosteric metal and binds to the sites that are different from the active site [30]. This character makes  $\text{Ca}^{2+}$  a better modulator to enzyme proteins since it has the capacity not only to activate but also to inhibit them [31]. Moreover,  $\text{Ca}^{2+}$  can also be controlled tightly, based on its characteristics: binding it reversibly, with particular concentration and appropriate

affinity to cellular proteins [32]. For these reasons,  $\text{Ca}^{2+}$  has been selected as a universal intracellular messenger that regulates numerous general biological processes to all cells, such as gene transcription, metabolic pathways, differentiation, and cell growth or death. It also regulates other cell-specific processes like neural regulation and muscle contraction [33].

### 1.2.2 $\text{Ca}^{2+}$ signaling in cardiomyocytes

Normal cardiac function and myofilament contraction rely on  $\text{Ca}^{2+}$  signaling which transfers the depolarizing current to the contractile force of each sarcomere [34]. In every cardiac cycle, a depolarizing action potential is derived from the sinoatrial (SA) node. This current spreads through the right to the left atrium and pauses for a 0.1-s period (in humans) when arriving the atrioventricular (AV) node, to complete the atrial systole. After the short stop, the depolarization spreads through the Purkinje fibers to the left right ventricular separately to finish the whole cardiac contraction cycle [35]. When depolarizing action current reaches the T-tubules, it triggers the L-type  $\text{Ca}^{2+}$  channels (LTCCs) to release small flows of  $\text{Ca}^{2+}$  (Con < 10  $\mu\text{M}$ , or “sparklet”) into the gap called dyad cleft between the cell membrane and the sarcoplasmic reticulum(SR)/endoplasmic reticulum(ER) [36]. The “sparklet  $\text{Ca}^{2+}$ ” induces the opening of ryanodine receptors 2(RyR2) which is plasmalemma-situated on the SR. It consequently mobilizes a large  $\text{Ca}^{2+}$  flow (Con >100  $\mu\text{M}$ ) releasing into the dyad cleft as known as “ $\text{Ca}^{2+}$  spark”, a phenomena described as “ $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) or  $\text{Ca}^{2+}$  coupling” [37]. During a single action potential, thousands of RyR2 sites are simultaneously activated by their corresponding L-type VOCCs provided “ $\text{Ca}^{2+}$ sparklet” in dyad cleft. This process produces an average global  $\text{Ca}^{2+}$  increase of 500 nM to  $\sim 1 \mu\text{M}$  [38]. This amount of  $\text{Ca}^{2+}$  subsequently engages the  $\text{Ca}^{2+}$ -binding component, troponin (TnC), which is sensitive over that range. It triggers the filaments shorting to provide the contractile force for pumping blood [39]. In the end, these unique junctional membrane complexes (JMCs) successfully transport an action current to create a synchronic sarcomere contraction of each article and ventricle. After triggering the contractile units,  $\text{Ca}^{2+}$  dissociates from the TnC and rapidly removes from the cytosol to prepare for the next round of CICR [40]. The efflux of dissociated  $\text{Ca}^{2+}$  is mainly taken back to the SR by sarco/endoplasmic

reticulum  $\text{Ca}^{2+}$  ATPase (SERCA2a) pump [41] and goes through extrusion by the cell membrane  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) [42].



**Figure 1:  $\text{Ca}^{2+}$  cycling in cardiomyocyte, cited from [43]**

### 1.2.3 $\text{Ca}^{2+}$ homeostasis in cardiomyocytes

Considering  $\text{Ca}^{2+}$  is imperative in the excitation-contraction (EC) coupling of each cardiomyocyte, it is of utmost importance to maintain a low intracellular diastole  $\text{Ca}^{2+}$  concentration, which guarantees a simultaneous sarcomere contraction in the next cardiac beating cycle [27]. Besides, the  $\text{Ca}^{2+}$  carrying signals modulate the enzyme process and other essential biochemical activities through its precisely controlled elevation in the cytosol and subcellular compartments [30]. So, after the transit elevation demanded by the contraction,  $\text{Ca}^{2+}$  concentration should return to the low/intermediate nM range. A protracted  $\text{Ca}^{2+}$  increase can trigger abnormal activities that the cells cannot tolerate [44]. In cytosol, the concentration of free  $\text{Ca}^{2+}$  is normally maintained to be as low as at 100 to 200 nM. SR luminal  $\text{Ca}^{2+}$  is one of the main sources, and it can raise the cytosol  $\text{Ca}^{2+}$  concentration to over 100  $\mu\text{M}$  during the CICR, as mentioned above [37]. Extracellular  $\text{Ca}^{2+}$  is another important source, in which the  $\text{Ca}^{2+}$  concentration fixed at 3mM, with

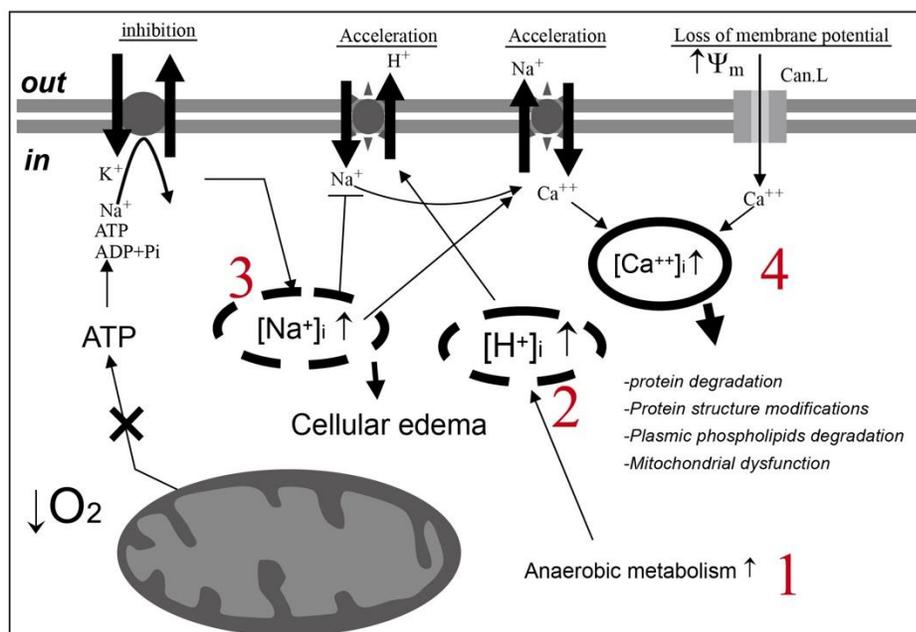
approximately half being ionized [45]. The luminal SR deposit ensured the fast reaction of mobilizable  $\text{Ca}^{2+}$ , which contributes to the sarcomere contraction, along with the high-concentration extracellular pool that creates a large gradient and electrochemical force on  $\text{Ca}^{2+}$  to entering the cell conveniently as a second messenger and modulator [39]. The transport of  $\text{Ca}^{2+}$  across membranes is precisely controlled by a set of different components in cardiomyocytes, including channels (L-type  $\text{Ca}^{2+}$  channels and RyR2 receptors), ATPases (sarco/endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase), and exchangers ( $\text{Na}^+/\text{Ca}^{2+}$  exchanger), all of which are directly engaged in the process of CICR [34]. Other components, such as store-operated  $\text{Ca}^{2+}$  entry channels (SOCEs), inositol-(1,4,5)-trisphosphate receptors (InsP3R), and plasma membrane  $\text{Ca}^{2+}$  ATPase (PMCA) also participate in cytosol  $\text{Ca}^{2+}$  regulation and stabilization [46]. Furthermore,  $\text{Ca}^{2+}$ -binding proteins are another crucial system maintaining intracellular  $\text{Ca}^{2+}$  homeostasis. Some of them are pure buffering proteins, like calsequestrin, which are contained in SR and determine the calcium storage [47]. Calsequestrin also coordinates with RyR2 for regulating  $\text{Ca}^{2+}$  release during the process of EC coupling [48]. Others named  $\text{Ca}^{2+}$  sensors, like calmodulin (CaM), not only regulate the  $\text{Ca}^{2+}$  concentration but also process and decode the  $\text{Ca}^{2+}$  signal. CaM mediates the action of cyclic ADP-ribose (cADPR), which acts as an endogenous regulator of CICR in cardiomyocytes [49]. Using immunoblot assays, Lee, Aarhus, and Graeff (1995) found that CaM was responsible for conferring the sea urchins eggs an increased sensitivity to cADPR, thus playing a critical role in calcium homeostasis [50].

Thanks to this strong adjusting and buffering system,  $\text{Ca}^{2+}$  is tightly regulated in cardiomyocytes. However, under some conditions of overwhelming stress and pathogenesis,  $\text{Ca}^{2+}$  could lose its homeostasis, and the most common situation is the cytosol could not maintain the low basal concentration [51]. The first impact of the  $\text{Ca}^{2+}$  dysregulation would be the contraction process. Termination of CICR is largely dependent on the depletion of calcium. Thus, if  $\text{Ca}^{2+}$  is not removed, CICR will remain at peak levels, and cardiac relaxation will not be achieved [52]. Apart from being a cellular powerhouse, mitochondrial ability to handle elevated cytosolic  $\text{Ca}^{2+}$  is critical to cellular processes and survival [53]. However, the excessive influx of  $\text{Ca}^{2+}$  could impair the ATP synthesis and production [54]. Endoplasmic reticulum (ER) is the major  $\text{Ca}^{2+}$  storage and

is sensitive to the  $\text{Ca}^{2+}$  environment changes [55]. An altered  $\text{Ca}^{2+}$  level would render the ER incapable of supporting cellular functions, such as assembly and folding proteins [56]. The impairment of calcium regulation would also induce over-activation of some enzymes or proteases, such as calpain, which causes further disruption and damage to the cells [57]. A disturbance in calcium homeostasis is, therefore, important in pathological development.

#### 1.2.4 $\text{Ca}^{2+}$ overload after ischemia/reperfusion

When ischemia occurs, intracellular pH declines progressively due to increased  $\text{H}^+$  that is released by anaerobic glycolysis. The increased  $\text{H}^+$  activates the primary extrusion mechanisms of the  $\text{Na}^+/\text{H}^+$ -exchanger (NHE) and the  $\text{Na}^+-\text{HCO}_3^-$  cotransporters [58]. The fast  $\text{Na}^+$  influx and the inhibited  $\text{Na}^+/\text{K}^+-\text{ATPase}$ , because of the insufficient ATP production, both contribute to the cytosolic  $\text{Na}^+$  overload [59]. After the rise of  $\text{Na}^+$ , the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) is converted into its reverse mode, which extrudes the  $\text{Na}^+$  but brings in the  $\text{Ca}^{2+}$  [60]. The low ATP concentration also impaired the sarcoplasmic reticulum  $\text{Ca}^{2+}-\text{ATPase}$  (SERCA), which further develops the intracellular  $\text{Ca}^{2+}$  substantial accumulation and overload [61] [Figure 2]. At the onset of reperfusion, extracellular  $\text{H}^+$  is quickly removed by the restored blood flow [62]. The accelerated  $\text{H}^+$  efflux, through the reactivated NHE and  $\text{Na}^+-\text{HCO}_3^-$  cotransporters, corrects the intracellular pH but also aggravates  $\text{Na}^+$  overload and the consequent  $\text{Ca}^{2+}$  overload [59]. The exacerbated  $\text{Ca}^{2+}$  overload causes lethal cell injury, mainly through hypercontraction, calpain-mediated proteolysis, and mitochondrial permeability transition [63]. These injuries have been demonstrated to cause necrotic cell death shortly after the reperfusion in cardiomyocytes close to the ischemic artery. However, the mechanism of late apoptotic cell death in surviving cardiomyocytes remains incompletely understood. These cardiomyocytes stay farther from the ischemic artery and suffer minor  $\text{Ca}^{2+}$  dysregulation [59, 62, 64]. The reason why they still undergo apoptosis later after I/R requires further investigation.



**Figure 2:  $\text{Ca}^{2+}$  overload after ischemia, cited from [65]**

## 1.3 Junctophilin-2

### 1.3.1 The biophysiology of JPH2

In the year 2000, junctophilin (JPH) was first discovered in rabbit skeletal muscle and defined as a crucial member of junctional membrane complexes (JMCs) [66]. Four different isoforms are found in the JPH family (JPH 1-4), in which JPH1 is mainly expressed in skeletal muscle, and JPH3 and JPH4 are widely expressed in the neurons and brain [67, 68]. Junctophilin-2 (JPH2) is the predominant cardiac isoforms. It is encoded by the JPH2 gene, which incorporates five coding exons at 20q13.12 [69]. The GO annotations associated with the JPH2 gene consist of phosphatidylinositol-4 and phosphatidylinositol-3-phosphate binding, as well as 5-bisphosphate binding [70]. Substitute splicing has been experienced at this locus and two variations coding different isoforms are defined [71]. In human cardiomyocytes, the JPH2 gene encodes a 74kDa protein JPH2 with 696 amino acids [72]. It has eight times repeated MORN motifs (14 amino acid residues) in the N-terminal domain, which binds to the plasma membrane or

sarcolemma (T-tubules). At the opposite side, its C-terminal transmembrane segment spans the SR/ER membrane. In a putative  $\alpha$ -helical region (~100 amino acids residues) forms the structural body basis for this bridging protein [66, 68, 70].

### 1.3.1.1 Role of JPH2 in JMCs construction

Junctional membrane complexes (JMCs), as mentioned above, were first observed in the mid-'50s by Porter and Palade [73]. They are found in all kinds of excitable cells [74, 75]. This unique channel's cross-talk structure is necessary for E-C coupling [52, 76]. It has been demonstrated that JPH2 is the key anchoring molecule, which mechanically maintains the fixed distance between the T-tubule and sarcoplasmic reticulum (SR) [43, 77]. The importance of JPH2 for JMCs in hearts was first discovered by Takeshima et al. in JPH2 knockout mice, which exhibited a defective coupling area with randomly irregular  $\text{Ca}^{2+}$  transients. Due to the contractile disability, these mice were embryonically lethal at E10.5 [66]. Wehrens's group, using inducible cardiac-specific JPH2 knockdown mice, confirmed the reduction of JMCs and further demonstrated a causal link between loss of JPH2 and defective CICR within JMCs. They crossed shJPH2 mice with  $\alpha$ -myosin heavy chain ( $\alpha$ MHC)-MerCreMer (MCM) mice to ensure cardiac-specific shRNA expression. MCM-shJPH2 mice with impaired EC coupling also developed acute contractile and heart failure [78]. Together, these studies show that JPH2 is an essential component that reinforces the structural stability of the JMCs formation, ensuring the CICR for stable EC coupling.

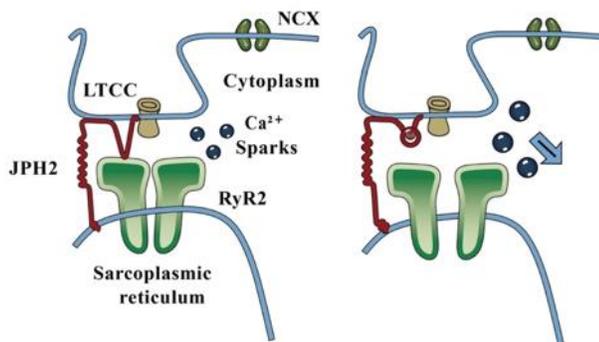
### 1.3.1.2 Role of JPH2 in T-tubule development

Transverse tubules (T-tubules) are physical extensions of the surface sarcolemma, which invaginate deep into the interior ventricular myocytes and along with the Z-line [79]. It transports the depolarizing current to  $\text{Ca}^{2+}$  signals via L-type  $\text{Ca}^{2+}$  channels (LTCCs), which initiate the process of CICR and excite the cardiomyocytes for contraction [80]. In neonatal mammalian cardiomyocytes, T-tubules are absent, and the EC coupling was conducted by direct  $\text{Ca}^{2+}$  influx through promoted sodium-calcium exchanger (NCX) at the periphery of the cell [81]. During the early postnatal stage, the development of the T-tubule network is one of the hallmarks of myocytes maturation, and JPH2 plays a critical

role in this process [82]. Studies from Chen et al. [83] and Reynolds et al. [84] both demonstrated that cardiac-specific silencing of JPH2 significantly reduced T-tubule organization in the heart during the postnatal stage. In contrast, overexpression of JPH2 accelerated T-tubule maturation by postnatal day 8 [84]. It is highly likely that JPH2 can anchor the invaginating sarcolemma to the SR, thereby triggering the development of mature T-tubules, which allows full EC-coupling for maximized contraction capacity.

### 1.3.1.3 Role of JPH2 in RyR2 stabilization

Ryanodine receptor 2 (RyR2) is a crucial component of JMCs in cardiomyocytes. It acts as the major SR/ER-stored calcium release channel, which directly participates in CICR, in charge of SR  $\text{Ca}^{2+}$  mobilization for myofilament contraction [76]. Emerging evidence suggests that JPH2 has a functional regulatory role in RyR2 stability in addition to the JMC structural integrity. Immunoprecipitation targeting RyR2 from mouse heart tissues "pulled down" JPH2, suggesting a potential direct interaction between RyR2 and JPH2 [85]. Recent super-resolution imaging studies of immunofluorescence staining on rat ventricle tissues confirmed a strong molecular-scale co-localization between JPH2 and RyR2, indicating a direct interaction between them [86, 87]. Studies on adult-onset JPH2 knockdown mice showed abnormal  $\text{Ca}^{2+}$  release through RyR2 and decreased EC-coupling gain, supporting a regulatory role of JPH2 in RyR2 [88]. Furthermore, Calcium imaging and single channel RyR2 recording studies in ventricular-isolated cardiomyocytes from JPH2 knockdown mice indicated that JPH2 was a negative regulator of RyR2-gating. The loss of JPH2 resulted in aberrant  $\text{Ca}^{2+}$  leakage from SR/ER through RyR2 [89] [Figure 3]. These data strongly support a new role of JPH2 in regulating RyR2 function in cardiomyocytes. However, its pathophysiological significance remains to be determined in the heart.



**Figure 3: Ca<sup>2+</sup> leakage through RyR2, cited from [77]**

### 1.3.2 JPH2 and heart diseases

#### 1.3.2.1 Mutations of JPH2 associated with hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a cross-age genetic disorder which enlarges a portion of myocardium without external stimulations [90]. It is the leading cause of sudden cardiac death of young athlete and mostly asymptomatic beforehand [91, 92]. The study from Matsuoka's group on murine genetic models first implicated JPH2 dysregulation to HCM and dilated cardiomyopathy [93]. This finding was supported by a clinical study where down-regulated JPH2 was observed in surgically resected myocardium from human HCM patients [94]. This study also looked into whether loss of JPH2 was sufficient to initiate the myocardial hypertrophy. JPH2 acute suppression in HL-1 cells increased the cellular size and induced hypertrophic process with up-expression of several transcriptional markers such as atrial natriuretic factor, brain natriuretic factor, myosin heavy chain, and skeletal actin. The JPH2 expression silencing also reduced the transient amplitude and altered Ca<sup>2+</sup> homeostasis [94]. Based on these findings, several studies have been conducted to identify whether JPH2 gene mutation contributes to the HCM. Three unique mutations JPH2-T141H, S101R, and S165F were first identified from 388 North American HCM referral patients [95]. These probands, which had the clear echocardiogram and other diagnosed evidence, were distinct from the

mutated genes in sarcomere that were traditionally associated with HCM development. Furthermore, the study demonstrated that JPH2-T141H, S101R, and S165F mutations were sufficient to induce hypertrophic remodeling in cardiac immortalized cells, along with ablated CICR amplitude and disrupted cellular ultrastructure [95]. Additionally, two genetic variants JPH2-R436C and G505S have been found in a limited cohort of Japanese patients with HCM [96]. However, further studies are required to provide in vitro or in vivo evidence to support the pathogenicity of these variants, for the reason that the case group was small and the specificity was questioned of these two variants to HCM [96, 97]. Recently, another JPH2 gene missense mutation annotated as E169K was uncovered from an Italian cohort of 203 diagnosed HCM referrals [88]. This mutation was also identified to be associated with the development of atrial fibrillation(AF) in a small family of HCM probands [88]. Subsequent study on Pseudo-knockin (PKI) mouse models expressing E169K mutant, which localized to the structural  $\alpha$ -helical domain of JPH2, showed a reduced RyR2-JPH2 binding area with increased RyR2-mediated SR  $\text{Ca}^{2+}$  leak. This alteration disrupted the JMC's stabilization, leading to aberrant spontaneous  $\text{Ca}^{2+}$  waves and enlarged spark frequency, which as a consequence has a higher incidence of induced AF in the mice cardiomyocytes [88]. These results suggest that defective JPH2 may be implicated in the development of HCM and disturbance of  $\text{Ca}^{2+}$  homeostasis in cardiomyocytes.

### 1.3.2.2 Role of JPH2 in T-tubule remodeling in failing hearts

Heart failure refers to the physiological state when cardiac output is no longer sufficient to keep up with the required demands of systemic metabolism. This end dysfunction stage could be progressively developed from cardiomyopathy, cardiac stress, or other chronic factors [98]. It has been demonstrated that severe reduction of myocardial contractility attributed to impaired  $\text{Ca}^{2+}$  handling is the key characteristic during the progression of heart failure and leads to the final cardiac death [99, 100].

Many studies have observed T-tubule remodeling, including T-tubule reduction, disorganization, and dilation, from different heart failure animal models and human patients with heart failure [101-103]. These studies provided compelling evidence that T-tubule structural remodeling is the key pathological alteration during the progression of

many forms of cardiac disease towards heart failure [104, 105]. There is a direct link between T-tubule remodeling and dysfunction of SR  $\text{Ca}^{2+}$  release [106-108]. Due to the structural disorganization of the T-tubule system, the propagation of the action potential from the cell surface to T-tubule network experiences some disturbance [109, 110]. Also, the structural disorganization state causes T-tubule remodeling to change the distribution and organization of the ion carriers resulting in alteration of the shape and the duration of action potentials [111-113].

As previously mentioned, JPH2 mechanically maintains the structural integrity of T-tubule/SR coupling and plays an important role in T-tubule development [43, 82]. Emerging evidence from recent studies suggest that down-regulation or mislocalization of JPH2 contributes to T-tubule remodeling in failing hearts. In a transgenic mouse model of  $\text{G}\alpha_q$ -dependent heart failure, JPH2 was cleavage by activated calpain and resulted in T-tubule disruption with defect  $\text{Ca}^{2+}$  transient. The treatment of calpain inhibitor or turning off  $\text{G}\alpha_q$  prevented calpain-dependent proteolysis of JPH2, and more importantly reversed the t-tubule remodeling and the development of heart failure [114]. Moreover, in a murine model of pressure overload-induced hypertrophy, the transportation of a kinesin-driven microtubule densification took part in JPH2 mislocalization, which correlated with T-tubule remodeling and led to heart failure [115]. On the other hand, in a transgenic mice model, cardiac-specific JPH2 overexpression did not alter the baseline cardiac function, but strengthened the T-tubules/junctional SR-coupled dyads, which protected the cardiomyocytes from pressure-overload induced T-tubule remodeling and heart failure development [116]. This effect was also confirmed by the AAV9-JPH2 mediated gene therapy in the transverse aortic constriction (TAC) mouse model. Enhanced JPH2 ameliorated T-tubule structural disruption and prevented SR  $\text{Ca}^{2+}$  leak, which improved the contractility of the failing hearts [117].

## 1.4 ER stress and ischemia/reperfusion injury

The ER/sarcoplasmic reticulum is an organelle involved in many cell functions, including the folding of protein molecules, the transport of synthesized proteins to the Golgi apparatus, and lipid metabolisms, etc. [118]. ER/sarcoplasmic reticulum is also a major storage site of  $\text{Ca}^{2+}$  in cells, and can contribute to cellular activities through  $\text{Ca}^{2+}$

signaling [119].  $\text{Ca}^{2+}$  concentrations within ER/sarcoplasmic reticulum are regulated by RyR2 and IP3 receptors which release  $\text{Ca}^{2+}$  from the ER/sarcoplasmic reticulum to the cytosol, and the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$  transport ATPase, which transfers  $\text{Ca}^{2+}$  from the cytosol to the ER/sarcoplasmic reticulum [120].

$\text{Ca}^{2+}$  homeostasis helps maintain normal ER function [121]. Depletion of ER  $\text{Ca}^{2+}$  impairs the processing and folding of newly synthesized proteins and thus, induces accumulation of unfolded proteins, leading to ER stress [122]. This stress triggers the unfolded protein response (UPR) which activates ER transmembrane sensors to initiate the adaptive responses [123]. The three well-described ER transmembrane sensors include protein kinase-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6) [124, 125]. Activation of the PERK pathway results in phosphorylation of eukaryotic translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), global translation attenuation, and subsequent translation of the transcription factor ATF4 [126, 127]. IRE1 activation leads to JNK1/2 activation and transcriptional induction and splicing of XBP-1 [128, 129]. Proteolysis of ATF6 facilitates its nuclear translocation and subsequent transcriptional action [130, 131]. In concert, these pathways lead to the induction of genes including chaperones GRP78 and GRP94, XBP1 and CHOP. If ER stress is prolonged or overwhelming, however, it can induce cell death mainly through the activation of JNK1/2, CHOP and caspase-12 pathways [132]. Many studies have reported the activation of ER stress in the ischemic heart [56, 125]. However, the role of ER stress in the ischemic myocardium is complex and controversial. Either ischemia or ischemia/reperfusion can potentially induce the adaptive and pro-apoptotic pathways of UPR.

Martindale et al. have demonstrated the activation of the ATF6 branch of ER stress and its protective effect in the heart against I/R injury [133]. In their study, transgenic mice with tamoxifen-activated ATF6 exhibit better myocardium recovery from ex vivo I/R, compared to the non-tamoxifen-treated mice hearts. They also found that the acute activation of ATF6 in cardiomyocytes significantly reduced necrosis and apoptosis, along with increased cytoprotective ER-resident chaperones, including GRP78 and GRP94 [133]. The cardioprotective role of the ATF6 arm was supported by another study where

the inhibition of ATF6 in mice using 4-(2-aminoethyl) benzenesulfonyl fluoride further developed cardiac remodeling in ischemic heart and cause higher mortality rate [134]. Another effector of the UPR that preserves the myocardium against ischemic injury is the transcription factor X-box binding protein-1 (XBP1). Thuerlauf et al., in their study using rat neonatal cultured cardiomyocytes, showed that hypoxia increased XBP1 mRNA splicing, as well as the expression of other UPR markers [135]. They infected the cells with a recombinant adenovirus encoding dominant-negative XBP1 and then subjected it to hypoxia/reoxygenation. Inhibition of XBP1 sharply increased H/R-induced apoptosis, demonstrating the cardioprotective role of the XBP1 arm against I/R injury [135]. Furthermore, GRP94, which is one of the cytoprotective chaperones, is up-regulated by XBP1 during ER stress [136, 137]. Overexpression of GRP94 in cultured H9c2 cardiomyocytes significantly reduced the necrosis caused by calcium overload or simulated ischemia, indicating that the XBP1-inducible proteins may contribute to the protective effect [138].

On the other hand, the pro-apoptotic effect of ER stress has also been demonstrated in the ischemic heart. Terai et al. showed that hypoxia induced CHOP expression and the cleavage of caspase-12 in neonatal rat cardiomyocytes [139]. In their study, they found a significantly reduced apoptosis rate after the inhibition of these two ER stress-specific apoptotic pathways by the small interfering RNA (siRNA) technique. Thus, ER-initiated apoptotic signaling is involved in cell death after a hypoxic insult [139]. Furthermore, Tao et al. reported the ER stress-dependent apoptosis in vivo rat I/R model, as evidenced by increased CHOP, caspase-12, and JNK activation [140]. Modification of ER stress by apelin infusion, which significantly attenuated the activation of all the three pro-apoptotic pathways, successfully reduced the caspase-3 activity (apoptosis marker) and the final infarct size after reperfusion [140]. In addition, the p53-upregulated modulator of apoptosis (PUMA), which is a pro-apoptotic member of the Bcl2 family, has been implicated in ER stress-dependent cardiomyocyte apoptosis. Nickson et al. reported that the pharmacological stressor-induced ER stress promoted the expression of PUMA in neonatal cardiomyocytes and increased apoptosis [141]. In Langendorff-perfused isolated mice hearts, the targeted deletion of PUMA improved cardiac function and protects cardiomyocytes from cell death during I/R periods [142].

The mechanisms of I/R-induced pro-apoptotic ER stress remain unclear, especially when oxygen and nutrients are restored after reperfusion. Disrupted intracellular  $\text{Ca}^{2+}$  homeostasis may play a fundamental role in it. Emerging evidence suggested that not only the depletion of  $\text{Ca}^{2+}$  in ER but also the concomitant  $\text{Ca}^{2+}$  elevation in cytosol contributes to the activation of pro-apoptotic ER stress events [123, 143]. Thus, the correction of I/R-induced aberrant  $\text{Ca}^{2+}$  release from SR/ER may prevent pro-survival ER stress shifting into pro-apoptotic. ER stress may represent an important therapeutic target for I/R injury and ischemic heart diseases.

## 1.5 Calpain/calpastatin system in ischemic heart

Calpains belong to a family of  $\text{Ca}^{2+}$ -dependent cysteine proteases. Fifteen isoforms have been reported in mammals. Among them, calpain-1 ( $\mu$ -form) and calpain-2 (m-form) are ubiquitously expressed in the cytoplasm, and other calpain family members have more limited tissue distribution [144]. Calpain-1 and calpain-2 are heterodimers, differing in their  $\text{Ca}^{2+}$  requirement for activation ( $\sim 50 \mu\text{M}$  for calpain-1 and  $\sim 1000 \mu\text{M}$  for calpain-2) [145]. They consist of a distinct large 80-kDa catalytic subunit encoded by the genes *capn1* and *capn2*, respectively, and a common small 28-kDa regulatory subunit encoded by *capn4*, also known as *capns1* [146]. The small subunit is indispensable for calpain-1 and calpain-2 assembly and activation [147]. Thus, deletion of *capn4* functionally knocks out calpain-1 and calpain-2 [148]. Both calpain-1 and calpain-2 are tightly regulated by the intracellular free  $\text{Ca}^{2+}$  [149]. They are also controlled by its endogenous inhibitor calpastatin. Calpastatin specifically inhibits calpain-1 and calpain-2, but not other cysteine proteases [150].

Accumulative evidence has suggested that calpain activation plays a critical role in ischemic heart diseases. The proteolytic activity of calpain during I/R targets  $\alpha$ -fodrin, which is a crucial component to the formation of a membrane's cytoskeleton [151]. Evidence indicated that the degradation of  $\alpha$ -fodrin increased the fragility of the sarcolemma [152, 153]. In cultured cardiomyocytes, the activation of calpain cleaved fodrin into two fragments of 150 and 145 kDa [154]. Immunohistochemical studies further confirmed this proteolysis that occurred at the sarcolemma and the intercalated discs following I/R in rat hearts [155]. In vivo administration of calpain inhibitor-1 in 15-

minute global ischemia and 60-minute reperfusion rat hearts successfully suppressed the degradation of  $\alpha$ -fodrin and prevented I/R-induced contractile dysfunction [156]. In another rat model with transient coronary occlusion, the intravenous infusion of calpain inhibitor-3 (MDL-28170) during the first minute of reperfusion preserved  $\alpha$ -fodrin degradation and reduced the final infarct size by 32% [157]. It has been implied that the degradation of this membrane's backbone could lead to disruptions on ion channels [158]. L-type  $\text{Ca}^{2+}$  channels (LTCC) are the indispensable membrane's signal transporters that conduct excitation-contraction coupling in cardiomyocytes [36]. Decreased activities of LTCC have been reported upon the disturbance of cytoskeletal proteins [155]. Calpain inhibitor-3 counteracted the proteolysis of LTCC in I/R-injured cardiomyocytes [159]. Moreover, a novel calpain inhibitor, SNJ-1945, has been shown to prevent I/R-induced  $\alpha$ -fodrin degradation and restore the total  $\text{Ca}^{2+}$  handling in excitation-contraction coupling [160]. These together indicated a possible regulating role of  $\alpha$ -fodrin to the basal activity of LTCC. Another ion channel that was affected by the degradation of  $\alpha$ -fodrin is  $\text{Na}^+/\text{K}^+$ -ATPase. It has been demonstrated that  $\text{Na}^+/\text{K}^+$ -ATPase binds to the fodrin-based membrane cytoskeleton through the sarcolemmal protein ankyrin [161]. An in-vitro study on isolated cardiomyocytes from rats identified the calpain activation that proteolyzed both  $\alpha$ -fodrin and ankyrin at the beginning of reperfusion. The  $\alpha$ -subunit of the  $\text{Na}^+/\text{K}^+$ -ATPase was found to be detached from its membrane anchorage, which resulted in the failure interaction of this channel [162]. The loss of the  $\text{Na}^+$ -pump compromises the normalization of cytosolic  $\text{Na}^+$  concentration at the onset of reperfusion and results in the further disruption of  $\text{Ca}^{2+}$  homeostasis [163]. Inhibition of calpain with MDL-28170 during reperfusion increased  $\text{Na}^+/\text{K}^+$ -ATPase activity and improved contractile recovery [162].

On the other hand, I/R-induced calpain proteolysis also disrupts  $\text{Ca}^{2+}$  handling by directly targeting key  $\text{Ca}^{2+}$  channels [164]. SERCA2a is an essential  $\text{Ca}^{2+}$ -ATPase that reuptakes  $\text{Ca}^{2+}$  from cytosol to the lumen of SR/ER [48]. Its dysregulation impairs SR-mediated cardiac contractile function [165]. I/R-induced calpain activation has been demonstrated to degrade SERCA 2a and the SERCA regulatory protein PLB in perfused rat hearts [164]. Calpain inhibition with calpain inhibitor-3 (MDL-28170) during I/R attenuated the degradation of both proteins and improved the cardiac contractility [166]. This was

supported by another study on Langendorff-perfused rat hearts, which were subjected to I/R in the presence and absence of calpain inhibitor: leupeptin. The presented data suggested that I/R-induced calpain activation was not only responsible for the degradation of SERCA2a but also for the cleavage of ryanodine receptor-2 (RyR2), the major  $\text{Ca}^{2+}$  release channel on SR. Treatment with leupeptin recovered SR function and regulation, which was consistent with improved cardiac contraction after I/R [167].

Furthermore, the damage of mitochondria during I/R has also been associated with calpain activation. In a model of Langendorff-perfused isolated rabbit hearts, Sonata et al using the skinned fiber technique, observed decreased state 3 respiration rate (a parameter of mitochondrial function) after global ischemia and reperfusion [168]. Reduced state 3 respiration reflects the impairment of all complex I in the electron transport chain (ETC) [169]. They showed that calpain inhibition (BSF 409425) significantly improved state 3 respiration, so for the first time, it involved calpain activation in I/R-caused mitochondrial injuries [168]. This was also supported by [170]. Treatment with either calpain inhibitor BSF 409425 or A-705239 also attenuated mitochondrial leak-respiration and state 4 respiration, which provided evidence that calpain inhibition could prevent the inner mitochondrial membrane from becoming permeable [168, 170]. Thus, the proteolytic activity of calpain can function as a cytolytic initiator for the mitochondrial permeability transition during I/R.

In addition to cytosol, calpains and their endogenous inhibitor, calpastatin, have also been reported that present in mitochondria [171]. It is believed that I/R-induced intracellular  $\text{Ca}^{2+}$  overload forced excessive  $\text{Ca}^{2+}$  influx into the mitochondria and triggered the activation of mitochondrial calpains [172]. One of the most serious consequences of the activation of mitochondrial calpain is the cleavage and release of the apoptosis inducing factor (AIF) [173]. AIF is a mitochondrial antioxidant that is mostly located within the mitochondrial intermembrane space [174]. It has been demonstrated that AIF could be released into cytosol to induce caspase-independent apoptotic cell death under stress circumstances [175]. However, before release from mitochondria, it requires the cleavage of its anchored site on the inner membrane [176]. Qun Chen et al. first identified that  $\mu$ -calpain presented in isolated and purified cardiac mitochondria. In this study, global I/R

on Langendorff-perfused mouse hearts triggered the activation of mito- $\mu$ -calpain and decreased AIF content in the isolated mitochondria [177]. Calpain inhibitor MDL-28170 preserved the AIF content within mitochondria, indicating the required role of mito- $\mu$ -calpain in AIF cleavage. Calpain inhibition also decreased myocardial injury during I/R, as reflected by the decreased LDH release when compared to untreated hearts [177]. Thus, they provided evidence that mitochondrial  $\mu$ -calpain contributed to I/R injury in cardiomyocytes by its proteolytic effect on AIF.

Recently, we discovered a novel pathway linking calpain activation to I/R-induced lethal injury in cardiomyocytes that involved the induction of ER stress and subsequent JNK1/2 activation [178]. Indeed, we found that ER stress and apoptosis could be directly induced by only up-regulation of calpain-1 in cultured cardiomyocytes, indicating the correlation between them. Inhibition of calpain-1 prevented the induction of ER stress, and JNK1/2 activation in hypoxia/reoxygenation (H/R) stimulated cardiomyocytes [178]. Importantly, it was also demonstrated that ER stress/JNK1/2 signaling mediates apoptosis in cardiomyocytes following H/R. Inhibition of ER stress protected cardiomyocytes against H/R- or calpain-1-induced apoptotic cell death [178]. In vivo, transgenic mice with overexpression of calpastatin (Tg-CAST) significantly attenuated ER stress and phosphorylated JNK1/2 after I/R, confirming the important role of calpain activation in ER stress-mediated apoptosis [178]. Thus, calpains have become attractive targets for the development of synthetic inhibitors for therapy to reduce I/R injury and ischemic heart disease.

## 1.6 Rationale

Myocardial infarction (MI) is a fatal ischemic heart disease and a leading cause of death in Canada [2]. Reperfusion remains the primary and effective therapy. However, reperfusion can induce damage to the ischemic myocardium, a condition described as ischemia/reperfusion (I/R) injury, which contributes to heart failure post MI [4, 19]. Over 500,000 Canadians are afflicted with heart failure, and up to 50% of them die within five years of diagnosis [18]. Unfortunately, effectively limiting myocardial I/R injury remains a major challenge and is in urgent need for further investigation.

Our lab has recently demonstrated an important role for calpain activation in myocardial I/R injury and MI [178]. We further reported that calpain cleaved and down-regulated junctophilin-2 (JPH2) protein in hearts following I/R [179]. These findings raise an intriguing question: does decreased JPH2 contribute to myocardial I/R injury?

JPH2 plays a role in modulating the gating of RyR2  $\text{Ca}^{2+}$  channel, preventing aberrant  $\text{Ca}^{2+}$  release via RyR2 in cardiomyocytes and thus, ensuring appropriate  $\text{Ca}^{2+}$  homeostasis [89, 180]. Importantly,  $\text{Ca}^{2+}$  homeostasis is disrupted in cardiomyocytes during I/R [59, 62]. It is known that perturbation of  $\text{Ca}^{2+}$  homeostasis results in the activation of downstream  $\text{Ca}^{2+}$  signaling, in particular calpain activation [151, 181]. Our lab reported that calpain activation induces ER stress in cardiomyocytes [178]. ER stress has been demonstrated to contribute to apoptosis in cardiomyocytes under stress [55, 132]. Thus, we reason that decreased JPH2 promotes cardiomyocyte I/R injury.

Understanding the role of JPH2 in myocardial I/R injury is clinically relevant because the protein levels of JPH2 are reduced in human diseased hearts [77, 94]. Thus, this study will help to identify therapeutic targets which may inform a future clinical trial of pharmacological approaches in patients with ischemic heart disease, with the goal of improved clinical outcomes in human ischemic heart disease.

## 1.7 Hypothesis

I/R induces down-regulation of JPH2 in cardiomyocytes. Decreased JPH2 promotes RyR2 dysfunction and perturbation of  $\text{Ca}^{2+}$  homeostasis, which triggers calpain activation and ER stress, leading to apoptosis in cardiomyocytes. Up-regulation of JPH2 prevents  $\text{Ca}^{2+}$  dysregulation and protects cardiomyocytes from apoptosis following I/R.

## Chapter 2

### 2 Materials and Methods

#### 2.1 Cell culture

H9c2 cells were purchased from the American Type Culture Collection (ATCC), and were employed within 10 generations for this study. H9c2 is a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissue. The cells were grown in a culture flask at 37 °C in a 5% CO<sub>2</sub> humidified incubator, and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Chicago, IL, USA) which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin 100 IU/mL, and streptomycin 10 µg/mL (Invitrogen, Chicago, IL, USA). Cultured H9c2 cells were split every three days. To passage the H9c2 cells, media was aspirated; cells were washed with D-Hank's solution (NaCl, KCl, KH<sub>2</sub>PO<sub>4</sub>, NaHCO<sub>3</sub>, D-glucose, phenol red), and incubated with 0.25% Trypsin (Gibco, Life Technologies Burlington, ON, Canada) at room temperature for 20 to 30 seconds. Fresh culture media was added to terminate the trypsinization process, and cells were divided and re-plated as required.

#### 2.2 Neonatal mouse cardiomyocyte isolation

Primary cardiomyocytes were isolated from neonatal mice born within 2 days. Briefly, after sterilizing using 70% ethanol, the neonatal mice's hearts were harvested and placed into D-Hank's solution (50 mL tube). Each heart was cut into 4–5 pieces using a surgical blade, and washed using D-Hank's solution. The heart pieces were then subjected to the following 3 rounds of digestion: (1) Heart tissues were incubated with 2 mL Liberase Blendzyme(10µg/mL) in a water bath at 37°C for 10 min. After stirring 3-5 times, the supernatant was discarded; (2) The tissues were incubated with 4 mL of fresh Liberase Blendzyme solution at 37°C. During the incubation, the minced hearts were gently swirled in every 5 minutes. After 15 minutes, the supernatant was collected and stored in DMEM containing 10% FBS (Invitrogen, Chicago, IL, USA); (3) Four mL of fresh Liberase Blendzyme solution were added into the heart tissues. The digestion was followed as described in (2). The supernatants from (2) and (3) were pooled and

centrifuged at 200g for 5 min. The resulting cell pellets were re-suspended in DMEM containing 10% FBS. Next, the suspended cells were subjected to pre-plating in a culture dish containing 6 mL of DMEM containing 10% FBS at 37°C for 120 min. The pre-plating allowed fibroblasts to adhere to the culture plate. After fibroblast adherence, cardiomyocytes were collected, and the cell number was counted. The cardiomyocytes were then seeded into a culture plate which has been already paved with 1% gelatin for 120 min in an incubator. Finally, the plate containing cardiomyocytes was placed in a CO<sub>2</sub> incubator at 37°C. After 18 hours, the cardiomyocytes were subjected to various experiments.

### 2.3 Adenoviral infection

Cultured H9c2 cells or primary neonatal mice cardiomyocytes were infected with recombinant adenoviral vectors containing human JPH2 (Ad-JPH2) or beta-gal (Ad-gal) as a control at a multiplicity of infection (MOI) of 100 plaque forming units per cell (100 PFU/cell). These adenoviral vectors were purchased from Vector Biolabs (USA). The adenoviral vectors were prepared in DMEM with 2% FBS and added to the cells in half the volume of cultural medium. After incubation for 2 h, the full volume of culture medium was supplied. All experiments were implemented after 24 h of adenoviral infection.

### 2.4 Hypoxia/reoxygenation (H/R)

For the induction of hypoxia, cardiomyocytes were placed in the plates, and the culture medium was reduced to half volume. Next, cardiomyocytes were placed in a sealed bag containing GENbag anaer (bioMérieux) at 37 °C for 24 h. A indicator (bioMérieux) was used to monitor hypoxia. Inside the bag containing the GENbag anaer, the O<sub>2</sub> concentration was rapidly reduced close to zero within 30 min. This experimental condition perfectly mimics the initial period of ischemia. Reoxygenation was achieved by replacing culture media with fresh one and returning the cells to normal culture conditions.

## 2.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Cells were lysed in a lysis buffer containing 1% Triton-X-100, 0.5 mM EDTA, 15 mM NaCl, and 2 mM Tris base (Invitrogen, Chicago, IL, USA). The cell lysates were then collected and centrifuged at 10,000g at 4°C for 10 min. After that, the supernatant was transferred into a clean tube, and the protein concentration was determined by the Bradford assay. The gel casting apparatus was assembled first with 1.5 mm spacers, and was tested for leaks to implement the SDS-PAGE. A concentration of 10% or 12% of separating gel was then prepared, depending on the size of the target protein in the sample [Table 1, For a 10mL separating gel].

**Table 1: Composition of separating gel**

| <b>Acrylamide percentage</b>                | <b>12%</b> | <b>15%</b> |
|---|------------|------------|
| H <sub>2</sub> O                            | 3.2 mL     | 2.2 mL     |
| Acrylamide/Bis-acrylamide<br>(30%/0.8% w/v) | 4 mL       | 5 mL       |
| 1.5M Tris(pH=8.8)                           | 2.6 mL     | 2.6 mL     |
| 10% (w/v) SDS                               | 0.1 mL     | 0.1 mL     |
| 10% (w/v) ammonium<br>persulfate (AP)       | 100 µL     | 100 µL     |
| TEMED                                       | 10 µL      | 10 µL      |

The mixture was then immediately transferred to the casting apparatus, with a water overlay to avoid the air interference. After the separating gel was solidified in 15-20 minutes, the stacking gel was made [Table 2, For a 5 mL stacking gel] and filled on top of the separating gel. A clean 10 wells comb was inserted to make sure that no residual bubbles were in the wells.

**Table 2: Composition of stacking gel**

|   |          |
|---|----------|
| H <sub>2</sub> O                            | 2.975 mL |
| 0.5 M Tris-HCl, pH 6.8                      | 1.25 mL  |
| 10% (w/v) SDS                               | 0.05 mL  |
| Acrylamide/Bis-acrylamide<br>(30%/0.8% w/v) | 0.67 mL  |
| 10% (w/v) ammonium<br>persulfate (AP)       | 0.05 mL  |
| TEMED                                       | 0.005 mL |

A total of 50 µg proteins from sample lysates were mixed with the loading buffer [Table 3, for a 10 mL Laemmli buffer 6X] (the volume ratio of 5:1). The sample mixtures were boiled at 95°C for 5 min and loaded into wells using gel loading tips. The gel assembly was taken out of the casting and put into the gel tank. Running buffer (0.025 M Tris base, 0.192 M Glycine, 0.1 % SDS) was then poured into the tank until above the top of the gel (~800 mL).

**Table 3: Composition of loading buffer**

|                        |         |
|------------------------|---------|
| ddH <sub>2</sub> O     | 2.10 mL |
| 0.5 M Tris-HCl, pH 6.8 | 1.20 mL |
| SDS                    | 1.20 g  |
| Bromophenol blue       | 6.00 g  |
| Glycerol               | 4.70 mL |
| DTT                    | 0.93 g  |

Next, the electrophoresis assembly was connected to the power supply set at 15mA, 200V, and 24W for about 1 hour or until the running front reached the end of the gel. After electrophoresis, proteins were separated and transferred to a polyvinyl difluoride (PVDF) membrane as follows: First, the PVDF membrane and the transfer cassette equipment was immersed in transfer buffer (20% v/v methanol, 0.19 M glycine, and 0.05 M Tris); Next, the cassettes were assembled in a layer of blotting paper, a sponge, PVDF membrane, electrophoresed gel, blotting paper, and a sponge; The cassette was then placed in the tank filled with transfer buffer at 30V in the fridge (4°C) for overnight.

In the next day, PVDF membranes were taken out from the cassette and kept with 5% non-fat milk in tris-buffered saline containing Tween 20 (TBST) at room temperature for one hour. This step was to block the non-specific binding sites. Following this, primary antibodies were diluted with 5% BSA (5ml), and applied to the membranes in a clean tube. The tube was then set on a rocking table and subjected to 4°C for overnight. After being incubated with primary antibodies, PVDF membranes were washed three times with TBST, with 10 minutes for each time. The membranes were then incubated with secondary antibodies in 5% non-fat milk at room temperature for one hour. Following incubation, the washing step was repeated again in TBST for three times, with 10 minutes each time. Finally, the membranes were immersed in ECL reagent for one minute. The signals were visualized using an enhanced chemiluminescence-detection system.

## 2.6 Caspase-3 activity measurement

Activated caspase-3 in cardiomyocytes was measured using a caspase-3 activity assay kit according to the manufacturer's protocol (BIOMOL Research Laboratories). Lysis buffer containing 50 mM HEPES (pH 7.4), 0.1% CHAPS, 5 mM DTT, 0.1 mM EDTA and 0.1% NP-40 (Invitrogen, Chicago, IL, USA) was added to cardiomyocytes. Cell lysates were collected and centrifuged at 10,000g at 4°C for 10 minutes. Then, the supernatant was transferred into a clean tube, and the protein concentration was determined by the Bradford assay. A total of 50µg protein was incubated with caspase-3 substrate Ac-DEVD-AMC or Ac-DEVD-AMC in the presence or absence of inhibitor AC-DEVD-CHO diluted in assay buffer at 37°C for 2 hours. The assay buffer contained 50 mM HEPES (pH 7.4), 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10 mM NaCl, and 10%

Glycerol. Caspase-3 targeted chromophore AMC and cleaved it from the peptide substrate's C-terminus. The fluorescence intensity of cleaved AMC was quantified by a fluorescent spectrophotometer with excitation 355 nm/emission 460 nm.

## 2.7 Cellular DNA fragmentation

DNA fragmentation was measured using a Cellular DNA Fragmentation ELISA kit (Roche Applied Science, Canada) according the manufacturer's instructions. H9c2 cells were pre-labeled with BrdU. After various treatments, cells were lysed and cell lysates were collected. After centrifuged at 250 g at 4 °C for 10 min, the supernatant was transferred to a clean tube, and the protein concentration was determined by the Bradford assay. A 96-well microplate was pre-coated with anti-DNA antibody at 4°C for overnight. The following day, incubation buffer was added to each sample well. The plate was left at room temperature for 30 minutes. After three washes of the pre-coated microplate with the washing buffer, samples were added into the microplate and stranded for 90 minutes at room temperature. This step allowed the BrdU-labeled DNA fragments to bind to the immobilized anti-DNA antibody. Next, the samples in the microplate were washed twice. For the third wash, the microplate with washing buffer was placed in a microwave for 5 minutes, followed by 10 minutes at -20 °C. BrdU-labeled DNA fragments were denatured and fixed on the bottom of the microplate in this step. In the next step, second-antibody anti-BrdU-peroxidase diluted in washing buffer was placed into each sample well of the microplate and stranded at room temperature for 90 minutes. Finally, 100 µL TMB substrate was added to each well, and the fluorescence intensity of peroxidase bound in the immune complex was determined by a fluorescent spectrophotometer with excitation of 355 nm/emission of 450 nm. Results were expressed as fold increases over control.

## 2.8 Calpain activity

Cells were lysed in a lysis buffer containing 20 mM Tris-HCL (pH 7.6), 150 mM NaCl, and 1% Triton X-100 (Invitrogen, Chicago, IL, USA). Cell lysates were then subjected to sanitation on ice for 10 seconds and centrifuged at 11,000g at 4°C for 10 min. The supernatant was transferred into a clean tube, and the protein concentration was

determined by the Bradford assay. A total of 30 $\mu$ g proteins were incubated with a fluorescence substrate N-succinyl-LLVY-AMC (Cedarlane Laboratories) diluted in reaction buffer (63 mM imidazole-HCl, pH 7.3, 10 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, and 10 mM EGTA) with or without calcium chloride at 37 C ° for 2 h. The fluorescence intensity of cleaved AMC was quantified with a multilabel reader (excitation, 360 nm; emission, 460 nm), and calpain activity was determined as the difference between calcium-dependent and calcium-independent fluorescence.

## 2.9 Single cell calcium photometry and SR leak

Cells were plated on the surface of a 35 mm glass coverslip within each well of a 24-well plate. On the experiment day, cells were loaded with 2 $\mu$ M fura-2 AM (Invitrogen, Chicago, IL, USA) at 37 °C in a 5% CO<sub>2</sub> humidified incubator for 40 min. Cells were then washed twice and replaced with 500 $\mu$ L normal Tyrode (NT, 140 mM NaCl, 4 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM glucose, and 5 mM HEPES, pH 7.4) and stood at 37 °C for additional 10 min. For the experiment, the glass coverslip with attached cells was placed in a perfusion chamber. 1 mL of extracellular buffer NT was provided to the chamber. Fluorescence intensity at 510 nm was measured with alternating 345 nm and 380 nm excitation (Ratio F<sub>345/380</sub>), by a Deltascan monochromator system (Photon Technology International, London, ON). Only one cell was tested from each coverslip. After a steady state of the ratio intensity, SERCA inhibitor 10  $\mu$ M cyclopiazonic acid (CPA) was slowly added to the bath by a pipette (close but not inserted into the surface of the medium). The increased intracellular Ca<sup>2+</sup> was measured and taken to analyze the SR leak. For the negative control, RyR2 inhibitor 3 $\mu$ M tetracaine (Invitrogen, Chicago, IL, USA) was administrated with NT before testing.

## 2.10 Statistical Analysis

All data were presented as mean  $\pm$  standard deviation (SD). Results were analyzed by two-way ANOVA followed by the Newman-Keuls test for multi-group comparisons. A student 's T-test was adopted for comparison between 2 groups. The value  $P < 0.05$  was considered statistically significant.

## Chapter 3

### 3 Results

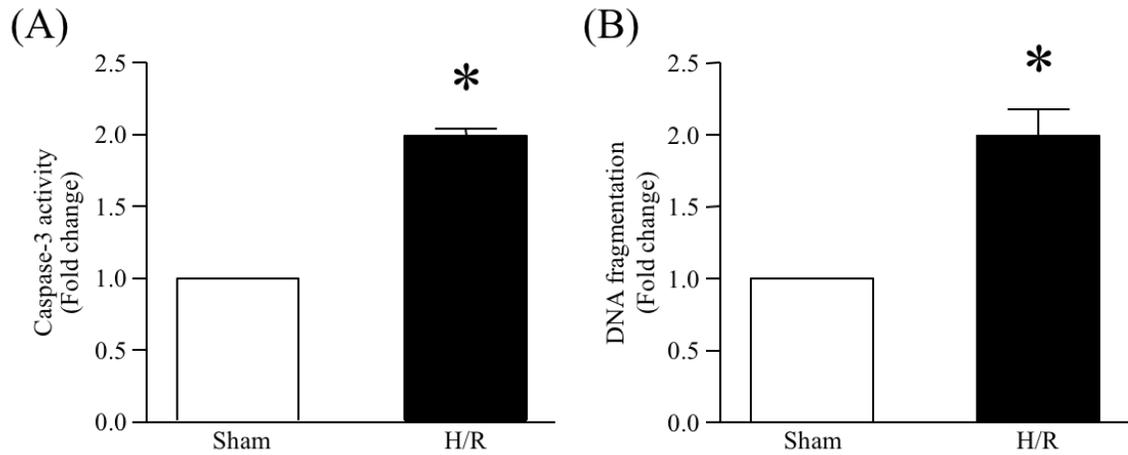
#### 3.1 Establishment of an in vitro model of Hypoxia/Reoxygenation injury

The in vitro experimental model of hypoxia/reoxygenation (H/R) in cultured cardiomyocytes has been widely used to mimic in vivo ischemia/reperfusion in the heart [182]. The H/R was induced as described in Method chapter. To confirm the hypoxic conditions, we added 300  $\mu$ L of culture media to each well in 24-well plate and measured  $O_2$  concentration in culture media at different time points after hypoxia. This volume of culture media was chosen because the same volume of culture media was used for cell culture during H/R. As shown in Table 4, the  $O_2$  concentration was dramatically reduced by up to 50% within 30 min and by greater than 80% after 6 hours. It is important to mention that the actual  $O_2$  concentration was lower after hypoxia because the culture media was exposed to normal air for a while before  $O_2$  measurement. This result verifies the hypoxic conditions in culture media.

**Table 4:  $O_2$  concentration in different time points after hypoxia**

| Hours | Ctrl,% | Hypoxia,% |
|-------|--------|-----------|
| 0.5   | 100    | 55        |
| 1.0   | 100    | 48        |
| 3.0   | 100    | 32        |
| 6     | 100    | 19        |
| 12    | 100    | 12        |
| 24    | 100    | 8         |

We next determined whether this H/R condition was sufficient to induce apoptosis in cardiomyocytes. To do this, we seeded H9c2 cells into 24-well plates. Twenty-four hours later, we exposed the cells to a hypoxic condition for 24 hours followed by reoxygenation for additional 24 hours. H9c2 cells were then assayed for caspase-3 activity, which is the most frequently activated death protease in the execution phase of apoptosis. H/R significantly increased the level of caspase-3 activity by one fold compared to the control group under normoxic condition (N/R group) [Figure 4A]. Apoptosis was further analyzed by measuring DNA fragmentation, which is also a biological hallmark of apoptosis. Similarly, H/R increased DNA fragmentation by one fold in comparison to the N/R group [Figure 4B]. These results demonstrate that H/R induces apoptosis in H9c2 cells, indicative of cell injury. Taken together, we successfully establish an in vitro model of H/R-induced injury in cardiomyocytes, which was employed for the following studies.

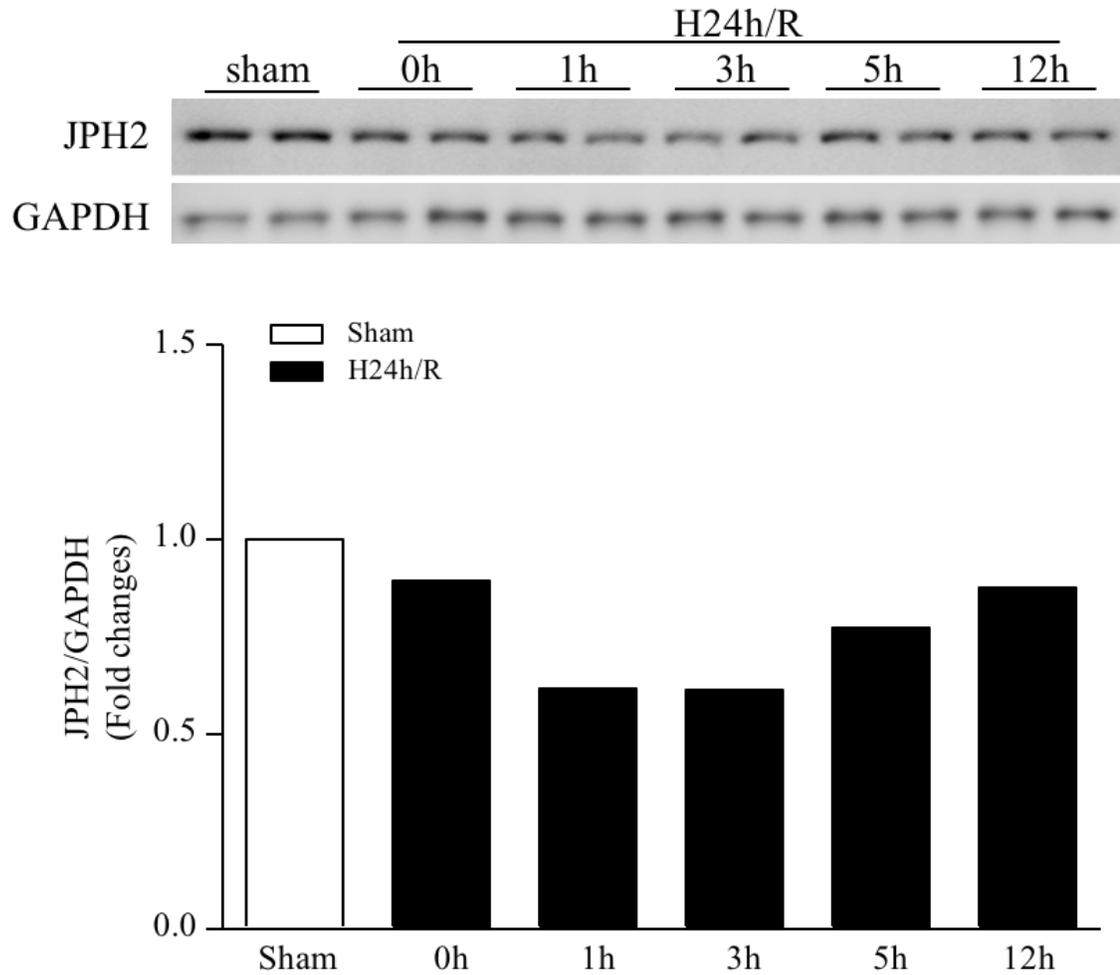


**Figure 4: Assessments of apoptosis on H9c2 cells following H/R**

H9c2 cells were subjected to 24h hypoxia, followed by 24h reoxygenation. Apoptosis was assessed by (A) measuring caspase-3 activity, and (B) DNA fragmentation. Data are normalized to control and expressed as means  $\pm$  SD from at least 3 different experiments. \*p < 0.05 analyzed by unpaired student's T-test.

### 3.2 H/R down-regulated JPH2 protein expression in cardiomyocytes

JPH2 is down-regulated in diseased hearts [77, 117]. We determined whether H/R decreased JPH2 protein expression in cardiomyocytes. At different time points after reoxygenation, we examined the protein levels of JPH2 by western blot analysis. Our result showed that the protein levels of JPH2 were reduced after hypoxia and further decreased during early reoxygenation within 3 hours. The protein levels of JPH2 were increased a little after 3 hours during reoxygenation but remained lower than those before H/R [Figure 5]. In contrast, the protein levels of GAPDH were not changed after H/R. This data demonstrates that H/R induces down-regulation of JPH2 protein in cardiomyocytes.

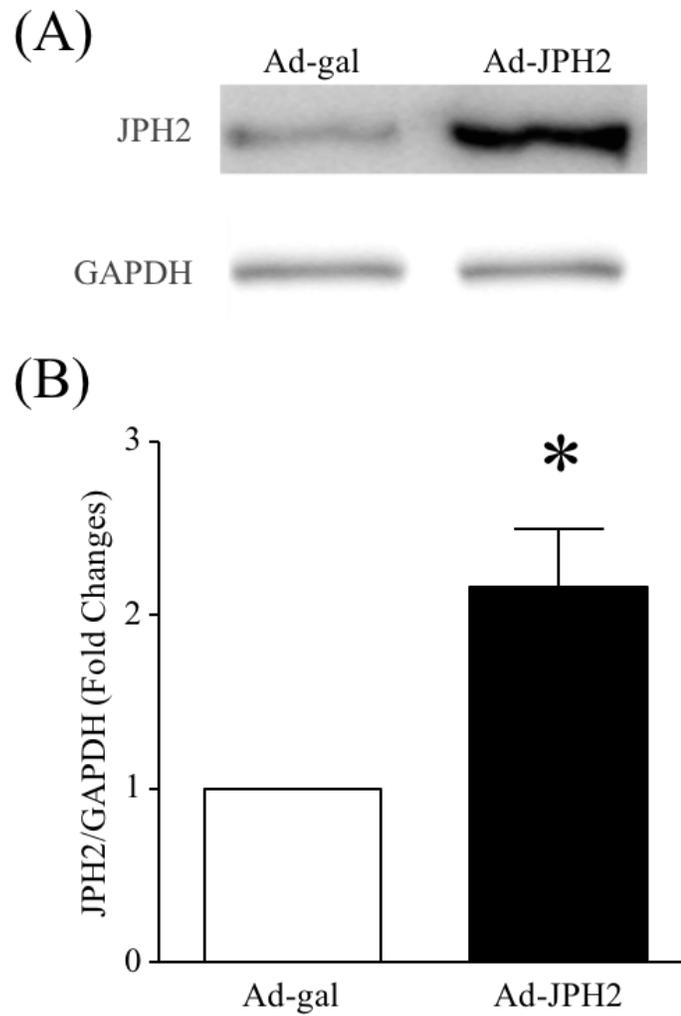


**Figure 5: Time course of JPH2 protein expression following H/R in H9c2 cells**

H9c2 cells were subjected to hypoxia for 24h, and were analyzed for JPH2 and GAPDH protein expression at different time points after reoxygenation. (A) A western blot for JPH2 and GAPDH protein expression from 1 experiment (not replicated). (B) Quantification of JPH2 protein expression relative to GAPDH.

### 3.3 Adenoviral vector mediated up-regulation of JPH2 expression in heart cells

To up-regulate JPH2 protein expression in cardiomyocytes, we infected H9c2 cells with adenoviral vectors containing human JPH2 gene (Ad-JPH2) or beta-gal (Ad-gal) as a control at a multiplicity of infection (MOI) of 100 plaque forming units (PFU) per cell. Twenty-four hour after adenoviral infection, western blot analysis was performed to determine the protein levels of JPH2 and GAPDH [Figure 6]. The protein levels of JPH2 increased significantly in Ad-JPH2 infected H9c2 cells, compared to Ad-gal group. These results indicate that infection with Ad-JPH2 induces up-regulation of JPH2 protein expression in H9c2 cells.



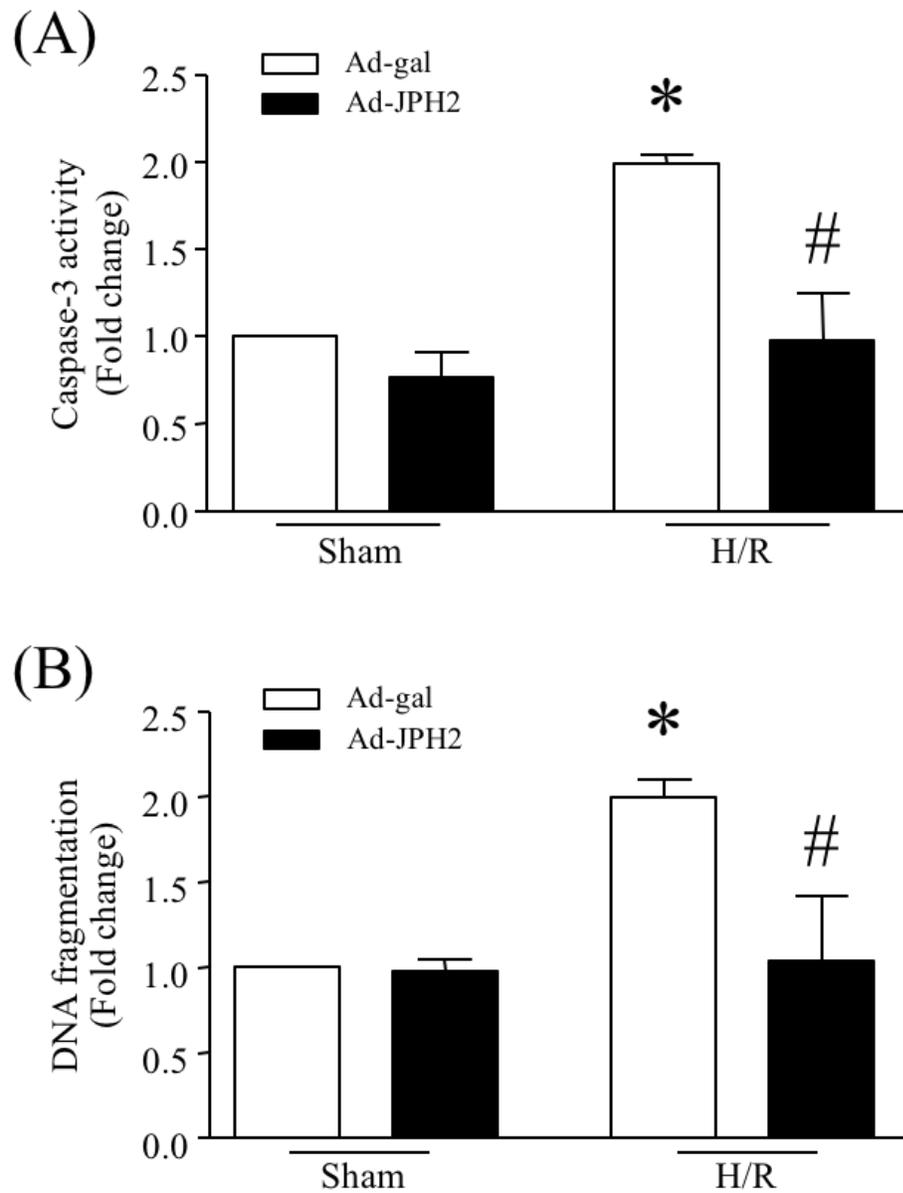
**Figure 6: JPH2 protein expression in Ad-JPH2 infected H9c2 cells**

H9c2 cells were infected with Ad-JPH2 or Ad-gal. (A) A representative western blot for JPH2 and GAPDH from three different experiments. (B) Quantification of JPH2 protein expression relative to GAPDH. Data are mean  $\pm$  SD from 3 different experiments.

\* $P < 0.05$  analyzed by unpaired student's T-test.

### 3.4 Up-regulation of JPH2 prevented apoptosis induced by H/R

To determine the role of JPH2 in H/R-induced apoptosis, we infected H9c2 cells with Ad-JPH2 or Ad-gal as a control. Twenty-four hours later, the cells were subjected into H/R or N/R. After 24 hours reoxygenation, apoptosis was determined by measuring caspase-3 activity and DNA fragmentation. As shown in Figure 7, H/R significantly increased caspase-3 activity and DNA fragmentation, indicative of apoptotic cell death. This effect of H/R on apoptosis was prevented by infection with Ad-JPH2. These results demonstrate that up-regulation of JPH2 protects cardiomyocytes against H/R-induced apoptosis.

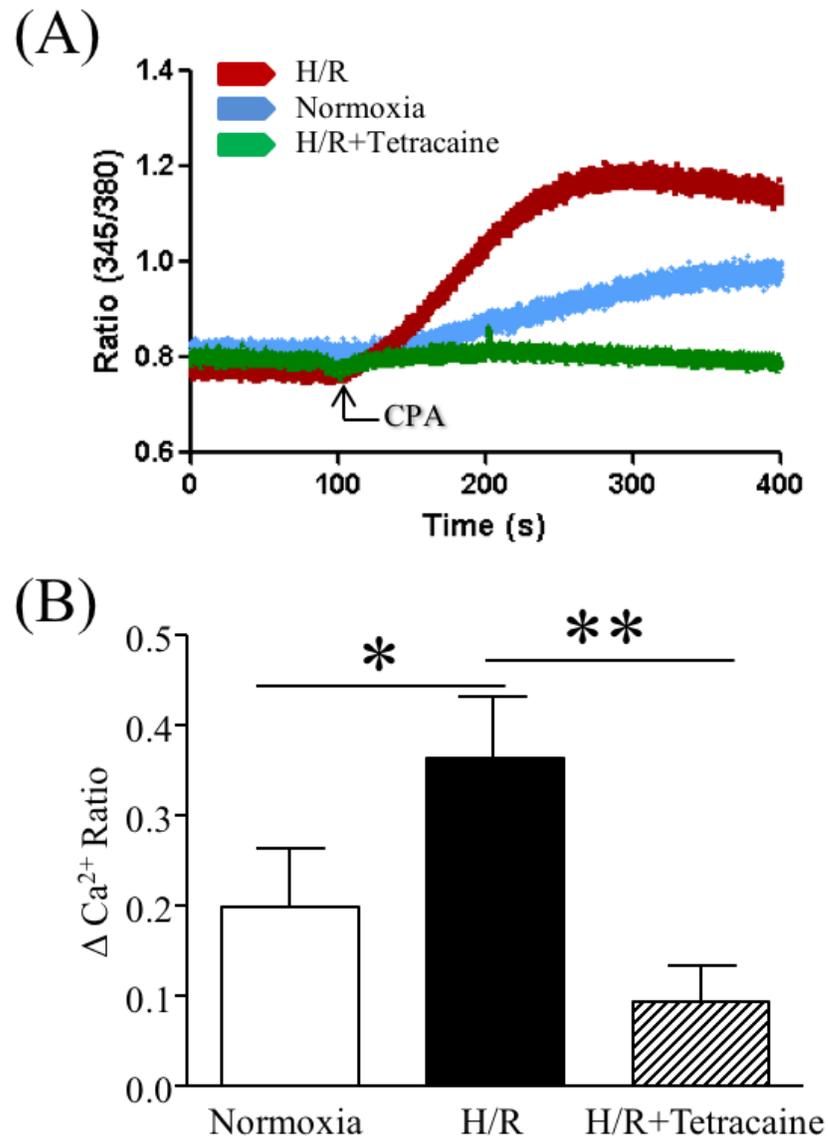


**Figure 7: Effect of JPH2 up-regulation on apoptosis in H9c2 cells after H/R**

H9c2 cells were infected with Ad-JPH2 or Ad-gal, and then subjected to H/R. Apoptosis was assessed by measuring caspase-3 activity (A) and DNA fragmentation (B). Data are mean  $\pm$  SD from 3 different experiments. \* $P < 0.05$  and # $P < 0.05$  analyzed by two-way ANOVA followed by the Newman-Keuls test.

### 3.5 JPH2 regulated RyR2-mediated aberrant Ca<sup>2+</sup> release from SR in H/R-stimulated cardiomyocytes.

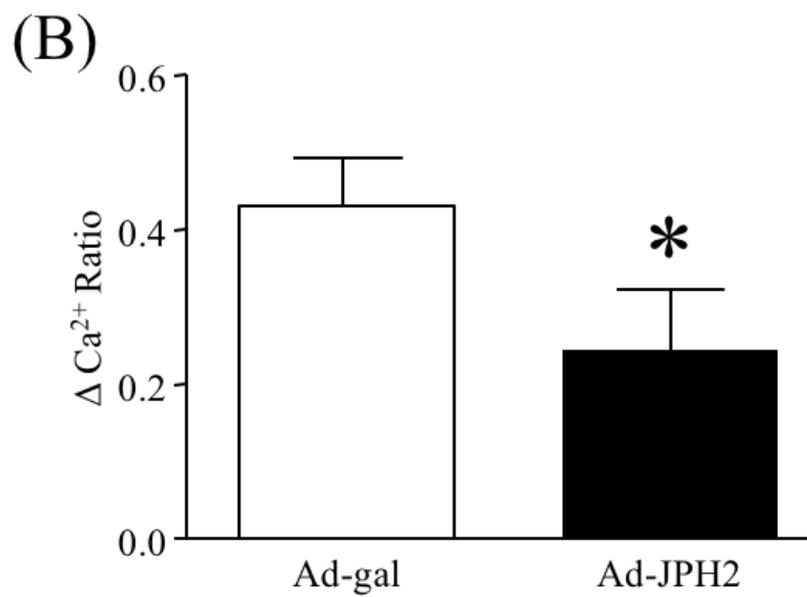
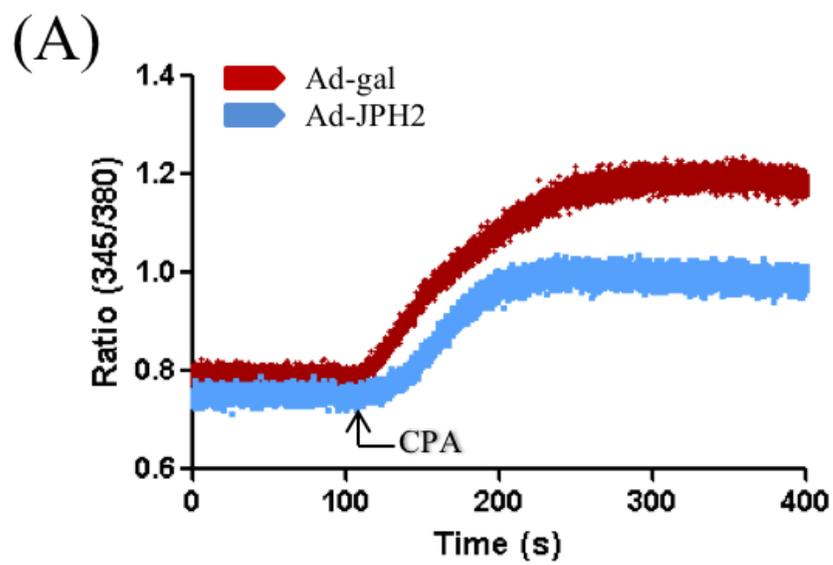
SR Ca<sup>2+</sup> leak has been implicated in ischemic heart disease [100, 180]. Recent studies suggested that JPH2 is a negative regulator of RyR2 gating [86, 89]. We investigated whether JPH2 regulated aberrant Ca<sup>2+</sup> release from SR via RyR2. Sarcoplasmic reticulum calcium-ATPase (SERCA) is a major cytosolic Ca<sup>2+</sup> efflux channel that reuptakes Ca<sup>2+</sup> to SR/ER. Its inhibition disrupted the dynamic balance of Ca<sup>2+</sup> cycling, leading to temporary Ca<sup>2+</sup> accumulation in cytosol. In the H9c2 cells under N/R, intracellular Ca<sup>2+</sup> level gradually increased after inhibition of SERCA by cyclopiazonic acid (CPA, 10 μM) [Figure 8]. However, compared to N/R, H/R enhanced intracellular Ca<sup>2+</sup> level in cytosol after incubation with CPA. This result suggests that H/R may promote Ca<sup>2+</sup> release from SR. To confirm this, we blocked RyR2 with its antagonist tetracaine (3 μM) in H9c2 cells and then added CPA. As shown in Figure 8, the rising Ca<sup>2+</sup> in H/R-stimulated H9c2 cells was prevented and the levels were even lower than that in N/R group, supporting that the CPA-induced Ca<sup>2+</sup> cytosolic Ca<sup>2+</sup> influx is from SR via RyR2. This result also eliminated the possibility that extracellular Ca<sup>2+</sup> or Ca<sup>2+</sup> stored in other organelle like mitochondria got involved in the CPA-stimulated intracellular Ca<sup>2+</sup> inflow. Taken together, these results indicate that H/R impairs RyR2 function and subsequently induces aberrant Ca<sup>2+</sup> release from SR.



**Figure 8: Measurement of SR Ca<sup>2+</sup> release in H9c2 cells following H/R**

After 24h hypoxia followed by 24h reoxygenation, H9c2 cells were loaded with Fura-2 AM (2 $\mu$ M) and shifted in normal Tyrode(NT) medium. Intracellular Ca<sup>2+</sup> changes were assessed by Delta Scan digital fluorescence photometry. (A) Representative Fura-2 ratio illustrating cytosolic Ca<sup>2+</sup> changes after administration of CPA in three different samples, which are normoxia, H/R and tetracaine pretreated H/R. (B) Quantification of increased Ca<sup>2+</sup> ratio in cytosol after administration of CPA. Data are means  $\pm$  SD of 11-16 cells for each condition from more than three independent preparations, \* $p < 0.05$  and \*\* $p < 0.05$  analyzed by two-way ANOVA followed by the Newman-Keuls test.

To determine whether JPH2 regulated H/R-induced aberrant SR  $\text{Ca}^{2+}$  release via RyR2, we infected H9c2 cells with Ad-JPH2 or Ad-gal as a control. Twenty-four hours after adenoviral infection, the cells were subjected into H/R. CPA-stimulated  $\text{Ca}^{2+}$  elevation was measured thereafter. Infection with Ad-JPH2 prevented RyR2-mediated abnormal  $\text{Ca}^{2+}$  efflux from SR [Figure 9]. Thus, up-regulation of JPH2 normalizes  $\text{Ca}^{2+}$  regulation in cardiomyocytes.

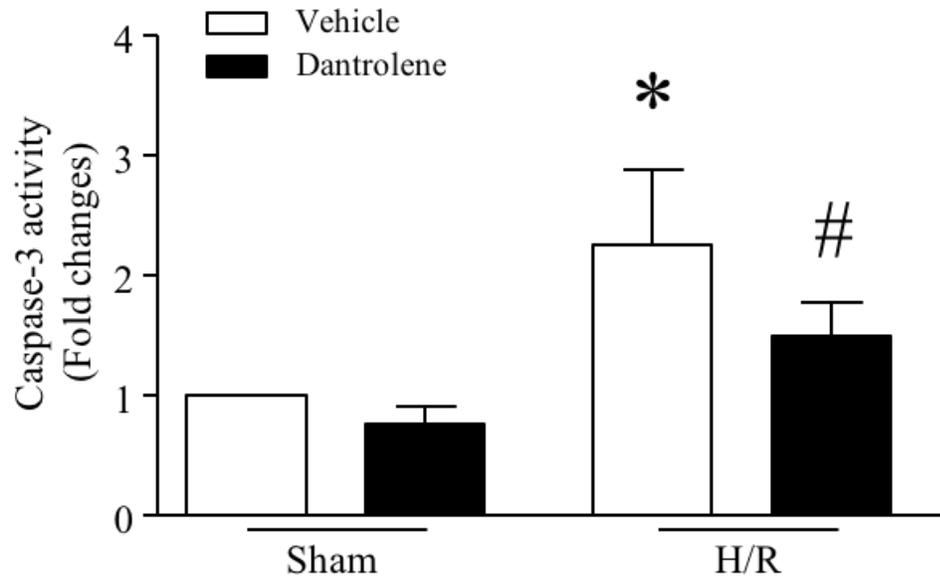


**Figure 9: Effects of JPH2 up-regulation on aberrant SR Ca<sup>2+</sup> release in H9c2 cells following H/R**

H9c2 cells were infected with Ad-JPH2 or Ad-gal, and then subjected to a 24-hour hypoxia followed by a 24-hour reoxygenation. Cells were then loaded with Fura-2 AM (2  $\mu$ M) and shifted in normal Tyrode(NT) medium. (A) Representative Fura-2 ratio illustrating cytosolic Ca<sup>2+</sup> changes after administration of CPA in H/R group. (B) Quantification of increased Ca<sup>2+</sup> ratio in cytosol after administration of CPA in H/R group. Data are means  $\pm$  SD from at least three independent preparations with 10-14 cells for each condition. \* $p < 0.05$  analyzed by unpaired student's T-test.

### 3.6 Blockage of RyR2 reduced apoptosis induced by H/R

To determine whether aberrant  $\text{Ca}^{2+}$  release via RyR2 played a role in apoptosis after H/R, we incubated H9c2 cells with dantrolene (100  $\mu\text{M}$ ), an antagonist of RyR2 which inhibits  $\text{Ca}^{2+}$  release from SR via RyR2 or vehicle, and performed H/R on these cells. Apoptosis was assessed by measuring caspase-3 activity. Consistently, H/R induced apoptosis in H9c2 cells, which was significantly reduced by dantrolene [Figure 10]. This result suggests that RyR2-mediated aberrant SR  $\text{Ca}^{2+}$  release contributes to H/R-induced apoptosis.

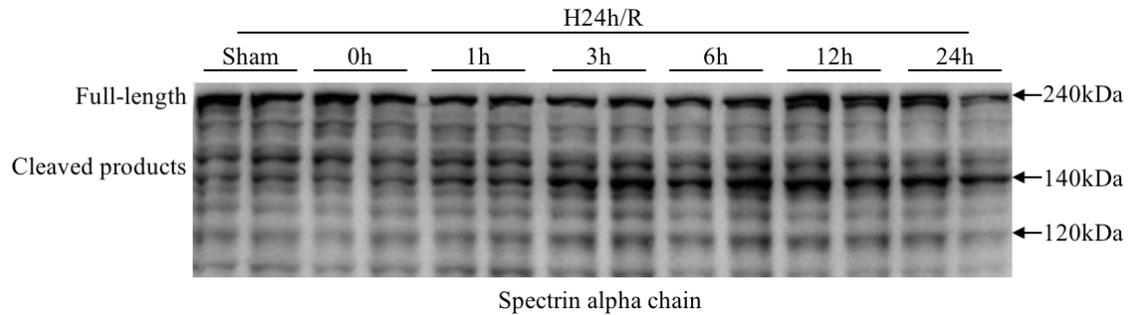


**Figure 10: Effect of dantrolene on apoptosis in H9c2 cells following H/R**

H9c2 cells were pretreated with dantrolene (100  $\mu$ M) for 30 minutes prior to being subjected to H/R. Apoptosis was assessed by measuring caspase-3 activity. Data are mean  $\pm$  SD from 3 different experiments. \* $P < 0.05$  and # $P < 0.05$  analyzed by two-way ANOVA followed by the Newman-Keuls test.

### 3.7 Up-regulation of JPH2 prevented calpain activation in H/R-stimulated cardiomyocytes.

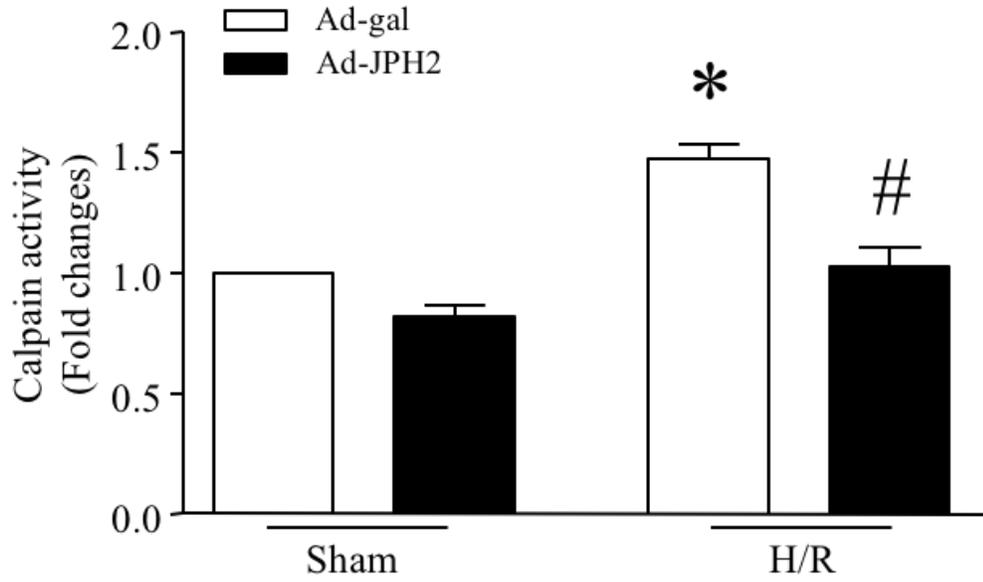
Dysregulation of  $\text{Ca}^{2+}$  induces calpain activation, which has been implicated in ischemic heart diseases [57, 181]. Since H/R induced aberrant  $\text{Ca}^{2+}$  leak from SR, we measured calpain activity in H/R-stimulated cardiomyocytes. H/R led to an increase in cleaved fragment of  $\alpha$ -spectrin (140kDa) after reoxygenation in a time-dependent manner [Figure 11], indicative of calpain activation as active calpain cleaves  $\alpha$ -spectrin and releases a 140KDa fragment.



**Figure 11: Measurement of calpain activation in H9c2 cells following H/R**

H9c2 cells were subjected to hypoxia for 24h, and were collected separately in different time point after reoxygenation. A western blot for the cleavage of  $\alpha$ -spectrin (140 kDa) from one experiment (not replicated) indicates calpain activation.

To further confirm the activation of calpain in H/R-stimulated cardiomyocytes, we measured its enzymatic activity. Consistently, calpain enzymatic activity was increased after H/R. Notably, upregulation of JPH2 by infection with Ad-JPH2 prevented calpain activation in H9c2 cells after H/R [Figure 12].

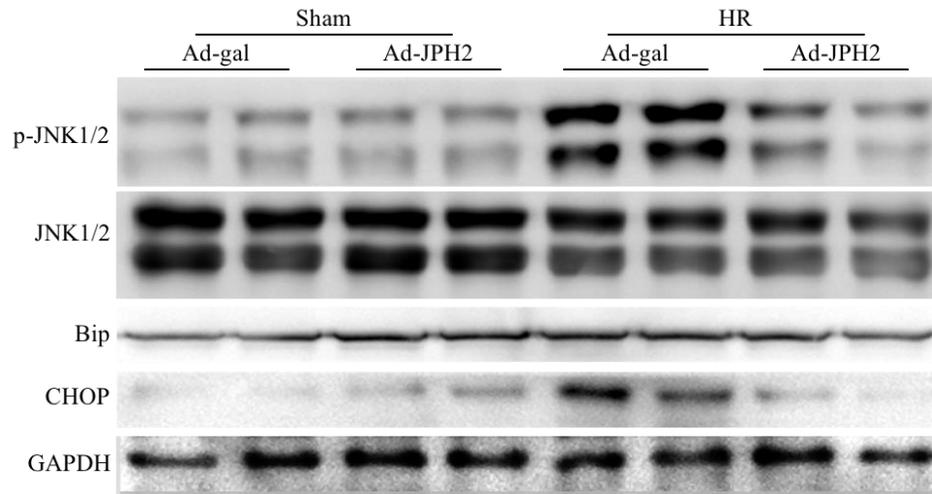


**Figure 12: Effects of JPH2 up-regulation on calpain activation in H9c2 cells following H/R**

H9c2 cells were infected with Ad-JPH2 or Ad-gal and then were subjected to a 24-hour hypoxia followed by a 24-hour reoxygenation. Calpain enzymatic activity was then measured in H9c2 cells. Data are mean  $\pm$  SD from 3 different experiments. \* $P < 0.05$  and # $P < 0.05$  analyzed by two-way ANOVA followed by the Newman-Keuls test.

### 3.8 Up-regulation of JPH2 attenuated ER stress in H/R-stimulated cardiomyocytes.

It has been well demonstrated that ER-stress promotes apoptosis and H/R induces ER stress in cardiomyocytes [132, 143, 178]. Having shown that JPH2 prevented apoptosis induced by H/R in cardiomyocytes, we hypothesized that JPH2 inhibited H/R-induced ER stress. To examine this hypothesis, we infected neonatal cardiomyocytes with Ad-JPH2 or Ad-gal and then performed H/R in these cells. H/R significantly increased the protein levels of GRP78, CHOP, and phosphorylated JNK1/2, indicative of ER stress. Upregulation of JPH2 by infection with Ad-JPH2 reduced their levels (CHOP and phosphorylated JNK1/2) in H/R-stimulated cardiomyocytes [Figure 13]. These results support our hypothesis that JPH2 inhibits ER stress induced by H/R.



**Figure 13: Effect of JPH2 over-expression on ER stress in primary neonatal cardiomyocytes following H/R**

Neonatal cardiomyocytes were infected with Ad-JPH2 or Ad-gal, and then subjected to a 24-hour hypoxia followed by a 24-hour reoxygenation. Representative western blots for ER stress markers from two different experiments.

## Chapter 4

### 4 Discussion, Limitation & Future direction

#### 4.1 Discussion

The major finding of this study is that JPH2 prevents H/R-induced apoptosis in cardiomyocytes. The anti-apoptotic role of JPH2 is associated with decreased  $\text{Ca}^{2+}$  leakage through RyR2 and inhibition of calpain activation, leading to prevention of ER stress in H/R-stimulated cardiomyocytes. To the best of our knowledge, this is the first report to demonstrate that JPH2 protects cardiomyocytes against H/R-induced apoptosis.

##### 4.1.1 JPH2 downregulation and its contribution to apoptosis in H/R injury

Down-regulation of JPH2 protein has been reported in various human heart diseases including hypotrophy, dilated cardiomyopathy and heart failure [77, 88, 95, 96], as well as in rodent models of heart diseases [93, 114, 115]. Several mechanisms have been demonstrated to contribute to the dysregulation of JPH2 in hearts under stress. First, early studies showed that upregulation of miR-24 was correlated with down-regulation of JPH2 protein in human diseased hearts and mouse heart tissues of pressure overload-induced cardiac hypertrophy [183]. Importantly, inhibition of miR-24 restored the protein levels of JPH2 in pressure over-load-induced mouse hearts, suggesting that miR-24 may target and repress JPH2 protein expression [183]. Indeed, there is a putative binding site in 3'-untranslational region of JPH2 mRNA for miR-24 and mutation of this putative binding site abrogated the inhibitory role of miR-24 in JPH2 expression [183]. Second, activation of calpain was reported to contribute to a reduction in JPH2 protein in cardiomyocytes following I/R and in ischemic hearts [184]. Incubation with calpain inhibitors increased the protein levels of JPH2 in cardiomyocytes following I/R. Further study identified R565T as the primary cleavage site of calpain-1 on the C-terminal of JPH2 [185]. Third, the functional defect of JPH2 has also been suggested. Several mutation regions in the gene encoding JPH2 have been affirmatively associated with the myocardial diseases heretofore, including S101R, Y141H, and S165F in hypertrophic cardiomyopathy (HCM)

[95], E169K in atrial arrhythmias [88], together with newly founded G505S and R436C [96], and [186].

The present study showed that H/R decreased the protein levels of JPH2 in cardiomyocytes. This result is consistent with previous reports. However, it remains unknown what causes the down-regulation of JPH2 in H/R-stimulated cardiomyocytes. Our data showed that H/R induced calpain activation in cardiomyocytes. Thus, it is possible that calpain activation may cleave JPH2 protein, leading to a reduction in JPH2 protein following H/R. Whether miR-24 plays a role in JPH2 expression in our system requires future investigations.

Although decreased JPH2 protein plays an important role in t-tubule remodeling in diseased hearts [114, 115, 117], an important finding of the present study is that JPH2 protects cardiomyocytes against H/R-induced apoptotic cell death. Several lines of evidence support this conclusion. First, decreased JPH2 protein was correlated with apoptosis in H/R-stimulated cardiomyocytes. Second, restoration of JPH2 protein inhibited caspase-3 activation and DNA fragmentation induced by H/R. Third, up-regulation of JPH2 prevented H/R-induced ER stress in cardiomyocytes. These data strongly support that decreased JPH2 contributes to H/R-induced cell death in cardiomyocytes. Given that apoptosis is implicated in myocardial injury and the development of heart failure following I/R, up-regulation of JPH2 may be a new approach to reduce I/R injury in hearts.

#### **4.1.2 Decreased JPH2 increases aberrant SR Ca<sup>2+</sup> release via RyR2, which contributes to H/R injury**

In addition to the important role of JPH2 in T-tubule system, recent studies have suggested that JPH2 might also have a regulatory role in RyR2 channel stability [85, 88, 89, 180]. However, whether JPH2 modulates RyR2-mediated aberrant SR Ca<sup>2+</sup> leakage during I/R remains unclear. In the present study, we show that H/R induces aberrant SR Ca<sup>2+</sup> release via RyR2 in cardiomyocytes, which agrees with previous report [187]. We further demonstrate that up-regulation of JPH2 reduces H/R-induced aberrant SR Ca<sup>2+</sup> release via RyR2 in cardiomyocytes. This result argues that JPH2 may stabilize RyR2

and reduce  $\text{Ca}^{2+}$  leak through RyR2. Increased cytosol  $\text{Ca}^{2+}$  has been implicated in apoptosis in cardiomyocytes through multiple mechanisms including  $\text{Ca}^{2+}$ /calmodulin [188, 189], calcineurin [190, 191] and calpain [57, 181]. Thus, it is highly possible that JPH2 prevents H/R-induced apoptosis in cardiomyocytes by stabilizing RyR2 and inhibiting  $\text{Ca}^{2+}$  leak through RyR2. In support of this view, we further showed that inhibition of RyR2 remarkably attenuated apoptosis and up-regulation of JPH2 prevented calpain activation in H/R-stimulated cardiomyocytes.

#### 4.1.3 Up-regulation of JPH2 prevents Calpain activation in H/R-stimulated cardiomyocytes

Calpain activation has been implicated in myocardial I/R injury [181, 192, 193]. Specifically, pharmacological and genetic inhibition of calpain reduces myocardial I/R injury in a global I/R model of isolated whole hearts and in vivo myocardial I/R animal models [156, 160, 166, 168, 170, 194]. Our recent study further demonstrated that calpain activation induces ER stress, which contributes to apoptosis in H/R-stimulated cardiomyocytes [178]. These studies provide strong evidence in support of the role of calpain in myocardial I/R injury. It is well known that elevation of intracellular  $\text{Ca}^{2+}$  induces calpain activation [144]. In the present study, we report that H/R increased  $\text{Ca}^{2+}$  release from SR via RyR2 and calpain activation in cardiomyocytes. Thus, it is possible that increased  $\text{Ca}^{2+}$  release from SR via RyR2 may result in elevation of intracellular  $\text{Ca}^{2+}$  concentration, which leads to calpain activation. In fact, our previous studies have demonstrated that blocking RyR2 channel prevents calpain activation in cardiomyocytes under septic and diabetic conditions as well as in response to norepinephrine [195-197]. Since up-regulation of JPH2 prevented  $\text{Ca}^{2+}$  release from SR via RyR2 in H/R-stimulated cardiomyocytes, we show that up-regulation of JPH2 decreased calpain activity. Thus, it is likely that JPH2 prevents H/R-induced calpain activation by inhibiting  $\text{Ca}^{2+}$  release from SR via RyR2 in cardiomyocytes.

Interestingly, our recent study revealed that calpain targeted and cleaved JPH2 in ischemic hearts [179]. This raises an intriguing possibility that active calpain can commence a positive feedback loop that potentiates  $\text{Ca}^{2+}$  dysregulation through JPH2

cleavage, increased intracellular  $\text{Ca}^{2+}$  and calpain activation, leading to lethal cell injury following I/R.

#### 4.1.4 Up-regulation of JPH2 attenuates the induction of ER stress

The sarcoplasmic reticulum(SR) collaborates with membrane T-tubules and is responsible for sarcomere contraction through  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) [37, 48]. In contrast, perinuclear endoplasmic reticulum (ER) in cardiomyocytes supports basic cellular functions such as protein synthesis, folding, post-translational modification and stress responses [198]. Many studies have implicated ER stress-initiated apoptosis in ischemic heart injury [56, 133, 135, 138-142]. In our previous study, we reported that ER stress induced apoptosis through JNK activation in H/R-stimulated cardiomyocytes [178]. In the present study, we found that up-regulation of JPH2 significantly reduced ER stress responses and prevented JNK1/2 activation in H/R-stimulated cardiomyocytes. Moreover, inhibition of RyR2 prevented ER stress and reduced apoptosis in H/R-stimulated cardiomyocytes. Thus, we argue that JPH2 inhibits H/R-induced ER stress by preventing RyR2-mediated  $\text{Ca}^{2+}$  leakage in cardiomyocytes.

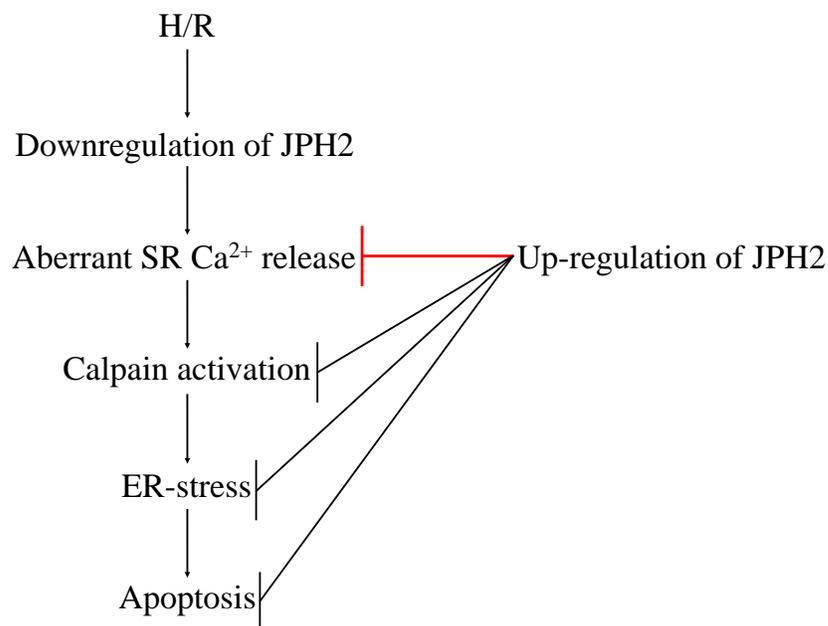
However, it is currently unknown how aberrant  $\text{Ca}^{2+}$  leakage via RyR2 contributes to ER stress in response to H/R. Our recent study demonstrated that calpain activation induced ER stress in cardiomyocytes and inhibition of calpain activation prevented H/R-induced ER stress [178]. Calpain has been shown to target and cleave  $\text{Ca}^{2+}$  regulatory proteins in SR. For example, calpain activation cleaves SERCA and RyR2 subunits, leading to depletion of  $\text{Ca}^{2+}$  in SR, which contributes to ER stress [122, 164-166]. Calpain may also activate caspase-12 [199, 200]. Interestingly, we show that up-regulation of JPH2 inhibited calpain activation in H/R-stimulated cardiomyocytes. Thus, inhibition of calpain activation may be a possible mechanism by which JPH2 prevents ER stress in H/R-induced cardiomyocytes.

In this study, we demonstrate a novel mechanism associating JPH2 to H/R-induced cell death. JPH2-RyR2 impairment acts as the main source of persistent  $\text{Ca}^{2+}$  dysregulation in the cardiomyocytes, which subsequently initiates a series of cellular alteration and ultimately leads to apoptosis. Since  $\text{Ca}^{2+}$  dysregulation is not exclusive to I/R injury, this

pattern is highly possibly involved in other kinds of cardiomyopathies, which require further investigation. Furthermore, the junctophilin family exists in all types of excitable cells and have been associated with different cell types of diseases. For instance, junctophilin-1 is mainly expressed in skeletal muscles and has been involved in eccentric contraction and prolonged muscle contraction when it loses its function [201, 202]. Though JPH3 and JPH4 distribute slightly differently in neural sites of the brain [203], studies have shown that their disruptions contribute to Huntington's disease-like 2 [204]. Based on the similarity of JMCs organized by junctophilin subtypes, the other members of junctophilins may also serve as regulators of their matched Ryanodine receptors. Therefore, other junctophilin family members (JPH1, JPH3 and JPH4) may provide similar protective roles other types of excitable cells.

#### 4.1.5 Concluding remarks

This thesis demonstrates that H/R induces JPH2 down-regulation and increases  $\text{Ca}^{2+}$  leakage from SR through RyR2, which consequently promotes calpain activation, leading to ER stress and apoptosis in cardiomyocytes. Thus, upregulation of JPH2 stabilizes RyR2 and reduces calpain activation by preventing the persistent aberrant  $\text{Ca}^{2+}$  release from SR, therefore protecting cardiomyocytes from ER stress and lethal cell injury in response to H/R. This study reveals a novel role of JPH2 in inhibiting apoptosis induced by H/R in cardiomyocytes and suggests that up-regulating JPH2 may be a potential therapeutic approach to reduce I/R-induced heart injury.



**Figure 14: Summary of the findings**

## 4.2 Limitations and future direction

Although H9c2 cells are originally derived from embryonic BDIX rat heart tissue sharing plenty biological similarities to cardiomyocytes and have been widely used for cardiac stress studies, this cell line lacks some morphological and electronic characteristics compared to the freshly isolated rat cardiomyocytes [205]. One of the differences is that H9c2 cells are absent of spontaneous or inducible beating. So, when it came to the Ca<sup>2+</sup> leakage experiment, we could not test whether the aberrant Ca<sup>2+</sup> release alters the Ca<sup>2+</sup> transients during the contraction process. Thus, for further investigations, we will conduct H/R and measure Ca<sup>2+</sup> transients on isolated rodent adult cardiomyocytes with observable spontaneous contraction.

Another limitation in Ca<sup>2+</sup> leakage experiment is about the administration of caffeine. Caffeine is known to mobilize store Ca<sup>2+</sup> drastically efflux from SR, which chalks up to its ability to reduce the release threshold of the RyR2 channel [206]. Caffeine has been

used to measure SR content [207]. Unfortunately, we have not yet been able to stimulate  $\text{Ca}^{2+}$  efflux in the H9c2 with caffeine.

$\text{Na}^+/\text{Ca}^{2+}$  exchanger(NCX) is a major cytosolic  $\text{Ca}^{2+}$  extrusion channel in cardiomyocytes, and its dysfunction has been demonstrated to contribute to  $\text{Ca}^{2+}$  overload in I/R injury [60]. Recent studies suggest that JPH2 may also co-localize with NCX and regulates its activity [89]. In our future study, we will investigate whether NCX dysfunction is involved in the JPH2-mediated  $\text{Ca}^{2+}$  dysregulation of I/R injury.

As mentioned before, caspase-12 correlates with calpain-mediated ER stress and JNK activation, but it has not been studied in I/R-stimulated cardiomyocytes. Thus, to clarify how calpain mediates ER stress, especially the pro-apoptotic JNK activation, we will examine caspase-12 in our future study.

In the future, we will extend to determine the protective role of JPH2 up-regulation in I/R-induced heart injury in vivo by using transgenic animals. Mechanistically, we will investigate the molecular mechanisms by which JPH2 protects RyR2 function in cardiomyocytes following I/R.

## Chapter 5

### 5 References

1. Mendis, S., P. Puska, and B. Norrving, *Global atlas on cardiovascular disease prevention and control*. 2011: World Health Organization.
2. Vanita Gorzkiewicz, J.L. and K. Kori, *Cardiac Care Quality Indicators: A New Hospital-Level Quality Improvement Initiative for Cardiac Care in Canada*. *Healthcare Quarterly*, 2012. **15**(1): p. 22-25.
3. Wong, N.D., *Epidemiological studies of CHD and the evolution of preventive cardiology*. *Nat Rev Cardiol*, 2014. **11**(5): p. 276-289.
4. Buja, L.M., *Myocardial ischemia and reperfusion injury*. *Cardiovascular Pathology*, 2005. **14**(4): p. 170-175.
5. Kalogeris, T., et al., *Cell Biology of Ischemia/Reperfusion Injury*. *International review of cell and molecular biology*, 2012. **298**: p. 229-317.
6. Reimer, K.A. and R.E. Ideker, *Myocardial ischemia and infarction: Anatomic and biochemical substrates for ischemic cell death and ventricular arrhythmias*. *Human Pathology*, 1987. **18**(5): p. 462-475.
7. Vakeva, A.P., et al., *Myocardial Infarction and Apoptosis After Myocardial Ischemia and Reperfusion*. *Circulation*, 1998. **97**(22): p. 2259.
8. Sanada, S., I. Komuro, and M. Kitakaze, *Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures*. *American Journal of Physiology - Heart and Circulatory Physiology*, 2011. **301**(5): p. H1723.
9. Allen, D.G. and C.H. Orchard, *Myocardial contractile function during ischemia and hypoxia*. *Circulation Research*, 1987. **60**(2): p. 153.
10. Buja, L.M., H.K. Hagler, and J.T. Willerson, *Altered calcium homeostasis in the pathogenesis of myocardial ischemic and hypoxic injury*. *Cell Calcium*, 1988. **9**(5-6): p. 205-217.
11. Buja, L.M. and M.L. Entman, *Modes of Myocardial Cell Injury and Cell Death in Ischemic Heart Disease*. *Circulation*, 1998. **98**(14): p. 1355.
12. Maroko, P.R., et al., *Coronary Artery Reperfusion: I. EARLY EFFECTS ON LOCAL MYOCARDIAL FUNCTION AND THE EXTENT OF MYOCARDIAL NECROSIS*. *The Journal of Clinical Investigation*, 1972. **51**(10): p. 2710-2716.
13. Ginks, W.R., et al., *Coronary Artery Reperfusion: II. REDUCTION OF MYOCARDIAL INFARCT SIZE AT 1 WEEK AFTER THE CORONARY OCCLUSION*. *The Journal of Clinical Investigation*, 1972. **51**(10): p. 2717-2723.
14. Van de Werf, F., *The history of coronary reperfusion*. *European Heart Journal*, 2014. **35**(37): p. 2510.
15. Rentrop, K.P. and F. Feit, *Reperfusion therapy for acute myocardial infarction: Concepts and controversies from inception to acceptance*. *American Heart Journal*, 2015. **170**(5): p. 971-980.
16. Jhund, P.S. and J.J.V. McMurray, *Heart Failure After Acute Myocardial Infarction*.

- Circulation, 2008. **118**(20): p. 2019.
17. Ross, H., et al., *Treating the right patient at the right time: Access to heart failure care*. Canadian Journal of Cardiology, 2006. **22**(9): p. 749-754.
  18. Blair, J.E.A., M. Huffman, and S.J. Shah, *Heart failure in north america*. Current cardiology reviews, 2013. **9**(2): p. 128-146.
  19. Garcia-Dorado, D., M. Ruiz-Meana, and H.M. Piper, *Lethal reperfusion injury in acute myocardial infarction: facts and unresolved issues*. Cardiovascular Research, 2009. **83**(2): p. 165.
  20. Hausenloy, D.J. and D.M. Yellon, *Myocardial ischemia-reperfusion injury: a neglected therapeutic target*. The Journal of Clinical Investigation, 2013. **123**(1): p. 92-100.
  21. Ibáñez, B., et al., *Evolving Therapies for Myocardial Ischemia/Reperfusion Injury*. Journal of the American College of Cardiology, 2015. **65**(14): p. 1454-1471.
  22. McCully, J.D., et al., *Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury*. American Journal of Physiology - Heart and Circulatory Physiology, 2004. **286**(5): p. H1923.
  23. Jaeschke, H. and J.J. Lemasters, *Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury*. Gastroenterology, 2003. **125**(4): p. 1246-1257.
  24. Gottlieb, R.A., et al., *Reperfusion injury induces apoptosis in rabbit cardiomyocytes*. Journal of Clinical Investigation, 1994. **94**(4): p. 1621.
  25. Eefting, F., et al., *Role of apoptosis in reperfusion injury*. Cardiovascular Research, 2004. **61**(3): p. 414.
  26. Hausenloy, D.J., et al., *Ischemic preconditioning protects by activating prosurvival kinases at reperfusion*. American Journal of Physiology - Heart and Circulatory Physiology, 2005. **288**(2): p. H971.
  27. Brini, M., et al., *Intracellular Calcium Homeostasis and Signaling*, in *Metallomics and the Cell*, L. Banci, Editor. 2013, Springer Netherlands: Dordrecht. p. 119-168.
  28. Petersen, O.H., M. Michalak, and A. Verkhratsky, *Calcium signalling: past, present and future*. Cell Calcium, 2005. **38**(3-4): p. 161-9.
  29. Clapham, D.E., *Calcium signaling*. Cell, 2007. **131**(6): p. 1047-58.
  30. Carafoli, E., *Calcium signaling: A tale for all seasons*. Proceedings of the National Academy of Sciences, 2002. **99**(3): p. 1115-1122.
  31. Berridge, M., P. Lipp, and M. Bootman, *Calcium signalling*. Current Biology, 1999. **9**(5): p. R157-R159.
  32. Clapham, D.E., *Calcium signaling*. Cell, 1995. **80**(2): p. 259-268.
  33. Bootman, M.D., et al., *Calcium signalling--an overview*. Semin Cell Dev Biol, 2001. **12**(1): p. 3-10.
  34. Fearnley, C.J., H.L. Roderick, and M.D. Bootman, *Calcium Signaling in Cardiac Myocytes*. Cold Spring Harbor Perspectives in Biology, 2011. **3**(11).
  35. Fukuta, H. and W.C. Little, *The Cardiac Cycle and the Physiological Basis of Left Ventricular Contraction, Ejection, Relaxation, and Filling*. Heart failure clinics, 2008. **4**(1): p. 1-11.
  36. Wang, S.-Q., et al., *Ca<sup>2+</sup> signalling between single L-type Ca<sup>2+</sup> channels and ryanodine receptors in heart cells*. Nature, 2001. **410**(6828): p. 592-596.

37. Fabiato, A., *Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum*. American Journal of Physiology-Cell Physiology, 1983. **245**(1): p. C1-C14.
38. Cheng, H. and W.J. Lederer, *Calcium Sparks*. Physiological Reviews, 2008. **88**(4): p. 1491.
39. Bers, D.M. and T.A.O. Guo, *Calcium Signaling in Cardiac Ventricular Myocytes*. Annals of the New York Academy of Sciences, 2005. **1047**(1): p. 86-98.
40. Shannon, T.R. and D.M. Bers, *Integrated Ca<sup>2+</sup> Management in Cardiac Myocytes*. Annals of the New York Academy of Sciences, 2004. **1015**(1): p. 28-38.
41. Lytton, J., et al., *Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps*. Journal of Biological Chemistry, 1992. **267**(20): p. 14483-14489.
42. Bridge, J.H., Smolley, and K.W. Spitzer, *The relationship between charge movements associated with I<sub>Ca</sub> and I<sub>Na-Ca</sub> in cardiac myocytes*. Science, 1990. **248**(4953): p. 376.
43. Garbino, A. and X.H. Wehrens, *Emerging role of junctophilin-2 as a regulator of calcium handling in the heart*. Acta Pharmacol Sin, 2010. **31**(9): p. 1019-21.
44. Carafoli, E., *The calcium-signalling saga: tap water and protein crystals*. Nature Reviews Molecular Cell Biology, 2003. **4**(4): p. 326-332.
45. Kretsinger, R.H. and D.J. Nelson, *Calcium in biological systems*. Coordination Chemistry Reviews, 1976. **18**(1): p. 29-124.
46. Carafoli, E. and C.B. Klee, *Calcium as a cellular regulator*. 1999: Oxford University Press, USA.
47. Schwaller, B., *Cytosolic Ca<sup>2+</sup> buffers*. Cold Spring Harbor perspectives in biology, 2010. **2**(11): p. a004051.
48. Yano, K. and A. Zarain-Herzberg, *Sarcoplasmic reticulum calsequestrins: structural and functional properties*. Molecular and cellular biochemistry, 1994. **135**(1): p. 61-70.
49. Chin, D. and A.R. Means, *Calmodulin: a prototypical calcium sensor*. Trends in cell biology, 2000. **10**(8): p. 322-328.
50. Lee, H.C., R. Aarhus, and R.M. Graeff, *Sensitization of calcium-induced calcium release by cyclic ADP-ribose and calmodulin*. Journal of Biological Chemistry, 1995. **270**(16): p. 9060-9066.
51. Røe, Å.T., M. Frisk, and W.E. Louch, *Targeting Cardiomyocyte Ca(2+) Homeostasis in Heart Failure*. Current Pharmaceutical Design, 2015. **21**(4): p. 431-448.
52. Bers, D.M., *Cardiac excitation-contraction coupling*. Nature, 2002. **415**(6868): p. 198-205.
53. Szabadkai, G. and M.R. Duchen, *Mitochondria: the hub of cellular Ca<sup>2+</sup> signaling*. Physiology, 2008. **23**(2): p. 84-94.
54. Rimessi, A., et al., *The versatility of mitochondrial calcium signals: from stimulation of cell metabolism to induction of cell death*. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 2008. **1777**(7): p. 808-816.
55. Glembotski, C.C., *Endoplasmic Reticulum Stress in the Heart*. Circulation Research, 2007. **101**(10): p. 975.

56. Minamino, T., I. Komuro, and M. Kitakaze, *Endoplasmic Reticulum Stress As a Therapeutic Target in Cardiovascular Disease*. Circulation Research, 2010. **107**(9): p. 1071.
57. Potz, B.A., et al., *Calpains and Coronary Vascular Disease*. Circ J, 2016. **80**(1): p. 4-10.
58. Garcarena, C.D., et al., *H<sup>+</sup>-activated Na<sup>+</sup> influx in the ventricular myocyte couples Ca<sup>2+</sup>-signalling to intracellular pH*. Journal of molecular and cellular cardiology, 2013. **61**: p. 51-59.
59. Garcia-Dorado, D., et al., *Calcium-mediated cell death during myocardial reperfusion*. Cardiovascular research, 2012. **94**(2): p. 168-180.
60. Chen, S. and S. Li, *The Na<sup>+</sup>/Ca<sup>2+</sup> exchanger in cardiac ischemia/reperfusion injury*. Medical science monitor: international medical journal of experimental and clinical research, 2012. **18**(11): p. RA161.
61. Kristián, T. and B.K. Siesjö, *Calcium in ischemic cell death*. Stroke, 1998. **29**(3): p. 705-718.
62. Dhalla, N.S. and T.A. Duhamel, *The paradoxes of reperfusion in the ischemic heart*. Heart Metab, 2007. **37**: p. 31-34.
63. Garcia-Dorado, D., M. Ruiz-Meana, and H.M. Piper, *Lethal reperfusion injury in acute myocardial infarction: facts and unresolved issues*. Cardiovascular research, 2009. **83**(2): p. 165-168.
64. Gill, C., R. Mestril, and A. Samali, *Losing heart: the role of apoptosis in heart disease—a novel therapeutic target?* The FASEB Journal, 2002. **16**(2): p. 135-146.
65. Gourdin, M. and P. Dubois, *Impact of Ischemia on Cellular Metabolism*. 2013.
66. Takeshima, H., et al., *Junctophilins: A Novel Family of Junctional Membrane Complex Proteins*. Molecular Cell, 2000. **6**(1): p. 11-22.
67. Nishi, M., et al., *Characterization of human junctophilin subtype genes*. Biochem Biophys Res Commun, 2000. **273**(3): p. 920-7.
68. Garbino, A., et al., *Molecular evolution of the junctophilin gene family*. Physiological Genomics, 2009. **37**(3): p. 175-186.
69. Huang, Y.-C., et al., *JPH2 is a novel susceptibility gene on chromosome 20q associated with diabetic retinopathy in a Taiwanese population*. ScienceAsia, 2013. **39**(2): p. 167.
70. Im, Y.J., et al., *The N-terminal membrane occupation and recognition nexus domain of Arabidopsis phosphatidylinositol phosphate kinase 1 regulates enzyme activity*. J Biol Chem, 2007. **282**(8): p. 5443-52.
71. Nishi, M., et al., *Characterization of Human Junctophilin Subtype Genes*. Biochemical and Biophysical Research Communications, 2000. **273**(3): p. 920-927.
72. Hoffmann, R. and A. Valencia, *Implementing the iHOP concept for navigation of biomedical literature*. Bioinformatics, 2005. **21**(suppl\_2): p. ii252-ii258.
73. Porter, K.R. and G.E. Palade, *Studies on the endoplasmic reticulum: III. Its form and distribution in striated muscle cells*. The Journal of biophysical and biochemical cytology, 1957. **3**(2): p. 269.
74. Friedman, J.R. and G.K. Voeltz, *The ER in 3D: a multifunctional dynamic membrane network*. Trends in cell biology, 2011. **21**(12): p. 709-717.

75. Rosenbluth, J., *Subsurface cisterns and their relationship to the neuronal plasma membrane*. The Journal of cell biology, 1962. **13**(3): p. 405.
76. Franzini-Armstrong, C. and F. Protasi, *Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions*. Physiological Reviews, 1997. **77**(3): p. 699-729.
77. Beavers, D.L., et al., *Emerging roles of junctophilin-2 in the heart and implications for cardiac diseases*. Cardiovasc Res, 2014. **103**(2): p. 198-205.
78. Van Oort, R.J., et al., *Disrupted junctional membrane complexes and hyperactive ryanodine receptors after acute junctophilin knockdown in mice*. Circulation, 2011: p. CIRCULATIONAHA-110.
79. Dibb, K.M., et al., *A functional role for transverse (t-) tubules in the atria*. Journal of molecular and cellular cardiology, 2013. **58**: p. 84-91.
80. Song, L.S., et al., *Calcium biology of the transverse tubules in heart*. Annals of the New York Academy of Sciences, 2005. **1047**(1): p. 99-111.
81. Brette, F. and C. Orchard, *T-Tubule Function in Mammalian Cardiac Myocytes*. Circulation Research, 2003. **92**(11): p. 1182.
82. Han, J., et al., *Morphogenesis of T-tubules in heart cells: the role of junctophilin-2*. Sci China Life Sci, 2013. **56**(7): p. 647-52.
83. Chen, B., et al., *Critical roles of junctophilin-2 in T-tubule and excitation-contraction coupling maturation during postnatal development*. Cardiovasc Res, 2013. **100**(1): p. 54-62.
84. Reynolds, J.O., et al., *Junctophilin-2 is necessary for T-tubule maturation during mouse heart development*. Cardiovasc Res, 2013. **100**(1): p. 44-53.
85. van Oort, R.J., et al., *Disrupted Junctional Membrane Complexes and Hyperactive Ryanodine Receptors Following Acute Junctophilin Knockdown in Mice*. Circulation, 2011. **123**(9): p. 979-988.
86. Jayasinghe, Isuru D., et al., *Nanoscale Organization of Junctophilin-2 and Ryanodine Receptors within Peripheral Couplings of Rat Ventricular Cardiomyocytes*. Biophysical Journal, 2012. **102**(5): p. L19-L21.
87. Hou, Y., et al., *Nanoscale analysis of ryanodine receptor clusters in dyadic couplings of rat cardiac myocytes*. J Mol Cell Cardiol, 2015. **80**: p. 45-55.
88. Beavers, D.L., et al., *Mutation E169K in junctophilin-2 causes atrial fibrillation due to impaired RyR2 stabilization*. Journal of the American College of Cardiology, 2013. **62**(21): p. 10.1016/j.jacc.2013.06.052.
89. Wang, W., et al., *Reduced junctional Na(+)/Ca(2+)-exchanger activity contributes to sarcoplasmic reticulum Ca(2+) leak in junctophilin-2-deficient mice*. American Journal of Physiology - Heart and Circulatory Physiology, 2014. **307**(9): p. H1317-H1326.
90. Maron, B.J., et al., *Prevalence of hypertrophic cardiomyopathy in a general population of young adults*. Circulation, 1995. **92**(4): p. 785-789.
91. Maron, B.J., *Sudden death in young athletes*. New England Journal of Medicine, 2003. **349**(11): p. 1064-1075.
92. Maron, B.J., S.E. Epstein, and W.C. Roberts, *Causes of sudden death in competitive athletes*. Journal of the American College of Cardiology, 1986. **7**(1): p.

- 204-214.
93. Minamisawa, S., et al., *Junctophilin type 2 is associated with caveolin-3 and is down-regulated in the hypertrophic and dilated cardiomyopathies*. *Biochem Biophys Res Commun*, 2004. **325**(3): p. 852-6.
  94. Landstrom, A.P., et al., *Junctophilin-2 expression silencing causes cardiocyte hypertrophy and abnormal intracellular calcium-handling*. *Circ Heart Fail*, 2011. **4**(2): p. 214-23.
  95. Landstrom, A.P., et al., *Mutations in JPH2-encoded junctophilin-2 associated with hypertrophic cardiomyopathy in humans*. *Journal of molecular and cellular cardiology*, 2007. **42**(6): p. 1026-1035.
  96. Matsushita, Y., et al., *Mutation of junctophilin type 2 associated with hypertrophic cardiomyopathy*. *J Hum Genet*, 2007. **52**(6): p. 543-8.
  97. P Landstrom, A. and M. J Ackerman, *Beyond the cardiac myofilament: hypertrophic cardiomyopathy-associated mutations in genes that encode calcium-handling proteins*. *Current molecular medicine*, 2012. **12**(5): p. 507-518.
  98. Kemp, C.D. and J.V. Conte, *The pathophysiology of heart failure*. *Cardiovascular Pathology*, 2012. **21**(5): p. 365-371.
  99. Hasenfuss, G. and B. Pieske, *Calcium cycling in congestive heart failure*. *Journal of molecular and cellular cardiology*, 2002. **34**(8): p. 951-969.
  100. Luo, M. and M.E. Anderson, *Mechanisms of altered Ca<sup>2+</sup> handling in heart failure*. *Circulation research*, 2013. **113**(6): p. 690-708.
  101. Kostin, S., et al., *The internal and external protein scaffold of the T-tubular system in cardiomyocytes*. *Cell and tissue research*, 1998. **294**(3): p. 449-460.
  102. Schaper, J., et al., *Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy*. *Circulation*, 1991. **83**(2): p. 504-514.
  103. Kaprielian, R.R., et al., *Distinct patterns of dystrophin organization in myocyte sarcolemma and transverse tubules of normal and diseased human myocardium*. *Circulation*, 2000. **101**(22): p. 2586-2594.
  104. He, J.-Q., et al., *Reduction in density of transverse tubules and L-type Ca<sup>2+</sup> channels in canine tachycardia-induced heart failure*. *Cardiovascular research*, 2001. **49**(2): p. 298-307.
  105. Balijepalli, R.C., et al., *Depletion of T-tubules and specific subcellular changes in sarcolemmal proteins in tachycardia-induced heart failure*. *Cardiovascular research*, 2003. **59**(1): p. 67-77.
  106. Shannon, T.R., S.M. Pogwizd, and D.M. Bers, *Elevated sarcoplasmic reticulum Ca<sup>2+</sup> leak in intact ventricular myocytes from rabbits in heart failure*. *Circulation research*, 2003.
  107. Bers, D.M., D.A. Eisner, and H.H. Valdivia, *Sarcoplasmic reticulum Ca<sup>2+</sup> and heart failure*. 2003, Am Heart Assoc.
  108. Ai, X., et al., *Ca<sup>2+</sup>/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca<sup>2+</sup> leak in heart failure*. *Circulation research*, 2005. **97**(12): p. 1314-1322.
  109. Bito, V., et al., *Crosstalk between L-type Ca<sup>2+</sup> channels and the sarcoplasmic reticulum: alterations during cardiac remodelling*. *Cardiovascular research*, 2008.

- 77(2): p. 315-324.
110. Lipp, P., et al., *Spatially non-uniform Ca<sup>2+</sup> signals induced by the reduction of transverse tubules in citrate-loaded guinea-pig ventricular myocytes in culture*. The Journal of Physiology, 1996. **497**(Pt 3): p. 589.
  111. Louch, W.E., et al., *Reduced synchrony of Ca<sup>2+</sup> release with loss of T-tubules—a comparison to Ca<sup>2+</sup> release in human failing cardiomyocytes*. Cardiovascular Research, 2004. **62**(1): p. 63-73.
  112. Hasenfuss, G., et al., *Relation between myocardial function and expression of sarcoplasmic reticulum Ca (2+)-ATPase in failing and nonfailing human myocardium*. Circulation research, 1994. **75**(3): p. 434-442.
  113. Litwin, S.E., D. Zhang, and J.H.B. Bridge, *Dyssynchronous Ca<sup>2+</sup> sparks in myocytes from infarcted hearts*. Circulation Research, 2000. **87**(11): p. 1040-1047.
  114. Wu, C.Y., et al., *Calpain-dependent cleavage of junctophilin-2 and T-tubule remodeling in a mouse model of reversible heart failure*. J Am Heart Assoc, 2014. **3**(3): p. e000527.
  115. Zhang, C., et al., *Microtubule-mediated defects in junctophilin-2 trafficking contribute to myocyte transverse-tubule remodeling and Ca<sup>2+</sup> handling dysfunction in heart failure*. Circulation, 2014. **129**(17): p. 1742-50.
  116. Guo, A., et al., *Overexpression of junctophilin-2 does not enhance baseline function but attenuates heart failure development after cardiac stress*. Proc Natl Acad Sci U S A, 2014. **111**(33): p. 12240-5.
  117. Reynolds, J.O., et al., *Junctophilin-2 gene therapy rescues heart failure by normalizing RyR2-mediated Ca<sup>2+</sup> release*. Int J Cardiol, 2016. **225**: p. 371-380.
  118. Garfield, S.A. and R.R. Cardell Jr, *Endoplasmic reticulum: rough and smooth*. Int. Rev. Cytol. Suppl, 1987. **17**: p. 255-273.
  119. Michalak, M., J.M.R. Parker, and M. Opas, *Ca<sup>2+</sup> signaling and calcium binding chaperones of the endoplasmic reticulum*. Cell calcium, 2002. **32**(5): p. 269-278.
  120. Görlach, A., P. Klappa, and D.T. Kietzmann, *The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control*. Antioxidants & redox signaling, 2006. **8**(9-10): p. 1391-1418.
  121. Pozzan, T., et al., *Molecular and cellular physiology of intracellular calcium stores*. Physiological reviews, 1994. **74**(3): p. 595-637.
  122. Mekahli, D., et al., *Endoplasmic-reticulum calcium depletion and disease*. Cold Spring Harbor perspectives in biology, 2011. **3**(6): p. a004317.
  123. Xu, C., B. Bailly-Maitre, and J.C. Reed, *Endoplasmic reticulum stress: cell life and death decisions*. The Journal of clinical investigation, 2005. **115**(10): p. 2656-2664.
  124. Dickhout, J.G., R.E. Carlisle, and R.C. Austin, *Interrelationship between cardiac hypertrophy, heart failure, and chronic kidney disease*. Circulation Research, 2011. **108**(5): p. 629-642.
  125. Groenendyk, J., et al., *Biology of endoplasmic reticulum stress in the heart*. Circulation research, 2010. **107**(10): p. 1185-1197.
  126. Shi, Y., et al., *Identification and characterization of pancreatic eukaryotic initiation factor 2  $\alpha$ -subunit kinase, PEK, involved in translational control*. Molecular and cellular biology, 1998. **18**(12): p. 7499-7509.

127. Ma, K., K.M. Vattem, and R.C. Wek, *Dimerization and release of molecular chaperone inhibition facilitate activation of eukaryotic initiation factor-2 kinase in response to endoplasmic reticulum stress*. Journal of Biological Chemistry, 2002. **277**(21): p. 18728-18735.
128. Cox, J.S., C.E. Shamu, and P. Walter, *Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase*. Cell, 1993. **73**(6): p. 1197-1206.
129. Shamu, C.E. and P. Walter, *Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus*. The EMBO journal, 1996. **15**(12): p. 3028.
130. Yoshida, H., et al., *Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins Involvement of basic leucine zipper transcription factors*. Journal of Biological Chemistry, 1998. **273**(50): p. 33741-33749.
131. Shen, J., et al., *Stable binding of ATF6 to BiP in the endoplasmic reticulum stress response*. Molecular and cellular biology, 2005. **25**(3): p. 921-932.
132. Sano, R. and J.C. Reed, *ER stress-induced cell death mechanisms*. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2013. **1833**(12): p. 3460-3470.
133. Martindale, J.J., et al., *Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6*. Circulation research, 2006. **98**(9): p. 1186-1193.
134. Toko, H., et al., *ATF6 is important under both pathological and physiological states in the heart*. Journal of molecular and cellular cardiology, 2010. **49**(1): p. 113-120.
135. Thuerauf, D.J., et al., *Activation of the unfolded protein response in infarcted mouse heart and hypoxic cultured cardiac myocytes*. Circulation research, 2006. **99**(3): p. 275-282.
136. Eletto, D., D. Dersh, and Y. Argon. *GRP94 in ER quality control and stress responses*. Elsevier.
137. Kapulkin, V., B.G. Hiester, and C.D. Link, *Compensatory regulation among ER chaperones in C. elegans*. FEBS letters, 2005. **579**(14): p. 3063-3068.
138. Vitadello, M., et al., *Overexpression of the stress protein Grp94 reduces cardiomyocyte necrosis due to calcium overload and simulated ischemia*. The FASEB Journal, 2003. **17**(8): p. 923-925.
139. Terai, K., et al., *AMP-activated protein kinase protects cardiomyocytes against hypoxic injury through attenuation of endoplasmic reticulum stress*. Molecular and cellular biology, 2005. **25**(21): p. 9554-9575.
140. Tao, J., et al., *Apelin-13 protects the heart against ischemia-reperfusion injury through inhibition of ER-dependent apoptotic pathways in a time-dependent fashion*. American Journal of Physiology-Heart and Circulatory Physiology, 2011. **301**(4): p. H1471-H1486.
141. Nickson, P., A. Toth, and P. Erhardt, *PUMA is critical for neonatal cardiomyocyte apoptosis induced by endoplasmic reticulum stress*. Cardiovascular research,

2007. **73**(1): p. 48-56.
142. Toth, A., et al., *Targeted deletion of Puma attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion*. American journal of physiology. Heart and circulatory physiology, 2006. **291**(1): p. H52-60.
143. Zhao, L. and S.L. Ackerman, *Endoplasmic reticulum stress in health and disease*. Current opinion in cell biology, 2006. **18**(4): p. 444-452.
144. Goll, D.E., et al., *The calpain system*. Physiological reviews, 2003. **83**(3): p. 731-801.
145. Cong, J., et al., *The role of autolysis in activity of the Ca<sup>2+</sup>-dependent proteinases (*mu*-calpain and *m*-calpain)*. Journal of Biological Chemistry, 1989. **264**(17): p. 10096-10103.
146. Davies, P.L., et al., *The effects of truncations of the small subunit on m-calpain activity and heterodimer formation*. Biochemical Journal, 1997. **326**(1): p. 31-38.
147. Arthur, J.S.C., P.A. Greer, and J.S. Elce, *Structure of the mouse calpain small subunit gene*. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1998. **1388**(1): p. 247-252.
148. Arthur, J.S.C., et al., *Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division*. Molecular and cellular biology, 2000. **20**(12): p. 4474-4481.
149. Suzuki, K., et al., *Calpain: novel family members, activation, and physiological function*. Biological Chemistry Hoppe Seyler, 1995. **376**: p. 523-523.
150. Crawford, C., *Protein and peptide inhibitors of calpains*. CRC Press. Boca Raton, FL, 1990: p. 75-89.
151. Neuhof, C. and H. Neuhof, *Calpain system and its involvement in myocardial ischemia and reperfusion injury*. World J Cardiol, 2014. **6**(7): p. 638-52.
152. Armstrong, S.C., et al., *Ischemic loss of sarcolemmal dystrophin and spectrin: correlation with myocardial injury*. Journal of molecular and cellular cardiology, 2001. **33**(6): p. 1165-1179.
153. Insete, J., et al., *Ischemic preconditioning attenuates calpain-mediated degradation of structural proteins through a protein kinase A-dependent mechanism*. Cardiovascular Research, 2004. **64**(1): p. 105-114.
154. Wang, K.K.W., *Calpain and caspase: can you tell the difference?* Trends in neurosciences, 2000. **23**(1): p. 20-26.
155. Yoshida, K.-i., et al., *Reperfusion of rat heart after brief ischemia induces proteolysis of caldesmon (nonerythroid spectrin or fodrin) by calpain*. Circulation Research, 1995. **77**(3): p. 603-610.
156. Yoshikawa, Y., et al., *Calpain inhibitor-1 protects the rat heart from ischemia-reperfusion injury: analysis by mechanical work and energetics*. Am J Physiol Heart Circ Physiol, 2005. **288**(4): p. H1690-8.
157. Hernando, V., et al., *Calpain translocation and activation as pharmacological targets during myocardial ischemia/reperfusion*. J Mol Cell Cardiol, 2010. **49**(2): p. 271-9.
158. Larsen, T.H., et al., *Membrane skeleton in cultured chick cardiac myocytes revealed by high resolution immunocytochemistry*. Histochemistry and cell

- biology, 1999. **112**(4): p. 307-316.
159. French, J.P., et al., *Exercise-induced protection against myocardial apoptosis and necrosis: MnSOD, calcium-handling proteins, and calpain*. The FASEB Journal, 2008. **22**(8): p. 2862-2871.
  160. Yoshikawa, Y., et al., *Cardioprotective effects of a novel calpain inhibitor SNJ-1945 for reperfusion injury after cardioplegic cardiac arrest*. Am J Physiol Heart Circ Physiol, 2010. **298**(2): p. H643-51.
  161. Rubtsov, A.M. and O.D. Lopina, *Ankyrins*. FEBS letters, 2000. **482**(1-2): p. 1-5.
  162. Inserte, J., et al., *Calpain-mediated impairment of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity during early reperfusion contributes to cell death after myocardial ischemia*. Circ Res, 2005. **97**(5): p. 465-73.
  163. Garcia-Dorado, D., et al., *Calcium-mediated cell death during myocardial reperfusion*. Cardiovasc Res, 2012. **94**(2): p. 168-80.
  164. Singh, R.B., et al., *The sarcoplasmic reticulum proteins are targets for calpain action in the ischemic-reperfused heart*. J Mol Cell Cardiol, 2004. **37**(1): p. 101-10.
  165. Temsah, R.M., et al., *Alterations in sarcoplasmic reticulum function and gene expression in ischemic-reperfused rat heart*. American Journal of Physiology-Heart and Circulatory Physiology, 1999. **277**(2): p. H584-H594.
  166. French, J.P., et al., *Ischemia-reperfusion-induced calpain activation and SERCA2a degradation are attenuated by exercise training and calpain inhibition*. Am J Physiol Heart Circ Physiol, 2006. **290**(1): p. H128-36.
  167. Gilchrist, J.S.C., et al., *Extensive autolytic fragmentation of membranous versus cytosolic calpain following myocardial ischemia-reperfusion*. Canadian journal of physiology and pharmacology, 2010. **88**(5): p. 584-594.
  168. Trumbeckaite, S., et al., *Calpain inhibitor (BSF 409425) diminishes ischemia/reperfusion-induced damage of rabbit heart mitochondria*. Biochemical pharmacology, 2003. **65**(5): p. 911-916.
  169. Otani, H., et al., *In vitro study on contribution of oxidative metabolism of isolated rabbit heart mitochondria to myocardial reperfusion injury*. Circulation research, 1984. **55**(2): p. 168-175.
  170. Neuhof, C., et al., *A novel water-soluble and cell-permeable calpain inhibitor protects myocardial and mitochondrial function in postischemic reperfusion*. Biological chemistry, 2003. **384**(12): p. 1597-1603.
  171. Chen, Q. and E.J. Lesnfsky, *Heart mitochondria and calpain 1: Location, function, and targets*. Biochim Biophys Acta, 2015. **1852**(11): p. 2372-8.
  172. Honda, H.M., P. Korge, and J.N. Weiss, *Mitochondria and ischemia/reperfusion injury*. Ann N Y Acad Sci, 2005. **1047**: p. 248-58.
  173. Yu, S.-W., et al., *Mediation of poly (ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor*. Science, 2002. **297**(5579): p. 259-263.
  174. Ozaki, T., et al., *Characteristics of mitochondrial calpains*. Journal of biochemistry, 2007. **142**(3): p. 365-376.
  175. Kar, P., et al., *Mitochondrial calpain system: an overview*. Archives of biochemistry and biophysics, 2010. **495**(1): p. 1-7.
  176. Norberg, E., et al., *Oxidative modification sensitizes mitochondrial apoptosis-*

- inducing factor to calpain-mediated processing*. Free Radical Biology and Medicine, 2010. **48**(6): p. 791-797.
177. Chen, Q., et al., *Activation of mitochondrial mu-calpain increases AIF cleavage in cardiac mitochondria during ischemia-reperfusion*. Biochem Biophys Res Commun, 2011. **415**(4): p. 533-8.
  178. Zheng, D., et al., *Calpain-1 induces endoplasmic reticulum stress in promoting cardiomyocyte apoptosis following hypoxia/reoxygenation*. Biochim Biophys Acta, 2015. **1852**(5): p. 882-92.
  179. Guo, A., et al., *Molecular determinants of calpain-dependent cleavage of junctophilin-2 protein in cardiomyocytes*. Journal of Biological Chemistry, 2015. **290**(29): p. 17946-17955.
  180. Hill, J.A. and A. Diwan, *Ca(2+) Leak in AF: Junctophilin-2 Stabilizes Ryanodine Receptor*. Journal of the American College of Cardiology, 2013. **62**(21): p. 10.1016/j.jacc.2013.07.054.
  181. Inserte, J., V. Hernando, and D. Garcia-Dorado, *Contribution of calpains to myocardial ischaemia/reperfusion injury*. Cardiovasc Res, 2012. **96**(1): p. 23-31.
  182. Portal, L., et al., *A model of hypoxia-reoxygenation on isolated adult mouse cardiomyocytes: characterization, comparison with ischemia-reperfusion, and application to the cardioprotective effect of regular treadmill exercise*. Journal of cardiovascular pharmacology and therapeutics, 2013. **18**(4): p. 367-375.
  183. Xu, M., et al., *Mir-24 regulates junctophilin-2 expression in cardiomyocytes*. Circ Res, 2012. **111**(7): p. 837-41.
  184. Murphy, R.M., et al., *Ca2+-dependent proteolysis of junctophilin-1 and junctophilin-2 in skeletal and cardiac muscle*. J Physiol, 2013. **591**(3): p. 719-29.
  185. Guo, A., et al., *Molecular Determinants of Calpain-dependent Cleavage of Junctophilin-2 Protein in Cardiomyocytes*. J Biol Chem, 2015. **290**(29): p. 17946-55.
  186. Sabater-Molina, M., et al., *Mutation in JPH2 cause dilated cardiomyopathy*. Clin Genet, 2016. **90**(5): p. 468-469.
  187. Fauconnier, J., et al., *Ryanodine receptor leak mediated by caspase-8 activation leads to left ventricular injury after myocardial ischemia-reperfusion*. Proceedings of the National Academy of Sciences, 2011. **108**(32): p. 13258-13263.
  188. Ozcan, L. and I. Tabas, *Pivotal role of calcium/calmodulin-dependent protein kinase II in ER stress-induced apoptosis*. 2010, Taylor & Francis.
  189. Timmins, J.M., et al., *Calcium/calmodulin-dependent protein kinase II links ER stress with Fas and mitochondrial apoptosis pathways*. The Journal of clinical investigation, 2009. **119**(10): p. 2925-2941.
  190. Wang, H.-G., et al., *Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD*. Science, 1999. **284**(5412): p. 339-343.
  191. Saito, S., et al.,  *$\beta$ -Adrenergic pathway induces apoptosis through calcineurin activation in cardiac myocytes*. Journal of Biological Chemistry, 2000. **275**(44): p. 34528-34533.
  192. Chen, M., et al., *Calpain and mitochondria in ischemia/reperfusion injury*. J Biol Chem, 2002. **277**(32): p. 29181-6.

193. Muller, A.L., L.V. Hryshko, and N.S. Dhalla, *Extracellular and intracellular proteases in cardiac dysfunction due to ischemia-reperfusion injury*. Int J Cardiol, 2013. **164**(1): p. 39-47.
194. Takeshita, D., et al., *A new calpain inhibitor protects left ventricular dysfunction induced by mild ischemia-reperfusion in in situ rat hearts*. The Journal of Physiological Sciences, 2013. **63**(2): p. 113-123.
195. Li, Y., et al., *Calpain activation contributes to hyperglycaemia-induced apoptosis in cardiomyocytes*. Cardiovascular research, 2009: p. cvp189.
196. Li, X., et al., *Over-expression of calpastatin inhibits calpain activation and attenuates myocardial dysfunction during endotoxaemia*. Cardiovascular research, 2009. **83**(1): p. 72-79.
197. Li, Y., et al., *Taurine prevents cardiomyocyte death by inhibiting NADPH oxidase-mediated calpain activation*. Free Radical Biology and Medicine, 2009. **46**(1): p. 51-61.
198. Michalak, M. and M. Opas, *Endoplasmic and sarcoplasmic reticulum in the heart*. Trends in cell biology, 2009. **19**(6): p. 253-259.
199. Tan, Y., et al., *Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis*. Journal of Biological Chemistry, 2006. **281**(23): p. 16016-16024.
200. Bajaj, G. and R.K. Sharma, *TNF- $\alpha$ -mediated cardiomyocyte apoptosis involves caspase-12 and calpain*. Biochemical and biophysical research communications, 2006. **345**(4): p. 1558-1564.
201. Corona, B.T., et al., *Junctophilin damage contributes to early strength deficits and EC coupling failure after eccentric contractions*. American Journal of Physiology-Cell Physiology, 2010. **298**(2): p. C365-C376.
202. Murphy, R.M., et al., *Ca<sup>2+</sup> - dependent proteolysis of junctophilin - 1 and junctophilin - 2 in skeletal and cardiac muscle*. The Journal of physiology, 2013. **591**(3): p. 719-729.
203. Nishi, M., et al., *Coexpression of junctophilin type 3 and type 4 in brain*. Molecular brain research, 2003. **118**(1): p. 102-110.
204. Seixas, A.I., et al., *Loss of junctophilin - 3 contributes to huntington disease - like 2 pathogenesis*. Annals of neurology, 2012. **71**(2): p. 245-257.
205. Hescheler, J., et al., *Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart*. Circulation research, 1991. **69**(6): p. 1476-1486.
206. Kong, H., et al., *Caffeine induces Ca<sup>2+</sup> release by reducing the threshold for luminal Ca<sup>2+</sup> activation of the ryanodine receptor*. Biochemical Journal, 2008. **414**(3): p. 441-452.
207. Klein, M.G., B.J. Simon, and M.F. Schneider, *Effects of caffeine on calcium release from the sarcoplasmic reticulum in frog skeletal muscle fibres*. The Journal of physiology, 1990. **425**: p. 599.

# Curriculum Vitae

Zijun Su

## EDUCATION

---

|             |      |  |
|-------------|------|--|
| 2015 -      | MSc  | Western University, London, Ontario                                    |
|             |      | Master of Science, Research based                                      |
|             |      | Field/Discipline: Pathology  |
|             |      | Supervisor: Dr. Tianqing Peng  |
|             |      | Co-supervisor: Dr. Stephen Sims  |
| 2007 - 2012 | MBBS | Guangzhou Medical University, Guangzhou city, Guangdong,<br>P.R. China |
|             |      | Bachelor of Medicine and Bachelor of Surgery                           |
|             |      | Field/Discipline: Clinical Medicine                                    |

## RESEARCH EXPERIENCE

---

- 2014 - 2017      MSc in Pathology and Laboratory Medicine,  
  
Western University, London, Ontario  
  
Supervisor: Dr. Tianqing Peng  
  
Co-supervisor: Dr. Stephen Sims  
  
Thesis project: The protective role of JPH2 in ischemia/reperfusion injury
- 2013 - 2014      Research Assistant in the Central Laboratory,  
  
Guangzhou General Hospital of Guangzhou Military Command of Chinese PLA,  
Guangzhou city, Guangdong, P.R China  
  
Supervisor: Dr. Hongbing Zhang  
  
Thesis project: Influence of miRNA upon condition of hepatitis B's  
  
Immune tolerance
- 2012 - 2013      Lab Shadowing Technician in the Key Lab for Shock and  
  
Microcirculation Research  
  
Southern Medical University. Guangzhou city, Guangdong, P.R China  
  
Supervisor: Dr. Rong Shi  
  
Responsibility: using RPMI-1640 10% fetal bovine medium to cultivate vero

---

cell, in charge of vero cell exchange, passage, planking cryopreservation and thawing, conducting initial denaturation.

2011 - 2012 Undergrad Research Assistance in Department of Pathophysiology,  
Guangzhou Medical University, Guangzhou city, Guangdong, P.R.China  
Supervisor: Dr. Benchang Shen  
Thesis project: Different Adjuvant'S Influences upon Th17 Cell

## WORK EXPERIENCE

---

2011 - 2012 Clinical Medicine Internship in the Second Affiliated Hospital of Guangzhou  
Medical University, Guangzhou city, Guangdong, P.R. China

## TECHNICAL EXPERIENCE

---

- Calcium Imaging: digital fluorescence imaging using  $\text{Ca}^{2+}$ -sensitive fluorescent dyes to monitor regional changes of calcium in single cell.
- Micro-pipet and muscle cells stimulation.
- Cellular and Molecular Biology: including Western blot, caspase-3 activity assay, ELISA DNA fragmentation assay, calpain activity assay and PCR.
- Neonatal cardiomyocytes isolation, adenoviral trisection and in vitro hypoxia/reoxygenation treatment.

- 
- Proficient in Microsoft Excel, Microsoft PowerPoint, SPSS statistics software and Graph Pad Prism

## EXTRACURRICULAR

---

- 2010 - 2011            Minister of Student Union
- Organizer of the School Dancing Competition, the College Sports Meeting Opening Ceremony and awarded as "Excellent Leader"
- 2009 - 2010            Vice Minister of Student Union
- Participated in organizing the School Singing Competition and awarded as "Excellent Leader"

## PUBLICATION AND ABSTRACT

---

- 2016                    Pathology and Laboratory Medicine Research Day, London, Ontario
- Abstract accepted: "Up-regulation of junctophilin-2 prevents ER stress and Up-regulation of junctophilin-2 prevents ER stress and apoptosis in hypoxia/reoxygenation-stimulated H9c2 cells"
- Poster presenting at April 7th

---

2015

Pathology and Laboratory Medicine Research Day, London, Ontario

Abstract accepted: "The protective role of JPH2 in ischemia/reperfusion injury"

Poster presented at April 7th