

**Myocardial Ischemia/Reperfusion Injury and Cardioprotection: Novel Roles
of Frataxin and Uridine Triphosphate**

by

Abdullah Fayez H. AlAsmari

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 06, 2017

Keywords: Frataxin, Cardioprotection, Ischemia/Reperfusion Injury,
P2Y Receptors, UTP, ERK1/2.

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Approved by

Jianzhong Shen, Chair, Associate Professor of Drug Discovery and Development
Timothy Moore, Department Head and Professor of Drug Discovery and Development
Vishnu Suppiramaniam, Professor of Drug Discovery and Development
Muralikrishnan Dhanasekaran, Associate Professor of Drug Discovery and Development
Juming Zhong, Professor of Anatomy, Physiology and Pharmacology

Abstract

Ischemia reperfusion (IR) injury in cardiac myocytes is well known to provoke membrane damage that is mediated by oxygen free radicals and that is associated with iron accumulation, energy dysregulation and opening of the mitochondrial permeability transition pore (MPTP). Nonetheless, the exact mechanism for how the oxygen free radicals develop in the mitochondria due to IR injury is currently ambiguous and need to be unraveled. In the present work, therefore, we explicitly focus upon understanding the role of frataxin in regulating mitochondrial damage which is associated with IR injury. Frataxin, which is a highly conserved small acidic mitochondrial matrix protein, has been recognized to regulate the mitochondrial iron metabolism and modulate the function of the electron transport chain (ETC) and production of reactive oxygen species (ROS). Loss of frataxin, in Friedreich's ataxia, is coupled with impaired mitochondrial iron homeostasis, increased ROS production and respiratory chain dysfunction which prompt tissue damages. In the first project, we propose that enhanced expression of frataxin will salvage cardiomyocytes against IR injury by promoting and preserving the myocardial bioenergetics and that will improve the anti-oxidants effect in response to IR injury. We reported for the first time that frataxin expression is increased during reoxygenation stage of IR injury in cardiomyocytes and that led to mitigation the mitochondrial iron accumulation, prevented the MPTP opening and ROS formation, which in turn preserved the integrity of the mitochondria and increased the cardiomyocyte viability. Furthermore, cells that over-express frataxin had improvement in the mitochondrial respiratory chain complexes I and IV and in the

cellular oxygen consumption compared to frataxin knockdown cells. Unexpectedly, we observed that enhanced frataxin expression revealed elevated levels of glutathione (GSH) and superoxide dismutase (SOD) and that protect the cardiomyocytes against IR injury. Together, our data present novel findings that over-expression of frataxin is cardioprotective against reperfusion injury-mediated cellular damage through its anti-oxidant effect, improving the mitochondrial bioenergetics and by inhibiting MPTP opening.

It is evident that cardiomyocytes express different types of P2 purinergic receptors. P2 receptors, which can be divided into P2X (ligand gated ion channels) and P2Y (G-protein coupled receptors) receptors, are known to be activated by extracellular nucleotides. Uridine triphosphates (UTP) is an extracellular nucleotide that is known to activate specific P2Y G-protein coupled receptor, including P2Y2 and P2Y4, in most cells to mediate numerous biological functions. It has been shown that UTP induced cardioprotection against IR injury and that protection was reported to be induced via P2Y2 and/or P2Y4 receptors. However, the exact mechanism how UTP is cardioprotective against hypoxic and/or IR injury conditions is still to be elucidated. In the second project, therefore, we examined the expression of P2Y2 receptors in cardiomyocytes and we tested the hypothesis that activation of P2Y2 receptors in cardiomyocytes by UTP is cardioprotective through the activation of mitogen activated protein kinases (MAPKs) signaling pathway. In the current study, we found that P2Y2 receptors were highly expressed in H9C2 cardiomyocytes as validated by gene analysis studies. Although P2Y2 receptors are highly expressed in these cells, extracellular nucleotides (UTP and ATP) pre-treatments could not induce any changes in the intracellular calcium mobilization, suggesting that these receptors are not Gq-coupled receptors that mediate the activation of PLC (phospholipase C). Unexpectedly, we found that UTP significantly induced the phosphorylation

of ERK1/2 MAPK in a time- and dose-dependent manner. Additionally, UTP triggered minimal but insignificant activation of p-p38 and p-JNK and had no effect on p-Akt. Surprisingly, ARC-118925XX (P2Y2 selective antagonist), MRS-2578 (P2Y6 selective antagonist) and pertussis toxin (PTX, Gi-protein inhibitor) did not inhibit the induction of p-ERK1/2 by UTP, suggesting that these receptors are not P2Y2, P2Y4 or P2Y6 receptors and possibly these are new P2Y-like receptors because UTP induced ERK1/2 phosphorylation was abolished by suramin, non-selective antagonist of all P2Y receptors. In addition, we provide evidence that activation of these receptors by UTP increased the cell viability of cardiomyocytes in response to serum starvation. Therefore, activation of these receptors could be a potential cardioprotective mechanism, especially under stress conditions.

Acknowledgments

In the name of Allah, the Most Gracious, the Most Merciful and the Most Beneficent I begin. First and with great reverence, all thanks, praises and glories to the Almighty Allah, the lord of the universe, on whom we completely and solely depend for guidance, sustenance and success. I thank him Almighty for giving me the strength, inspiration and blessing to finish this work and I continue to pray for his guidance and blessing in my future.

I would like to express my deepest gratitude and thanks to my family, my parents Fayeze AlAsmari and Seeda AlAsmari (may Allah bless them), my brothers Fatimah, Sulaiman, Ahmed, Sara, Mohammed and Yousef, my wife Amnah and my daughter Rena for their prayers, endless love and motivations to finish my work and pursue my degree. It stands to reason that none of this work would have been achieved without their marvelous sacrifice, help, encouragement and patience and therefore I dedicate this success to all of them.

During my study here in Auburn and during the preparation of my dissertation, many people in USA have helped and supported me and to whom I am so thankful and grateful. Particularly and above all, it is my proud privilege to express my heartfelt thanks and sincere appreciation and gratitude to my major supervisor Dr. Jianzhong Shen, Dept. of Drug Discovery and Development, for his constant guidance, helpful suggestions and boundless encouragements. His invaluable help of continuous support, endless patience and constructive comments have contributed to the success of this work. Sincerely, I could not have imagined having a better advisor and mentor than him and I will never forget his help for the rest of my life.

I owe a deep sense of gratitude and gratefulness to Dr. Vishnu Suppiramaniam, Dept. of Drug Discovery and Development and the Assistant Dean for Research and Graduate Programs, for his guidance, endless support and extraordinary effort to offer every possible help to finish my work in Auburn University. It is a great privilege to have him in my dissertation committee. I am thoroughly grateful and greatly indebted to Dr. Murali Dhanasekaran, Dept. of Drug Discovery and Development, for his constant support and for his invaluable and extreme scientific assistance in my experiments. I am profusely thankful to Dr. Juming Zhong, Dept. of Anatomy, Physiology and Pharmacology, for helping me to understand adult cardiomyocytes isolation techniques and for his valuable time to serve on my dissertation. Special thanks and appreciations to Dr. Timothy Moore, Department head and professor of Drug Discovery and Development, for his valuable time to be in my dissertation committee, Dr. Ya-Xiong Tao, Dept. of Anatomy, Physiology and Pharmacology, to be the official university examiner for my dissertation and Dr. Raj Amin for his contribution in the first project. Also, I would like to extend my sincere appreciation to Dr. John Quindry, Dept. of Kinesiology, for conducting ischemia reperfusion surgeries in mice. I am extremely thankful to Dr. David Riese and Dr. Moore for their leadership and for their great help and kind care during my hard times. My sincere appreciation extends to my friends and staff in Harrison School of Pharmacy for their attentive supports, especially Dr. Gayani, Dr. Shravanthi, Dr. Yiwei, Ms. Pattanin, Dr. Lingxin, Mr. Chuan, Mr. Thamer and Mr. Mohammed. Special thanks, appreciation and respect to all people and friends who supported, encouraged and motivated me in one way or another and their names do not appear here. Finally, I would like to thank King Saud University and the government of Saudi Arabia represented by Saudi Arabian Cultural Mission to U.S. for funding my graduate studies in Auburn University.

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List of Abbreviations

AC	Adenylate Cyclase
ACC	American College of Cardiology
AHA	American Heart Association
AMI	Acute Myocardial Infarction
AP	Action Potential
AV	Atrioventricular Node
CDC	Centers for Disease Control
CHD	Coronary Heart Diseases
CHF	Congestive Heart Failure
ChIP	Chromatin Immunoprecipitation
CVD	Cardiovascular Diseases
DM	Diabetes Mellitus
EC	Endothelial Cell
ECG	Electrocardiograph
EHH	European Heart House
EPO	Erythropoietin
ESC	European Society of Cardiology
ETC	Electron Transport Chain Complexes
Fe-S	Iron-sulfur

FRDA	Friedreich's Ataxia
FXN	Frataxin
FXN-KD	Frataxin Knockdown Cells
FXN-OE	Frataxin Overexpressed Cells
GLUT	Glucose Transporter
GPCR	G Protein Coupled Receptors
GSH	Glutathione
HDL	High Density Lipoprotein
HIF-1	Hypoxia Inducible Factor-1
HK	Hexokinase
HRE	Hypoxia Response Element
IHD	Ischemic Heart Disease
IPC	Ischemic Preconditioning
IR	Ischemic reperfusion
ISC	Iron Sulfur Cluster
LDL	Low Density Lipoprotein
LPO	Lipid Peroxidation
LV	Left Ventricle
MCSF	Macrophage Colony Stimulating Factor
MPTP	Mitochondrial Permeability Transition Pore
MAPK	Mitogen Activated Protein Kinase
NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drug

NSTEMI	Non-ST Segment Elevation Myocardial Infarction
OXPPOS	Oxidative Phosphorylation
PAD	Peripheral Arterial Disease
PHD	Prolyl Hydroxylase
pVHL	Von Hippel-Lindon Tumor Suppressor Protein
RAAS	Renin Angiotensin Aldosterone System
RASI	Renin Angiotensin System Inhibitor
ROS	Reactive Oxygen Species
SA	Sinoatrial Node
SCA	Sudden Cardiac Arrest
SERCA	Sarco-Endoplasmic Reticulum Calcium ATPase Pump
ShRNA	Short Hairpin Ribonucleic Acid
SMC	Smooth Muscle Cell
SOD	Super –Oxide Dismutase
STEMI	ST Segment Elevation Myocardial Infarction
SV	Stroke Volume
TCA	Tri-Carboxylic Acid Cycle
TG	Tri-Glyceride
UTP	Uridine Tri-Phosphate
WHF	World Health Federation
WHO	World Health Organization
VEGF	Vascular Endothelial Growth Factor
$\Delta\Psi M$	Mitochondrial Membrane Potential

CHAPTER 1:

INTRODUCTION

Despite the recent advances and therapeutic interventions for the ischemic heart diseases (IHDs) in the last decades, ischemic heart diseases including acute myocardial infarction (AMI) and Congestive heart failure (CHF) are the most common types of cardiovascular diseases (CVDs) which account for high morbidity and mortality rates in the United States. CVDs continue to be considered the leading cause of death in the United States and worldwide and accounts for more than 600,000 deaths (1 in every 4 deaths or 25% of all cases of death) and costs over \$108 billion every year for the direct health care ¹⁻³. Unfortunately, cardiovascular diseases are anticipated to be the leading cause of death globally in 2030 according to different released reports ^{1,4}. In the United States, it is projected in 2030 that almost 24.5 million (32.5%) people will die from cardiovascular diseases based on recently released reports ^{1,4}. Prognosis and mortality rates from MI depend on the infarction size, which also determines the rate of progression to heart failure ⁵. Increased rates of mortality among people due to ischemic injury identify the lack in knowledge for recognizing the molecular signaling mechanisms that potentiate heart failure. Therefore, understanding the fundamental molecular signaling mechanisms in the myocardium due to MI would be of paramount importance in order to reveal novel and effective therapeutic targets to protect the ischemic hearts from progression to failure and then to death.

Myocardial ischemia is an acute occlusion or severe damage of the coronary blood supply to the myocardium which is mostly resulted from thrombosis or other serious modifications of the atherosclerotic plaques in the coronary arteries. Myocardial reperfusion, however, is basically restoration of coronary blood flow to the myocardium ⁶. Although the restoration of coronary blood flow after transient ischemia reduces the ischemic injury or damage and necrosis, it may be associated with further pathological, biochemical, morphological and functional changes that eventually result in reversible or irreversible cell damage which can influence the cardiomyocytes viability and this is termed as “ischemia-reperfusion (IR) injury” or we can call it reperfusion injury ⁶⁻⁹. Consequently, irreversible cell damage because of the IR injury can clinically result in arrhythmia, endothelial and microvascular injury (no reflow), myocardial stunning and myocyte death ⁹⁻¹¹. It should be noted that the severity of the myocardial damage during reperfusion stage depends on the severity and duration of the ischemic period. Indeed, if the duration of the ischemic period is less than twenty minutes, cardiomyocytes can tolerate the ischemic injury and the pathological and functional changes can be treated as the damage resulted is reversible. Nonetheless, if the ischemic period persists for more than 20 minutes, irreversible cardiac damage, impaired cardiac functions, increased infarction size and necrosis will be observed ^{8,10-15}. The absence of oxygen during ischemia shifts the cellular metabolic axis from oxidative phosphorylation (OXPHOS) towards anaerobic glycolysis that can result in decrease in the intracellular and extracellular pH, depolarization of the mitochondrial membrane potential, Adenosine Triphosphate (ATP) depletion, loss of the myocardial contractile functions and alteration in the echocardiogram (ECG) readings ¹⁶⁻¹⁹. Although the restoration of blood supply to the ischemic regions in the heart can rescue the heart from the damage by restoring the oxygen supply and substrates that are required to adjust ATP

production and balancing the cellular pH, reperfusion period is attributed to the damage developed during IR injury by promoting different signaling pathways that trigger apoptosis and necrosis and thus diminish cardiomyocyte viability. Indeed, generation of reactive oxygen species (ROS) during the reoxygenation stage leads to ATP depletion, Ca^{2+} overload, opening of the mitochondrial permeability transition pores (MPTP) and contractile dysfunction which can result in cardiomyocytes death ²⁰⁻²⁷. Reportedly, cardiomyocytes cell death occurs via three different mechanisms or patterns; apoptosis, necrosis and autophagy which will be further discussed in details in the following chapter ^{7,28,29}. Therefore, investigating and targeting these cellular mechanisms and signaling cascades contributing to cardiac damage in response to IR injury are essential to foster the development of novel effective therapeutic interventions.

Though the majority of the cardiac damage occurs during reperfusion period, early reperfusion remains the most effective intervention to minimize the severity of IR injury and infarction size ³⁰⁻³². For several years, ischemic preconditioning (IPC) is considered the most effective therapeutic intervention to protect the heart from IR injury. IPC can be defined as induction of 2-3 short periods of ischemia (each period from 2-5 minutes) followed by short periods of reperfusion prior to prolonged or sustained ischemia. This induction of short periods of ischemia is not long enough to prompt tissue damage, but rather it will enhance the tolerance of the heart to more maintained ischemia. Different studies provide confirmatory evidence that IPC can afford reduction in the infarction size and prevent the depletion of ATP production in response to IR insult when it compared to non-preconditioned hearts. Over years, IPC has been comprehensively studied and investigated to identify practical and sustainable pharmacological mechanisms to reduce IR injury. Some of these studies have revealed many drugs (both agonists and antagonists) that can triggers different signaling pathways in the mitochondria to protect the

heart against IR injury. These mediators and signaling pathways include protein kinase C (PKC), heat shock proteins (HSP), cyclic GMP dependent protein kinase (PKG), ERK and AKT pathways³³⁻⁴⁰.

Mitochondria are highly abundant, powerful, dynamic and vital intracellular organelles. They have the capability to modulate and regulate their functions, size and shape to rectify any physiological changes in the cell. They play critical roles in regulating oxidative phosphorylation (OXPHOS), calcium homeostasis, heme biosynthesis and metabolism, iron-sulfur cluster (ISC) biogenesis and apoptosis^{26,41-43}. As the mitochondria occupy nearly 35% of the cardiomyocyte volume, produce more than 90% of the ATP, consider the largest single intracellular source of superoxide anion radicals ($O_2^{\cdot-}$) and play a pivotal role in the development of cardiac damage and consequently progression to heart failure following IR injury, conservation of the functions and structures of the mitochondria especially during the reperfusion period is crucial for cardio-protection against IR injury. The highly conserved nuclear encoded mitochondrial matrix protein frataxin (FXN) has been found to promote and regulate the mitochondrial energetics and OXPHOS and accordingly maintains the mitochondrial integrity⁴⁴. FXN is highly expressed in mitochondrial-rich tissues, for instance, cardiomyocytes, neurons, brown fat, liver, and pancreas⁴⁵. Notwithstanding that the exact function of the mitochondrial iron binding protein FXN remains relatively ambiguous, there is evidence that FXN emerges a principal regulator of mitochondrial iron homeostasis. Furthermore, previous research has demonstrated that FXN may act as a chaperone for iron-sulfur cluster (ISC) formation and heme biosynthesis⁴⁶⁻⁴⁹. Disruption of FXN expression, in Friedreich's ataxia (FRDA), is associated with mitochondrial iron overload, increased reactive oxygen species (ROS) formation and cellular damage. Friedreich's ataxia (FRDA) is a rare autosomal recessive inherited neuro and cardio-degenerative disease that

is characterized by hypertrophic cardiomyopathy⁵⁰⁻⁵². FRDA affects approximately 1:50000 people in the United States and it considered the common type of hereditary ataxia. It is manifested by limb ataxia, loss of coordination, progressive gait and hypertrophic cardiomyopathy. The homozygous mutation of a guanine–adenine–adenine (GAA) trinucleotide repeats in the first intron of FXN gene leads to transcriptional silencing of the FXN gene and subsequently reduced the protein levels in FRDA patients. Severity of the FRDA, duration, age of onset and cardiomyopathy are correlated with the smaller GAA repeat mutation⁵³⁻⁵⁵.

Previously, FXN has been thought of as a chaperone protein because of its role in regulating iron sulfur cluster biogenesis. Nevertheless, evidence from recent publications have indicated that FXN has major role in iron regulation because it can serve as an iron storage protein, iron sensor, scavenger of ROS and regulates mitochondrial energetics^{56,57}. Furthermore, the results obtained by Ristow and his colleagues demonstrate that over-expression of FXN stimulates OXPHOS, increases tricarboxylic acid (TCA) cycle activity, and mitochondrial membrane potential⁴⁴. Nonetheless, little attention has been devoted to the impact of FXN in the field of the protection of the heart against IR insult. To the best of our knowledge, there are no studies that have investigated the role of FXN on the heart in response to IR injury especially during the reperfusion stage. Therefore, in this dissertation we explicitly hypothesize that increased expression of FXN will offer protection to the cardiomyocyte against IR injury by regulating myocardial energetics and by improving the anti-oxidant capacity in cardiomyocytes. In this study, we show that FXN expression is increased during reperfusion and provides cardio-protection against IR injury. Further, the increased expression of FXN protects the mitochondria from iron overload and the ensuing ROS formation, which are known to compromise the mitochondrial integrity by increasing the anti-oxidants capacity levels. Finally, we proved that

FXN over-expression increases cell viability, ATP production and the mitochondrial membrane potential during the reperfusion and therefore protects the cardiomyocyte from IR injury. Together, these findings help to understand the protective role of FXN and could help to treat diseases where FXN is altered in expression such as FRDA, diabetic cardiomyopathy and doxorubicin mediated cardiac hypertrophy.

CHAPTER 2:

REVIEW OF LITERATURE

2.1. Coronary Heart Diseases (CHD)

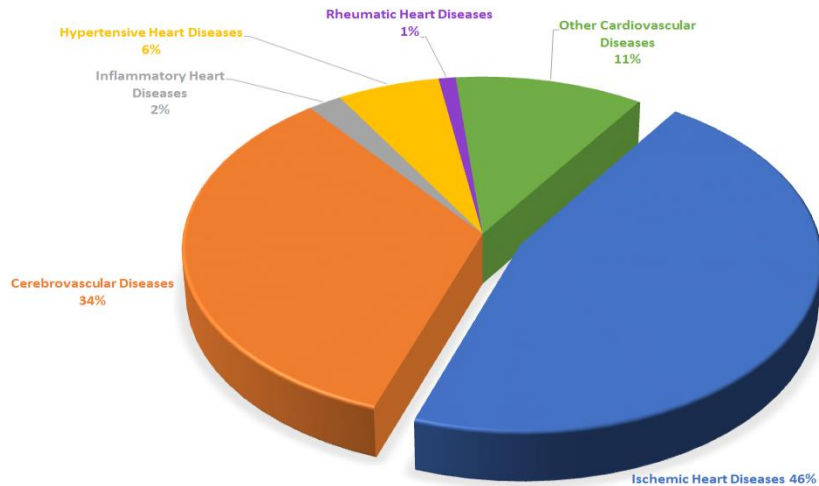
Cardiovascular diseases (CVDs) are a group of conditions that are triggered by any abnormalities in the structure, function or physiology of the heart and blood vessels. It encompasses ischemic heart diseases (IHDs), ventricular arrhythmia, rheumatic heart disease, cardiac arrest, abdominal aortic aneurysm, hypertension and heart failure. Globally, CVDs come to be pandemic and it continues to be a major and challenging healthcare issue to the international healthcare systems at present. Notwithstanding the recent dramatic advances and developed therapeutic interventions for the CVDs in the last decades, CVDs, which are the most predominant noncommunicable diseases, account for high morbidity and mortality rates in the United States⁵⁸⁻⁶⁰. The preponderance of CVDs manifest as ischemic heart diseases (IHDs).

Ischemic heart diseases are primarily comprise of coronary heart diseases (CHDs), which clinically manifest as angina, myocardial infarction (MI) and sudden cardiac arrest (SCA), and it accounts for high mortality rates in most developed counties and in many developing countries. Formation of atherosclerotic plaques in the inner lining (intraluminal) of coronary blood vessels which lead to partial or total occlusion of the coronary artery is the principal mechanism or pathology of SCA and MI. It should be noted that prognosis and mortality from MI depends on the infarction size and that also can control the rate of progression to cardiac failure⁶¹⁻⁶⁴.

2.2. Prevalence of Coronary Heart Diseases

According to recently released reports from the World Health Organization (WHO), cardiovascular diseases, including myocardial infarction and heart failure, are the top leading cause of death globally. Indeed, CVDs evidently attribute to more than 17 million cases of death, which represent approximately 32% of all global cases of death^{59,65}. The majority of these deaths (more than 80%) occur in middle and low income countries. Out of the 17 million cases of deaths, nearly 8 million deaths resulted from coronary heart diseases alone. Moreover, CHDs cause more than 46% of deaths in men and almost 38% of death among women. Furthermore, 34% of cardiovascular deaths among men and about 37% of deaths in women resulted from cerebrovascular disease, which is the second cause of death after ischemic heart diseases (figure 2.2). Mathers and his colleague Loncar predicted that ischemic heart diseases including coronary heart diseases will be the single leading cause of death globally by 2030 and it will result in more than 23.6 million cases of death in high and low-income countries¹.

(A)



(B)

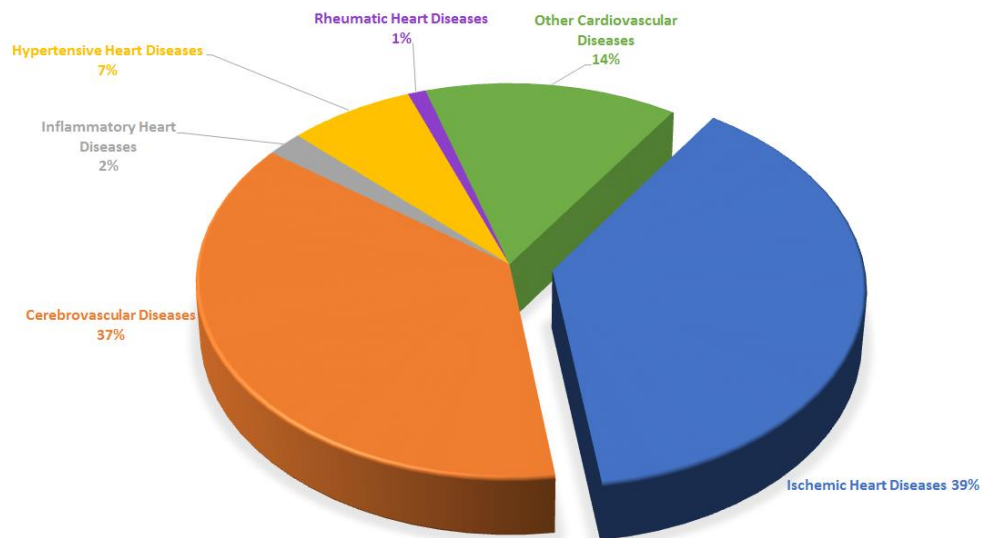


Figure 2.2: Percentages of Cardiovascular deaths caused by different types of cardiovascular diseases (Ischemic heart diseases, Rheumatic heart diseases, Hypertensive heart disease, inflammatory heart diseases, cerebrovascular diseases and other cardiovascular diseases) in 2011 in (A) men and (B) women. Reproduced with the permission of the WHO (publisher) from Mendis, S., *et al.* 2011. Global Atlas on Cardiovascular Disease Prevention and Control. *WHO* 2–14, figures 4 & 5, http://whqlibdoc.who.int/publications/2011/9789241564373_eng.pdf?ua=1, (Accessed on August 8th 2016).

Current reports issued from the United States (U.S.) Centers for Disease Control and Prevention (CDC) revealed that IHDs are the major leading cause of death for both men and women in the U.S. and it accounts for more than 610,000 deaths (that means 25% of all cases of death) and costs over \$108 billion for the direct healthcare annually ^{67,68}. Furthermore, it is reported that nearly 85 million Americans live with some form of cardiovascular diseases and about 2,000 people die each day because of cardiovascular diseases. Additionally, it is estimated that around 720,000 Americans have heart attacks annually (of those 520,000 people have heart attack for the first time) and that result in almost 120,000 deaths among them ⁶⁷⁻⁷⁰. As reported by Heidenreich and his colleagues in their paper which entitled “*Forecasting the Future of Cardiovascular Disease in the United States*”, almost half the population of the U.S. (approximately 120 million people) are projected to have some form of cardiovascular diseases by 2030. Further, the direct healthcare costs of cardiovascular diseases will incredibly rise to \$818 billion and the indirect medical care costs to \$275 billion in 2030 ⁴. These alarming statistics of increased rates of morbidity and mortality among people because of ischemic heart diseases along with high cost of caring for individuals with these chronic diseases are one of the most pressing issues in healthcare system at present. Therefore, finding new therapeutic targets to prevent the cardiac damage is of preeminent importance and it will also help to reduce the morbidity and mortality rates. In order to do that, nevertheless, we have first to understand the molecular signaling mechanisms and the pathophysiology of the ischemic heart diseases.

2.3. Pathophysiology of Coronary Heart Diseases

Thrombus formation in the inner lining (intima) of the coronary blood vessels resulting in partial or total occlusion of the coronary artery is the principal mechanism or pathology of SCA and MI. Indeed, this process which leads to the occlusion of the coronary arteries is known as atherosclerosis. Atherosclerosis comes from the Greek words ‘athere’ which means gruel (or accretion of lipids) and ‘sclerosis’ which means hardening (hardening or thickening of the arteries) ^{63,71}. Atherosclerosis can be defined as a chronic, multifocal, immuno-inflammatory arterial disease that is manifested by the accretion of lipids in the intima of medium and large size arteries. Approximately 60% of all cases of stroke are caused by atherosclerotic diseases and about 20% of that is caused by carotid atherosclerosis. Research indicates that atherosclerosis affects men more than women and it is more common in white men than in black men. It should be noted that atherosclerosis initiates and advances for years before the progress and the development of the occlusion ^{63,72,73}.

Over the past 5 decades, different studies have investigated the etiology and the risk factors that might cause and progress atherosclerosis. Those factors could be of genetic origins or of environmental origins. It includes, for instance, diabetes mellitus (DM), elevated cholesterol levels (hypercholesterolemia), hyperfibrinogenemia, hypertension, smoking, gender, proliferation of smooth muscle cells (SMCs), increase in the inflammatory markers (C-reactive protein and cytokines), infiltration of macrophages, family history (genetic factor) and formation of the thrombus. Moreover, many studies have linked the increase in the concentration of amino acids that contain homocysteine (hyperhomocysteinemia) to atherosclerotic diseases as it cause endothelial damage by promoting atherogenesis ^{61,62,74–76}. Many studies have indicated that

atherosclerotic lesions (atheroma) can start early in life (childhood) and it appears as fatty streaks, which is considered as the initial visible lesion of atherosclerosis, in the aorta. After that (in the second decade), the disease can be observed in the coronary arteries and that can cause thickening of the innermost layer of the coronary arteries which can lead to narrowing (occlusion) of that arteries and cause myocardial infarction, whereas its appearance in the cerebral arteries in the third or fourth decade of life can result in cerebral ischemia^{63,71,74}.

Endothelial cells (ECs) play an important role in the progression of atherosclerosis. It regulates inflammation, vascular remodeling and thrombosis as it can selectively permeate the transfer of molecules between blood and tissues. This importance of ECs in atherosclerosis has been investigated and validated as the removal of these cells led to migration and proliferation of the SMCs. There are many factors and forces that act on ECs, but blood shear stress is the foremost factor that significantly modulates the morphology of ECs. For example, in areas where the blood flow is constant and even such as tubular regions, ECs have an ovoidal shape. However, ECs have hexagonal shape in areas where the blood flow is not uniform such as areas in the vessels branching (ECs in such regions are intact, leaky, activated, have less elastin layer and dysfunctional) and these areas are the most likely and favorable areas for the formation and development of lesions and atherosclerosis (Falk 2006; Hansson 2005; Singh et al. 2002).

High levels of Low Density Lipoproteins (LDL) in the circulation, which is transported by apolipoprotein B100 (ApoB100), and the aggregation of it in the ECs are the first events in originating atherosclerosis. There are compelling evidences that LDL can freely diffuse and penetrate through EC junctions and as a consequence of the binding of ApoB100 containing LDL to matrix proteoglycans, LDL is retained in the blood vessels wall along with other lipoproteins like lipoprotein (a) and stimulate atherosclerosis. In the blood vessels wall, the

accumulated LDL has been reported to be modified by lipolysis, oxidation and proteolysis with oxidation (either by lipoxygenases and myeloperoxidase enzymes or by reactive oxygen species) being considered as one of the most significant modification process that affects LDL and that contributes to the formation of foam cells and inflammation. It is appreciated that LDL (but not oxidized LDL) is formed during the initial phases of atherosclerosis, while oxidized LDL is formed in the advanced phases and it is much more atherogenic than LDL and that result in enrollment and retention of the macrophages at the site of inflammation. Opposite to LDL, high density lipoproteins (HDL) have protective roles and considered an important factor against atherosclerosis as they remove the high levels of cholesterol from arterial intima to the liver, inhibit the oxidation modification that occur to LDL, inhibit the migration of monocyte adhesion molecules to the intima and stimulate the cell repair and proliferation ^{71,74,77-79}.

The increase in the accumulation of oxidized LDL (OxLDL) in the arterial walls potentiates the atherosclerotic lesions (plaques) in the ECs to release pro-inflammatory molecules and cytokines such as adhesion molecules and MCSF (macrophage colony stimulating factor) and that cause activation of ECs. Further, oxidized LDL can cause inhibition of the vasodilator and the anti-atherogenic factor, nitric oxide (NO), which has been reported that mice that lack NO synthase, an enzyme that catalyze the production of NO from L-arginine, developed atherosclerosis ^{72,73,77} (figure 2.3). It has been reported that recruitment of leukocytes into the activated ECs (site of inflammation) is mediated by chemotactic cytokines (chemokines) and cell adhesion molecules ^{74,79}. Monocytes, T-cells, B-cells, mast cells, dendritic cells and to some extent neutrophils are different types of cells that are recruited to the atherosclerotic lesions. Rolling of leukocytes along the ECs is the first step, which is facilitated by ICAM-1 (Intercellular Adhesion Molecule-1), integrin VLA-4 (very late antigen-4), selectins (P and E-

selectins), which binds to ligands on leukocytes, and monocyte chemoattractant protein (MCP-1) (figure 2.3). The following steps are arrest, firm adhesion and transmigration through the ECs (diapedesis) where the monocytes can proliferate and differentiate into macrophage in the intima. The highly oxidized LDL is then taken up (engulfed) by macrophages through a group of receptors like SR-A (scavenger receptor-A1) and CD-36, which has been shown that these receptors are regulated by PPAR- γ (peroxisome proliferator activated receptor), and that result in the formation of foam cells^{73,74,78,79}.

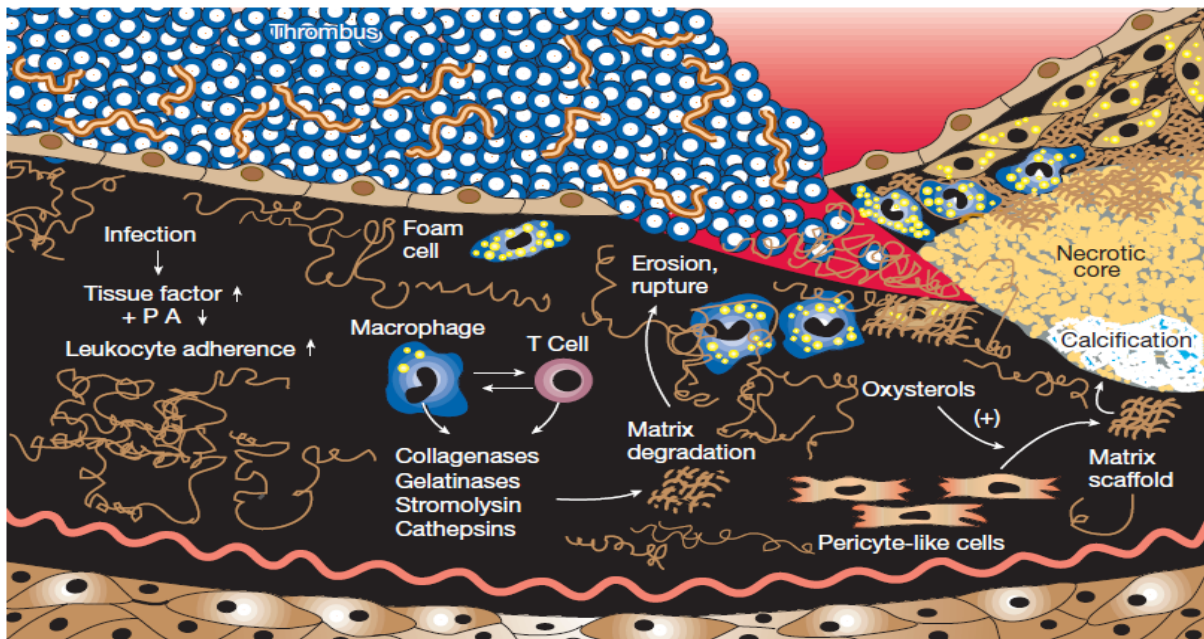


Figure 2.3: Initiation of atherosclerotic plaque. *Adapted by permission from Macmillan Publishers Ltd: Nature 407, 233-241; copyright (14 September 2000).*

The atherosclerotic lesions or plaques are characterized by increasing levels of cholesterol, inflammatory cells, cellular detritus and accumulation of SMCs, in which its migration and proliferation are controlled by macrophages and T-cells. The interaction of CD-40 with its ligand CD-154 are shown to be important for the progression of the atherosclerotic plaques as CD-40 is expressed in ECs, SMCs and macrophages and play a crucial role in the release of inflammatory cytokines and adhesion molecules^{71,74,76,79-81}. Generally, atherosclerotic plaques are composed of fibrous tissue (fibrous thin cap), calcium (Ca^{+2}), foam cells and necrotic lipid rich core (figure 2.4). Indeed, once the atherosclerotic plaques (lesion) advanced, ECs, SMCs and macrophages undergo either apoptosis or necrosis and this will lead to the formation of the lipid rich core and fibrous thin cap plaques, which then can be calcified (cytokines and oxysterols control calcification) and be responsible of some diseases like stable angina^{71,74,79-82}. Infections (like cytomegalovirus), hormones (oestrogen), hypertension, neovascularization (formation of new blood vessels) and increased levels of homocysteine are different factors that have been linked to the progression of the atherosclerotic plaques^{71,74}. At that step when the atherosclerotic plaques advanced and calcified, arterial remodeling occurs in order to adapt that anomaly. Because the atherosclerotic plaques are composed of fibrous thin and fragile cap, they usually rupture (mainly at the lesion edges as they are abounding in foam cells and activated immune cells) and that is considered the main cause of coronary artery thrombosis (related to more than 75% of all cases of heart attack) (figure 2.4)^{63,73,74,78,79}.

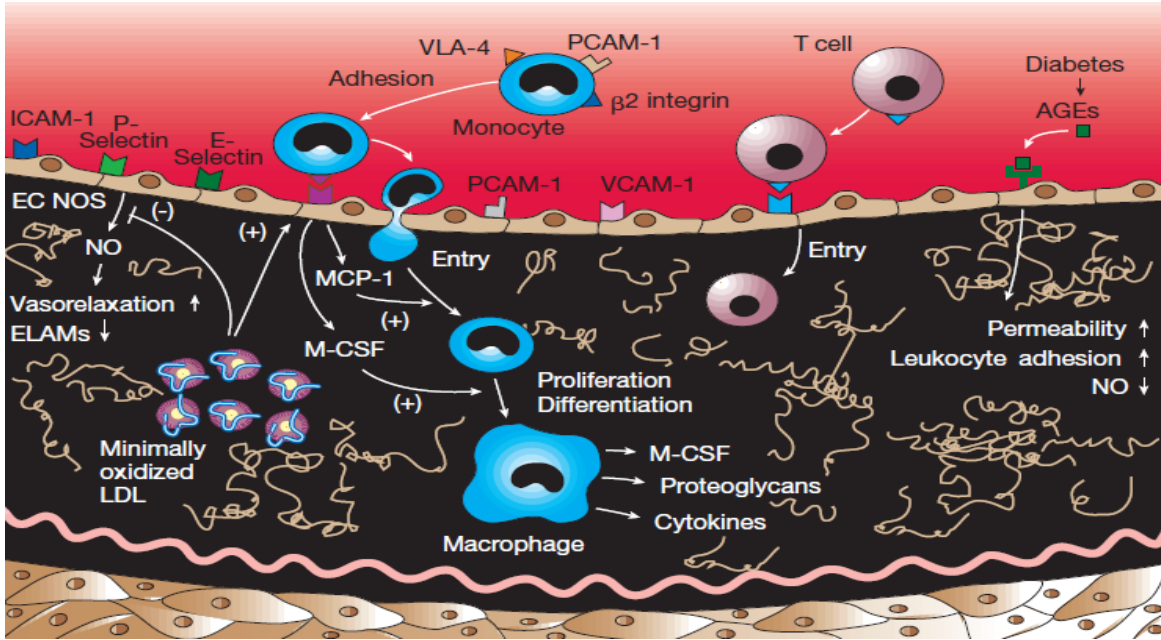


Figure 2.4: Development and progression of atherosclerotic plaque. *Adapted by permission from Macmillan Publishers Ltd: Nature 407, 233-241; copyright (14 September 2000).*

Plaques rupture occur because the lipid rich core of the plaques release proteolytic enzymes and various inflammatory molecules which can enfeeble the plaques making it much more fragile and prone to rupture which subsequently result in thrombus formation (this mechanism supports the proposition that thrombus formation is preceded by atherosclerotic plaques rupture)^{77,83,84}. It should be noted that the composition of the atherosclerotic plaques, plaques volume and the plaque susceptibility to rupture are the main factors that can affect the thrombus formation in the arteries (causes occlusion of the arteries). As it is reported in previous studies, atherosclerotic lesion can be categorized based on their morphology into different types (type I to type IV)^{71,85,86}. Non-atherosclerotic intimal lesions, or type I, have two subtypes; intimal thickening and intimal xanthoma, where intimal thickening contains abundant SMCs (but

not thrombosis, foam cells or lipids) and intimal xanthoma is rich in foam cells. Type II which is progressive atherosclerotic lesions is further classified into fibroatheroma, thin-cap fibroatheroma, plaque fissure and pathological intimal thickening. Pathological intimal thickening is characterized by the absence of thrombosis and the accumulation of SMCs in a proteoglycan matrix, while fibroatheroma is manifested by the presence of necrotic core and by the lack of thrombosis. Plaque fissure, however, is characterized by the occurrence of thrombus and hemorrhage and it encompasses large necrotic core with neovascularization. Thin-cap atheroma has a thin-fibrous cap with no SMCs in the absence of thrombosis. Lesions with acute thrombi is the third type of atherosclerotic lesions and this type can be subdivided into plaque erosion, plaque rupture and calcified nodule. Thrombosis is present in all of these subtypes with the thrombosis might be not occlusive in plaque erosion and calcified nodule and occlusive in the plaque rupture. Type IV is healed atherosclerotic lesions and it contains SMCs, collagen (type III), proteoglycans and inflammatory cells with no thrombosis ^{71,85-88}.

➤ **Atherosclerosis: Current therapy and clinical trial**

As we mentioned early in the previous section (*section 2.3*), approximately more than 75% of all cases of myocardial infarction, peripheral ischemic diseases and stroke are caused or developed because of atherosclerosis. Thus, targeting the process of development of the atherosclerotic plaques is essential in order to reduce the incredible mortality rates. Currently, there are different established pharmacological interventions for treating atherosclerosis including statins, nicotinic acid, non-steroidal anti-inflammatory drugs (NSAIDs), anti-platelet, beta adrenergic receptors inhibitors (β -blockers) and renin angiotensin system inhibitors (RASi).

Although statins are considered the primary and the most effective treatment for atherosclerosis, statins are related with severe adverse events including diabetes mellitus (DM), neurological and skeletal symptoms⁸⁹⁻⁹². According to recently published study by Thompson and his colleagues, statins are reported to induce myopathy, myalgia, rhabdomyolysis, myositis, myonecrosis, mitochondrial dysfunction, dementia (resolved after withdrawing statins), decrease in hippocampal volume, liver enzymes imbalance, acute kidney injury and Interstitial lung disease (ILD)⁸⁹. Therefore, investigating new therapeutic approaches to overcome adverse events of some of the current pharmacological drugs would be of great benefit to prevent cardiovascular events. In that manner, numerous molecular signaling pathways, strategies and drugs are in developing stages and being tested and evaluated in clinical trials as putative future targets. The current conventional treatments as well as some strategies in clinical trial are summarized in table 2.3.

Table 2.3 Current approaches and investigational trials for the treatment of atherosclerosis

Drug / Strategy	Mechanism	Active Status	Evidence
A) <u>Conventional treatment</u>			
Statins (ex. atorvastatin)	HMG-CoA reductase inhibitors (inhibit cholesterol synthesis), anti-oxidants (inhibit AT-1 receptors and stimulate NO release) and anti-inflammatory	Established treatment. Approved from ACC/AHA as primary and secondary prevention	75,79,93
β-blockers (ex. betaxolol)	Anti-hypertensive	Established treatment (secondary prevention)	94,95
Anti-platelets (ex. clopidogrel) and NSAIDs (ex. aspirin)	Anti-Platelet (inhibit platelet aggregation)	Established treatment (secondary prevention)	96-98
Nicotinic Acid, Vitamin B3	Reduce cholesterol, LDL and TG.	Established treatment	99-103

(ex. niacin)	Induce HDL. Anti –oxidant and anti-inflammatory	(secondary prevention)	
B) <u>Potential therapeutic strategies in clinical trials</u>			
PCSK9 inhibitors (ex. Alirocumab)	PCSK9 inhibitors (reduce circulating LDL)	Clinical trial phase 3	104–106
apolipoprotein C-III, ApoC-III (ex. Antisense apo C-III or ASO apo C-III)	Reduce triglycerides levels (TGs)	Clinical trial phase 2	105,107,108
HDL inducers (ex. Cholesteryl ester transfer protein inhibitors (CETPIs) such as evacetrapib, HDL mimetics such as apoA-I Milano, apolipoprotein A-I inducers such as RVX-208 and microRNA such as miR-33)	Increase HDL levels	CETPIs (Clinical trial phase 3), apoA-I Milano (Clinical trial phase 1), RVX-208 (Clinical trial phase 2) and miR-33 (Pre-Clinical)	109–117
Targeting atherogenesis and inflammation (ex. sPLA2 inhibitors such as varespladib, LpPLA2 inhibitor such as darapladib, 5-lipoxygenase inhibitors such as VIA-2291 and methotrexate (anti-inflammatory)	Reduce atherogenesis and inflammation	sPLA2 (Clinical trial phase 3), LpPLA2 (Clinical trial phase 3), VIA-2291 (Clinical trial phase 2) and methotrexate (Clinical trial phase 3)	118–126

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2.4. Myocardial Infarction (MI)

Myocardial infarction (MI), which is the first and the major manifestation of coronary heart diseases, is the leading cause of morbidity and mortality globally. MI can be defined and recognized from a number of several aspects relevant to pathological, clinical, biochemical, electrocardiographic, imaging and epidemiological features. Diagnosis of myocardial infarction has significant and extreme social, psychological and legal repercussions as MI is a final outcome parameter in observational studies and clinical trials. Precise data about MI attack cases and mortality rates can offer invaluable information about the disease prevalence in and between populations and that can help in the epidemiological studies¹²⁷⁻¹²⁹. In order to use MI data in clinical and observational studies, monitoring of epidemiological parameters, coding of MI diagnostic cases and MI classification, we should first define MI accurately. Precise and exact definition of MI would be of paramount importance as it can grant correct and reliable data that can be used in disease monitoring, different types of MI studies, economic analysis, healthcare costs and quality certainty^{127,130}.

A few decades ago, there was a general agreement and a common acceptance to the definition of the term 'myocardial infarction' by the world health organization (WHO). Indeed, this definition by WHO in 1971 did not include any cardiac-specific biomarkers of myocyte necrosis because of the lack in reproducibility and specificity¹³¹. At that time and according to WHO definition, MI can be diagnosed by the manifestation of two out of three features: clinical symptoms of MI (chest pain or discomfort), fluctuations in electrocardiographic (ECG) pattern (development of Q waves or constant T waves) and soar in non-specific cardiac markers (creatine kinase) (table 2.4)¹³¹.

Table 2.4 World Health Organization (WHO) criteria for defining myocardial infarction (MI)

World Health Organization definition of MI	
Criteria No.	Definite acute MI
1	Certain ECG variations or;
2	Typical or atypical symptoms jointly with possible ECG changes or abnormal markers levels or;
3	Typical symptoms with abnormal enzymes with ischemia or non-codable or not available ECG or;
4	Fatal case (sudden or not sudden) with naked eye appearance of fresh MI, recent coronary occlusion found at necropsy, or both.

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Because of the evolution in the development of highly sensitive and cardiac-specific biomarkers (ex. Troponin C, T and I, CKMB activity and CKMB mass) in the early 1990s and because of the advances in producing highly specific and sensitive imaging techniques, detection of even very small amounts of necrosis or infarcts (weighing less than 0.9g) become much more easier and sensitive. Therefore, clinical trials, epidemiological and observational studies, healthcare systems, clinical practices, physicians and patients require a re-evaluation of the existing definition of MI by WHO in order to define MI much more precisely.

In July 1999 and for that reason, the European Society of Cardiology (ESC) and the American College of Cardiology (ACC) convoked a consensus conference at the European Heart House (EHH) to re-define jointly the existing definition of MI ¹²⁸. The new definition of MI by ESC/ACC committee denotes that any necrosis caused by ischemia should be considered and

categorized as MI, therefore, any patient who was diagnosed previously with unstable or stable angina pectoris will be diagnosed now as he/she had MI; consequently, more cases of MI will be identified. The use of the highly specific biomarkers that mentioned in this definition, however, will minimize the diagnosis of false positive MI ^{127,128,132}. In addition to updating the definition, the ESC/ACC committee studied the implications of the new definition of MI from different aspects (electrocardiographic, biochemical, pathological, imaging, public policy and clinical trials) and they provided different criteria to characterize MI, such as infarct size (extent of cardiomyocytes cell loss), time of myocardial necrosis and triggering of MI. This definition of MI by ESC/ACC was re-evaluated and re-defined in 2007 by the ESC, ACC, the American Heart Association (AHA) and the World Heart Federation (WHF), global MI task force, to include five new criteria to characterize MI and that was called ‘Universal Definition of Myocardial Infarction’ ^{129,133}. Nonetheless, the advances in the development of selective and sensitive biomarkers to accurately detect MI and the improvement in the diagnosis and managements of MI raise the need to further revise the existing definition. Therefore, the global MI task force (ESC, ACC, AHA and WHF) has updated the definition of MI in 2012 and that meeting resulted in ‘Third Universal Definition of Myocardial Infarction’ ¹³⁰. This definition includes new criteria to define MI with the increase/decrease of sensitive cardiac biomarker is the cornerstone of this definition (table 2.5).

Table 2.5 Third Universal Definition of MI by global MI task force

Definition of Myocardial Infarction	
Criteria for Acute Myocardial Infarction	
The term acute myocardial infarction (MI) should be used when there is evidence of myocardial necrosis in a clinical setting consistent with acute myocardial ischemia. Under these conditions any one of the following criteria meets the diagnosis for MI:	
Criteria No.	Definite acute MI
1	Detection of a rise and/or fall of cardiac biomarker values [preferably cardiac troponin (cTn)] with at least one of the following criteria
2	Symptoms of ischemia
3	New or presumed new significant ST-segment-T wave (ST-T) changes or new left bundle branch block (LBBB)
4	Development of pathological Q waves in the ECG
5	Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality
6	Identification of an intracoronary thrombus by angiography or autopsy

Adapted with permission from Oxford University Press: Thygesen K., Third universal definition of myocardial infarction, Eur. Heart J., copyright 2012, (33), 2551-2567

Therefore, we can define MI pathologically as injury and necrosis of the cardiac cells because of prolonged ischemia (more than 20 minutes). Myocardial ischemia is initiated due to the imbalance between oxygen demand and oxygen supply, and that is the first event in developing MI. Furthermore, MI can be identified biochemically by the increase in specific cardiac biomarkers such as cardiac troponin, cTN, (I or T) or CKMB (MB fraction of CK) and clinically by the presence of some symptoms (not always an indication of MI) such as chest, arm,

epigastric, jaw and shoulder pain (lasts over 20 minutes), fatigue, dyspnea and syncope^{130,134}. ECG (ST-T segment abnormalities and pathological Q waves are indication of MI) and advanced imaging techniques, which measure cardiac wall thickening, wall motion irregularity, and cardiac cell viability, can also be used to identify MI^{128,129}. Based on pathology, biochemistry and clinical criteria, the third global MI task force classified MI into six different groups (table 2.6)^{130,133}. Further, they classified MI based on diagnostic criteria into ST elevation MI (STEMI) and non-ST elevation MI (NSTEMI)¹³⁰. In the following sub-sections, we will discuss the changes in the cardiac functions, adaptations and ECG in further details.

Table 2.6 Third global MI task force classification of MI

Clinical Classification of MI	
Type 1	Spontaneous myocardial infarction
Spontaneous myocardial infarction related to atherosclerotic plaque rupture, ulceration, fissuring, erosion, or dissection.	
Type 2	Myocardial infarction secondary to an ischemic imbalance
Myocardial infarction secondary to ischemia due to either increased oxygen demand or decreased supply (e.g. coronary artery spasm, coronary embolism, anemia, arrhythmias, hypertension, or hypotension).	
Type 3	Myocardial infarction resulting in death when biomarker values are unavailable
Sudden cardiac death with symptoms suggestive of myocardial ischemia and presumed new ischemic ECG changes but death occurring before blood samples could be obtained.	
Type 4a	Myocardial infarction related to percutaneous coronary intervention (PCI)
Myocardial infarction associated with PCI is defined by elevation of cTn values >5 x 99 th percentile URL	
Type 4b	Myocardial infarction related to stent thrombosis
Myocardial infarction associated with stent thrombosis as detected by coronary angiography or autopsy.	

Type 5**Myocardial infarction related to coronary artery bypass grafting (CABG)**

Myocardial infarction associated with CABG is defined by elevation of cardiac biomarker values $>10 \times 99^{\text{th}}$ percentile URL.

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2.4.1. MI and Cardiac Remodeling

Acute myocardial injury, increase in cardiac wall stress and the acute necrotic loss of myocardium during myocardial infarction (MI) result in cellular, molecular, biochemical and gene expression changes that lead to cardiac remodeling (ventricular remodeling), which is considered to play an important role in the development of congestive heart failure (CHF) and can be a predictor of mortality¹³⁵⁻¹³⁷. Ventricular remodeling is a physiologic (adaptive process throughout normal growth) and pathologic (due to MI, myocarditis, cardiomyopathy, pressure overload or valves heart diseases) condition that result in changes in the function, shape, size and volume of the ventricles and that are controlled by genetic and neurohormonal (beta adrenergic system and renin-angiotensin-aldosterone-system 'RAAS') factors. Because of the myocardial cell death during MI, different cellular and molecular signaling cascades as well as inflammatory reactions will be activated and that can result in ventricular dilatation, hypertrophy, thinning of the infarcted area (because of cell stretching and decrease in the intracellular space) and collagen scar formation (fibrous tissues) at the site of infarction (figure 2.5). It is evident that ventricular dilatation in response to MI is a normal compensatory mechanism (to restore normal functions

and normal stroke volume) and a pathological mechanism that progress heart to failure ^{135,136,138–141}.

Ventricular remodeling and infarct progression (expansion) following MI begin with the stimulation of the release of matrix metalloproteinases (MMPs) from neutrophils, which degrade coronary vasculatures and extracellular matrix (contains fibroblasts and type I and III collagen). This expansion of the infarction occurs within the first few hours after the myocardial injury and continues to progress over weeks and that can result in thinning of the infarcted zone, increase in the cardiac wall stress (it stimulates hypertrophy) and ventricular dilatation (figure 2.5). Coupled with the infarcted myocardium, infarction progression can cause deterioration of the border zones and non-infarcted areas ‘remote areas’ resulting in vascular remodeling, alterations in the Frank-Starling law mechanism, formation of interstitial fibrosis, eccentric hypertrophy and hyperkinesia ^{135,141–144}. Although eccentric hypertrophy in the remote areas is beneficial as it can compensate for the cardiomyocytes loss in the infarcted regions, it has deleterious effect over time as the increase in the ventricular size lead to an increase in the wall stress and that results in increase in oxygen demand ¹⁴⁵. These abnormalities in the infarcted regions along with the formation of interstitial fibrosis and the reduction in the capacity of hypertrophied cardiomyocytes in the non-infarcted areas result in decrease in the efficiency (performance) of the ventricles and that can progress the heart to failure (HF) ¹³⁹.

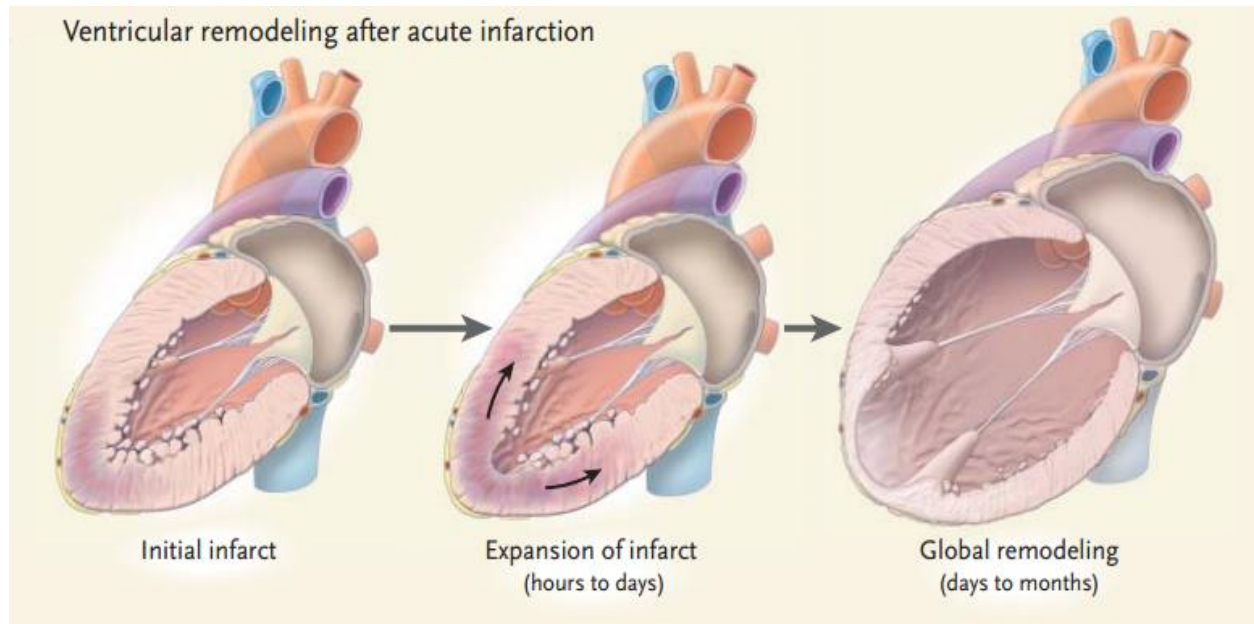


Figure 2.5: Cardiac remodeling in response to myocardial infarction (MI). *Reproduced with permission from Jessup M. et al, Heart Failure, NEJM 348, 2007-2018, Copyright (15 May 2003) Massachusetts Medical Society.*

2.4.2. Cardiac Functional Changes after MI

Although cardiac remodeling that involves wall thinning of the infarcted zones, dilation of the ventricles chambers and eccentric hypertrophy of the remote non-infarcted areas (we discussed it in details in the previous sub-section 2.4.1) can restore cardiac functions by restoring stroke volume and pumping efficiency of the ventricles, it can result over time in pathological conditions that can consequently lead to cardiac dysfunction and then failure¹⁴⁰. Previous and current research on MI have devoted much attention to the study of left ventricular end-systolic pressure (LVESP) and volume (LVESV), left ventricular end-diastolic pressure (LVEDP) and volume (LVEDV), left ventricular ejection fraction (LVEF) and stroke volume (SV) as they can

be used to evaluate the cardiac functions following any incidence of cardiac injury such as MI¹⁴⁶. However, LVEF cannot be used always in all patients diagnosed with MI to measure cardiac functions as it has been reported that hyperkinetic patients, notwithstanding the substantial cardiac damage, have normal LVEF levels. Moreover, the site of myocardial infarction has an impact on LVEF value, for example, anterior myocardial infarction has remarkable decrease in the LVEF compared to inferior myocardial infarction with the same size of infarction, which means LVEF (in some cases) might not be the optimal parameter to measure to assess the cardiac function. Therefore, the use of wall motion score index (WMSI) can be used, alternatively, to measure the global ejection fraction¹⁴⁷⁻¹⁵⁰.

Using recent and highly advanced imaging techniques, different studies have reported that both LVESV and LVEDV exceedingly increased with noticeable decrease in LVEF in MI patients. Further, formation of ROS and sustained activation of the neurohormonal systems have been shown to be upregulated in ischemic compared to non-ischemic hearts. Therefore, we can conclude that the increase in LVESV and LVEDV and the decrease in LVEF index are the major changes in the cardiac function in patients with MI and therefore, improvement of LVEF can result in improvement in the cardiac function and that can protect the heart from the progression to failure^{138,140,151-156}.

2.4.3. Electrocardiography (ECG) in MI

Electrocardiogram, which is referred to as ECG or EKG, is the best, gold standard and most commonly used technique to analyze the cardiac rhythm during a cardiac cycle through the measurement of the differences in the electrical impulses that are conducted through the heart.

These electrical impulses (action potentials (AP)) that generated from the sinoatrial node (SA) and conducted through the heart result in the depolarization and repolarization of the heart and that cause excitation and contraction (mediated by excitation-contraction coupling (ECC)) in each heartbeat (Ashley and Niebauer 2004; Becker 2006; Noble, Hillis, and Rothbaum 1990; Trägårdh et al. 2007). To clearly understand the mechanism of cardiac conductance of impulses in each cardiac cycle and to easily and precisely interpret the information obtained from ECG in order to correctly diagnose different diseases, we should understand the ECG of the normal sinus rhythm, the principal concept of the cardiac AP and the ECC in each cardiac cycle.

In the heart, the myocardial action potential (AP) originates in the sinoatrial node (SA), which is the natural pacemaker of the heart, and then conducted along the atria into the atrioventricular node (AV). These electrical impulses at the AV node are delayed for a short period of time (to complete the atrial contraction and to enable ventricular filling with blood). From the AV node, these impulses will be transmitted to the Bundle of His and Purkinje fibers prompting the ventricles to contract (figure 2.6). It is worthy to note that there are two different types of the myocardial action potential; pacemaker (SA & AV node) and non-pacemaker (atria, ventricles and Purkinje fibers) action potentials (figure 2.7A)¹⁶¹⁻¹⁶⁶.

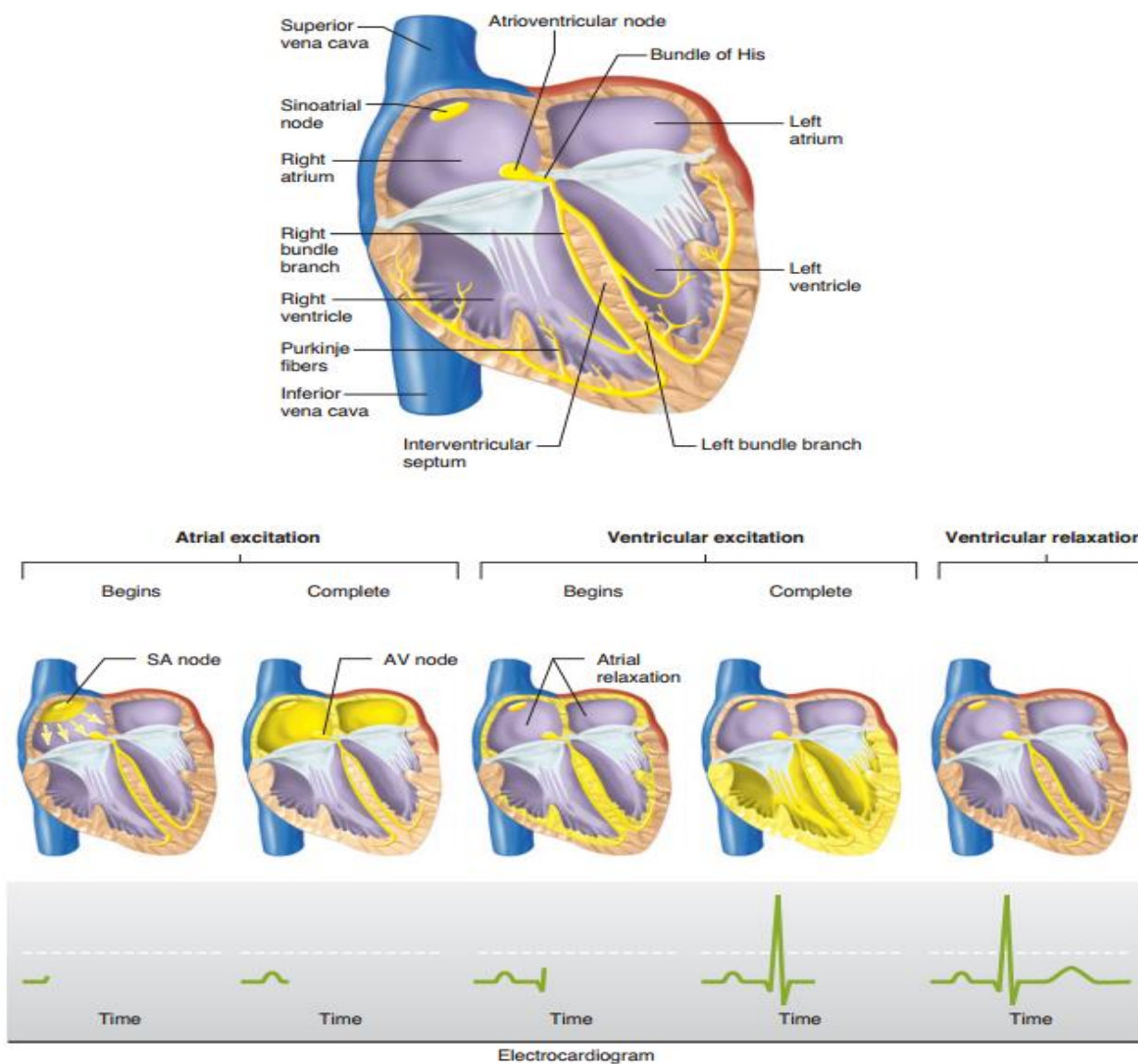


Figure 2.6: Cardiac conduction system. *Adapted from Barrett K. et al, Ganong's review of medical physiology, 23rd edition, 2009. Copyright © 2010 by The McGraw-Hill Companies Inc. All rights reserved.*¹⁶⁷

➤ **Action potential of Non-pacemaker cells:**

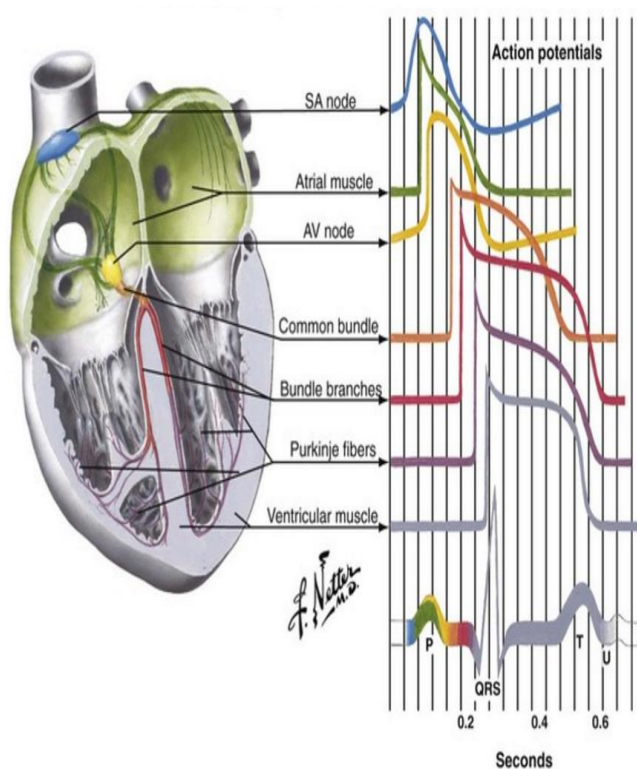
It's also called fast response cells as these cells have almost very rapid depolarization. Unlike pacemaker cells, AP of these cells composed of five different phases and it has true resting potential (phase 4). **Phase 0:** in this phase, there is a rapid depolarization of cells (from -90mV to -70mV) because of the transient increase in the fast Na^+ channels that cause increase in the Na^+ conductance to the cells. **Phase 1:** Because of the opening of special type of K^+ channels (transient outward K^+ channels), repolarization is taking place in this phase. **Phase 2:** "Plateau phase". At a membrane potential near -40mV, voltage dependent Ca^{+2} channels (L-type) open and that lead to increase Ca^{+2} inward to cells. This plateau phase prolongs the AP, which is the unique and the most distinguishable feature of the cardiac AP from other cell's AP. The plateau phase seen in the AP of these cells is because there is an increase in inward Ca^{+2} at the same time of K^+ outward that causes the repolarization to delay. **Phase 3:** This phase represents repolarization, which happens when K^+ conductance (g_{K^+}) and K^+ current (I_{K}) increase and at the same time Ca^{+2} conductance ($g_{\text{Ca}^{+2}}$) decreases as the Ca^{+2} channels close. **Phase 4:** Cells in this phase will be highly negative (-90mV) as both K^+ conductance (g_{K^+}) and K^+ current (I_{K}) are high because K^+ channels are open and both Ca^{+2} and Na^+ channels are closed. This phase occur after the repolarization is complete. It should be noted that the AP in these cells start and end at phase 4 (figure 2.7A) ^{159,160,165,166,168}.

➤ **Action potential of pacemaker cells:**

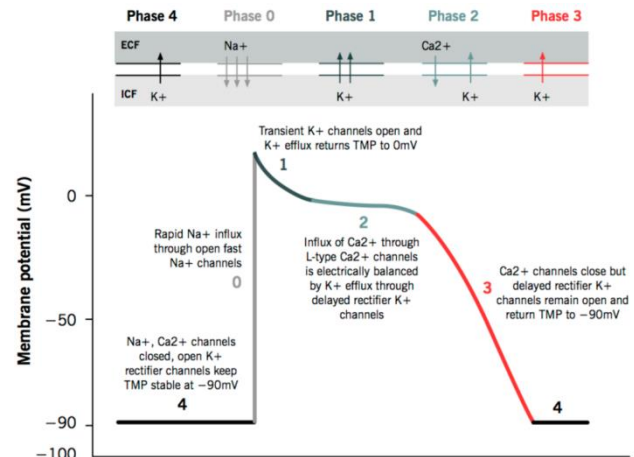
SA and AV nodes are the primary pacemaker cells. These cells have no true resting potential and they generate regular and spontaneous action potentials. Furthermore, there is no fast Na^+ currents operate in SA nodal cells. It should be noted that these cells have the ability to initiate its AP and they do not require external stimulation to initiate AP. **Phase 4:** It is the

spontaneous depolarization phase. Although it's unclear how that is happening, slow Na^+ currents and changes in $g_{\text{Ca}^{+2}}$ and g_{K^+} are likely the mechanism that responsible for this phase. When the membrane potential is around -50mV , T-type Ca^{+2} channels will open and cause Ca^{+2} to enter the cell. **Phase 0:** in this phase, depolarization is caused as Ca^{+2} conductance ($g_{\text{Ca}^{+2}}$) increases due to the opening of L-type Ca^{+2} channels. In this phase also Ca^{+2} conductance is occurred through L-type not T-type Ca^{+2} channels as the Ca^{+2} conductance through T-type Ca^{+2} channels are declined. **Phase 3:** Repolarization occurs because K^+ channels are open (increase K^+ conductance (g_{K^+})), which cause K^+ to go out the cells, and that help cells to repolarize as the Ca^{+2} channels are closed (figure 2.7B) ¹⁶⁸⁻¹⁷².

A



B



C

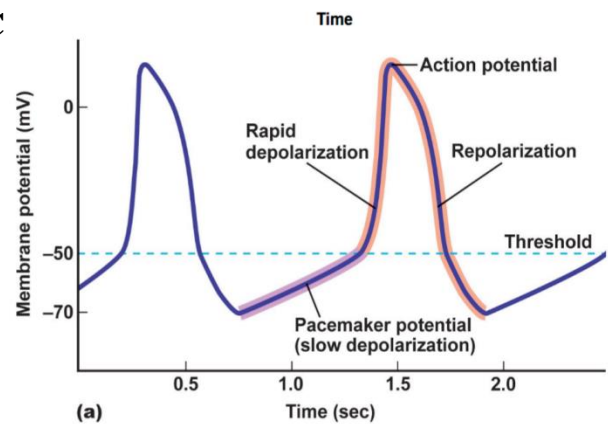


Figure 2.7: Cardiac action potential. (A) Action potential of different regions in the heart. (B) Non-pacemaker action potential. (C) Pacemaker Action potential. *These images are adapted from (A) Netter images (www.netterimages.com) © Elsevier Inc. All rights reserved 2016, (B) Ikonnikov G. et al, Physiology of cardiac conduction and contractility, McMaster Pathophysiology Review, 2013 ¹⁷³, Copyright © 2012-2016 McMaster Pathophysiology Review (MPR). All rights reserved (C) Pearson education Inc., Copyright © 2011 Pearson Education, Inc. All rights reserved.*

➤ **Cardiac Excitation-Contraction Coupling (ECC):**

Cardiac excitation-contraction coupling (ECC) can be defined as the process that couples the cardiac electrical impulses (action potential “AP”) with the cardiac contraction. In other words, it is the process that couples the depolarization of the cardiac cells to the Ca^{+2} release from the sarcoplasmic reticulum (SR), which is the intracellular store of Ca^{+2} . In ECC and during phase 2 (plateau phase), L-type Ca^{+2} channels (voltage gated Ca^{+2} channel located on the SR and the plasma membrane) will open and that will potentiate Ca^{+2} to enter the cell. This increase in the Ca^{+2} inward subsequently lead to the stimulation of the release of the stored Ca^{+2} in the sarcoplasmic reticulum (SR) through RYR (ryanodine receptor) (figure 2.8). This process called “calcium induced calcium release (CICR)” ¹⁷⁴⁻¹⁷⁸. After Ca^{+2} are released from SR, the intracellular Ca^{+2} concentration will dramatically increase (10^{-7} to 10^{-5} M). This will cause the binding of Ca^{+2} to troponin-C (TN-C) which then induce conformational changes to troponin-I (TN-I) and that will promote the binding of myosin ATPase, which is located on the myosin head, to actin. This binding of myosin ATPase to actin results in hydrolysis of the ATP and that hydrolyzed ATP can be used to induce contraction (myosin-actin complex). After phase 2 is

completed and because Ca^{+2} channels are inactivated (closed) at phase 3, Ca^{+2} inward to cells will be declined and Ca^{+2} ions will be sequestered or removed from the cytoplasm through different mechanisms, including sarcoplasmic reticulum ATP-dependent calcium pump called sarco-endoplasmic reticulum calcium-ATPase (SERCA) which re-sequester Ca^{+2} ions into the SR (Ca^{+2} storage organelle), sodium-calcium exchangers (NCX) which removes Ca^{+2} ions from the cytosol by exchanging one Ca^{+2} molecule from inside the cell with 3 molecules of Na^{+} from outside the cell and plasma membrane calcium ATPase pump which remove Ca^{+2} from the cells through the sarcolemma. Consequently, intracellular (cytoplasmic) Ca^{+2} concentration will sharply decrease and that can inhibit the binding of Ca^{+2} to TN-C (figure 2.8) ^{174-176,178-184}.

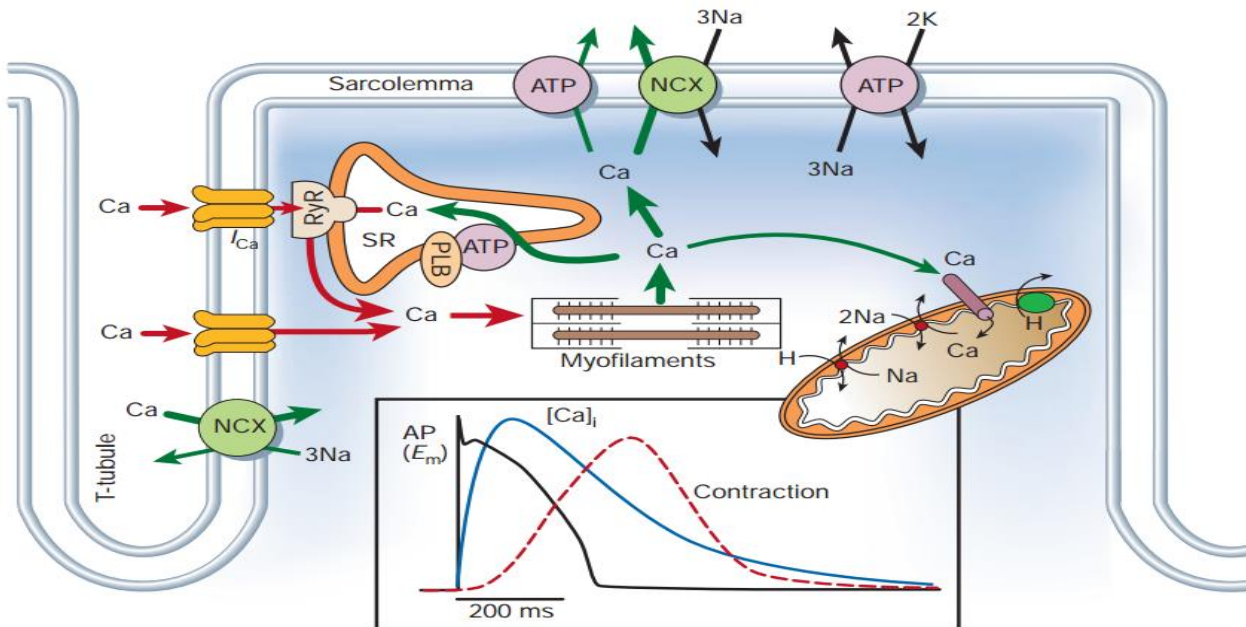


Figure 2.8: Cardiac excitation contraction coupling (ECC). *Adapted by permission from Macmillan Publishers Ltd: Bers D., Nature 415, 198-205, copyright 2002*

Based on that, these electrical impulses (action potentials), which are generated and transmitted throughout the heart, are measured by ECG through different electrodes placed on certain regions on the skin and then represent these impulses graphically. It should be noted that the ECG has 10 electrodes: six placed on different areas on the chest, one electrode on each leg and one electrode on each arm (three bipolar and three unipolar leads) ¹⁵⁸⁻¹⁶⁰. Each bipolar lead measures the differences in potentials between 2 electrodes. For instance, lead I measures the difference in the impulses between the right and left arms, while lead II measures the potential differences between the left leg and the right arm. Lead III, however, measures the potential differences between the left leg and the left arm. The three unipolar leads (also known as “the augmented leads”) basically measure the differences in the potentials between 1 electrode and zero potential ^{158,160,185}.

The movement of the electrical activities can affect the direction of the deflections, for example, the upward deflection is caused by the movement of the electrical activity to the lead, whereas its movement out from the lead results in downward deflection ¹⁵⁸. In the ECG tracing, the baseline (the isoelectric line) represents the resting membrane potential. The ECG tracing begins with a small deflection (upward deflection) called “P wave” and this wave represents the atrial depolarization (figure 2.9). Depolarization of the ventricles is represented by QRS wave complex, where the Q wave indicates the depolarization of the ventricular septum, R wave represents the ventricular mass depolarization and S wave indicates the last depolarization of the ventricles. T wave in the ECG represents the ventricular repolarization (either upward or downward deflection). The time between the appearance of the P wave (first deflection) and the appearance of the QRS complex is called “PR interval”, while the time from the end of the QRS

wave complex to the beginning of the T wave is called “ST interval or ST segment” (figure 2.9)

158–160,186–188

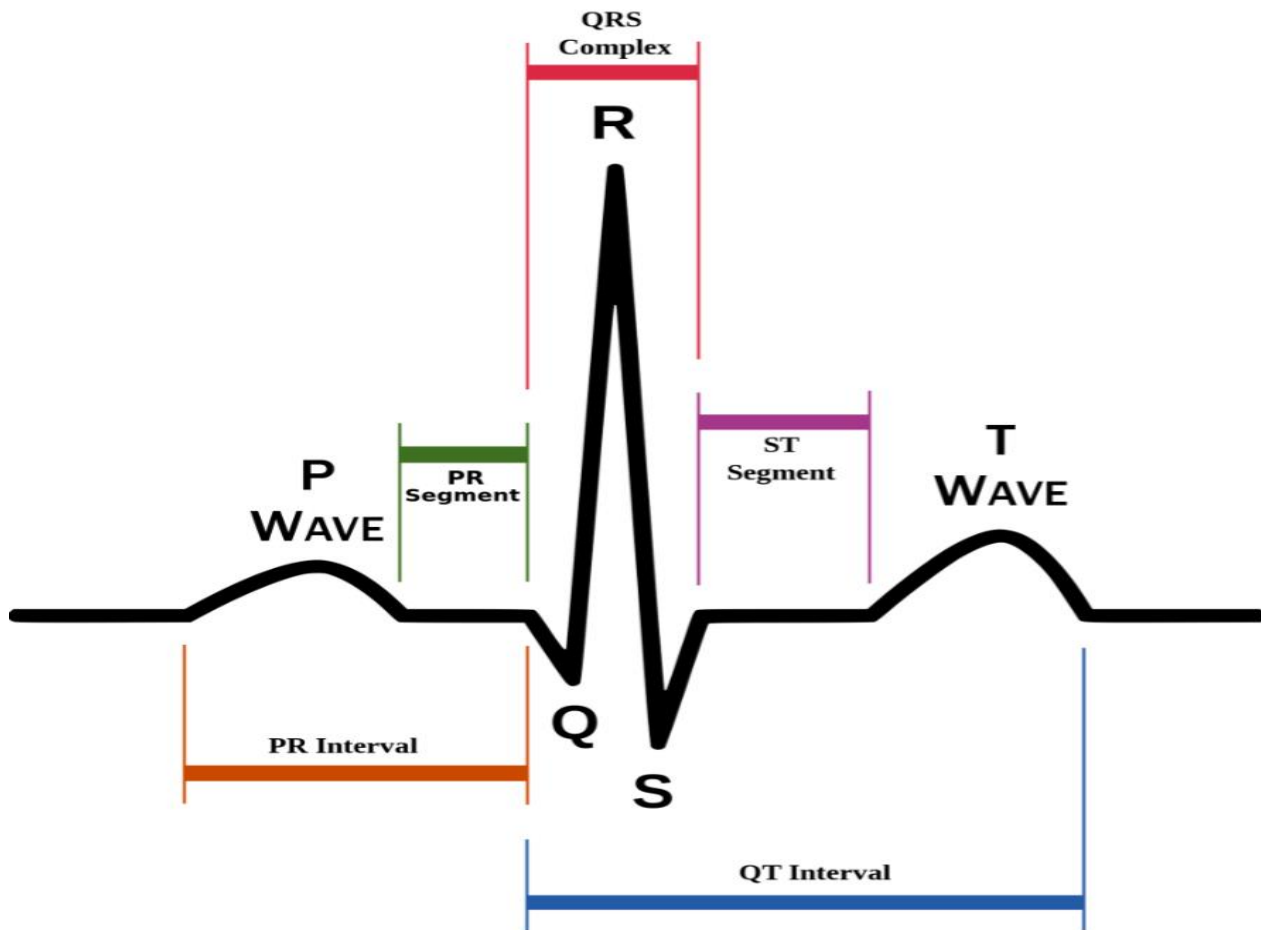


Figure 2.9: Normal Sinus rhythm obtained by electrocardiography (ECG). *Adapted from* <http://dev.laptop.org/pub/content/wp/en/Electrocardiogram.html>

Electrocardiography (ECG) is a central technique that can be used along with other techniques to accurately diagnose MI according to the third global MI task force. In that meeting, global MI task force has indicated that patients with symptoms of MI should go through ECG procedures immediately (within 15 minutes) upon their initial presentation and it should be

repeated more than one time with 10-20 minutes gap between each recording¹³⁰. Changes in the QRS complex, PR segment, T waves and ST segment in addition to signs of atrioventricular and intraventricular conduction delays and cardiac arrhythmia are some of the ECG abnormality features (signs) of MI. The major ECG characteristics of MI are changes in the ST segment (ST segment elevation) and T waves. It should be noted that ST segment elevation is preceded by increase in the amplitude of T waves (T waves may become tall, symmetric, pointed or inverted). Resolving of the elevation of the ST segment and the inverted T waves may take several weeks or even months. ST segment depression (which is an indication of large infarction), generation of transient pathological Q wave and the absence of the R wave pattern from the QRS complex can be detected in progressed MI^{130,189,190}. Furthermore, formation of a single and giant R wave, due to the fusion of QRS complex, T wave and ST segment, can be an indication for the developing of MI. Furthermore, posterior infarction can be diagnosed and displayed on the ECG as ST segment depression with positive T waves (upward deflection)^{130,190,191}. Collectively, we can conclude that ECG provides valuable information about the myocardium and its pathophysiology during MI and it helps the clinicians to easily diagnose and treat patients with symptoms of MI promptly.

2.5. Myocardial Ischemia Reperfusion (IR) Injury

In the early nineteenth century, the term “ischemia” was originally used to indicate the lack (loss) of blood supply to tissues as a result of occlusion of the arterial blood flow. For several decades, considerable and boundless endeavor has been devoted to understand the

cellular and molecular mechanisms that result in tissue damage after an ischemic period in order to salvage tissues from ischemia-induced damage ^{6,8,17}. Over the past 50 years, numerous reported studies have markedly participated in expanding our understanding of the cellular and molecular mechanisms of ischemia and reperfusion and how that can lead to tissue damage, more specifically cardiac damage ⁶. In 1960, the famous paper by Jennings *et al.* depicted for the first time, using a model of coronary artery occlusion in dogs, the term “ischemia-reperfusion (IR) injury” ¹⁹². In this landmark paper, Jennings and his colleagues reported that the size of the necrotic zone was hugely escalated during the reperfusion period and this increase in the necrotic size and the histological changes observed after 60 minutes of IR injury were exceedingly equivalent to the degree of damage usually observed after 24 hours of sustained coronary ligation ¹⁹². Results of this paper foster the discussion that continued for many years whether reperfusion is independently responsible for tissue damage (injury) or it indirectly promotes and accelerates tissue damage ^{193,194}. The results obtained by Braunwald and Kloner in 1985 revealed a proof that myocardial reperfusion is directly responsible for the tissue damage that observed in response to IR and they described the reperfusion in this paper as “a double-edged sword” because of its beneficial and harmful effect ¹⁹⁵. Furthermore, the discovery of IPC (ischemic preconditioning) by Murry and Reimer in 1986 helped to elucidate the difference between the independent effect of ischemia and reperfusion ³³. Despite the rigorous and the comprehensive investigation for many decades, current knowledge about IR injury and the molecular mechanisms that promote tissue damage in addition to the negative results obtained from clinical trials make us far away from thoroughly understanding the principal mechanism of IR ^{8,9,196}. Therefore, understanding the underlying molecular mechanism of IR by testing different hypotheses that target different molecular pathways would help us to accurately unravel the

principal mechanism of IR. In order to do that, we should first recognize the current notion about the mechanistic progression of IR injury. In that regard, we can define myocardial ischemia as an acute occlusion or severe damage of the coronary blood supply to the myocardium which is mostly resulted from thrombosis or other serious modifications of the atherosclerotic plaques in the coronary arteries. Myocardial reperfusion, however, is basically restoration of coronary blood flow to the myocardium ⁶. Although the restoration of coronary blood flow after transient ischemia reduces the ischemic injury or damage and necrosis, it may be associated with further pathological, biochemical, morphological and functional changes that eventually result in reversible or irreversible cell damage which can influence the cardiomyocytes viability and this is termed as “ischemia-reperfusion (IR) injury” ⁶⁻⁹. It is appreciated that the majority of the cardiac damage occurs during reperfusion period not during the ischemic period ³⁰⁻³². Therefore, the severity of the myocardial damage during reperfusion stage depends on the severity and duration of the ischemic period. Indeed, if the duration of the ischemic period is less than twenty minutes, cardiomyocytes can tolerate the ischemic injury and the pathological and functional changes can be treated as the resulted damage is reversible. Nonetheless, if the ischemic period persists for more than 20 minutes, irreversible cardiac damage, impaired cardiac functions, increased infarction size and necrosis will be observed (figure 2.10) ^{8,10-15}.

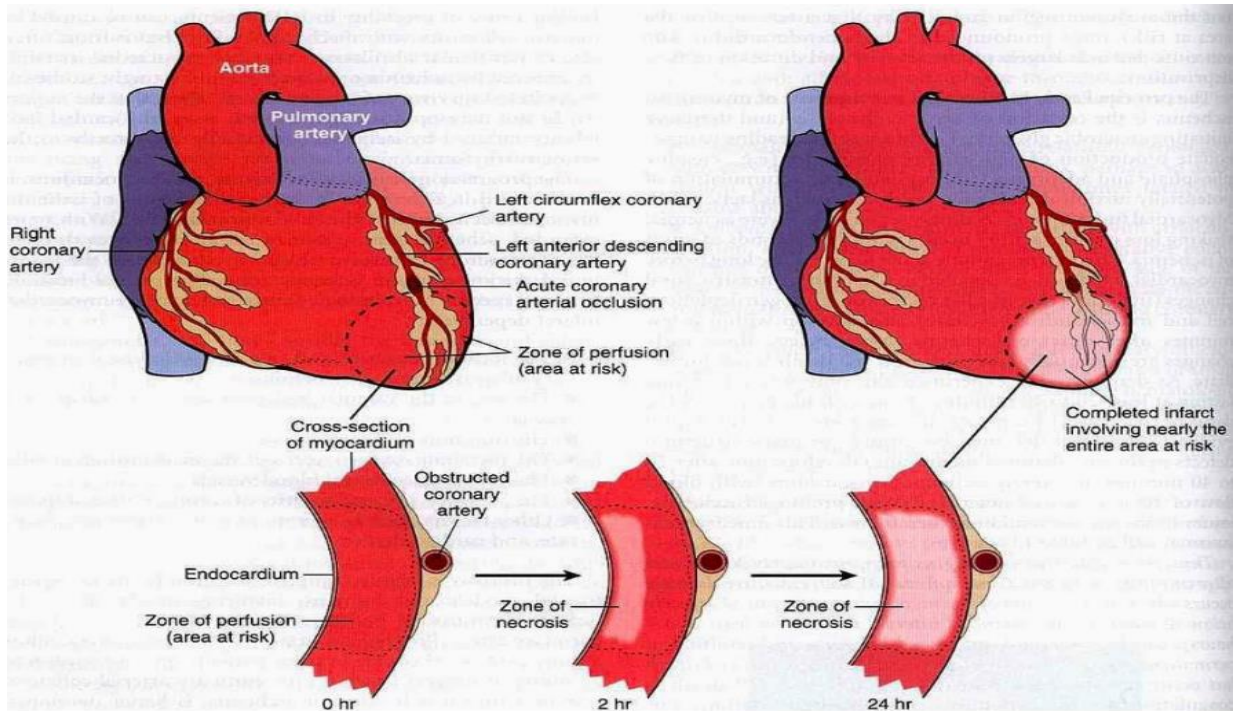


Figure 2.10: Myocardial necrosis development after coronary occlusion for 0, 2 and 24 hours.

Adapted from Elsevier Inc. All rights reserved 2005.

2.5.1. Clinical Consequences of Myocardial IR Injury

The cardiac cell damage developed because of the IR injury can be clinically reversible (arrhythmia, endothelial and microvascular dysfunction (no reflow) and myocardial stunning) or it can be irreversible and that results in cardiac myocyte death (apoptosis and necrosis)⁹⁻¹¹. Here in this section, we will discuss each of these clinical consequences in further details.

➤ **Myocardial stunning:**

Myocardial stunning, which was first defined by Heyndrickx *et al* in 1975, is a reversible prolonged post-ischemic depression of the cardiac contraction function of the viable myocardium that rescued (salvaged) during reperfusion^{17,18,25,197}. It results from the deleterious effect of oxidative stress and calcium imbalance (overload) during reperfusion of the ischemic myocardium. It can be developed even after less than 15 minutes of ischemia, which is insufficient period to induce cardiac myocytes necrosis, as it has been reported that 15 minutes of ischemia in dogs induced myocardial contractile dysfunction that persist for 24 hours^{11,198,199}. Nonetheless, when the coronary occlusion sustained for longer period, more than 3 hours, the myocardial stunning will be much more severe and it will persist for longer time. Reperfusion of a regionally (percutaneous coronary intervention ‘PCI’, unstable angina and thrombolysis) or globally (cardiac arrest) ischemic myocardium can result in myocardial stunning. It should be noted that the full functional recovery of the stunned myocardium requires a longer period of time; therefore, constant incidents of cardiac ischemia and reperfusion can result in heart failure²⁰⁰⁻²⁰³.

➤ **Arrhythmia:**

Sudden restoration of the blood supply to the ischemic myocardium can potentially result in lethal ventricular arrhythmia^{11,204,205}. Accelerated idioventricular arrhythmia, ventricular fibrillation and ventricular tachycardia are the most common arrhythmia that can cause sudden

cardiac death in response to reperfusion. It has been proposed that ventricular arrhythmia is developed because of oxidative stress (ROS) that is generated after IR injury^{206–208}.

➤ **Microvascular dysfunction (no-reflow):**

Microvascular dysfunction is a manifestation of the IR injury. It was first described in 1966 by Krug et al as the failure to perfuse the ischemic region during reperfusion (no-reflow)^{11,17,209}. The occurrence of no-reflow is characterized by alteration in the vascular structure and decrease in the resting cardiac blood flow. This phenomenon has been revealed using myocardial contrast echocardiography, positron emission tomography, magnetic resonance imaging (MRI) and nuclear scintigraphy^{210–218}. Several studies have linked microvascular dysfunction to partial ST segment recovery and that microvascular injury can induce myocardial infarction and death in some cases^{219–224}. Moreover, the presence of microvascular dysfunction is connected with a larger infarction size, negative LV remodeling and decrease in the EF^{218,223,225–227}. Endothelial cell dysfunction, microvascular obstruction, de novo thrombosis, micro-embolism of platelets, neutrophil capillary plugging, leukocyte activation, oxidative stress, edema, and increase in fluid and protein extravasation into the interstitium are the major contributing pathogenesis of microvascular injury during reperfusion^{17,227–230}. Therefore, insufficient perfusion to the infarcted zones of the myocardium during reperfusion can result in severe and serious damage and that can result in increase in the wall stress and subsequently to wall rupture. Unfortunately, there is no effective treatment available currently available to treat or minimize the microvascular injury^{11,17}.

➤ **Endothelial dysfunction:**

For many decades, the impact of the IR injury on the heart has been extensively studied. However, the impact of the IR injury on the arteries (endothelial cells) hasn't attracted much attention of the researchers¹⁷. It was not until 1982 when Ku *et al* has reported, for the first time, the development of endothelial dysfunction after he induced ischemia for 90 minutes followed by 2 hours of reperfusion²³¹. Endothelial dysfunction can be manifested by the deficiency in the endothelium vasodilation, increase in the endothelium vasoconstriction and increase in the production and release of potent vasoconstrictor such as oxygen free radicals and endothelin-1, which can reduce the blood flow¹⁷. It has been reported that endothelial dysfunction can be developed early during reperfusion (within 5 minutes) and it will recover but after long time (up to 12 weeks)²³²⁻²³⁵. Different studies reported that ischemia (for 2-4 hours) with no reperfusion will result in slight changes in the endothelial vasodilation with the prolonged ischemia (from 4-6 hours) can result in endothelial dysfunction similar to what is observed after IR^{234,236}.

➤ **Cardiac myocyte death:**

It has been reported that cardiac cells can tolerate short period of ischemia (less than 15 minutes) without any loss in the myocardium viability (no cell death). This tolerance of the myocardium can be observed in balloon angioplasty, angina and coronary vasospasm^{200,202}. However, longer period of ischemia (more than 20 minutes) can result in cardiac cell death. Cardiomyocyte cell death during the re-oxygenation period is called 'oncosis'. There are

different mechanisms that can lead to cardiac cells death that we will discuss in further details in this section ^{17,237}.

2.5.2. Biochemical and Metabolic Changes During Ischemia

It is well-established and well-known that oxygen is an important and crucial factor that is required by all cells of most organisms to synthesize sufficient amounts of ATP that is needed for different cellular functions. It has an important role in the respiratory chain complex as it the acceptor of the final electron. Because of the importance of oxygen, it is not surprising that any deficiency or shortage in the oxygen supply that caused by different mechanisms and/or diseases such as ischemia/hypoxia of human tissues and cells can result in enduring damage and subsequently tissue death. Cells naturally have numerous mechanisms to modulate the deficiency in the oxygen supply in order to maintain the viability of cells through the synthesis of ATP by targeting and stimulating many genes and pathways ²³⁸⁻²⁴¹.

Under the normal physiological conditions, cardiac myocytes have specific and limited tolerance (susceptibilities) to a brief period of ischemia. Because the normal heart consumes more than 90% of the energy that are required to maintain the normal function (such as contraction, ions homeostasis and normal metabolic processes) as ATP through the aerobic fatty acid oxidation (mitochondrial oxidative phosphorylation 'OXPHOS') and because the coronary blood supply to the myocardium can deliver the required energy (ATP) and oxygen (O₂) and clear the cells from the waste such as lactate and carbon dioxide (CO₂), any change or shortage in the oxygen blood supply can lead to changes in the normal functions of the heart and

subsequently to biochemical and metabolic changes^{194,238,240,242}. Indeed, during ischemia, lack of oxygen blood supply to the myocardium (deficiency of oxygen in the mitochondrial respiratory chain) results in depolarization of the mitochondrial membrane, reductions of ATP levels and inhibition of the cardiac contraction. This cause cells to shift from OXPHOS (Oxidative phosphorylation) to anaerobic glycolysis to maintain ATP production, which results in the production of lactic acid and accumulation of hydrogen ions (H^+) and that lead to a decrease in both intracellular and extracellular pH^{6,8,21,237,242}. Depleted ATP levels inactivate Na^+/K^+ ATPase pump leading to Na^+ accumulation in the myocyte. Furthermore, Na^+/H^+ exchanger brings Na^+ into the cell while pushing out H^+ in order to adapt acidosis (figure 2.11). This will lead to increase in the intracellular Na^+ concentration, which cause the Na^+/Ca^{+2} exchanger to function in reverse mode carrying Na^+ out of the cell and Ca^{+2} inside the cell^{11,18,243–245}. Ca^{+2} also enters through the sarcolemmal voltage-gated Ca^{+2} channel (L-type) resulting in Ca^{+2} induce Ca^{+2} release from the SR (sarcoplasmic reticulum) and the combined increase in intracellular Ca^{+2} levels (Ca^{+2} overload) leads to hyper-contraction and that can stimulate cellular damage through the activation of different degrading enzymes such as nucleases, phospholipases and proteases (calpains) that participating in the damage of the membrane structure and that can cause cell death if the ischemic period is prolonged. Ca^{+2} ions that transported into the cells via L-type Ca^{+2} channel are removed from the cell primarily by NCX (Na^+/Ca^{+2} exchanger), while the Ca^{+2} released from the SR is re-sequestered into the SR via SERCA (sarcoendoplasmic reticulum calcium transport ATPase). The acidic conditions that developed during ischemic period prevent opening of the MPTP (mitochondrial permeability transition pores) (figure 2.11)^{13,32,245–249}. It should be noted that ischemia induces reduction of glutathione (GSH) level, which is a known potent anti-oxidant enzyme that helps to protect cells against oxidation events, and

that can result in increase in the toxic effect of the oxidants. The duration of the ischemic period and the degree of the decrease in the coronary blood supply have a huge impact on tissue injury and on the biochemical and metabolic changes that occur in response to ischemia⁷.

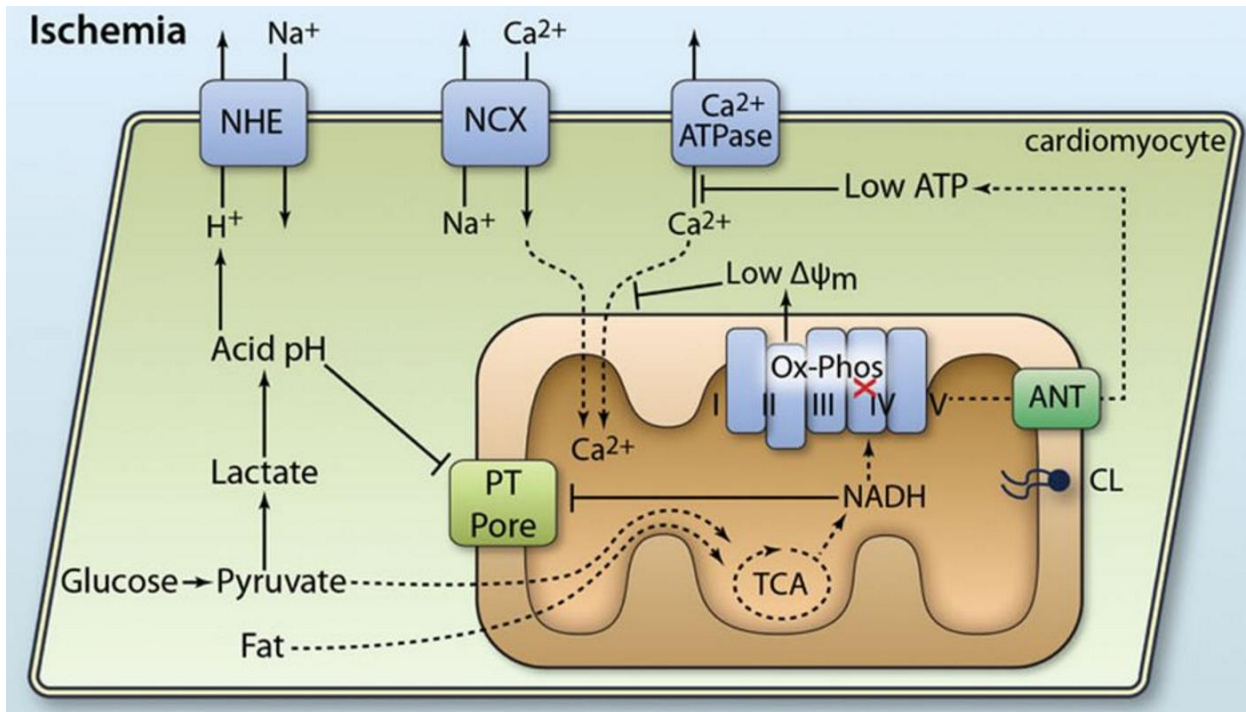


Figure 2.11: Myocardial pathological changes (biochemical and metabolic) during ischemia. Adapted with permission from Wolters Kluwer: *Circulation Research*, Walters A. et al, *Mitochondria as a Drug Target in Ischemic Heart Disease and Cardiomyopathy*, 111 (9); 1222-1236, 2012.

2.5.3. Biochemical and Metabolic Changes During Reperfusion

The re-oxygenation of the cardiac cells during reperfusion, however, leads to activation of the electron transport chain which is inefficiently synthesizing ATP, loss of membrane phospholipids, decrease in the production of nitric oxide (NO) and increase in the formation of ROS (reactive oxygen species). These ROS mediate myocardial reperfusion injury by inducing the opening of the MPTP and mediating dysfunction of the sarcoplasmic reticulum (SR) (figure 2.12). Reactivation of the Na^+/K^+ pump by ATP slowly restores the sodium gradient leading to normal cation fluxes with the NCX. Reperfusion and reactivation of the Na^+/H^+ exchanger result in washout of lactic acid, leading to rapid restoration of physiological pH, which causes MPTP opening and cardiomyocyte hyper-contraction. The increase in the intracellular and the mitochondrial calcium content induces MPTP opening, which strongly contributes to the cardiomyocytes hyper-contraction, apoptosis and necrosis (figure 2.12). Therefore, successful clinical intervention during the reperfusion period can help to salvage the cardiac cells as successful intervention can minimize the infarction size by more than 40%.

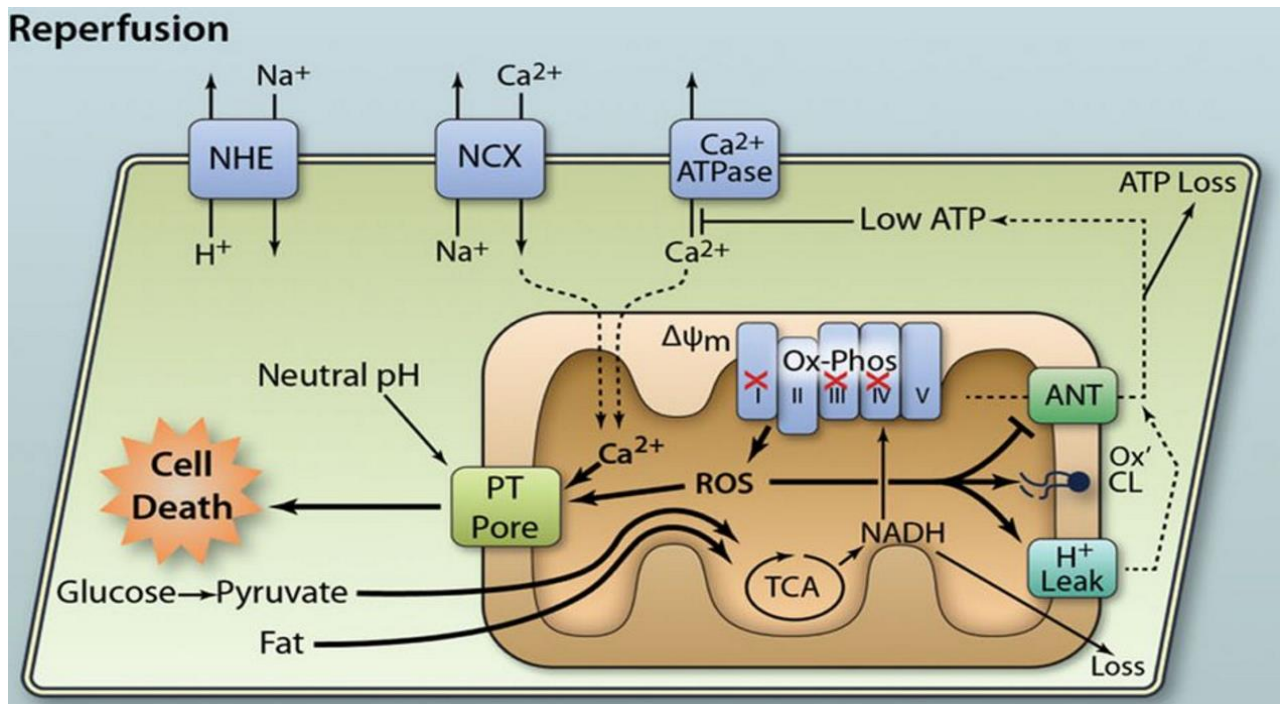


Figure 2.12: Myocardial pathological changes (biochemical and metabolic) during reperfusion. Adapted with permission from Wolters Kluwer: *Circulation Research*, Walters A. et al, *Mitochondria as a Drug Target in Ischemic Heart Disease and Cardiomyopathy*, 111 (9); 1222-1236, 2012.

2.6. Cellular and Pathophysiological Mechanisms of IR Injury

Despite many decades of intensive research regarding ischemia-reperfusion injury, the fundamental pathophysiological mechanisms of reperfusion injury still have not been fully explained. However, different mechanisms have been proposed recently to be involved essentially in the IR injury. It should be noted that the probability that any of the proposed mechanisms alone can induce myocardial injury is little or unlikely, but this probability increase dramatically when many mechanisms occur at the same time. These mechanisms are multifactorial and complex, and involve oxidative stress (oxygen free radicals), Ca^{+2} overload (induce hyper-contraction) and mitochondrial permeability transition pores (MPTP).

➤ Oxidative stress:

The formation of the oxygen free radicals (ROS such as peroxynitrite, hydroxyl radicals and superoxide anion) during ischemia and first few seconds of reperfusion have been associated with most of the damage developed in cardiomyocytes according to different published studies. The main sources of these ROS are cellular oxidases (NADPH oxidases and xanthine oxidases), mitochondrial respiration complexes (complexes I, III and IV), activated neutrophils and nitric

oxide producing synthases, which reversibly inhibits cytochrome oxidase and generate ROS instead of NO^{13,26,250-252}. Furthermore, ROS can be also generated through different cellular and biochemical pathways such as catecholamine oxidation (MAO, monoamine oxidases), cyclooxygenases, white blood cells (WBCs) breakout of phagocytes and iron and copper metals reactions. Although Ca⁺² overload and oxidative stress during IR injury are 2 independent events, it has been shown that the cellular accumulation of calcium significantly stimulates and potentiates ROS formation, which give an indication of how these 2 major cellular events can somehow related to each other under IR injury. Indeed, function of the Ca⁺² ATPase pump and Na⁺-K⁺ ATPase pump can be modulated by peroxidation in the sarcolemma. Furthermore, the sequestering properties of the SERCA have been shown to be diminished during reperfusion because of the high levels of ROS. The three major mechanisms are related tightly to each other as Ca⁺² overload in addition to ROS formation can lead to opening of the MPTP, which is the third major mechanism that lead to IR injury^{7,253-257}.

Different studies have demonstrated that oxidative stress mediates damage in the structure, function and integrity of the mitochondria and can oxidize the cellular components of the cells such as lipids in the cell membrane (by lipid peroxidation), proteins and nucleic acids. Oxidation of certain amino acids of some proteins can modulate its functions and its structure by exposing the thiol group and that can result in development of carbonyl side chain on these amino acids (oxidized amino acids)^{7,258-262}. Furthermore and as described early, ROS can affect the integrity and the function of mitochondria and also the mitochondrial DNA through inhibition of the mitochondrial protection mechanism that mediated by histone and through the inhibition of the repairing mechanisms. These mechanisms together during the re-oxygenation period can lead to opening of the mitochondrial permeability transition pores (MPTP) and that

can result in myocardial damage and dysfunction. In the same context, myocardial stunning, which is a reversible contractile dysfunction after IR injury in the heart, is considered as a manifestation of excessive oxidative stress and it can be measured by myocardial contrast echocardiography, myocardial perfusion nuclear scanning or contrast-enhanced cardiac MRI. Furthermore, free radicals in the perfused heart can be measured by electron spin resonance by grinding frozen samples or by using fluorescent indicators^{261,263-267}.

➤ **Calcium overload:**

Different studies have reported that there is an increase in both cytosolic and mitochondrial calcium levels in response to ischemia reperfusion insult²⁶⁸⁻²⁷³. However, the first study that reported the increase in intracellular calcium levels in response to IR injury was published in 1972 by Shen and Jennings *et al*^{274,275}. In their study and using a canine heart, they have observed an increase in the cytosolic calcium levels during the reperfusion period. Currently, it is appreciated that IR injury promotes Ca^{+2} entry via the L-type Ca^{+2} channel and that can result in Ca^{+2} induced Ca^{+2} release (CICR) from the sarcoplasmic reticulum and that leads to cardiac contraction (hyper-contraction). The significant increase in calcium levels intracellularly lead to modifications in the sensitivity of the myofilaments to calcium (desensitization) and that can result in tissue damage and cardiac dysfunction in response to IR insult. Furthermore, calcium overload intracellularly can activate the calcium/calmodulin-dependent protein kinases (CaMK) resulting in cardiac dysfunction and tissue damage (death)^{7,17,32,276}. During reperfusion and because of the imbalance in the calcium levels, arrhythmias can be prompted and that cause death in response to IR insult. Furthermore, many studies have demonstrated that both oxidative stress and calcium overload compromised the mitochondrial

integrity in the cardiomyocytes and that can cause damage of the cardiac myocytes by opening MPTP ^{17,237,247} .

Using Mn^{+2} to reduce cytosolic Ca^{+2} , mitochondrial Ca^{+2} levels in perfused hearts were measured by a group of researchers using indo-1 and surface fluorescence. MPTP opening during IR injury can be measured by fluorescence microscopy of isolated cardiac myocytes, by measuring the loss of mitochondrial NAD^{+} (as both mitochondrial and cytosolic NAD^{+} are lost during IR injury and that cause opening of MPTP), by measuring the lactate dehydrogenase levels and by measuring radioactive markers such as 3H in the mitochondria ^{16,26,277-279} .

➤ **MPTP:**

In recent years, a great attention has been devoted to study the important role of mitochondrial permeability transition pores (MPTP) during IR injury. Results of different studies have revealed that opening of the MPTP during reperfusion period results in the cardiac damage and death as the opening of the MPTP lead to decrease in the mitochondrial membrane potential ($\Delta\psi M$), generation of more ROS, ATP depletion and rupture of the mitochondria ^{8,16,26,246,276,280} . Furthermore, inhibition of the MPTP opening during reperfusion period has been found to be effective therapy against IR injury ²⁸¹⁻²⁸⁴ .

MPTP is a non-selective pore protein that remains closed under normal conditions and it opens in response to cellular stresses such as IR injury. It is located in the inner membrane of the mitochondria and it facilitates the transfer of molecules between the cytoplasm and the mitochondrial matrix in order to regulate the mitochondrial function and structure by maintaining the $\Delta\psi M$ ^{26,280,282} . Although the molecular components (proteins) that form this pore still to be elucidated, different reports have proposed that cyclophilin D (mitochondrial peptidyl prolyl),

adenine nucleotide transporter or translocator (ANT) and mitochondrial phosphate carrier “PIC”, which is a voltage dependent anion channel, that are located in the mitochondrial matrix, inner mitochondrial membrane and outer mitochondrial membrane, respectively, are forming this mitochondrial pore^{285–287}. Of note that calcium overload, increased levels of inorganic phosphate (iP), oxidative stress, reduced $\Delta\psi_M$ and ATP loss are the key regulators of the opening of the MPTP during IR injury²⁸².

During ischemia, MPTPs have been reported to remain closed despite the increase in the ROS, ATP loss and increased levels of iP because the cellular pH is less than 6.5 (acidic) which can also decrease the calcium uptake during the ischemic period^{288–290}. During reperfusion period, however, the restoration of the physiological pH in addition to the increase in the formation of ROS due to the re-introduction of oxygen, calcium overload, ATP depletion and rise in NADH levels lead to opening of the MPTP. Opening of these pores during reperfusion will cause the $\Delta\psi_M$ to collapse and that will result in modification of the mitochondrial bioenergetics (loss of ATP and NAD^+) and permeation to molecules (bigger than 1.5 KDa) through the mitochondrial membrane and that can result in mitochondrial swelling and rupture of the mitochondrial membrane leading to release of the pro-apoptotic factors (Cyt c) which prompts apoptosis and that can result in cell death^{23,278,291–293}. Therefore, it is not surprising that targeting these key regulators of MPTP opening during reperfusion is important and considered as one of the most important cardioprotective mechanisms against IR injury^{294–297}.

2.7. Main Mechanisms of Tissue Death in IR Injury

The heart is a greatly energetic and active organ that has partial ability for regeneration and restoration of normal physiological functions when subjected to stresses. It is well appreciated that the heart has high rate of ROS because of the high energetics demand. It has been believed, for long time, that the cardiac cell death in response to IR injury arise through necrosis and other factors, such as ATP depletion, Ca^{+2} overload and ROS formation. However, programmed cell death (apoptosis and autophagy) is currently being appreciated to induce cell death after myocardial IR injury. Different studies have reported that myocardial cell death after IR injury occurs via apoptosis, necrosis and autophagy. Therefore, we will discuss each of these death mechanisms in details in this section.

2.7.1. Apoptosis:

Apoptosis is a programmed cell death mechanism that can happen as a part of normal physiological process or under pathological conditions in response to stimuli (external or internal). Apoptosis is characterized by cell shrinkage, DNA fragmentation, gene activation, activation of caspases, cytochrome C release and presence of apoptotic bodies, which contain cellular components^{276,298,299}. In this type of cell death, there is no inflammatory reaction due to the absence of the intracellular components release¹³. The mechanism of apoptosis can be initiated by intrinsic (mitochondrial pathway) and extrinsic (death receptor pathway) pathways. In the extrinsic pathway, activation of the death receptors, such as Fas, TRAIL (TNF-related apoptosis-inducing ligand) and TNF- α leads to recruitment of the FADD (Fas associated death domain) and TRADD (TNF receptor associated death domain) proteins to form a receptor-protein complex which activates the caspases (caspase-8 and -3) and that causes cleavage and

fragmentation of the DNA and other vital cellular components^{276,300-302}. In the intrinsic pathway, nevertheless, the presence of stimuli (e.g., IR, ROS or toxic compounds) causes the members of Bcl2 protein family (Bak and Bax) to translocate into the mitochondrial outer membrane and that results in MPTP opening and modification in the mitochondrial membrane potential resulting in release of pro-apoptotic proteins (cytochrome C and serine proteases) which in turn activate caspases 3 and 9 to induce DNA fragmentation and then cell death. This apoptotic pathway (intrinsic pathway) is considered the principal mechanism of apoptotic cell death in the heart after IR injury³⁰⁰⁻³⁰⁵. It is worthy to note that after permanent occlusion of coronary arteries in the heart (ischemia), apoptosis is the major mechanism of cell death and it is peaked at 5 hours and it is further intensified (more apoptotic cell death) during reperfusion²⁹⁹.

For many years, there has been an indecisive debate regarding the inhibition of caspases and the cardiac infarction size after IR injury. For instance, it has been reported that inhibition of the pro-apoptotic factors, Bax, and stimulation or overexpression of anti-apoptotic factors, Bcl2, can minimize cardiac cell death after IR injury. Furthermore, it has been observed that caspases inhibitors can decrease the infarction size significantly after IR injury³⁰⁶⁻³¹². In contrast, it has been reported that inhibition of caspases resulted in insignificant reduction in the cardiac infarction size in response to IR when it compared to the infarction size after overexpression of anti-apoptotic factors³¹³. These studies suggested that overexpression of anti-apoptotic factors can reduce the cell death after IR injury.

2.7.2. Autophagy:

Autophagy is a highly conserved physiological mechanism of transportation, degradation and disposal (removal) of damaged or denatured proteins and organelles, such as mitochondria

and ER, through the autophagosome (double-membrane structure) to the lysosomes. It is an important dynamic mechanism for cells to maintain homeostasis, survival and adaptation in response to stress conditions (e.g. hypoxia, ROS and starvation) through the disposal of damaged protein to the autophagosome to the lysosome to degrade it^{29,299,303,314-318}. Although autophagy is considered as the cellular defensive mechanism under pathological conditions, it is also recognized as a cell death mechanism especially if this process is un-controlled¹³. It has been proved that autophagy has a pivotal role in cardioprotection against IR injury when it is induced (stimulated), but when it is inhibited, it has a deleterious effect on the cardiomyocytes and that can stimulate cardiac cell death³¹⁸⁻³²². This cardioprotective role of autophagy can be explained by the role of Beclin-1 (autophagy stimulating protein) on Bax as it has been shown that inhibition of Beclin-1 cause activation of the pro-apoptotic factor Bax and the stimulation of Beclin-1 inhibits the activation of Bax^{321,322}. Furthermore, depletion of ATP during ischemia leads to an increase in the ratio of AMP which results in activation of AMPK (adenosine monophosphate protein kinase) and inhibition of mTOR (mammalian target of rapamycin) proteins and that can induce autophagy to protect the heart against the ischemic injury^{316,318,323-326}. It has been shown that activation of autophagy happen within 30 minutes of ischemia²⁹⁹. Contrary, it has been suggested that autophagy is another form of cellular death and it plays an important role in cardiac cell death in response to IR injury. Therefore, inhibition of autophagy could protect the heart against the IR injury and that means inhibition rather stimulation of autophagy has beneficial effect on the heart¹³.

2.7.3. Necrosis:

Necrosis can be characterized and distinguished from other forms of cell death by its unique morphological features, which include organelles and cells swelling, rupture of the plasma membrane, mitochondrial dysfunction and ATP and other cellular contents leakage. In contrast to apoptosis, release of intracellular contents can trigger the inflammatory response and that can further lead to pathological consequences^{13,276,299,302}. It has been proposed that the release of creatine kinase (CK) and troponin during IR injury is because of necrosis-mediate intracellular components release²⁷⁶. It has been widely accepted that necrosis is a random and uncontrolled mechanism that result in unintentional cells death in response to different stresses³²⁷. However, recent research has shown that necrosis is a controlled process and it can be regulated by different signaling pathways. This term of controlled necrosis was then called “necroptosis or programmed necrosis”^{328–332}.

During ischemia, as we mentioned early in sub-section 2.5.1, there is a metabolic shift from OXPHOS to anaerobic glycolysis in order to preserve cellular ATP production that is needed for cells to survive and that can lead to cellular and mitochondrial accumulation of Ca^{+2} ions. This buildup of Ca^{+2} ions results in increase in ROS formation, decline in NADH levels and ATP depletion^{13,299,302,303}. Consequently, mitochondrial swelling and rupture can occur and that lead to release of the intracellular content which can be further exaggerated during the reperfusion period as reperfusion cause opening of the MPTP and that will result in apoptotic and necrotic cell death. It has been reported that necrosis peaks at 24 hours after IR injury and this process (necrosis) is mainly irreversible process^{299,328,333,334}. However, inhibition of the MPTP opening especially during reperfusion period was shown to reduce apoptotic and necrotic

ell death after IR injury demonstrating that targeting the mitochondria could be beneficial to protect cardiomyocytes from damage and death in response to IR insult^{285,286,335–340}.

2.8. Therapeutic Interventions for Reducing IR Injury

➤ **Ischemic Preconditioning (IPC):**

For many decades, ischemic preconditioning (IPC) is considered the most effective therapeutic intervention to protect the heart from IR injury. IPC was first described and reported by Murry et al in 1986 as an induction of 4 short periods of ischemia (each period for 5 minutes) followed by short periods of reperfusion (each period for 5 minutes) prior to prolonged or sustained ischemia³³. Following the discovery of IPC, different protocols have been implemented to reduce IR injury, but all of these protocols have the same concept, which is repetitive cycles of short periods of ischemia and reperfusion followed by prolonged ischemia¹³. This induction of short periods of ischemia is not long enough to prompt tissue damage; rather it will enhance the tolerance of the heart to more maintained ischemia. Different studies provide confirmatory evidence that IPC can afford reduction in the infarction size and prevent the depletion of ATP production (decrease ATP consumption rate and prevent ATP hydrolysis) in response to IR insult when it compared to non-preconditioned hearts. Furthermore, it has been demonstrated that IPC hearts have higher intracellular pH, less lactate levels and acidosis (decrease in the anaerobic glycolysis) during the ischemic period compared to non-IPC hearts^{310,341–346}. Over years, IPC has been comprehensively studied and investigated to identify practical and sustainable pharmacological mechanisms to reduce IR injury. Some of these studies have revealed many drugs (both agonists and antagonists) that can triggers different signaling

pathways in the mitochondria to protect the heart against IR injury. These mediators and signaling pathways include protein kinase C (PKC), heat shock proteins (HSP), cyclic GMP dependent protein kinase (PKG), ERK and AKT pathways³³⁻⁴⁰. Despite the protective effect of IPC, the fact that the cardioprotective effect of IPC declines with time (usually 1-2 hours) raises the need to find more effective and sustainable treatment to reduce the cardiac damage after IR injury³⁴⁷⁻³⁴⁹.

➤ **Ischemic Postconditioning:**

Although IPC can protect the heart in many clinical cases, IPC is not the choice of treatment in AMI patients (STEMI patients) as they have occlusion in the coronary arteries at the time of clinical presentation. Different studies have reported that ischemic postconditioning, which is a brief cycles or periods of ischemia during the reperfusion period (immediately after an ischemic period), is another cardioprotective mechanism that can reduce the infarction size after IR injury³⁵⁰⁻³⁵⁶. In contrast to IPC, ischemic postconditioning has different protocols depending on the species, duration of ischemia and the time of reperfusion as it has been reported that ischemic postconditioning has no or minimal effect if the ischemic period was for more than 45 minutes³⁵⁷. Furthermore, it has been shown that induction of ischemic postconditioning after 10 minutes of reperfusion has no beneficial effect (not cardioprotective)³⁵⁴. Although different mechanisms have been proposed to be responsible for the protection that offered by ischemic postconditioning, delaying the restoration or normalization of cellular pH at the time of reperfusion is the main mechanism of ischemic postconditioning as that can lead to inhibition of Na⁺/H⁺ exchanger and conservation of NO-cGMP-PKG signaling pathway^{358,359}. Similar to IPC, ischemic postconditioning has been shown to induce its cardioprotective effect by triggering

different signaling pathways in the mitochondria to protect the heart against IR injury, including ERK, Akt, PKG and PI3K ^{360–363}.

➤ **Pharmacological Interventions:**

For several decades, myriad of pharmacological treatments have been discovered and implemented for the treatment of IR injury. Unfortunately, some of these pharmacological interventions have failed to give positive results and protect the heart in response to IR insult. This failure could reflect the shortage in our understanding of the underlying mechanism of IR or the incompatibility of the design and the strategy used to treat the patients ^{7,17,21,32,246}. However, numerous studies have determined that treatment or prevention of the IR injury can be successfully achieved if the pharmacological interventions are introduced at the beginning of reperfusion ¹⁷. Table 2.7 summarizes the current and the most effective pharmacological treatments against IR injury.

Table 2.7 Current therapeutic interventions for reducing IR injury

Drug / Strategy	Mechanism	Evidence
Statins (ex. Simvastatin and atorvastatin)	HMG-CoA reductase inhibitor, reduce the infarction size by activating the PI3K/Akt (phosphatidyl inositol 3-kinase) signaling pathway, activating NOS pathway and prevent the activation of neutrophils.	^{364–368}
Atrial Natriuretic Peptide (ANP)	Activating the cGMP/PKG signaling pathway	^{369,370}

Glucose-insulin-potassium (GIK)	Phosphorylation and activation of eNOS-PI3k-Akt signaling pathway	371-373
Adenosine	Phosphorylation and activation of eNOS-PI3k-Akt signaling pathway, opening of the mitochondrial K_{ATP} channels, induces vasodilation and inhibition of MPTP opening.	374-377
Glucagon-like peptide-1 (ex. Exenatide).	Activating the eNOS-cGMP-PKG signaling pathway	378-381
MPTP opening inhibitors (ex. Cyclosporine A)	Inhibit the opening of the MPTP	336
Anti-Oxidants (ex. SOD and CAT) Xanthine oxidase inhibitors (allopurinol)	Breakdown superoxide and hydrogen peroxides (reduce ROS levels) and thus protect the heart against IR injury.	382-386
Calcium Antagonists	Reduce reperfusion arrhythmias and stunning, reduce the activation of proteases and prevention of mitochondrial damage.	387,388
Na^+/H^+ exchanger inhibitors	Inhibit Na^+/H^+ antiporter pump	389,390
Na^+/Ca^{+2} exchanger inhibitors	Inhibit Ca^{+2} overload (influx) and activate K_{ATP} channel	391,392
ACEIs (angiotensin converting enzyme inhibitors)	Inhibit the breakdown of bradykinin, stimulate the production of prostaglandin and NO.	393,394

2.9. Hypoxia Inducible Factor-1 (HIF-1) and IR Injury

It is well established that oxygen is an important and crucial element that is required by all cells of most organisms to maintain their normal systemic and cellular functions and to synthesize sufficient amounts of ATP that is needed for different cellular functions²³⁸. Because of the importance of oxygen, it is not surprising that any deficiency or shortage in the oxygen supply that caused by different mechanisms and/or diseases such as ischemia/hypoxia to human tissues and cells can result in enduring damage and subsequently tissue death. Therefore, it is critical to precisely maintain the cellular oxygen concentration within a narrow range to protect the cells from the damage that can be initiated from deficiency in the oxygen supply (hypoxia) or from excess oxygen supply (hyperoxia)³⁹⁵.

Cells have numerous adaptive mechanisms to modulate the deficiency in the oxygen supply by targeting and stimulating regulatory genes and pathways. For instance, it has been shown that hypoxic/ischemic conditions lead to cellular metabolic switch from OXPHOS to anaerobic glycolysis to preserve cellular ATP production. This metabolic shift toward anaerobic glycolysis is achieved through the activation and upregulation of different genes that encode for glucose transporters and glycolytic enzymes, such as hexokinases (HK)³⁹⁶. The induction of these genes is controlled by the activation of the oxygen dependent transcription factor, hypoxia inducible factor-1 (HIF-1).

Hypoxia inducible factor-1 (HIF-1), which is a heterodimer complex that is composed of α and β subunits, is a master transcription factor and a major regulator of oxygen homeostasis within cells. As a transcription factor, it affects and regulates the expression of different genes involved in maintaining homeostasis and survival as oxygen concentrations change in the

affected cells. HIF-1 expression in response to ischemia/hypoxia helps normal tissues to survive under hypoxic conditions by targeting myriad of genes that are important for survival^{397–399}. However, expression of HIF-1 in cancer cells has been linked to the development and growth of tumors and stimulation of angiogenesis⁴⁰⁰. So far, more than 40 target genes have been found to be regulated by HIF-1. It should be noted that the activity of HIF-1 is measured by the level of the expression and the activity of HIF-1 α subunit not the β -subunit as the β -subunit is constitutively expressed in the nucleus regardless of the condition of oxygenation, while α -subunit is constantly synthesized and degraded (by proteasomal degradation) under normoxic conditions and stabilized under hypoxic conditions^{399,401}.

In 1991, the study of the structure of erythropoietin (EPO) and the molecular mechanism that regulate the expression and activation of EPO under hypoxic conditions has resulted in the discovery of HIF-1^{402,403}. In this study, Semenza and his colleagues have discovered a short DNA sequence (~ 50 bp) in the 3'-flanking region of the human EPO gene known as 'hypoxia response element (HRE)'. Using a mouse EPO gene, Pugh *et al* has reported the same results and identified the HRE in the 3'-flanking region⁴⁰⁴. Further investigational experiments have revealed the consensus sequence of this HRE and that sequence is (5'-RCGTG-3'); where R could be either A or G. The same group of researcher has identified that HIF-1 binds to the HRE in the regulatory region of the EPO gene under hypoxic conditions and that leads to the activation of the targeted gene. Nonetheless, mutation of this HRE sequence resulted in a total loss of the activation of the targeted gene because of the loss in the activity of HRE (HIF-1 failed to bind to the HRE)^{405–407}.

As we mentioned early, HIF-1 is a heterodimeric complex or transcription factor that is ubiquitously expressed mostly in all mammalian cells. It is composed of oxygen-dependent

(regulated) α subunit and constitutively expressed β subunit (also known as aryl hydrocarbon nuclear translocator (ARNT))^{397,400}. HIF-1 α expression is tightly regulated by ubiquitination and subsequent proteasomal degradation under normoxia (normal oxygen supply) resulting in basically no measurable HIF-1 α protein⁴⁰¹. Under situations of low or reduced oxygen supply (hypoxia), nevertheless, HIF-1 α will be stabilized and the proteasomal degradation will be inhibited and that allows HIF-1 α to translocate to the nucleus and heterodimerizes with HIF-1 β subunit and then binds to the HRE on the targeted gene in the presence of the co-activator factors. Both subunits contain basic helix-loop-helix (bHLH) domain and Per-ARNT-Sim (PAS) domain that are important for the DNA binding and dimerization. In addition to that, HIF-1 α gene has 2 transactivation domains (TAD) at the C- and N- terminals (found from amino acids 813-826 and from amino acids 531-575, respectively) which are needed for HIF-1 α to target different genes on the hypoxia response element (HRE). The C-terminal of the TAD is important for the binding of different co-activator proteins, namely CBP, p300, TIF-2 and SRC-1, which are essential to stabilize the dimerization of the HIF-1 α and β subunits and to activate HIF-1. Furthermore, HIF-1 α gene has 2 oxygen-dependent degradation domains (ODD) that span from residue 401 to residue 531 and from residue 575 to residue 603, respectively. These polypeptide regions are crucial for the stability of HIF-1 under hypoxic conditions. More explicitly, under situations of sufficient oxygen availability (normoxia), proline residues in the ODD at positions 402 & 564 (Pro402 & Pro564) are hydroxylated by prolyl hydroxylases (PHD1, 2 and 3) in the presence of oxygen, 2-oxoglutarate (2-OG), iron and ascorbate (figure 2.13)^{238,398-400,408,409}. Knockdown studies using small interference RNA (siRNA) revealed that PHD-2, but not PHD-1 or PHD-3, is the most effective and efficient enzyme that induces HIF-1 α degradation under normoxic conditions as the inhibition of PHD-2 led to marked stabilization of HIF-1 α under

oxygenated situations⁴¹⁰⁻⁴¹³. Hydroxylation of proline residues under normal oxygenation levels provokes the binding of the product of von Hippel-Lindau tumor suppressor gene (pVHL). The binding of pVHL leads to the ubiquitination of HIF-1 α by E3 ligase and that results in degradation by proteasomes (26S) (figure 2.13). Recent research has demonstrated that acetylation of the lysine residue at the position 532 (Lys532) in ODD and hydroxylation of the asparagine residue at position 803 (Asp803) in the C-terminal TAD can trigger proteasomes degradation of HIF-1 α adding new mechanisms for how HIF-1 α gene is degraded under normoxic conditions (figure 2.13)^{400,408,409}. Under hypoxia, however, prolyl hydroxylases are inhibited and thus HIF-1 α expression stabilizes and translocate to the nucleus. In the nucleus, it binds to HIF-1 β forming HIF-1 in the presence of the co-activators CBP/p300 (figure 2.13). Then, it transcriptionally regulates the genes associated with protective physiological processes including angiogenesis and metabolism by binding to hypoxia response element (HRE) sequence in these targeted genes (figure 2.13). Regulation of these different genes results in modulated cellular responses to oxygen deprivation^{397,399,401,409}.

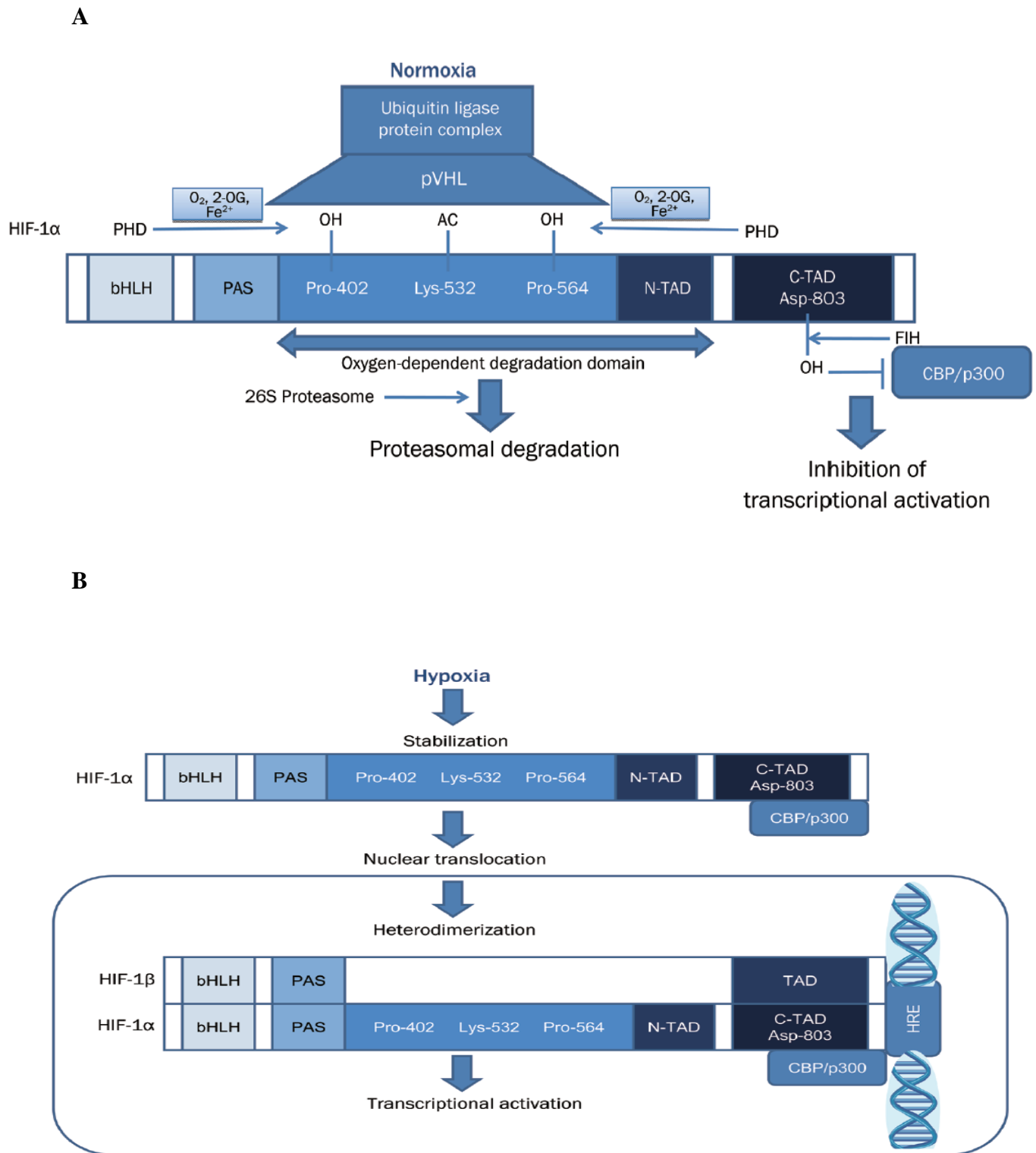


Figure 2.13: HIF-1 α regulation under (A) hypoxic and (B) normoxic conditions. Adapted with permission from Macmillan Publisher Ltd: *Acta Pharmacologica Sinica*, Tekin D. et al, *Hypoxia Inducible Factor 1 (HIF-1) and cardioprotection*, 31 (9); 1085-1094, 2010, Copyright 2010.

2.10. HIF Therapeutic Strategies

Cumulative evidence has demonstrated that HIF-1 is vital for the normal development of the brain, cardiovascular system, thymocytes and for embryogenesis. It has been reported that mouse embryos that have HIF-1 α knockout gene died early (approximately embryonic day 11) as a result of the development of acute cardiovascular malformations, abnormal neuronal growth and mesenchymal cell death. Furthermore, HIF-1 α knockout mice showed severe reduction of spatial memory, severe reduction in neuron cells, development of hydrocephalous, abnormal B-cell development and myeloid cells dysfunction^{400,401,414}.

Stimulation and eventually upregulation of HIF-1 α in response to any stress conditions (ex. hypoxia or ischemia) is a normal and essential physiological response that has a pivotal role in the protection and survival of the damaged organ or tissue. HIF-1 α regulates the genes that are associated with protective physiological processes including angiogenesis and metabolism resulting in modulated cellular responses to oxygen deprivation. For instance, HIF-1 α directly regulates the vascular endothelial growth factor (VEGF) to stimulate the migration of endothelial cell to the hypoxic area to supply blood and oxygen by forming new blood vessels^{400,401,408}. Furthermore, HIF-1 α regulates many genes that are important for oxygen delivery to the cells such as erythropoietin (EPO), nitric oxide synthase-2 (NOS2), transferrin and transferrin receptor. Furthermore, it has been shown that HIF-1 α regulates a wide range of genes, such as glucose transporter-1 (GLUT-1), hexokinase-I and II (HK-I & II) and insulin like growth factor (IGF-1) to shift the cells toward anaerobic glycolysis to maintain the energy needed for cells and to induce cell proliferation^{238,399,415}.

Stabilization or over-expression of HIF-1 α using different approaches (PHD inhibition, mutation of proline sites or genetically over-express HIF-1 α) have been shown to be beneficial to treat and improve different diseases such as myocardial infarction (MI), anemia, arterial diseases and gastrointestinal disease. In addition, induction of HIF-1 α in response to IR injury has been reported to mitigate iron accumulation, ROS formation and improve mitochondrial membrane potential as well as decrease the infarction size. Because stabilizing HIF-1 α is essential for cardioprotection and also for treating other diseases, we will address the different approaches that can be used to stabilize HIF-1 α .

➤ **Cobalt Chloride (CoCl₂):**

Recent research by Xi L. and his colleagues has revealed that CoCl₂ treatment (single low dose) induced sustained cardioprotection (~ 8 hours) through the stimulation and stabilization of HIF-1 α expression and iNOS, but not NF- κ B (nuclear factor-kappa B) ⁴¹⁶. Further, these results were confirmed by other group of researcher using IR injury model. In their study, they reported that single dose of CoCl₂ reduced the cardiac cells apoptosis through the reduction in the expression of Bax (pro-apoptotic protein) and stimulation in the anti-apoptotic proteins Bcl2 and phosphorylated Akt ⁴¹⁷.

➤ **Prolyl hydroxylases inhibitors:**

Another approach to stabilize HIF-1 α under normoxic conditions is to inhibit the HIF-1 α inhibitors, such as prolyl hydroxylase-2 (PHD-2). It has been shown that the treatment of GSK360A, a potent orally active PHD-2 inhibitor, for 28 days protected the heart from IR injury, induced long-term (3 months) improvement in the ventricular contractility and remodeling,

improved the ejection fraction and increased the levels of hemoxygenase-1 (HO-1) gene ⁴¹⁸. Furthermore, recent studies have demonstrated that preconditioning the heart with the potent PHD-2 inhibitor, DMOG (dimethyloxalylglycine), before the IR injury resulted in attenuation of the IR injury in the rabbit hearts ⁴¹⁹⁻⁴²¹. Besides, treatment with the iron chelator, deferoxamine, causes blockage of PHD-2 leading to stabilization of HIF-1 α expression and consequently lead to improvement in the calcium transient and contractile function in cardiomyocytes after IR injury ^{420,422}.

➤ **Small interfering RNA (siRNA):**

Recently, a new effective technique to knockdown PHD-2 was introduced by Natarajan and his group in 2006. In their experiments, they knocked down PHD-2 gene using siRNA method and they reported that knocking down PHD-2 using this technique induced HIF-1 α expression, protected the heart from IR injury, reduced the inflammatory responses in post-ischemic myocardium and reduced the post-ischemic injury ⁴²³⁻⁴²⁵.

➤ **Other approaches:**

Other approaches including gene therapy, micro RNAs (miRNAs) and exercise have been shown to induce HIF-1 α expression in response to IR injury and that can grant cardioprotection. Indeed, it has been proved that gene therapies for HIF-1, such as injection of naked DNA encoding the N-terminal DNA-binding and dimerization domains of HIF-1 α attached to the transactivation domain of the herpes virus VP16 (HIF-1 α /VP16 hybrid), was expressed for 14 days after administration and was not expressed or detected after 28 days post-injection and it

was reported that injection of HIF-1 α /VP16 hybrid to hearts subjected to IR injury induced protection against IR injury^{426,427}.

2.11. Targeted Genes of HIF-1

In the last 2 decades, a great effort has been devoted to study the regulatory network of HIF-1 α in order to reveal the target genes for HIF-1 α and their roles in promoting physiological adaptive response to stresses. In order to unravel these target genes, different techniques have been utilized, including chromatin immunoprecipitation (ChIP) assay, luciferase reporter assay, electrophoretic mobility shift (EMSA) assay and microarray analysis. Using these methods, different genes that are involved in oxygen utilization and supply (oxygen homeostasis), angiogenesis, glucose utilization and metabolism, inflammation, apoptosis, stemness, autophagy, redox homeostasis, iron metabolism, invasion and metastasis, cell proliferation, cell death and erythropoiesis have been discovered. So far, more than 100 genes have been found to be directly regulated by the transcription factor HIF-1 α ^{414,428–430}. Recently, we have discovered a new putative target for HIF-1 α . Our luciferase promoter activity assay and chromatin immunoprecipitation (ChIP) assay confirmed the presence of a functional hypoxia response element (HRE) in the frataxin promoter region (~ 2.5kb)⁴³¹. In this study and using IR injury model, we provided evidences that HIF-1 α transcriptionally regulates the expression of the mitochondrial protein frataxin in response to hypoxia and the increased expression of frataxin mitigated mitochondrial iron overload and subsequent ROS production, thus preserving the mitochondrial membrane integrity and viability of cardiomyocytes offering a cardioprotective mechanism against ischemic injury⁴³¹.

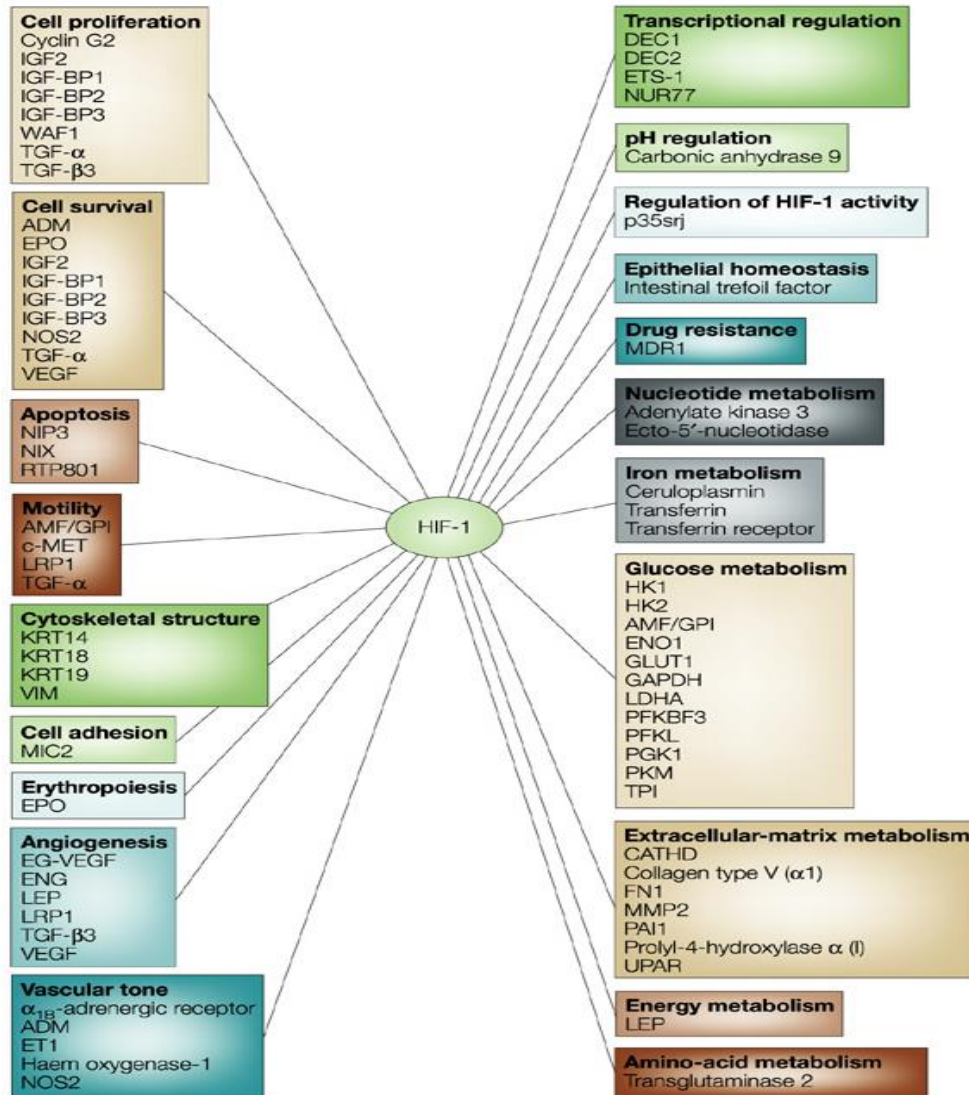


Figure 2.14: HIF-1 α target genes. Adapted with permission from Macmillan Publisher Ltd: *Nature Reviews Cancer*, Semenza G., *Targeting HIF-1 for cancer therapy*, 3 (10); 721-732, Copyright 2003.

2.12. Frataxin

Frataxin (FXN) is a highly conserved nuclear encoded mitochondrial matrix protein that is found in most prokaryotes and eukaryotes. It is highly and abundantly expressed in mitochondrial-rich tissues including cardiomyocytes, neurons, brown fat, liver, and pancreas. The exact molecular function of the mitochondrial iron binding protein FXN remains relatively unclear, however, it has been found to play a key role in iron homeostasis during iron-sulfur (Fe-S) clusters biogenesis⁴³²⁻⁴³⁴. Recently, it has been reported that FXN has multiple roles besides its role in iron regulation. Certainly, it has been shown that FXN can act as a chaperone for iron-sulfur cluster (ISC) formation and heme biosynthesis, as an iron storage protein in conditions of excess iron (iron overload), can promote restoration of oxidatively damaged aconitase, can abate the levels of ROS under stress conditions and regulation of the mitochondrial iron homeostasis, mitochondrial energetics and OXPHOS and thus maintains the mitochondrial integrity^{46,56,57,434,435}. It should be noted that this mitochondrial protein has a direct influence on the mitochondrial function and respiration, as most of the mitochondrial electron transport chain (ETC) complexes contain ISC⁴³⁶. Therefore, it is obvious that disruption or deficiency in the production of FXN results in different diseases, such as Friedreich's ataxia (FRDA), that are characterized by imbalance in the cellular iron homeostasis and mitochondrial dysfunction.

Friedreich's ataxia (FRDA) is a rare autosomal recessive inherited neuro and cardio-degenerative disease that affects approximately 2-4:100000 people in Europe and around 9000 individuals in the United States and it is considered to be the most common type of hereditary ataxia. FRDA is caused by a deficiency in the expression of the mitochondrial protein FXN and it is manifested by muscular weakness, limb ataxia, loss of coordination, progressive gait and

hypertrophic cardiomyopathy^{49,54,437}. Attenuated FXN levels in FRDA patients have been found to cause cardio-metabolic and contractile dysfunctions as well as cardiac hypertrophy. It has been reported that the disruption in iron homeostasis is associated with the slow progression of the cardiac, neurological and muscular symptoms⁴³². The pathogenic homozygous mutation (expansion) of a guanine–adenine–adenine (GAA) trinucleotide repeats in the first intron of FXN gene (chromosome 9q13) leads to transcriptional silencing of the FXN gene and subsequently reduced the FXN protein levels in FRDA patients. Severity of the disease and duration, age of onset and age of death and cardiomyopathy are related to the shorter GAA repeat expansion^{50,437–439}. It should be noted that all FRDA patients have some FXN, even though it is not within the normal range, as it has been reported that complete loss of FXN results in embryonic lethality⁴³⁷.

Fratxin (FXN) is an acidic small protein (210 amino acids) that is highly conserved from yeast to human. It is synthesized in the cytosol as a precursor protein (pro-peptide) of 210 amino acids (23KDa) which is then targeted or imported to the mitochondria for maturation and processing by the mitochondrial processing peptidase- β (MPP- β) through a two sequential cleavage steps (figure 2.15). These 2 steps maturation by MPP- β result in the generation of 19KDa intermediate protein (42-210 amino acids) and 2 mature proteins 17KDa and 15KDa (residues 56-210 and 81-210, respectively), with the later mature form (81-210 amino acids) is considered the main mature form of FXN as validated using frataxin over-expressing cell lines (FXN-OE) (figure 2.15). Furthermore, 15KDa mature form of FXN was shown to salvage or retrieve FXN deficiency in FRDA patients^{49,437,439–441}.

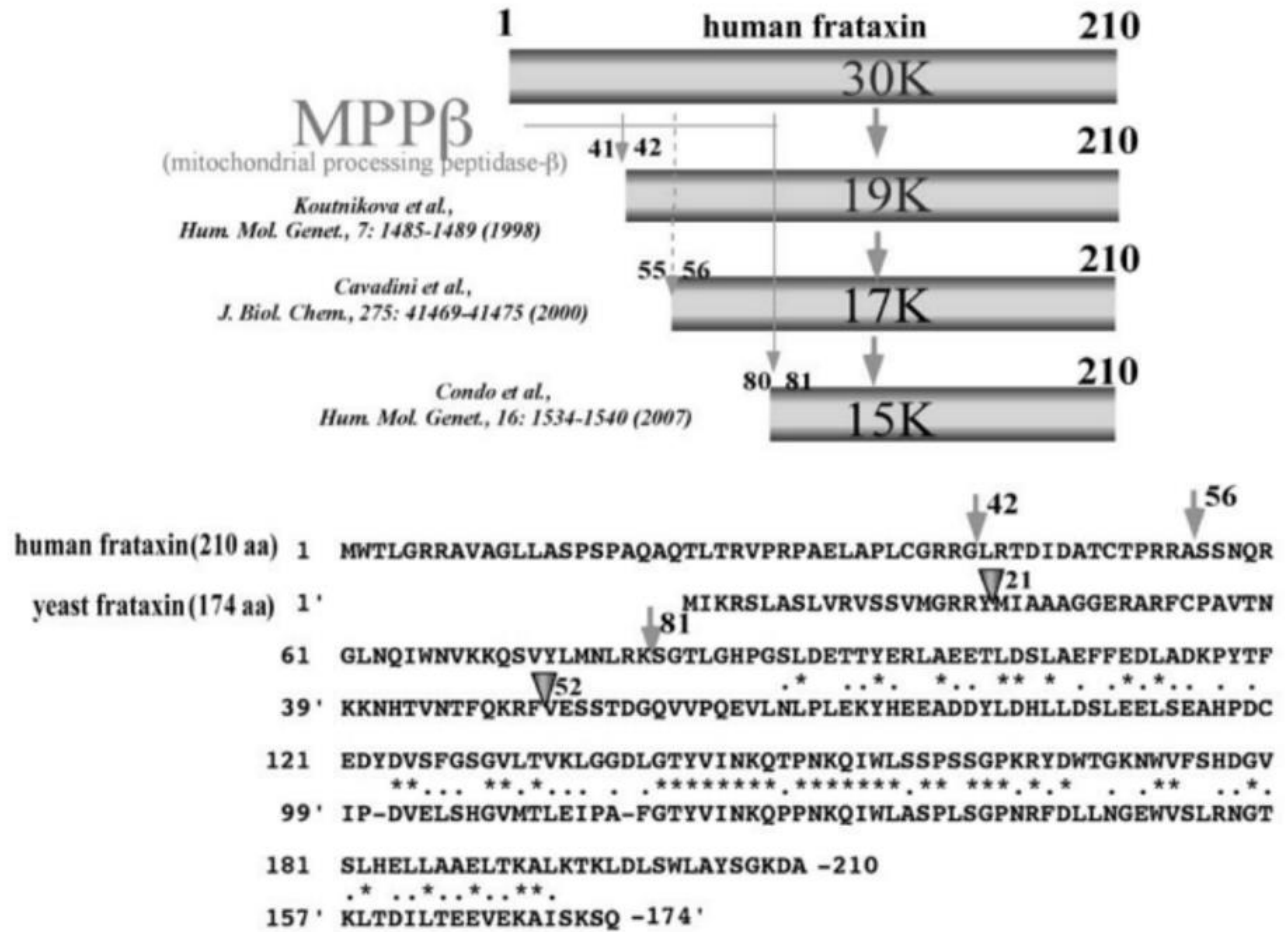


Figure 2.15: Maturation of frataxin. Adapted with permission from Macmillan Publisher Ltd: *Antioxid Redox Signal., Mackenzie E., Intracellular Iron Transport and Storage: from Molecular Mechanisms to Health Implications, 10 (6); 997-1030, Copyright 2008.*

2.13. Frataxin, Iron-Sulfur cluster Assembly and Cellular Iron Metabolism

Accumulated data show that FXN plays a critical role in heme biosynthesis as FXN is considered the main iron chaperone that transports ferrous ion (Fe^{+2}) to ferrochelatase, which is the final step in the heme synthesis pathway, therefore, stimulating heme biosynthesis^{442,443}.

These findings were further confirmed by different groups of researchers using FXN null yeast strains. Using these strains, they observed a decrease in ferrochelatase, cytochrome b and cytochrome c oxidase and the induction of ferrochelatase using plasmid with multi-copy of ferrochelatase did not rescue the heme synthesis^{45,444-447}. These results give an indication that FXN is important for heme biosynthesis and its deficiency can inhibit heme synthesis.

In addition to its crucial impact on heme biosynthesis, FXN has a direct role in iron-sulfur cluster (ISC) biogenesis (assembly) $[4\text{Fe-4S}]^{+2}$. ISC is essential for different cellular functions, such as electron transport chain (ETC) complexes in the mitochondria, iron uptake and gene regulation and it is one of the most complicated prosthetic group that consists of iron and sulfur ions⁴⁴⁷⁻⁴⁵⁰. Different studies have reported that cysteine desulfurase (Nfs-1), Nfs-1 binding protein (ISD-11), FXN and iron-sulfur cluster scaffold unit (ISCU1/2) are the essential components that lead to the assembly of the ISC $[4\text{Fe-4S}]^{+2}$ upon their direct binding⁴⁵¹⁻⁴⁵⁵. In yeast mitochondria, as a model, ISC biogenesis commences with the interaction and formation of a disulfide bond between the sulfur donor, Nfs-1, and the free mitochondrial cysteine to release alanine. It has been shown that ISD-11 protein is imperative to stabilize Nfs-1. Moreover, ISD-11 can act as a connector between Nfs-1 and ISCU1/2 to facilitate the release of sulfur from Nfs-1^{453,456-458}. The Nfs-1, ISD-11, FXN and ISCU1/2 complex are then interacting to assemble ISC $[4\text{Fe-4S}]^{+2}$ (figure 2.16). It has been reported that FRDA patients, FXN knockout mouse model as well as bacterial system that lack FXN have ISC deficiency, mitochondrial iron overload and respiratory complexes deficiency suggesting that FXN has a direct and important role in ISC biogenesis (figure 2.16)^{447,449,450,459,460}. In contrast, different reports have revealed that FXN has no essential direct role in ISC assembly; rather, it increases the efficiency of the ISC biogenesis⁴⁶¹. This concept did not get much attention because there is mounting evidence demonstrates

that there is a direct interaction between FXN and the ISC assembly machinery ISCU1/2 and Nfs-1/ISD-11 and that FXN is essential for ISC biogenesis $[4\text{Fe-4S}]^{+2}$ (figure 2.16) ^{453,462-465}.

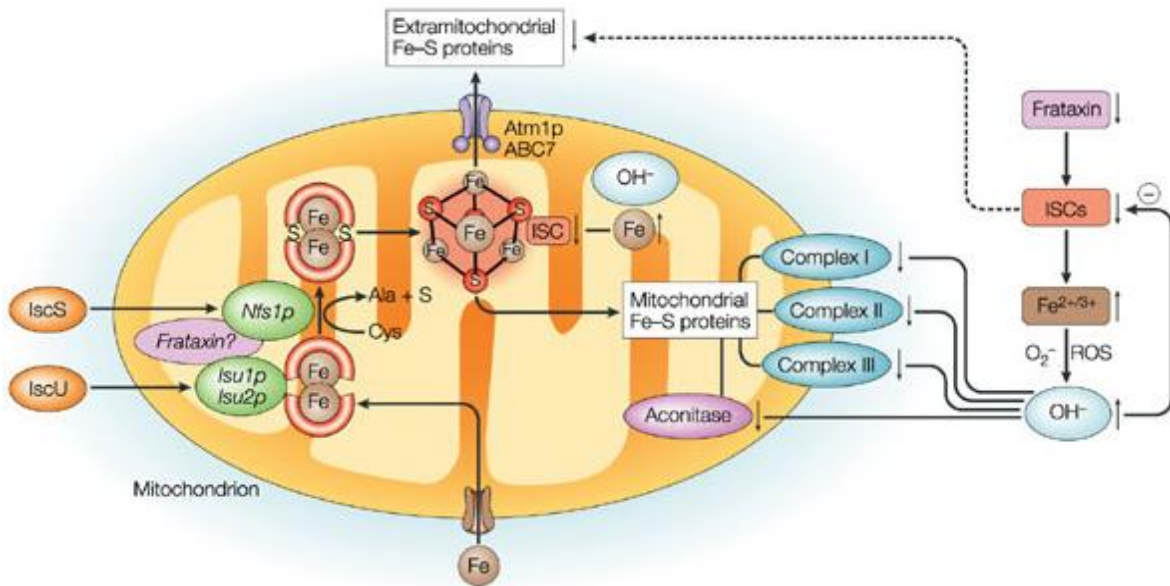


Figure 2.16: Iron-Sulfur Cluster (ISC) biogenesis. Adapted with permission from Macmillan Publisher Ltd: Nature Reviews Neuroscience, Taroni F., Pathways to motor incoordination: the inherited ataxias, 5 (8); 641-655, Copyright 2004.

Recently, several published reports have related the deficiency in aconitase enzyme to the deficiency in FXN protein ^{447,459,466}. Aconitase is an essential enzyme in the tricarboxylic acid cycle (also known as Krebs-cycle or citric acid cycle) that catalyze the conversion of citrate to isocitrate. It has been shown that FXN is important for the normal function of aconitase as FXN can deliver ferrous ion (Fe^{+2}) to aconitase to repair the oxidatively damaged ISC to convert it

from $[3\text{Fe-4S}]^+$ to $[4\text{Fe-4S}]^{+2}$. Furthermore, Bulteau and his colleagues have shown that FXN can directly interact with aconitase to protect the ISC $[4\text{Fe-4S}]^{+2}$ from the dis-assembly, degradation and/or irreversible inactivation under conditions of elevated ROS levels ^{55,467,468}. Moreover, reduction in aconitase results in up-regulation of the iron importer mechanisms and that can lead to mitochondrial iron overload. Therefore, it is not surprising that damage to aconitase because of the deficiency in FXN can result in oxidative damage, iron overload and ROS generation ⁴⁴⁸. In a recent study that investigated the effect of induction of FXN on aconitase activity, Schulz *et al* has found that up-regulation of FXN resulted in significant increase in aconitase, cellular respiration and ATP content ⁴⁶⁹. Together, these data propose another defensive mechanism offered by FXN under stress conditions through the interaction with aconitase to protect the ISC from damage.

Numerous published reports have investigated the exact role of FXN based on its importance in ISC assembly and aconitase function and they proposed that FXN can act as an iron chaperone, iron sensor, metabolic switch and as a scavenger of oxidative stress ⁴⁹. Mounting evidence proposes that FXN can act as an iron chaperone protein based on the assembly of ISC. As we described early, the formation of the ISC $[4\text{Fe-4s}]^{+2}$ requires the presence of sulfur, which is provided by Nfs-1 and the presence of iron, which is provided by an unknown provider. Investigational studies on the assembly of ISC have proved that the unknown provider of iron is FXN protein ⁴⁷⁰⁻⁴⁷². Furthermore, using experimental and bioinformatics analysis, these results were further supported as it has been found that FXN act as an iron donor (Fe^{+2}) in the heme synthesis process through the delivery of Fe^{+2} to protoporphyrin IX ^{47,473}. In addition to this proposed function, different studies have hypothesized that FXN can act as an iron storage protein, like ferritin. This hypothesis is based on the fact that yeast FXN showed a considerable

iron-binding ability (approximately 50 atoms of iron per monomer) and convert the highly reactive iron (Fe^{+2}) to the redox inactive state (Fe^{+3})^{474,475}. However, this concept did not get much attention as it is described as a non-physiological mechanism because the normal mitochondrial levels of magnesium and calcium would prevent the oligomerization capacity of FXN and that will prevent the binding of iron. Furthermore, chemical analysis and crystallization of the human FXN structure did not show any selectivity for iron binding, therefore, this hypothesis would not be considered as an exact function of FXN as these data contest this concept^{44,476–478}.

➤ **Fratxin (FXN) and hypoxia:**

To the best of our knowledge, not many studies are found in the literature discussing the role of FXN against ischemia-reperfusion injury (hypoxia/reoxygenation conditions). In one study, Oktay and his colleagues have reported that HIF-2 α rather than HIF-1 α is accountable for regulating the FXN levels in mice liver under hypoxic conditions⁴⁷⁹. Recently, we have reported a new cardioprotective mechanism offered by HIF-1 α , not HIF-2 α , against IR injury through the direct activation of the mitochondrial protein FXN⁴³¹. In this study, we have demonstrated a significant rise in FXN expression in the isolated hearts of control mice that subjected to IR injury. However, this phenomenon was diminished in cardiac-specific HIF-1 α knockout (KO) mice. When it compared to control group, these knockout mice (HIF-1 α KO) maintained substantial or considerable cardiac damage in response to IR injury comparing to the control mice. Furthermore, employing RNAi technique to attenuate HIF- α levels has resulted in reduction of FXN expression in response to hypoxic conditions *in-vitro*. Thus, we hypothesized

that HIF-1 α transcriptionally regulates FXN expression in response to hypoxia and that pathway present a new cardioprotective mechanism against ischemic injury⁴³¹. In order to validate this hypothesis, we have used promoter activity assay and chromatin immunoprecipitation (ChIP) assay. Results from these assays confirmed the presence of a functional hypoxia response element (HRE) in the human FXN promoter. Moreover, our data indicate that increased FXN expression led to modification of mitochondrial iron accumulation and ensuing ROS production, thus conserving the mitochondrial membrane potential and viability of cardiac myocytes⁴³¹.

To the extent of our knowledge, there is no study investigating the modulation of FXN expression during the reperfusion period after IR injury. Furthermore, would be the upregulation of FXN during the reoxygenation period cardioprotective or not remains elusive. Therefore, in my current dissertation I will present a new findings regarding the expression of FXN during reperfusion and how that can protect the heart form the damage after IR insult.

2.14. Purinergic receptors

Extracellular nucleotides (purines and pyrimidines) are complex molecules that are essential and important for numerous biological functions and signaling mechanisms. It can be found as monomers or polymers, and in this case it called nucleic acids (DNA or RNA)⁴⁸⁰⁻⁴⁸². All nucleotides composed of a phosphate group that is linked to a pentose sugar (either ribose or deoxyribose) by a phosphoester bond and that is linked to an organic nitrogen base (when there is no phosphate group, it called nucleosides). Indeed, all nucleotides are identical and share the same structure, but they differ in the nitrogen bases. Adenine and guanine are purines, while cytosine, thymine and uracil are pyrimidines⁴⁸¹⁻⁴⁸⁴.

Pyrimidines (UTP and UDP) and purines (ATP, ADP and Adenosine) are important extracellular signaling molecules that have different effect on biological processes, such as endo and exocrine secretion, neurotransmission, smooth muscle contraction, inflammatory responses, modulation of cardiac functions and pain ⁴⁸⁵. It is widely accepted that both purines and pyrimidines are exerting their effect through the binding to purinergic receptors. In 1929, Drury and Szent-Györgyi were the first to report the signaling mechanisms of purines as they reported that adenosine and AMP have biological actions on the heart, namely lowering blood pressure, heart block and arterial dilatation ⁴⁸⁶. This discovery led to the findings that adenosine, AMP and ATP have different actions and implicate the presence of different purine receptors ⁴⁸⁷. However, it was not until 1976 when Burnstock and his colleagues discovered the purinergic receptors ^{488,489}. Two years later (1978) and because of the findings that adenosine, ATP, ADP and AMP have different potencies and because of the use of antagonism, Burnstock and his colleagues divided the purinergic receptors into P1-purinoceptors (the natural ligand is adenosine) and P2-purinoceptors (where ATP and ADP are the natural ligands) ⁴⁹⁰. This classification of purine receptors was updated in 1985 to be P1 and P2, where P2 can be further divided into P2X and P2Y based on the molecular structure and the pharmacological properties ⁴⁹¹. Later on, it has been confirmed that purine receptors respond to pyrimidines (ex. UTP) as much as purines ⁴⁹². Currently, there are 8 different types of P2Y receptors and 7 different types of P2X receptors ^{485,493–495} (table 2.8).

Table 2.8 Purinergic receptor classification with its agonists

Receptor	Agonists	Transduction mechanism
P1		
P1A ₁	Adenosine	Gi/o → ↓cAMP
P1A _{2A}	Adenosine	Gs → ↑cAMP
P1A _{2B}	Adenosine	Gs → ↑cAMP
P1A ₃	Adenosine	Gq/11, Gi/o → ↓cAMP, ↑IP3
P2X		
P2X1-P2X7	ATP	intrinsic cation channel (mainly Ca ⁺²)
P2Y		
P2Y ₁	ATP	Gq/G11; PLC activation
P2Y ₂	UTP > ATP	Gq/G11 and possibly Gi; PLC activation
P2Y ₄	UTP > ATP	Gq/G11 and possibly Gi; PLC activation
P2Y ₆	UDP > UTP > ATP	Gq/G11; PLC activation
P2Y ₁₁	ATP	Gq/G11 and possibly Gs; PLC activation
P2Y ₁₂	ADP > ATP	Gi/o → ↓cAMP
P2Y ₁₃	ADP > ATP	Gi/o → ↓cAMP
P2Y ₁₄	UDP	Gq/G11; PLC activation

Adapted from Cellular and molecular life sciences: Burnstock G., Purine and Pyrimidine receptor, copyright 2007, (64), 1471-1483

P2X receptors are ligand-gated (ATP-gated) cationic channels that facilitate ions influx (Na⁺ and Ca⁺²) and efflux (K⁺) in excitable tissues causing depolarization of the cell membrane. These receptors are widely distributed in the glial cells, neurons, endothelium, bones and

muscles. P2X receptors are responsible of origination of pain signaling, neurotransmitter release and fast synaptic transmission in the central and peripheral nervous systems⁴⁹⁶⁻⁴⁹⁸. P2Y receptors, nevertheless, are G-protein coupled receptors (GPCRs) with a characteristic or distinctive heptahelical transmembrane (7-TM) motif. In contrast to P2X receptors, P2Y receptors have high sensitivity and binding ability to both purines (ATP and ADP) and pyrimidines (UTP and UDP)^{496,497}. Many of P2Y receptors exert their effect through G-protein receptors that are coupling to stimulate PLC (phospholipase C) and subsequently produce IP3 (inositol triphosphate). Consequently, MAPK (mitogen activated protein kinase) signaling pathway, PKC (protein kinase C) signaling pathway, NOS (nitric oxide synthase) pathway and PLA2 (phospholipase A2) and voltage gated Ca^{+2} channels will be activated⁴⁹⁶. However, some of P2Y receptors are G-protein receptors that are coupling to G_i which will lead to the inhibition of AC (Adenylate cyclase) and that can lead to the inhibition of cAMP. P2Y receptors can be classified based on their structure into two groups; group I which includes P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 and group II which includes P2Y12, P2Y13 and P2Y14. Group I of P2Y receptors are coupled to G_q (PLC and IP3) proteins and that lead to the increase in IP3 and calcium mobilization, except for P2Y11 receptor which is coupled to both G_q and G_s (increase AC, therefore, increase cAMP). Group II P2Y receptors, however, are coupled to both G_i (decrease AC, therefore, decrease cAMP) and G_s (at higher concentrations of ADP)⁴⁹⁶⁻⁴⁹⁸. Nonetheless, P2Y receptors can be also classified pharmacologically (based on the ligand) into 4 groups; 1) adenine nucleotide receptors (favoring ATP and ADP), which includes human and rodent P2Y1, P2Y12 and P2Y13, and human P2Y11 (also respond to UTP); 2) uracil nucleotide receptors (favoring UTP and UDP), which includes human P2Y4 and P2Y6; 3) adenine and uracil nucleotide receptors (mixed selectivity), which includes human and rodent P2Y2 and

P2Y4 and human P2Y11; and 4) receptors that are responding merely to UDP-glucose and UDP-galactose receptors, which include P2Y14^{496,497}.

➤ **Sources of naturally occurring ligands (nucleotides): Release and breakdown**

In the last few years, a great effort has been devoted to the study of nucleotides and their effect on different tissues and organs. Purinergic receptors and its ligands (purines and pyrimidines) have gained most of the attention and numerous studies have been investigated their roles. However, to better understand the roles of different nucleotides on different tissues, one must first understand the mechanism of the release and breakdown of these nucleotides. It has been reported that ATP is released in the extracellular milieu as an autocrine/paracrine molecule under physiological (neuronal stimulations) pathophysiological conditions, such as hypoxia, platelet aggregation and stress⁴⁹⁷. The concentration of naturally occurring ligands of purinergic receptors (ATP, ADP, UTP, UDP and UDP-glucose) is within the range of 0.1 – 10 μ M and this concentration is required for their activity. ATP is found in the cytoplasm of each cell in millimolar concentration (3-10 mM) and this is the steady state concentration of ATP that is required to maintain normal physiological functions. Nevertheless, because ATP and other nucleotides have negative charge, they can not penetrate through the cell membrane to exert their effect, thus, they require a transport mechanism to be transported to the extracellular milieu to bind and act on the targeted receptors⁴⁹⁷.

Numerous studies have reported that levels of nucleotides have been detected in various cell types under normal conditions (unstimulated conditions) and these levels were markedly elevated under stress conditions (stimulated conditions) followed by restoration of its levels to

the normal levels immediately^{499,500}. It should be noted that the normal levels (resting levels) of ATP and UTP are preserved by equilibrium between release and metabolism. This means that both ATP and UTP are constitutively released under unstimulated conditions and the levels of these nucleotides are progressively increased upon the inhibition of their metabolism^{499,501,502}. In excitatory and secretory tissues, such as neurons, mast cell, endocrine cells, platelets and chromaffin cells (in adrenal medulla), ATP and other nucleotides are stored in vesicles (granules) at higher concentration ranging from 150-1000 mM. The stimulation of these tissues/cells engenders the fusion of these vesicles to the plasma membrane; consequently, this will lead to the release of the granular contents (nucleotides) into the extracellular space (exocytosis) in a Ca^{+2} -dependent mechanism^{497,503,504}. In non-excitatory/secretory tissues (smooth muscle cells, endothelial cells, fibroblasts, monocytes, lymphocytes and astrocytes), however, the mechanism of release of ATP remains elusive and complicated and was proposed to involve many mechanisms^{505,506}. In the heart and endothelial cells, it has been reported that hypoxia stimulated the release of nucleotides (ATP) to play a vital role in reactive hyperemia and regulation of the function of epithelial cells. Besides these mechanisms of nucleotides release, nucleotides can be released from the intracellular compartments into the extracellular milieu upon tissue/cell damage or lysis⁵⁰⁷⁻⁵¹⁰.

Different classes of enzymes are responsible of degradation of nucleotides, specifically ecto-nucleotide triphosphate diphosphohydrolase (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), ecto-5'-nucleotidase, adenylate kinase, alkaline phosphatase and nucleotide converting enzyme ecto-NDPK. Ecto-nucleotides enzymes are expressed mainly in all cell types and it can be found either in soluble or membrane associated forms and they are responsible to degrade and convert the extracellular nucleotides^{497,511,512}.

➤ **Purinergic Receptors and Cardioprotection:**

In 1929, Drury and Szent-Györgyi have reported that extracellular AMP can induce coronary arteries dilation by acting on the coronary arteries of guinea pig, dogs, cat and rabbits⁵¹³. Later on, it has been found that adenosine and ATP are not beneficial for the treatment of heart diseases as it induced heart block and paroxysmal tachycardia in guinea pigs^{514,515}. However, in 1940 and for two decades, ATP and AMP were used for the treatment of angina pectoris and coronary insufficiency. After the discovery of the types of purinergic receptors by Burnstock in 1972, the interest in the extracellular nucleosides and nucleotides has markedly increased⁵¹³.

Recently, it has been reported that ATP induces positive inotropic effects on the heart and that is interceded by P2Y₁₁ receptors. Furthermore, it has been found in growing rats that both P2Y₂ and P2Y₄ receptors are regulating the contractility of the heart^{516,517}. Of all P2Y receptors, P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ were found to be expressed in neonatal rat cardiomyocytes and they are producing their effect through G-protein receptors coupled to the activation of PLC (G_{q/11})⁵¹⁸. In the heart, ATP and tentatively UTP are released in variable amounts (10-40 nM) and these levels are considerably elevated under hypoxic and/or ischemic conditions. The major source of ATP release in the heart is red blood cells (RBCs), especially under hypoxic conditions (when oxygen demand surpasses oxygen supply). Other sources include endothelial cells, smooth muscle and activated platelets⁵¹³.

In the past two decades, purinergic receptors and extracellular nucleotides signal transduction have attracted intense attention, especially against IR injury and hypoxic and/or

ischemic conditions both *in-vivo* and *in-vitro* ⁵¹³. In more than one study, adenosine has been shown to induce activation of kinase pathways (phosphorylation of Akt and ERK1/2) during the reperfusion period and that resulted in inhibition of the MPTP opening ^{13,39,519}. Furthermore, another group of researcher has found that adenosine exerts its cardioprotective effect through the activation of PKC ⁵²⁰. In addition to ATP, pre-administration of UTP was shown to be cardioprotective (ischemic pre-conditioning) ⁵²¹. As we mentioned early, the ATP release increases under hypoxic/ischemic conditions and it was reported that this increase in ATP release regulates cardiomyocytes death probably by activating P2Y2 receptors, which in turn activate ERK1/2 and/or Akt pathways ⁴⁸². Similar to ATP, UTP release during ischemia can protect cardiomyocytes and can reduce the post-ischemic myocardial damage by activating P2Y2 and P2Y4 receptors. Indeed, UTP protects cardiomyocytes against the ischemic injury by restoring the electron flow in ETC complexes, restoring the heart function, restoring mitochondrial membrane potential and prevent the ATP loss ⁵²¹⁻⁵²⁵. In contrast to these results, it has been reported that UTP increased the rate of myocardial cell death under hypoxic conditions by activating P2Y2 receptors. Furthermore, David Erlings and his colleagues have found that UTP is released during myocardial ischemia and it is not protective as it induced arrhythmia in response to ischemia ^{523,526}. Therefore, the necessity to comprehensively investigate the role of extracellular nucleotides (ATP and UTP) and purinergic receptors on the heart has risen, especially under hypoxic and ischemic conditions in order to reveal the precise mechanism that is offered by different nucleotides.

CHAPTER 3:

Cardioprotective Mechanisms of Frataxin in Ischemia Reperfusion Injury in

Cardiomyocytes

3.1. Abstract:

Oxygen free radicals associated with ischemia-reperfusion (IR) injury in cardiomyocytes is known to cause mitochondrial damage. However, the exact mechanism for how the oxygen free radicals develop in the mitochondria due to IR stress is currently unclear. In the current study, we focus upon understanding the role of frataxin (FXN) in regulating mitochondrial damage associated with IR injury. FXN, a nuclear encoded mitochondrial matrix protein, has been observed to regulate mitochondrial iron homeostasis and thus mitochondrial energy regulation. Loss of FXN, in Friedreich's ataxia, is associated with mitochondrial iron overload, increased ROS formation and cellular damage. In this study, we hypothesize that FXN protects cardiomyocytes against IR injury by preventing the dysregulation of myocardial bioenergetics. We identified for the first time that FXN expression is increased during the reperfusion stage of IR injury resulting in regulation of mitochondrial iron homeostasis and the ensuing mitochondrial ROS formation. Most surprisingly, we observed that enhanced FXN expression displayed elevated levels of glutathione (GSH) and superoxide dismutase (SOD). Furthermore,

these findings were supported in our FXN over-expressing and knock down cells under the same IR condition. Together, these results demonstrate that increased expression of FXN is cardioprotective against reperfusion injury through its anti-oxidant effect and by improving mitochondrial energetics.

3.2. Introduction:

Ischemic heart diseases including acute myocardial infarction (AMI) and congestive heart failure (CHF) are the leading causes of death worldwide ¹. In the United States, coronary heart disease accounts for approximately 25% of all cases of death and costs over \$150 US million dollars for the direct health care ^{2,3}. These findings indicate the critical need to discover novel molecular signaling mechanisms for potential therapeutic interventions that can prevent morbidity and mortality from ischemic injury and prevent progression of the ischemic heart to failure.

Previous studies have illustrated that ischemia reperfusion (IR) injury compromises the mitochondrial integrity of cardiomyocytes ^{21,24–27,527}. The lack of oxygen during ischemia shifts the cellular metabolic axis from oxidative phosphorylation (OXPHOS) to anaerobic glycolysis leading to depolarization of the membrane potential, ATP depletion and myocardial contractile dysfunctions ¹¹. During the reperfusion stage of the IR injury, generation of reactive oxygen species (ROS) leads to ATP depletion, Ca²⁺ overload, opening of the mitochondrial permeability transition pores (MPTP) and contractile dysfunction that can result in cardiomyocytes death ^{21,24–27,527}. Furthermore, damage from IR injury is associated with reperfusion period by promoting different signaling pathways that trigger apoptosis and necrosis and thus diminish cardiomyocyte viability ²¹. As the mitochondria occupy nearly 35% of the cardiomyocyte volume and produce more than 90% of the ATP, the conservation of the functions and structures of the mitochondria during the reperfusion period is crucial for cardio-protection against IR injury.

The highly conserved nuclear encoded mitochondrial matrix protein frataxin (FXN) has been found to promote and regulate the mitochondrial energetics and OXPHOS and thus maintains the mitochondrial integrity⁴⁴. Friedreich's ataxia (FRDA), a rare autosomal recessive inherited neuro and cardio-degenerative disease, is caused by a deficiency in the expression of FXN and is characterized by hypertrophic cardiomyopathy⁵⁰. Attenuated FXN levels in FRDA patients have been found to display cardio-metabolic and contractile dysfunctions as well as cardiac hypertrophy⁴³⁸.

Although the exact function of the mitochondrial iron binding protein FXN remains relatively unknown, it has been found that FXN plays a key role in mitochondrial iron homeostasis⁴³¹. Furthermore, it has been suggested that FXN may act as a chaperone for iron-sulfur cluster (ISC) formation and heme biosynthesis^{46,56,435}. FXN is highly expressed in mitochondrial-rich tissues including cardiomyocytes, neurons, brown fat, liver, and pancreas⁴⁵. This mitochondrial protein has a direct influence on the mitochondrial function and respiration, as most of the mitochondrial electron transport chain (ETC) complexes contain ISC⁴³⁶.

Previously, FXN has been thought of as a chaperone protein because of its role in regulating iron sulfur cluster biogenesis. However, recently it has been proposed that FXN has multiple roles in iron regulation because it can serve as an iron storage protein, iron sensor, scavenger of ROS and regulates mitochondrial energetics^{56,57}. Furthermore, Ristow and colleagues have reported that over-expression of FXN promotes OXPHOS, increased tricarboxylic acid (TCA) cycle activity, and mitochondrial membrane potential⁴⁴. We therefore hypothesize that increased expression of FXN protects the cardiomyocyte against IR injury by regulating myocardial energetics and improves the anti-oxidant capacity in cardiomyocytes. In this study, we show that FXN expression is increased during reperfusion and provides cardio-

protection against IR injury. Further, the increased expression of FXN protects the mitochondria from iron overload and the ensuing ROS formation, which are known to compromise the mitochondrial integrity by increasing the anti-oxidants capacity levels. Finally, we offer that FXN over-expression increases cell viability, ATP production and the mitochondrial membrane potential during the reperfusion and therefore protects the cardiomyocyte from IR injury.

3.3. Materials and Methods

3.3.1. Animals:

The breeding protocols and the experimental procedures were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Animals were housed in Auburn University's Biological Research Facility and maintained in a controlled environment (23°C; 12 hours light/ dark cycles) with free access to water and standard chow diet. Male C57Bl/6 wild type mice at an age of 12 weeks were used in this study.

3.3.2. Ischemia Reperfusion studies:

IR surgeries were performed on 12-week-old male C57Bl/6 mice. In general, mice were anesthetized with sodium pentobarbital and ventilated mechanically. The hearts were exposed by left thoracotomy and the proximal left anterior descending (LAD) coronary artery was reversibly ligated for 30 minutes with an 8.0 silk suture mounted on a tapered needle. Reperfusion was achieved by loosening the ligature for 2 hours. The sham operated mice underwent the same procedure without LAD occlusion. After that, hearts were enucleated and the infarcted and non-infarcted regions were then separated and snap frozen for further analysis.

3.3.3. Cell Line and Culture:

H9C2 ventricular embryonic rat cardiomyocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin solution. Cells were maintained at 37°C, 5% CO₂ levels with humidity. Hypoxia was achieved by exposing the cells to 1% O₂ gas in an airtight Billups Rothernberg hypoxia chamber with adequate humidity at 37°C for 18 hours. Re-oxygenation was achieved by incubating the cells at 37°C, 5% CO₂ and 20% O₂ levels with humidity for 0, 2, 6, 12 and 24 hours after 18 hours of hypoxia.

3.3.4. Cryopreservation of Cell Line

To cryopreserve the cell lines for long time, different cell lines were incubated at 37°C and 5% CO₂ levels with humidity until it reached approximately 90% confluency. Then, cells were collected and centrifuged at 1000 rpm for 5 minutes to make a pellet. The formed pellet were then re-suspended in 2ml of the complete DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin solution and 10% (v/v) DMSO (dimethyl sulfoxide), the freezing media, and then transferred to cryovials. The cryovials were kept in an isopropanol freezing container (Thermoscientific cat.# 5100-0001) and kept in -80°C overnight. The day after, the cryovials were transferred into liquid nitrogen tank (-196°C).

3.3.5. Production of stable frataxin over-expressed cardiomyocyte cell line:

Stable frataxin over-expressed cardiomyocytes (FXN-OE) cell lines were developed by utilizing a lentiviral system based upon the gateway system. Briefly, FXN cDNA was cloned into a gateway donor vector. Using LR clonase, this vector was then cloned into a puromycin resistant destination vector pLX-302, which is a lentiviral mammalian expression destination vector. Transformation of the cells was achieved using *E. coli* strain DH5 α and FXN plasmid was isolated using the PureYield Plasmid Midiprep System (Promega, Cat #A2495). The purified gateway vector was then co-transfected with lentiviral packaging vector (psPAX2) and envelope vector (pMD2.G) into human embryonic kidney 293 (HEK 293T) cell line. Three days post-transfection, the media containing the virus was collected and was filtered using a cartridge system. After that, the H9C2 cells were incubated with a mixture of the purified media and complete DMEM (1ml and 2ml, respectively) for 24 hours. On the second day, the media was changed and the cells were incubated with fresh complete DMEM media. To promote selection of FXN, cells were incubated with puromycin (1 μ g/ ml). Presence of FXN was measured by PCR (genomic DNA) and western analysis (protein) of the V5 tag which was incorporated into the FXN vector.

3.3.6. Production of stable frataxin knockdown cardiomyocytes cell line:

The lentiviral system containing shRNA for FXN was used to construct stable frataxin knockdown cell lines (FXN-KD). This shRNA lentiviral vector has the PLKO.1 backbone, which is a kind gift from David Root's lab ⁵²⁸ and was obtained from Addgene (plasmid

#10878). ShRNA oligos were developed according to the protocol mentioned in Addgene website (<http://www.addgene.org/tools/protocols/plko/>). The constructed oligos in addition to the PLKO.1 backbone were treated with the restriction enzymes Age1 and EcoR1 (New England Biolabs) and ligated using Quick ligation kit (New England Biolabs). The ligated vector was then transformed into competent bacterial plasmids, and isolated using a Promega pure yield plasmid midi prep system. Using the packaging plasmids described previously, the purified vector was transfected into HEK293T cells. The virus containing media was collected three days after transfection and purified by passing the media through 0.45µm syringe filter. H9C2 cells were then infected with a mixture of the 1ml purified media and 2ml complete media. Media was then changed and cells were incubated with fresh complete DMEM. For selection, cells were incubated with media containing 1µg/ ml puromycin.

3.3.7. Production of mCherry-FXN vector:

The mCherry-FXN vector was constructed using the gateway system. Detailed description of the generation of frataxin donor vector is included in the construction of FXN-OE cell lines mentioned above. The mammalian expressed mCherry destination vector was purchased from Addgene which was deposited by Robin Shaw's lab (plasmid #31907)⁵²⁹. The FXN donor vector was cloned to the mCherry destination vector using LR clonase, and the resulting expression vector was transformed in to DH5alpha competent bacteria. The expression vectors were subsequently purified using Promega pure yield plasmid midi prep system.

3.3.8. Western blots:

The proteins from H9C2, FXN-OE and FXN-KD cells were extracted using the cell lysis buffer from Cell Signaling (9803) with protease cocktail inhibitor (Thermo-Fisher). The protein lysates from different cell lines and from heart tissue were denatured using Laemmli buffer with DTT and heated at 95°C for 5 minutes. The proteins were resolved in 12% SDS-PAGE with the standard protein marker and transferred to polyvinylidene fluoride (PVDF) membranes and immunoblotted with the following primary antibodies overnight at 4°C; anti-rabbit anti-FXN (Santa Cruz, C2913) with a concentration of 1:750 and anti-alpha tubulin (Developmental studies Hybridoma Center at University of Iowa) with a dilution of 1:2000. Blots were washed on the second day with Tris-Buffered Saline and Tween 20 (TBS-T) and incubated with horseradish peroxidase (HRP) conjugated secondary antibody (Rockland, 31833) with concentration of 1:2000, for 2 hours and imaged using a BioRad gel dock system. Protein bands were analyzed using ImageJ software. Results were standardized to α -tubulin and presented as fold change from control.

3.3.9. Fluorescence microscopy assay:

H9C2 cardiomyocytes were cultured in complete DMEM until they were approximately 70% confluent. Cells were co-transfected with the mCherry-FXN plasmid (see production of mCherry-FXN vector section) using Jetprime transfecting reagent (Polyplus). Four hours later, media was changed and replaced with fresh complete media. One day post transfection, cells were introduced to 18 hours of hypoxia followed by different time point of re-oxygenation (0, 2,

6, 12 and 24 hours). Mitochondria were then visualized using MitoTracker Green FM fluorescent stain (100 nM) according to the manufacturer's protocol (Invitrogen, M-7514) and visualized using a Nikon Ti fluorescence microscope.

3.3.10. MTT assay:

The cell viability was assessed using MTT cell viability assay kit (Biotium, 30006) and the assay was conducted according to the manufacturer's protocol. The absorbance was measured at 570 nm using Bio-Tek plate reader. Values were standardized to a Sulforhodamine B colorimetric assay.

3.3.11. ATP assay:

The ATP levels were measured using the ATPLite Luminescence Assay kit from PerkinElmer (6016941), which is an ATP determining system based on firefly luciferase. Cells were plated in 96-well plates and introduced to hypoxia for 18 hours and then for 0, 2, 6, 12, 24 and 48 hours of re-oxygenation. The assay was conducted as per the manufacturer's protocol. In brief, the presence of the luciferase in addition to D-Luciferin and ATP emit light, which is proportional to ATP concentrations. Plate reader was used to measure the emitted bioluminescence.

3.3.12. Mitochondrial isolation:

The mitochondria were isolated as previously described⁵³⁰. Briefly, cells were washed twice with ice cold PBS and were incubated in mitochondrial isolation buffer (sucrose 250 mM, EDTA 1 mM, Hepes 10 mM supplemented with protease cocktail inhibitor) and disrupted with a syringe (27.5 gauge). The supernatant was collected after centrifugation at 600g for 5 minutes to remove the debris and then centrifuged again at 7000g for 10 minutes. The pellets were re-suspended in mitochondrial isolation buffer and centrifuged at 7000g for 10 minutes. The pellet was dissolved in the mitochondrial isolation buffer supplemented with 1% Triton X – 100.

3.3.13. Mitochondrial iron assay:

Mitochondria were isolated as indicated above for the Ferrozine colorimetric assay⁵³¹. Briefly, cells were introduced to hypoxia (18 hours) and exposed to re-oxygenation environment for variable times (0, 2, 6, 12 and 24 hours). Ferrozine - iron complex was measured by reading the absorbance at 550 nm in the plate reader (Bio-Tek instruments). Results were standardized to protein concentration and presented as fold change from control.

3.3.14. Mitochondrial ROS assay:

Mitochondrial reactive oxygen species (ROS) were measured using the mitochondrial specific indicator dihydro-rhodamine (DHR) (Biotium). DHR is an uncharged non-fluorescent ROS indicator that accumulates in the mitochondria and shifts into the oxidized form, the

cationic rhodamine123, which exhibits a green fluorescence. H9C2 cells and FXN stable cell lines were stained according to the manufacture's protocol. The multispectral-fluorescent plate reader (Bio-Tek) was used to measure the fluorescence at 505/ 534 nm ($\lambda_{Ex}/ \lambda_{Em}$) and imaged using a Nikon-TiS inverted confocal microscope.

3.3.15. Mitochondrial membrane potential assay:

Using the Tetramethylrhodamine ethyl ester (TMRE) dye, the mitochondrial membrane potential was measured in H9C2, FXN-OE and FXN-KD cell lines after introducing the cells to hypoxia re-oxygenation injury. TMRE (Biotium) is a positively charged red-orange dye that accumulates in active mitochondria. The staining of the cells was accomplished according to the manufacture's protocol and the signals were detected using a Nikon Ti fluorescence microscope at $\lambda_{Ex}/ \lambda_{Em}$ at 548/ 575 nm.

3.3.16. Cellular oxygen consumption studies:

Cellular respiration was measured as described previously⁵³². In brief, cells were washed twice with ice cold PBS and centrifuged at 800g for 3 minutes. Cell pellets were re-suspended in 400 μ l PBS containing 10 mm glucose, 10 mm Hepes and 0.2% BSA (pH 7.45) and were maintained at 37°C. Then, 300 μ l of glucose supplemented PBS and 350 μ l of the cell suspension solution were added to the oxygen chamber (Hansatech Instruments) and equilibrated for approximately 3 minutes. Basal respiration rate was recorded for 5 minutes. Then, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which uncouples mitochondrial oxidative

phosphorylation, was added to the chamber and oxygen consumption was monitored for 1.5 minutes. Results of basal and maximal respiration were normalized to protein concentration.

3.3.17. Complex I activity assay:

Mitochondria from H9C2, FXN-OE and FXN-KD cells were isolated as described above. NADH activity was measured according to the previously described protocol⁵³³. Oxidation of NADH by NADH dehydrogenase was measured spectrophotometrically at 340 nm in isolated mitochondria. Briefly, isolated mitochondria were mixed with the reaction mixture (10µl of 8.4 mM NADH, 5µl of 2.3 mM coenzyme Q10 and 200µl of 0.01 M PBS) and oxidation of NADH was measured by monitoring the decrease in absorbance at 340 nm. Known concentration of NADH was used to obtain standard curves for NADH at 340 nm. Changes in absorbance indicate the amount of NADH being oxidized. The mitochondrial complex I activity is expressed as percentage change from control.

3.3.18. Complex IV activity assay:

Complex IV activity is based on the oxidation of cytochrome C, which is measured spectrophotometrically at 550 nm⁵³⁴. In general, mitochondrial suspensions were added to the reaction mixture containing equal volume of cytochrome C (50 µM) and 0.01 M phosphate buffered saline (PBS). The absorbance was measured at 550 nm for 2 minutes and the enzyme activity was expressed as percentage change from control.

3.3.19. Glutathione (GSH) content assay:

Glutathione (GSH) content was measured fluorimetrically as described elsewhere⁵³⁵. In this assay, o-phthalaldehyde (OPA) was used with GSH to generate a fluorescent product. After the precipitation of the protein by 0.1 M phosphoric acid, cellular supernatant was added to the assay mixture, which was composed of equal volumes of 0.1% OPA and 0.01 M PBS. This mixture was incubated for 20 minutes and readings were taken at excitation and emission wavelengths at 340 and 420 nm, respectively. Using commercially available GSH, a standard curve was plotted. The GSH content was calculated as nmol of GSH per μg of protein.

3.3.20. Superoxide dismutase (SOD) activity assay:

Total superoxide dismutase (SOD) activity was determined using the pyrogallol assay⁵³⁶. This assay is based on the competition between pyrogallol oxidation and superoxide dismutation by superoxide radicals and SOD, respectively. Briefly, cell lysate was added to the assay mixture composed of 50 mM Tris-Cl buffer and 0.2 mM pyrogallol (dissolved in 0.01N HCl). The absorbance of pyrogallol was monitored at 420 nm. Results were standardized to protein concentrations and expressed as percentage change from control.

3.3.21. Lipid peroxidation content assay (TBARS):

The content of lipid peroxidation was measured by measuring the content of thiobarbituric acid reactive substances (TBARS)⁵³⁷. Briefly, supernatant from cells were incubated with ice cold TCA (10% w/v) for 15 minutes on ice. The mixture was then centrifuged

at 2200g for 15 minutes. Supernatants were then collected and 200 μ l of 0.67% TBA was added. The assay mixture was then incubated in boiling water for 20 minutes and cooled to room temperature with absorbance measured at 532 nm. A standard curve of TABARS was carried out using increasing concentrations of the mixture and levels of TBARS were calculated. Results were presented as percentage change from control.

3.3.22. Statistical analysis:

Data were expressed as mean \pm SEM. Results were normalized to the basal values and analyzed by a student two-tailed t-test (comparison between 2 groups) and one-way ANOVA (to compare more than 2 groups) analysis with Tukey's post hoc analysis for significances between groups. The level of significance was set for $p < 0.05$. All statistical calculations were performed on a computer using GraphPad Prism software (version 5.04 for windows, La Jolla, California, USA). All cell culture studies were based upon a minimal of 3 independent experiments, unless otherwise specified.

3.4. Results

3.4.1. FXN expression is significantly increased during the reperfusion of the ischemic hearts

Previously, it was reported that 18 hours of hypoxia significantly increased the expression of FXN (more than 2-fold) in H9C2 cardiomyocytes when compared to normoxic conditions⁴³¹. These findings suggest a cardio-protective role for the up regulation of FXN expression in response to ischemia. In the current study and to further explain the regulation of FXN in the reperfusion stage, we introduced mice to either sham or ischemia for 30 minutes and then to 2 hours of reperfusion. The extracted protein lysates from perfused (P) and ischemic (I) tissues revealed an elevation in the expression of FXN in the ischemic regions when compared to the perfused regions (Fig. 3.1A & B). To better understand the role of FXN during reperfusion, we constructed FXN over-expressed (FXN-OE) cell line using a lentiviral system. When we introduced both FXN-OE and H9C2 cells to 18 hours of hypoxia and then to increasing times of re-oxygenation, we observed a significant increase in FXN expression after 0 and 6 hours of re-oxygenation (Fig. 3.1C & D). Further, in both cell lines (FXN-OE and H9C2 cells), the greatest increase in FXN expression was observed at six hours of re-oxygenation compared to control H9C2 cells under normoxic condition (Fig. 3.1C & D). Surprisingly, we observed a reduction in FXN expression after 12 and 24 hours of re-oxygenation in H9C2 cardiomyocytes (Fig. 3.1C & D). These findings indicate that FXN expression is considerably increased in mice introduced to

IR injury when compared to sham treated mice. Further, this increase maintains during the reperfusion stages of ischemia reperfusion injury as further demonstrated in our in-vitro studies.

Furthermore, our fluorescence cell imaging revealed that under normoxic condition (20% O₂), FXN (red) is primarily located in the cytosol of H9C2 cardiomyocytes (Fig 3.1E). After 18 hours of hypoxia (at 0 hour of re-oxygenation), a little FXN was localized into the cytosol (red) and some was found in the mitochondria (yellow in the merged image). This pattern changed dramatically after 6 hours of re-oxygenation where most of the FXN was found to be associated in the mitochondria (yellow in the merged image) (Fig. 3.1E). Surprisingly, this pattern changed dramatically after 12 hours of re-oxygenation, where FXN was localized mostly in the cytosol and reverted to normoxic conditions after 24 hours of re-oxygenation (red in the merged image) (Fig. 3.1E).

3.4.2. Mitochondrial FXN mitigates iron accumulation and ROS formation

FXN is known as a key regulator for mitochondrial iron metabolism. Therefore, we postulated that over-expression of FXN will mitigate mitochondrial iron accumulation in response to hypoxia/re-oxygenation stress. To further investigate this cardio-protective mechanism, we constructed FXN over-expressed (FXN-OE) and FXN knockdown 2 & 3 (FXN KD-2 and FXN KD-3) stable cell lines using a lentiviral system. FXN KD-2 has approximately 40% reduction in the expression of FXN, while FXN KD-3 has almost 80% reduction in FXN expression (Fig. 3.2A-C). These stable cell lines were characterized by polymerase chain reaction (PCR) analysis of genomic DNA (Fig. 3.2A) and western analysis (Fig. 3.2B & C). We observed that FXN-OE cell lines showed approximately 2-fold increase in FXN expression

compared to H9C2 cells (Fig. 3.2B & C). Construction of FXN knockdown cell lines using shRNA sequences are shown in Fig. 3.2D. Strand 1 did not suppress the expression of FXN successfully, while strands 2 and 3 knockdown FXN expression 40% and 80%, respectively. In the current study, we utilized FXN KD-3 rather than FXN KD-2 due to the relative knock down efficiency observed from the application of FXN KD-3 in cells and we will refer to it as FXN-KD cells in the current study.

To better understand the role of mitochondrial iron regulation by FXN under hypoxia/re-oxygenation stress, we conducted the ferrozine colorimetric assay on the isolated mitochondria from our H9C2 cardiomyocyte cell lines that were introduced to 18 hours of hypoxia followed by increasing times of re-oxygenation. Our data in H9C2 cells suggested no significant changes in mitochondrial iron levels between 0, 2, 6 and 12 hours of re-oxygenation and the control H9C2 under normoxic conditions (Fig. 3.3A). However, iron levels significantly increased after 24 hours after re-oxygenation and that may be explained by the decrease in FXN expression at 24 hours of re-oxygenation (Fig. 3.3A). Furthermore, FXN-KD cells showed marked mitochondrial iron accumulation immediately after re-oxygenation was initiated. In stark contrast, the frataxin over-expressing cells (FXN-OE) displayed no increase in mitochondrial iron accumulation after 6 hours of re-oxygenation (Fig. 3.3A). Together these data suggest that FXN expression levels are significantly involved in regulating mitochondrial iron levels.

It has been suggested that the increase in mitochondrial iron accumulation and the defect in the expression of cytochrome c and heme, which are present in the respiratory complexes I and IV, results in increased mitochondrial reactive oxygen species (ROS) formation^{51,538,539}. To investigate this during re-oxygenation, we conducted mitochondrial ROS formation assay using a Dihydro rhodamine (DHR) dye. Our results were consistent with what we found previously. In

H9C2 cells, ROS generation started to increase at 12 and at 24 hours of re-oxygenation it increased significantly (Fig. 3.3B & C). FXN-KD cells showed increased levels of ROS formation immediately at the initiation of re-oxygenation (0 hour of re-oxygenation). However, these findings were not observed in FXN-OE cells which showed relatively no change in ROS levels over time after hypoxia (Fig. 3.3B & C).

Cardiomyocytes are known to have the highest mitochondrial volume of all tissues in the body, and therefore have a higher capability for generating reactive oxygen species (ROS). The accumulation of ROS caused by oxidative damage, results in an increase in lipid peroxidation (LPO), as an observed consequence from ischemia reperfusion (IR) injury⁵⁴⁰⁻⁵⁴². Therefore, protection of cardiomyocytes from increased levels of lipid peroxidation (LPO) can help in the recovery from IR injury. In this manner, we observed relatively little change in lipid peroxidation in H9C2 cells in response to hypoxia/re-oxygenation insult until 12 hours of re-oxygenation (Fig. 3.3D). Further, we also observed a significant increase in the LPO contents in FXN-KD cells throughout the re-oxygenation (Fig. 3.3D). However, the FXN-OE displayed a cardio-protective mechanism against the development of increased levels of LPO and thus protects the cardiomyocytes from further damage that might be caused by re-oxygenation (Fig. 3.3D).

Several studies have demonstrated that cardiomyocytes cell death occurs in response to decreased mitochondrial membrane potential ($\Delta\Psi_M$) and increased opening of the mitochondrial permeability transition pores (MPTP) found during the reperfusion stages of IR injury^{27,527,543,544}. Therefore, protection against alteration of the mitochondrial membrane potential ($\Delta\Psi_M$) is critical for cardiomyocytes survival from IR injury because $\Delta\Psi_M$ is considered the driving force for the ATP synthesis. In the current study, we measured the $\Delta\Psi_M$ in our cells by using a mitochondrial fluorescent dye TMRE. Cells that have reduced level of FXN (FXN-KD)

displayed the lowest $\Delta\psi_M$ after the hypoxia followed by re-oxygenation for 24 hours (Fig. 3.4A & B). Normal H9C2 cardiomyocytes had an increase in the $\Delta\psi_M$ after 0-6 hours and decreased after 12 and 24 hours of re-oxygenation when compared to the same cells under normoxic conditions (Fig. 3.4A & B). FXN-OE, however, protected the cardiomyocytes from changes in $\Delta\psi_M$ observed in the other cell lines during re-oxygenation (Fig. 3.4A & B).

Taken together, increased expression of FXN prevents iron accumulation in response to IR injury, as well as formation of ROS and LPO. In addition increased FXN expression preserves the mitochondrial integrity by maintaining mitochondrial membrane potential.

3.4.3. Increased expression of FXN preserves the mitochondrial bioenergetics and protects cardiomyocytes from re-oxygenation mediated damage

We investigated the changes in cardiomyocyte bioenergetics during the re-oxygenation by measuring oxidative phosphorylation (OXPHOS) and electron transport chain (ETC) complex activities. Deficiency of FXN expression in FXN-KD cells was associated with a marked decrease in the activity of complexes I & IV, but increased expression of FXN significantly preserved complexes I and IV enzymatic activities (Fig. 3.5A & B). These results were consistent with previously published findings on the role of FXN expression and bioenergetics ^{44,545}.

Under basal conditions, FXN-OE cell lines showed a significant increase in oxygen uptake after 2 and 6 hours of re-oxygenation when compared to H9C2 cardiomyocytes under similar conditions (Fig. 3.6A). Further, FXN-KD cells had significantly reduced basal oxygen consumption rates when compared to H9C2 and FXN-OE cells (Fig. 3.6A). Furthermore,

following the addition of the uncoupling agent FCCP, we observed marked increase in oxygen consumption rates in FXN-OE cells, suggesting an elevated mitochondrial oxidative capacity in these cells when compared to both H9C2 and FXN-KD cells (Fig. 3.6B).

Based on these findings, we proposed that the improvement in respiration seen in FXN-OE cardiomyocytes may improve the overall ATP levels and thus maintain cardiomyocyte viability following IR injury. To better investigate this, we conducted multiple assays to assess the metabolic activity and health of the cells. As ATP is formed in the mitochondria and is the primary source of energy for living systems, we measured ATP levels in FXN-OE and H9C2 cell lines. Our data revealed that the levels of ATP were improved 6 hours after re-oxygenation in both cell lines and maintained at elevated levels in FXN-OE cells but not H9C2 cells up to 24 hours of re-oxygenation (Fig. 3.6C).

These data suggest that over-expression of FXN preserves the mitochondrial bioenergetics. Further, it preserves the mitochondrial integrity and thus protects against cellular oxidative stress during the re-oxygenation.

3.4.4. FXN over-expression promotes anti-oxidant defense mechanism via glutathione and superoxide dismutase

To investigate whether over-expression of FXN leads to an increase in the level of anti-oxidants and thus mitigates re-oxygenation mediated damage to cardiomyocytes, we measured the content of glutathione (GSH) and the activity of Superoxide dismutase (SOD). Interestingly, content of GSH, which is a vital cellular protective anti-oxidant, and activity of SOD were markedly elevated in FXN-OE cells in comparison to H9C2 and to the other cell line (Fig. 3.7A & B). FXN-KD cardiomyocytes, however, had noticeably much lower levels than the levels

observed in the H9C2 or in the FXN-OE cells (Fig. 3.7A & B). Although the levels of SOD and GSH were maintained at 0, 2 and 6 hours of re-oxygenation, the GSH content and SOD enzymatic activities in H9C2 cardiomyocytes were decreased after 24 hours of re-oxygenation (Fig. 3.7A & B).

Based on these findings, we proposed that the increase in FXN expression helps maintain cardiomyocytes viability against hypoxia/reoxygenation damage. To further investigate the role of FXN on cell viability during re-oxygenation, we carried out MTT assays for cell viability. Our findings indicate that H9C2 cells displayed significant decrease in the cell viability immediately after the re-oxygenation started and then improved with time (Fig. 3.7C). FXN-OE cells, paradoxically, showed significant increase after the re-oxygenation started by 6 hours and then maintained the cell viability at 12 and 24 hours of re-oxygenation (Fig. 3.7C).

Taken together, over-expression of FXN mediates cardio-protection against the hypoxia/re-oxygenation induced cardiomyocytes injury and that can be explained by the antioxidant property of FXN on the glutathione and superoxide dismutase enzymes and the increase in the cells viability, which gave an indication that increased expression of FXN is cardioprotective.

3.5. Discussion

Myocardial damage developed from IR injury is mostly associated with reperfusion and due to the cellular mechanisms involving ROS formation and increased MPTP activity^{11,26}. Therefore, many published studies have investigated cardioprotective signaling pathways that mitigate ROS formation and alter MPTP activity and thus offer protection to the heart against ischemia reperfusion injury. However, the mechanisms that explain how mitochondrial damage occurs during reperfusion remain relatively unknown. In the current study, we identified a novel signaling mechanism involving the regulation of FXN expression during reperfusion resulting in maintaining cellular energetics and anti-oxidant levels and thus limiting myocardial reperfusion injury.

Recently, it was reported that FXN expression significantly increased in response to hypoxia. Furthermore, hypoxia inducible factor 1 alpha (HIF-1 α) was reported to transcriptionally regulates FXN as confirmed by promoter activity and chromatin immunoprecipitation assays⁴³¹. Our recent findings add to the current knowledgebase for understanding the role that FXN plays during IR injury. To the best of our knowledge, we are the first to report that FXN increases in expression after six hours of re-oxygenation in cardiomyocytes. Interestingly, we also observed that in response to reperfusion, FXN localizes in the mitochondria, thus suggesting the increased mitochondrial localization to be critical for cardioprotection against IR injury.

Although the exact molecular function of FXN is not fully known, it has been proposed to be involved in regulating various iron required energy regulating pathways in the mitochondria. Further, we as well as others have observed in pathological conditions (diabetic cardiomyopathy and doxorubicin mediated cardiac hypertrophy) reduced levels of FXN in the heart that are associated with mitochondrial iron overload and cellular damage ⁴⁹. Furthermore, we observed that over-expression of FXN promotes mitochondrial iron homeostasis observed during re-oxygenation and thus protects the mitochondria from oxidative damage. Conversely, FXN knockdown cardiomyocytes displayed significant increase in mitochondrial iron accumulation throughout the re-oxygenation period and suggests that FXN plays an integral part in mitochondrial iron homeostasis. Further, increased levels of mitochondrial iron accumulation and defect in FXN expression result in elevated mitochondrial ROS levels via the Fenton reaction, resulting in the inactivation of the iron-sulfur cluster (ISC) protein activity, hypersensitivity to oxidative stress and increased mitochondrial damage ^{51,447,538,539,546}.

Previous studies have observed that numerous intracellular and extracellular processes can generate ROS. In cardiomyocytes, however, the most appropriate site for the generation of ROS is the mitochondria associated from the mitochondrial respiratory chain activity ^{26,263,547,548}. Elevated ROS levels at the beginning of reperfusion have been proposed to induce opening of the mitochondrial permeability transition pore (MPTP). This protein is a non-selective pore protein that remains closed under normal conditions and opens in response to cellular stresses. MPTP is located in the inner membrane of the mitochondria and facilitates the passage of molecules between the cytoplasm and the mitochondrial matrix in order to regulate the mitochondrial function and structure by maintaining the $\Delta\Psi_M$ ^{16,549}.

During ischemia, mitochondrial OXPHOS is significantly reduced due to oxygen requirements and therefore energy regulation shifts to anaerobic glycolysis leading significantly to reduce ATP levels. In addition, ROS levels increase during ischemia, which then leads to a decrease in GSH levels. Furthermore, the demise of the mitochondrial membrane potential, pH and the increase in lipid peroxidation levels further intensify the mitochondrial damage. During the reperfusion stage, however, the increase in the mitochondrial levels of ROS intensifies the mitochondrial damage. The opening of MPTP in reperfused cardiomyocytes is followed by significant modifications of the cellular bioenergetics, improved mitochondrial permeability to molecules with molecular weights greater than 1.5 kDa, disruption of the mitochondrial membrane potential, ATP depletion, inhibition of the movement of electrons through the respiratory chain and resulting in death of the cardiomyocyte (Fig. 3.8)^{11,26}.

During reperfusion, increased MPTP activity progresses the cardiomyocyte to cell death, and mediates cardiac injury. Therefore inhibition of MPTP opening is crucial for cardio-protection against ischemia reperfusion injury. The mitochondrial membrane potential ($\Delta\psi_M$) can be considered as an important indicator of mitochondrial function and its loss will result in opening of MPTP and the ensuing cell death. Our data revealed that FXN-OE cells prevented ROS production and preserved $\Delta\psi_M$ throughout the re-oxygenation when compared to normal H9C2 cells under normoxia or even when it compared to both H9C2 and FXN-KD cells under the same condition. Conversely, our ferrozine and TMRE data demonstrated that FXN-KD had the lowest $\Delta\psi_M$ and induced the highest ROS production in response to the re-oxygenation of the hypoxic cardiac myocytes and may be due to the elevated mitochondrial iron accumulation.

Further, we observed that in re-oxygenation, FXN deficiency in FXN knockdown cardiomyocytes led to marked decrease in the activity of respiratory chain complexes I and IV,

decrease in mitochondrial oxygen consumption and increase in lipid peroxidation. Furthermore, progressive depletion of ATP production was observed in H9C2 cells introduced to re-oxygenation. Nonetheless, FXN over-expression restored the mitochondrial complex activities and mitigates lipid peroxidation thus increasing the ATP production and accordingly increased the cardiomyocyte viability during re-oxygenation.

Shoichet and colleagues observed that the anti-oxidant effect of FXN is defined by its indirect stimulation of identified enzymes like glutathione peroxidase but not SOD²⁵. Our data in FXN-OE cells, nevertheless, showed significant increase in both GSH and SOD levels, which were impaired in FXN knockdown cardiomyocytes. Further, over-expression of FXN increased the OXPHOS and simultaneously protects cardiomyocytes from damage during re-oxygenation. These results explain the role of FXN in maintaining the structure and function of the mitochondria in response to ischemia reperfusion injury and described how enhanced expression of FXN is cardioprotective. In this manner, we have observed that the normal cellular response to re-oxygenation results in increased FXN and anti-oxidant levels. The consequence of these findings are validated in our FXN-OE cell line studies and offer a novel cardioprotective signaling mechanism against ischemia reperfusion injury.

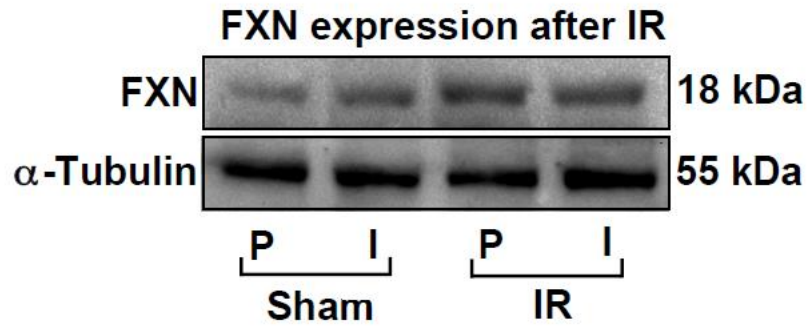
One limitation of the current study is that we used ventricular H9C2 cell line in our experiments, which is an embryonic rat cardiomyocytes cell line. However, various studies have observed that H9C2 cells can be used as a valuable *in-vitro* model to study heart disease^{550,551}. Second, we utilized H9C2 cells to create stable over expressing and knockdown models of frataxin to better understand the role of frataxin in response to reperfusion stress. Also, we used an *in-vitro* hypoxia re-oxygenation model to mimic the *in-vivo* ischemia reperfusion injury (IR).

Nonetheless, Portal and colleagues reported that the hypoxia re-oxygenation model in mice cardiomyocytes have similar features to the *in-vivo* model of ischemia reperfusion in mice⁵⁵².

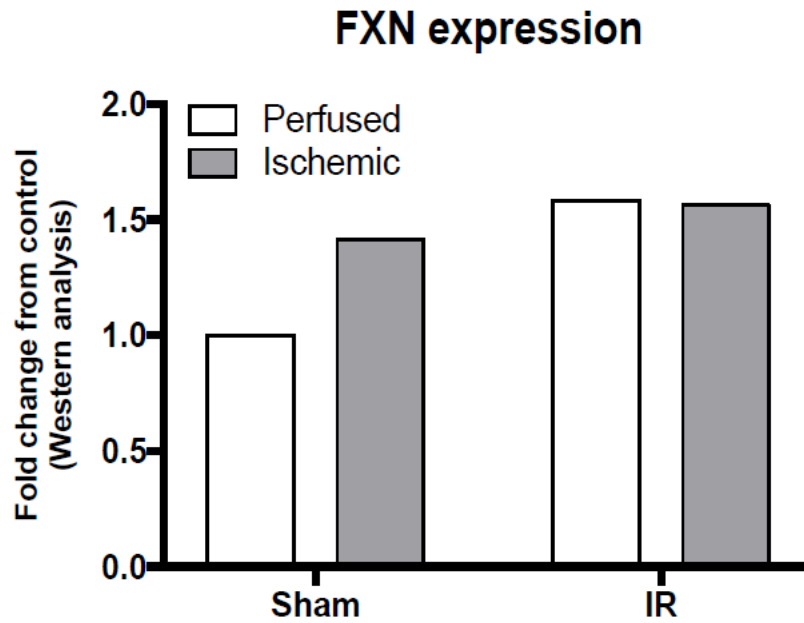
In conclusion, we provide a novel finding that the normal myocardial cellular response to reperfusion requires an increase in FXN expression resulting in increased antioxidant and energy regulating properties. More specifically, the increased FXN expression mitigates mitochondrial iron accumulation, MPTP opening and ROS formation, which in turn preserved the integrity of the mitochondria and increased the cardiomyocyte viability. Together, these findings help to understand the protective role of FXN and treat diseases where FXN is altered in expression such as FRDA, diabetic cardiomyopathy and doxorubicin mediated cardiac hypertrophy.

3.6. Figures and Figure Legends

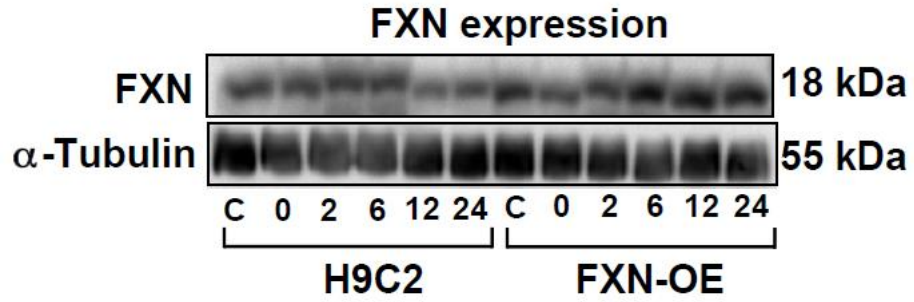
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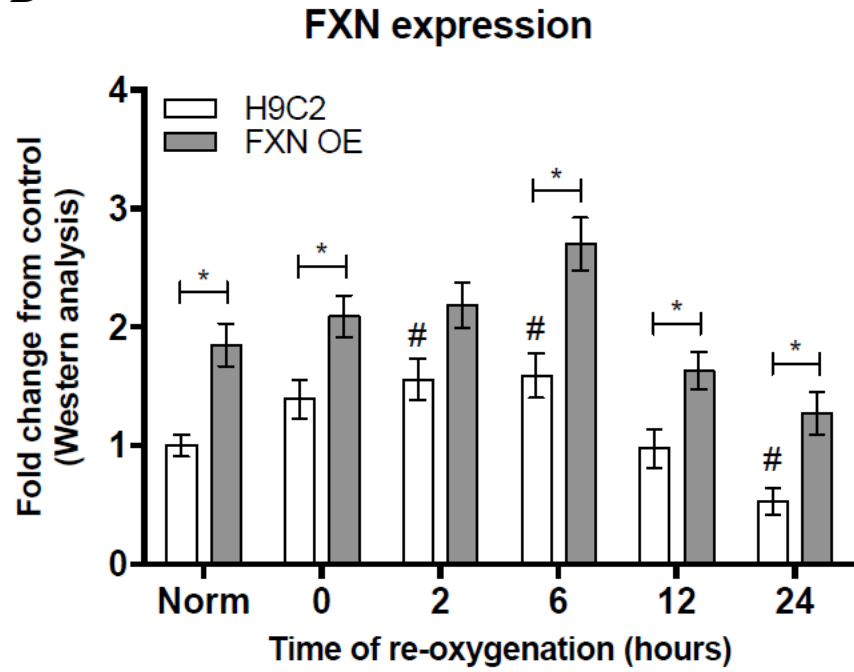
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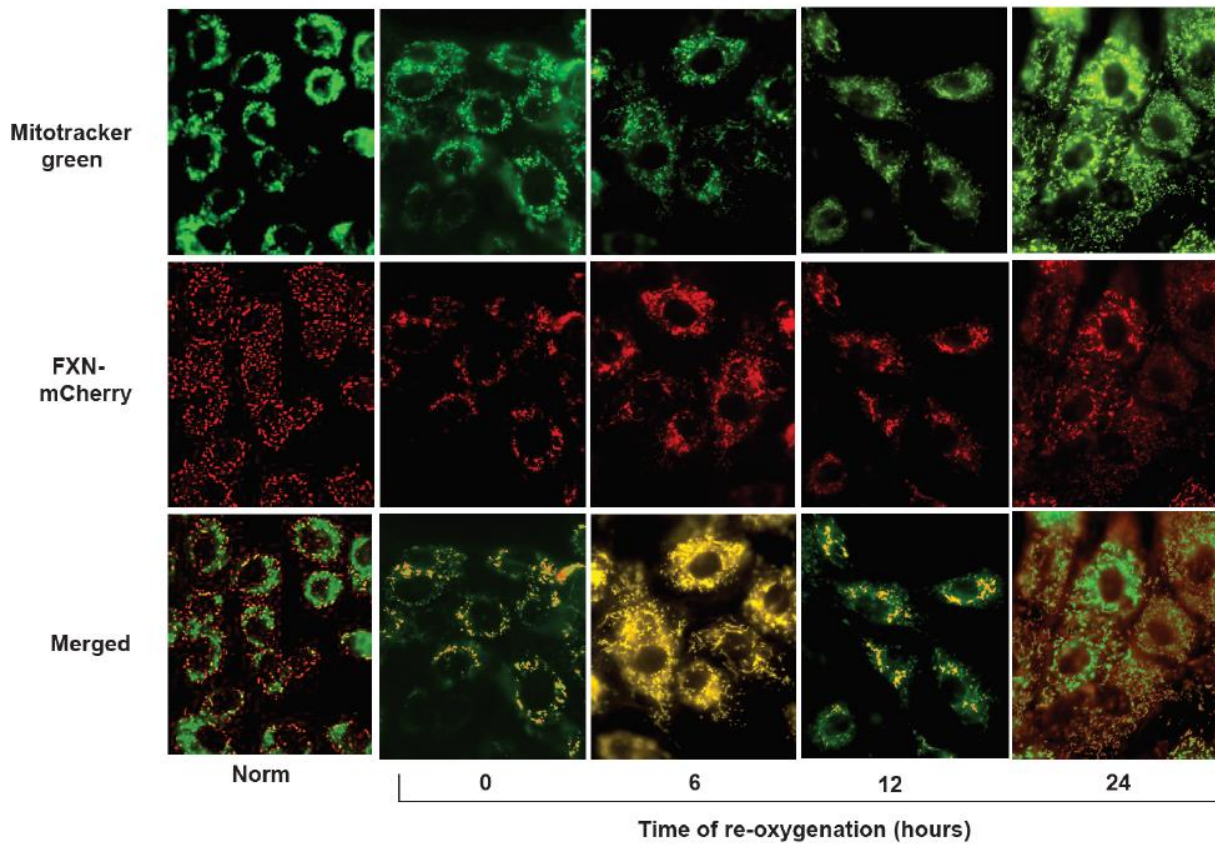
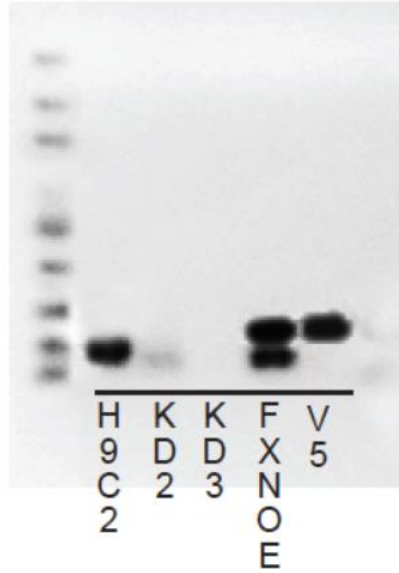


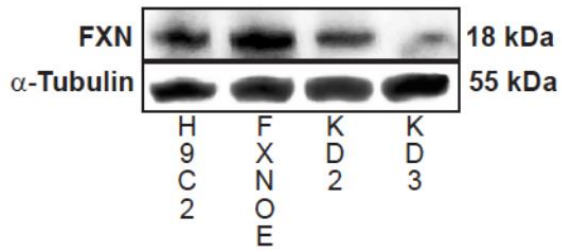
Figure 3.1. Frataxin expression increases in response to IR injury. (A) Representative image of western immunoblot for FXN protein expression from control mice hearts that underwent either sham surgeries or ischemia for 30 minutes followed by 2 hours of reperfusion. Protein lysates from ischemic (I) and perfused (P) regions were resolved to determine the changes in FXN protein expression. (B) Densitometric analysis of average values of FXN protein expression as determined by western analysis from control mice hearts. Values were digitized and standardized to α -Tubulin levels. (C) Representative blot demonstrates changes in FXN levels in response to hypoxia/re-oxygenation. Changes in FXN protein levels were determined by western analysis in H9C2 control cells and FXN over expressed cells (FXN-OE) under

normoxic condition (Norm), 20% O₂, in comparison to the hypoxic condition, 1% O₂, for 18 hours followed by different time of re-oxygenation (0, 2, 6, 12 & 24 hours). (D) Bar graph represents the average densitometric values obtained for FXN expression from H9C2 and FXN-OE cells exposed to normoxia (20% O₂) and hypoxia (1% O₂) for 18 hours and then to increasing time of re-oxygenation. (E) Immunofluorescence analysis of H9C2 reveal that hypoxia followed by re-oxygenation induced FXN localization into the mitochondria. H9C2 cardiomyocytes were introduced to normoxia (20% O₂) or to 18 hours of hypoxia (1% O₂) and then to different time of re-oxygenation. FXN-mCherry (red) represents the localization of FXN in the cytosol and Mitotracker green were used to label the mitochondria. Localization of FXN in the mitochondria during re-oxygenation showed as yellow color in the merged images. The graphical analysis reflects averages from 3 independent experiments, *, #; P<0.05, where comparisons to H9C2 cells under normoxic conditions are represented by #. Values are expressed as mean ± SEM.

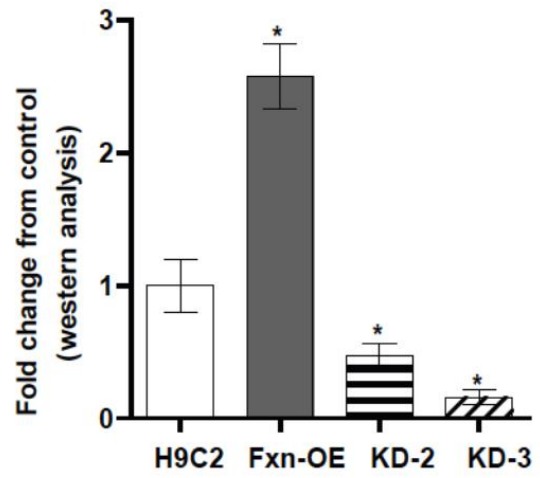
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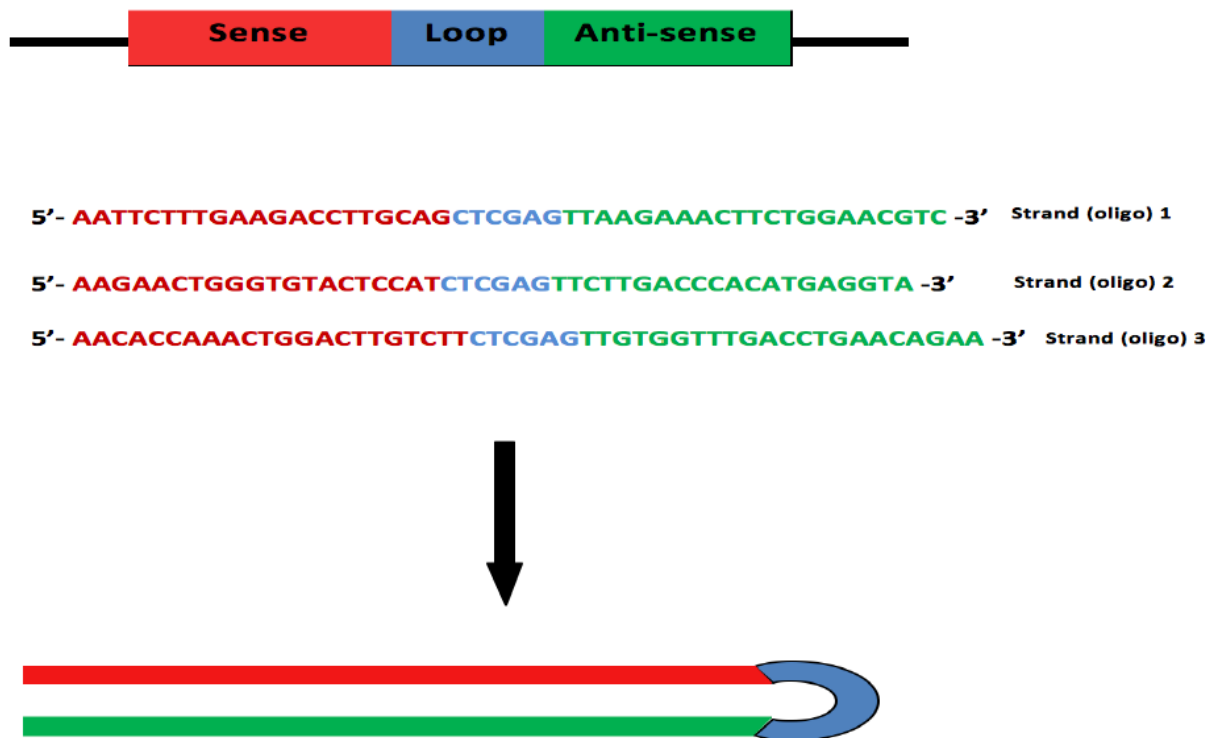
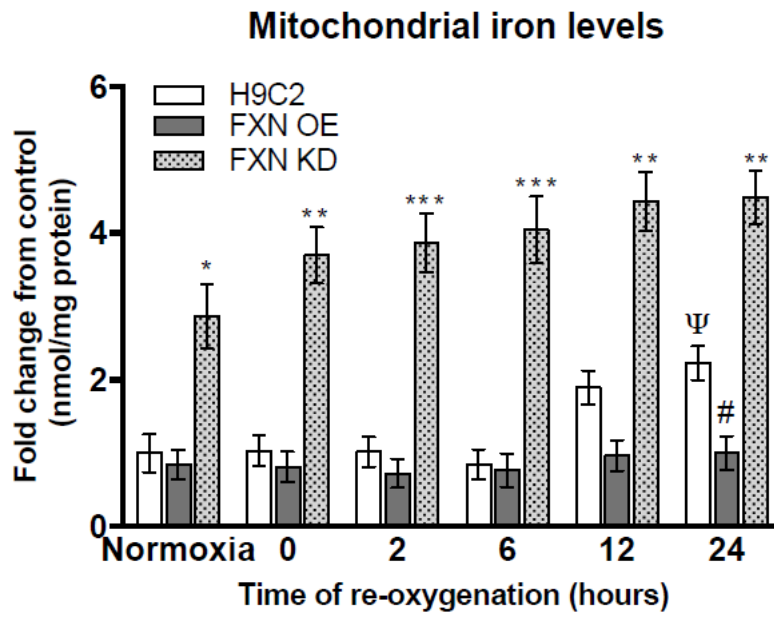
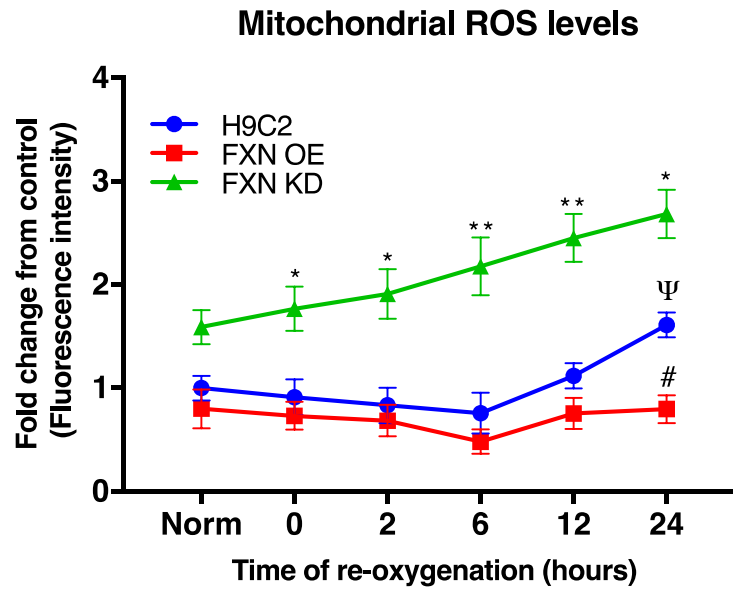


Figure 3.2. Identification and characterization of frataxin knockdown and over-expressing cell lines. (A) Total DNA from H9C2, FXN knockdown-2 (KD-2), FXN knockdown-3 (KD-3) and FXN over-expressed (FXN-OE) cell lines were analyzed for FXN expression using the same set of primers by polymerase chain reaction (PCR). V5 tag primers (V5) were used to detect the FXN over-expressed cell line. (B) Protein lysates from these cell lines were analyzed for FXN expression by western blotting (WB) and standardized by α -tubulin expression levels. (C) Graphical representation of average densitometric values of FXN as determined by western analysis. Values are based upon averages from 3 independent experiments and compared to control H9C2 cardiomyocytes. Where *; $P < 0.05$. (D) Sequences of the strands (oligos) constructed to develop the lentiviral vectors for FXN knockdown cell construction. Strands 2 and 3 were used to develop KD-2 and KD-3 FXN knockdown cells, respectively. Strand 1 failed to attenuate the FXN protein expression significantly (data not shown).

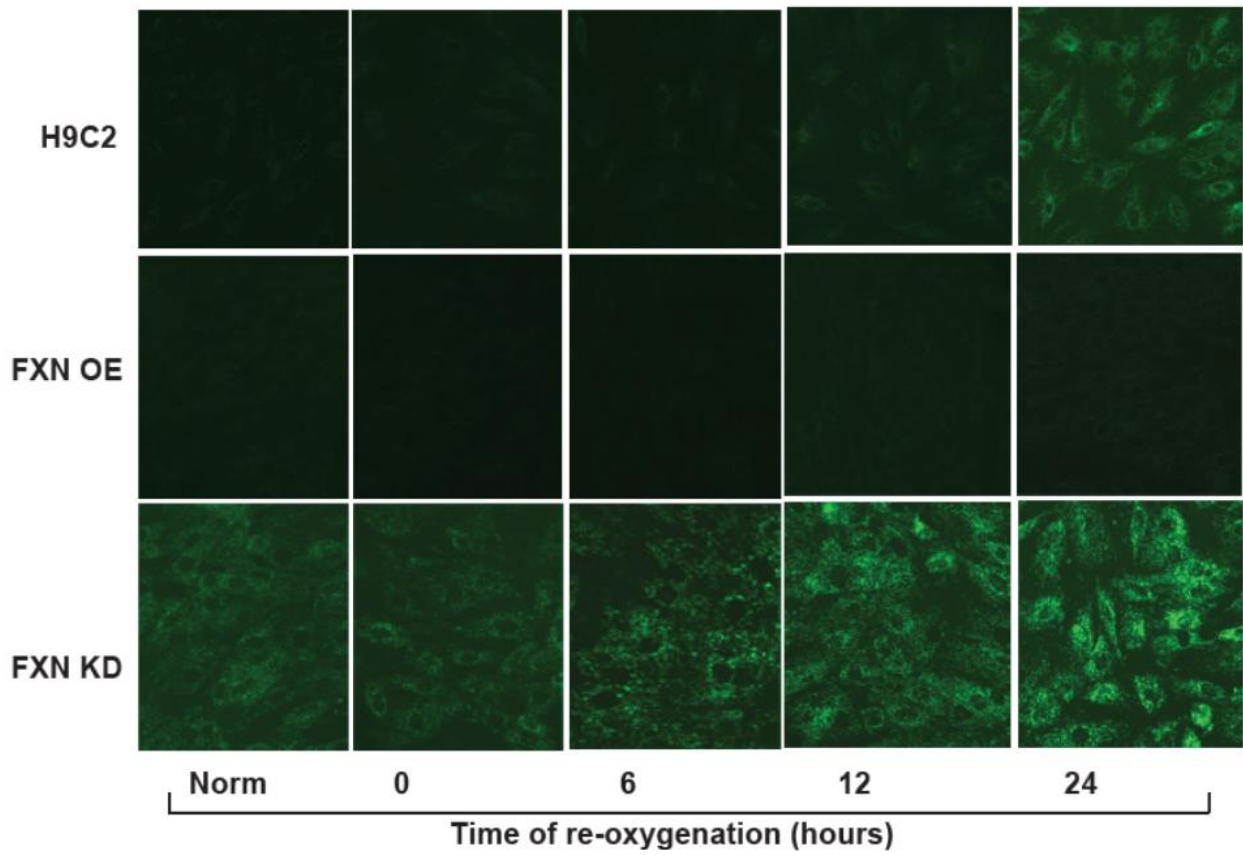
A



B



C



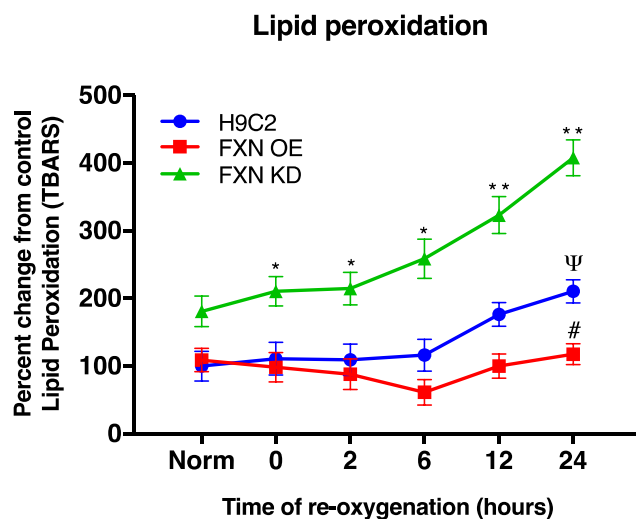
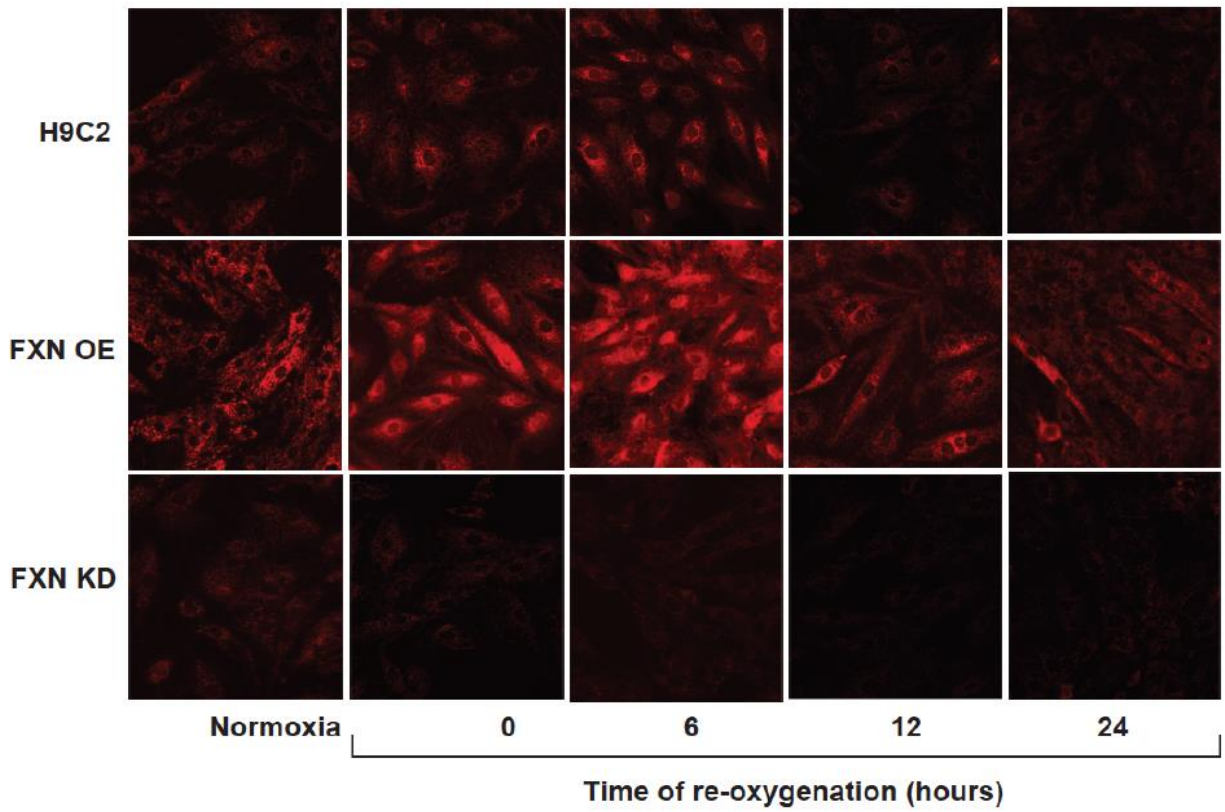
D

Figure 3.3. Over-expression of FXN mitigates iron accumulation, ROS formation and lipid

production. (A) Levels of mitochondrial iron in H9C2, FXN-OE and FXN-KD cells under normoxia or hypoxia for 18 hours followed by variable times of re-oxygenation were determined by Ferrozine assay. (B) Representative images showing FXN-KD cells that underwent hypoxia (1% O₂ for 18 hours) followed by variable times of re-oxygenation displayed increased mitochondrial ROS formation as measured by mitochondrial specific dihydro-rhodamine (DHR) dye and imaged by fluorescence microscopy when compared to control H9C2 cells. However, FXN-OE cells did not display increased mitochondrial ROS formation in response to hypoxia/re-oxygenation insult. (C) Graphical analysis of ROS images from panel (B) are represented as fold change from normoxic (Norm) treated H9C2 cells. (D) Change in levels of thiobarbituric acid reactive substance (TBARS), a lipid peroxidation product, in H9C2, FXN-OE and FXN-KD cells under normoxia or hypoxia followed by re-oxygenation are presented as percentage change from normoxic H9C2 cells. Values are expressed as mean \pm SEM and were normalized to normoxic control H9C2 cells. Where N=3 independent experiments. *, #, Ψ P<0.05; **P<0.01; ***P<0.001; where comparisons to control H9C2 cells under normoxic conditions are represented by Ψ . Comparisons between FXN-OE and H9C2 cells in each time point are represented by #, while the comparisons between FXN-KD and H9C2 cells at every time point are represented by *.

A



B

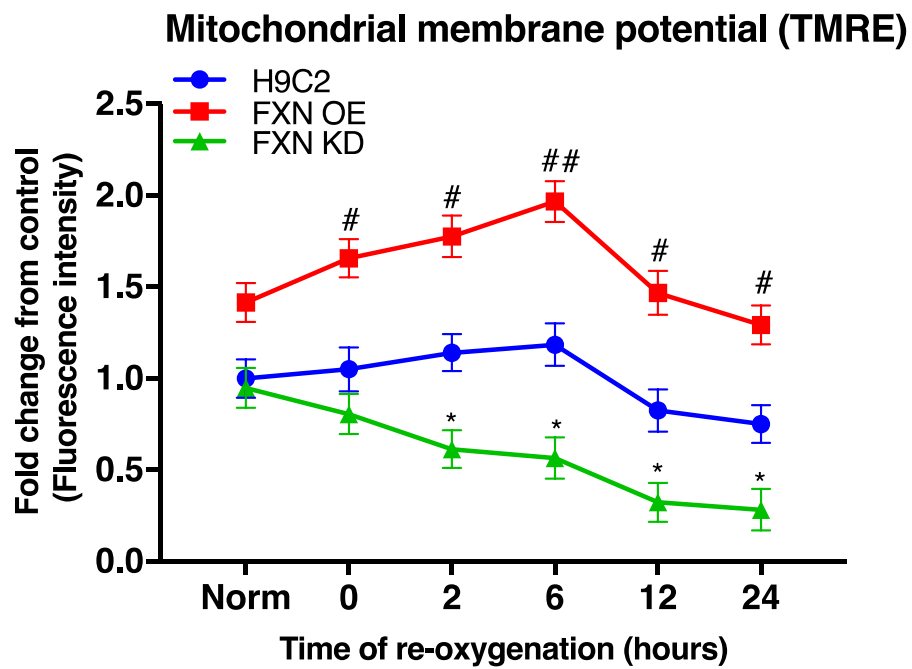
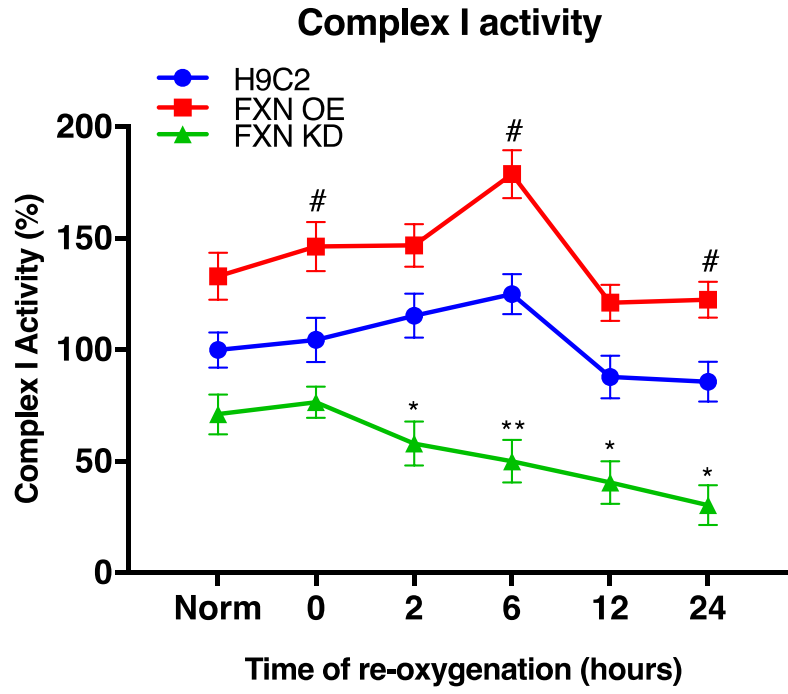


Figure 3.4. Increased FXN expression preserves the mitochondrial integrity. (A) Change in the mitochondrial membrane potential ($\Delta\psi_M$) in H9C2, FXN-OE and FXN-KD cells that exposed to normoxia (Norm) or to 18 hours of hypoxia followed by 0, 2, 6, 12, 24 hours of re-oxygenation using mitochondrial specific TMRE stain. FXN-OE cells show an increase in the $\Delta\psi_M$ during re-oxygenation compared to H9C2 and KD. (B) Graphical representation of $\Delta\psi_M$ measurements in different cell lines. Values are normalized to the control normoxic H9C2 and expressed as mean \pm SEM from 3 independent experiments, where * \cdot #P<0.05; ##P<0.01; where the comparisons between FXN-OE and H9C2 cells in each time point are represented by #, while the comparisons between FXN-KD and H9C2 cells at every time point are represented by *.

A



B

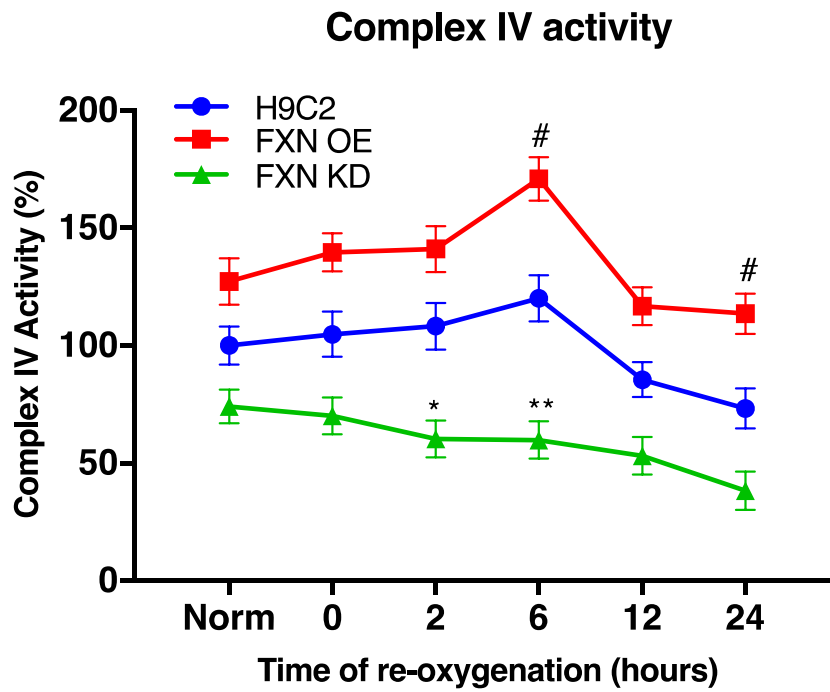
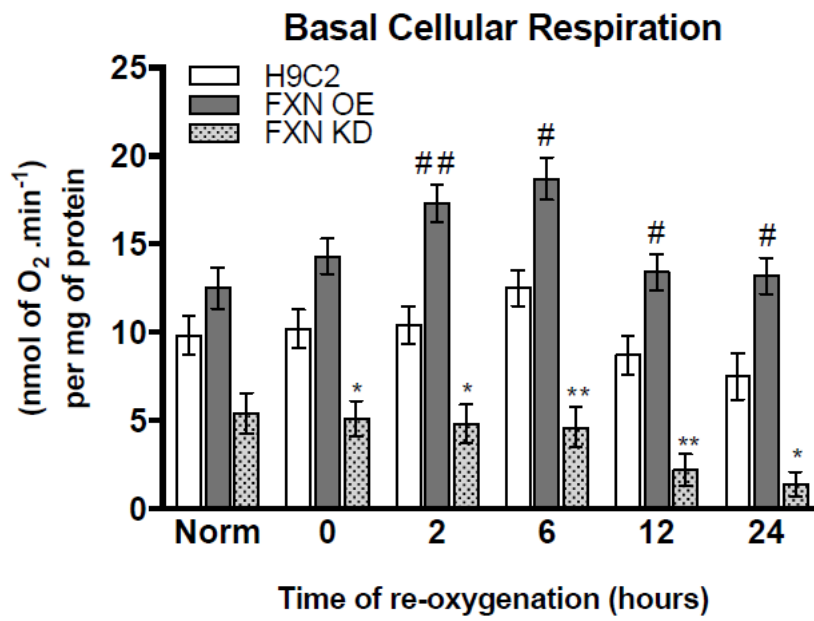


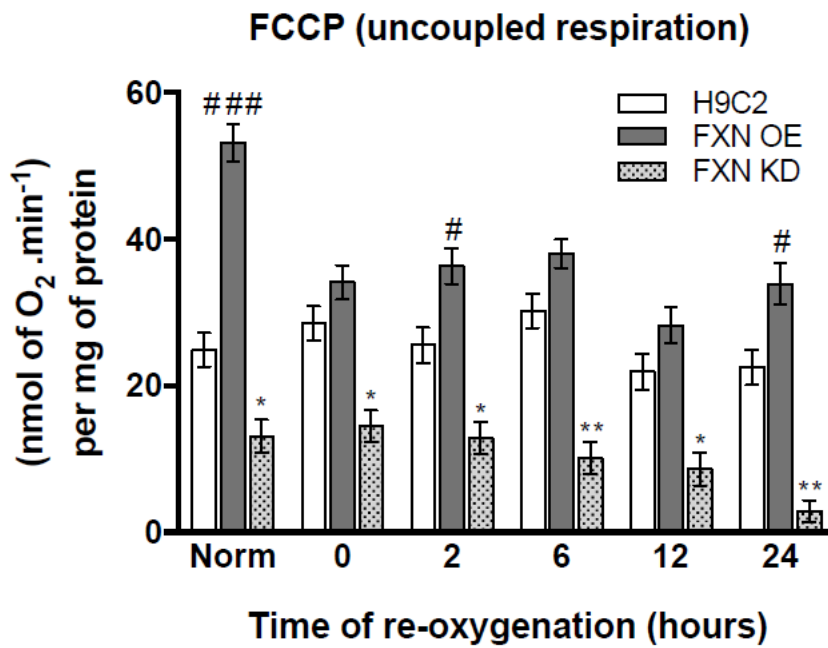
Figure 3.5. Attenuation of FXN expression reduces the enzymatic activity of the ETC. (A)

Percentage of complex I activity in H9C2, FXN-OE and FXN-KD cardiomyocytes. Cells introduced to either normoxia (20% O₂) or 18 hours of hypoxia (1% O₂) and then to increasing time of re-oxygenation. Values were standardized to total protein concentrations and are represented as percentage change from the control normoxic H9C2 cells. (B) Graphical representation of percentage of complex IV activity in different cell lines. Values are normalized to the control normoxic (Norm) H9C2 and represented as mean ± SEM from 3 independent experiments, where *[#]P<0.05; **P<0.01. Comparisons between FXN-OE and H9C2 cells in each time point are represented by #, while the comparisons between FXN-KD and H9C2 cells at every time point are represented by *.

A



B



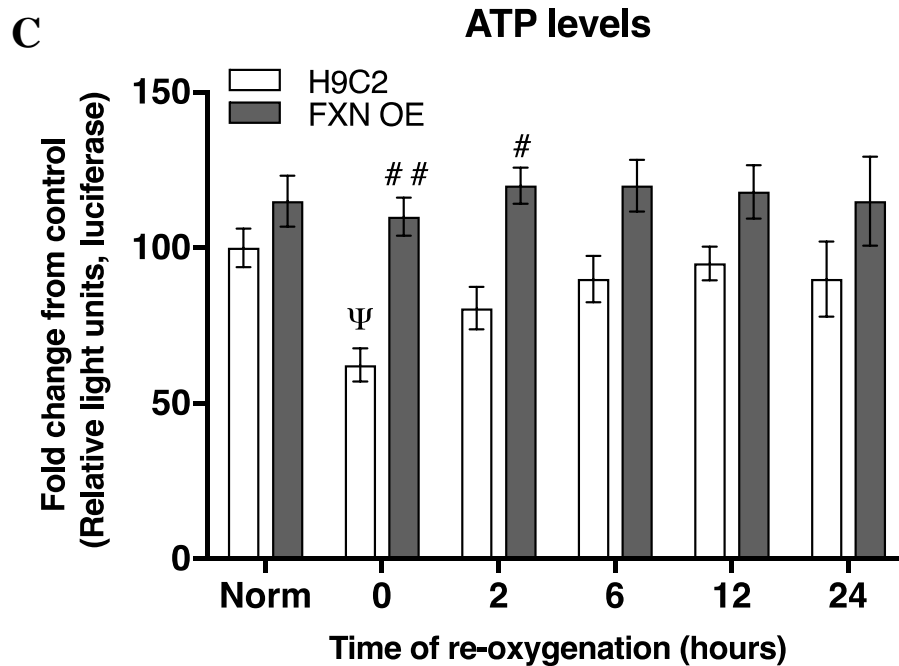
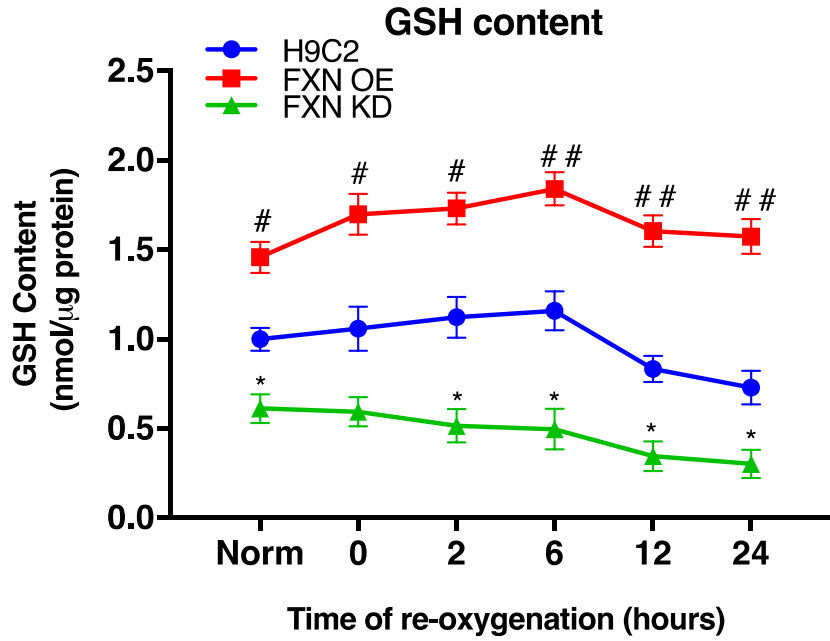
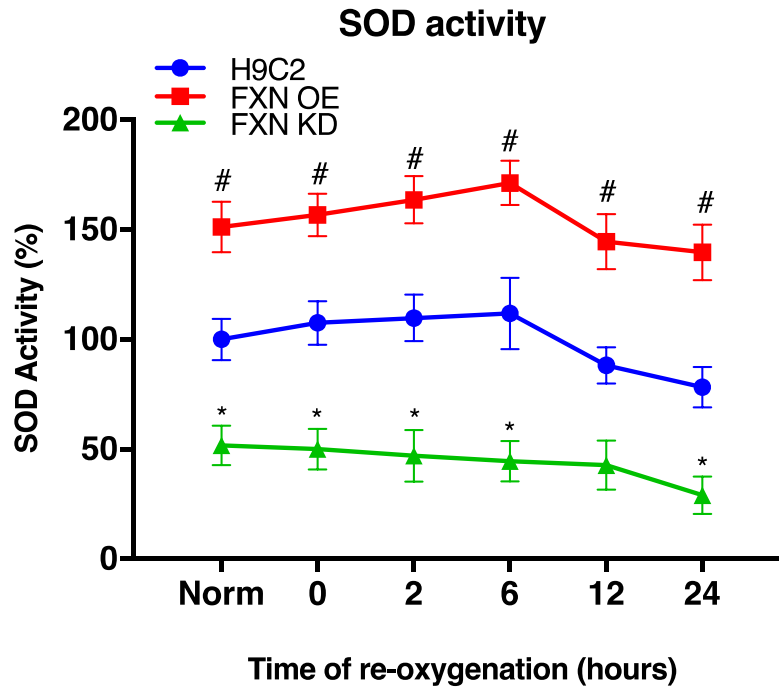


Figure 3.6. FXN deficient cells alter the mitochondrial bioenergetics. (A) Basal cellular respiration levels in H9C2, FXN-OE and FXN-KD cells that were exposed to normoxia (Norm) or hypoxia followed by re-oxygenation. (B) Cellular respiration of different cell lines after the addition of FCCP (mitochondrial un-coupler). Values are normalized to the protein concentration. (C) ATP levels in H9C2 and FXN-OE cardiomyocytes that experienced normoxia (Norm) or hypoxia for 18 hours followed by different times of re-oxygenation. Values are normalized to control normoxic H9C2 cells and expressed as mean \pm SEM from 3 independent experiments, where \ast , #, ψ $P < 0.05$; $\ast\ast$, $\ast\ast\ast$ $P < 0.01$; $\ast\ast\ast$ $P < 0.001$. Comparisons to control H9C2 cells under normoxic conditions are represented by ψ . Comparisons between FXN-OE and H9C2 cells in each time point are represented by #, while the comparisons between FXN-KD and H9C2 cells at every time point are represented by \ast .

A



B



C

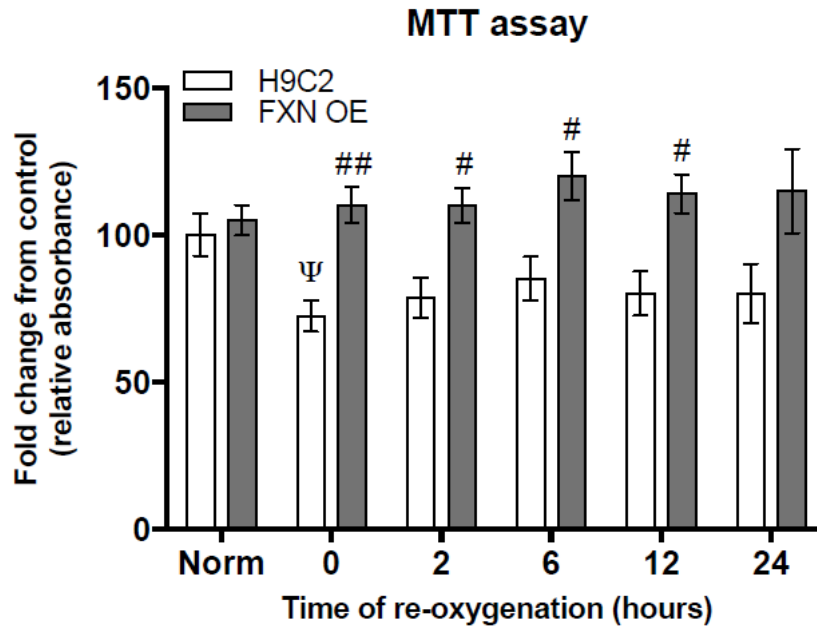


Figure 3.7. FXN over-expression increases GSH content and SOD activity. (A) Total glutathione (GSH) levels in H9C2, FXN-OE and FXN-KD cells. Both FXN-OE and H9C2 cells display elevated glutathione levels in response to hypoxia and re-oxygenation for 6 hours. Values are normalized to the protein concentration. (B) Graphical representation of percentage change in superoxide dismutase (SOD) activity in different cell lines in response to hypoxia/ re-oxygenation stress. (C) Results from MTT assays revealed that FXN-OE cells retained cell viability when they were introduced to 18 hours of hypoxia and increasing times of re-oxygenation when compared to H9C2 cells. Values are normalized to the control normoxic H9C2 and represented as mean \pm SEM from 3 independent experiments, where *, #, ψ $P < 0.05$; ## $P < 0.01$. Comparisons to control H9C2 cells under normoxic conditions are represented by ψ . Comparisons between FXN-OE and H9C2 cells in each time point are represented by #, while the comparisons between FXN-KD and H9C2 cells at every time point are represented by *.

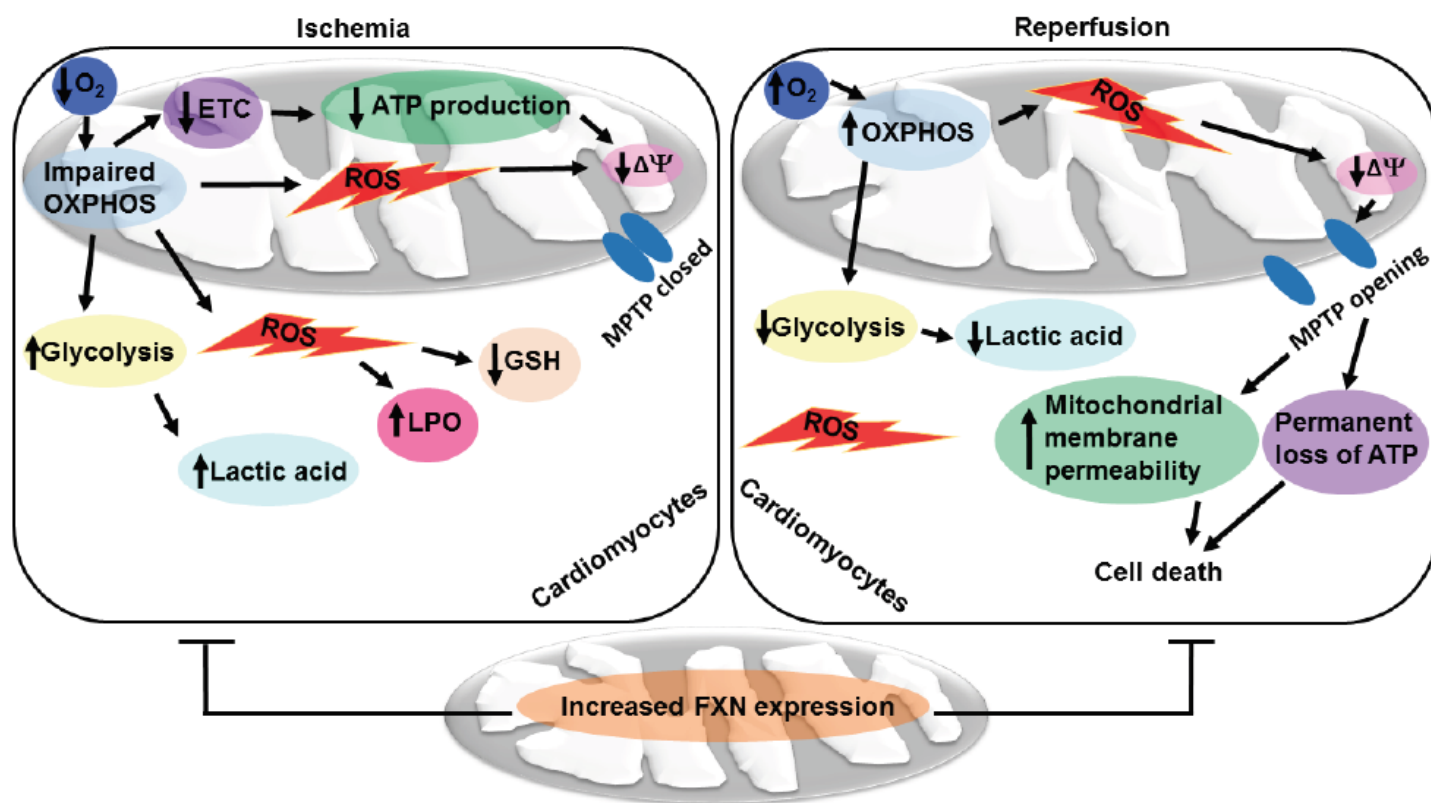


Figure 3.8. Schematic model for the cardioprotective role of FXN against IR injury. During ischemia, oxygen absence shifts cardiomyocyte cellular energy flux from OXPHOS to anaerobic glycolysis, resulting in decreased adenosine triphosphate (ATP) levels. As a consequence of increased glycolysis, lactic acid production increases. The elevation of ROS levels during ischemic injury results in increased lipid peroxidation (LPO) and the ensuing depletion of glutathione (GSH) levels (Tanoue *et al.*, 1996; Paradies *et al.*, 1999; Anderson *et al.*, 2012). During reperfusion, however, the increases in ROS lead to a decrease in the mitochondrial membrane potential ($\Delta\Psi_M$) and increase in the mitochondrial permeability transition pore (MPTP) activity. The resulting attenuated ATP levels and increase in the mitochondrial membrane permeability leads to pronounced mitochondrial damage and the eventual cell death. Our findings indicate that, increased expression of FXN serves as a protective mechanism for cardiomyocytes

against IR injury by preventing ROS production, LPO accumulation, mitochondrial membrane potential decline, ATP depletion and MPTP opening. Furthermore, increased expression of FXN helps maintain the anti-oxidant potential by improving GSH and increase the activity of superoxide dismutase (SOD). Further, we propose that the increase in FXN expression protects the cardiomyocyte during reperfusion injury as we observed in our MTT assay.

CHAPTER 4:

Uridine Triphosphate (UTP) Induces ERK1/2 Activation in Cardiomyocytes by the Activation of new P2Y-like Receptors

4.1. Abstract:

It is evident that cardiomyocytes express different types of P2 purinergic receptors. P2 receptors, which can be divided into P2X (ligand gated ion channels) and P2Y (G-protein coupled receptors) receptors, are known to be activated by extracellular nucleotides. Uridine triphosphates (UTP) is an extracellular nucleotide that is known to activate specific P2Y G-protein coupled receptors, including P2Y2, P2Y4 and P2Y6, in most cells to mediate numerous biological functions. It has been shown that UTP induced cardioprotection against ischemia/reperfusion (IR) injury and that protection was reported to be induced via P2Y2 and/or P2Y4 receptors. However, the exact mechanism how UTP is cardioprotective against hypoxic and/or IR injury conditions is still to be elucidated. In this project, therefore, we will examine the expression of P2Y2 receptors in cardiomyocytes and we will test the hypothesis that activation of P2Y2 receptors in cardiomyocytes by UTP is cardioprotective through the activation of mitogen activated protein kinases (MAPKs) signaling pathway. In the current study, we found that P2Y2 receptors are highly expressed in H9C2 cardiomyocytes as validated by gene analysis studies.

Although P2Y2 receptors are highly expressed in these cells, extracellular nucleotides (UTP and ATP) pre-treatments could not induce any changes in the intracellular calcium mobilization, suggesting that these receptors are not Gq receptors that coupled to the activation of PLC (phospholipase C). Unexpectedly, we found that UTP significantly induced the phosphorylation of ERK1/2 MAPK in a time-dependent and dose-dependent manner. Additionally, UTP triggered minimal but insignificant activation of p-p38 and p-JNK and had no effect on p-Akt. Surprisingly, ARC (P2Y2 selective inhibitor), MRS2578 (P2Y6 selective inhibitor) and PTX (Gi-protein coupled receptors inhibitor) compounds did not abolish the induction of p-ERK1/2 by UTP, proposing that these receptors are not P2Y2, P2Y4 or P2Y6 receptors and possibly these are new P2Y-like receptors. In addition to that, we provide evidence that activation of these receptors by UTP increased the cell viability of cardiomyocytes; therefore, activation of these receptors could be a potential cardioprotective mechanism, especially under stress conditions.

4.2. Introduction:

Extracellular nucleotides, purines (ex. ATP) and pyrimidines (ex. UTP), are complex molecules that are essential for numerous biological functions and signaling mechanisms, such as endo and exocrine secretion, neurotransmission, smooth muscle contraction, vasoconstriction and modulation of cardiac function⁴⁸⁵. It is widely accepted that both purines and pyrimidines are exerting their effect through the binding to purinergic receptors. In 1929, Drury and Szent-Györgyi reported, for the first time, the signaling mechanisms of purines. They reported that adenosine and AMP have biological actions on the heart, namely lowering blood pressure, heart block and arterial dilatation⁴⁸⁶. Later on (in 1976), Burstock and his colleagues classified purinergic receptors into P1 and P2, where P2 can be further divided into P2X and P2Y based on the molecular structure and the pharmacological properties⁴⁹¹. Currently, there are 8 different types of P2Y receptors and 7 different types of P2X receptors^{485,493-495}.

P2Y receptors have high sensitivity and binding ability to both purines (ATP and ADP) and pyrimidines (UTP and UDP)^{496,497}. Many of P2Y receptors are G-protein coupled receptors that stimulate PLC (phospholipase C) and subsequently produce IP3 (inositol triphosphate). Consequently, MAPK (mitogen activated protein kinase) signaling pathway, PKC (protein kinase C) signaling pathway and other signaling pathways will be activated⁴⁹⁶. However, some of P2Y receptors are G-protein receptors that are coupled to Gi which will lead to the inhibition of AC (Adenylate cyclase) and that can lead to decrease intracellular level of cAMP. P2Y2 and

P2Y4 receptors are activated by UTP, while P2Y6 is activated by UDP and P2Y11 and P2Y14 are activated by ATP and UDP-glucose, respectively.

It has been shown that UTP induces positive inotropic effects on the heart and that is mediated by P2Y receptors. Furthermore, it has been found in growing rats that both P2Y2 and P2Y4 receptors are regulating the contractility of the heart ^{516,517}. Of all P2Y receptors, P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 were found to be expressed in neonatal rat cardiomyocytes and they are producing their effect through G-protein coupled receptors coupled to the activation of PLC (Gq) ⁵¹⁸. In the heart, ATP and UTP are released in variable amounts (10-40 nM) and these levels are considerably elevated under stress conditions, such as hypoxic and/or ischemic conditions (IR).

It is well known that cells can respond coordinately to different extracellular stimuli by activating specific signaling cascades and intracellular programs, such as mitogen activated protein kinases (MAPKs). By engaging and activating MAPKs, cells can regulate and control embryogenesis, cell proliferation, differentiation and death, gene expression, metabolism and survival ⁵⁵³⁻⁵⁵⁵. MAPKs family, which are serine-threonine kinases, include extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNKs), p-38, ERKs 3 and 4, and ERK5 ^{553,554}. In the heart, three of these MAPKs have been studied under stress conditions, such as IR injury, and these MAPKs are ERK1/2, p-38 and JNKs ⁵⁵⁶⁻⁵⁵⁸. These studies have demonstrated that activation of p-38 and JNKs enhanced apoptosis, while activation and phosphorylation of ERK1/2 induced cardioprotection against IR injury ^{556,559}. Furthermore, it has been reported that inhibition of ERK1/2 through the inhibition of its phosphorylation state significantly enhanced apoptosis in response to IR injury ⁵⁶⁰⁻⁵⁶³. Therefore, enhancing this

signaling pathway in the heart by increasing its phosphorylation activity, especially under stress conditions (IR injury), could be a promising therapeutic strategy.

Conflicting studies have been published recently regarding the role of UTP on cardiomyocytes. Erlinge and his colleagues have found that UTP is released during myocardial ischemia and consequently it induces arrhythmia in response to ischemia (Erlinge et al. 2005). Conversely, UTP was reported to protect cardiomyocytes against the ischemic injury by restoring the electron flow in ETC complexes, restoring the heart function, restoring mitochondrial membrane potential and preventing the ATP loss^{521-525,564}. However, none of these studies provided evidences to explain the exact mechanism how UTP offered cardioprotection effect against hypoxic and/or IR injury conditions (in vitro and in vivo, respectively). Hence, there is a necessity to investigate the role of extracellular nucleotides (ATP and UTP) and purinergic receptors (P2Y) on cardiomyocytes to reveal the precise mechanism that is offered by these nucleotides. In this project, therefore, we will examine the expression of P2Y2 receptors in cardiomyocytes and we will test the hypothesis that activation of P2Y2 receptors in cardiomyocytes by UTP is cardioprotective through the activation of ERK signaling pathway. In the current study, we found that P2Y2 receptors are highly expressed in our H9C2 cardiomyocytes as validated by gene analysis studies. Although P2Y2 receptors are highly expressed in these cells, extracellular nucleotides (UTP and ATP) pretreatments could not induce any changes in the calcium signaling (intracellular calcium mobilization) suggesting that these receptors are not Gq receptors that coupled to the activation of PLC (P2Y2 receptors are Gq receptors). Unexpectedly, we found that UTP significantly induced the phosphorylation of ERK1/2 MAPK in a time-dependent and dose-dependent manner. Additionally, UTP triggered minimal activation of p-p38 and p-JNK and had no effect on p-Akt. Surprisingly, P2Y2 and

P2Y6 selective inhibitors did not abolish the induction of p-ERK1/2 by UTP, proposing that these receptors are not P2Y2 nor P2Y6 receptors and possibly these are new P2Y-like receptors. In addition to that, we provide evidence that activation of these new P2Y-like receptors by UTP increased the cell viability of cardiomyocytes; therefore, activation of these receptors can be a potential cardioprotective mechanism especially under stress conditions, such as hypoxia/ischemia.

4.3. Materials and Methods:

4.3.1. Chemicals and reagents:

Adenine-5'-Triphosphate (ATP) and Uridine-5'-Triphosphate (UTP) were obtained from Sigma-Aldrich (Cat# A7699 and U6625, respectively), while Uridine-5'-Diphosphate (UDP) was obtained from Alfa Aesar company (Cat# 27821). Positive control compound for calcium signaling assay (A-23187) was purchased from Thermo-Scientific Fisher (Cat# A1493). SYBR green master mix for qRT-PCR was obtained from Biotool Company (Cat # B21202). ARC-118925XX, ATP- γ -S and UTP- γ -S, MRS-2578 and suramin hexasodium salt were purchased from Tocris Company (Cat # 4890, #4080, #3279, #2146 and #1472, respectively), while Pertussis Toxin (PTX) was obtained from Enzo Life Sciences (Cat#70323).

4.3.2. Cell Lines and Cell Culture:

H9C2 ventricular embryonic rat cardiomyocytes were obtained from ATCC (Cat# CRL-1446). According to the provider's instructions, these cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS, HyClone from Fisher Scientific) and 1% Penicillin-Streptomycin solution (P/S, HyClone from Fisher Scientific). Cells were maintained at 37°C, 5% CO₂ levels with humidity. These cells were passaged for at least three times before it was used in the experiments. To seed cells for different experiments, cells were harvested using 3ml of trypsin (HyClone from Fisher Scientific) and then incubated for about 5 minutes at 37°C, 5% CO₂ levels with humidity and then collected in

complete DMEM. After centrifugation at approximately 1000 rpm for 5 minutes, pellets were re-suspended in complete DMEM and 1ml of cell suspension were cultured in 6-well plate in proper confluency (~70%). The day after, cells were starved by replacing the complete DMEM with starvation media (incomplete media, which is DMEM with 1% P/S and without 10% FBS) for overnight. Drug treatments (either agonists or antagonists) were added at different time points according to the experiment's design. Cells were pretreated with PTX for overnight, while ARC was added 40 minutes before harvesting the cells.

4.3.3. Cells Cryo-preservation:

To cryopreserve H9C2 cells for long time storage, cells were incubated at 37°C and 5% CO₂ levels with humidity until it reached approximately 90% confluency. Then, cells were collected and centrifuged at 1000 rpm for 5 minutes to make a pellet. The formed pellet was then re-suspended in 2ml of the complete DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin solution and 10% (v/v) DMSO (dimethyl sulfoxide), the freezing media, and then transferred to cryovials. The cryovials were kept in an isopropanol freezing container (Thermo-Scientific Cat# 5100-0001) and kept in -80°C for overnight. The day after, the cryovials were transferred into liquid nitrogen tank (-196°C) for long storage.

4.3.4. Western blot:

To perform the western blot experiemnts, different chemicals and reagents were used and these chemicals are listed in table 4.1 below.

Table 4.1 Chemicals and Reagents used in Western Blot Assay

Reagent	Company	Catalog Number
10x Tris/Glycine/SDS running buffer	Bio-Rad	161-0732
10x Tris/Glycine transfer buffer	G-Biosciences	786-478
10x Phosphate Buffer Saline (PBS)	Amresco	J373
10x Tris-Buffered Saline (TBS)	Alfa Aesar	J62938
Blotting non-fat dry milk	Bio-Rad	170-5016
bovine serum albumin	VWR	0332
western blotting Lightning [®] Plus-ECL	Perkin-Elmer	NEL122001EA
2x Laemmli buffer	Thermo-Scientific Fisher	161-0374
Precision Plus Protein™ Dual Color Standards	Bio-Rad	161-0374

All the 10x solutions were diluted to 1x solution using Milli-Q-ultrapure water before each experiment. TBST buffer was made by diluting the 10x TBS buffer into 1x and then mixed it with 0.1% Tween20. To block the membrane, 5% non-fat dry milk (blocking buffer) diluted in TBST was used. Primary antibodies were dissolved in 5% BSA in TBST buffer.

H9C2 cells were seeded in 6-well plate (in 2ml DMEM) for one day or until it reaches ~ 85% confluency. Then, cells were subjected to starvation for overnight. In some experiments when PTX compound was used, cells were pretreated with 100ng/ml at the same time of replacing the media to starvation media and kept for overnight. ARC compound (3 μ M), MRS compound (3 μ M) and suramin (100 μ M) were added in some experiments 40 minutes before harvesting cells. ATP, UTP and UDP, UTP- γ -S and ATP- γ -S were added 10 minutes before harvesting the cells, unless otherwise specified. To harvest the cells and collect the proteins, the 6-well plated were immersed in ice-cold PBS for about 5 times and then cells were lysed with 300 μ l of 2x laemmli buffer and scrapped using rubber policeman scrappers. After that, lysates were collected

in 1.5ml centrifuge tubes and heated in boiling water for 5 minutes and then kept at -20°C for further use. The protein lysates were resolved in 10% Mini-Protean[®] TGX stain free gels (Bio-Rad Cat# 4568033) with the standard protein marker (Bio-Rad Cat# 1610374) was used as a molecular weight marker. Gels were transferred to polyvinylidene fluoride (PVDF) membranes using a semi-dry transfer apparatus (Bio-Rad) for 30 minutes. Membranes were blocked in 5% non-fat dry milk for 1 hour and then washed with TBST for 4 cycles each for 10 minutes and then incubated at 4°C for overnight with the following primary antibodies: p-ERK, p-JNK, p-p38, p-Akt, t-ERK, t-JNK, t-p38 and t-Akt. The dilution of all the primary antibodies were (1:2000) and all of the antibodies obtained from cell signaling company. The day after, membranes were washed with TBST for 4 cycles each for 10 minutes and then incubated with the secondary anti-rabbit antibody (HRP, Cell Signal) for 1 hour and then washed with TBST for 4 cycles each for 10 minutes. After that, the conjugated proteins were detected by incubating the membrane with the ECL reagent (Perkin-Elmer) for 1 minute and image was developed on a classic X-ray film (Research Products International corp.) and visualized by using medical film processor machine (Konica Minolta Medical and Graphic Inc.). Stripping off the proteins for further incubation with other antibodies was obtained by washing the membrane briefly with TBST and then incubating the membrane with stripping buffer (Thermo-Scientific) for 20-25 minutes. Then, the membrane was washed with TBST for 1 minute and then re-blocked with 5% non-fat dry milk in TBST for 1 hour and finally incubated with the new primary antibody for overnight.

4.3.5. qRT-PCR analysis:

H9C2 cells were cultured in 6-well plates until it reached the optimal confluency. Total RNA and total DNA were then extracted using RNeasy and DNeasy assay kits (Qiagen), respectively. 1µg of the total isolated RNA was used to synthesize cDNA using TaqMan reverse transcription reagents kit (Applied Biosystems) according to the manufacturer's protocol. Then, PCR technique was used to amplify the synthesized cDNA in the presence of SYBR Green Master Mix (Biotool), reverse and forward primers. All pairs of primers that were used are listed in table 4.2.

Table 4.2 List of Forward and Reverse Primers for different genes that used in qRT-PCR Analysis

Gene	Forward Primer	Reverse Primer
P2Y2 (rat)	5'-ATGGCAGCAGGCCTGGACTCC-3'	5'-CTATAGACGAATGTCCTTAGTCTC-3'
P2Y2 (rat)	5'-CTGAGCATCCAAGAGCACCT-3'	5'-AGGCTGCGTAGAGAGAGTCA-3'

The PCR reaction conditions are: heat activation step at 95°C for 5 minutes followed by 40 cycles of denaturation step at 95°C for 1 minute, annealing step at 60°C for 1 minute and extension step at 72°C for 2 minutes. The resulted PCR products were resolved in 1% agarose gel that contains 1% ethidium bromide. The gel was visualized using a BioRad gel dock system.

4.3.6. Calcium Signaling Assay:

FLUOFORTE® Calcium Assay Kit from Enzo (Cat# ENZ-51016) was used to conduct calcium signaling assay. By measuring the fluorescence, this calcium assay kit detects the intracellular calcium mobilization to give an indication about stimulation (agonists) and inhibition (antagonists) of calcium signal transduction (calcium signaling). To perform the assay, 100µl of cell suspension were seeded in 96-well black plates at 80% confluency for overnight.

The day after, media was replaced to starvation media for overnight. On the third day, the media was removed and 100µl of FLUOFORTE loading dye (includes buffer A, B and C) was added to each well and kept at 37°C for 45 minutes. After the total incubation of the plate at 37°C (45 minutes) the plate was kept in room temperature in dark place for another 15 minutes. After that and according to the experimental design, 20µl of different drug treatments with different concentrations were added (UTP 10, 100 and 300µM, ATP 10 and 100µM, UTP-γ-S 10µM, ATP-γ-S 10µM, Endothelin-1 (ET-1) 1 and 10µM, Angiotensin-II (Ang-II) 1 and 10µM and positive control A-23187 10µM). The fluorescence was measured at Ex=490nm and Em=525nm for 9 minutes.

4.3.7. Cell Viability assay:

To measure the cardiomyocytes cell viability after UTP treatments, we used cell counting kit-8 (CCK-8) from Biotools Company (Cat # B34304) according to the manufacturer's protocol. In general, we seeded the H9C2 cells in 96-well plate for 24 hour. On the day after, we changed the complete media to starvation media and incubated the plates at 37°C for overnight. On the following day, we added different drug treatments, which include UTP 1µM, UTP 10µM, UTP 100µM and 10% FBS (used as positive control) in addition to control cells (HAD NO TREATMENTS) and incubated the plates at 37°C for 48 hours. It should be noted that some wells had only media without cells to be used as blank. After that, we added 10µl from the CCK-8 solution to each well and incubated the plates for 4 hours until the color turned orange and measured the absorbance at 450 nm. The calculations were conducted as per the manufacturer's instructions.

4.3.8. Statistical Analysis:

Data are expressed as mean \pm S.E.M. Results were analyzed by a student two-tailed t-test (comparison between 2 groups) and one-way ANOVA analysis for comparison between 3 groups or more with post hoc analysis were conducted to compare the mean of each group with control group using Dunnett test. The level of significance was set for $p < 0.05$. All statistical calculations were performed on a computer using GraphPad Prism software (version 5.04 for windows, La Jolla, California, USA). All cell culture studies were based upon a minimal of 3 independent experiments, unless otherwise specified.

4.4. Results

4.4.1. Expression of P2Y2 Receptors in H9C2 Cardiomyocytes

It has been reported that different type of P2Y receptors are expressed in the human heart, namely P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 with the expression of P2Y2 is being the highest⁵⁶⁵. Neonatal rat cardiomyocytes, however, express the same type of receptors that are expressed in the human hearts^{518,524}. Based on that and because we are interested in investigating the expression of P2Y2 in our H9C2 cells, we measured the gene expression of these receptors using qRT-PCR technique and then ran the PCR product on 1% agarose gel to detect it (Figure 4.1). Our results indicate that P2Y2 receptors are highly expressed (~1020 bp) in our cells as it is shown in Figure 4.1 and that prove the presences and expression of these receptors.

4.4.2. Activation of P2Y2 Receptors by UTP Did Not Induce Any Changes In The Intracellular Calcium Mobilization (Signaling)

As we mentioned early in section 2.14, P2Y2 receptors are activated by extracellular nucleotides, explicitly UTP and ATP (P2Y2 receptors have higher affinity to UTP than ATP; UTP>ATP). Furthermore, it is reported that P2Y2 receptors are G-protein receptors (GPCRs) that are coupled via Gq/11 and Gi. Upon activation of these receptors by UTP, these receptors will stimulate the activation of PLC, which will breakdown phospholipids and

Phosphatidylinositol 4,5-bisphosphate (PIP₂), leading to the formation of IP₃ and DAG (Diacylglycerol) and that will cause an increase in the intracellular calcium mobilization (signaling) and activation of different signaling pathways, such as MAPK, PKC, PLA₂ and NOS, respectively. Therefore and in order to investigate the activity of P₂Y₂ receptors and whether these receptors are coupled to G_q proteins, we measured the intracellular calcium mobilization after activation of these receptors by UTP and ATP using FLUOFORTE® Calcium Assay Kit. Unexpectedly, UTP did not induce any changes in the calcium signaling in these cells when it compared to both control cells and A23187 compound (10 μM), which is a positive control that is known to induce the calcium mobilization, even with high concentrations of UTP (10, 100 and 300 μM) (figure 4.2A, B and C). Additionally, ATP also had no effect on these receptors as our results indicate that ATP did not cause any changes in the intracellular calcium mobilization (figure 4.3A and B). Because extracellular nucleotides (UTP and ATP) are degraded rapidly to di-phosphates and mono-phosphate forms (UDP, ADP and UMP and AMP)^{511,565-567} and because we would like to investigate whether the degradation of UTP to UDP might be the cause for the absence of calcium signaling that we observed as UDP activates P₂Y₆ receptors (P₂Y₆ receptors are also coupled to G_q proteins), we used enzymatically stable non-degradable forms of UTP and ATP (UTP-γ-S and ATP-γ-S, respectively) in addition to UDP (to investigate the role of P₂Y₆ receptors), endothelin-1 (ET-1) and Angiotensin II (Ang-II), which are known agonists for G_q-protein coupled receptors⁵⁶⁸. Similarly, UTP-γ-S (10 μM), ATP-γ-S (10 μM), UDP (10 μM), ET-1 (10 μM) and Ang-II (10 μM) did not affect the intracellular calcium levels suggesting that these receptors are not coupled to G_q proteins in our H9C2 cells (figure 4.4A and B) and probably these receptors are not P₂Y₂ nor P₂Y₆ receptors.

4.4.3. UTP Induces ERK1/2 Activation in a Time- and Dose-Dependent Manner

Different studies have reported the UTP preconditioning (pre-treatment) can increase ATP formation, membrane potential and decrease apoptosis and that could be cardioprotective^{521,523–525,564}. These reports, nonetheless, did not provide evidence for the exact mechanism of this protective role of UTP. It has been shown that induction and phosphorylation of ERK1/2 is cardioprotective against IR injury^{556,559–561}. Therefore, we hypothesize that the activation of these P2Y receptors in our H9C2 cells by UTP could increase the phosphorylation state of ERK1/2 and other MAPKs, such as c-JNK and p-38. To validate this hypothesis, we pre-treated our cells with 10 and 100 μM of UTP and 10% FBS (used as positive control) for 10 minutes and then collect the protein lysates to run a western blot to investigate the MAPKs signaling pathways. We found a marked increase in the phosphorylation form of ERK1/2 at 10 and 100 μM concentrations of UTP when it compared to control cells (figure 4.5 A & B). Furthermore, we could not observe such a high increase in the phosphorylation states of p-38 and c-JNK MAPKs (figure 4.5 A, D & E). In addition to the MAPKs signaling pathway, p-Akt has been reported to be activated by UTP and that can induce cardioprotection against hypoxic conditions⁵⁶⁹. Our results indicate that there is no change in the p-Akt after UTP stimulation for 10 minutes and even with high concentration of UTP (100 μM) (figure 4.5 A & C).

Based on these results, we investigated the increase in p-ERK1/2 after UTP stimulation. We found that UTP pre-treatment increased the p-ERK1/2 in a time-dependent fashion as it increased immediately within 5 minutes of UTP stimulation and peaked after 10 minutes and gradually decreased to basal levels after 60 minutes of stimulation (figure 4.6 A & B). Furthermore, UTP pre-treatment dose-dependently induced the p-ERK1/2 when it compared to

control cells (figure 4.6 C & D).

To examine that the induction of p-ERK1/2 is mediated by UTP not by UDP, we used the enzymatically stable non-degradable forms of UTP and ATP (UTP- γ -S and ATP- γ -S, respectively). To do so, we treated our cells with UTP, UDP, UTP- γ -S and ATP- γ -S (10 μ M) for 10 minutes and then detect the p-ERK1/2. We found that all the drug treatments significantly induced p-ERK1/2 (figure 4.6 E & F) giving an indication that these receptors could be P2Y2, P2Y6 or new P2Y-like receptors as these receptors are induced by UTP and UDP but are coupled via Gq- proteins.

4.4.4. UTP-Induced ERK Phosphorylation is Not Mediated by P2Y2 Nor P2Y6 Receptors

Our results revealed that these receptors are activated by UTP, ATP and UDP and that led to the activation of p-ERK1/2. However, activation of these receptors did not induce any changes in the intracellular calcium mobilization. Different reports have reported that both P2Y2 and P2Y4 receptors are G-protein receptors that are coupled via both Gq and Gi^{495,570}. Therefore, we investigated whether these receptors are coupled to Gi or not in order to include or exclude P2Y2 and P2Y4 receptors. To validate that, a known Gi-protein inhibitor, PTX (Pertussis Toxins), was used and we activated the receptors using UTP and found that this inhibitor, PTX, did not inhibit the increase in the p-ERK1/2 that induced by UTP (figure 4.7 A & B). These results in addition to our results from calcium signaling assay proved that these receptors are not coupled to Gq and Gi-proteins and that strongly suggest these receptors are not P2Y2 nor P2Y4 receptors (figure 4.7 A & B). To further validate our results, selective P2Y2 inhibitor (ARC compound) was used. Similarly, ARC compound (3 μ M) did not abolish the activation of p-ERK1/2 that is induced by UTP (figure 4.7 C-F). Therefore, we concluded that these receptors neither P2Y2 nor P2Y4

receptors.

To further validate the role of P2Y6 receptors and whether these receptors are P2Y6, we used a selective P2Y6 antagonist (MRS compound). Surprisingly, MRS compound (3 μM) significantly induced p-ERK1/2 and that induction in the p-ERK1/2 was further increased when cells pre-treated with UTP and UDP (10 μM) (figure 4.8 A & B). Suramin, which is a non-selective antagonist of P2Y receptors and a known inhibitor of G-protein coupled receptor, was used. As we expected, Suramin (100 μM) completely inhibit the activation of p-ERK1/2 even when cells stimulated with UTP and UDP (10 μM) (figure 4.8 C & D).

Together, these results clearly indicate that these receptors are not P2Y2, P2Y4 nor P2Y6 receptors. Furthermore, our results revealed that these receptors are G-protein coupled receptors as validate by the use of Suramin. Finally and most interestingly, we think H9C2 cells express new P2Y-like receptors as they are induced by UTP, ATP and UDP but are not coupled to Gq nor Gi and not inhibited by P2Y2 and P2Y6 inhibitors, but inhibited by suramin.

4.4.5. Effect of UTP on H9C2 Cardiomyocytes Viability

To examine the role of UTP on these P2Y-like receptors and to examine if the activation of p-ERK1/2 by UTP can induce cardioprotection by increasing the viable cells, we conducted cell viability assay using CCK-8 kit. We found that UTP pre-conditioning (1, 10 and 100 μM) protected the cardiomyocytes and significantly increased the cardiac cells viability when it compared to control cells (figure 4.9). These results revealed a new cardioprotective mechanism mediated by the activation of new P2Y-like receptors by UTP and that could be of paramount importance especially under stress conditions like IR injury in the heart.

4.5. Discussion

In this study, we report, for the first time, that embryonic rat cardiomyocytes cell lines (H9C2 cell line) express new P2Y-like receptors as validated by our calcium signaling assay and western analysis. Indeed, we demonstrated that activation of these receptors by extracellular nucleotides (UTP) induced activation of p-ERK1/2 in a dose- and time-dependent fashion and this response was not diminished by P2Y2 and P2Y6 selective inhibitors. Consequently, this led to the increase of cell viability of H9C2.

Purinergic receptors have been divided into P1-purinoceptors (the natural ligand is adenosine) and P2-purinoceptors (where ATP and UTP are the natural ligands), where P2 was further divided into P2X and P2Y based on the molecular structure and the pharmacological properties. Currently, there are 8 different types of P2Y receptors and 7 different types of P2X receptors^{488,490,491}. P2Y receptors are G-protein coupled receptors (GPCRs) with the intracellular signaling transduction or cascades are the main mechanism of communication with the regulatory targets in cells. It has been reported that embryonic rat cardiomyocytes (E12) had no response to P2Y agonists and it did not induce any changes in the calcium mobilization. However, embryonic rat cardiomyocytes (E14 to E18) had response to P2Y agonists and accordingly it induced the calcium mobilization⁵⁷¹. Measuring mRNA transcripts of different P2Y receptors, Webb and his colleague have reported that P2Y1, P2Y2, P2Y4 and P2Y6 receptors are expressed in neonatal rat cardiomyocytes. However, P2Y4 mRNA transcripts could not be detected and were not expressed in adult rat cardiomyocytes, giving an indication that

there are active changes in the expression of different P2Y receptors during the heart development⁵⁷¹. Similarly, P2Y1, P2Y2 and P2Y6 were expressed in the adult human hearts with P2Y4 expression were not detected and P2Y2 receptors were the most abundant receptors⁵⁶⁵. In this study, our results demonstrate that H9C2 cells (E13) highly express P2Y2 receptors as validated by mRNA analysis.

It was shown that UTP effects on the heart are mediated mainly via P2Y receptors⁵⁷². Up to date, UTP is shown to selectively act on and activate P2Y2, P2Y4 and P2Y6, where P2Y6 has high affinity and response to UDP than UTP (UDP > UTP)⁵⁷². Numerous studies have reported that P2Y2 and P2Y4 receptors have high affinity and response to UTP than ATP⁵⁷³. Furthermore, P2Y2 and P2Y4 are G-protein receptors that are coupled via Gq and possibly via Gi⁴⁹⁵. Therefore, to examine if the high expression of P2Y2 receptors we found in our cells are coupled to Gq, we carried out calcium mobilization assay. As we mentioned early in the results section, we could not observe any changes in the calcium signaling even with high concentrations of ATP and UTP. As UTP is rapidly degraded to UDP, which can activate P2Y6 receptors, making our finding of calcium mobilization regarding P2Y2 receptor's inaccurate and making receptor characterization difficult, we used stable and non-degradable form of UTP and ATP (UTP- γ -S and ATP- γ -S, respectively). Similar to what we found early, UTP- γ -S and ATP- γ -S in addition to UDP, ET-1 and Ang-II did not cause in up-regulation in the intracellular calcium levels. Et-1 and Ang-II are known agonists for Gq-protein coupled receptors and known to induce hypertrophy in H9C2 cells and neonatal rat ventricular myocytes^{550,568}. Therefore, these results give an indication that these receptors, which are activated by UTP and UDP, are not coupled to Gq.

It's well known that cells can respond coordinately to different extracellular stimuli by activating specific signaling cascades and intracellular programs, such as mitogen activated protein kinases (MAPKs). By engaging and activating MAPKs, cells can regulate and control embryogenesis, cell proliferation, differentiation and death, gene expression, metabolism and survival ⁵⁵³⁻⁵⁵⁵. MAPKs family, which are serine-threonine kinases, include extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNKs), p-38, ERKs 3 and 4, and ERK5 ^{553,554}. In the heart, three of these MAPKs have been studied under stress conditions, such as IR injury, and these MAPKs are ERK1/2, p-38 and JNKs ⁵⁵⁶⁻⁵⁵⁸. These studies have demonstrated that activation of p-38 and JNKs enhanced apoptosis, while activation and phosphorylation of ERK1/2 induced cardioprotection against IR injury ^{556,559}. Furthermore, it has been reported that inhibition of ERK1/2 through the inhibition of its phosphorylation state significantly enhanced apoptosis in response to IR injury ⁵⁶⁰⁻⁵⁶³. Therefore, we investigated whether the induction of these receptors by UTP can induce activation of MAPKs. In this study, we show that UTP pre-treatment induced activation of p-ERK1/2 in a time-dependent and dose-dependent manner. Furthermore, UTP treatment (10 and 100 μ M) induced a slight but not significant increase in p-38 and p-JNK, while it had no effect on p-Akt as we observed. To determine if this response is mediated by P2Y2, P2Y4 or P2Y6, we used UTP- γ -S, ATP- γ -S and UDP and we found that all drug treatments in addition to UTP induced the p-ERK1/2 to a similar level.

Because different reports have demonstrated that P2Y2 receptors could be coupled via Gi-proteins not Gq-proteins and that lead to the inhibition of cAMP, we thought that these receptors in our cells could be P2Y2 receptors that are coupled via Gi not Gq ^{495,574}. It has been found that rat adrenal medulla cells (PC12) express P2Y2 receptors that are coupled to Gi and

upon UTP activation, they observed increase in p-ERK1/2. Furthermore, they found that Gi-protein receptors are sensitive to PTX (pertussis toxins) and this compound inhibits Gi-protein and that led to the inhibition of the activation of MAPKs that were activated by UTP and ATP⁵⁷⁴. Therefore, we investigated the coupling of P2Y2 receptors to Gi using Gi-protein inhibitor compound called PTX (Pertussis Toxins), which is shown to inhibit Gi^{568,574}. We report that PTX (100 ng) could not abolish the activation in the p-ERK1/2 that we observed after UTP treatment, suggesting that the involvement of P2Y2 and P2Y4 receptors are unlikely. To further confirm our findings, we used P2Y2 selective inhibitor compound, ARC (3 μ M) and stimulated our cells using UTP and found that ARC compound did not diminish the p-ERK1/2 activation induced by UTP. These results together made us to exclude the involvement of P2Y2 and to less extent P2Y4 receptors and the response that we found (increase in p-ERK1/2) may be induced by P2Y6 or new P2Y-like receptors.

To study the contribution of P2Y6 receptors, we used selective P2Y6 antagonist (MRS2578) in addition to suramin, which is a non-selective inhibitor of P2Y receptors and a known inhibitor of G-protein coupled receptor. Surprisingly, we found that MRS2578 compound induced p-ERK1/2 not inhibiting it and this induction was further increased when cells stimulated with UTP. One explanation for the increase that we observed in p-ERK1/2 instead of the inhibition is that MRS2578 compound might be a biased agonist of these new P2Y-like receptors independent of its antagonizing effect on P2Y6 receptors. Recently, it has been reported that agouti-related peptide (AgRP), which is an antagonist of neural melanocortin receptors (MCRs), can induce biased signaling on MC4R as it activates p-ERK1/2, p-Akt and p-AMPK suggesting that AgRP is a biased agonist of MC4R independent of its antagonizing effect⁵⁷⁵⁻⁵⁷⁷. As we expected, suramin completely blocked the response even with UTP

treatment. Several factors have led us to hypothesize that these cells express a new P2Y-like receptor. These factors are; 1) both P2Y2 and P2Y4 receptors are coupled via Gq and possibly via Gi and our results showed that the receptors that are expressed in our cells are not coupled to Gq nor Gi-proteins, so the presence of P2Y2 or P2Y4 is less likely, 2) both P2Y2 and P2Y4 are selectively activated by UTP and UTP- γ -S, not UDP and we found a significant increase in the p-ERK1/2 with UDP and that also led us exclude the P2Y2 and P2Y4, 3) selective antagonist for P2Y2 receptors could not prevent the induction of p-ERK1/2, which strongly support our hypothesis, 4) we excluded the role of both P2Y1 and P2Y11 even though these receptors are reported to be expressed in rat hearts because these receptors are activated by ATP and ADP not by UTP and the response we found was mediated by UTP and UDP not ATP, 5) selective P2Y6 antagonist did not inhibit the increase in the phosphorylation of ERK1/2, rather it induced it significantly, which is an unexpected results that we found and because of that we excluded the role of P2Y6 receptors, 6) the data from suramin experiment revealed that these receptors are G-protein coupled receptors as the response was inhibited when cells treated with suramin and that led us to further stick to the hypothesis that these receptors are new P2Y-like receptors because these receptors are activated by extracellular nucleotides, explicitly UTP and UDP and are g-protein coupled receptors and these characterizations are the exact characterization of P2Y receptors and 7) because all reports, up to date, have revealed that adult rat and human cardiomyocytes express P2Y1, P2Y2, P2Y4, P2Y6 and/or P2Y11 and as we discussed above that we excluded the involvement of P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 based on our results, we think that these cells (H9C2 cells) express new P2Y-like receptors and these cells lead to induction of p-ERK1/2 upon activation by UTP and that induced cardiomyocytes viability as we demonstrated.

Results of this project in addition to the first project add to the current knowledge about cardioprotection against IR injury. Furthermore, we think that over-expression of FXN simultaneously with the activation of these receptors by UTP and the induction of p-ERK1/2 pathway could be beneficial and that could protect the heart in response to IR injury. Therefore, discovering new and effective approaches or drugs that can over-express and induce both FXN and UTP, respectively, would be of paramount importance, especially to prevent IR injury. In the future projects, we will investigate the role of activation of these receptors on FXN expression during hypoxia/reoxygenation conditions and whether the activation of these receptors can lead to an increase in FXN expression and induction of p-ERK1/2 signaling pathway.

In summary, we found P2Y2 receptors to be expressed highly in our H9C2 cells, but these receptors are not responsible for the induction of p-ERK1/2 that we observed after UTP pre-treatment. In addition, pre-treatment of UTP induced slight increase in p-38 and p-JNK but this increase was insignificant. P-Akt was not changed even with high doses of UTP. P2Y2 and P2Y6 antagonists failed to inhibit the induction of p-ERK1/2, suggesting these receptors are not P2Y2, P2Y4 or P2Y6 receptors. Finally, we report that UTP pre-treatment preserved the cardiomyocytes viability as demonstrated by our cell viability assay. These results will open the door for future research to investigate furthermore the presence of new P2Y-like receptors and the use of UTP preconditioning to treat different diseases, such as IR injury in the heart.

4.6. Figures and Figure Legends

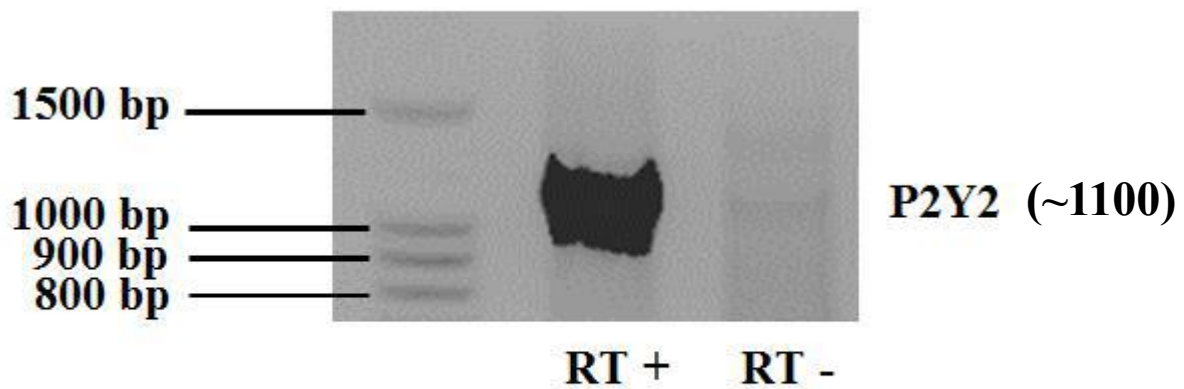
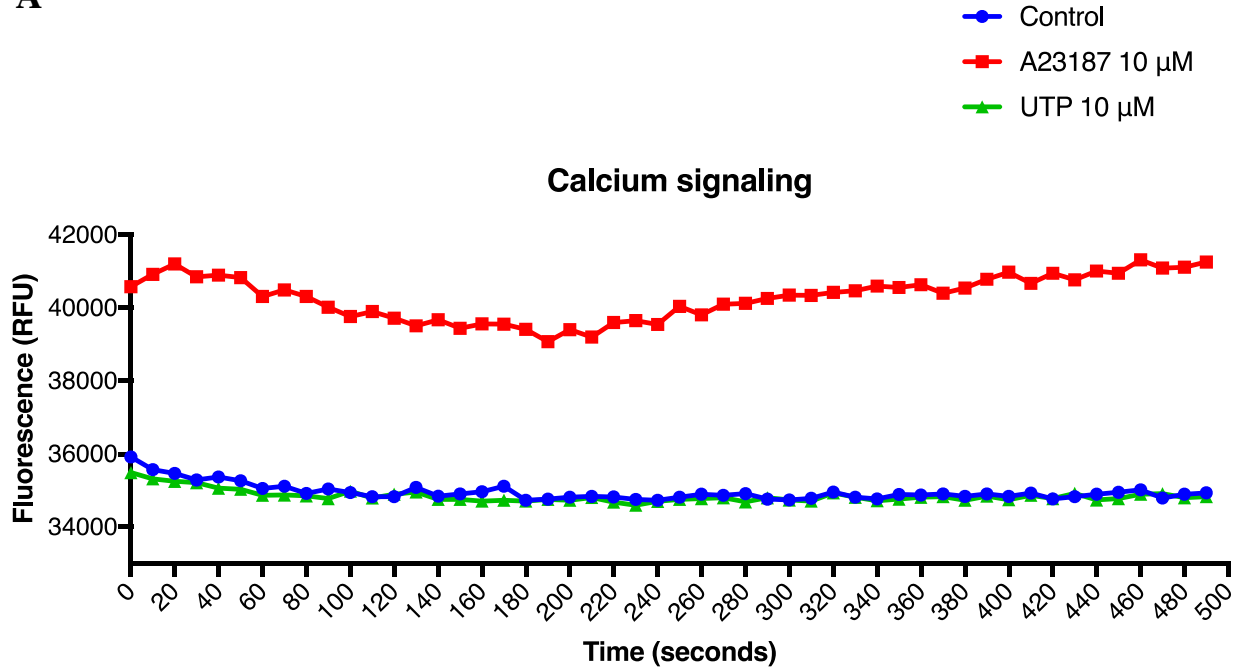
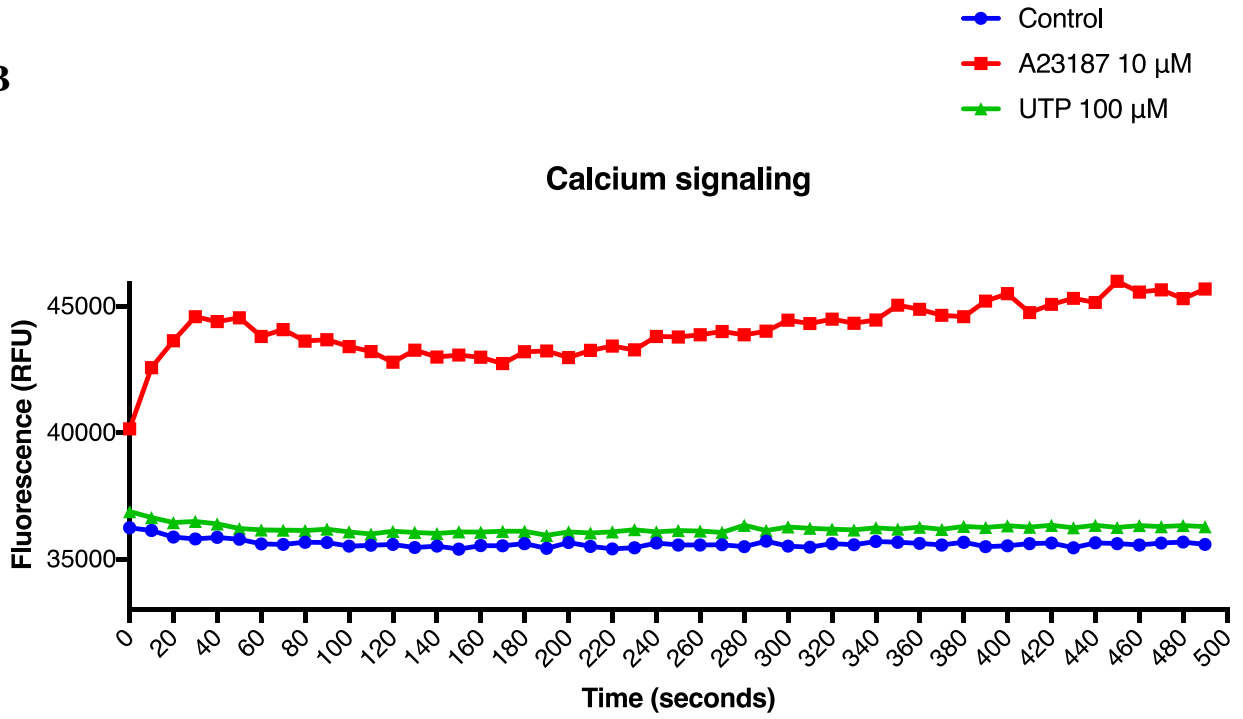


Figure 4.1. H9C2 Cardiomyocytes Express P2Y2 Receptors. H9C2 cells were starved for 24 hours before total RNA was isolated to synthesize cDNA. The qRT-PCR product was then resolved in 1% agarose gel to visualize the band. P2Y2 band appeared at ~ 1125bp based on the designed primer pairs.

A



B



C

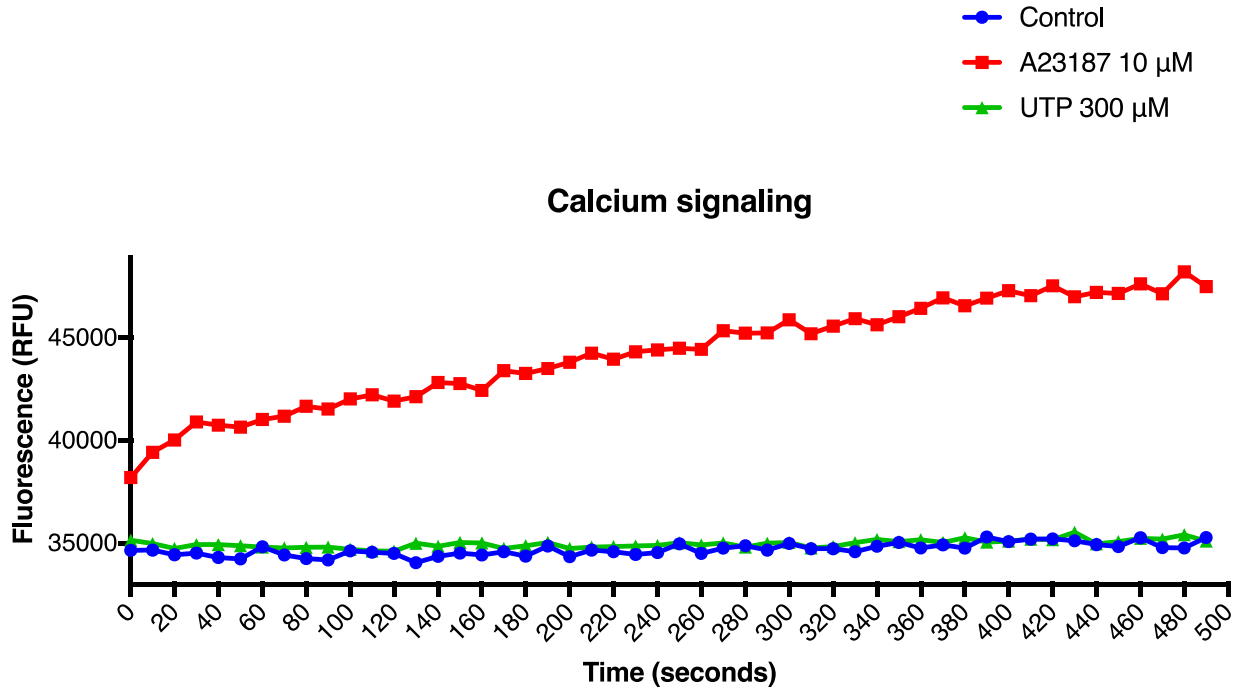
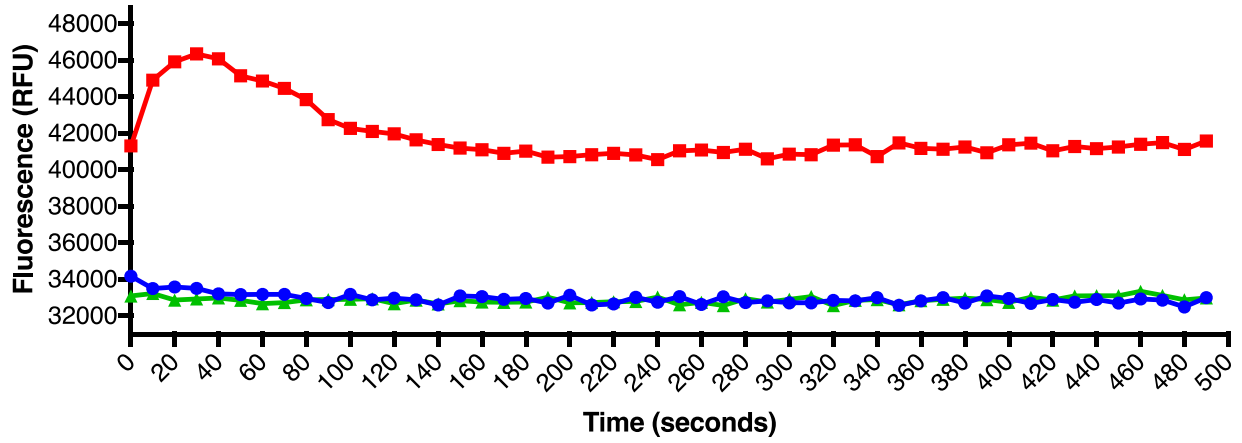


Figure 4.2. UTP had no Effect on the Intracellular Calcium Mobilization. Cells were starved for overnight in 96-well plate with black bottom before it was incubated with the calcium signaling dye for 45 minutes. After that, 20μl from Buffer C (added to the control cells), positive control (A23187) and UTP (10, 100 and 300 μM) were added (3A, B and C, respectively). Data are presented as relative fluorescence unit (RFU) per seconds (n=3).

A

- Control
- A23187 10 μ M
- ATP 10 μ M

Calcium signaling



B

- Control
- A23187 10 μ M
- ATP 100 μ M

Calcium signaling

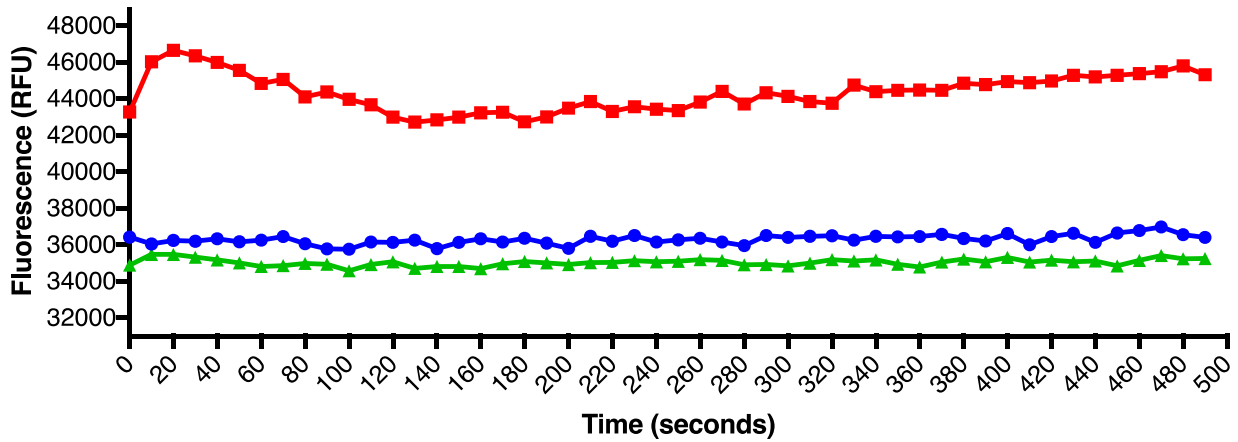
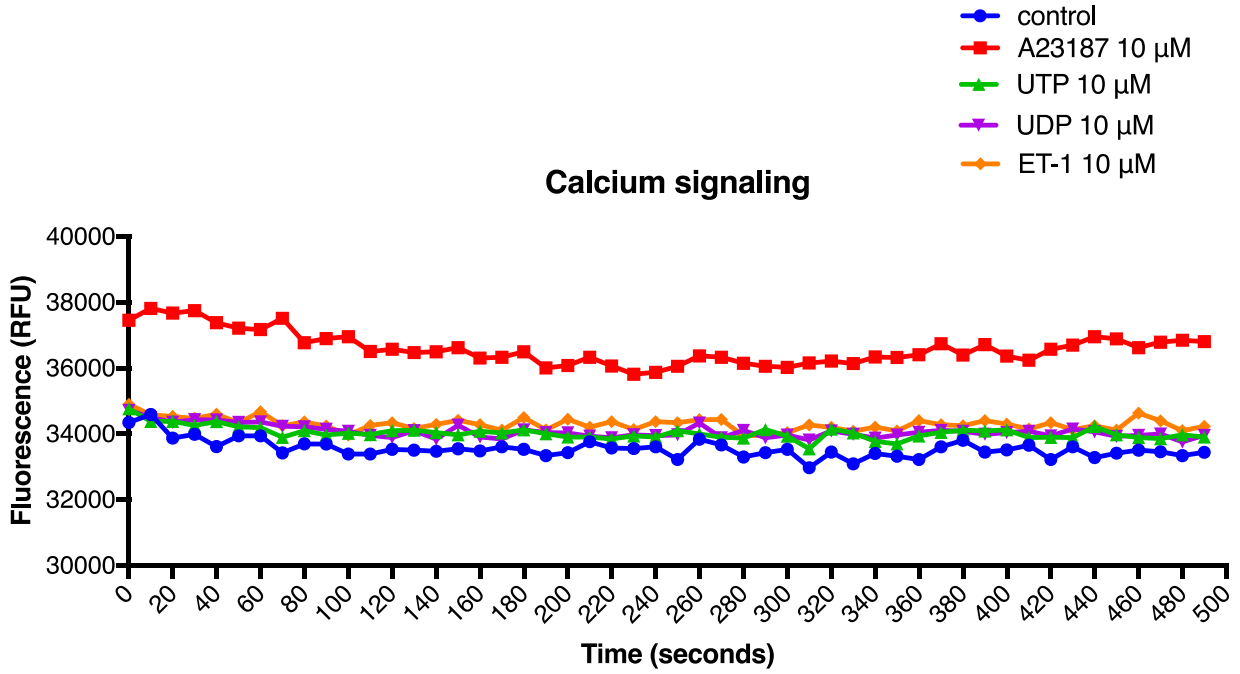


Figure 4.3. Effect of ATP on the Calcium Signaling. Cells were seeded in completed DMEM for one day before it was starved for overnight in 96-well plate with black bottom. The day after, Starvation media was replaced with calcium signaling dye for 45 minutes. After that, 20 μ l from Buffer C (added to the control cells), positive control (A23187) and ATP (10 and 100 μ M) were added (4.3 A and B, respectively). Data are presented as relative fluorescence unit (RFU) per time unit (seconds). Data were obtained from 3 independent replicates.

A



B

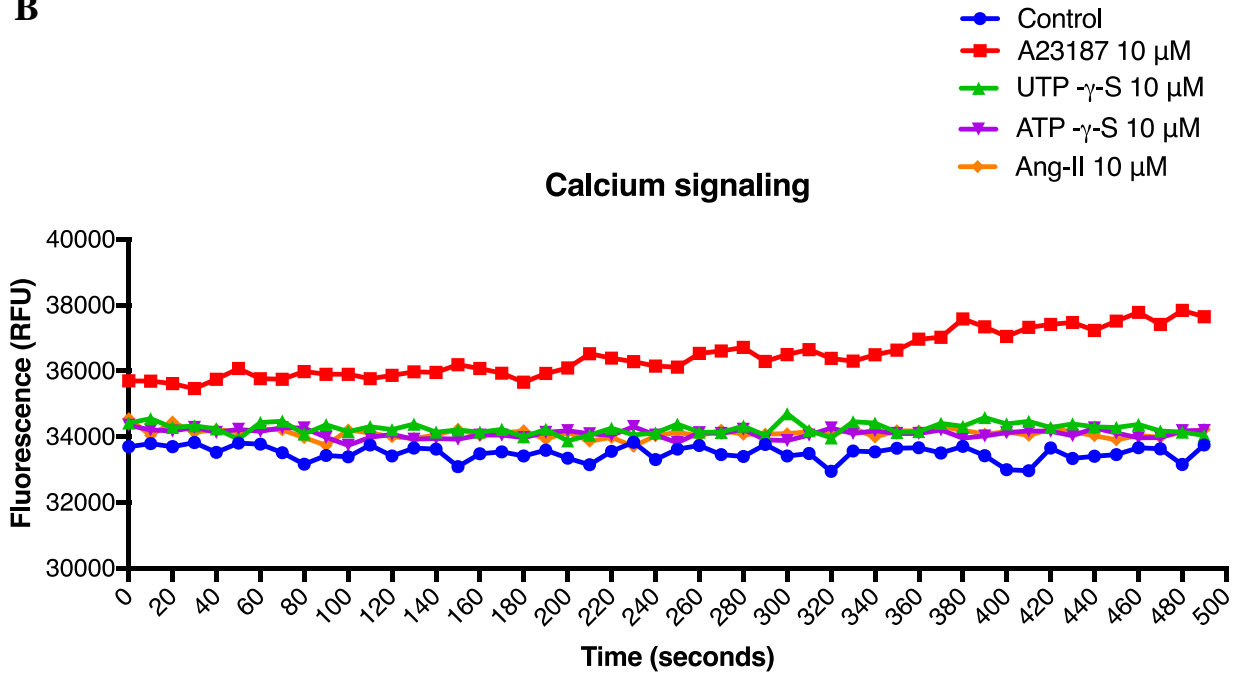
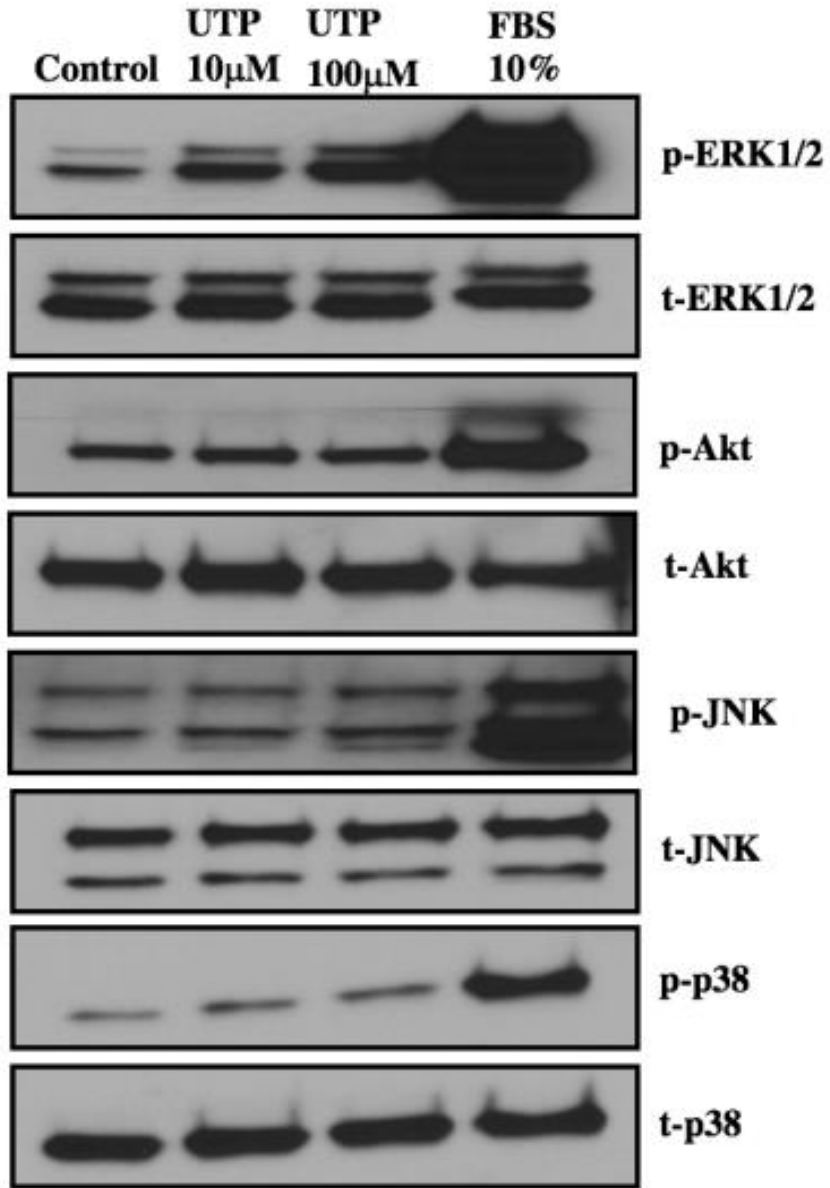


Figure 4.4. UTP- γ -s and ATP- γ -S Did Not Cause Any Changes in the Intracellular Calcium Levels. Cells were seeded in completed DMEM for one day before it was starved for overnight in 96-well plate with black bottom. The day after, Starvation media was replaced with calcium signaling dye for 45 minutes. After that, 20 μ l from (A) Buffer C (added to the control cells), positive control (A23187), UTP, UDP and ET-1 (10 μ M) and (B) UTP- γ -S, ATP- γ -S and Ang-II (10 μ M) were added. Data are presented as relative fluorescence unit (RFU) per time unit (seconds).

A



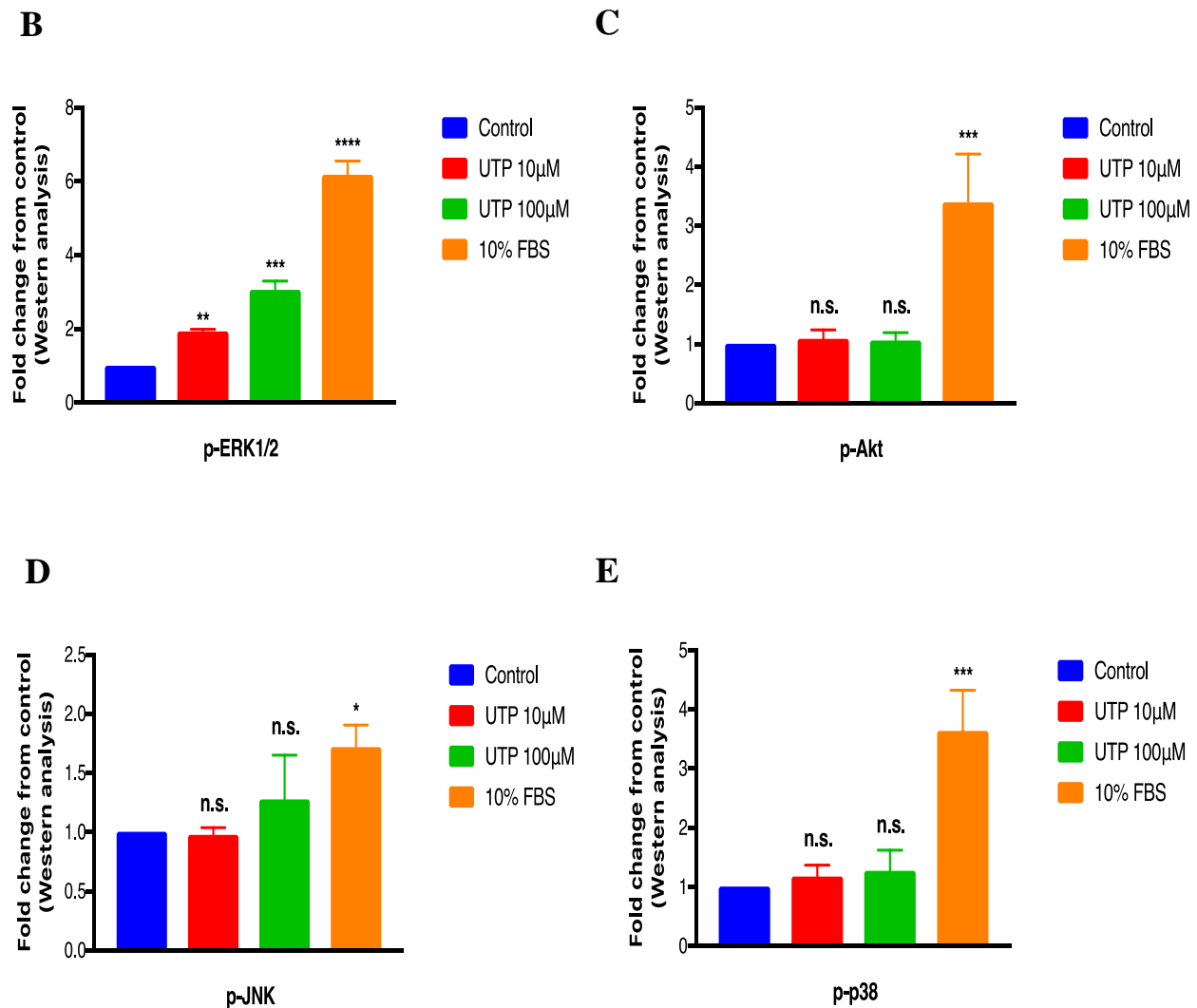
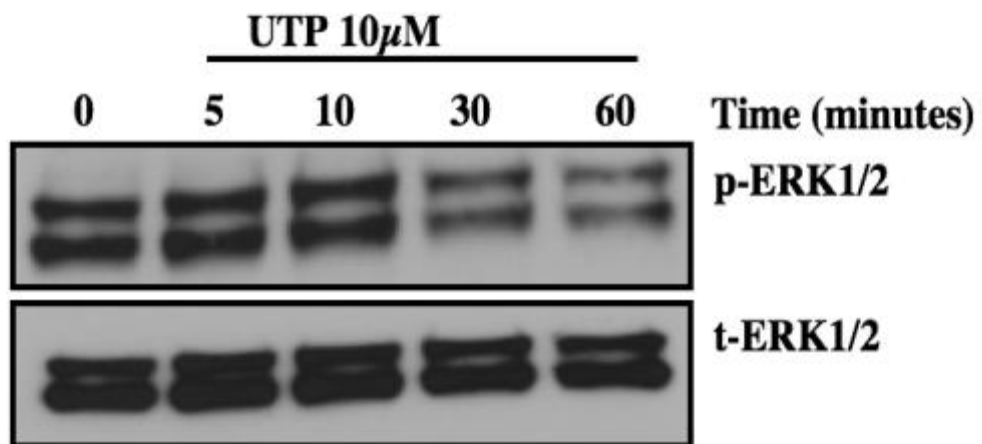
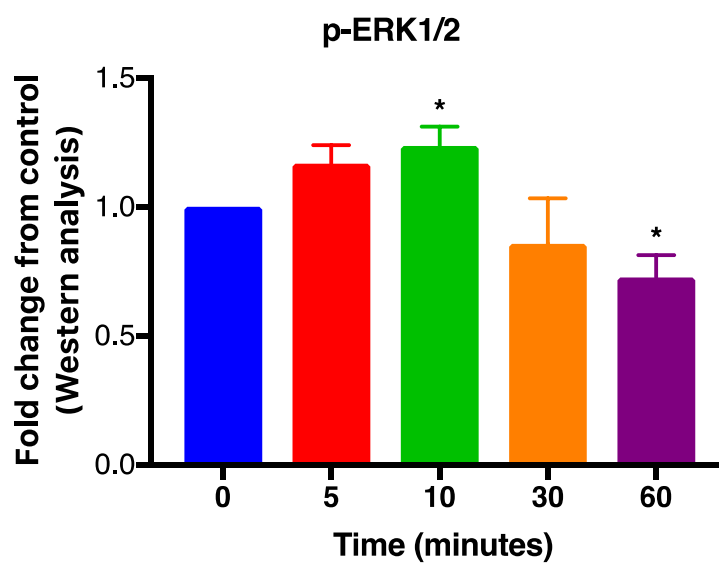


Figure 4.5. Effect of UTP on MAPKs and Akt Activation. (A) Representative blots demonstrate changes in cellular phosphorylation levels of ERK1/2, Akt, JNK and p-38 as determined using western blot assay after stimulating the H9C2 cells with UTP (10 and 100 µM) and 10% FBS for 10 minutes. (B-E) Bar graphs represent the average densitometric values obtained for p-ERK1/2, p-Akt, p-JNK and p-p38, respectively. The graphical analysis reflects averages from 3 independent experiments, n.s. $P>0.05$, * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. Values are expressed as mean \pm SEM.

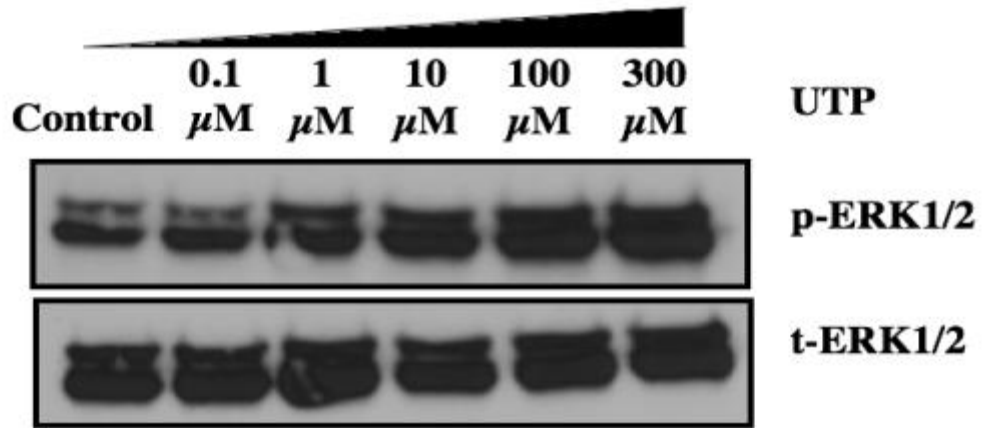
A



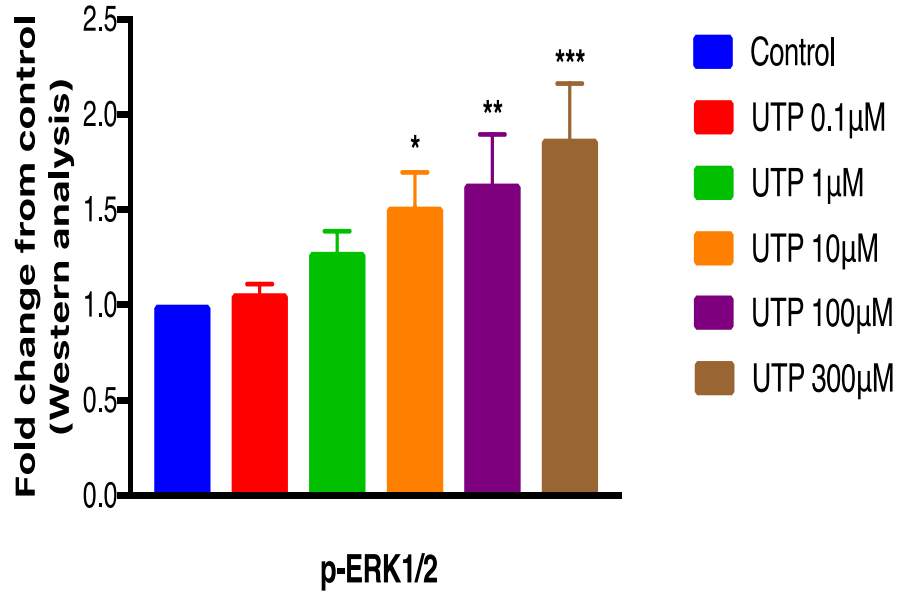
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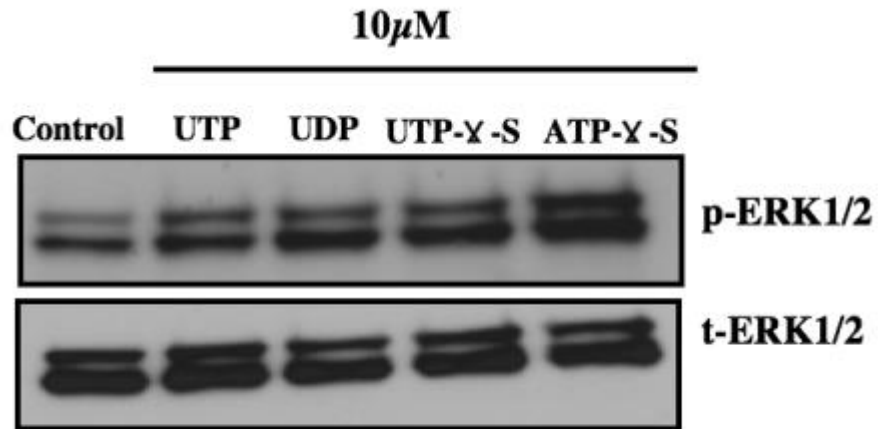
C



D



E



F

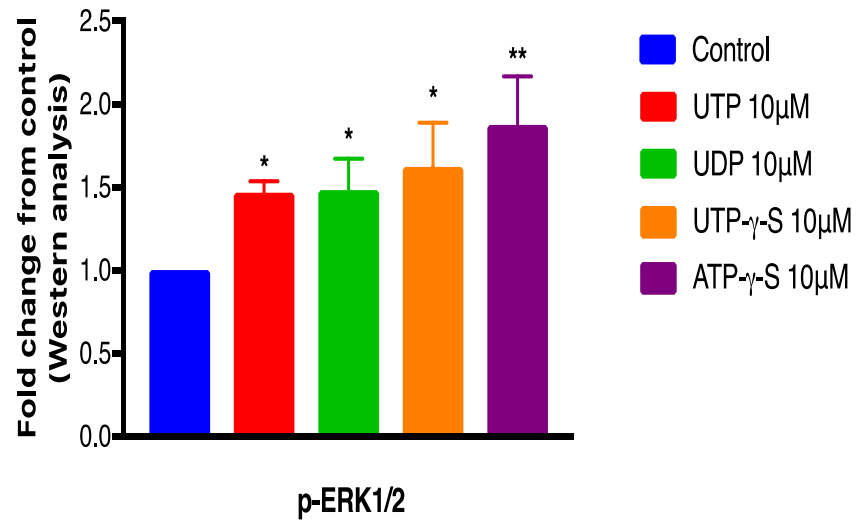
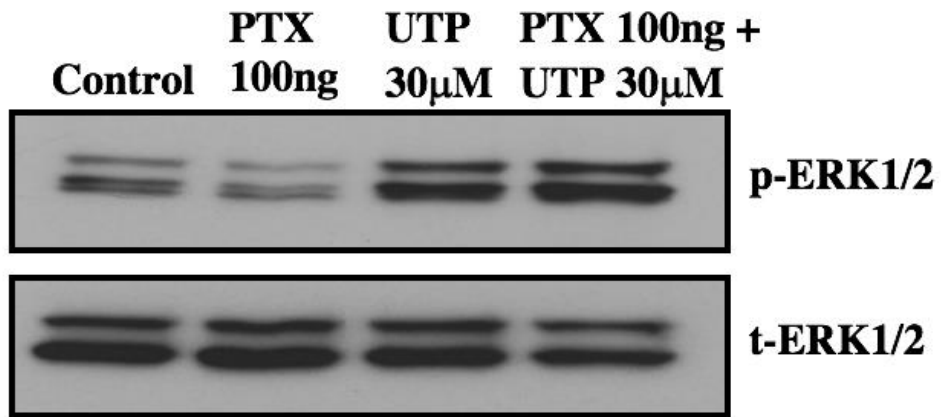


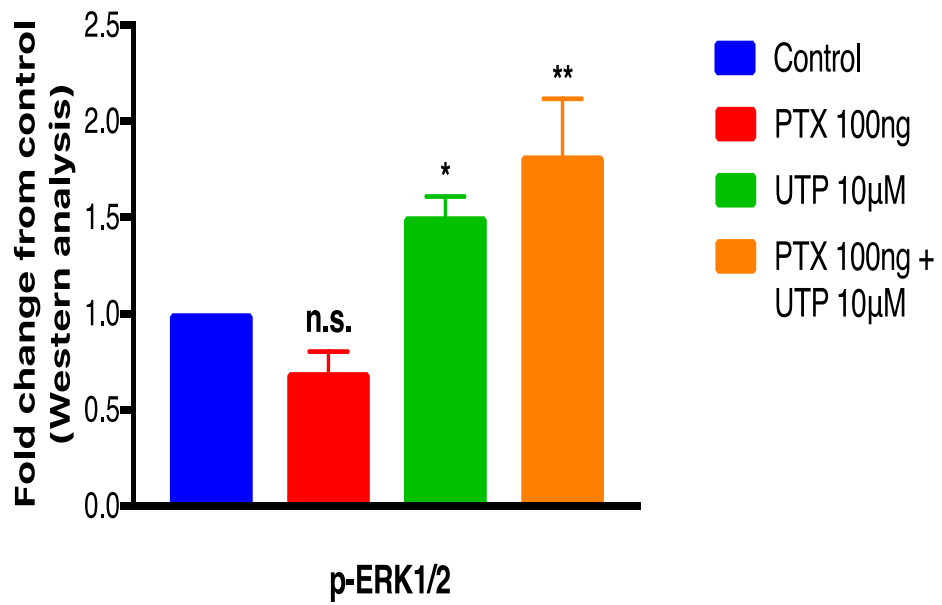
Figure 4.6. UTP Induces ERK1/2 Activation in a Time- and Dose-Dependent Fashion. (A)

UTP (10 μ M) induced p-ERK1/2 when cells were stimulated for 10 minutes and this induction was in a time-dependent manner as it peaked after 10 minutes and returned to basal levels after 60 minutes. (B) Densitometric analysis of average values of p-ERK1/2 levels as determined by western analysis from panel (A). (C) UTP (0.1, 1, 10, 100 and 300 μ M) dose-dependently induced p-ERK1/2 as validated by western blot analysis. (D) Densitometric analysis of average values of p-ERK1/2 levels from panel (C). (E) UTP, UDP, UTP- γ -S and ATP- γ -S (10 μ M) induced p-ERK1/2 to a similar level. (F) Bar graph represents the average densitometric values obtained for p-ERK1/2 from panel (E). Graphical analysis reflects averages from 3 independent experiments; where *P<0.05, **P<0.01 and ***P<0.001. Values are expressed as mean \pm SEM.

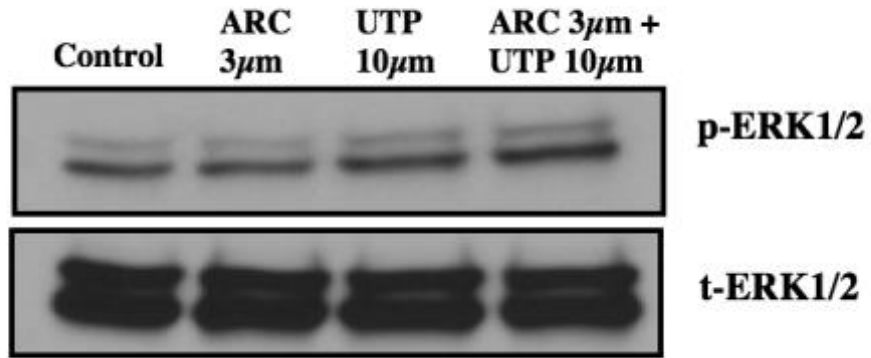
A



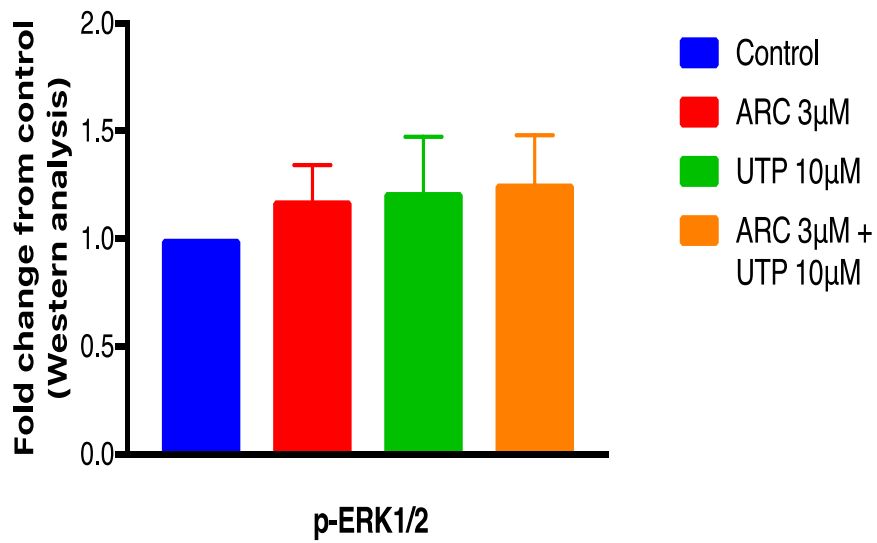
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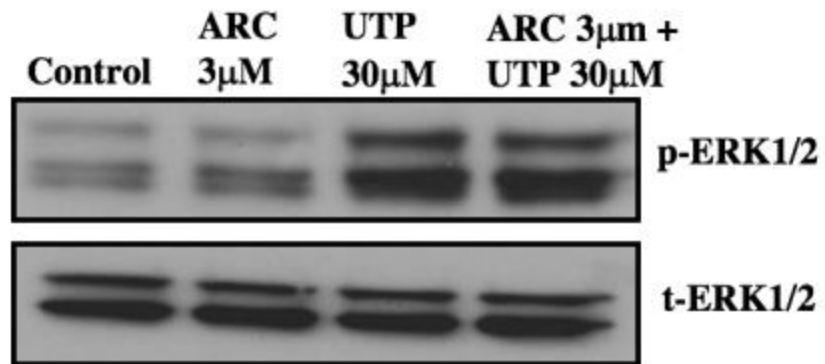
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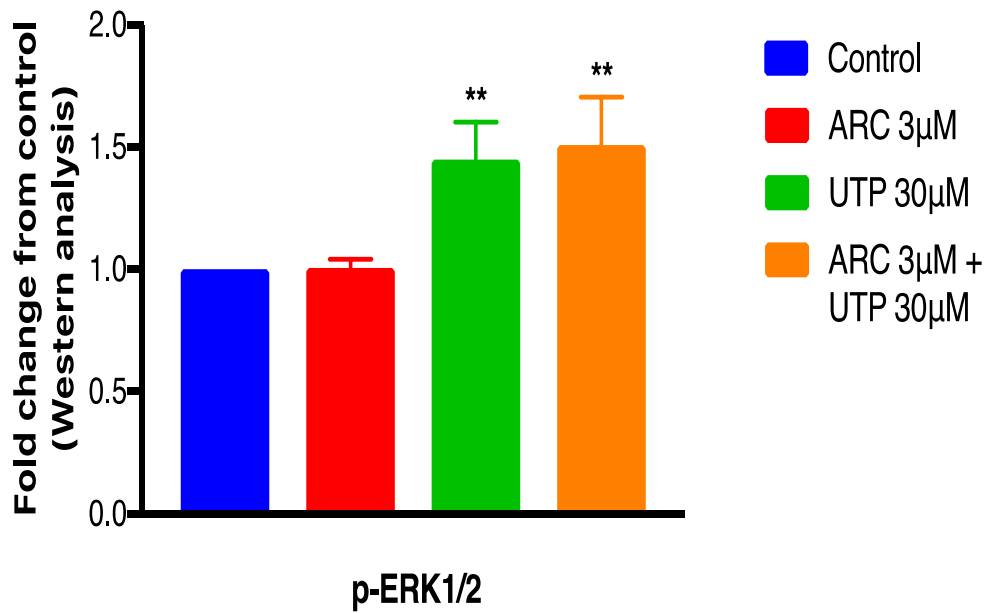
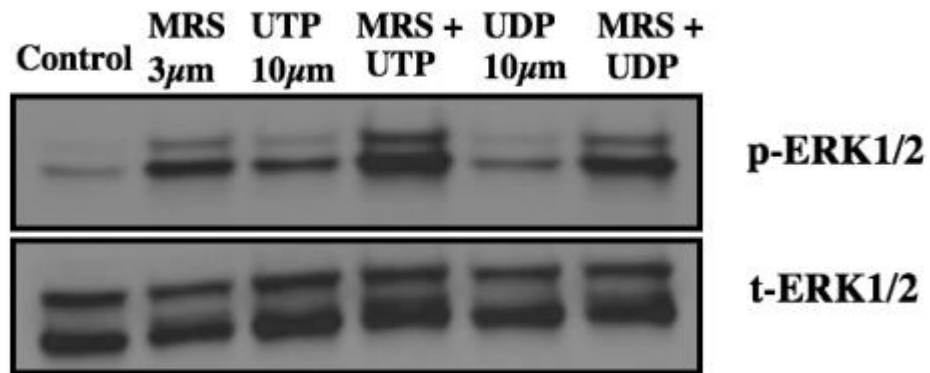
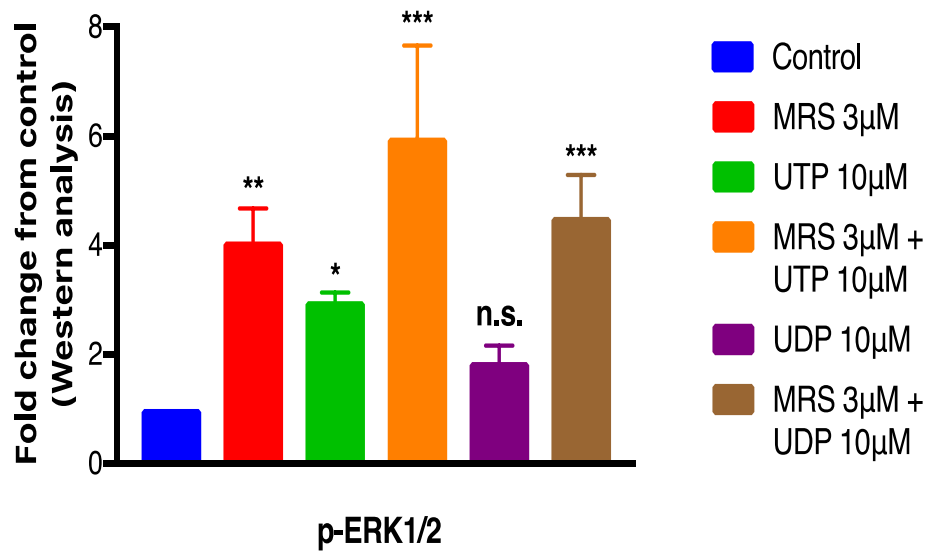


Figure 4.7. Induction of p-ERK1/2 by UTP is not Mediated by P2Y2 Receptors. (A) Pertussis toxin (PTX 100ng) did not inhibit the UTP-induced ERK1/2 activation as validated by western analysis. (B) Bar graph represents the average densitometric values obtained for p-ERK1/2 from panel (A). (C) ARC compound (3 μ M), which is selective antagonist for P2Y2, did not inhibit the effect that induced by 10 μ M UTP. (D) Bar graph represents the average densitometric values obtained for p-ERK1/2 from panel (C). (E) ARC compound (3 μ M) did not inhibit the effect that induced by 30 μ M UTP. (F) Bar graph represents the average densitometric values obtained for p-ERK1/2 from panel (E). PTX was incubated with cells for overnight and ARC compound were added 40 minutes before isolating the protein, while UTP was added 10 minutes before isolating the protein. Graphical analysis reflects averages from 3 independent experiments; where n.s. $P > 0.05$, * $P < 0.05$ and ** $P < 0.01$. Values are expressed as mean \pm SEM.

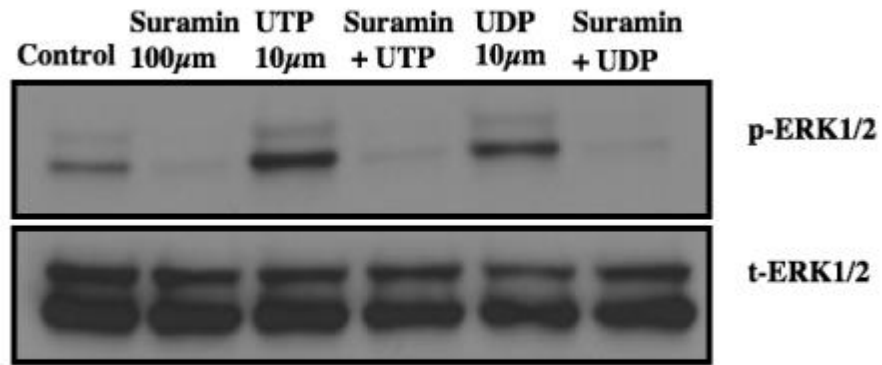
A



B



C



D

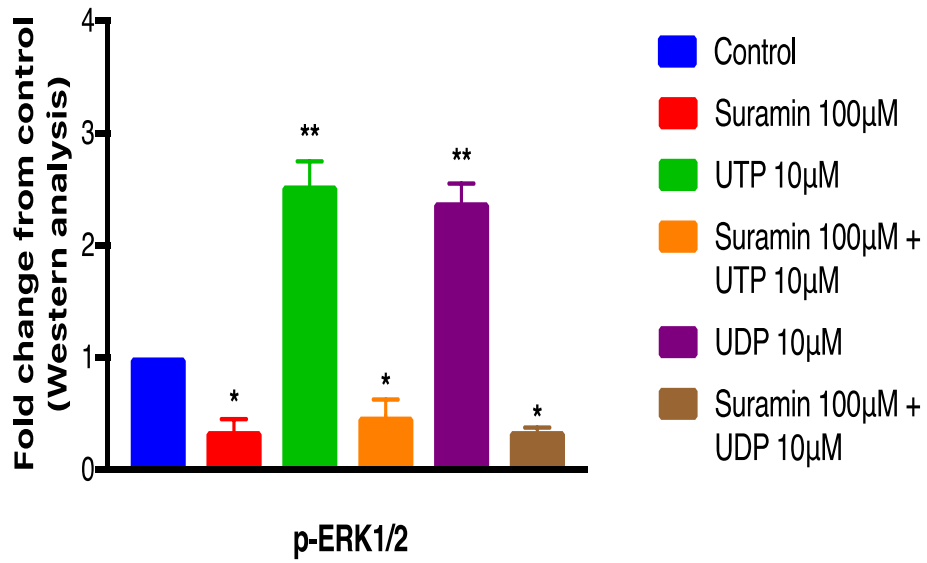


Figure 4.8. P2Y6 Selective Antagonist Induced p-ERK1/2. (A) MRS compound (3 μ M), which is a selective antagonist of P2Y6 receptors, surprisingly induced p-ERK1/2 after cells were treated for 10 minutes with UTP (10 μ M) and (UDP 10 μ M). (B) Densitometric analysis of average values of p-ERK1/2 levels as determined by western analysis from panel (A). (C) Non-selective P2Y receptor antagonist (suramin 100 μ M) completely inhibits the induction of p-ERK1/2 by UTP (10 μ M). (D) Densitometric analysis of average values of p-ERK1/2 levels as determined by western analysis from panel (C). MRS and suramin were added 40 minutes before extracting the protein, whereas UTP stimulated the cells for 10 minutes before isolating the protein. Graphical analysis reflects averages from 3 independent experiments; where n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Values are expressed as mean \pm SEM.

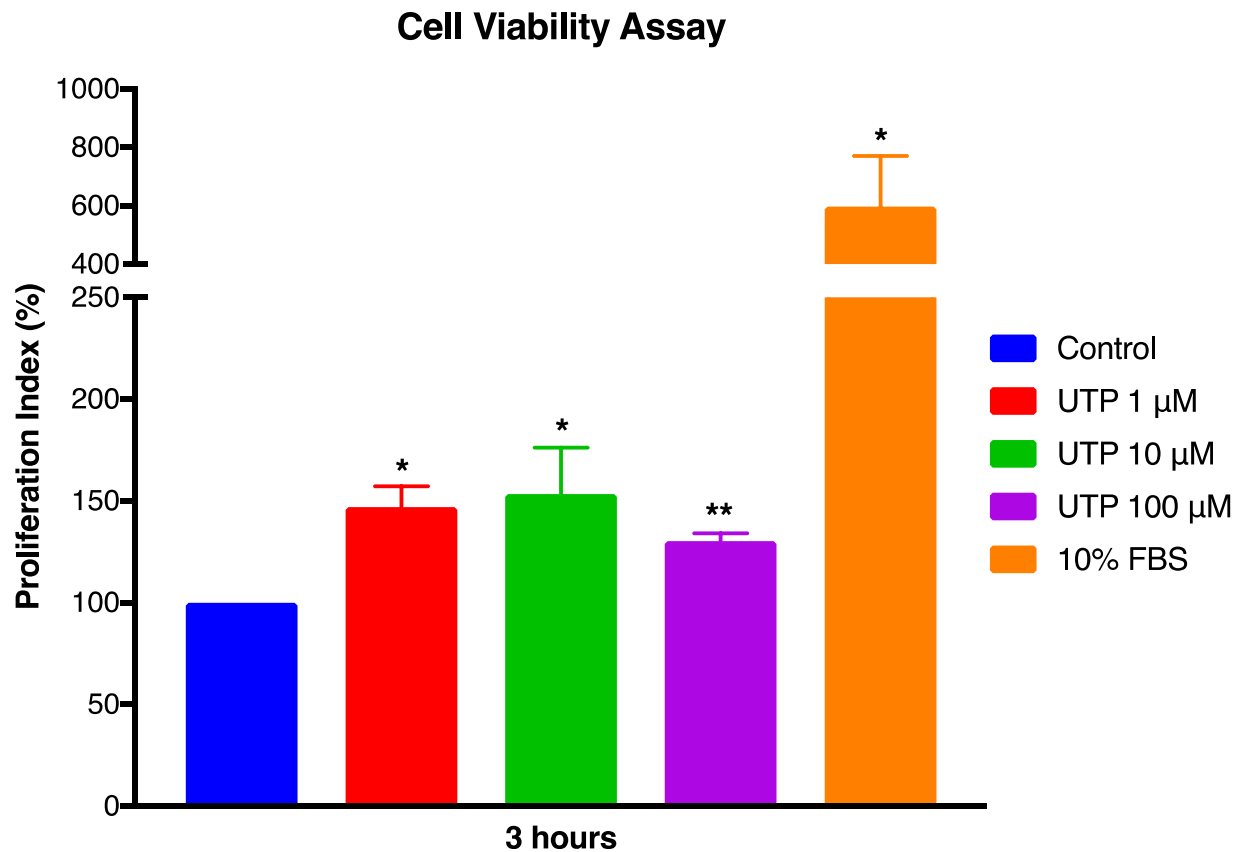


Figure 4.9. UTP Induced Cardiomyocytes Viability. Cardiomyocytes viability was assessed using CCK-8 kit. Cells were seeded in 96-well plate for 1 day and starved after that for overnight. On the day after, cells were stimulated with different concentration of UTP (1, 10 and 100 μ M) and 10% FBS for 2 days. On the third day, 10 μ l of the CCK-8 solution were added to each well and incubated for 4 hours. Data are presented as proliferation index % after 3 hours of incubation with CCK-8 solution. Data were normalized to control cells from 3 independent replicates, where * $P < 0.05$ and ** $P < 0.01$. * represents comparison between each bar and control cells.

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