Proteomic analysis of the heart under aerobic condition and after ischemia/reperfusion

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Pharmacology

University of Saskatchewan

Saskatoon

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ABSTRACT

Cardiovascular disease is one of the main causes of mortality and one of the significant burdens to society. Major cardiovascular diseases such as acute myocardial infarction (heart attack), heart failure and cardiac arrhythmia often result in the development of ischemia/reperfusion (I/R) injury.

Untreated I/R injury is known to cause cardiac contractile dysfunction. It is established that matrix metalloproteinase-2 (MMP-2) is activated and degrades contractile proteins during I/R, and many other factors including metabolic enzymes, kinases and structural proteins are affected by I/R. However, the molecular mechanisms responsible for these changes are unclear.

Since MMP-2 is known to its broad spectrum of action, I hypothesize that, in addition to contractile proteins, proteins related to regulation of energy metabolism are MMP-2 targets during I/R, and protein kinase such as myosin light chain kinase (MLCK) is also involved in this process. The use of proteomics in studying heart injury triggered by I/R will reveal new potential targets for pharmacological protection of heart from I/R induced contractile dysfunction. In addition, selective inhibition of MMP-2 using MMP-2 siRNA protects the heart from I/R injury.

In this study, we investigated the protein modulation during I/R using proteomic approach. In order to study the effect of protein kinases (MLCK) and MMP-2, their selective inhibitors were used to inhibit those factors and evaluate the changes in energy metabolic proteins during I/R.

Proteomic analysis revealed that six proteins are involved in energy metabolism: ATP synthase β subunit, cytochrome b-c1 complex subunit 1, 24-kDa mitochondrial NADH dehydrogenase, NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, cytochrome c oxidase subunit, and succinyl-CoA ligase subunit, resulting in decreased levels in I/R hearts. The data suggests that energy metabolic proteins, especially the metabolic enzymes involved in the electron transport chain in the mitochondria may contribute to I/R injury. In addition, our data provides evidence that the right and left ventricles of the heart respond differently to I/R injury, in terms of the regulation of contractile proteins and energy metabolic enzymes.

Studies using MLCK inhibitor, ML-7, and MMP-2 inhibitor, MMP-2 siRNA to investigate the effect of myosin light chain kinase (MLCK) and MMP-2 in energy metabolic proteins have shown that succinyl-CoA ligase and ATP synthase are affected by MLCK and MMP-2 respectively. These results demonstrate that the effect of inhibition of the MLCK

and MMP-2 involves optimization of energy metabolism in I/R injury, likely resulting in increased energy production. Hence, the observed proteins increase in cardiac recovery after I/R. Also, inhibition of MLCK and MMP-2 by ML-7 and MMP-2 respectively shows cardio protective effect during I/R.

In summary, this study provides a novel pathogenesis in the development of I/R-induced cardiac contractile dysfunction. Moreover, we suggest a new therapeutic approach whereby using MMP-2 siRNA can be a promising gene therapy in the development of new preventive or treatment strategies against I/R injury.

ACKNOWLEDGEMENTS

Finally, I have the chance to express myself. These four years is the most memorable journey in my life.

I would like to thank my PhD advisory committee members for all their guidance, patience, support, availability and precious advice. Thanks to Drs. Bekar, Desai, Gopal, Liu, Majewski, Richardson.

Thanks to the College of Graduate Study and Research and the College of Medicine for the financial support through all four years. (2010-2014).

Very special thanks to Grzegorz (Greg) Sawicki and Jolanta Sawicka. They are like my parents in the lab (Even though you guys may not accept such a special grant kid). Whenever I have done something wrong, always, I do mean always, either of you will stand on my side and fight with the other. Greg as my supervisor and my mentor, he helped me every single time when I felt down and rough during these four years, not only in academia but also in life. I am so sorry that I did let you guys disappoint for many times, but you always forgive me and guide me how to face it and do it right. I am sure that you guys will have a great impact in my future life.

To Our lab:
Virgilio Cadete (Montreal), Steven Arcand, Keshav Sharma (Edmonton), Matthew Martyniuk (Regina), Bikram Sra (Vancouver), Preston O'Brien and Abigael Oludeyi. Thanks for all the help in experiments and writing. And of course, the most important thing is the companionship from all of you guys, and I hope I will have a place to stay when I come to visit you.

To Drs. Mieczyslaw Wozniak, Francisco Cayabyab, Mike Moser and Zhecheng Chen. Thanks for their help in experimental support and manuscripts writing.

Thanks to ABCDE, A (Arlene Drimmie), B (Bob Wilcox), C (Cindy Wruck), D(Donna Dodge) and E (Evelyn Bessel): thanks for all your help in everything, which makes the Department of Pharmacology a sweet place for everyone.

To all my friends who support and help me throughout these four years, you know who you are!

To everyone at Louis' pub, Thanks for four great years of work and friendship. Always remember that we are the most educated pub in Saskatoon.

To my family:
My parents, Tiao-huan Lin and Jie-ying Mai. My sister, Queena Yan-na Lam. My brotehr-in-law, Eddy Sau-wai Yiu. Thanks for their love and support for all these years.

To everyone I forgot to mention here but you know you are important to me.

Last, but not least:
To my wife, Jia-min Li (Kamin Lee), you almost gave up everything to support my goal. The beginning of the new life in Canada is tough. Thanks for holding on every day. I am sure that we will have a much better life in the near future with our first kid. Kardy Lam, I hope that you will have a happy life.

DEDICATION

To my parents

Tiao-huan Lin and Jie-ying Mai

To my wife

Jia-min Li (Kamin Lee)

To my kid

Kardy Ho Lam

Thanks for their endless support and love to help me fulfill my dream.

予：

我的父母

林眺寰　麥結英

我的妻子

李嘉敏

我的孩子

林昊 (林兢甡)

感謝他們給予我的支持和鼓勵，使我從不放棄自己的夢想。

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2-DE	two-dimensional electrophoresis
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
CF	coronary flow
CHF	congestive heart failure
CK	creatine kinase
CO	cardiac output
CoA	coenzyme A
Cr	creatine
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELC	essential light chain (also MLC1)
ERK	extracellular-signal-regulated kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GTP	guanosine triphosphate
HPLC	high pressure liquid chromatography
H-R	hypoxia-reoxygenation
HR	heart rate
I/R	ischemia/reperfusion
LC/MS	liquid chromatography/mass spectrometry
LV	left ventricle
LVDP	left ventricular developed pressure
MHC	myosin heavy chain
MI	myocardial infarction

MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MMP	matrix metalloproteinase
MMP-2	matrix metalloproteinase-2
MS	mass spectrometry
MT-MMP	membrane-type metalloproteinase
PAGE	polyacrylamide gel electrophoresis
PDH	pyruvate dehydrogenase
PTMs	posttranslational modifications
RLC	regulatory light chain (also MLC2)
RPP	rate pressure product
TnI	troponin I
TnT	troponin T
IEF	isoelectrofocusing
IgG	immunoglobulin G
MAP	mean arterial pressure
NCBI	national center for biotechnology information
PKC	protein kinase C
PTM	post-translational modification
Q-TOF	quadruple time of flight
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
siRNA	small interfering RNA
SV	stroke volume
TEMED	N,N,N’,N’-tetra-methyl-ethylenediamine

TIMP	tissue inhibitor of metalloproteinase
VM	ventricular myocyte

1. INTRODUCTION

1.1 Cardiovascular disease

1.1.1 Facts of cardiovascular disease

Cardiovascular diseases include dysfunctional conditions of the heart and the vascular system supplying oxygen and nutrients to vital, life-sustaining areas of the body, such as the brain, the heart itself and other organs. The likelihood of developing cardiovascular disease increases with different risk factors, such as aging, smoking, stress, physical inactivity, overweight, excessive alcohol consumption, air pollution, high blood pressure (hypertension), high blood cholesterol and diabetes. (2). The global epidemic of cardiovascular disease still remains the number one cause of death around the world (3). In the year of 2012, an estimate of 17.3 million people died from cardiovascular diseases, contributing to about 30% of all deaths in the world (4). By the year 2030, it is predicted that around 23.3 million people will die from cardiovascular diseases (5). In Canada, heart disease and stroke are two of the three leading causes of death. Every 7 minutes, at least one Canadian dies from heart disease and stroke (1, 4, 6)**.** Cardiovascular disease is also the leading economic burden of disease in Canada, with a total direct cost (hospital care, drugs, physician care, other institutional care, etc.) of CDN$6.8 billion and an indirect cost (relating to mortality, and short and long term disability) of CDN$11.6 billion (7-8). Therefore, cardiovascular disease is a major burden on society, accounting for more deaths, disability and health care costs than any other disease.

1.1.2 Classification of cardiovascular diseases

Several methods have classified cardiovascular diseases into different groups. A commonly used classification demonstrates that cardiovascular diseases manifest in six forms including:

- coronary heart disease – disorder of the blood vessels supplying the heart muscle;
- cerebrovascular disease - disorder of the blood vessels supplying the brain;
- peripheral arterial disease – disorder of blood vessels supplying the arms and legs;
- rheumatic heart disease – damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria;
- congenital heart disease – prenatal malformations of heart structure;
- deep vein thrombosis and pulmonary embolism – blood clots in the leg veins, which can be dislodged and can move to the heart and lungs. (4)

Among these six forms of cardiovascular diseases, coronary heart disease is the most common type covering 60% of all cardiovascular disease, causing the most death in the world (9). Coronary heart disease is caused by the narrowing or blockage of coronary arteries and thus the reduction in blood supply of the heart muscle. There are different pathological reasons causing the narrowing and blockage of the blood vessels including arteriosclerosis, vasospasm and atherosclerosis. However, atherosclerosis is the major cause of coronary heart disease. Atherosclerosis results from the accumulation of cholesterol, triglycerides and low-density lipoprotein in the arteries, triggering a chronic inflammatory response to generate plaques, and hence, narrowing the arteries. With the age increases, the plaques become thicker and eventually cause the blockage of the artery, and ischemia of the heart occurs. Untreated ischemia of the heart leads to severe damage to the cardiac tissue. Even after reperfusion, the damaged myocardium cannot be recovered. This is the classic pathway that I/R injury induces myocardium infarction, and causes contractile dysfunction, and this eventually leads to heart failure. Most of the cardiologists believe that it is the major reason that patients die from coronary heart disease.

1.2 Ischemia/reperfusion injury (I/R injury)

Ischemia/reperfusion (I/R) injury is a multifaceted event that encompasses several distinct mechanisms leading to a worsening of cardiac function (10). There are two pathological processes in I/R injury, which are ischemic injury and reperfusion injury. Ischemia is characterized by the lack of an efficient supply of oxygenated and nutrient-rich blood to a tissue or organ. Reperfusion is the restoration of blood supply to the previously ischemic area. Some studies indicate that I/R injury only means reperfusion injury (11), while others think that it is incorrect to make that assumption (12). There are different mechanisms between ischemic injury and reperfusion injury in terms of reactive oxygen species generation (13), inflammatory response (14) and damage of energy metabolism system (13). Therefore, ischemia/reperfusion injury should be considered as two different processes with a close relationship to each other.

1.2.1 Ischemic injury

The general definition of ischemia is the reduction in blood flow in the tissue. Many pathological conditions can trigger ischemia, such as atherosclerosis and thrombus blockage.

Reduction in the blood flow leads to oxygen transportation dysfunction and causes the shortage of oxygen in the tissue and cells. When oxygen concentration in the tissue drops significantly, oxidative phosphorylation cannot occur, and this will result in reduced ATP formation in the mitochondria. Cardiomyocytes have much more mitochondria than most of the cell types in the body because of the great need of energy to perform their function. ATP formation is the most vital part for the cardiomyocyte contraction and survival. During ischemia in the heart, the lack of oxygen in the myocardium will result in respiratory chain dysfunction, energy production disorder and reactive oxygen species (15) generation, which will then eventually lead to severe, irreversible damage to the cardiac tissue and a serious impact on the cardiac contractile function. A common therapeutic strategy for ischemic injury is the reperfusion therapy. However, both reperfusion therapy and self-reperfusion would potentially cause another different pathological process, namely reperfusion injury.

1.2.2 Reperfusion injury

Reperfusion injury is the tissue damage caused by newly returning blood flow to previously ischemic tissue. When the blood returns to the ischemic tissue, the restoration of the blood flow further damages the ischemic tissue. The absence of oxygen and nutrients during ischemia creates a condition in which the restoration of oxygen and nutrients result in inflammation and oxidative damage through the induction of oxidative stress. In the therapeutic strategy for I/R injury, such as coronary artery disease induced myocardium infarction (MI), reperfusion therapy is still the fundamental treatment for more than twenty years (16). It has been widely accepted that ischemic injury is more severe than reperfusion, in terms of the tissue damage, causing that reperfusion injury has been overshadowed by ischemic injury. There are only a few studies or clinical trials focus on the treatment and prevention of reperfusion injury (17). However, ischemic injury and reperfusion injury should be considered as two inseparable processes and therapeutic approaches should be developed to treat both and thus may result in more effective recovery for the patient.

1.2.3 Mechanism of ischemia/reperfusion injury

Ischemia/reperfusion injury has been studied for more than half a century; however, the underlying molecular mechanisms involved in the pathogenesis of myocardial I/R injury are complicated and involve the interactions of different pathways that are still unclear. A multitude of mediators triggered by ischemia and/or reperfusion have been identified and range from inflammation (14), energy metabolism (13), cell death (18-19), and many others.

Limited supply of oxygen and nutrients for the maintenance of normal cardiac function during ischemia and the generation of oxidative stress during reperfusion might be the main reasons for the following consequence. There is a growing body of evidence over the last decade pointing out that the degradation of functional proteins triggered by I/R-dependent ROS generation could be the fundamental molecular mechanism for ischemia/reperfusion injury. It has been shown that several mediators and pathways, such as contractile proteins (20-21), MMPs (22), kinase (23) and energy metabolism proteins (24) are involved in this mechanism. However, more studies are still needed in search of better targets for the therapeutic strategies of ischemia/reperfusion injury.

1.3 Cardiac contractile proteins

In the cardiovascular system, the heart is most important organ for the circulation and is responsible for supplying blood flow to meet the metabolic demands of the organs and removing metabolic waste. The heart has been considered as a pump in the circulation, and its physiological function highly depends on the contractile machinery of the heart. In order to study the function and mechanism of cardiac contractile machinery, investigators focus on the cardiac sarcomere in the heart. The cardiac sarcomere is the basic unit of muscle contraction in the heart. The sarcomere is composed of thick and thin filaments. Physical and chemical interaction between the thick and thin filaments result in the shortening of the sarcomere, and that is what we call the contraction of the heart (25). There are different proteins performing various functions in both thick and thin filaments. The thick filament is mainly comprised of myosin, and the thin filament is comprised of troponin, actin and tropomyosin. These cardiac contractile proteins have been studied for decades and the physiological functions of each protein have been identified (26). However, how cardiac contractile proteins are regulated in the pathological conditions is still unclear. Recently, the regulatory subunits of myosin and myosin light chains have been investigated as a biomarker of heart function in pathological conditions, and this topic has been given increasing attention.

1.3.1 Myosin light chains (MLCs)

Myosin is a large and complicated molecule which contains different types of subunits. Most of myosin molecules are composed of two head domains, two neck domains and one tail domain. The head domain is composed of myosin heavy chains, which is the motor protein of the thick filament. Myosin heavy chain has the ATPase activity and triggers

the sliding between thick and thin filaments and the contraction. The neck domain is composed of myosin light chains, which are myosin light chain 1 and myosin light chain 2 (27-29). These myosin light chains play both structural and regulatory roles. Myosin light chain 1 and myosin light chain 2 bind in the tandem to the domain of the myosin head that attaches to the myosin tail (30), stabilizing both the myosin head and the myosin tail. Myosin light chain 1 is known as the essential light chain, which is present in the hinge of the myosin head for stability purpose. Myosin light chain 2 is the regulatory light chain which performs the modulation in between the myosin head and myosin tail. It has been shown that interaction between myosin light chain 1 and actin play an important role in the regulation of contraction (27, 31-39). Myosin light chain 1 knockdown model shows a ~50% of reduction of contractility (33). The regulatory light chain, myosin light chain 2 is also involved in the regulation of contraction. It has been demonstrated that phosphorylation of myosin light chain 2 regulates Ca-dependent contraction (40-42). Because of the presence of myosin light chains, the myosin structure maintains stable and the contraction process remains regular in the physiological condition, which indicates the normal function of the cardiac sarcomere. However, a different story is presented in the pathological condition.

1.3.2 Myosin light chains in ischemia/reperfusion injury

The underlying molecular mechanism of ischemia/reperfusion injury still remains unclear. Protein damage within the cardiomyocyte has been considered a key event during ischemia/reperfusion injury. However, the main protein-protein interactions or substrate-enzyme reactions in this pathological process are still poorly understood. It is almost certain that ischemia/reperfusion injury is a complex process, which includes various factors, such as structural protein, energy metabolic proteins, kinase and proteolytic enzyme. It has been shown that myosin light chains are one potential target for the protein degradation during ischemia/reperfusion. Myosin light chain modification such as phosphorylation, modulates contractile force generation during ischemia/reperfusion (43). The correlation between the function of the heart and the alteration in phosphorylation of myosin light chain has been reported (44-45). In addition, some studies have demonstrated that both myosin light chain 1 and myosin light chain 2 are released into the effluent of an ischemic heart (45), indicating that myosin light chains could be a target for the pathological heart. However, no conclusion has been made on what proteolytic enzyme is responsible for the degradation of myosin light chains, and the molecular mechanism of the release of this degraded protein is still under investigation. Our studies have shown that myosin light chains are degraded by matrix

metalloproteinase-2 (MMP-2) during I/R (22, 46-47), and protein kinases, such as myosin light chain kinase (MLCK) and Rho kinase, are partially involved in this process (48). Despite there are many unanswered questions about the molecular basis of I/R injury in the heart, myosin light chains has become one of the key biomarkers for heart injury.

1.4 Kinase pathways

Kinase is a type of enzyme that catalyzes the phosphorylation of molecules, and the phosphorylation can change the molecules' activity and ability to react with other molecules to perform different cellular responses. Therefore, kinases are crucial in metabolism, cell signaling, protein regulation and many other physiological functions. Beside the vital role of kinases in the physiological condition, it has been widely shown that kinases also play an important role in pathological conditions. There is a group of more than 500 protein kinases (49), which are an intricate part of ischemia/reperfusion. These vascular and cellular events alter the response of many kinases, leading to subsequent cell injury and dysfunction. Studies have reported that MLCK, protein kinase C (PKC) and Rho kinase, are at least, partially related to I/R injury (48). The recognition that these kinases are involved in I/R will permit us to find better therapeutic methods to control these ubiquitous and highly recognizable important enzymes.

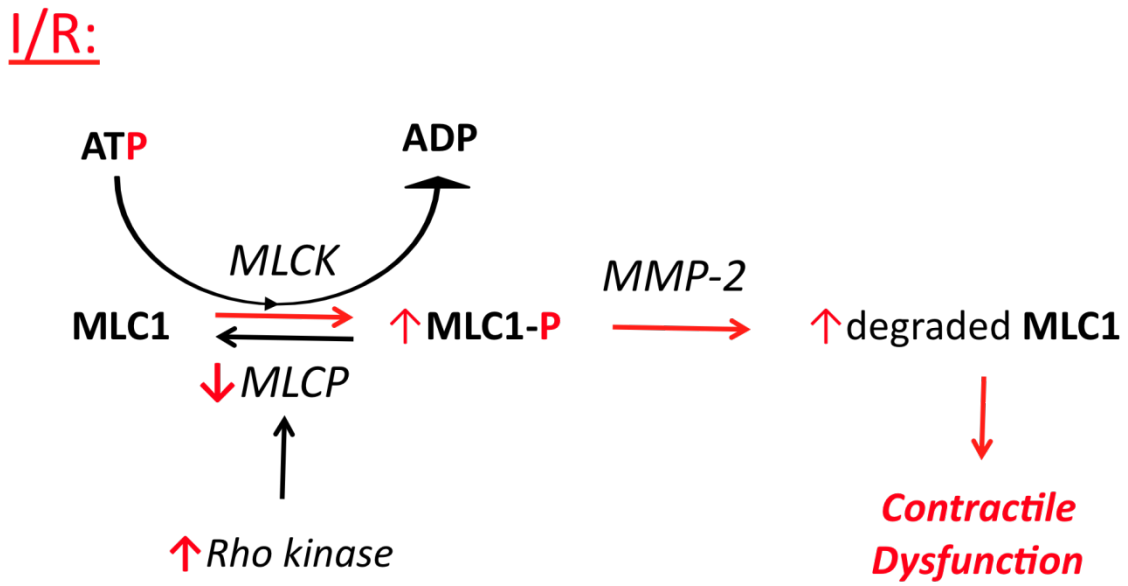

Figure 1.1
Proposed mechanism triggered by reactive oxygen species (ROS) that leads to cardiac contractile dysfunction. MLC1 – myosin light chain 1; MLCK – myosin light chain kinase; MLCP – myosin light chain phosphatase; MLC1-P – phosphorylated MLC; MMP-2 – matrix metalloproteinase-2 (use with permission)

1.4.1 Myosin light chain kinase (MLCK) pathway

Currently, the role of the myosin light chain kinase (MLCK) pathway in the development of cardiovascular disease and I/R injury has gained growing attention (50). MLCK, also known as MYLK, is a Ca^{2+}/calmodulin activated serine/threonine-specific protein kinase that phosphorylates the regulatory light chain of myosin (51-52). Phosphorylation of cardiac myosin heavy chains and light chains by a kinase, such as MYLK3, potentiates the force and the rate of cross-bridge recruitment in cardiomyocytes (53). The MLCK is activated by the extracellular-regulated kinase (54-55). The MLCK pathway has been reported to be involved in the pathology of several cardiovascular disorders (56-57). Studies have shown that, in response to I/R, MLCK activity is increased and this increase in activity is mainly associated with phosphorylation of MLC1, and inhibition of MLCK protects heart from I/R injury by regulation of phosphorylation of MLC (55, 58). These data suggest that inhibition of MLCK's increased phosphorylation of MLC1 can be a valid, therapeutic target. However, the knowledge of the effectors of the MLCK pathways is still limited, and the understanding of the molecular mechanism of MLCK pathway is fundamental for the therapeutic strategy development.

1.4.2 Rho kinase pathway

Rho kinase (ROCK) is one of the major downstream mediators of the GTPase RhoA and plays an important role in the cytoskeleton (59). It is involved in a wide range of crucial cellular functions, such as migration, contraction, proliferation, motility and adhesion (60). Moreover, ROCK regulates contractile force generation by phosphorylating several contractile proteins. It has been well demonstrated that MLC is one of the targets for the phosphorylation by Rho kinase pathway (23). When ROCK is activated by the active form of Rho, ROCK phosphorylates the myosin binding subunit of myosin phosphatase inhibiting its activity, and thus increases the phosphorylation of MLC of myosin II (61-63). Evidence has shown that Rho kinase pathway plays a critical role in the pathogenesis of several cardiovascular disorders (64-66). It is well established that the inhibition of ROCK protects I/R hearts from contractile dysfunction (67). Our research lab has recently indicated that the mechanism of protection of I/R heart, in addition to inhibition of ROCK, may also up-regulate energy metabolic protein such as lactate dehydrogenase (LDH) and glyceraldehyde-3-phosphate dehydrogenase, which is involved in an increase of energy production (67), suggesting that kinase pathways in the I/R heart might also regulates metabolic process.

1.4.3 Protein kinase C (PCK) pathway

Protein kinase C (PKC) is a group of protein serine/threonine kinase that are related to the regulation of protein function via phosphorylation of the proteins. PKC has been classified as 1) the conventional PKCs, the diacyglycerol (DAG), calcium-dependent PKCs; 2) the novel PKCs, which require DAG, but not calcium; 3) the atypical PKCs, which are not stimulated by DAG or calcium, but are stimulate by lipid-derived second messengers (68). PKCs are expressed in most of the tissues and play crucial role in many signal transduction cascades such as muscle contraction (69), platelet aggregation (70) and neuronal excitation (71). It has been shown that there are abundant expression of PKCs in human and rat cardiomyoscytes (72-74). Different isozymes of PKCs might have different effects in response to physiological and pathological conditions. For example, activation of δPKC and εPKC shows a protective effect in ischemic heart (75-77), in contrast, depletion of αPKC by gene knock –out increased myocardial contractility (78). Pharmacological tools have been developed to study the role of PKCs in the pathogenesis of heart injury. These include PKC activator and various PKC inhibitors. However, most of these agents are not selective for the individual PKC isozymes (79-82). In addition, most of the substrate or targets of PKCs have not yet been identified. It is important to identify the proper PKC isozymes and it targets to develop therapeutic strategies for the treatment of pathological cardiac diseases.

1.4.4 Kinase inhibitor

Since it has been found that protein kinases play a significant role in the pathogenesis of cardiovascular disease including I/R injury, hypertension, coronary and vascular vasospasm (83-84), various protein kinases have been targeted for therapeutic potential.

In recent years, several MLCK inhibitors have been reported, including K-252a (85), ML-7 (86), ML-9 (87) and peptide 18 (88). These MLCK inhibitors are newly developed small molecule inhibitors. Small molecule inhibitors allow researchers to have better condition to study multiple aspects of the molecule mechanisms, especially the signaling pathways within the cells. Among these reported inhibitors, ML-7 is one of the most widely used MLCK inhibitors. ML-7 has been reported to be able to protect the heart in I/R injury (50, 89) and have beneficial effects in brain injury (90) and glaucoma (91). Our studies have shown that the cardio protective effects of ML-7's inhibition of MLCK in I/R injury are mainly associated with the protection of contractile function, via regulating the phosphorylation of myosin light chain 1 (MLC1), which in turn, regulate MLC1 degradation

by MMP-2. This data suggests that the inhibition of MLCK (with ML-7, for example) represents a potential for new therapeutic strategies aiming to regulate MLC phosphorylation levels in the treatment of I/R injury.

Other studies suggest that ROCK is a promising pharmacological target for I/R injury. Inhibition of ROCK has been shown to have cardio protective effect for the treatment of I/R injury and myocardial infarction (MI) (92-99). Y-27632, it is one of the well-known ROCK inhibitors, has been reported to be able to reduce the I/R injury, infarct size in response to experimental myocardial infarction (MI) rat model (100). Similar results have shown that administration of Y-27362 results in a more than 80% of functional recovery in the heart subjected to I/R in our research (23). Additionally, potential targets for ROCK have been identified, which includes contractile proteins and metabolic proteins (23, 101).

Protein kinase activation in the cardiovascular diseases induce cellular signaling pathways which have both a beneficial and harmful effect. To date, neither the signaling pathways of protein kinases in pathological heart, nor the molecular mechanism of kinase inhibition treatment are fully investigated. Thus, more studies are necessary for the understanding of protein kinases, and it is the fundamental stage for the development of therapeutic strategy.

1.5 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes involved in the degradation of protein in the matrix (102). MMPs contain zinc ion in their active site, and zinc is indispensable for proper catalytic function (103). It has been described that MMPs are involved in not only the physiological processes such as, platelet aggregation, wound healing (104), bone formation (105) and angiogenesis (106), but also in the pathological processes, such as rheumatoid arthritis (107), multiple sclerosis (108), tumor cell invasion (109), atherosclerosis, myocardial infarction, heart failure, ventricular remodeling and myocardial I/R injury (110-113). To date, among all these MMPs, matrix metalloproteinase-2 (MMP-2) is probably one of the most widely studied types.

1.5.1 Matrix metalloproteinase-2 (MMP-2)

MMP-2 is also known as type IV collagenase and gelatinase A, and it has been extensively described for its extracellular functions. However, a growing number of studies show that it also has important intracellular functions, mainly in pathological conditions (46,

89). One of these intracellular functions is the negative effect of MMP-2 in cardiovascular disease. MMP-2 has been reported to contribute to cardiac dysfunction by degrading intracellular contractile structural proteins, such as myosin light chain 1 (22), troponin I (114), titin (115) and alpha-actinin (116). Under the normal physiological conditions, matrix metalloproteinase-2 activity is regulated at multiple levels. MMP-2 is expressed as a pro-enzyme. Classical activation of MMP-2 involves proteolytic cleavage of the N-terminal propeptide by a membrane-type MMP. Therefore, the key cysteine thiol residue with active zinc site can be exposed for binding. Additionally, there is non-classical activation of MMP-2, for example, MMP-2 can also be activated by the reactive oxygen/nitrogen species, such as peroxynitrite (47,46,117,118). Another mechanism for the regulation of MMP-2 activity is the inhibition by tissue inhibitors of metalloproteinases (TIMPs) (103, 119). Both activated and pro-MMP-2 can be inhibited by TIMPs, and TIMPs can inhibit the membrane-type MMP, and prevent the activation of the MMPs. Besides, TIMPs can directly form a complex with the active MMPs and inhibit its activity (120). In the heart, TIMP-4 is the major isoform expressed and inhibits MMP-2, preventing the cleavage of the propeptide domain of MMP-2 and the consequent activation of MMP-2 (121). Also, MMP-2 can be regulated by the gene transcription and translation or post-translational modification, such as gluathiolation and phosphorylation (119). Moreover, active MMP-2 can be inhibited by many different pharmacological compounds against MMPs.

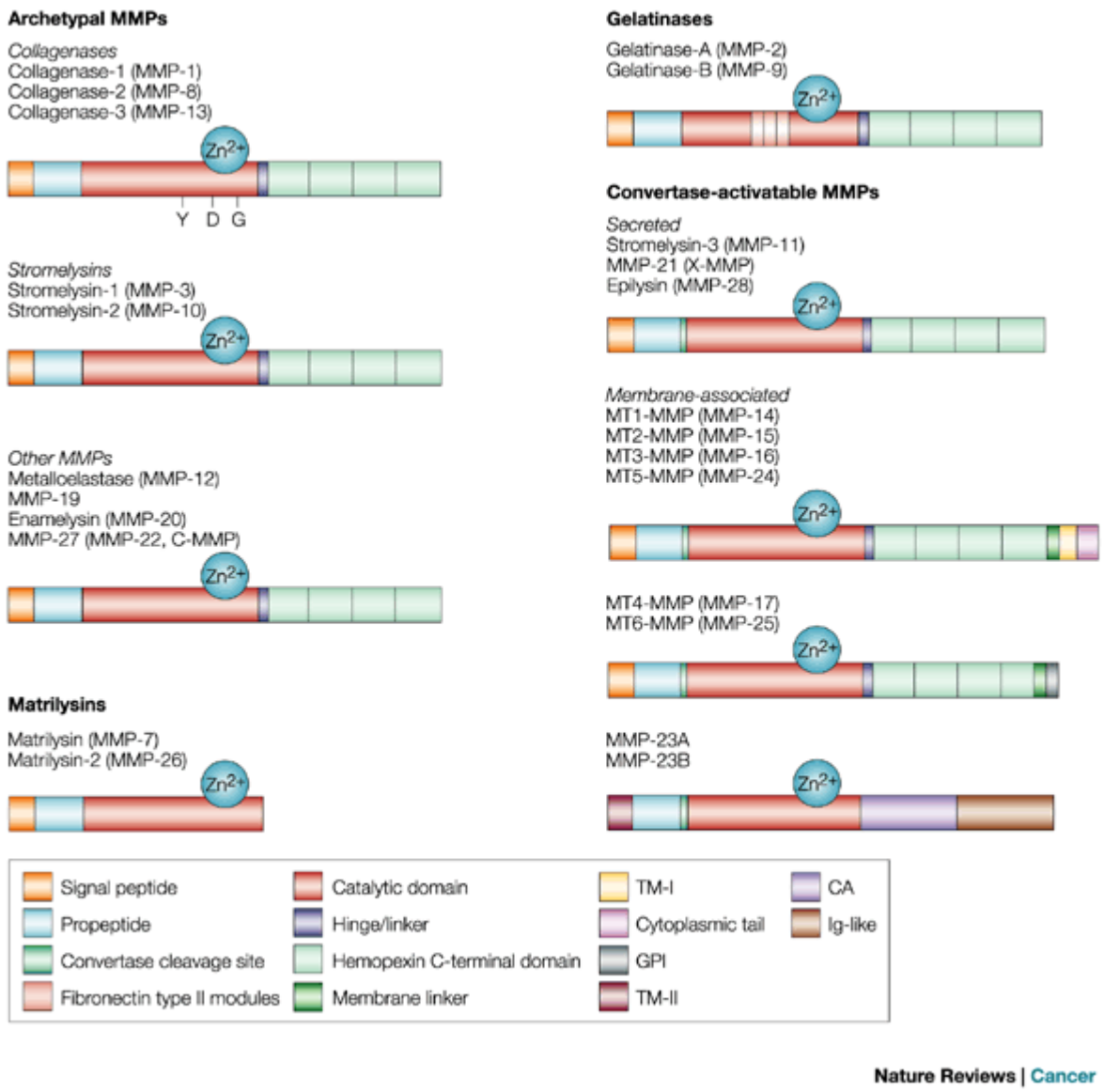

Figure 1.2
Classification of human MMPs. Schematic representation of the structure of the 24 human matrix metalloproteinases (MMPs), which are classified into four different groups on the basis of domain organization. (Overall CM, Lopez-Otin C. Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer. 2002 Sep, use with permission)

1.5.2 Matrix metalloproteinase in ischemia/reperfusion injury

I/R injury is comprised of a series of complicated processes, and these processes range from metabolic to morphological and contractile adaption in response to ischemia/reperfusion. Studies have shown that MMP-2 contributes to acute cardiac dysfunction caused by I/R injury (47, 122). One of the mechanisms by which MMP-2 gets involved in cardiac contractile dysfunction is the degradation of cardiac contractile proteins (22). As mentioned above, contractile machinery proteins, such as myosin light chain 1, myosin light chain 2, troponin I and titin are important for normal cardiac muscle contraction.

The degradation of these important contractile proteins, by MMP-2, in response to conditions of increased oxidative stress, such as I/R, has been discussed previously. Taking together the data suggests that the inhibition of the deleterious intracellular actions of MMP-2 can be an important therapeutic target for the prevention of oxidative stress induced cardiac contractile dysfunction.

1.5.3 Matrix metalloproteinase inhibitors

Pharmacological approaches have been developed against MMPs for more than 30 years. MMP inhibitors, such as batimastat (123), marimastat (124) , ONO-4817 (115), PD-166793 (125) and Ilomastat (126) have been frequently used in different experimental models of pathological conditions. Among the many MMPs inhibitors that are commercially available, few are classically used in cardiovascular studies. Among these inhibitors, phenanthroline and doxycycline have been shown to protect hearts from I/R injury (127) (128).

Phenanthroline is a heterocyclic organic compound that exhibits chelating actions by forming stable complexes with most metal ions, including zinc, a metal ion present in the MMP active site. It is this chelation of zinc that inhibits MMPs activity. Studies on I/R injury have shown that using phenanthroline, as a MMP-2 inhibitor, can prevent the degradation and the release of proteins during reperfusion (122). However, one of the main problems with the use of this compound is its toxicity, due to the fact that the chelation of metal ions is indiscriminate and can affect many other metalloproteins. In addition, phenanthroline is also a broad spectra MMP inhibitor and not specific to MMP-2.

The tetracycline class of antibiotics has been proven to be one of the most clinically useful tools in MMP inhibition, especially doxycycline. Tetracyclines can inhibit MMPs' activity through their ability to chelate the catalytic zinc essential for MMP activity (129). Doxycycline has been used in the experimental model of I/R injury to inhibit MMP-2 and has been shown to improve acute contractile dysfunction (122). Based on the promising results from pre-clinical studies using doxycycline, several clinical studies are currently investigating the efficacy of doxycycline in post MI remodeling and doxycycline in cardio protection during, and following, the coronary artery bypass grafting(130).

It is known that MMP-2 have both extracellular and intracellular functions in pathological condition, and inhibition of MMPs is protective. However, the physiological role of MMP-2 is also important. Thus, the development of a selective MMP-2 inhibitor is crucial to understanding of the molecular mechanisms involved in regulating the

physiological and pathological roles of MMP-2. Moreover, the understanding of how MMP-2 actions contribute to both normal physiology and developing pathology is the key to the development of highly efficient therapies aiming at inhibition of MMP-2.

1.6 Energy metabolic proteins

Cardiomyocyte is the basic functional cell unit in the cardiac tissue, and it is responsible for the contraction of the cardiac muscle. One of the most important features of cardiomyocytes is the high density of mitochondria in the cytoplasm, allowing cardiomyocyte to produce ATP effectively, making them highly resistant to fatigue. Mitochondrion is the factory where aerobic respiration transforms the biochemical energy form nutrients into ATP. Aerobic respiration is constituted of three metabolic processes, which are involved with a variety of metabolic proteins. Most of these metabolic proteins are enzymes which perform different catalytic role through the whole ATP generating process. During physiological conditions, cardiac tissue is abundant in oxygen, and aerobic respiration performs as usual and produces enough ATP for maintaining the normal function of the heart. However, under pathological conditions, such as I/R, lack of oxygen and nutrients leads to the disorder of energy production. Alteration and modulation of metabolic processes and metabolic proteins will occur and complicate this pathological event. Studies focusing on the metabolic protein in the I/R heart have been reported since 1980s (131-132). However, the underlying mechanism in which the energy metabolic proteins contribute to the development of myocardial I/R injury is still unclear. More studies are needed for the understanding of the pathogenesis of myocardial I/R injury regarding to metabolic process.

1.6.1 Glycolysis and citric acid cycle

Glycolysis and citric acid cycle are the first two steps of aerobic respiration. Neither of them requires oxygen, but they act differently in aerobic and anaerobic conditions. Glycolysis is a sequence of biochemical reactions that converts glucose into pyruvate with the concomitant production of a relatively small amount of ATP. Glycolysis is an especially important pathway for organisms that ferment sugars. Glycolysis also serves as a source of raw materials for the synthesis of other compounds. After glycolysis, pyruvate is oxidized to acetyl-CoA, and enters the citric acid cycle. Citric acid cycle is also known as the tricarboxylic acid cycle, or the Krebs cycle (133). Citric acid cycle is a series of biochemical reactions involving different enzymes and co-enzymes, and oxidizes acetyl-CoA to form

high-energy reactive molecules, NADH and FADH, which will be used in the electron transport chain to produce ATP. However, when there is not enough oxygen, the citric acid cycle will not occur, and pyruvate will be fermented into lactic acid. This process is known as anaerobic glycolysis, which has been considered as one of the important energy source for the ischemic heart.

1.6.2 Electron transport chain

Electron transport chain is also known as respiratory chain and oxidative phosphorylation. It is the last and the most important step of the aerobic respiration. This metabolic pathway takes place in the mitochondria and uses its special structure, enzymes and the high-energy reactive molecules released from the oxidation of pyruvate to produce ATP. This whole process is carried out by a set of complexes including NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome bc1 complaex (complex III), cytochrome c oxidase (complex IV). These complexes transport electrons from electron donors to electron acceptors through redox reaction, and couple the electron with protons (H^+) across the mitochondria inner membrane. The protons accumulate in the mitochondrial intermembrane matrix and generate an electrochemical proton gradient, which can perform ATP synthesis via the ATP synthase (complex V), within the electron transport chain. There are five large molecules complex composing the electron transport chain. During aerobic condition, these complexes transfer the electrons to the final electron acceptor oxygen and produce ATP, via ATP synthase, using the proton gradient, and thus supplying most of the physiological demands. However, when oxygen is absent, the electron transport chain cannot perform its normal function. In contrast, complex located in the mitochondrial membrane generates reactive oxygen species (15) during anaerobic condition and cause mitochondria fragmentation, which would eventually lead to cell death. This process has been claimed to play a crucial role in the I/R injury (134).

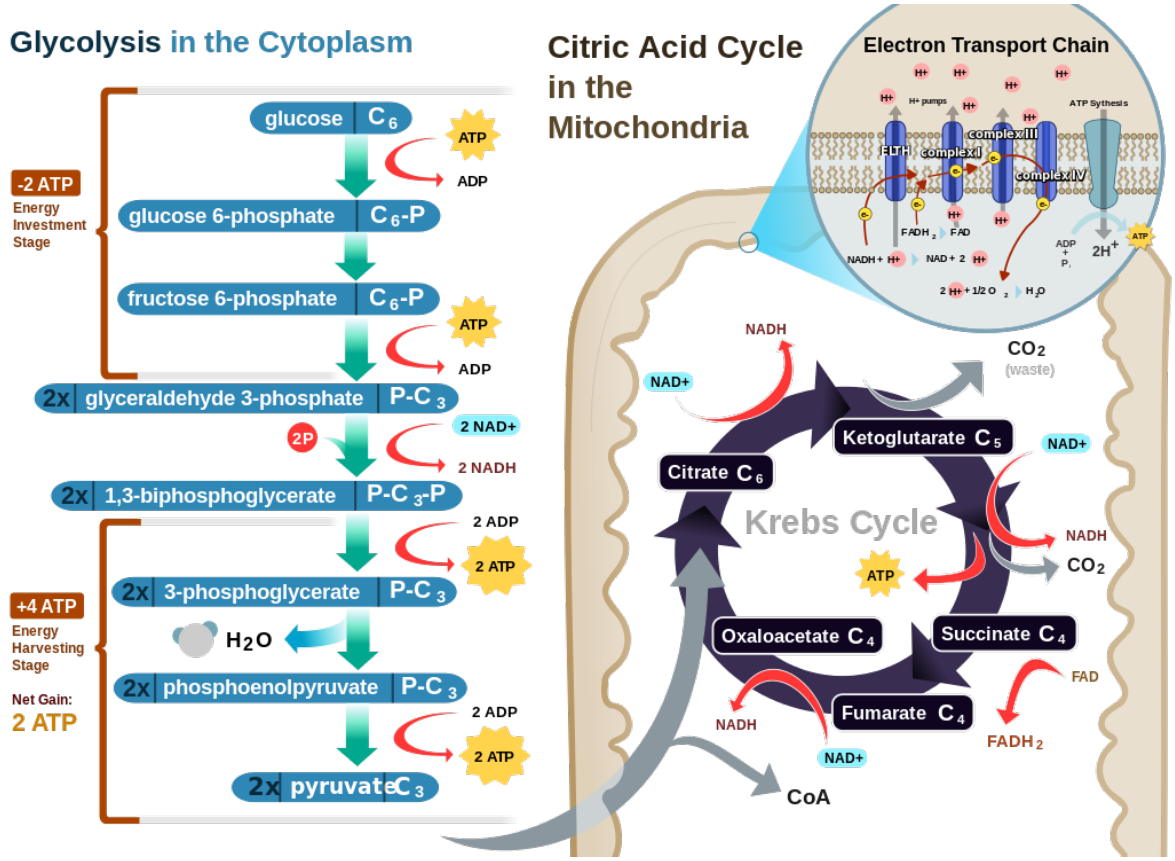

Figure 1.3
A diagram of cellular respiration including glycolysis, Krebs cycle (AKA citric acid cycle), and the electron transport chain (RegisFrey March 2007 Creative Commons CC Attribution-ShareAlike BY-SA 3.0, use with permission)

1.6.3 Energy metabolic pathways in ischemia/reperfusion injury

It has been suggested that electron transport chain is interrupted in anaerobic condition. Interruption of electron transport chain causes an increasing production of ROS, and ROS further activates harmful proteolytic enzyme and kinases, which contribute to the damage and eventually leads to mitochondrial fragmentation and cell death (135). During reperfusion, ROS generation also enhances, due to the suddenly increased oxygen concentration (11). One of the important proteolytic enzymes has been identified, which is matrix metalloproteinase-2 (MMP-2). It has been shown that ROS activates MMP-2, and MMP-2 contributes to the development of ventricle remodeling and other pathological process in the heart (136). On the other hand, mitochondrial antioxidants are overexpressed, preventing the mitochondrial damage from ROS and improving the cardiac tolerance from I/R (137-138). Therefore, these pathways suggest that mitochondria play a significant role in the pathological process during I/R, and acts like a double-edged sword. In addition, during anaerobic condition, for example, in ischemia, lactic acid glycolysis is the major resource for the energy production, since citric acid cycle and oxidative phosphorylation cannot occur. It

has been reported in our study that during I/R, two important enzyme in lactic acid glycolysis, lactate dehydrogenase (LDH) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are up-regulated, which provides the heart with a supplemental energy generation (23). Moreover, another process of energy production, citric acid cycle, has rarely been focused. Even though citric acid cycle does not require oxygen, pyruvate will not be oxidized to acetyl-CoA and enter the citric acid cycle. During I/R, the role of citric acid cycle is still unclear. Some suggest that it could be an important ATP source (139) and others claim that it may contribute to ROS generation (140). It is intricate how the energy metabolic pathways are modulated in the pathological process during I/R, and the understanding of these molecular mechanisms is crucial for the development of innovative treatments for cardiovascular diseases.

1.7 Proteomic approach

"PROTEOME" was introduced in 1994, and has been given great attention for two decades. As we know that proteins are the basic functional unit in the cells and the most common diagnostic and therapeutic targets in medicine. The search of proteome can discover new diagnostic and therapeutic targets, which leads to the development of new strategies for diagnosis and treatments. Proteome is the protein expression profile of cells, tissues or the intact organs under a certain condition, such as normal physiological condition, pathological condition and experimental condition. Proteomics is the dynamic changes of the protein expression profile under these different conditions. Proteomics approach is one of the most common methods to search and discover the new diagnostic and therapeutic target for different diseases. The use of proteomics to study cardiovascular diseases has allowed the characterization of global alterations in comparative protein expression and identifies the biomarkers and the treatment target for its pathogenesis. Two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) are the most fundamental techniques in proteomics research. In most of the cases, the individual proteins and their fragments or modifed forms are separated by their isoelectric point and molecular weight using 2-DE. After 2-DE, quantitative analysis of protein spots intensity from 2-DE was measured which are then further identified and analyzed by MS. Proteomic methodology has been developing with the improvement of bioinformatics methods to correlate enzymatic digest mass fingerprints and peptide fragmentation data arising from mass spectrometry analysis with genome sequence. The combination of physical protein data from MS with genome data has made unambiguous identification of proteins from 2-DE gels much more rapid and much cheaper than

conventional peptide sequencing (141). Many proteome studies have been done for the comparative protein expression among different physiological and pathological conditions in the heart, and more than 50 proteins in heart diseases have been identified. Studies suggest that these proteins are important in the regulation of the heart function (142-143). To date, more proteomic research focusing on the cardiovascular diseases are still under operation all over the world, and hence it can be predicted that more data will be generated in the upcoming future.

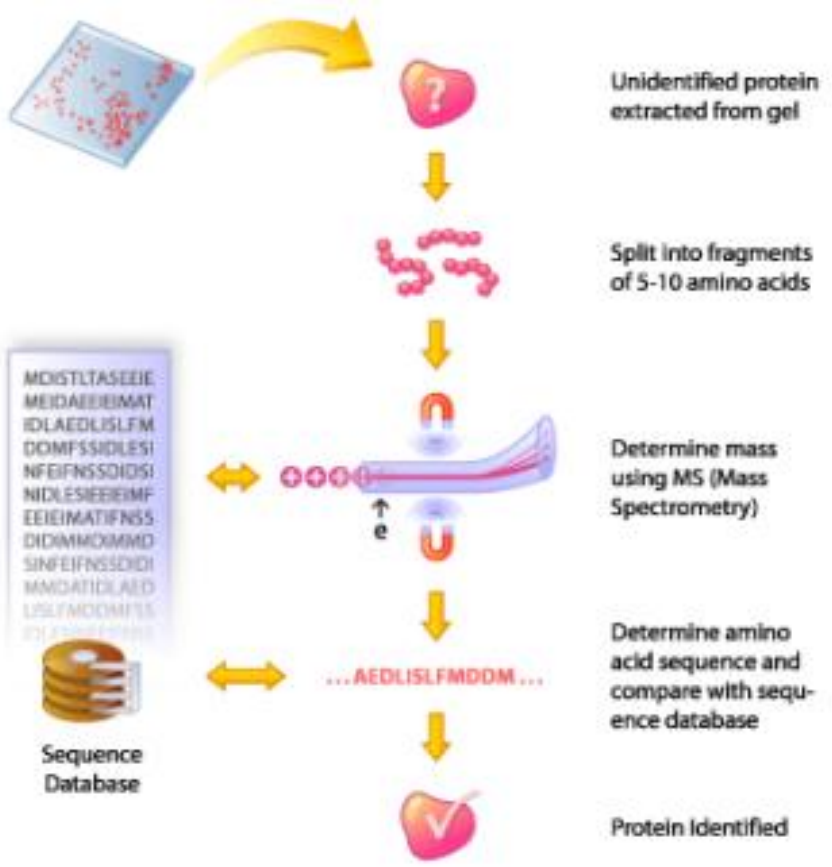

Figure 1.4
A proteomic workflow diagram, showing sample separation by gel matrix, protein digestion, mass analysis by a mass spectrometer, validated search analysis, and protein identification. (Vincent Shen, Mistaken identities in proteomics, Biotechniques 2011January, use with permission)

1.8 Hypothesis

It is established that MMP-2 is activated and degrades contractile proteins during I/R, and many other factors including metabolic enzymes, protein kinases and structural proteins are affected by I/R. However, the molecular mechanisms responsible for these changes are still not clear. Since MMP-2 is known for its broad spectrum of action, I hypothesize that, in addition to contractile proteins, proteins related to regulation of energy metabolism are MMP-2 targets during I/R, and protein kinase such as myosin light chain kinase (MLCK) is also involved in this process. The use of proteomics in studying heart injury triggered by I/R will reveal new potential targets for pharmacological protection of heart from I/R induced contractile dysfunction. In addition, selective inhibition of MMP-2 using MMP-2 siRNA will protect the heart from I/R injury.

1.9 Objectives

My PhD project had five main objectives:

1. To identify proteomic profile in the heart subjected to I/R injury
2. To compare the proteomic profile in right and left ventricles from the heart subjected to ischemia/reperfusion injury
3. To determine the effect of the Myosin Light Chain Kinase inhibitor ML-7 on the proteome of hearts subjected to I/R injury
4. To analyze the inhibition of MMP-2 gene expression with small interfering RNA in isolated cardiomyocytes by proteomic analysis, with the focus on the degradation of energy metabolic proteins by MMP-2
5. To determine the cardioprotective effect of inhibition of MMP-2 using MMP-2 siRNA on isolated cardiomyocytes.

The underlying mechanism of I/R-induced heart contractile dysfunction is still unclear. Many factors have been shown to contribute to the development in the I/R injury. Using proteomics approaches provide a wide range and comprehensive profiles of the dynamic alteration of the proteins. This will give the investigators evidence, links and clues to integrate the mechanism. From the wide range of the protein profile, we have mainly focused on the energy metabolic proteins in this study. It has been demonstrated that energy metabolic proteins are involved in the development of I/R injury, however, the underlying molecular mechanisms of the modulation of those proteins, especially the pathways of protein-protein interaction among energy metabolic proteins, kinases and proteolytic enzymes, are still poorly understood. We considered that the study of changes in proteomic profile of energy metabolic proteins may be a key to reveal the pathological mechanisms of I/R injury. The protection of the heart from degradation of contractile proteins by inhibition of myosin light chain kinase using pharmacological compound ML-7 has been shown in our studies. However, we do not know whether energy metabolic proteins are related to the myosin light chain kinase pathway, or if they are protected by ML-7 during I/R. In this study, we investigated the effect of myosin light chain kinase inhibitor ML-7 on the proteome of heart subjected to I/R injury. We have also shown that the inhibition of MMP-2 protects the heart from I/R injury. So far, we have only revealed that MMP-2 degrades contractile proteins, but no one knows whether MMP-2 degrades energy metabolic proteins. In the other words, no one knows whether inhibition of MMP-2 would affect the energy metabolic proteins. We

started to study the proteomics of inhibition of MMP-2 in cardiomyocyte. The major propose of this study is to conceive a basic frame of the regulatory pathways of energy metabolic enzyme during I/R, in order to develop novel therapeutic strategies aimed at energy production during I/R.

2. Proteomic analysis of right and left cardiac ventricles under aerobic conditions and after ischemia/reperfusion

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# These authors contributed equally to this study

VJJC – Experimental design, experimental work, manuscript writing

H-BL –Experimental work, experimental design, manuscript writing

JS – Experimental work

MW – manuscript writing

GS – Hypothesis generation, experimental design, manuscript writing

2.1 Preface

The manuscript presented here has been published in the journal *Proteomics (Cadete and Lin et al.2012)*. This manuscript addressed objectives 1 and 2 of my PhD project.

1. To identify proteomic profile in the heart subjected to I/R injury
2. To compare the proteomic profile in right and left ventricle from heart subjected to ischemia/reperfusion injury

This manuscript is the first approach of the project. We demonstrated the proteomic profile of the I/R heart from a completely different angle, namely we evaluated proteomic alteration in different regions of the heart. Since the studies of right ventricle have been overshadowed by numerous studies of left ventricle and the right ventricle is often neglected on cardiac research, in this study, we compared for the first time the proteomic alteration in right and left ventricle from heart subjected to I/R injury. Our finding determined for the first time that there are different protein expressions between left and right ventricles response to I/R injury. In addition, results show that there are many energy metabolic proteins affected in I/R injury. This finding gave us some new ideas of the mechanism of I/R injury, namely not only are contractile proteins effected in I/R, but also energy metabolic proteins are involve in I/R injury. This study provided the research foundation for pursuing the underlying molecular mechanism of energy metabolic proteins regulation in I/R injury.

2.2 Abstract

Ischemia/reperfusion (I/R) injury is a major consequence of cardiovascular interventions. The study of changes of the left and right ventricle proteomes from hearts subjected to I/R may be key to revealing the pathological mechanisms underlying I/R-induced heart contractile dysfunction.

Isolated rat hearts were perfused under aerobic conditions or subjected to 25 min global ischemia and 30 min reperfusion. At the end of perfusion right and left ventricular homogenates were analyzed by 2-dimensional electrophoresis (2-DE).

Contractile function and coronary flow were significantly reduced by I/R. 2-DE followed by mass spectrometry identified 10 protein spots whose levels were significantly different between aerobic left and right ventricles, 8 protein spots whose levels were different between aerobic and I/R left ventricle, 10 protein spots whose levels were different between aerobic and I/R right ventricle 10 protein spots whose levels were different between the I/R groups. Among these protein spots were ATP synthase β subunit, myosin light chain 2, myosin heavy chain fragments, peroxiredoxin-2 and heat shock proteins, previously associated with cardiovascular disease.

These results reveal differences between proteomes of left and right ventricle both under aerobic conditions and in response to I/R which contribute to a better understanding of I/R injury.

2.3 Introduction

The heart can be viewed as two independent pumps, the one delivering blood to the lungs, the other to the rest of the body. The right heart, containing right atrium and ventricle, pumps blood through the pulmonary circulation. The left heart comprised of the left atrium and ventricle and pumps blood through the systemic circulation. Because the contractile work performed by each side of the heart is different the anatomy of right and left ventricles (the most important functional parts of the heart) is also different. The left ventricular wall is thicker than the right due to the fact that resistance and pressures are higher in the systemic branch of the circulatory system, in comparison to the pulmonary circulation. Although the knowledge and understanding of cardiac anatomy and function is abundant, very little is known about differences in protein profiling and protein levels between different regions of the heart, including ventricles. Differences have been shown in protein expression levels between left and right ventricles in physiological conditions (144), but nothing is known about differences in protein expression levels between right and left ventricle from injured heart. Few studies have shown changes in cardiac protein expression but are limited to analysis of left ventricle (46, 145) or mitochondria (146).

Acute or chronic reduction in oxygen supply and/or nutrients can lead to severe, irreversible damage to the cardiac tissue and seriously impact cardiac contractile function. Studies of protein levels in myocardial tissue exhibiting contractile dysfunction have shown changes in protein expression in different regions of the heart that can be dependent on protein degradation, or changed expression of protein levels due to protein synthesis or release from the cell (147). Studies have shown that during ischemia, significant protein degradation is associated with contractile dysfunction (22, 46-47, 145). The study of the proteomes of the damaged heart due to ischemia/reperfusion (I/R) injury is key to a better understanding of the pathogenic mechanisms underlying I/R-induced contractile dysfunction.

The development of I/R injury, such as occurs in myocardial infarction (MI), has been attributed to several factors. Many of these factors have been identified either by using whole heart homogenates or left ventricle samples. The structural consequences of MI in adults are mostly dictated by left ventricular remodeling, leading to heart failure (148). Failure of proper oxygen delivery caused by a decrease in blood flow together with increased oxygen demand by the heart results in tissue ischemia. Severe ischemia can cause the cardiac muscle to die due to prolonged hypoxia, such as during MI.

Functional studies of the right ventricle have been overshadowed by studies of left ventricle function and the right ventricle is often ignored on cardiovascular studies. The role of the right ventricle was later recognized in certain conditions, such as in the follow-up of some corrected and non-corrected congenital heart diseases, in valvular heart diseases, in acute and chronic coronary arterial diseases, in pulmonary hypertension and in congestive heart failure (149).

Since different cardiac pathologies resulting in ischemia affect different regions of the heart, it is important to understand the molecular mechanisms by which different regions of the heart respond to ischemia. In this project, we focused on comparative studies of proteomes of left and right ventricles in physiological (aerobic perfusion) and pathological (I/R) conditions. Here we show that the right and left ventricles exhibit different aerobic proteomes. Moreover, when subjected to ischemia, the proteomic response of the right and left ventricles is also different.

2.4 Materials and Methods

The experimental procedures described below conform to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

2.4.1 Isolated heart perfusions

Male Sprague-Dawley rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The hearts were rapidly excised and briefly rinsed by immersion in ice-cold Krebs-Henseleit buffer. Spontaneously beating hearts were placed in a water-jacketed chamber (EMKA Technologies) to maintain their temperature at 37°C. Hearts were perfused in the Langendorff mode at a constant pressure of 60 mm Hg with modified Krebs-Henseleit buffer at 37ºC containing (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (3.0), $NaHCO_3$ (25), glucose (11), and EDTA (0.5), and gassed continuously with 95% O2/5% CO2 (pH 7.4).

2.4.2 Ischemia/reperfusion protocol

Control hearts (aerobic control, n=5 were perfused aerobically for 45 minutes. Ischemic hearts (I/R, n=4) after 25 min at aerobic perfusion were subjected to 20 minutes global no- flow ischemia by closing of aorta inflow line, followed by 30 min reperfusion. At the end of the perfusion protocol ventricular free wall biopsies from right and left ventricles were isolated and quickly snap frozen in liquid nitrogen and kept at -80˚ C until further analysis was perform.

2.4.3 Preparation of heart protein extracts

Protein samples for 2-dimensional electrophoresis (2DE) were prepared at room temperature by mixing frozen (-80°C), powdered heart tissue (40 to 60mg wet weight) with 200 µL rehydration buffer (8 mol/L urea, 4% CHAPS, 10 mmol/L DTT, 0.2% Bio-Lytes 3/10 [BioRad]) at room temperature. Samples were sonicated twice for 5 seconds and centrifuged for 10 minutes at 10,000g to remove any insoluble particles. Protein content of the heart extract in rehydration buffer was measured with the BioRad protein assay.

2.4.4 Two-Dimensional electrophoresis (2-DE)

Because we could not analyze more than 12 samples in 2DE setting (see explanation below) for analysis of ventricle proteomes we used 3 ventricular samples (n=3) for each of

the 4 groups.

Protein (0.4 mg) from ventricular samples (n=3/group) was applied to each of 11 cm immobilized linear pH gradient (5 to 8) strips (IPG, BioRad), with rehydration for 16–18 h at 20ºC. For isoelectrofocusing (IEF), the BioRad Protean IEF cell was used with the following conditions at 20ºC with fast voltage ramping: step 1: 15 min with end voltage at 250 V; step 2: 150 min with end voltage at 8000 V; step 3: 35 000 V-hours (approximately 260 min). After IEF, the strips were equilibrated according to the manufacturer's instructions. Second dimension of 2-DE was then carried out with Criterion pre-cast gels (8 – 16%) (BioRad). After separation, proteins were detected with Coomassie Briliant Blue R250 (BioRad). To minimize variations in resolving proteins during the 2-DE run, 12 gels were run simultaneously using a Criterion Dodeca Cell (BioRad). Because of this limitation for 2-DE analysis we used 3 hearts from each group. All the gels were stained in the same bath and next scanned with calibrated densitometer GS-800 (BioRad). Quantitative analysis of protein spots intensity from 2-DE was measured with PDQuest 7.1 measurement software (BioRad). The protein spot sensitivity threshold used to determine significant changes in protein spot size and density is based on 4 parameters: minimum peak value sensitivity, smallest spot area, largest spot area, and a noise filter level. Using these criteria for protein resolution and staining, we are able to obtain high reproducibility to analyze both a single protein from the same sample run in different gels (150) and a specific protein from different heart samples (22). Only protein spots with relative intensity between 2 and 500 arbitrary units were considered for analysis.

2.4.5 Mass Spectrometry (MS)

Protein spots of interest were manually excised from the 2-DE gel. These spots were then processed using a MassPrep Station from Micromass using the methods supplied by the manufacturer. Briefly, the excised gel fragment containing the protein spot was first destained in 200 µl of 50% acetonitrile with 50 mM ammonium bicarbonate at 37°C for 30 minutes, reduced, alkylated, digested with trypsin and extracted overnight at room temperature with 50 µL of a mixture of formic acid, water, and isopropanol (1:3:2, vol:vol). The resulting tryptic digests were then analyzed by mass spectrometry (MS). For electrospray, quadruple time-of-flight (Q-TOF) analysis, 1 µl of the solution was used. Liquid chromatography/mass spectrometry (LC/MS) was performed on a CapLC high-performance liquid chromatography unit (151) coupled with Q-TOF-2 mass spectrometer (Micromass). A mass deviation of 0.2 was tolerated and one missed cleavage site was allowed. Resulting values from mass

spectrometry (MS/MS) analysis were used to search against the NCBInr and SwissProt databases with Mammalia specified. We used the Mascot (www.matrixscience.com) search engine to search the protein database.

2.4.6 Immunoblot analysis

Frozen heart tissue powder was homogenized on ice in 150 mM NaCl, 50 mmol/L Tris-HCl (pH 7.4) containing protease inhibitor cocktail (Sigma, St Louis, MO, USA) and 0.1% Triton X-100. Homogenates were centrifuged at 10 000*g* at 4°C for 10 minutes, and the supernatant was collected and stored at -80°C until use.

Protein extracts from the same hearts used for 2DE (30 μg protein) from each of the groups were separated by SDS-PAGE (n=3/group). After electrophoresis proteins were transferred to a PVDF (polyvinylidene difluoride) membrane (BioRad). Membranes were probed with rabbit polyclonal anti-peroxiredoxin II (Santa Cruz Biotechnology), rabbit polyclonal anti-MLC2 (AbCam), rabbit polyclonal anti-enoyl-CoA hydratase (Santa Cruz Biotechnology), mouse monoclonal anti-heat shock protein-70 (Santa Cruz Biotechnology) according to the supplier's instructions. Goat anti-rabbit and goat anti-mouse secondary antibodies, tagged with Alexa Fluor647 and Alexa fluor488 respectively (Invitrogen), were used and membranes developed with VersaDoc5000 using appropriate filters. Band densities were determined with Quantity One software (BioRad).

2.4.7 Statistical Analysis

The protein spot levels were analyzed using PDQuest measurement software and evaluated by Kruskal Wallis and Mann-Whitney U test. The proteins of interest were also analyzed with unpaired t-test followed by identification by mass spectrometry. ANOVA or Kruskal-Wallis test was used in functional studies. Data are expressed as mean±SEM.

2.5 Results

2.5.1 Cardiac function

Contractile function and coronary flow were used as a measurement of cardiac function. Contractile function is shown as rate pressure product (product between left ventricle developed pressure and heart rate) – RPP. Hearts subjected to 25 min of global, no-flow ischemia followed by 30 min of reperfusion showed a nearly zero recovery of contractility during reperfusion in comparison to time-matched, aerobically perfused hearts (Figure 2.1, top). Also, coronary flow was significantly reduced (approximately 75 %) during reperfusion (Figure 2.1, bottom).

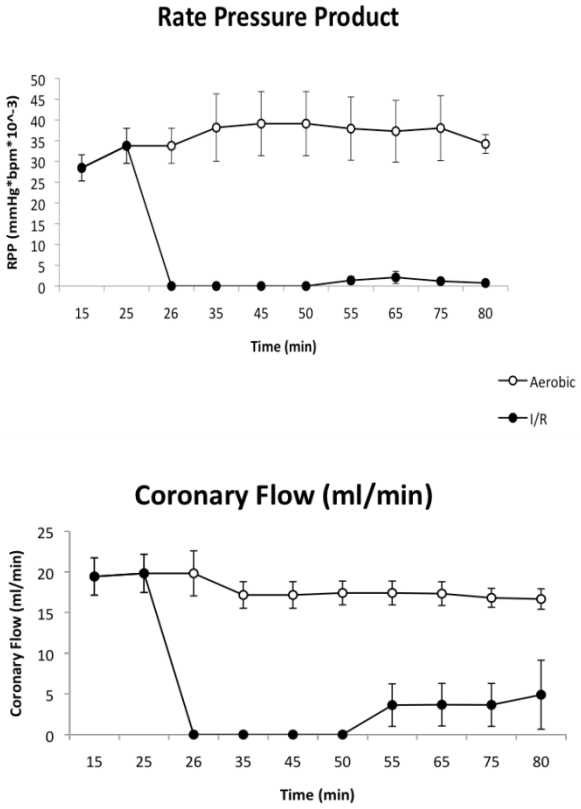

Figure 2.1
Mechanical function of rat hearts perfused aerobically or subjected to 25 min of global, no-flow ischemia followed by 30 min reperfusion. Contractile function was assessed as RPP (rate pressure product) and calculated as the product of left ventricular developed pressure by heart rate (top panel). Coronary flow (bottom panel) was evaluated by measurement of Krebs-Henseleit buffer flow through coronary arteries in milliliters per minute. n= 4/5.* $p<0.05$ vs. Aerobic controls

2.5.2 Right and left ventricle proteomes from aerobic hearts

In order to determine changes in protein abundance in left and right ventricles of the heart and the effect of ischemia on the heart tissue, we have compared the 2-D electrophorograms of left ventricular and right ventricular myocardial tissue samples from

aerobic and ischemic tissues. Representative 2-D electrophorograms from each analyzed group are shown in figure 2.2.

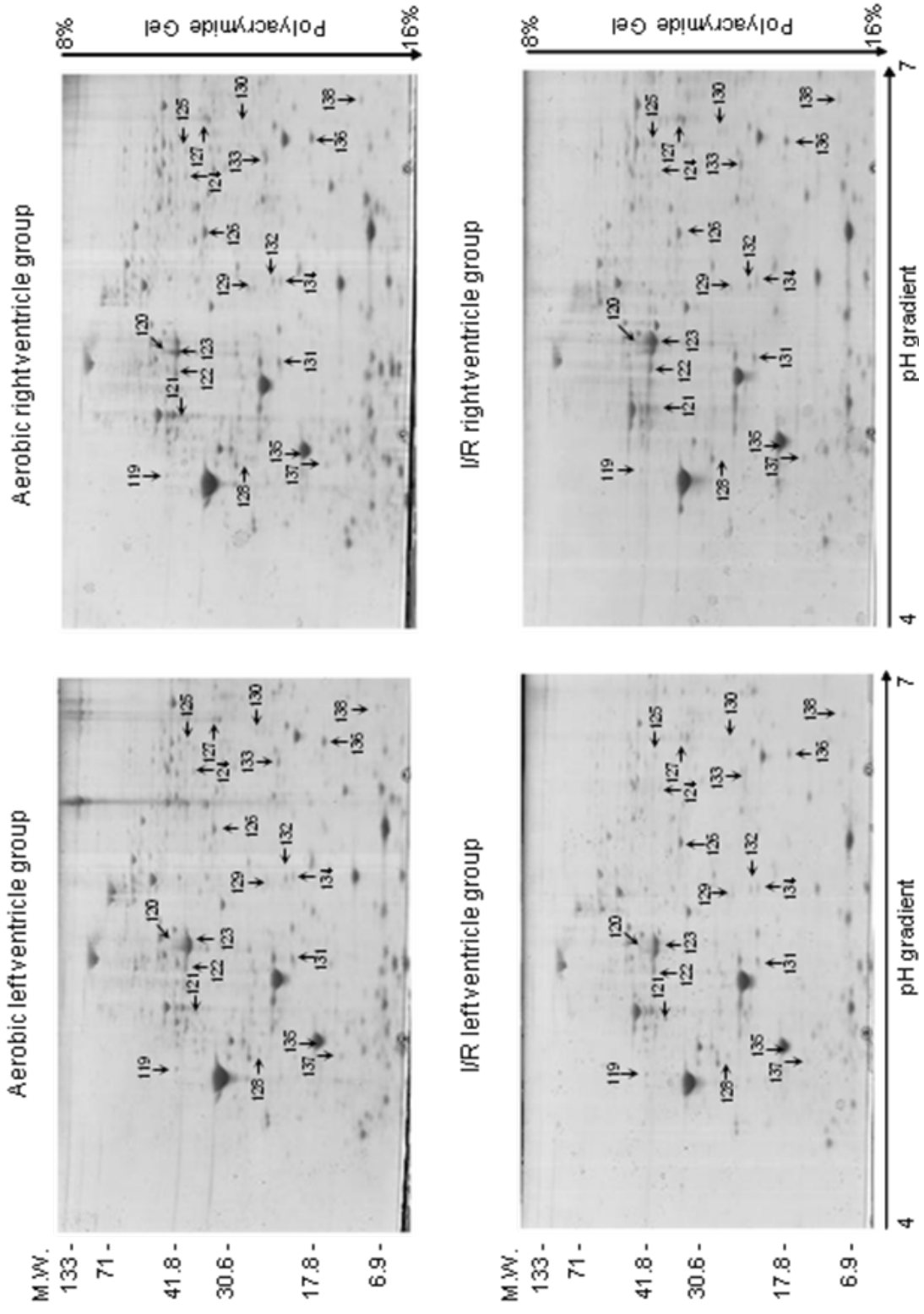

Figure 2.2
Two-dimensional electrophoresis (2-DE). Representative 2-DE gels from each of the analyzed groups. Protein spots found to be significantly changed between groups are indicated with arrows and the respective protein spot number.

Comparison of the 2D gels from right and left ventricles revealed significant differences between the analyzed proteomes. The protein identity, accuracy of protein identification and number of matched peptides are summarized in tables 2.1-2.3. Proteins whose levels showed statistically significant differences in between left and right ventricles (aerobic or I/R) or between aerobic and ischemic heart tissues were included in the tables. For better visual presentation the altered proteins are grouped in 3 groups according to their abundance; 2-20, 20-80 and 80-500 arbitrary units (Fig. 2.3-2.6). Analysis of right and left ventricle proteomes from aerobically perfused hearts revealed 10 proteins showing different protein levels (Fig. 2.3). Of the 10 protein spots 9 proteins were identified by mass spectrometry. Structural proteins (vimentin, cardiac myosin light chain 2) and unknown protein (the 10^{th} protein) showed higher level in left ventricle. Also, the levels of proteins involved in energy metabolism (mitochondrial F1-APTase β subunit, isovaleryl-CoA dehydrogenase and chain a, 2-enoyl-CoA hydratase) were higher in left ventricle in comparison to right ventricle. In contrast, the level of other proteins also involved in energy metabolism, such as subunits of enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, cytochrome b-c1 complex and of ATP synthase, were higher in right ventricle. In addition peroxiredoxin 3, an antioxidant protein, was identified and its level was higher in the right ventricle (Fig. 2.3).

Table 2.1 Identification of protein spots from aerobic left ventricle and aerobic right ventricle heart homogenates – differences in protein profile between left and right ventricle perfused under aerobic conditions.

Protein spot NO.	Fold change	Mowse score a)	Queries matched	Sequence coverage (%)	pI/MW(kDa)	Identified protein
Energy metabolism						
127 ↓b)	-1.8	310	5	8%	9.11/83.20	mitochondrial long-chain enoyl-CoA hydratase/3-hydroxycyl-CoA dehydrogenase α-subunit
125 ↑	2	108	4	6%	8.03/48.86	isovaleryl-CoA dehydrogenase, mitochondrial precursor
128 ↑	2.9	398	7	31%	5.07/38.74	F1-ATPase β subunit
130 ↑	1.3	373	7	31%	6.41/28.55	chain A, 2-enoyl-CoA hydratase
132 ↓	-1.4	474	7	14%	5.57/53.50	cytochrome b-c1 complex subunit 1, mitochondrial precursor
134 ↓	-1.3	117	2	11%	6.17/18.81	ATP synthase subunit d, mitochondrial
Structural proteins						
119 ↑	1.9	704	13	31%	5.06/53.76	vimentin
135 ↑	1.2	891	8	42%	4.86/18.88	myosin light chain 2
137 ↑	3.3	162	3	25%	5.48/20.90	unknown protein
Others						
133 ↓	-1.6	494	6	24%	7.14/28.57	peroxiredoxin 3

a) Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 37 indicate identity or extensive homology ($p<0.05$).

b) The arrows (↑↓) indicate statistically significant changes in protein levels (*versus* aerobic right ventricle)

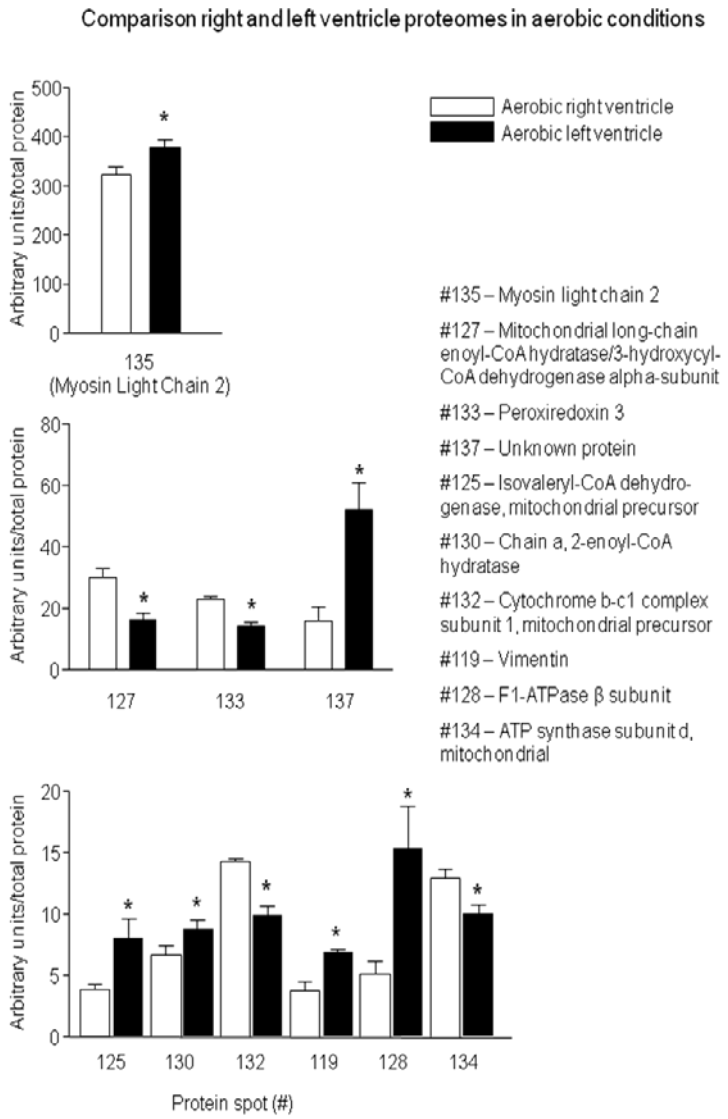

Figure 2.3
Comparison right and left ventricle proteomes from aerobic hearts. For better visual presentation the altered proteins are grouped in 3 groups. Each panel represents one protein group according to the level of abundance. n=3/group * $p<0.05$ vs. aerobic right ventricle.

2.5.3 Effect of I/R on protein profile in left ventricle

Ischemia induced several changes in protein levels in left ventricle (Fig. 2.4). Eight proteins were altered by I/R. The levels of structural proteins involved in contractile function, such as two isoforms of α-actin and myosin heavy chain 5, as well as dnaK-type molecular chaperone hsp72-ps 1 (also known as a heat shock protein 70) were elevated. In contrast heat shock protein β-6, myosin light chain 2, β subunit of F1-ATPase and an unknown protein were significantly reduced in response to ischemia (Fig. 2.4).

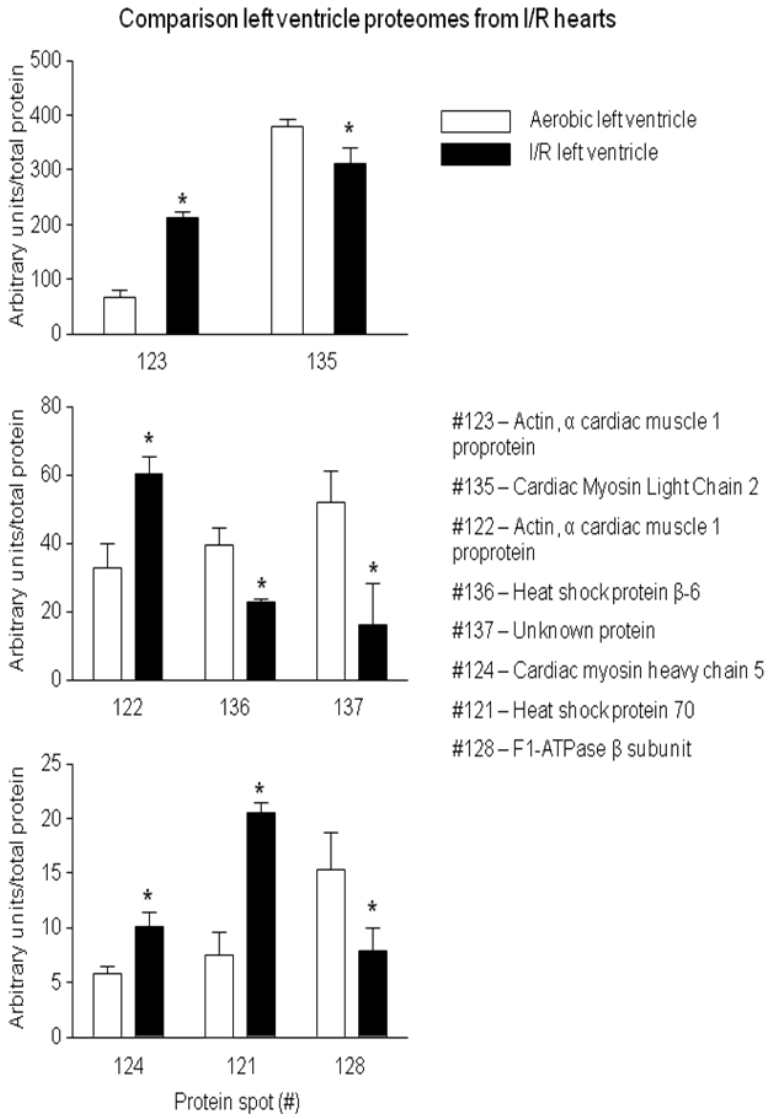

Figure 2.4
Effect of ischemia/reperfusion on ventricle proteomes. For better visual presentation the altered proteins are grouped in 3 groups. Each panel represents one protein group according to the level of abundance. n=3 * $p<0.05$ vs. Aerobic left ventricle

Table 2.2 Identification of protein spots from aerobic left ventricle and ischemic left ventricle heart homogenates – Effect of ischemia on protein profile in left ventricle.

Protein spot NO.	Fold change	Mowse score a)	Queries matched	Sequence coverage (%)	pI/MW(kDa)	Identified protein
Energy Metabolism						
128 ↓	-1.9	398	7	31%	5.07/38.74	F1-ATPase β subunit
Structural proteins						
122 ↑	1.8	751	12	34%	5.23/42.33	actin, α cardiac muscle 1 proprotein
123 ↑	3.1	792	11	36%	5.23/42.33	actin, α cardiac muscle 1 proprotein
135 ↓	-1.2	891	8	42%	4.86/18.88	myosin light chain 2
124 ↑	1.7	328	7	16%	5.52/47.88	cardiac myosin heavy chain 5
Others						
121 ↑	2.7	468	8	13%	5.43/71.11	heat shock protein 70
136 ↓	-1.7	415	6	32%	6.05/17.55	heat shock protein β-6

a) Ions score is -10*Log (P), where P is the probability that the observed match is a random event.
Individual ions scores > 37 indicate identity or extensive homology ($p<0.05$).

b) The arrows (↑↓) indicate statistically significant changes in protein levels (*versus* aerobic left ventricle)

2.5.4 Effect of I/R on protein profile in right ventricle

Ischemia resulted in alteration of 10 protein levels (Fig. 2.5). Of 5 proteins whose levels were increased, two are involved in fatty acid metabolism namely isovaleryl-CoA dehydrogenase and enoyl-CoA hydratase. In contrast the levels of subunit d of ATP synthase were decreased. The other 3 proteins whose levels were increased are: heat shock proteins 70 and β-1 and the antioxidant protein peroxiredoxin-2.

The 5 proteins whose levels were decreased include 2 enzymes involved in energy metabolism (B chain of L-lactate dehydrogenase and subunit 1 of cytochrome b-c1 complex,precursor). The levels of heat shock protein β-6 and histidine triad nucleotide-binding protein 1 were also reduced (Fig. 2.5).

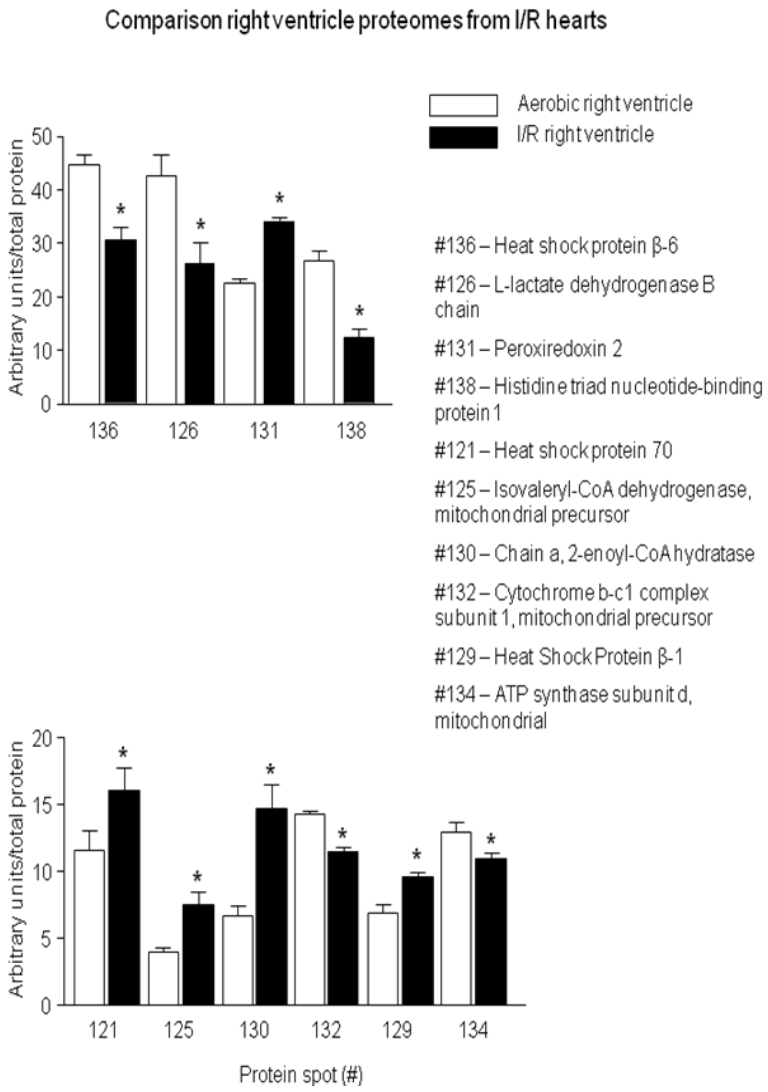

Figure 2.5
Effect of ischemia/reperfusion on protein profile in right ventricle. For better visual presentation the altered proteins are grouped in 2 groups. Each panel represents one protein group according to the level of abundance.n=3 * $p<0.05$ vs. Aerobic right ventricle

Table 2.3 Identification of protein spots from aerobic and ischemic right ventricle heart homogenates – Effect of ischemia on the protein profile in right ventricle.

Protein spot NO.	Fold change	Mowse score a)	Queries matched	Sequence coverage (%)	pI/MW(kDa)	Identified protein
Energy metabolism						
125 ↑b)	1.9	108	4	6%	8.03/48.86	isovaleryl-CoA dehydrogenase, mitochondrial precursor
126 ↓	-1.6	768	14	46%	5.70/36.87	L-lactate dehydrogenase B chain
130 ↑	2.2	373	7	31%	6.41/28.55	chain A, 2-Enoyl-Coa hydratase
132 ↓	-1.2	474	7	14%	5.57/53.50	cytochrome b-c1 complex subunit 1, mitochondrial precursor
134 ↓	-1.2	117	2	11%	6.17/18.81	ATP synthase subunit d, mitochondrial
Other						
121 ↑	1.4	468	8	13%	5.43/71.11	heat shock protein 70
129 ↑	1.4	589	9	42%	6.12/22.94	heat shock protein beta-1
131 ↑	1.5	573	7	31%	5.34/21.91	peroxiredoxin 2
136 ↓	-1.4	415	6	32%	6.05/17.55	heat shock protein beta-6
138 ↓	-2.2	180	4	42%	6.36/13.88	histidine triad nucleotide-binding protein 1

a) Ions score is -10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores > 37 indicate identity or extensive homology ($p<0.05$).
b) The arrows (↑↓) indicate statistically significant changes in protein levels (*versus* aerobic right ventricle)

2.5.5 Right and left ventricle proteomes from I/R hearts

Comparison of the 2D gels of samples from right and left ventricles of I/R hearts revealed 8 proteins spots which were significantly different. Levels of 5 proteins were decreased (Fig. 2.6) and these proteins were identified as: α actin, heat shock proteins β-1 and β-6, proxiredoxin 2 and chain a of 2-enoyl-CoA hydratase. The group of proteins that

revealed an increase in their levels includes: myosin heavy chain 5, heat shock protein 70 and vimentin (Fig. 2.6).

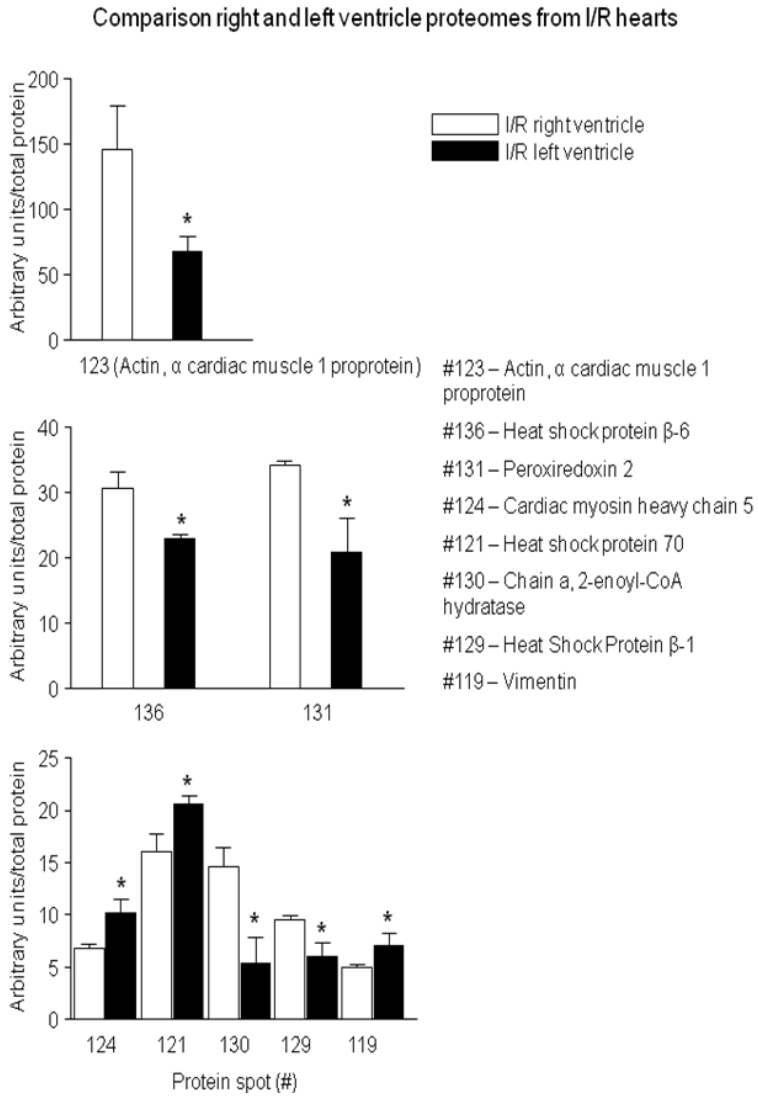

Figure 2.6
Changes in right and left ventricle proteomes from hearts subjected to ischemia reperfusion. For better visual presentation the altered proteins are grouped in 3 groups. Each panel represents one protein group according to the level of abundance. n=3/group * $p<0.05$ vs. I/R right ventricle

2.5.6 Validation of 2DE results

Protein level changes observed from 2DE for some proteins, was verified by immunoblotting (according to commercial availability) (Fig. 2.7). Myosin light chain 2 (MLC2) and 2-enoyl-CoA hydratase were identified as significantly increased in the left ventricle, in comparison to the right, by 2DE. Immunoblotting analysis confirmed the observations made by 2DE (Fig. 2.7A). Similarly, the observed decrease of peroxiredoxin II level in right ventricles from I/R hearts in comparison to left ventricles of I/R heart (by 2DE) was confirmed by immunoblot analysis (Fig. 2.7B). To validate the 2DE results from aerobic

and I/R left ventricular samples, we measured MLC2 protein levels by immunoblotting (Fig. 2.7C). Although statistical significance was not achieved, the trend of change observed was similar to what was observed with 2DE. The same was observed when peroxiredoxin II level from right ventricles of aerobic and I/R hearts was measured by immunoblot analysis (Fig 2.7D, p=0.07).

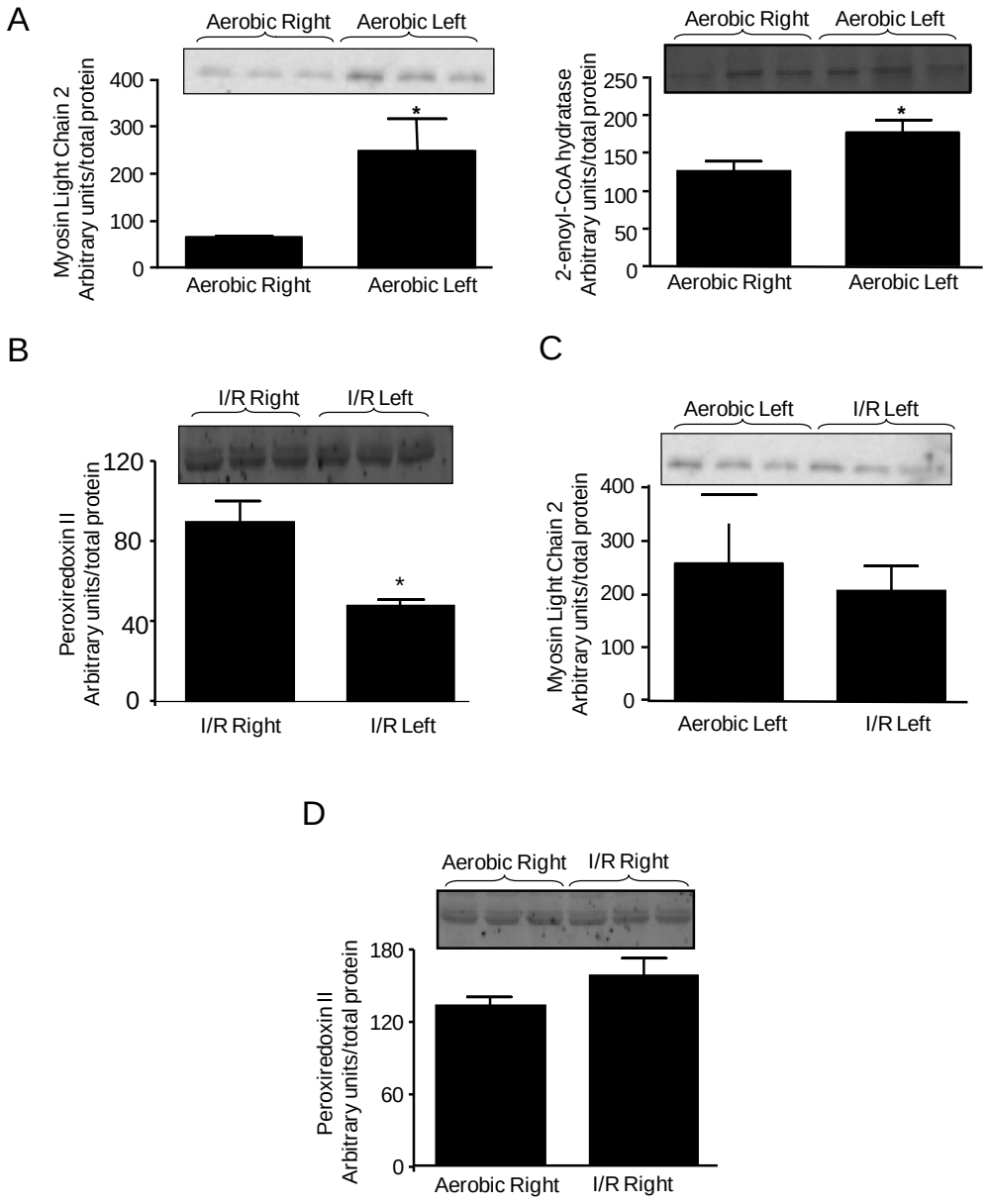

Figure 2.7
Validation of 2DE results by immunoblotting. The results obtained by 2DE were validated using immunoblotting. (A) Protein levels of myosin light chain 2 and 2-enoyl-CoA hydratase in right and left ventricle samples from aerobic hearts; (B) Protein levels of peroxiredoxin II in right and left ventricle samples from I/R hearts; (C) Protein levels of myosin light chain 2 in left ventricle samples from aerobic and I/R hearts; (D) Protein levels of peroxiredoxin II in right ventricle samples from aerobic and I/R hearts. n=3/group * $p<0.05$

2.6 Discussion

Proteomics provides a powerful experimental approach for observing the global changes in protein expression (level and profile) in cells, tissues or whole organs, in both physiological and pathological conditions (152-153). Using a proteomics approach, several studies have examined differences in protein expression developed in different subcellular compartments of hearts and alteration in the protein expression due to I/R induced injury in isolated hearts (54, 154-158). However, very little is known about differences between left and right ventricle proteomes. Up to date, only one study on proteomes of left and right ventricle in physiological conditions has been reported (144).

In the majority of the studies using a proteomics approach, two-dimensional electrophoresis (2-DE) is a basic technique. 2-DE is the technique of choice due to its relative simplicity and low cost. 2-DE is able to resolve approximately 1,000 proteins, depending on the gel size (159). However, the number of measurable proteins visible in 2-DE depends on the dynamic range of protein concentration in the available sample and is estimated to be approximately 10,000 (160). Because measurement of levels of high and low abundant proteins in 2-DE has a high associated error (the level of protein spots are either saturated or close to the detection limit), we have analyzed only proteins with expression levels between 2 and 500 arbitrary units.

In our study of left and right ventricular proteomes from I/R heart, significant differences in protein expression profile were found. In aerobic conditions sarcomeric proteins, such as MLC2, α-actin and myosin heavy chain 5, are differently expressed between left and right ventricles, with MLC2 levels being higher in the left ventricle. MLC2 has been described to be an important contractile protein for the regulation of contractility and force of contraction (43) and the left ventricle responsible for the systemic circulation. Hence it is logical to assume that the higher level of MLC2 in the left ventricle is associated with a need to regulate contraction, in contrast to the right ventricle which has a lower contractile work demand. Also, in I/R left ventricle, the level of MLC2 was decreased while the level of a fragment of myosin heavy chain 5 was increased. The decrease of the level of the contractile proteins can be explained by the degradation of MLC-2 by MMP-2 (145), and the degradation of myosin heavy chain by MMP-9 (161). These findings are in line with the paradigm that I/R causes contractile dysfunction. The loss of a key contractile regulatory protein (MLC2), associated in increased degradation of myosin heavy chain 5 (important

structural and kinetic protein) provides insights as what the molecular mechanisms of left ventricular contractile dysfunction might be.

Interestingly, no changes in the levels of sarcomeric proteins in right ventricle from I/R hearts in comparison to aerobic ventricle were observed. Again, this can be explained by fact that the contractile workload of the right ventricle is lower than the left ventricle. Under aerobic conditions, the left and right ventricle proteomes differ by expressing different levels of the mitochondrial antioxidant defensive protein peroxiredoxin 3, and the cytoskeleton protein vimentin. Peroxiredoxin 3 is less abundant in the left ventricle. Since peroxiredoxin 3 is a mitochondrial antioxidant protein and the left ventricle has very high metabolic and respiratory rates, the decreased levels may result from the different metabolic and respiratory rates between the two ventricles. Interestingly, peroxiredoxin 2 was upregulated in right I/R ventricle, in comparison to aerobic right ventricle. Nonetheless the levels of peroxiredoxin 2 in I/R conditions are still lower in the right than in the left ventricle. It appears as though left and right ventricle responses to oxidative stress by peroxiredoxin are isoform specific, with the left ventricle preferring peroxiredoxin 3 while the right ventricle relies on peroxiredoxin 2.

Vimentin is more abundant in the left ventricle and it can be explained by the higher contractile work demand. In addition, vimentin is associated with lipid metabolism which correlates with higher lipid metabolism for energy production in the left ventricle (162-163).

Two isoforms of α actin were significantly increased in the left ventricle in response to I/R. Actin is an important contractile protein. Although the general paradigm is that I/R causes degradation of cardiac contractile proteins, it is possible that the observed increase in actin protein levels is associated with an adaptive mechanism by the cardiomyocytes to decreased contractility and contractile efficiency.

In our study we observed changes in the levels of heat shock proteins (HSP) 70 and β-6. The level of HSP 70 decreased in left I/R ventricle but increased in the right I/R ventricle. HSP β-6 levels increased in both left and right I/R ventricles. We observed higher level of HSP 70 in left ventricle whereas HSP β-6 level was lower when the levels of these proteins from left and right ventricles of I/R hearts were compared. It has been reported that HSP 70 is upregulated during development of human heart failure (164). Our observations also have shown the upregulation (higher level) of HSP 70 in I/R injury, mainly in the left ventricle.

During myocardial ischemia, myocyte stress leads to mitochondrial damage. An increase of the permeability of mitochondrial pores leads to swelling of the mitochondrial matrix and subsequent rupture of the outer membrane and development of irreversible injury

(165). The mechanism of ischemic injury that induces mitochondrial protein level changes is complex. It may include the release of mitochondrial proteins (158, 166), changes in turnover of these proteins (balance between protein synthesis and protein degradation) (167-169) or post-translational modification that significantly change the pI or MW of the proteins (156). It is interesting to note the similarities and differences between right and left ventricles in response to ischemia. The level of HSP 8, HSP β -6, and subunits of ATP synthase were down after I/R stress in both right and left ventricles. However, while the left ventricle proteome responds to ischemia by an increase in the levels of structural proteins (such as α actin and cardiac myosin heavy chain 5), the right ventricle proteome adapts by modification of a group of mitochondrial proteins involved in energy metabolism.

Van Eyk and co-workers subjected rabbit ventricular myocytes to conditions mimicking ischemia and the protein analysis revealed alteration in protein pattern especially in proteins involved in mitochondrial energetics, chaperoning and stress-response (156). Similarly, separate studies of mitochondrial proteomes have been performed in perfused rabbit hearts subjected to I/R (157). The authors identify 25 proteins involved in mitochondrial respiratory chain and energy metabolism differentially expressed in the I/R heart (157). Of the 25 proteins described by the authors to be changed by I/R, 3 were also identified by us to be changed by I/R or differentially expressed in right and left ventricles. These proteins were enoyl-CoA hydratase, ATPsynthase and α-actin. Also, the pattern of protein level changes in our study is identical to the one reported.

Generally, our results are consistent with the reports describing the alteration of the protein expression in heart tissue in response to ischemic stress. Similar to the alteration in protein expression in human heart affected by ischemia (170), we have found that the altered proteins belong to three categories: (i) mitochondrial proteins involved in energy metabolism, (171) cytoskeletal (e.g. myosin light chain proteins), and (iii) stress response (e.g. HSP).

To our knowledge, this is the first report concerning the comparison of the protein expression profile in left ventricle versus right ventricle of isolated heart, together with the study of the alteration of protein expression profile under ischemic stress.

The significant difference in protein abundance between left and right ventricle and between aerobic and ischemic heart tissue has occurred in wide range of protein levels.

Our findings are particularly important when interpreting results from cardiovascular studies that use either whole rat heart or isolated rat cardiomyocytes. While the use of whole heart homogenates for studying the molecular mechanism involved in cardiovascular

pathologies is a common procedure, our data raises the question of how accurate these observations are, in light of the proteomic difference of right and left ventricles.

On the other hand the isolated adult cardiomyocytes are commonly used for the study of single cell contractility and calcium signaling. Due to the morphology of the cells, researchers have opted out to isolate right ventricle cardiomyocytes since it provides a more homogeneous population. Our data suggest that the observations made in isolated cardiomyocytes cannot be transposed to left ventricle or whole heart processes since the proteomes of left and right ventricles are distinct under aerobic conditions and respond very differently to I/R.

Our data provide evidence that the right and left ventricles of the heart respond differently to I/R. This is particularly important when dealing with clinical scenarios of global or regional ischemia. Although many studies have examined I/R injury and possible therapeutic strategies, the exact molecular and cellular mechanisms continue to be elusive and require more study.

3. Effect of the myosin light chain kinase inhibitor ML-7 on the proteome of hearts subjected to ischemia–reperfusion injury

Han-bin Lin, Virgilio J. J. Cadete, Jolanta Sawicka, Mieczyslaw Wozniak, Grzegorz Sawicki

H-BL –Experimental work, experimental design, manuscript writing

VJJC – experimental work, manuscript writing

JS – Experimental work

MW – Manuscript writing

GS – Hypothesis generation, experimental design, manuscript writing

3.1 Preface

The manuscript presented here has been published in the journal *Journal of Proteomics (Lin et al. 2012)*. This manuscript addressed objectives 1 and 3 of my PhD project.

1. To identify proteomic profile in the heart subjected to I/R injury

3. To determine the effect of the Myosin Light Chain Kinase Inhibitor ML-7 on the proteome of hearts subjected to I/R injury

As we found in the previous study that energy metabolic proteins are regulated during I/R, and it has been shown the myosin light chain kinase (MLCK) is involved in the development of I/R injury. In this study, we show that myosin light chain kinase (MLCK) inhibitor ML-7 potentially protects the heart against I/R injury. Decreased level of energy metabolism proteins associated with ATP synthesis were identified in I/R group. This decrease suggests that ATP synthesis is inhibited during I/R, which might be related to protein kinase, such as MLCK. The changes in metabolic enzymes and structural proteins' levels and their regulation by ML-7 suggest that the cardioprotective effect of ML-7 involves energy production and structural modulation.

3.2 Abstract

In the development of ischemia/reperfusion (I/R) injury, the role of the myosin light chain (MLC) phosphorylation has been given increased consideration. ML-7, a MLC kinase inhibitor, has been shown to protect cardiac function from I/R, however the exact mechanism remains unclear.

Isolated rat hearts were perfused under aerobic conditions (controls) or subjected to I/R in the presence or absence of ML-7. Continuous administration of ML-7 (5 μM) from 10 min before onset of ischemia to the first 10 min of reperfusion resulted in significant recovery of heart contractility. Analysis of gels from two-dimensional electrophoresis revealed eight proteins with decreased levels in I/R hearts. Six proteins are involved in energy metabolism: ATP synthase beta subunit, cytochrome b-c1 complex subunit 1, 24-kDa mitochondrial NADH dehydrogenase, NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, cytochrome c oxidase subunit, and succinyl-CoA ligase subunit. The other two proteins with decreased levels in I/R hearts are: peroxiredoxin-2 and tubulin. Administration of ML-7 increased level of succinyl-CoA ligase, key enzyme involved in the citric acid cycle.

The increased level of succinyl-CoA ligase in I/R hearts perfused with ML-7 suggests that the cardioprotective effect of ML-7, at least partially, also may involve increase of energy production.

3.3 Introduction

Myocardial infarction (MI), congestive heart failure and atherosclerosis are the main co-morbidities of ischemic heart disease, and are among the most common causes of death (172-173). Coronary reperfusion therapy is a well-established therapeutic strategy for the treatment of MI for over twenty years (174). However, newly returning blood flow to previously ischemic myocardium damages the cells within the myocardial tissues in what is called ischemia/reperfusion (I/R) injury, and up until now, the management of MI has mainly focused on the acute reperfusion of the previously ischemic myocardium (175). Present therapeutic strategies have few effective treatments focused on reducing the damage of I/R injury. Therefore, from a therapeutic point of view, it would be vital to target the active mediators of I/R injury and regulate these mediators in order to improve cell function and survival. In summary, the underlying mechanisms involved in the pathogenesis of myocardial I/R injury are complicated and involve the interactions of different pathways.

Currently, the role of the myosin light chain kinase (MLCK) pathway in the development of cardiovascular disease and I/R injury has gained attention (50). MLCK also known as MYLK is a Ca^{2+}/calmoduline activated serine/threonine-specific protein kinase that phosphorylates the 20kD regulatory light chain of myosin (51-52). Phosphorylation of cardiac myosin heavy chains and light chains by a kinase, such as MYLK3, potentiates the force and the rate of cross-bridge recruitment in cardiac myocytes (53). The MLCK is activated by the extracellular-regulated kinase (ERK) (55). The MLCK pathway has been reported to be involved in the pathology of several cardiovascular disorders (56-57), and it has been shown that the inhibition of MLCK protects heart from I/R injury by regulation of phosphorylation of MLC (55, 58). MLCK can be inhibited by ML-7, a membrane permeable agent (55). It has been reported that ML-7 protects the heart against I/R injury (50), and has beneficial effects in heart failure (55), brain injury (90) and glaucoma (91).

The phosphorylation of MLC also is regulated by Rho kinase pathway. The active form of Rho, GTP-Rho activates Rho kinase (ROCK). ROCK phosphorylates the myosin binding subunit of myosin phosphatase inhibiting its activity, thus increasing phosphorylation of MLC2 of myosin II (61-63). It is well established that the inhibition of ROCK protects I/R hearts from contractile dysfunction (67). This research lab has recently indicated that the mechanism of protection of I/R hearts, in addition to inhibition of ROCK may up-regulate the protein involved in an increase of energy production (67).

Considering the limited information about the effectors of the MLCK pathway and the wide spectrum of action for MLCK inhibitors action in I/R injury we hypothesized that the beneficial effects of ML-7 are well beyond regulation of contraction through phosphorylation or dephosphorylation of MLCs. The objective of this study is to identify possible additional molecular targets involved in the mechanism of cardioprotection by ML-7 with focus on energy production.

3.4 Materials and methods

The experimental procedures described below conform to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

3.4.1 Heart perfusion and I/R protocol

Male Sprague–Dawley rats (weighing 250–300 g) were anesthetized with pentobarbital (60 mg/kg). Hearts were excised and perfused through the aorta utilizing the Langendorff method at constant pressure (60 mmHg) with Krebs–Henseleit buffer at 37°C as previously described (127). Hemodynamic function was monitored during the entire protocol. Left ventricular developed pressure was calculated as the difference between systolic and diastolic pressures from the left ventricular pressure trace. The rate-pressure product (RPP) was calculated as the product of the spontaneous heart rate and left ventricular developed pressure. Control hearts (n=6) were aerobically perfused for 75 min. The ischemic hearts (I/R, n=6) were aerobically perfused for 25 min, followed by 20 min of global no-flow ischemia by closing of aortic inflow line (Emka Langendorff perfusion system, Paris, France) and 30 min of aerobic reperfusion. ML-7, when present, was infused for 10 min before the onset of ischemia and during the first 10 min of reperfusion at concentrations of 1, 3 and 5 μM (n=6/group). The hearts were subsequently frozen for biochemical studies.

3.4.2 Myosin light chain kinase (MLCK) inhibitor

ML-7 (Sigma Aldrich, St Louis, MO, USA), 1-(5-iodonaphthalene-1-sulphonyl) 1H-hexahydro 1, 4-diazepine hydrochloride, a selective inhibitor of myosin light chain kinase

3.4.3 Preparation of heart extracts for two-dimensional electrophoresis (2-DE)

Protein samples for 2-DE were prepared by mixing frozen (-80^{o} C), powdered heart tissue (40–60 mg wet weight) with 200 mL sample/rehydration buffer (8 mol/L urea, 4% CHAPS, 10 mmol/L DTT, 0.2% Bio-Lytes 3/10 (Bio-Rad, Hercules, CA, USA)) at room temperature. Samples were sonicated twice for 5 seconds and centrifuged for 10 min at 10 000 g at 4^{o} C to remove any insoluble particles. Protein content of the heart extract in sample/rehydration buffer was measured with the Bio-Rad protein assay. For other biochemical studies, frozen heart tissue powder was homogenized on ice in 150 mM NaCl, 50 mmol/L Tris-HCl (pH 7.4) containing protease inhibitor cocktail and 0.1% Triton X-100.

Homogenates were centrifuged at 10,000 g for 10 min, and the supernatant was collected and stored at -80^{o} C until further use.

3.4.4 Two-dimensional electrophoresis

Heart extract protein (400 mg) was applied to 11 cm immobilized pH gradient (5-8) strips (IPG, Bio-Rad) and equilibrated for 16 to 18 hours at 20^{o} C in rehydration buffer. For isoelectrofocusing, the Bio-Rad Protean isoelectrofocusing cell was used as previously described (22, 166). Next, 2-DE was carried out using Criterion precast gradient gels with 8–16% acrylamide (Bio-Rad). To minimize variations in resolving proteins during the 2-DE run, 12 gels were run simultaneously using a Criterion Dodeca Cell (Bio-Rad). The reproducibility of 2-DE and quality of protein loading has been previously verified by us (22, 150, 176). After separation, proteins were detected with Coomassie Brilliant Blue R-250 (Bio-Rad). All the gels were stained in the same bath. Developed gels were scanned with the GS-800 Calibrated Densitometer (Bio-Rad). Quantitative analysis of spot intensity from 2-DE was measured with PDQuest 8.01 software (Bio-Rad), and intensities of the separate bands from immunoblotting were analyzed and expressed in arbitrary units with Quantity One 4.4 measurement software (Bio-Rad). An arbitrary protein spot sensitivity threshold was used to detect changes of protein levels. This threshold determines significant changes in spot size, and intensity and is based on four parameters: largest and smallest spot area, minimum peak value sensitivity and noise filter level. The arbitrary threshold chosen by us eliminates from analysis low abundant protein spots that may result in an inaccurate identification by MS. Equal protein loading was additionally verified by measurement of tropomyosin level in 2-D gels.

3.4.5 Mass spectrometry (MS)

Protein spots whose levels were changed by I/R and further affected by ML-7 (decreased, normalized or increased) were manually excised from the 2-DE gel and processed using a MassPrep Station from Micromass (Waters, Milford, MA, USA) according to the methods supplied by the manufacturer. Briefly, the excised gel fragment containing the protein spot was first destained in 200 mL of 50% ACN with 50 mM ammonium bicarbonate at 37^{o} C for 30 min. Next, the gel was washed twice with water. The protein extraction was performed overnight at room temperature with 50 mL of a mixture of formic acid: water: isopropanol (1:3:2, v:v:v). The resulting solution was then subjected to trypsin digestion and

MS analysis. For electrospray, quadruple time-of-flight analysis, 1 mL of the solution was used. LC/MS/MS was performed on a CapLC highperformance liquid chromatography unit (151) coupled with a quadruple time-of-flight-2 mass spectrometer (Micromass from Waters). A mass deviation of 0.2 was tolerated and one missed cleavage site was allowed. Resulting values from MS/MS analysis were used to search against the NCBInr and Swiss-Prot databases for Rattus norvegicus. We used the MASCOT (www.matrixscience.com) search engine to search the protein database and identify the protein. Mowse scoring algorithm was used to justify accuracy of protein identification, which is incorporated in the MASCOT search engine.

3.4.6 Western blot analysis

Protein (30 mg) from heart homogenate was separated using 12% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad). Protein level of SUCLA2 (succinate-CoA ligase, ADPforming, β subunit) was measured using mouse monoclonal anti-SUCLA2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), NDUFV2 (NADH dehydrogenase [ubiquinone] flavoprotein 2) was measured using rabbit polyclonal anti-NDUFV2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), peroxiredoxin-2 was measured using rabbit polyclonal anti- peroxiredoxin-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Band densities were measured using Versa Doc 5000 and Quantity One 4.6 software (Bio-Rad). Equal protein loading was additionally verified by measurement of tropomyosin level with mouse monoclonal anti-tropomyosin antibody (abcam, Cambridge, MA, USA)

3.4.7 PKC activity assay

Protein kinase C (PKC) activity was determined using a commercially available PKC assay kit from Promega (Madison, WI, USA) according to the supplier’s instructions. Briefly, crude heart homogenates from frozen heart samples were homogenized in PKC extraction buffer using a sonicator. PKC was semi-purified using a 1 ml DEAE cellulose column. The PKC enriched fraction (9 μg protein/reaction) was incubated with PepTag® C1 peptide and incubated for 90 min at 30°C. The reaction was stopped by placing tubes in a boiling water bath. After cooling, the phosphorylated and non-phosphorylated peptides were separated in a 0.8% agarose gel at 100 V for 30 min. Detection of PepTag® C1 peptide was performed using the VersaDoc 5000 and Quantity One software (BioRad, Hercules, CA, USA). The decrease in non-phosphorylated PepTag® C1 peptide was used as an indicator of increased

PKC activity.

3.4.8 Statistical analysis

The protein spot levels were analyzed using t-test and Mann–Whitney U-test, which is incorporated in the PDQuest measurement. The proteins of interest were identified by MS. ANOVA and Kruskal–Wallis test were used in functional studies. Data are expressed as the mean ± SEM.

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3.5 Results

3.5.1 Protection of cardiac mechanical function by ML-7

Hearts subjected to 20 min global no-flow ischemia and 30 min reperfusion showed a significant decrease of mechanical function. The rate-pressure product (RPP) was decreased by approximately 60% (Figure 3.1A) and coronary flow decreased by 65% (Figure 3.1B). Administration ML-7 (MLCK inhibitor) significantly improved the RPP and coronary flow of isolated heart (Figure 3.1). The pharmacological effect of ML-7 was dose-dependent, and treatment with 5 μM ML-7, the highest dose used in our experimental conditions, elevated the recovery of RPP up to 75% and 100% of coronary flow in comparison to aerobic hearts (Figure 3.1).

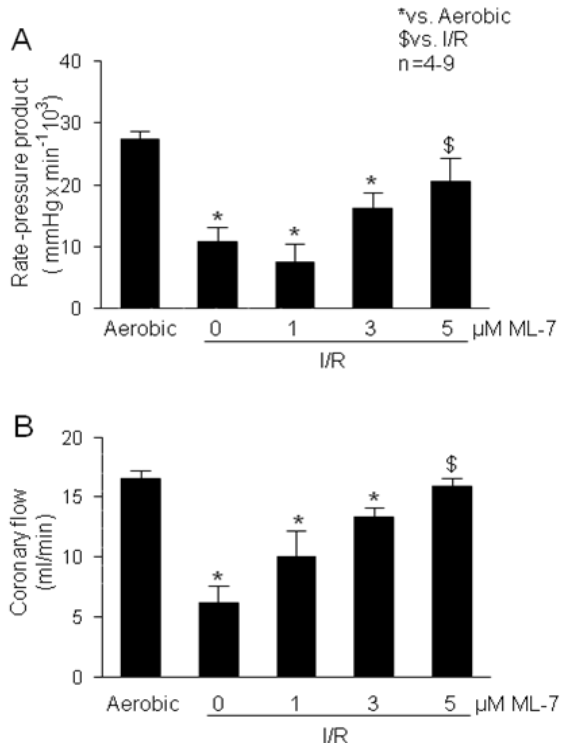


Figure 3.1
Hemodynamic parameters of retrograde rat heart perfusioned with or without MLC kinase inhibitor ML-7. (A) Mechanical function presented as rate-pressure product (RPP) and (B) coronary flow at the end of perfusion protocol.*p<0.05 versus aerobic, $p<0.05 versus I/R.

3.5.2 Identification of protein spots from aerobic versus I/R protocol group

Protein extracts from hearts perfused aerobically (control) with or without I/R protocol were separated by 2-dimensional electrophoresis (2-DE) (Fig. 3.2). Analysis of the protein spot intensity variation (using an arbitrary threshold) detected nine protein spots with significantly decreased level in the I/R hearts, in comparison to the control group (Fig. 3.3). All nine protein spots were identified by mass spectrometry (MS) (Table 3.1). Six protein

spots were identified as mitochondrial enzymes involved in energy production and included: ATP synthase beta unit, cytochrome b-c1 complex subunit 1, 24-kDa NADH dehydrogenase precursor, NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, cytochrome c oxidase subunit 5A, precursor, and succinyl-CoA ligase[ADP-forming] subunit beta, mitochondrial. Two protein spots were identified as peroxiredoxin-2 and selenium-binding protein. The ninth decreased protein spot was tubulin, a structural protein (Fig.3.3).

Usually the measurement of level of actin, glyceraldehyde-3-phosphate dehydrogenese (GAPDH) or tubulin is used as an indicator of equal protein loading in electrophoretic techniques. Previously, we showed that I/R injury in isolated hearts altered the level of glyceraldehyde-3-phosphate dehydrogenese (GAPDH). The level of actin is significantly decreased in the heart with myocardial infarction when measured by immunobloting (177). In this study we observed changes in tubulin level. The level of tropomyosin was not affected in 2-DE and immunobloting analysis. From this we used the measurement of tropomyosin levels as a control of protein loading (Fig. 3.3B, 3.5B, 3.6C and 3.7B).

Table 3.1Identification of protein spots from aerobic VS I/R protocol group

Protein spot NO.	Mowse score a)	Queries matched	Sequence coverage (%)	pI/MW (kDa)	Identified protein
Energy production					
187 ↓b)	490	15	15%	4.92/51.171	ATP synthase beta subunit
195 ↓	106	2	2%	5.57/ 53.500	cytochrome b-c1 complex subunit 1, mitochondrial precursor
196 ↓	668	24	34%	6.00/ 26.854	24-kDa mitochondrial NADH dehydrogenase precursor
197 ↓	635	13	13%	5.87/ 24.411	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial
198 ↓	317	11	13%	6.08/ 16.347	cytochrome c oxidase subunit 5A, mitochondrial precursor
204 ↓	309	13	16%	7.57/ 50.616	succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial
Structural proteins					
185 ↓	82	2	2%	4.79/ 50.225	tubulin beta-2C chain
Others					
194 ↓	151	8	14%	5.34/ 21.941	peroxiredoxin-2
203 ↑	314	15	22%	6.10/53.069	selenium-binding protein 1

a) Ions score is -10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores > 37 indicate identity or extensive homology ($p<0.05$).
b) The arrows (↑↓) indicate statistically significant changes in protein levels (versus aerobic protocol.

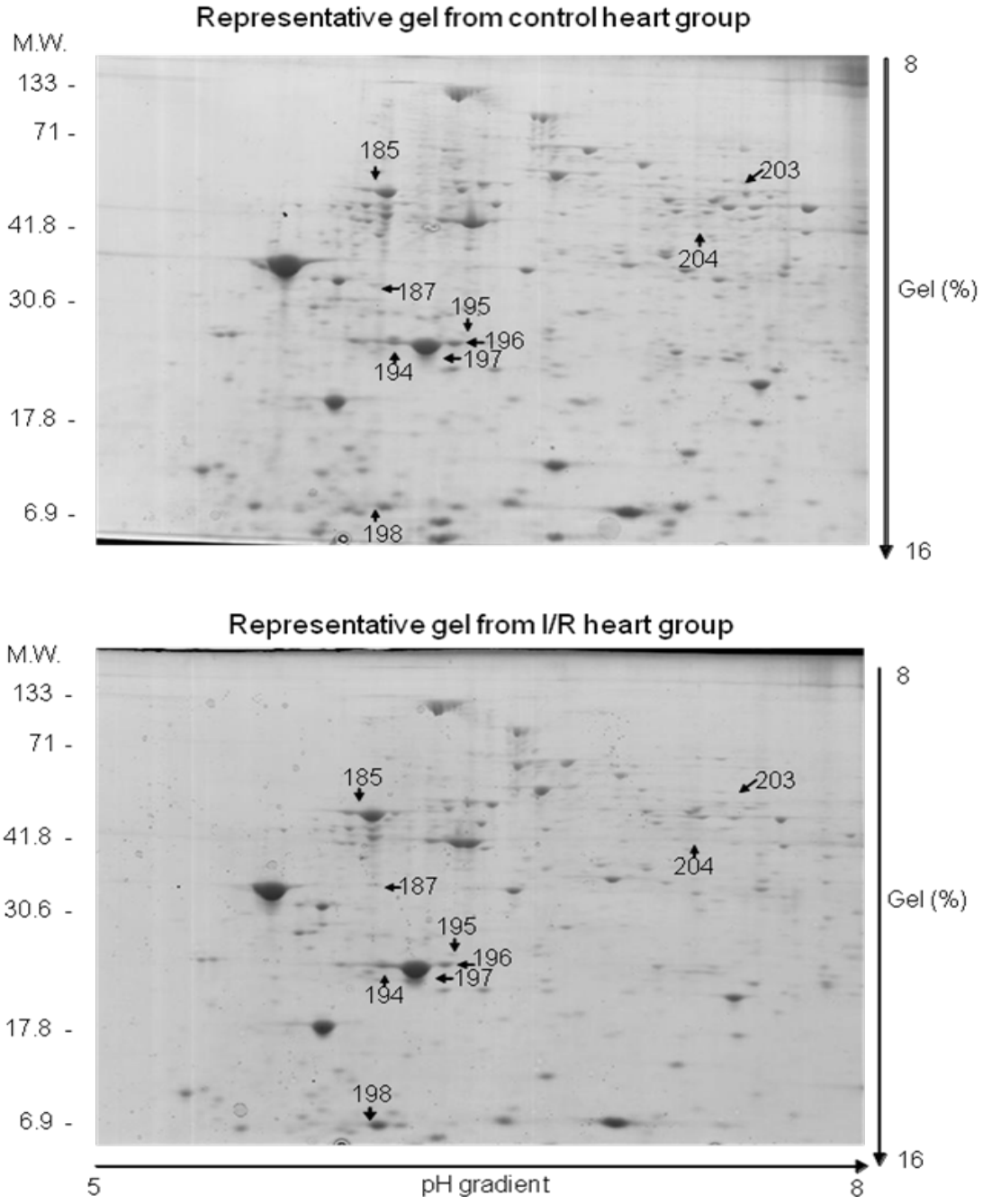


Figure 3.2
Two-dimensional electrophoresis (2-DE) of heart homogenates. Representative gels of 2-DE of protein extracts from aerobically (control) perfused hearts and I/R hearts. The arrows indicate the protein spots significantly changed by I/R.

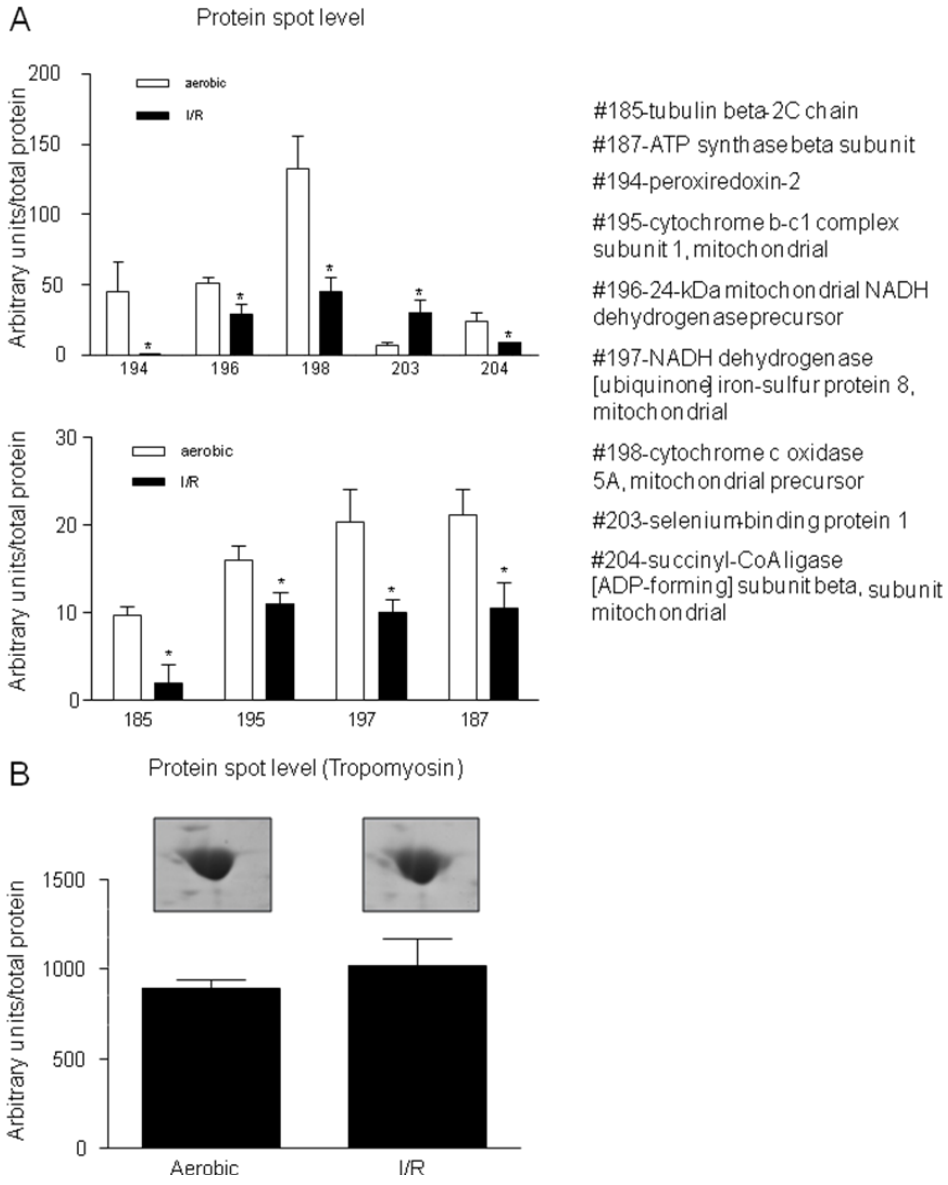


Figure 3.3
Densitometric analysis of protein spots from 2-DE gels from aerobically (control) perfused hearts and I/R hearts. (A) Results of densitometric analysis of protein spots affected by I/R. (B) Level of tropomyosin in aerobic and I/R hearts (control of equal protein loading). *p<0.05 versus aerobic, $p<0.05 versus I/R.

3.5.3 Effects of ML-7 treatment on the proteome of hearts subjected to I/R

Heart protein extracts from aerobic control, I/R and I/R treated with 5 μM of ML-7 were analyzed by 2-DE (Fig 3.4). Using an arbitrary threshold for variation, we detected three protein spots affected by ML-7 treatment and we identified them (Table 3.2). These spots were identified as structural proteins tubulin beta-2 C chain, vimentin and an energy production modulator mitochondrial succinyl-CoA ligase [ADP-forming] subunit beta. All of them revealed a similar profile of change being significantly increased in I/R hearts treated

with ML-7 (Fig. 3.5). The protein level of vimentin was increased only in hearts treated with ML-7. The tubulin level was normalized by ML-7. In contrast, the protein level of succinyl-CoA ligase in ML-7 treated I/R hearts was significantly increased in comparison to control and I/R hearts (Fig. 3.5).

Table 3.2 Identification of protein spots from I/R protocol VS I/R plus ML-7 protocol group

Protein spot NO.	Mowse score a)	Queries matched	Sequence coverage (%)	pI/MW (kDa)	Identified protein
Energy production					
204 ↑b)	309	13	16%	7.57/ 50.616	succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial
Structural proteins					
183 ↑	144	6	10%	5.06/ 53.757	vimentin
185	82	2	2%	4.79/ 50.225	tubulin beta-2C chain

a) Ions score is -10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores > 37 indicate identity or extensive homology ($p<0.05$).
b) The arrows (↑↓) indicate statistically significant changes in protein levels (versus I/R protocol)

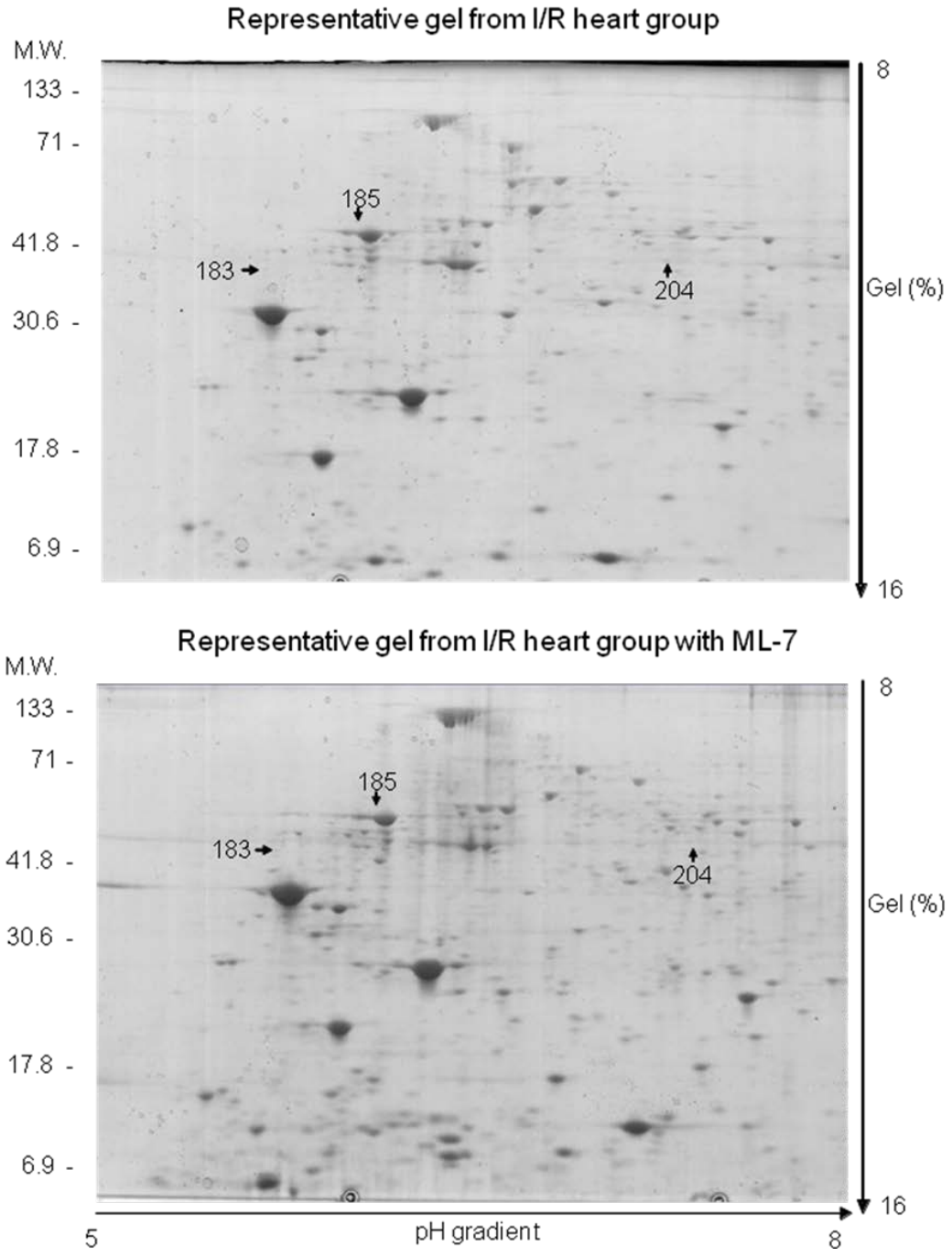


Figure 3.4
Effect of ML-7 treatment on the cardiac proteome. Representative 2-DE gels from I/R and I/R+ML-7 hearts. Arrows indicate the protein spots were significantly changed by ML-7 treatment.

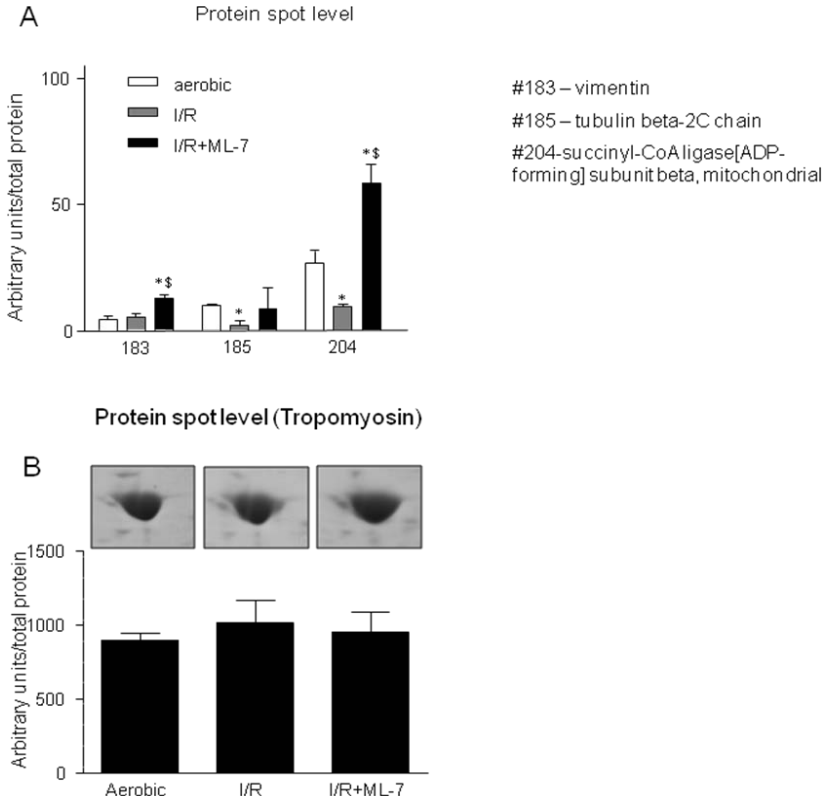


Figure 3.5
Densitometric analysis of protein spots from 2-DE gels from aerobically (control) perfused hearts and I/R hearts with and without ML-7. (A) Results of analysis of ML-7 on protein spot levels. (B) Level of tropomyosin in analyzed hearts (control of equal protein loading). *p<0.05 versus aerobic, $p<0.05 versus I/R.

3.5.4 Western blot analysis of identified protein

To verify results from 2-DE we performed immunoblotting for some of proteins. Immunoblot analysis of peroxiredoxin-2 and NADH dehydrogenase [ubiquinone] flavoprotein 2 (NDUFV2) showed decreased level of these proteins in I/R hearts what is similar to the results from obtained from 2-DE (Fig. 3.6). Analysis of changes of SUCLA2 levels in all 3 groups of hearts also showed similar results to these obtained from 2-DE (Fig. 3.7).

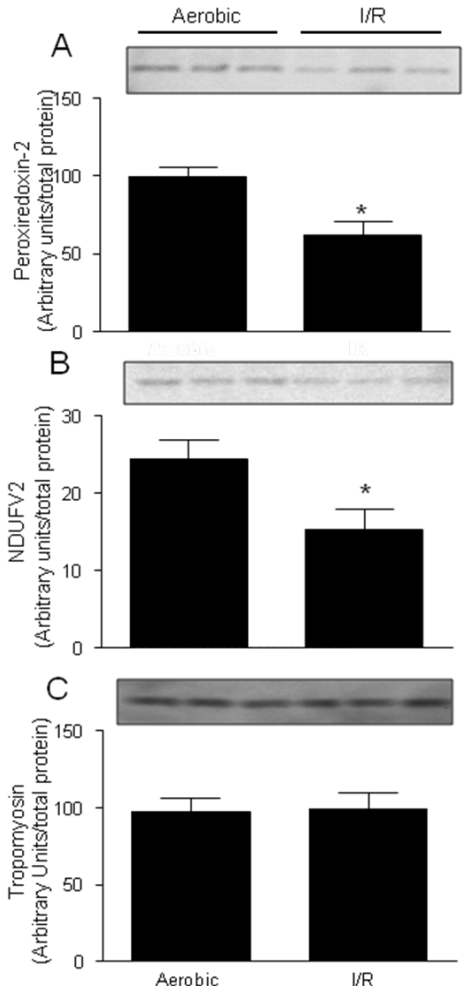


Figure 3.6
Verification of results from 2-DE by analysis of changes of protein level with immunoblotting: (A)Peroxiredoxin-2; (B) NADH dehydrogenase [ubiquinone] flavoprotein 2; (C) Tropomyosin, (control of equal protein loading). n=3/group; *p<0.05 versus aerobic.

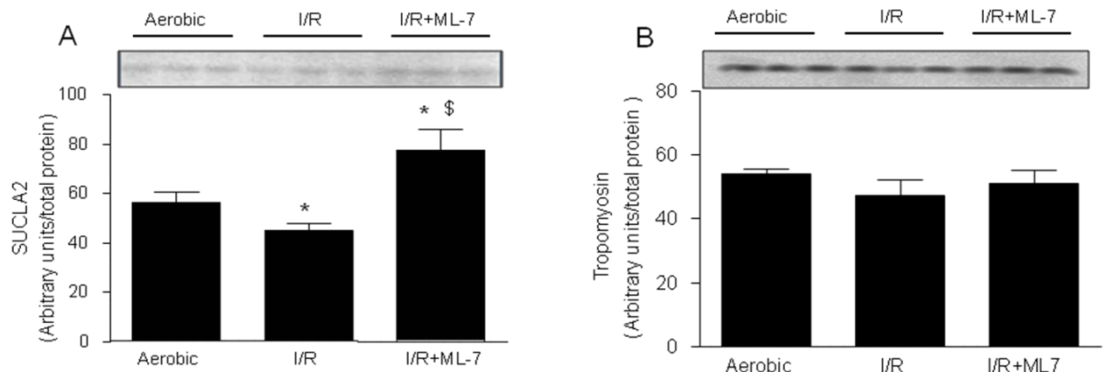


Figure 3.7
Analysis of Succinate-CoA ligase level s by immunoblotting (A) and (B) Tropomyosin, (control of equal protein loading) in control, I/R and I/R + ML-7 hearts. *p<0.05 versus aerobic, $p<0.05 versus I/R.

3.5.5 Protein kinase C (PKC) activity analysis

It has been reported that activation of PKC epsilon is central to cardioprotection against ischemic stress (178) and that ML-7 inhibits PKC activity (179). In our study we observed a significant increase of PKC activity in response to I/R injury, however 5 μM ML-7 did not affect PKC activity in hearts subjected to I/R (Fig. 3.8).

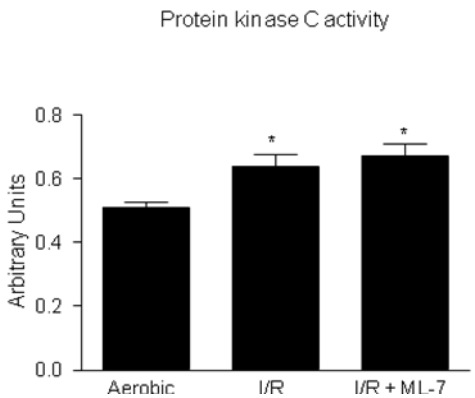


Figure 3.8
Protein kinase C activity in control hearts or subjected to I/R in the presence or absence of ML-7. (n=4). *p<0.05 versus aerobic

3.6 Discussion

Despite a large number of studies on heart injury by ischemia/reperfusion (I/R), the molecular basis of cardiac dysfunction caused by I/R is still unclear. Utilizing a proteomics approach, some studies have demonstrated differences in protein expression in different subcellular compartments of hearts and changes in the protein expression due to I/R injury in isolated hearts (155, 176). Recently, studies have shown that myocardial I/R injury, with the contribution of reactive oxygen species (15), activate some protein kinases (48, 98, 180-181). The central importance of MLC phosphorylation in contractile state of cardiomyocyte has been demonstrated previously. Phosphorylation of MLC at Ser19/Thr18 by activated MLC kinase (MLCK) or inhibition of MLC phosphatase (MLCP) plays a critical role in the development and regulation of contractile forces within cells (182). The cardioprotective effects of inhibition of the MLCK pathway by ML-7 have been described in different animal models but have been mainly associated with protection of contractile function via the regulation of the phosphorylation status of myosin. It was shown that ML-7 is specific, albeit a relatively weak inhibitor of smooth muscle MLCK. Mitogen and stress activated protein kinase 1 (MSK 1) was also inhibited by ML-7, although much less potent (179). The specificity of ML-7 inhibitory action depends on the origin MLCK. There is a cMLCK expressed only in cardiac muscle similar to the tissue-specific expression of skeletal and smooth muscle MLCK. The cardiac regulatory chain of myosin is not a good substrate for smooth muscle MLCK which probably phosphorylates non-muscle cytoplasmic myosin IIB (58).

Previously, we found that I/R treatment of isolated hearts resulted in reduction of protein levels. The most affected were the structural proteins, such as MLC1 and mitochondrial proteins involved in energy metabolism such as ATP synthase subunits, were shown to be affected by I/R (166). In this study, we found that I/R injury resulted in a similar pattern of protein alteration in comparison with aerobic control group. In the I/R affected group, six of the energy metabolism proteins were identified as significantly different from aerobic control: ATP synthase beta subunit (electron transport chain complex V), cytochrome b-c1 complex subunit (electron transport chain complex III), 24-kDa mitochondrial NADH dehydrogenase (electron transport chain complex I), cytochrome c oxidase subunit (electron transport chain complex IV),NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 (electron transport chain complex I) and succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial. Five of these proteins are involved in the most important process in ATP

production, the electron transport chain. Some reports have shown that global ischemia down-regulates genes and proteins related to mitochondrial function, energy production, co-factor catabolism, and the generation of precursor metabolites of energy (183). During myocardial ischemia, the electron flow along the respiratory complexes in mitochondria is inhibited, and the mitochondrial oxygen consumption as well as ATP production is decreased (183). Increased ROS formation during I/R injury leads to ischemic mitochondrial damage and is causally involved in cell death. Release of ROS or pro-apoptotic factors from damaged mitochondria enhances cardiomyocyte injury. Taken together, I/R injured mitochondria (e.g. due to limited oxidative phosphorylation), mitochondria themselves may contribute to myocardial injury.

The level of peroxiredoxin-2 decreased during I/R, in comparison with aerobic control group. Peroxiredoxins are a ubiquitous family of antioxidant enzymes which also control cytokine-induced peroxide levels and mediate signal transduction in mammalian cells. Similar trend was observed in the tissue of human failing hearts where the level of peroxiredoxin-2 was down-regulated (~1.5 fold) in contrast to peroxiredoxin-6 which was 2-fold up-regulated (184).

We also indentified the up-regulation of selenium-binding protein. Selenium is an essential trace mineral element involved in protection against oxidative damage through selenium-dependent glutathione peroxidases and other selenoproteins, such as selenium-binding protein (185).

Previously, we have shown that the inhibition of ROCK pathway of MLC1 phosphorylation partially protected the mechanical function of heart during I/R (67). Recently, the inhibition of MLCK pathway of MLC1 phosphorylation revealed a similar level of protection on the mechanical function of the I/R heart, but the pattern of proteins altered by I/R plus ML-7 treatment was different. Inhibition of ROCK pathway in the isolated heart resulted in up-regulation of two glycolytic enzymes, LDH-B and GAPDH whereas ATP synthase subunits and mitochondrial CK levels were normalized (67). Inhibition of the MLCK pathway revealed the up-regulation of some structural and involved in energy production proteins. The reason for the differences in inhibitory action on ROCK and MLCK pathways may had occurred because it has been demonstrated the central importance of MLCK in regulating the contractile state not only in cardiomyocytes but also plays a role in endothelial cell barrier functions (186).

We implicate the MLCK pathway in the regulation of the level of the proteins involved mainly in energy production and structural regulation. Inhibition of this pathway can

contribute to changes of energy production and structural regulation during I/R. In comparison with I/R group, ML-7 treated hearts subjected to I/R resulted in increased levels of several energy metabolism proteins and structural proteins. Two of those proteins were significantly increased, which are mitochondrial succinyl-CoA ligase [ADP-forming] subunit beta and vimentin. Succinyl-CoA ligase also is known as succinyl-CoA synthetase, which is an enzyme that catalyzes the reversible reaction of succinyl-CoA from succinate. This enzyme facilitates the coupling of the reaction to the formation of GTP or ATP. It plays a key role as one of the important catalysts involved in the citric acid cycle, located within the mitochondrial matrix of a cell. Vimentin plays a key role in anchoring and supporting the position of the organelles in the cytosol. Vimentin is attached to the nucleus, mitochondria, and endoplasmic reticulum (ER) either laterally or terminally (187). Vimentin Clips offer three different clips that show vimentin movement inside the cell. Vimentin is more abundant during I/R and it can be explained by the higher contractile work demand. In addition, vimentin is associated with lipid metabolism which correlates with higher lipid metabolism for energy production in I/R. Therefore, we conclude that the cardioprotective effect of inhibition of the MLCK pathway involves optimization of energy metabolism in I/R likely resulting in increased energy production, hence the observed increase in cardiac recovery after I/R. Despite the cardioprotective effect of ML-7 on the contractile protein MLC1 the additional pharmacological effect of ML-7 should be consider because not only ROS but asymmetric dimethylarginine (ADMA) significantly contributed to I/R heart injury. ADMA is the endogenous NOS inhibitor and can induce the MLCK synthesis in ischemic heart (188). The use of ML-7 can reduce the ADMA-induced heart injury.

Another important observation from this study is fact that tropomyosin level is stable and is not affected by I/R and drug treatment. This feature allows use tropomyosin as a control of protein loading. The level of commonly used housekeeping proteins, such as glyceraldehyde-3-phosphate dehydrogenese (GAPDH) and actin, are affected in the model of I/R (67) or MI (177). Tubulin another protein used as a housekeeping protein was decreased in this studies and its level were normalized by ML-7.

In summary, this study provides new insights into the molecular mechanisms of a broad variety of heart pathologies related to I/R injury. Also, we have shown the functional importance of MLCK during I/R, and as a result, the potential kinase inhibitors hold promise as novel strategies reducing the impact of cardiovascular disease. In addition, we propose ML-7 as a potential drug for new therapeutic strategies for treatment of MI.

4. Inhibition of MMP-2 expression affects metabolic enzyme expression levels: Proteomic analysis of rat cardiomyocytes

Han-Bin Lin, Keshav Sharma, Dariusz Bialy, Magdalena Wawrzynska, Randy Purves, Francisco S. Cayabyab, Mieczyslaw Wozniak, Grzegorz Sawicki

H-BL –Experimental work, experimental design, manuscript writing

KS – Experimental work

DB – Experimental design, manuscript writing

MW – Experimental design, manuscript writing

RP – Experimental work

FC – Experimental work, experimental design, manuscript writing

MW – Manuscript writing

GS – Hypothesis generation, experimental design, manuscript writing

4.1 Preface

This manuscript presented here has been published in the journal *Journal of proteomics (Lin et al. 2014).* This mauscript addressed the objective 4 in my PhD project.

4. To analyze the inhibition of MMP-2 gene expression with small interfering RNA in isolated cardiomyocytes by proteomic analysis, with the focus on the degradation of energy metabolic proteins by MMP-2

We have also shown that the inhibition of MMP-2 protects the heart from I/R injury. So far, we have only studied that MMP-2 degrades contractile proteins, but no one knows whether MMP-2 degrades energy metabolic proteins, or in the other words, inhibition of MMP-2 affects the energy metabolic proteins. This is why we started to study the proteomics of inhibition of MMP-2 in cardiomyocyte under aerobic condition. And we found that MMP-2 is involved in the regulation of the energy metabolic proteins such as ATP sythase in normal physiological condition.

4.2 Abstract

In this study we examined the effect of inhibition of MMP-2 expression, using siRNA, on the cardiomyocyte proteome.

Isolated cardiomyocytes were transfected with MMP-2 siRNA and incubated for 24 hours. Control cardiomyocytes from the same heart were transfected with scrambled siRNA following the same protocol. Comparison of control cardiomyocyte proteomes with proteomes from MMP-2 suppressed cardiomyocytes revealed 13 protein spots of interest (9 protein spots increased; 4 decreased). Seven protein spots were identified as mitochondrial enzymes involved in energy production and represent: ATP synthase β subunit, dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, cytochrome c oxidase subunit 5A, electron transfer flavoprotein subunit beta, NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 5 and a fragment of mitochondrial precursor of long-chain specific acyl-CoA dehydrogenase. Furthermore, precursor of heat shock protein 60 and Cu-Zn superoxide dismutase were identified. Two protein spots corresponding to MLC1 were also detected. In addition, ATP synthase activity was measured and was increased by approximately 30%.

Together, these results indicate that MMP-2 inhibition represents a novel cardioprotective therapy by promoting alterations in the levels of mitochondrial enzymes for improved energy metabolism and by preventing degradation of contractile proteins needed for normal excitation-contraction coupling

4.3 Introduction

Cardiac ischemia followed by reperfusion often results in the development of cardiac contractile dysfunction that limits the recovery and prognosis of patients. A number of pharmacological therapies are developed against ischemia/reperfusion (I/R) injury, however, the underlying molecular mechanisms of I/R injury and myocardial infarction (MI) remain unclear.

Matrix metalloproteinases (MMPs) have been shown to play important roles in protein degradation in heart diseases including I/R injury [reviewed in (189)], but the targets and mechanisms are still unclear. Currently there are 20 known MMPs, but identifying which isoform contributes to the mechanisms underlying I/R injury requires further investigation. However, studies on the role of MMPs in cardiovascular diseases suggest that MMP-2 is a significant contributor and plays a major role in I/R heart injury [reviewed in (190)]. It has been shown that α-actinin, a structural protein (116), and contractile proteins, such as troponin I (114) , myosin light chain 1(MLC1) (22), titin (115), and MLC2 (145), are degraded by MMP-2 and this causes contractile dysfunction of I/R hearts. Furthermore, it has been shown that inhibition of MMP-2 action protects these proteins and I/R hearts from contractile dysfunction [reviewed in (191)].

Despite extensive research on the role of MMP-2 in heart disease, the understanding of the biology of MMP-2 is far from satisfactory due to the lack of selective inhibitors. The MMP-2 inhibitors currently available are either broad inhibitors of all metalloproteinases or selective to one family of metalloproteinases (192). The synthetic compounds currently used to inhibit MMP-2 include o-phenathroline, doxycycline or derivatives of hydroxamic acid such as Batimastat, Marimastat or Ilomastat. These compounds often affect the action of other proteolytic enzymes in addition to inhibiting MMPs.

Endogenous protein inhibitors of MMP (TIMPs) are more suitable, however they still demonstrate a broad spectrum of MMP inhibition. Consequently, for the purpose of studying the biology of MMP-2 it is important to develop new strategies for selective/specific MMP inhibition. With the use of isolated cardiomyocytes, inhibition or reduction of MMP-2 expression appears to be a very promising strategy to study the role of MMP-2 in heart.

Small interfering RNA (siRNA) has been shown *in vitro* to specifically reduce or totally inhibit MMP-2 synthesis (193). To selectively inhibit MMP-2 action and identify additional molecular targets of MMP-2, this study used MMP-2 siRNA to inhibit MMP-2

expression in isolated myocytes. Proteomic analysis was performed on isolated cardiomyocytes treated with or without MMP-2 siRNA.

4.4 Experimental procedures

The experimental procedures described below conform to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

4.4.1 Isolation of cardiomyocytes

Male Sprague-Dawley rats (weighing 100–150 g) were anaesthetized with sodium pentobarbital (60 mg/kg i.p.) and hearts were removed. Right ventricular myocytes were obtained by enzymatic digestion as previously described (194-195). After isolation, the cells were suspended in DMEM medium supplemented with 10% fetal bovine serum and antibiotics (100U/ml penicillin and 100μg/ml streptomycin). The quality of myocyte preparations was assessed by measurement of cell viability and cell contractility. The viability of cardiomyocytes was assessed by the trypan blue exclusion test.

4.4.2 Primary culture of cardiomyocytes and siRNA transfection

Preparations of cardiomyocytes were incubated in 35 x 10mm cell culture plates (Nunc, Roskilde, Denmark) at concentrations of 2×10^5 cells per plate in DMEM medium (Lonza, Walkersville, MD, USA) supplemented with 10% (v/v) fetal bovine serum and antibiotics (100U/ml penicillin and 100μg/ml streptomycin) in a 95% air and 5% CO_2 incubator at 37°C.

A mixture containing a pool of 3 target-specific 19-25 nucleotide small interfering RNAs (MMP-2 siRNA) designed to knock down rat MMP-2 gene expression (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was resuspended in RNAse-free water to make a 10 μM solution in 10 mM Tris-HCl, pH 8.0, 20 mM NaCl, and 1 mM EDTA buffered solution. This solution was stored at -20°C. As a control, a scramble of siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used in the same conditions.

After a 7 h stabilization period, cardiomyocytes were washed with siRNA transfection medium (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Next 0.8 μM of MMP-2 siRNA or scrambled siRNA in 200μl of transfection medium was incubated for 24 h at 37°C according to the manufacturer's protocol. siRNA was removed and cells were further incubated for 17 h in DMEM medium. The control cardiomyocytes from the same heart were incubated for a total 48 h in DMEM medium. The experimental protocol and analyzed cell groups are shown in Figure 1.

For assessment of transfection efficiency, plasmid containing cDNA with green fluorescent protein (GFP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was transfected in the same way as the siRNA.

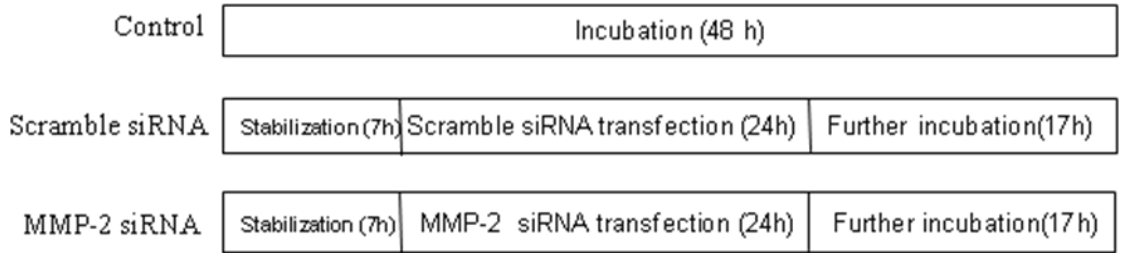


Figure 4.1
Experimental protocol outlining MMP-2 siRNA transfection. Isolated cardiomyocytes were divided into 3 groups. Control group (incubated for 48 hours without any intervention) scramble siRNA group, (control to the MMP-2 siRNA group), and MMP-2 siRNA transfected group. Total incubation time for all groups was 48 hours.

4.4.3 Measurement of contractility of cardiomyocytes

Cardiomyocyte contractility was evaluated at the end of transfection. Control myocytes were incubated for the same period of time as cells transfected with siRNA. The contractility was measured from 100 µl of the cell suspension using IonOptix system and IonWizard 6.0 software (IonOptix, Milton, MA USA). After a stabilization period the chamber containing the cells was perfused with an oxygenated buffer at a constant temperature of 37°C. Cells were continuously paced with 1 Hz and 5 V (IonOptix MyoPacer, Milton, MA, USA). The contractility of cardiomyocytes is shown as a percent of peak shortening in comparison to the total non-contracted length. On average, 8-10 cells per sample were analyzed for 10 min to determine contractile function.

4.4.4 Preparation of cell extracts

After the transfection procedure (48 h), cardiomyocytes were harvested and stored at -80°C. For biochemical studies frozen cardiomyocytes were sonicated on ice for 5 seconds (twice) in 50 mM Tris-HCl (pH7.4) containing 3.1 mM sucrose, 1 mM DTT, 10 µg/mL leupeptin, 10 µg/mL soybean trypsin inhibitor, 2 µg/mL aprotinin, and 0.1% Triton X-100. Homogenates were then centrifuged at 10 000g at 4°C for 10 minutes. The supernatant was collected and stored at -80°C until further use.

4.4.5 Measurement of MMP-2 activity

Gelatin zymography was performed as described (122, 127) . Briefly, homogenates from cardiomyocyte preparations containing 30 μg of protein were applied to 8% polyacrylamide gel copolymerized with 2 mg/mL gelatin. After electrophoresis, gels were rinsed three times for 20 minutes in 2.5% Triton X-100 to remove SDS. The gels were then washed twice in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM NaCl and 0.05% NaN_3) for 20 minutes at room temperature and then placed in incubation buffer at 37°C for 24 hours. The gels were stained using 0.05% Coomassie Brilliant Blue G-250 in a mixture of methanol:acetic acid:water (2.5:1:6.5, v:v:v) and destained in aqueous solution of 4% methanol and 8% acetic acid (v:v). Developed gels were scanned with GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA) and MMP-2 activity was measured using Quantity One 4.6 software (Bio-Rad, Hercules, CA, USA).

4.4.6 Immunoblot analysis

Protein (30 μg) from cardiomyocyte homogenate was separated using a 12% SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Myosin light chain 1 (MLC 1) was measured using mouse monoclonal anti-MLC1 antibody (Abcam, Cambridge, MA, USA) while MMP-2 was measured using rabbit monoclonal anti-MMP-2 antibody (Abcam, Cambridge, MA, USA). Band densities were calculated using Versa Doc 5000 and Quantity One 4.6 software (Bio-Rad, Hercules, CA, USA). Equal protein loading was additionally verified by measurement of tubulin level with mouse monoclonal antibody (Abcam, Cambridge, MA, USA).

4.4.7 Immunocytochemistry

For immunocytochemistry, cardiomyocytes were seeded on polylysine-coated coverslips and followed the same siRNA transfection protocol. After permeabilization with 0.25% Triton X-100 and fixation, cells were blocked for 1 h with PBS containing 5% bovine serum albumin (Sigma, St. Louis, MO, USA). Proteins of cardiomyocytes were labeled by overnight incubation (at 4°C) with rabbit anti-MMP-2 (Abcam, Cambridge, MA, USA) antibody diluted at 1:200 in blocking buffer followed by brief wash (three times; 10 min each) and incubation with Alexa Fluor 555-conjugated goat anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA, USA) at 1:1000 for 1 h. After Hoechst (Sigma, St. Louis, MO, USA) staining, the cover slips were mounted on newly cleaned slides using Prolong Gold Antifade Reagent (Invitrogen, Carlsbad, CA, USA) and observed with a LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and

analyzed with the Zeiss Zen 2009 software, version 5.5 SPI (Carl Zeiss, Oberkochen, Germany).

4.4.8 Two-dimensional electrophoresis (2-DE)

Protein samples for 2-DE were prepared by mixing frozen (-80ºC) cardiomyocytes (40–60 mg wet weight) with 0.2 mL sample/rehydration buffer (8 mol/L urea, 4% CHAPS, 10 mmol/L DTT, 0.2% Bio-Lytes 3/10 (Bio-Rad, Hercules, CA, USA) at room temperature. Samples were sonicated twice for 5 seconds and centrifuged for 10 min at 10 000 g at room temperature to remove any insoluble particles. Protein content of the cardiomyocytes homogenates in sample/rehydration buffer was measured with the Bio-Rad protein assay after suitable dilution of the homogenate.

For other biochemical studies, frozen cells were homogenized on ice in 150 mM NaCl, 50 mM Tris-HCl (pH 7.4) containing protease inhibitor cocktail and 0.1% Triton X-100. Homogenates were centrifuged at 10,000 g for 10 min, and the supernatant was collected and stored at -80ºC until further use. Protein content of the cardiomyocyte extract in homogenized buffer was also measured with the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Cardiomyocyte extract protein (400 μg) was applied to 11 cm immobilized pH gradient (3-10) strips (Bio-Rad, Hercules, CA, USA) and equilibrated for 16 to 18 hours at 20ºC in rehydration buffer. For isoelectrofocusing, the Bio-Rad Protean isoelectrofocusing cell was used as previously described (22, 166). Next, 2-DE was carried out using Criterion precast gradient gels with 8–16% acrylamide (Bio-Rad, Hercules, CA, USA). To minimize variations in resolving proteins during the 2-DE run, 12 gels were run simultaneously using a Criterion Dodeca Cell (Bio-Rad, Hercules, CA, USA). The reproducibility of 2-DE and quality of protein loading has been previously verified by us (22, 150, 176). After separation, proteins were detected with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA, USA). All the gels were stained in the same bath. Developed gels were scanned with the GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA). Quantitative analysis of spot intensity from 2-DE was measured with PDQuest 8.01 software (Bio-Rad, Hercules, CA, USA), and intensities of the separate bands from immunoblotting were analyzed and expressed in arbitrary units with Quantity One 4.4 measurement software (Bio-Rad, Hercules, CA, USA). An arbitrary protein spot sensitivity threshold was used to detect changes in protein levels. This threshold determines significant changes in spot size and intensity, and is based on four parameters: largest and smallest spot area, minimum peak value sensitivity and

noise filter level. The arbitrary threshold chosen by us eliminates from analysis low abundant protein spots that may result in an inaccurate identification by mass spectrometry.

4.4.9 Mass spectrometry (MS)

Protein spots were manually excised from the 2-DE gel and processed using a MassPrep II Proteomics Workstation (Micromass, UK) following the procedure described by Sheoran et al (196). Briefly, gel bands are destained twice (10 minute incubations) with 100 µL of 1:1(v/v) ammonium bicarbonate:acetonitrile. Protein reduction is performed with the addition of 10 mM DTT prepared in 0.1M ammonium bicarbonate. Gels are washed and dehydrated before being saturated with trypsin and prepared in ammonium bicarbonate. Digestion was carried out at 37ºC. Peptides were extracted with 30 µL 0.1% trifluoroacetic acid/3% acetonitrile, and then twice with 24 µL, 0.1% trifluoroacetic acid/50% acetonitrile. The combined extracts were dried in a speed vac (Labconco, Fishier Scientific, Waltham, MA, USA). Samples were reconstituted in 40 µL of 0.1% trifluoroacetic acid/3% acetonitrile for LC-ESI MS analysis. For LC-ESI MS analysis, 2 µL of the solution was used. LC/MS/MS was performed on Quadrupole Time-Of-Flight (Q-TOF) Global Ultima mass spectrometer (Micromass, Manchester, UK) equipped with a nano-electrospray (ESI) source and interfaced with a nanoACQUITY UPLC solvent delivery system (Waters, Milford, MA, USA). Resulting values from analysis were used to search against the NCBInr and Swiss-Prot databases for Rattus norvegicus. We used the MASCOT (version 2.4, www.matrixscience.com) search engine to search the protein database and identify the protein. For MS/MS ions search we assumed: 1) incomplete protein digestion by trypsin (1 missed cleavage level). Also thresholds of analysis ±50 ppm (fraction expressed as parts per million) and ±200 mmu (absolute milli-mass units) error windows on experimental peptide mass and for fragment mass tolerance were used. Mandatory alkylation and reduction of cysteines with iodoacteamide, and variable oxidation of methionine were assumed. Mowse scoring algorithm (197) was used to justify accuracy of protein identification, which is incorporated in the MASCOT search engine. The Mowse score is the probability that the observed match is a random event, and is presented as $-10^{*}\log_{10}(P)$ where P is the absolute probability.

4.4.10 Measurement of ATP synthase activity

ATP synthase activity was determined using a commercially available ATP synthase assay kit (Abcam, Cambridge, MA, USA) according to the supplier's instructions. This assay

determines the activity of ATP synthase in a human or rat sample. Briefly, the ATP synthase is immunocaptured and the enzyme activity is measured by monitoring the decrease in absorbance at 340 nm, which is caused by the conversion of ATP to ADP by ATP synthase. This conversion is coupled with oxidation of NADH to NAD^+ which is associated with reduction in absorbance at 340 nm.

4.4.11 Subunit beta of ATP synthase and MMP-2 sequence analysis

With the use of LALING peptide sequence comparison program (http://www.ch.embnet.org/software/LALIGN_form.html), the primary sequence of rat mitochondrial ATP synthase subunit beta (accession # P10719) for UniProtKB/Swiss-Prot protein Knowledgebase) was compared with 14 known MMP-2 cleavage recognition sites (10 amino acids each) (192). Sites that had a >60% homology for all 10 amino acids were considered in this analysis.

4.4.12 Statistical analysis

The protein spot levels were analyzed using t-test and Mann–Whitney U-test, which is incorporated in the PDQuest measurement. The proteins of interest were identified by MS. ANOVA and Kruskal–Wallis test were used in functional studies. Data are expressed as the mean ± SEM.

4.5 Results

4.5.1 The effect of siRNA transfection on cardiomyocyte viability and contractility

After 48 hours incubation the viability of control cardiomyocates was approximately 60%. Transfection of scrambled siRNA did not further decreased cell viability and was similar to the control level (Figure 4.2A). Similarly, the contractility of cardiomyocytes transfected with scramble siRNA was comparable to the contractility of the control cells (Figure 4.2B).

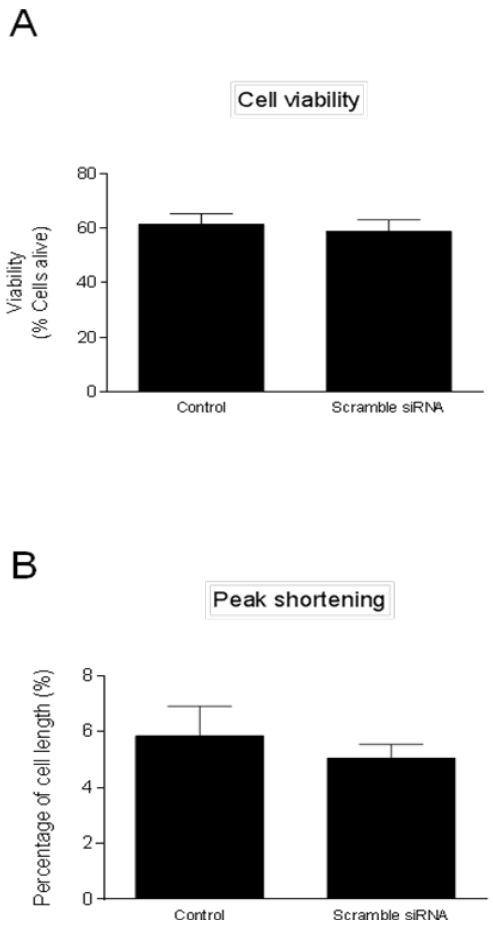


Figure 4.2
Effect of siRNA transfection on cardiomyocytes viability (A) and contractility (B). Results are presented as percentage of live cells and contractility is presented as percent of cell shortening. n=5/group; *p<0.05

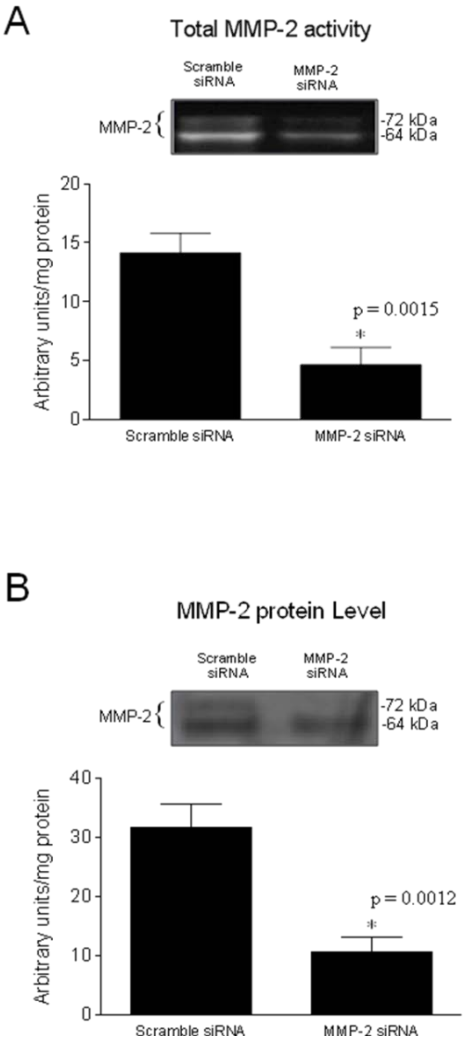
4.5.2 MMP-2 activity and MMP-2 expression in cardiomyocytes with MMP-2 knockdown cells

Because control cardiomyocytes and cardiomyocytes transfected with scrambled siRNA did not differ in viability and contractility (Figure 4.2), in all experiments the cells

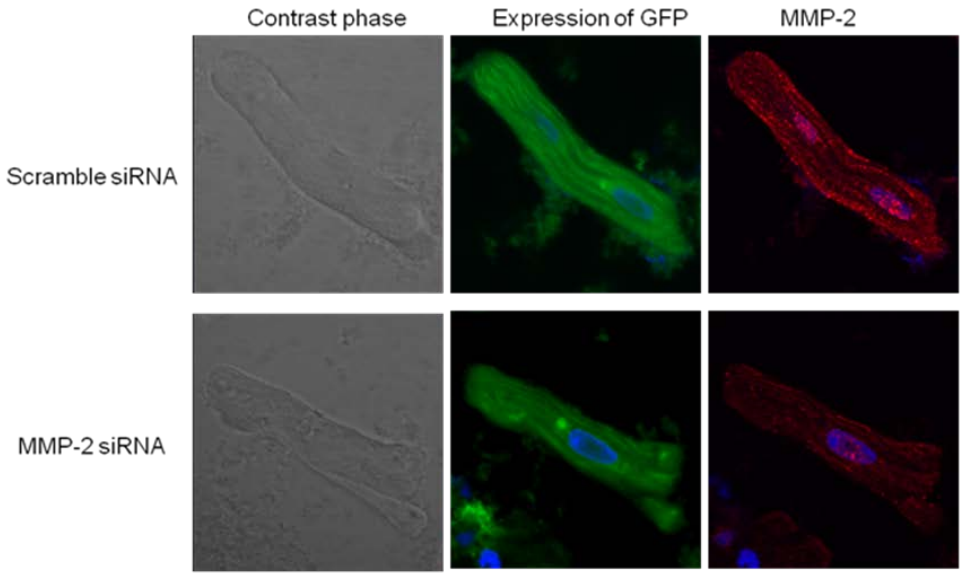
transfected with scrambled siRNA were used as a control to MMP-2 siRNA transfected cells.

Efficiency of silencing of MMP-2 expression by MMP-2 siRNA transfection was evaluated by three different methods: gelatin zymography, immunoblot analysis, and confocal microscopy (Figure 4.3). Total MMP-2 activity; determined by gelatin zymography, was reduced by approximately 70% in comparison to control cells (Figure 4.3A). Similar decrease was observed when an MMP-2 protein level was determined by immunoblotting (Figure 4.3B). The pro MMP-2 form (72 kDa) was almost no detectable in both techniques (Figure 4.3A and B). Confocal microscopy showed that the level of MMP-2; evaluated by immunstaining, decreased by approximately 40% in the cardiomyocytes transfected with MMP-2 siRNA, in comparison to scramble siRNA transfection (Figure 4.3C).

Transfection efficiency was determined by the measurement of fluorescence from GFP tagged siRNA which was co-transfected with either scramble or MMP-2 siRNA (Figure 4.3C). GFP fluorescence revealed that transfection efficiency was approximately 95% (data not shown).



C



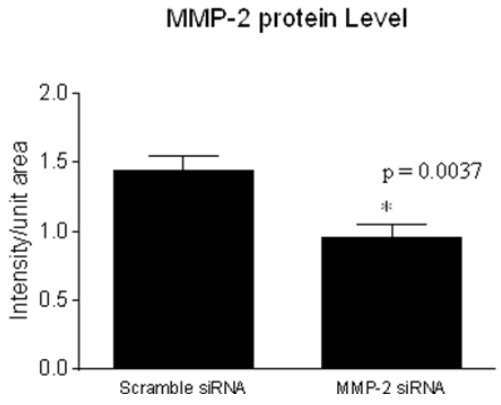


Figure 4.3
Inhibition of cardiomyocyte MMP-2 expression by MMP-2 siRNA. (A) Analysis of MMP-2 activity by zymography. (B) Analysis of protein level of MMP-2 by immunoblotting. (C) Evaluation of transfection efficiency. The efficiency was determined by measurement of fluorescence from GFP tagged siRNA which was co-transfected with MMP-2 siRNA or scrambled siRNA. n=6-13/group; *p<0.05

4.5.3 Effect of inhibition of MMP-2 expression on cardiomyocyte viability and contractility

Cardiomyocyte viability and contractility were measured after a total of 48 hours including stabilization, transfection and incubation periods. Transfection of MMP-2 siRNA improved cell viability by approximately 15% (Figure 4.4A) and increased three fold cardiomyocyte contractility in comparison to the group of cells transfected with scrambled

siRNA (Figure 4.4B).

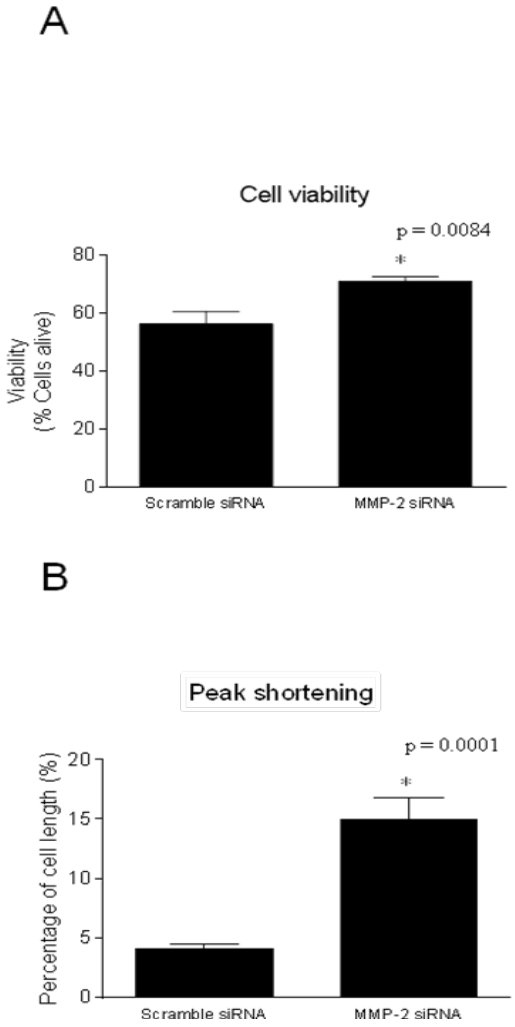


Figure 4.4
Effect of MMP-2 siRNA transfection on cardiomyocytes viability (A) and contractility (B). Scramble siRNA was used as a control of MMP-2 siRNA. Results are presented as percentage of live cells and contractility is presented as percent of cell shortening. n=5/group; *p<0.05

4.5.4 2-DE of protein extracts from siRNA transfected cells

Protein extracts from the scrambled and MMP-2 siRNA groups were separated by 2-dimensional electrophoresis (2-DE) (Figure 4.5). Analysis of the protein spot intensity variation (using an arbitrary threshold) detected 13 protein spots affected by inhibition of MMP-2. Nine protein spots showed an increase (Figure 4.6A) and four protein spots showed a decrease following MMP-2 siRNA treatment (Figure 4.6B).

The identification of these 13 protein spots is shown in Table 4.1. Seven protein spots were identified as mitochondrial enzymes involved in energy production; 5 of these are significantly increased in the MMP-2 siRNA group and include ATP synthase beta unit,

dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, cytochrome c oxidase subunit 5A, electron transfer flavoprotein subunit beta and NADH dehydrogenase (ubiquinon) 1 alpha subcomplex subunit 5. Two of them are reduced and include ATP synthase beta subunit (cleaved form of full length molecule) and long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor. Two protein spots (245 and 237) were identified as the contractile protein myosin light chain 1 (MLC1) (see Table 4.1). Protein spot 245 was identified as intact MLC1 and was significantly higher in the MMP-2 siRNA group (Figure 4.6A). Spot 237 was identified as a truncated form of MLC1 and its level was approximately 2 fold lower in the MMP-2 siRNA group (Figure 4.6B). Other protein spots were identified as heat shock protein 60 precursor and Cu-Zn superoxide dismutase.

Because MLC1 is a substrate for MMP-2 (22, 89), and changes in its level in cardiomyocytes perfused with MMP-2 inhibitors are described (47), to validate results from 2-DE we measured level of MLC1. In addition to MLC1 levels measured in 2-DE, we evaluated its level by immunobloting. The MLC1 level, similarly to the results from 2-DE, was increased (Figure 4.7) in immunoblotting as well. Measurement of tubulin level was used as a control of protein loading.

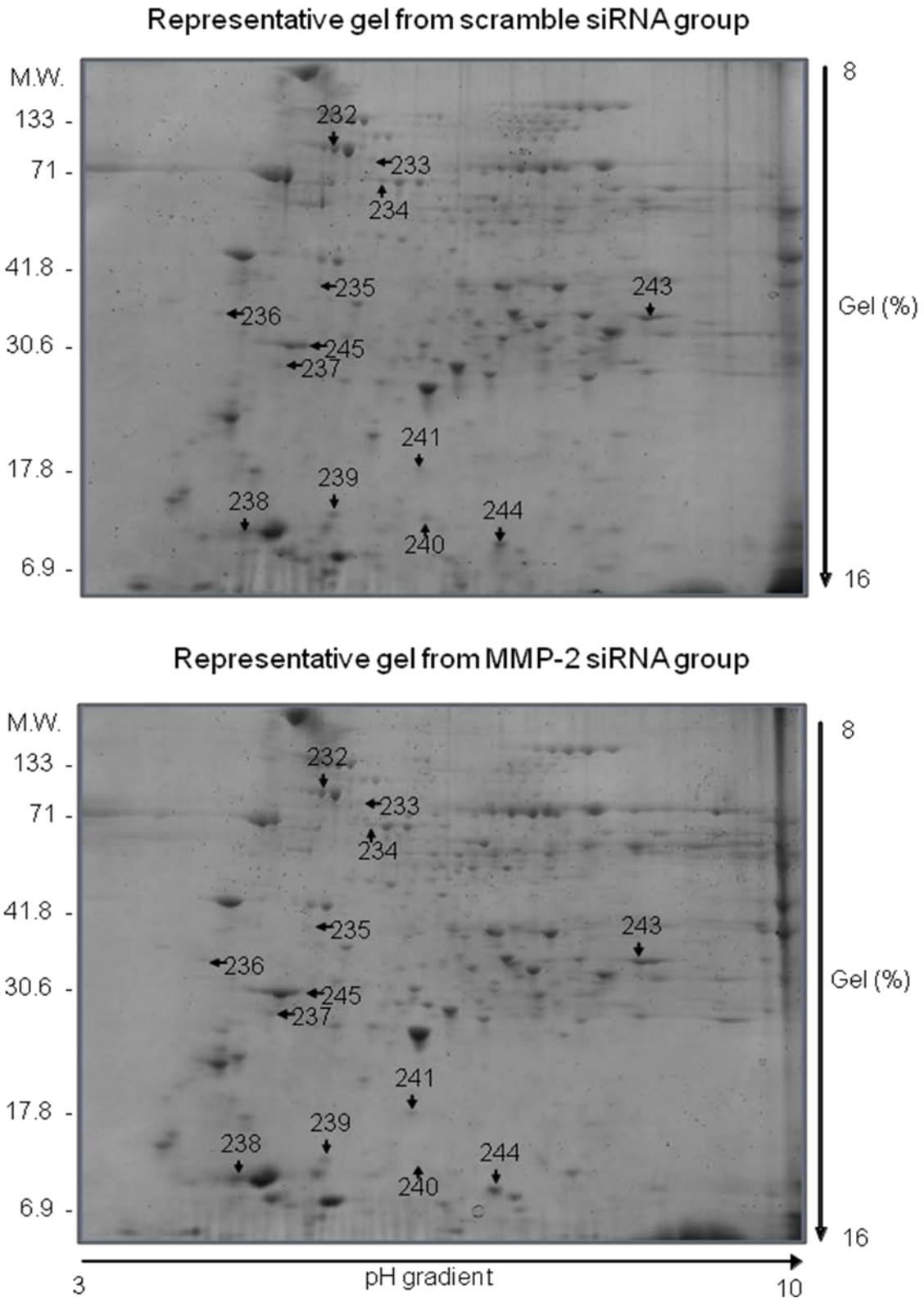


Figure 4.5
Two-dimensional electrophoresis (2-DE) of cardiomyocyte homogenates. Representative gels of 2-DE of protein extracts from control (scramble siRNA) group and MMP-2 siRNA group are showed. The arrows indicate protein spots significantly changed by MMP-2 inhibition. n=6/group; *p<0.05

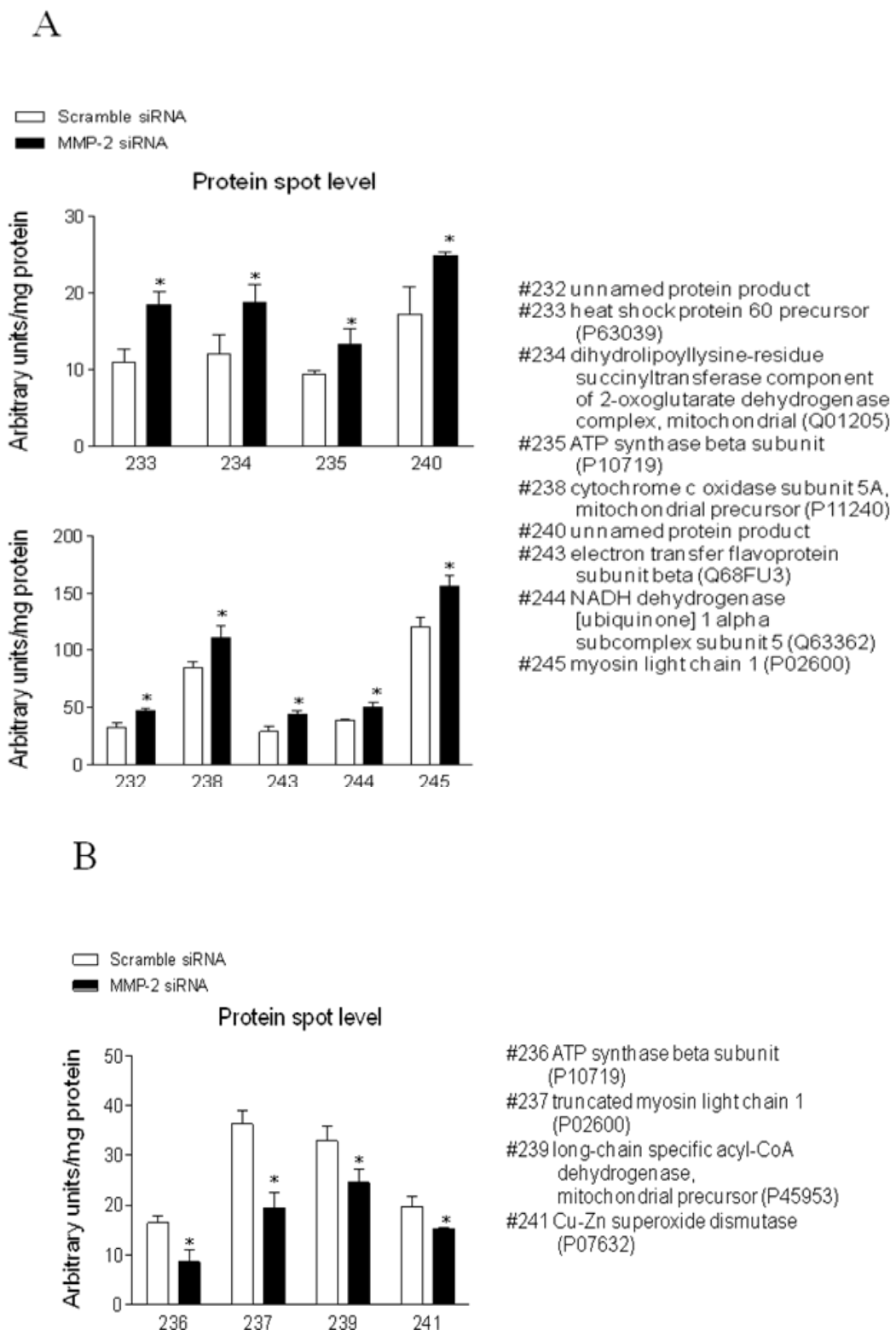


Figure 4.6
Effect of MMP-2 siRNA transfection on cardiomyocyte proteome. For better visual presentation, the altered proteins are grouped in three groups. Each panel represents one protein group according to the level of abundance. n=6/group *p<0.05 versus control group

Table 4.1 Identification of protein spots from control group VS MMP-2 siRNA group
Exp, experimental

Protein spot NO. (Accession NO.)	Mowse score [a]	Queries matched	Sequence coverage (%)	pI(Exp)/MW(Exp) (kDa)	Identified protein	Fold change	p value
Energy production							
234 ↑ [b] (Q01205)	765	16	31	8.89 (6.0)/49.236 (66)	Dihydro-lipoyllysine-residue succinyltrans-ferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	1.6	0.042
235 ↑ (P10719)	1149	26	39	4.92(5.2)/ 51.171(38)	ATP synthase beta subunit fragment	1.4	0.016
236 ↓ (P10719)	1115	30	44	4.92 (4.2)/ 51.171(36)	ATP synthase beta subunit fragment	-1.9	0.033
238 ↑ (P11240)	389	11	34	6.08 (4.3)/16.347 (13)	Cytochrome c oxidase subunit 5A, mitochondrial precursor	1.3	0.045
239 ↓ (P45953)	190	6	10	7.63 (5.2)/ 48.242 (15)	Long-chain specific acyl-CoA dehydrogenase mitochondrial precursor	-2.0	0.047
243 ↑ (Q68FU3)	1459	31	49	7.60 (8.4)/27.898 (36)	Electron transfer flavoprotein subunit beta	1.5	0.037
244 ↑ (Q63362)	1366	24	68	6.84 (7.1)/13.460 (10)	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	1.3	0.045
Structural protein							
237 ↓ (P02600)	293	8	40	5.03 (4.9)/ 22.256 (27)	Myosin light chain 1	-1.9	0.006
245 ↑ (P02600)	219	6	18	5.03 (4.9)/ 22.256 (31)	Myosin light chain 1	1.3	0.033
Others							
240 ↑ (CAA32441)	116	8	26	5.42 (6.3)/15.856 (14)	Unnamed protein product	1.4	0.045
232 ↑ (CAA37664)	2261	47	69	5.35 (5.3)/58.06 (84)	Unnamed protein product	1.4	0.020
233 ↑ (P63039)	1070	26	48	5.91 (5.9)/ 61.098 (78)	Heat shock protein 60 precursor	1.8	0.025
241 ↓ (P07632)	338	8	32	5.88 (6.3)/ 15.871 (18)	Cu-Zn superoxide dismutase	-1.3	0.003

[a] Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 31 indicate identity or extensive homology ($p<0.05$).
[b] The arrows (↑↓) indicate statistically significant changes in protein levels (versus control group)

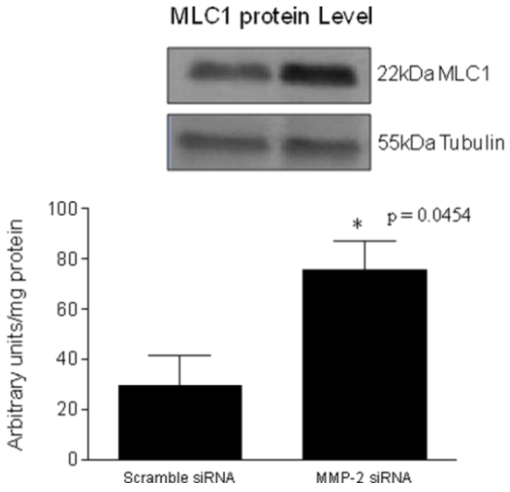


Figure 4.7
Validation of 2-DE results by immunobloting. Protein level of MLC1 was used to validate the results obtained by 2-DE. Tubulin was used as a protein loading control. n=5/group; *p<0.05

4.5.5 ATP synthase protein level and its activity

The analysis of protein spot 235 and 236 showed that these protein spots represent two different molecular forms of beta subunit of ATP synthase (Figure 4.8A). The level of the first protein spot (#235) was increased in MMP-2 siRNA group whereas the level of the second protein spot (#236) was decreased (Figure 4.8B)

Analysis of tryptic peptides identified by MS analysis (indicated in red in figure 4.9A) showed that the protein spot 235 represents the whole protein, whereas the second (spot 236) represents the truncated form of ATP synthase beta subunit with a lower molecular weight (Figure 4.9A). Comparison of molecular sizes of both forms of beta subunit (Figure 4.8A) and amino acid sequences (Figure 4.9A) shows that spot 236 is smaller than spot 235 by approximately 5 kDa.

Comparison of the ATP synthase beta subunit amino acid sequence with various MMP-2 cleavage recognition sequences revealed one site of interest localized between threonine 421 (T421) and leucine 430 (L430) and is indicated by the green box in figure. The site at amino acid 421 to 430 showed 60% homology to the MMP-2 cleavage consensus sequence (Figure 4.9B). The sequence of amino acids in P1-P6 positions shows over 80% homology (Figure 4.9B). In proteolytic enzyme research, the capital letters P and P' describe

localization of amino acid residue in the cleavage site region. The P amino acid residues are localized on left side of the cleavage site and P' residues are localized on the right side. The number indicates the location of the amino acid residue relative to the cleavage site. In addition, a low abundant tryptic peptide FLSQPFQVAEVFTGHMGK localized between amino acids phenylalanine 408 (F408) and lysine 236 (K236) was found in spot 236 (this peptide is underlined in red in figure 4.9A). The identified peptides and localization of cleavage site in the beta chain of ATP synthase by MMP-2 shows that the product of MMP-2 action will be shorter by 48 amino acids (approximately 5kDa). The localization of the recognized sequence by MMP-2 is shown in green in Figure 4.9A.

In addition to expression level, ATP synthase activity was evaluated using an ATP synthase enzyme activity assay. In the MMP-2 siRNA group, ATP synthase activity was increased by approximately 30% (Figure 4.10).

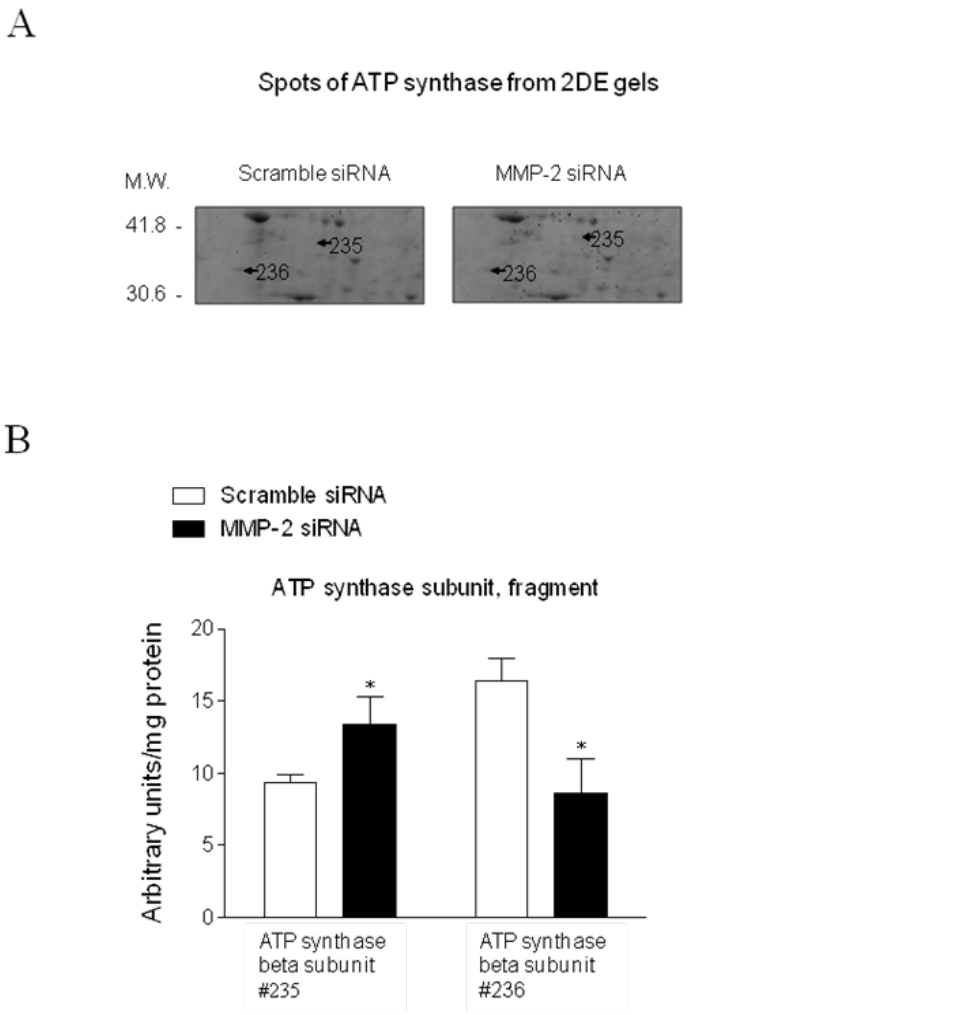


Figure 4.8
Effect of MMP-2 inhibition on beta subunit of ATP synthase. (A) Fragments of representative 2-DE gels from control (scramble siRNA) group and MMP-2 siRNA. Arrows indicate protein spots of ATP synthase subunits beta. (B) Densitometric analysis of affected protein spots. n=6/group; *p<0.05

A

Spot 235ATP synthase beta subunit

```
  1 EFRIATGQIV AVIGAVVDVQ FDEGLPPILN ALEVQGRESR LVLEVAQHLG
 51 ESTVRTIAMD GTEGLVRGQK VLDSGAPIKI PVGPETLGRI MNVIGEPIDE
101 RGPIKTKQFA PIHAEAPEFI EMSVEQEILV TGIKVVDLLA PYAKGGKIGL
151 FGGAGVGKTV LIMELINNVA KAHGGYSVFA GVGERTREGN DLYHEMIESG
201 VINLKDATSK VALVYGQMNE PPGARARVAL TGLTVAEYFR DQEGQDVLLF
251 IDNIFRFTQA GSEVSALLGR IPSAVGYQPT LATDMGTMQE RITTTKKGSI
301 TSVQAIYVPA DDLTDPAPAT TFAHLDATTV LSRAIAELGI YPAVDPLDST
351 SRIMDPNIVG SEHYDVARGV QKILQDYKSL QDIIAILGMD ELSEEDKLTV
401 SRARKIQRFL SQPFQVAEVF TGHMGKLVPL KETIKGFQQI LAGDYDHLPE
451 QAFYMVGPIE EAVAKADKLA EEHGS
```

Spot 236 ATP synthase beta subunit

```
  1 EFRIATGQIV AVIGAVVDVQ FDEGLPPILN ALEVQGRESR LVLEVAQHLG
 51 ESTVRTIAMD GTEGLVRGQK VLDSGAPIKI PVGPETLGRI MNVIGEPIDE
101 RGPIKTKQFA PIHAEAPEFI EMSVEQEILV TGIKVVDLLA PYAKGGKIGL
151 FGGAGVGKTV LIMELINNVA KAHGGYSVFA GVGERTREGN DLYHEMIESG
201 VINLKDATSK VALVYGQMNE PPGARARVAL TGLTVAEYFR DQEGQDVLLF
251 IDNIFRFTQA GSEVSALLGR IPSAVGYQPT LATDMGTMQE RITTTKKGSI
301 TSVQAIYVPA DDLTDPAPAT TFAHLDATTV LSRAIAELGI YPAVDPLDST
351 SRIMDPNIVG SEHYDVARGV QKILQDYKSL QDIIAILGMD ELSEEDKLTV
401 SRARKIQRFL SQPFQVAEVF TGHMGKLVPL KETIKGFQQI LAGDYDHLPE
451 QAFYMVGPIE EAVAKADKLA EEHGS
```

B

```
Sequence recognized by MMP-2    SGHMHKA↓LTA
                                .::: :   :

ATP synthase beta subunit  421 TGHMGK-↓LVPL 430
(10 amino acid fragment)        ▲   ▲  ▲
                                P6  P1 P1'

↓MMP-2 cleavage site
```

Figure 4.9
(A) Analysis of tryptic digests of ATP synthase subunit beta. The sequence coverage of beta subunit with experimentally obtained peptides is highlighted in red. Cleavage site for MMP-2 is showed in green. (B) Analysis for possible MMP-2 cleavage site within beta subunit of ATP synthase. The red line shows the C-terminus peptide detected in truncated form of ATP synthase. Amino acids of beta subunit was compared with various MMP-2 cleavage recognition sites. Two dots denote the same amino acid whereas one dot denotes a similarity. The letters P and P' describe localization of amino acid residues in the cleavage site region. The P amino acid residues are localized on left side of the cleavage site and P' residues are localized on the right site. The number indicates the location of the amino acid residue from to the cleavage site.

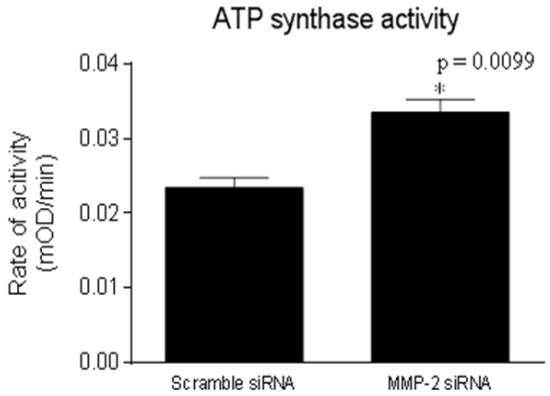


Figure 4.10
Effect of MMP-2 siRNA transfection on activity of ATP synthase in cardiomyocytes. n=6/group; *p<0.05

4.6 Discussion

Ischemia /reperfusion (I/R) injury of the heart is a complex processes ranging from molecular to functional adaptations in response to ischemia and/or reperfusion. The contribution of each phenomenon to the development of lethal injury remains a matter of controversy [reviewed in (198)].

In general, it is accepted that protein degradation within the heart is a major component of the regulatory mechanism in both the physiology and pathology of the heart. For decades it was commonly acknowledged that cardiac protein degradation was mediated by three main pathways such as the ubiquitin-proteasome pathway, autophagy/lysosomal degradation, and the calpain pathway (199). Research from the last decade shows that matrix metalloproteinase-2 (MMP-2) is another proteolytic enzyme involved in contractile dysfunction in I/R hearts [see review (101)]. In addition, it has been suggested that MMP-2 can be involved in physiological regulation, such as regulation of normal turnover of cardiac proteins (46). Rate of protein turnover in heart tissue varies considerably. It has been estimated that about 30% of newly synthesized protein in the heart is degraded in a period of 10 minutes. In the I/R heart, that period can be shorter (200). In a normal heart; in the case of sarcomeric proteins, the turnover is considered to be much slower, and is estimated to be 5-8 days (201).

Despite a large number of studies on the role of MMP-2 in heart injury, the physiological role of MMP-2 in regulation of cardiac contractility is still unclear. We have already shown that in I/R injury, MMP-2 degrades contractile proteins (22, 89). However, other mechanisms involving regulation of energy metabolism have been suggested (23, 202). Among a variety of work, including ours, it has been shown that contractile protein such as troponin I (114), myosin light chain 1 (MLC1) (22) and 2 (MLC2) (145), and titin (115) are degraded by MMP-2 during cardiac injury, and inhibition of MMP-2 shows cardioprotective effects mainly related to the protection of contractile proteins (203).

All of these observations have been made in intact isolated hearts undergoing rapid injury processes (minutes) and appear to be independent of changes in collagen content (204-205). However, many cell types in the heart express MMP-2 activity, including vascular endothelial cells. Therefore, it is possible that the MMP-2 from endothelial cells may have a paracrine effect on cardiac myocytes to effect changes in their contractility, or the MMP-2 may have an autocrine effect on the same myocytes. The use of isolated myocytes as a biological model should provide evidence whether the intracellular action of MMP-2 in a

single cell directly modulates protein level. This is why we decided to utilize a proteomic approach to investigate the cardiomyocyte protein profile affected by MMP-2 inhibition using isolated myocytes perfused under aerobic physiological conditions.

There are a few approaches developed for inhibition of MMPs, however none of those is specific against MMP-2. To overcome this problem we decided to knockout MMP-2 expression with small interfering RNA. Small interfering RNA (siRNA), also known as silencing RNA, is a class of double stranded RNA molecules, which can specifically inhibit protein synthesis by interfering with mRNA. In this study, we evaluated the effect of MMP-2 siRNA transfection on cardiomyocyte contractility and proteomic profile.

In our studies we inhibited near complete synthesis of the pro-form of MMP-2 (72 kDa) with siRNA. This inhibition is indicated by lack of the 72 kDa form in zymography and immunoblot analysis. The detection of the 64 kDa form of MMP-2 in both techniques is due to the nature of the activation process of MMP-2, which occurs on the surface of cell membranes (206-208). The cell surface MMP-2 is not accountable for degradation of intracellular proteins. The surface cell distribution of MMP-2 in MMP-2 siRNA transfected cells is visible by immunocytochemistry with confocal microscopy.

In the present study we observed that the contractility of cardiomyocytes is significantly higher in the myocytes transfected with MMP-2 siRNA in comparison to the control group. The damaging role of MMP-2 in a heart subjected to oxidative stress is already established [reviewed in (209-210)]. Additionally, the role of MMP-2 in normal heart physiology has also been postulated (210). Based on the postulated role of MMP-2 in normal heart physiology we hypothesize that MMP-2 regulates turnover of sarcomeric proteins. The increased level of intact form of MLC1 and decreased level of truncated form of MLC1 in MMP-2 siRNA transfected cells were expected as previously reported (5,20, 21). The changes in the levels of both forms of MLC1 in MMP-2 siRNA group resembled those changes observed with pharmacological inhibition of MMP-2. Therefore the changes in MLC1 levels (intact or truncated form) were a good control of our experimental design to study MMP-2 activity in the isolated cardiomyocytes.

We have found that the inhibition of MMP-2 expression affected the levels of 6 proteins involved in energy metabolism. It has already been shown that MMP-2 accumulates in cardiac mitochondria and that increased MMP-2 activity is associated with mitochondrial dysfunction (211). Changes in the level of subunits of mitochondrial ATP synthase in heart injury were previously reported in several models of heart injury (176, 202, 212). It has also been suggested that inhibition of Rho kinase with Y-27632 normalizes the level of ATP

synthase in I/R hearts (23). Despite these previous reports, however, speculation pertaining to the precise mechanism responsible for this normalization is lacking. For the first time, our results show that the mitochondrial dysfunction caused by MMP-2 might depend on metabolic enzyme regulation, including the ATP synthase.

To explore the effect of MMP-2 on energy production in cardiomyocytes, we focused on ATP synthase. Two molecular forms of subunit beta of ATP synthase were affected by inhibition of MMP-2. Peptide analyses of the beta subunit of ATP synthase, showed increased levels with MMP-2 siRNA and this form likely represented the full length protein. We also observed a beta subunit of ATP synthase with decreased levels after MMP-2 siRNA, which likely represented the truncated form with lower molecular weight and more acidic pI. In support of this notion, examination of the amino acid sequence of beta subunit ATP synthase revealed an MMP-2 consensus cleavage site that gives rise to a 5kDa peptide fragment and the truncated ATP synthase. The negative effect of MMP-2 on ATP synthase is additionally supported by the fact that ATP synthase activity in cardiomyocytes with inhibited MMP-2 expression is increased. Taken together, our present results implicate MMP-2 as a significant contributor to the degradation of beta subunit of ATP synthase and, therefore, regulates the ATP synthase activity. However, more studies are needed to study the role of MMP-2 in mitochondrial dysfunction.

Heat shock protein 60 (HSP 60) levels were also increased when MMP-2 expression was inhibited. The heat shock protein family are proteins that aid in protein folding and are involved in protein-protein interactions. If MMP-2 degrades HSP 60, the proper folding and functional expression of proteins may lead to alterations in cardiomyocyte function, for instance, by decreasing contractility (from MLC1 degradation) or by increasing mitochondrial dysfunction (from ATP synthase degradation) . Additional experiments focused on HSP 60 are necessary to determine a possible role of MMP-2-HSP 60 pathway in myocyte damage.

The decrease of Cu-Zn superoxide dismutase (171) level in MMP-2 knockout cells has no simple explanation, however it has been shown that over-expression of SOD is associated with activation of MMP-2 (213). Similar to HSP 60, more studies are needed to elucidate the role of MMP-2 in regulating SOD expression and activity.

In summary, this is the first report which shows that intracellular inhibition of MMP-2 in cardiomyocytes increases contractility of aerobically perfused myocytes. In addition, we showed for the first time that this inhibition is also associated with increased activity of ATP synthase. An association of ATP synthase with MMP was already indicated in studies on

regulation of ATPase activity of transglutaminase 2 by MT1-MMP (214). We also show that changes in metabolic enzyme levels are likely associated with proteolytic processing by MMP-2. The unanswered question is how this degradation/processing is regulated under physiological conditions? It is well established that phosphorylation is an important mechanism of regulation of energy metabolism (215). We have also shown that post-translational modifications (PTMs) such as nitration, nitrosylation and phosphorylation regulate MMP-2 activity under pathological conditions (145). We expect that similar processes may provide a basis for regulatory mechanisms under physiological conditions.

In conclusion, it appears that enzymes involved in energy production, including ATP synthase, are additional intracellular substrates for MMP-2. Thus, regulation of energy metabolism can be additionally achieved by modulation of intracellular action of MMP-2.

5. Inhibition of MMP-2 expression with siRNA increases baseline cardiomyocyte contractility and protects against simulated ischemic reperfusion injury

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H-BL –Experimental work, experimental design, manuscript writing

VJJC – Experimental design, experimental work, manuscript writing

BS – Experimental work

JS – Experimental work

ZC – Experimental work

LKB – Experimental design, manuscript writing

FC – Experimental work, experimental design, manuscript writing

GS – Hypothesis generation, experimental design, manuscript writing

5.1 Preface

This manuscript has been published in the journal *Biomed research international (Lin et al. 2014).* This manuscript addressed the last objective of my PhD project.

5. To determine the cardioprotective effect of inhibition of MMP-2 using MMP-2 siRNA on isolated cardiomyocytes.

From our studies, we conclude that MMP-2 degrades not only contractile proteins but also energy metabolic proteins, and this could be the key event of the development of ischemia/reperfusion injury. Developing a strategy for inhibition of MMP-2 may be a key therapy for ischemia/reperfusion injury. However, lack of selective MMP-2 inhibitor is the main barrier. Therefore, we decided to specifically inhibit MMP-2 by using MMP-2 siRNA, and we found that the inhibition of expression of MMP-2 protects cardiomyocytes from contractile dysfunction after ischemia/reperfusion. The key points of this study are: 1) prove again that MMP-2 plays a vital role in degradation of contractile and metabolic protein in ischemia/reperfusion injury; 2) develop a effective strategy for inhibition of MMP-2 by using MMP-2 siRNA to knockdown the expression of MMP-2; 3) this is the first study directly indicates the intracellular function of MMP-2, which is involve in contractile dysfunction in cardiomyocytes subjected to ischemia/reperfusion.

5.2 Abstract

Matrix metalloproteinases (MMPs) significantly contribute to ischemia reperfusion (I/R) injury, namely by the degradation of contractile proteins. However, due to the experimental models adopted and the lack of isoform specificity of MMP inhibitors, the cellular source and the identity of the MMP(s) involved in I/R injury remain to be elucidated. Using isolated adult rat cardiomyocytes, subjected to chemically induced I/R-like injury, we show that the specific inhibition of MMP-2 expression and activity by MMP-2 siRNA significantly protected cardiomyocyte contractility from I/R, associated with increased myosin light chains 1 and 2 (MLC1/2) expression levels, in comparison to scramble siRNA transfection. Moreover, the positive effect of MMP-2 siRNA transfection on cardiomyocyte contractility and MLC1/2 expression levels was also observed in the control group, suggesting an important additional role for MMP-2 in physiological sarcomeric protein turnover.

This study clearly demonstrates that autocrine intracellular expression of MMP-2 plays a significant role in sarcomeric protein turnover, namely MLC1 and MLC2, under aerobic (physiological) conditions. In addition, this study identifies intracellular/autocrine, cardiomyocyte-produced MMP-2, rather than paracrine/extracellular, as responsible for the degradation of MLC1/2 and consequent contractile dysfunction in cardiomyocytes subjected to I/R injury.

5.3 Introduction

The pathological role of matrix metalloproteinases (MMPs), including MMP-2, during the development of oxidative stress-mediated cardiac injury and contractile dysfunction has been shown previously (189, 191, 216). We and others have shown increased activity of MMP-2 in ischemic reperfusion (I/R) injury (127, 217-218), hypoxia-reoxygenation injury (219) and infusion of reactive oxygen species, namely peroxynitrite ($ONOO^-$) (125, 128). Furthermore, increased intracellular MMP-2 activity (22, 114-115, 145) is associated with degradation of contractile proteins such as troponin I (114), titin (115), myosin light chain 1 (MLC1)(22, 46, 89) and myosin light chain 2 (MLC2) (145). All these observations have been made in intact, isolated hearts during a relatively short time-course (minutes) and appear to be independent of changes in collagen content (204-205), suggesting preponderant acute intracellular action of MMP-2.

MMP-2 can be found in most cardiac cell types, including vascular endothelial cells (220), smooth muscle cells (221), fibroblasts (222) and cardiomyocytes (47, 114). The majority of MMP-2 synthesized is secreted (~60%) acting in a paracrine manner; with the remaining 40% being targeted to the cytosol (223) or mitochondrial associated membranes (224). Therefore, it is probable that MMP-2 originated in endothelium, smooth muscle cells or fibroblasts can be upregulated in response to oxidative stress and acting in a paracrine manner on cardiomyocytes, aiding in the development of I/R injury and cardiac contractile dysfunction. Hence, the understanding of MMP-2's mechanism of action (paracrine vs. autocrine) and determination of the cellular source/targets of MMP-2 is crucial in the development of novel and more selective drug design.

Greater than 20 MMPs have been described to date with all of them showing similarities in substrate specificities and response to known MMP inhibitors (225) limiting the clinical application of these broad-spectra drugs. The vast majority of studies, looking at the roles of MMPs in the development of I/R injury, have been performed in whole heart or whole cardiac tissue homogenates, without the discrimination between cell types. Moreover, the pharmacological approaches used to modulate MMPs' activity rely on the use of broad spectra MMP inhibitors (eg. doxycyline, ortho-phenathroline) not being possible to clearly identify isoform specific effects. Inhibition of MMP-2 has been shown to protect isolated cardiomyocyte contractility in response to oxidative stress (47, 226). Nonetheless, clinical usage of subantimicrobial doses of doxycyline in patients undergoing cardiopulmonary bypass surgery failed to show a protective effect on cardiac function despite the inhibition of

MMP-2 (227). Consequently, despite the wide body of pre-clinical evidence supporting inhibition of MMP-2 in cardiac pathologies, the failure of clinical translation makes it crucial to determine the physiological and pathological roles of MMP-2 to properly develop therapeutic strategies targeting MMP-2.

Here we unequivocally show that specific, autocrine, intracellular action of MMP-2 on cardiomyocytes regulates not only contractile protein turnover under physiological conditions but also the development of I/R-induced cardiac contractile dysfunction, *via* increased degradation of contractile proteins. These observations, made with the use of a specific inhibition of MMP-2 with siRNA, provide novel and important knowledge on the role of MMP-2 in I/R injury and indicate potential therapeutic alternatives to the prevention and treatment of I/R injury.

5.4 Materials and methods

This investigation conforms with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

5.4.1 Cardiomyocyte isolation

Male Sprague-Dawley rats (weighing 100–150 g) were anaesthetized with sodium pentobarbital (60 mg/kg i.p.) and hearts were removed. Right ventricular myocytes were used as they provide a consistently higher ratio of live cardiomyocytes to contaminating fibroblasts and endothelial cells in comparison to preparations from the left ventricle (191). Right ventricular myocytes were obtained by enzymatic dissociation as previously described (194).

5.4.2 Primary culture of cardiomyocytes

Isolated cardiomyocytes were seeded in 35x10 mm cell culture plates (Nunc, Roskilde, Denmark) at 2×10^5 cells per plate in DMEM medium (Lonza Walkersville, MD USA) supplemented with 10% FBS and incubated in a 95% air-5% CO_2 incubator at 37°C for 7 hours to stabilize cells. Viability and contractility of the isolated cardiomyocytes were assessed after isolation, transfection with siRNA and at the end of reperfusion (Figure 5.1).

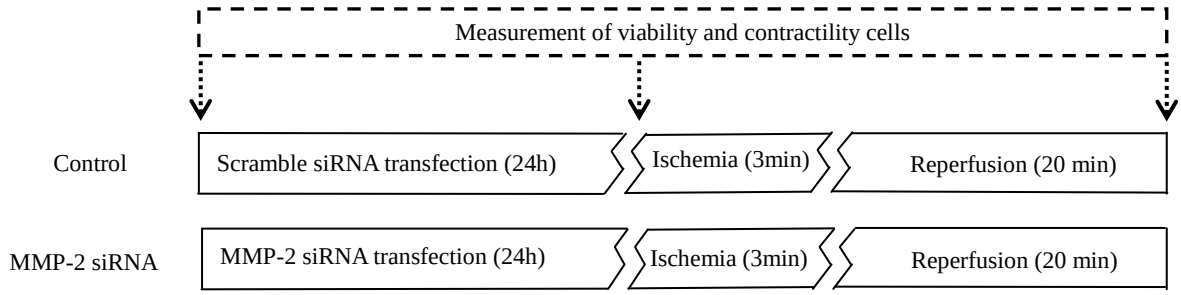


Figure 5.1
Schematic representation of the perfusion protocol for isolated cardiomyocytes. Scrambled siRNA was used as a control of MMP-2 siRNA. Arrows indicate when cell contractility was measured: (1) before siRNA transfection, (2) before ischemia, (1) at the end of reperfusion.

5.4.3 siRNA transfection

A mixture containing a pool of 3 target-specific 19-25 nucleotide small interfering RNAs (MMP-2 siRNA) designed to knock down rat MMP-2 gene expression (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was resuspended in RNAse-free water to a final 10 μM stock concentration in a buffer containing 10 μM Tris-HCl, 20 mM NaCl, and 1 mM EDTA at pH 8.0. This solution was stored at -20°C. As a control, scrambled siRNA (Santa

Cruz Biotechnology, Santa Cruz, CA, USA) was used under the same conditions.

Following a 7 hour stabilization period in DMEM and 10% FBS, cardiomyocytes were washed with siRNA transfection medium (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated for 24 hours at 37°C in 200μL of transfection medium containing 0.8 μM of MMP-2 siRNA or scrambled siRNA (Figure 1) according to the manufacturer's protocol. For assessment of transfection efficiency, green fluorescent protein (GFP) was co-transfected with siRNA.

5.4.4 Simulated ischemia/reperfusion protocol

After 24 hours of transfection with siRNA, cardiomyocytes were subjected to chemical ischemia, as previously described (228). Briefly, the transfection medium was removed and replaced with a solution containing 5mM 2-deoxyglucose to inhibit glycolysis and 4 mM NaCN to inhibit mitochondrial respiration (ischemia). After 1, 3, 5 and 7 minutes of incubation the solution was removed and replaced with culture medium without FBS (reperfusion).

5.4.5 Measurement of viability and contractility of cardiomyocytes

Cardiomyocyte viability and contractility were evaluated at three time points throughout the experimental protocol: after cell isolation, after transfection with siRNA (before ischemia), and at the end of reperfusion (Figure 1). The viability of cardiomyocytes was assessed by the trypan blue exclusion test (229-231). Cardiomyocyte contractility was measured using IonOptix system and the IonWizard 6.0 software (IonOptix, Milton, MA USA). After a stabilization period the chamber containing the cells was perfused with an oxygenated buffer at a constant temperature of 37°C. Cells were continuously paced at 1 Hz and 5 V (IonOptix MyoPacer, Milton, MA, USA). The assessment of cardiomyocyte contractility was made by the measurement of peak shortening, maximal velocity of cell shortening and maximal velocity of cell relengthening (47) on 8-10 cardiomyocytes per independent experiment, over a 10 min period to give an average measure per sample.

5.4.6 Preparation of cell extracts

Cardiomyocytes were collected and stored at -80°C. For biochemical studies, frozen cardiomyocytes were thawed and sonicated on ice twice for 5 seconds in 50 mM Tris-HCl buffer (pH 7.4) containing 3.1 mM sucrose, 1 mM DTT, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin, and 0.1% Triton X-100. Homogenates were

then centrifuged at 10000g at 4°C for 10 minutes and the supernatant was collected and stored at -80°C until further use. Protein content of the cardiomyocyte extract was measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

5.4.7 Measurement of MMP-2 activity

Gelatin zymography was performed as previously described (122, 127, 166). Briefly, homogenates from cardiomyocyte preparations containing 30 μg of protein were applied to 8% polyacrylamide gel copolymerized with 2 mg/mL gelatin. After electrophoresis, gels were rinsed three times for 20 minutes in 2.5% Triton X-100 to remove SDS. The gels were then washed twice in incubation buffer (50 mM Tris-HCl, 5 mM CaCl2, 150 mM NaCl and 0.05% NaN3) for 20 minutes at room temperature and incubated in incubation buffer at 37°C for 24 hours. The gels were stained using 0.05% Coomassie Brilliant Blue G-250 in a mixture of methanol:acetic acid:water (2.5:1:6.5, v:v:v) and destained in aqueous solution of 4% methanol:8% acetic acid (v:v). Developed gels were scanned with an GS-800 calibrated densitometer and MMP-2 activity was measured using Quantity One 4.6 software (Bio-Rad, Hercules, CA, USA).

5.4.8 Western blot analysis

Protein (30 μg) from cardiomyocyte homogenates were separated using 12% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Myosin light chain 1 and 2 (MLC 1 and MLC2) were identified using mouse monoclonal anti-MLC1 antibody and rabbit polyclonal anti-MLC2 antibody, respectively (Abcam, Cambridge, MA, USA), MMP-2 was identified using rabbit monoclonal anti-MMP-2 antibody (Abcam, Cambridge, MA, USA). Membranes were developed using Versa Doc 5000 and band densities were measured with Quantity One 4.6 software (Bio-Rad, Hercules, CA, USA). Equal protein loading was additionally verified by measurement of tubulin level with mouse monoclonal antibody (Abcam, Cambridge, MA, USA).

5.4.9 Immunocytochemistry

For immunocytochemistry, cardiomyocytes were seeded on polylysine-coated coverslips and follow the same siRNA transfection protocol. After permeabilization with 0.25% Triton X-100 and fixation, cells were blocked for 1 h with PBS containing 5% bovine serum albumin (BSA, Sigma, St Louis, MO, USA). Proteins of cardiomyocytes were labeled by overnight incubation (at 4°C) with rabbit anti-MMP-2 (Abcam, Cambridge, MA, USA)

antibody diluted at 1:200 in blocking buffer followed by brief wash (three times; 10 min each) and incubation with Alexa Fluor 555-conjugated goat anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA, USA) at 1:1000 for 1 h. After Hoechst (Sigma, St Louis, MO, USA) staining, the coverslips were mounted on newly cleaned slides using Prolong Gold Antifade Reagent (Invitrogen, Carlsbad, CA, USA) and observed with an LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Images were acquired using a Zeiss Plan-Apochromat 63X/1.6 oil objective lens and analyzed with the Zeiss Zen 2009 software (version 5.5 SPI).

5.4.10 Immunoprecipitation

The immunoprecipitation of MMP-2 with MLC1 or MLC2 was initiated by incubating 200 µg of total protein extract with 10 µg mouse anti-MLC1 IgG or 10 µg rabbit anti-MLC2 IgG in a total volume of 500 µl RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM PMSF) overnight at 4 °C. This buffer was chosen because of its known high stringency to avoid unspecific binding. As a negative control, unrelated IgG was used instead of anti-MLC IgG. Following the initial incubation, 100 µl of slurry of protein A–Sepharose beads were added and the resulting mixture incubated overnight at 4 °C. After overnight incubation the mixture was washed three times with 0.5 ml of RIPA buffer at 4 °C and 20 µl of sample buffer were added. Determination of co-localization of MLC1 or MLC2 with MMP-2 was determined by gelatin zymography of the immunoprecipitates.

5.4.11 Statistical analysis

For contractility measurements, at least three independent experiments (myocyte preparations from different hearts) were run. Each experiment was performed in triplicate (myocytes from the same heart). ANOVA with Kruskal–Wallis post-hoc analysis or Student's t-tests were used in this study. A $P < 0.05$ indicated statistical significance. Data are presented as the mean ± SEM.

5.5 Results

5.5.1 Cell contractility and duration of ischemia

The effect of the duration of ischemia on cardiomyocyte viability and contractility was determined. One minute of ischemia decreased cardiomyocyte viability by approximately 10%, in comparison to aerobic control cells, with longer durations reducing viability further (Figure 5.2A). Cardiomyocyte contractility, namely peak shortening and maximal velocity of myocyte relengthening, was decreased after 1 minute of ischemia; however maximal velocity of myocyte shortening was unaffected (Figure 5.2B). Three minutes of ischemia reduced contractility by 50% in all three measured parameters. Longer periods of ischemia (5 and 7 min) further reduced contractility to approximately 70-80% of aerobic values (Figure 5.2B). Based on cellular viability and contractility, 3 minutes of ischemia was chosen for further experiments.

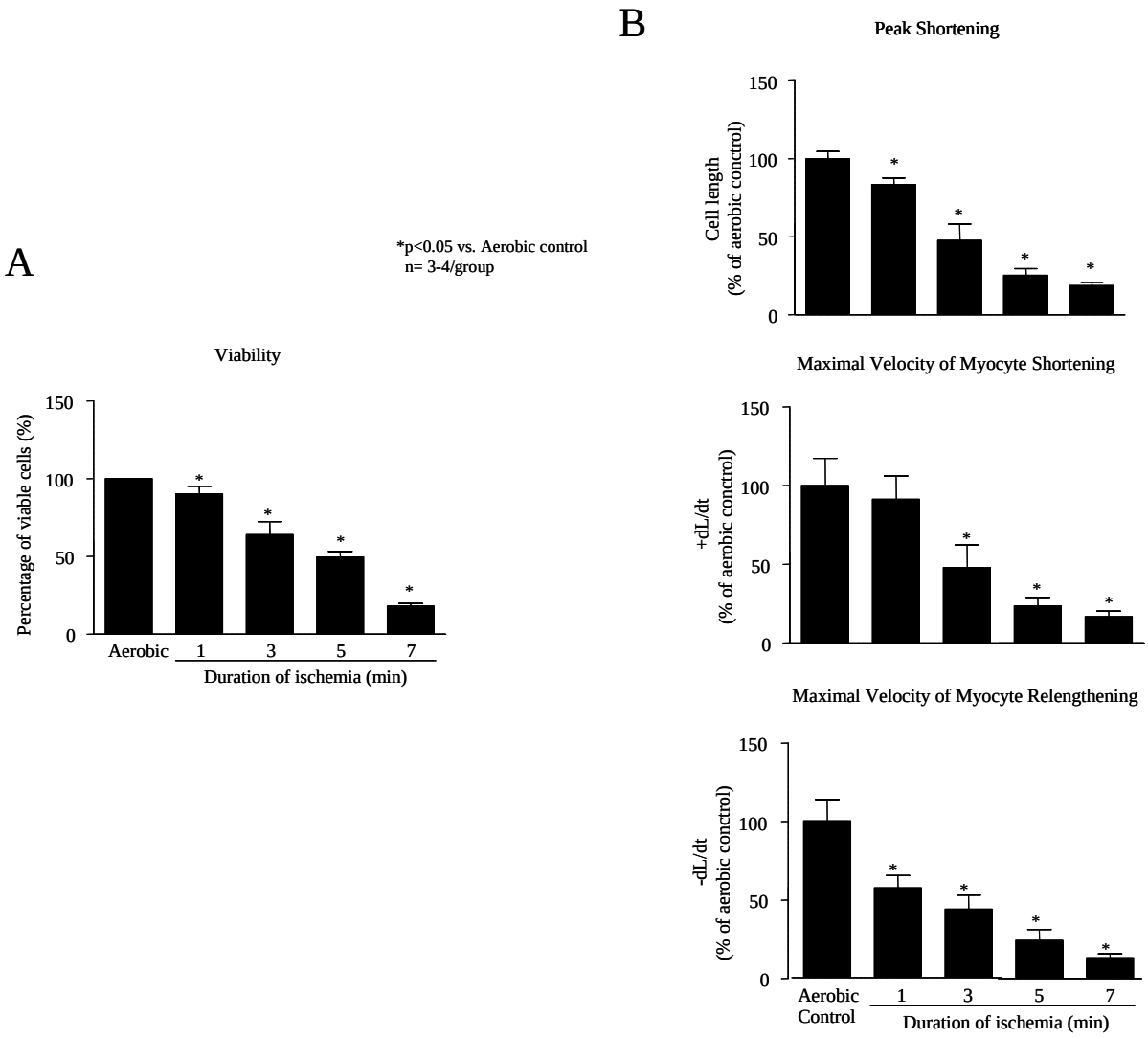


Figure 5.2
Effect of the duration of ischemia on cardiomyocyte viability (A) and contractility (B). The mean total number of alive cardiomyocytes in the control group is considered as 100%. n= 3-4 heart preparations per group, $^{*}p<0.05$ versus aerobic control.

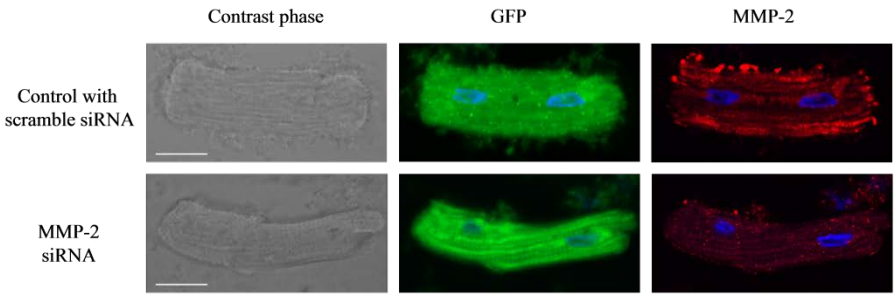
5.5.2 MMP-2 expression and activity in cardiomyocytes transfected with MMP-2 siRNA

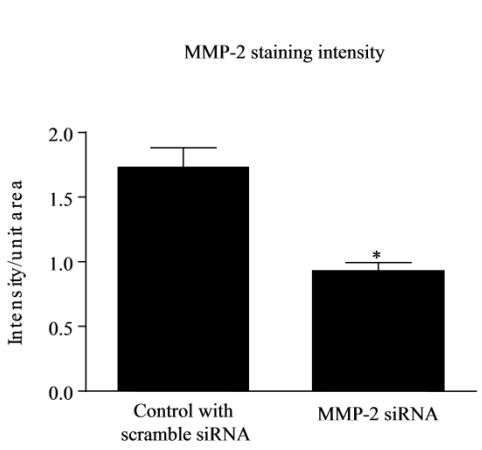
MMP-2 siRNA transfection silencing of expression was evaluated by gelatin zymography, immunoblot analysis, and immunocytochemistry using confocal microscopy (Figure 5.3).

Transfection efficiency, determined by the measurement of GFP-tagged siRNA fluorescence that was co-transfected with either scrambled or MMP-2 siRNA (Figure 5.3A, top pannel), was approximately 95%. This efficiency of overall transfection was associated with a 50 % decrease in the levels of MMP-2, evaluated by immunocytochemistry, in cardiomyocytes transfected with MMP-2 siRNA, in comparison to control cells transfected with scramble siRNA (Figure 5.3A).

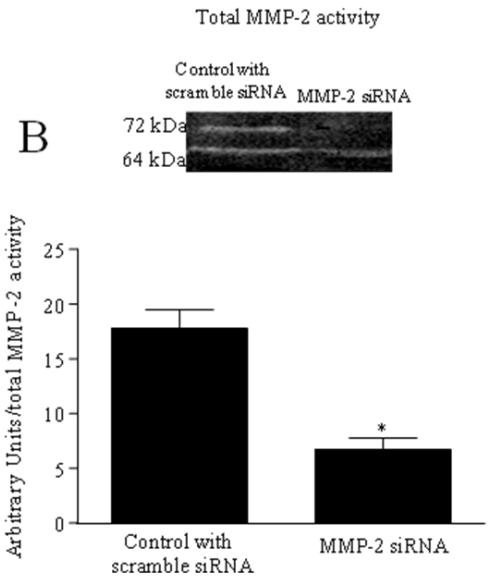
Total MMP-2 activity, as determined by gelatin zymography, was reduced by approximately 70% in comparison to control cells transfected with scrambled siRNA (Figure 3B). Although the cleaved 64 kDa form of MMP-2 was detected after knocking down MMP-2 mRNA, the pro MMP-2 form (72 kDa) was undetectable (Figure 5.3B). A similar decrease was observed for MMP-2 protein level determined by immunoblotting (Figure 5.3C).

A


Contrast phase
GFP
MMP-2
Control with scramble siRNA
MMP-2 siRNA


*p<0.05 vs. Control
n=25/group
MMP-2 staining intensity
Intensity/unit area
2.0
1.5
1.0
0.5
0.0
*
Control with scramble siRNA
MMP-2 siRNA

B


Total MMP-2 activity
Control with scramble siRNA
MMP-2 siRNA
72 kDa
64 kDa
Arbitrary Units/total MMP-2 activity
25
20
15
10
5
0
*
Control with scramble siRNA
MMP-2 siRNA

C

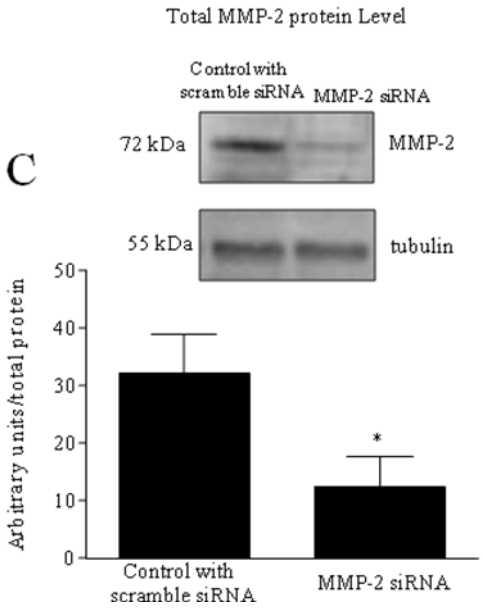

Total MMP-2 protein Level
Control with scramble siRNA
MMP-2 siRNA
72 kDa
MMP-2
55 kDa
tubulin
Arbitrary units/total protein
50
40
30
20
10
0
*
Control with scramble siRNA
MMP-2 siRNA

Figure 5.3
Effect of MMP-2 siRNA transfection on MMP-2 expression in isolated cardiomyocytes. (A) Efficiency of siRNA transfection and MMP-2 protein levels measured by immunocytochemistry. Scale bar, 50 μm. (B) Measurement of MMP-2 gelatinolytic activity by zymography. (C) MMP-2 protein level. (A) n=25 cells from 3 different hearts per group; n= 9-10 heart isolates per group for MMP-2 activity (B), in (C) n=4 heart isolates per group, and in , $^{*}p<0.05$ versus aerobic control.

5.5.3 MMP-2 knockdown effects on cardiomyocyte contractility before and after I/R injury

The effect of siRNA transfection on cell viability and contractility was evaluated using scrambled siRNA to control for possible effects independent of inhibition of MMP-2. Transfection of scrambled siRNA (control) did not impact either cardiomyocyte viability (Figure 5.4A) or contractility (Figure 5.4B).

Transfection of cardiomyocytes with MMP-2 siRNA resulted in an increase in the levels of the sarcomeric proteins myosin light chain 1 and myosin light chain 2 (MLC1 and MLC2, respectively) in comparison to control (Figure 5.5A). This increase in MLC1 and MLC2 was accompanied by a decrease in the formation of the protein complexes MMP-2–MLC1 and MMP-2–MLC2 (Figure 5.5B). These observations at the protein level were associated with an increase in contractile function of aerobically perfused, MMP-2 siRNA transfected cardiomyocytes (before ischemia) in comparison to cells transfected with scrambled siRNA (Figure 5.5C).

The contractile function of cardiomyocytes, transfected with scrambled siRNA, in response to I/R was significantly decreased, whereas transfection with MMP-2 siRNA fully protected contractile function against I/R (Figure 5.6A). However, it should be noted that baseline contractility was higher in the MMP-2 knockdown cells than those with scrambled transfection (Figure 5.5). The protective effects of MMP-2 inhibition by siRNA transfection were associated with levels of MLC1 and MLC2 that were 3 and 2 fold higher, respectively, in comparison to control scramble siRNA transfected cells under aerobic conditions (Figure 5.6B).

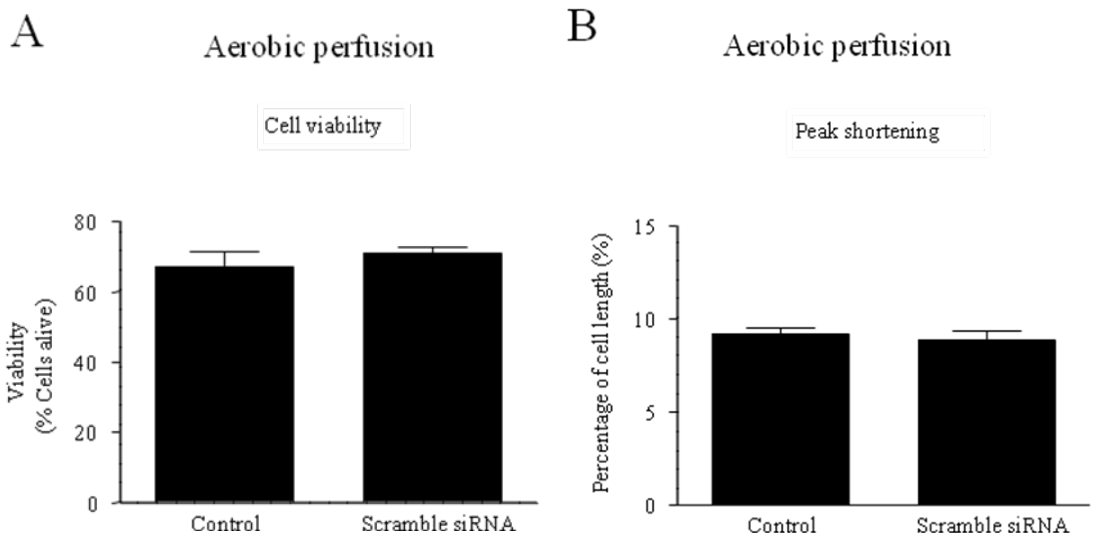
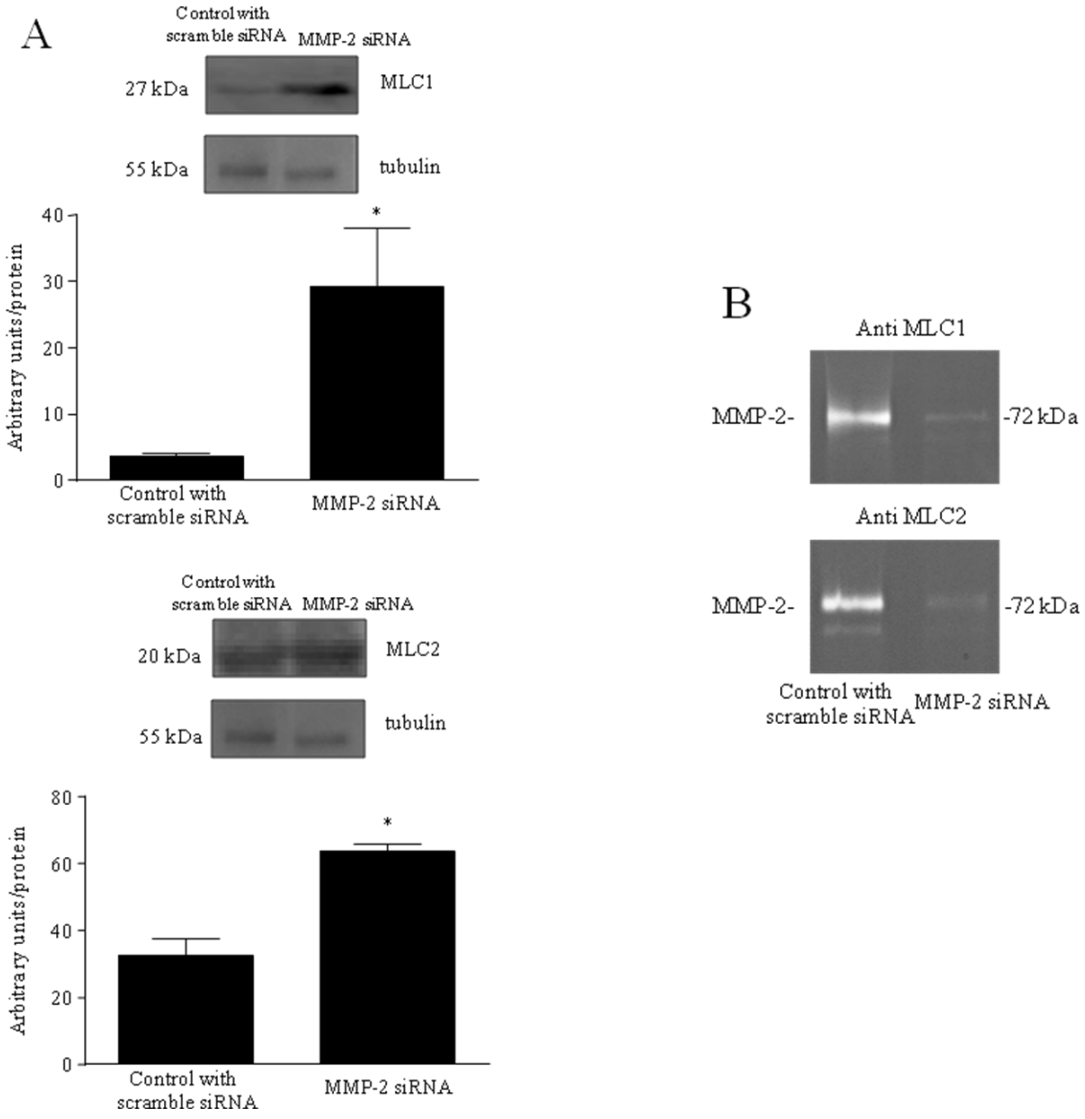


Figure 5.4
Effect of scrambled siRNA transfection on cardiomyocyte viability (A) and contractility (B). Control cells were transfected with scrambled siRNA. n=4 heart isolations per group.



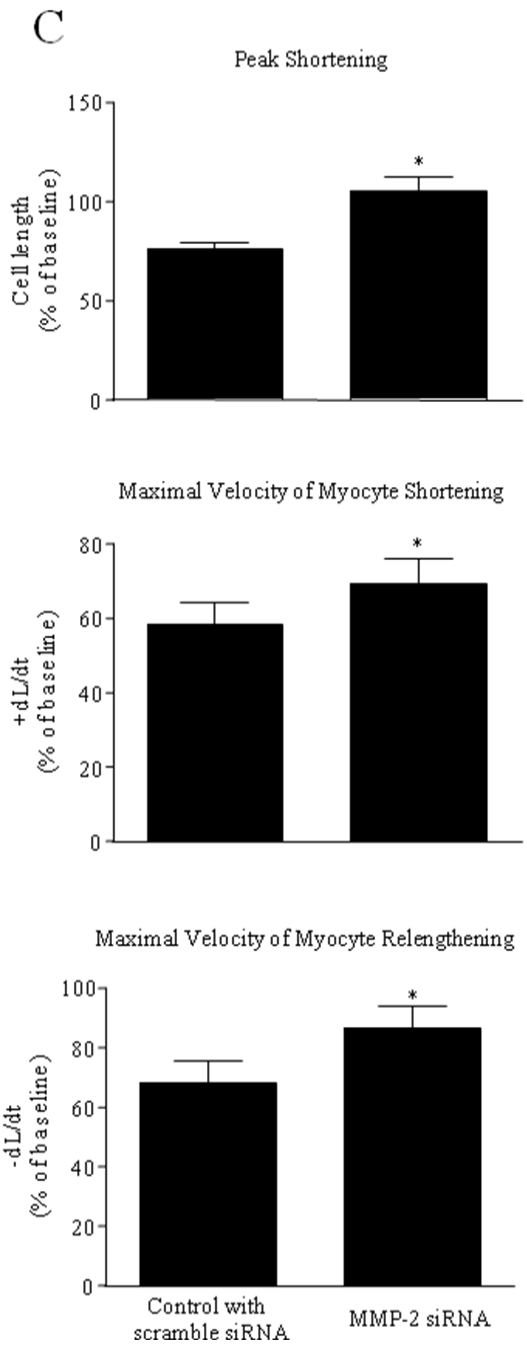


Figure 5.5
Effect of MMP-2 siRNA transfection on the level of the contractile proteins MLC1 and MLC2 (A), the formation of the complex between MMP-2 and MLC1 or MLC2 (B) and cardiomyocyte contractility (C). As a protein loading control the tubulin level was measured. Control cells were transfected with scrambled siRNA. n=4-6 heart preparation isolations per group. n=11 per group for contractility measurement and n=4-6 for measurement of protein levels. $^{*}p<0.05$ versus Control

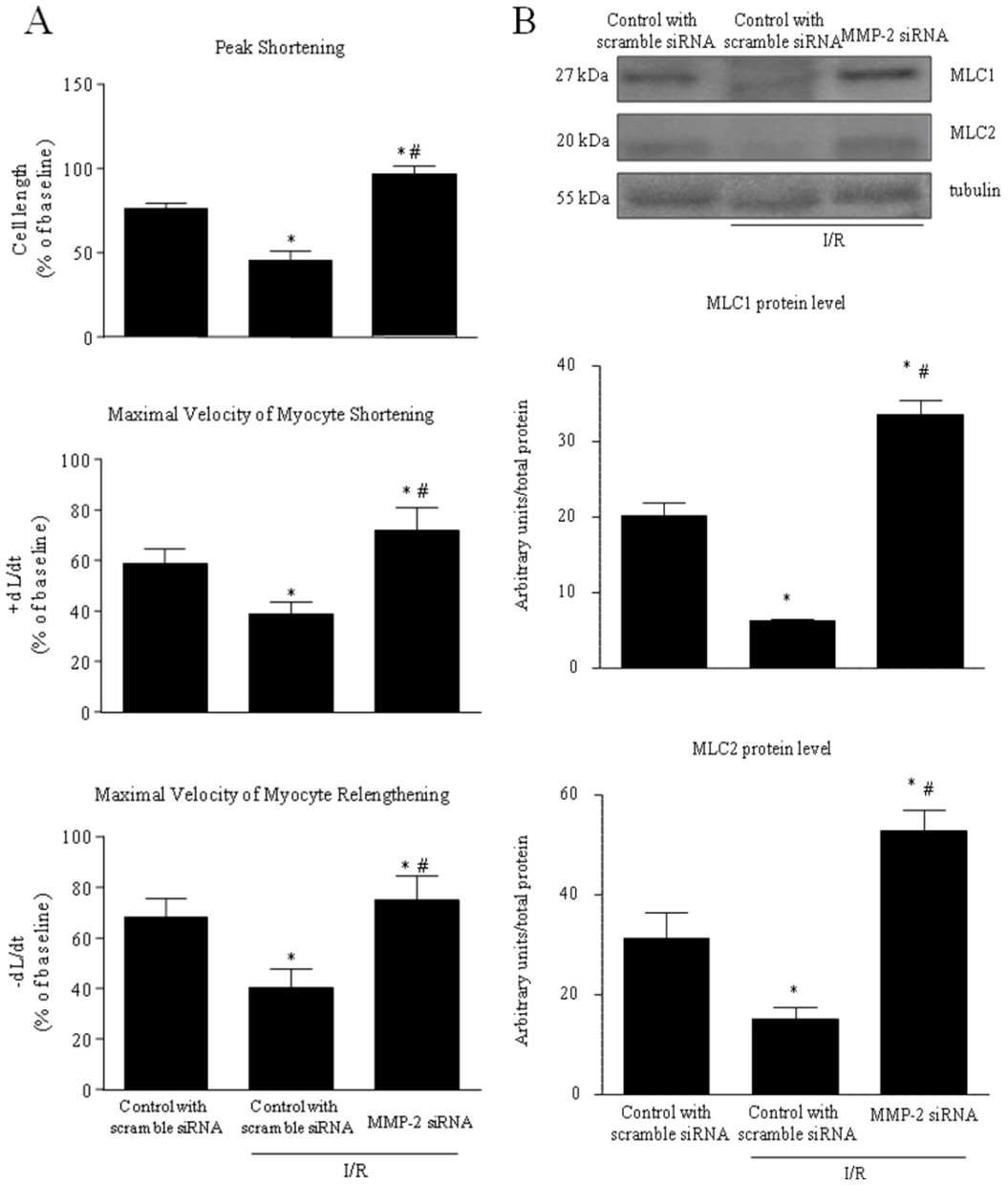


Figure 5.6
Effect of MMP-2 siRNA transfection on cardiomyocyte contractility (A) and MLC1 and MLC2 in cardiomyocytes subjected to I/R. As a protein loading control the tubulin level was measured. Control cells were transfected with scrambled siRNA. n=11 per group for contractility measurement and n=4 for measurement of protein levels. $^{*}p<0.05$ versus Control, $^{\#}p<0.05$ versus I/R.

5.6 Discussion

While the cellular mechanisms of I/R injury are complex and not entirely understood, the degradation of contractile proteins is considered to be a major cause of heart injury (22, 232), with matrix metalloproteinase-2 (MMP-2) playing a significant role in contractile protein degradation (22, 114, 127, 145). To the best of our knowledge, this study is the first to demonstrate that, under physiological conditions, MMP-2 regulates MLC1 and MLC2 protein turnover. In response to I/R MMP-2 activity increases leading to degradation of these contractile proteins and decreased cardiomyocyte contractility. Inhibition of MMP-2 by siRNA transfection and reduction in protein levels protects MLC1, MLC2 and cardiomyocyte contractility from I/R. Moreover, our study clearly demonstrates that MMP-2 acts in an autocrine and intracellular fashion to regulate contractile protein turnover under physiological conditions or mediate I/R-induced injury. Furthermore, our study suggests that, in contrast to broad spectra MMP inhibitors, the use of MMP-2 siRNA to specifically modulate MMP-2 activity can be of clinical relevance in the prevention and treatment of I/R injury and contractile dysfunction associated with loss of contractile proteins.

MMPs are proteolytic enzymes which are known to play a role in maintaining the structural integrity of the extracellular matrix (233). However, studies over the last decade strongly suggest that MMP-2, in addition to the role in remodeling and degradation of extracellular matrix, is also involved in increased intracellular degradation of contractile proteins in heart subjected to oxidative stress (191, 234-235). This increased degradation reduces sarcomeric integrity resulting in contractile dysfunction of the injured heart (89). The study of the roles of MMP-2 in the heart has, almost exclusively, focused on pathological conditions. We have previously reported that MMP-2 may be involved in the physiological regulation of contractile proteins, namely MLC1 and MLC2 (46, 145). Here we show that the specific inhibition of MMP-2 protein levels and activity, in cardiomyocytes, with siRNA reduces the formation of the protein complex between MMP-2 and MLC1/2, resulting in an increase in MLC1/2 protein levels, in cells cultured under aerobic conditions. Importantly, the increase in MLC1/2 protein levels is associated with increased cardiomyocyte contractility. This observation suggests that inhibition of MMP-2 can potentially be a useful strategy to increase cardiac contractility in cardiac pathologies characterized by depressed cardiac function, such as heart failure (236).

Biological studies of specific MMP actions, including MMP-2, have been challenging due to the lack of selectivity and specificity of commercially available synthetic inhibitors.

Synthetic inhibitors of MMPs exhibit a broad spectrum of action across MMP families, with K_i in the nM range (237). The lack of selectivity and specificity are also evident by cross reactivity with other proteinases with distinct catalytic mechanisms from MMPs. Another drawback associated with synthetic inhibitors is toxicity. In fact cytotoxicity is a major limitation and is observed with the currently available MMP inhibiting drugs when higher doses are used (127, 238). The better alternative to a synthetic inhibitor could be the use of natural endogenous tissue inhibitors of MMP (TIMPs). However, despite having no interaction with other proteinases with different mechanism of action, they show poor selective within the MMP group (239). Moreover, genetic knockdown of MMP-2 has failed to provide an ubiquitously adequate model for the study of both the physiological and the pathological roles of MMP-2, since compensatory mechanisms have been observed to occur. For instance, a study on autoimmune encephalomyelitis it has been shown that MMP-2 KO mice exhibit a more severe disease than did their wild-type counterparts, that can be explained by a 3-fold increase in expression of MMP-9 (240) (an inducible MMP belonging to same group as MMP-2 (233)). With this in mind we decided to modulate MMP-2 activity by the use of small interfering RNA (siRNA). This genetic manipulation causes only a transient, but significant, reduction in MMP-2 protein expression and consequent overall enzymatic activity (Figure 3), minimizing the role of compensatory mechanisms that are usually triggered in germline knockouts as early as embryogenesis (241-242).

Small interfering RNA has proven to be an effective method for reducing gene expression through the use of a small piece of anti-sense RNA complementary to a gene of interest (243). In addition, siRNA has been successfully used in pre-clinical studies focused on cardiac tissue protection (244-245). By using MMP-2 siRNA we show that selective inhibition of intracellular MMP-2 protects the levels of myosin light chain 1 and 2 (MLC1/2) and contractility of cardiomyocytes subjected to I/R. Also, we show that the autocrine and intracellular actions of MMP-2 are responsible for contractile dysfunction and MLC1 degradation in I/R injured cardiomyocytes, independent from paracrine and extracellular MMP-2 actions since no other cell types are present. Although we cannot exclude the involvement of other proteolytic enzymes or non-proteolytic pathways in regulating sarcomere contractility and protein turnover, we believe that the observed effects result solely from MMP-2 selective silencing, without the enabling of adaptive mechanisms.

In summary, this study provides clear evidence that intracellular MMP-2 plays a crucial role in the heart under both physiological and pathological conditions, namely at the level of regulation of contraction. The separation of intracellular from extracellular roles of

MMP-2 has the potential to provide new directions for studying mechanisms underlying several cardiac pathologies, including heart failure. Furthermore, due to the potential for the use of siRNA therapies in clinical practice, these results can have a significant impact on the development of new approaches for the protection of hearts from reperfusion injury due to myocardial infarction or coronary revascularization.

6. GENERAL DISCUSSION

The epidemic of cardiovascular diseases is one of the main burdens to society. Two thirds of cardiovascular diseases are related to coronary heart disease. The narrowing or blockage of the coronary arteries causes ischemia/reperfusion followed by myocardium infarction and contractile dysfunction, which eventually leads to heart failure. During I/R, it has been shown that many factors such as ROS(246), proteolytic enzymes(136), contractile proteins(247), protein kinases(48) and energy metabolic enzymes(248-249) are involved in the development of I/R injury. However, the protein-protein interaction within these factors and the underlying molecular mechanisms of the development of I/R injury are still poorly understood. In order to develop novel effective therapeutic strategies for ischemia/reperfusion-induced contractile dysfunction, a better understanding of the mechanisms of I/R injury is required. In this study, we utilized proteomic methods to provide new insight into the molecular mechanisms of I/R injury.

The proteomic approach is becoming a powerful tool for profiling diseased states. Using proteomics to study the molecular mechanism of cardiac injury provides the characterization of alterations in comparative protein expression and information in protein-protein interaction during cardiac injury. The results from proteomic approach allows investigators to have a comprehensive protein profile in certain pathological conditions. In addition, biomarkers and therapeutic targets can also be identified. Proteomic studies have been done for the comparative protein expression among different physiological and pathological conditions in the heart including ischemia preconditioning(250-251), congestive heart failure(252) and hypertrophy(253), and more than 400 proteins which are effected in cardiovascular diseases have been identified(44, 54, 254-256). Studies suggest that these proteins are important in the regulation of the heart function as well as the pathogenesis of the cardiovascular diseases. (142-143).

Most of proteomic studies focus only on proteins in the heart that display differential responses to different physiological or pathological conditions. There is few studies targeting comparison of different regions of the heart as it responds to various conditions. The left and right ventricles are two vital parts of the heart which perform different missions in the circulation. Despite both the left and right ventricles being well studied in their physiological role, the pathological roles of the ventricles are yet to be well demonstrated. In addition, since most of the studies mainly focus on the left ventricles in the pathological heart, the study of

the right ventricle has been overshadowed by the left ventricle. Here, we demonstrate the comparison of the proteomic profile in the left and right ventricles of the I/R heart. The study presented in this thesis compares for the first time the changes of the left and right ventricle proteomes from hearts subjected to I/R injury. We demonstrated that there are proteomic differences between left and right ventricle in response to I/R injury. Those identified proteins participate in several different biological processes, such as, electron transport system (ATP synthase and cytochrome b-c1 complex), glycolysis (L-lactate dehydrogenase), stress management (peroxiredoxin 2 and peroxiredoxin 3), and contractile function (Myosin light chain 2, actin and myosin heavy chain 5). Our results suggest different regulation of these pathological processes, depending on the stage and the region of the cardiac injury, and are important for therapeutic design in cardiovascular research. The development of cardiovascular disease due to I/R injury results in significant alterations in the protein expression profile of the myocardium tissue. Further investigation of the relationship between these differentially expressed proteins and the development of the diseases would be very important for the understanding of the molecular mechanisms of the disease , the development of clinical therapies for cardiovascular disease, and improvement of the surgical procedures

Numerous energy metabolic enzymes have been identified in three of our studies (67, 257-258). We found in this study that energy metabolic enzymes such as : ATP synthase beta subunit (electron transport chain complex V), cytochrome b-c1 complex subunit (electron transport chain complex III), 24-kDa mitochondrial NADH dehydrogenase (electron transport chain complex I), cytochrome c oxidase subunit (electron transport chain complex IV),NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 (electron transport chain complex I) and succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial decrease significantly under I/R condition. All of these enzymes are related to the most important process in ATP production, electron transport chain and citric acid cycle. This suggests that down-regulation of energy metabolic proteins and reduction of ATP generation are the key events in the development of I/R injury. We also found that MLCK effects energy metabolic enzymes such as succinyl-CoA ligase. Studies have already shown that MLCK phosphorylates contractile proteins such as MLC1 and MLC2(259-260), which plays an important regulatory role in pathological conditions. In this study, we implicate that the MLCK pathway in the development of I/R-induced contractile dysfunction is mainly involved in the regulation of energy production and contractile structural regulation. Inhibition of MLCK pathway using MLCK inhibitor ML-7 can reduce the phosphorylation of

energy metabolic proteins and contractile proteins. Hence, it improves the energy production and contractility during I/R.

Increasing activity of MMP-2 is implicated in a variety of pathological condition including atherosclerosis(261), ischemic heart diseases(262) and heart failure(263). Most of the MMP-2 studies have primarily focused on the extracellular function. It has already been shown that inhibition of MMP-2 protects the heart from I/R injury(264). In the present study, we used selective MMP-2 inhibitor MMP-2 siRNA to inhibit the MMP-2 protein expression. Using MMP-2 siRNA as tool we evaluated the pathological role of MMP-2 in the development of I/R injury. Isolated cardiomyocytes model was developed to study the intracellular function of MMP-2. We then investigated the proteomics of inhibition of MMP-2 in isolated cardiomyocyte under aerobic condition, and we found that MMP-2 is involved in the regulation of the energy metabolic enzymes in normal physiological condition. It has been reported that energy metabolic enzymes are modulated in several cardiac disorder (140, 155). However, the process of energy metabolic re-programming in the pathological heart still remians unclear. In this proteomic study, up-regulation of crucial energy metabolic enzyme such as cytochrome c oxidase, NADH dehydrogenase and ATP synthase were identified in the MMP-2 siRNA. Inhibition of MMP-2 increases the ATP synthase activity as well as the contractility of cardiomyocyte. In addition, using an isolated cardiomyocyte model, this study provides the first direct evidence for the intracellular function of MMP-2. This suggests that MMP-2 may degrade energy metabolic proteins in the mitochondria within the cardiomyocyte.

Taken together, our studies reveal different responses of the left and right ventricles in the I/R heart. These responses are mainly targetting the modulation of contractile proteins and energy metabolic enzymes. It has been deminstrated that contractile proteins are regulated by MLCK and MMP-2(101). In this study, in order to investigate the regulatory role of MLCK and MMP-2 in energy metabolism during I/R, a combination method of selective inhibitors (ML-7 and MMP-2 siRNA) and proteomic approach is applied. Proteomic analysis of the I/R models with the inhibition of MLCK or MMP-2 allows investigators to evaluate the protein expression profile in I/R condition and find out which proteins are effected by the inhibition of MLCK and MMP-2. Our results show that proteins related to regulation of energy metabolism are MMP-2 targets during I/R, and protein kinase such as myosin light chain kinase (MLCK) is also involved in this process.

7. CONCLUSION

Our previous studies have demonstrated that the cardiac contractile proteins, such as MLC1 and MLC2, are subjected to posttranslational modifications (PTM) in response to I/R. The modification of these contractile proteins marks the protein for degradation by MMP-2 with a consequent worsening of cardiac contractile dysfunction(20, 89). In our studies, energy metabolic proteins have been shown to contribute to cardiac I/R injury, which might be also associated with MLCK and MMP-2. Our data provided a novel insight into the molecular mechanisms of cardiac I/R injury: during I/R, activated MLCK phosphorylates contractile proteins and energy metabolic proteins, and these modifications increase their degradation by intracellular MMP-2, resulting in reduction of ATP production and contractility, eventually leading to contractile dysfunction.

We expect that results from our studies will have implications in the clinical research of I/R injury from different pathological conditions. In addition, selective MMP-2 inhibitor MMP-2 siRNA and MLCK inhibitor ML-7 show cardioprotective effects in response to I/R injury, suggesting novel promising strategies to reduce the impact of ischemic heart disease. Also, we have shown how the proteomic approach can be used to evaluate protein expression in the heart and to study the protein modulation related to cardiac dysfunction.

My PhD project has five main objectives:

1. To identify proteomic profile in the heart subjected to ischemia/reperfusion injury
2. To compare in proteomic profile in right and left ventricle from heart subjected to ischemia/reperfusion injury
3. To determine the effect of the Myosin Light Chain Kinase inhibitor ML-7 on the proteome of hearts subjected to ischemia/reperfusion injury
4. To analyze the inhibition of MMP-2 gene expression with small interfering RNA in isolated cardiomyocytes by proteomic analysis, with the focus on the degradation of energy metabolic proteins by MMP-2
5. To determine the cardioprotective effect of inhibition of MMP-2 using MMP-2 siRNA on isolated cardiomyocytes

Objectives are focusing on the alteration of proteome of the heart in the development of I/R injury. Our finding presented here support that the modulation of energy metabolic

proteins play a key role in the development of I/R injury. This can supplement our working mechanism of cardiac ischemia/reperfusion injury (101). Moreover, a new strategy for inhibition of MMP-2 and MLCK were developed, and shows an effective protection from ischemia/reperfusion-induced cardiac contractile dysfunction.

8. LIMITATION AND FUTURE DIRECTIONS

There are a few limitations in this study. First, the proteomic approach relies on the reproduction of two dimension electrophoresis, equal loading conditions and the software selection (such as PDQuest software) of significant protein targets. In order to gain consistent positive and reproductive results, numbers of experiments need to be repeated and high criteria of exclusion setting are required. When the target proteins are identified by MS, a more standard biochemistry analysis is necessary to verify these associations. To date, one of the main problems in the proteomic study is lack of follow-up research. Numbers of proteins are identified by proteomic analysis. However, most of the research groups do not intend to do the further research. McGregor et al has provided a comprehensive review indicating major of limitations within the proteomics approach used in the cardiovascular research (265-266). Second, purification/verification of cardiomyocyte has been performed in the *in vitro* model. However, contamination of other cell types still possibly affect the experimental results. Therefore, improvement of isolated cardiomyocyte *in vitro* model is necessary for further experiments.

Further direction for this study involves utilizing proteomics to investigate new insight into the molecular mechanism of cardiac injury which is important for the discovery of diagnostic and prognostic biomarker for heart disease. The potential pharmacological target for developing new therapeutic strategies can also be identified by the proteomic approach. In addition, the proteomic study constantly provides unexpected and exciting results with which we can have new directions to study the mechanisms of cardiac injury.

We have already demonstrated in the present and previous studies that MMP-2 and MLCK play significant roles in the development of I/R injury (202, 267-268). Inhibition of MMP-2 and MLCK using different pharmacological compounds has been shown to protect the heart from I/R injury. We suggest that the use of combination of pharmacological compounds targeting different factors would be an effective therapeutic strategy for the prevention and treatment of cardiovascular diseases. Based on the molecular mechanism of I/R injury, we have developed a combination of comprising inhibitor of MMP-2, MLCK, NOS and Rho kinase. This “drug cocktail” could act on separate factors and processes within the pathogenesis of the I/R injury, and show a powerful cardio protective effect. The clinical trial for using these drug combinations during cardiac surgery may be a promising development and could lead to improvement in the operative and postoperative complications.

As a long term study, *ex vivo* heart model and *in vitro* isolated cardiomyocyte model are necessary, but not enough. Developing an *in vivo* model should be the further stage for our study on pharmacological design for the treatment of cardiac injury. The *in vivo* model would be a vital step to verify the results from the *ex vivo* and *in vitro* model, especially the cardio protective effect from pharmacological compounds. With all the comprehensive knowledge gained from three different models, clinical studies could lead to the next direction of therapeutic strategies.

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