Ischemia/Reperfusion-induced Changes in Cardiac Contractile Proteins

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2. Abstract

Heart disease, often results in the development of ischemia/reperfusion (I/R) injury, and is one of the major causes of morbidity and mortality. I/R injury is usually characterized by the development of cardiac contractile dysfunction, which is one of the major indicators of recovery. The understanding of the mechanisms underlying the development cardiac contractile dysfunction in response to I/R is crucial for the prevention and treatment of I/R injury.

Using a proteomics approach to an isolated heart model we identified myosin light chain 1 (MLC1) as an important contractile protein, phosphorylated by myosin light chain kinase (MLCK) and degraded by matrix metalloproteinase-2 (MMP-2) due to I/R.

Prevention of MLC1 phosphorylation, with an MLCK inhibitor (ML-7) or a myosin light chain phosphatase – MLCP – stimulator (Y-27632), and degradation with an MMPs inhibitor (doxycycline) protects the heart from contractile dysfunction, associated with a preservation of MLC1 protein levels. The combined use of these three drugs (ML-7, Y-27632 and doxycycline) at subthreshold concentrations resulted in a full protection of both contractile function and MLC1 in hearts subjected to I/R.

The MLCP indirect activator Y-27632 (a Rho kinase inhibitor) showed the best degree of protection of contractile function at full protective doses, independent of MLC1 preservation. A proteomics approach revealed that the administration of Y-27632 to hearts subjected to I/R results, not only in a protection of MLC1 from phosphorylation and degradation, but also a modulation of enzymes involved in energy production (lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase). This modulation of energy metabolism is potentially an importa effect of Rho kinase inhibitors, secondary to direct protection of cardiac contractile proteins from degradation in response to I/R.

The data presented here show a novel paradigm in the development of I/R-induced cardiac contractile dysfunction. Moreover, we establish a new therapeutic approach (multidrug treatment) that can be of crucial importance in the development of new preventive or treatment strategies against cardiac injury.

3. Acknowledgements

So this is my chance to make a Hollywood speech, just before I grab the Oscar, and move on to write other plots.
Disclaimer: the author reserves the right to abandon scientific English writing, at any point during the following pages, to embrace poetic multilingual freedom and thank appropriately to all that made this journey memorable.

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6. Abbreviations

2-DE	two-dimensional electrophoresis
ADP	adenosine diphosphate
AMPK	5'-AMP activated protein kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CF	coronary flow
CHF	congestive heart failure
CK	Creatine kinase
CO_2	carbon dioxide
CoA	coenzyme A
Cr	creatine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELC	essential light chain (also MLC1)
ERK	extracellular-signal-regulated kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GTP	guanosine triphosphate
H^+	proton
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-2	matrix metalloproteinase-2
MS	mass spectrometry
Na^+	sodium ion
NAD+/NADH	nicotinamide adenine dinucleotide
PAGE	polyacrylamide gel electrophoresis
PCr	phosphocreatine
PDH	pyruvate dehydrogenase
PKC	protein kinase C
PTMs	posttranslational modifications
Q-TOF	quadrupole time of flight
RLC	regulatory light chain (also MLC2)
ROS	reactive oxygen species
RPP	rate pressure product

TnI	troponin I
TnT	troponin T

DEDICATION

To my grandparents Maria Eulalia, Aquiles de Jesus e Maria Alice,
To my uncles Paulo e Rui:

Wherever you may be, this one is for you!

To my parents:

Simply, Thank you!

Para os meus avós Maria Eulália, Aquiles de Jesus e Maria Alice,
Para os meus tios Paulo e Rui:

Onde quer quer estejam esta é para voçês!

Para os meus pais:

Obrigado!

1. Introduction

1.1. Background

Cardiovascular disease remains one of the major burdens to society and one of the major causes of mortality in Western societies. The vast majority of cardiovascular diseases can lead to the development of underperfusion of the myocardium limiting the supply of oxygen and nutrients essential for maintenance of normal cardiac function. Acute or chronic decrease in supply of oxygen and/or nutrients can cause severe and irreversible damage to the cardiac tissue and seriously compromise cardiac contractile function. Myocardial infarction (MI) refers to an acute decrease or suppression of the supply of oxygen and nutrients to the myocardium either by coronary artery spasm or atherosclerotic plaque rupture. The gold standard for management of MI is reperfusion therapy (Antman, Anbe et al. 2004) by which blood flow is restored to the occluded area, hence restoring the supply of oxygen and nutrients. However, restoration of blood flow to previously occluded myocardial tissue can result in the development of injury due to a burst in reactive oxygen species (ROS). This can increase the extent of injury to the myocardium and often results in the development of cardiac contractile dysfunction, severely compromising recovery. The study of the mechanisms involved in ischemia/reperfusion (I/R)-induced contractile dysfunction is key to the development of new therapeutic strategies targeting the protection of contractile function following reperfusion.

The development of I/R injury has been attributed to a myriad of factors ranging from oxidative stress, metabolic uncoupling, disruption of ion homeostasis, inflammation, platelet aggregation, endothelial dysfunction and protein degradation (Turer and Hill 2010). Although extensive research has been performed on the development of I/R injury and several mechanisms proposed, the true contribution of each mechanism to the overall development of injury remains to be elucidated. One of the common features indicating development of I/R injury is the presence of cardiac contractile dysfunction. Cardiac contractile dysfunction has been attributed to decreased calcium sensitivity by the contractile apparatus and degradation of contractile proteins.

We have previously demonstrated that during global ischemia significant degradation of proteins associated with contractility occur (Sawicki, Leon et al. 2005; Sung, Schulz et al. 2007; Doroszko, Polewicz et al. 2009; Doroszko, Polewicz et al. 2010; Polewicz, Cadete et al. 2010). Troponin I, myosin light chain 1 (MLC1) and myosin light chain 2 (MLC2) are key components

of the contractile machinery that have been reported to be degraded during I/R (Sawicki, Leon et al. 2005; Sung, Schulz et al. 2007; Doroszko, Polewicz et al. 2009; Doroszko, Polewicz et al. 2010; Polewicz, Cadete et al. 2010). Recently, Ali et al. have demonstrated that titin, a key protein complex within the sarcomere and essential in the elasticity and stability of the sarcomere, is degraded during I/R (Ali, Cho et al. 2010). Importantly, degradation of these key contractile elements is strongly correlated with worsening of contractile function. Moreover, degradation has been attributed to matrix metalloproteinase-2 (MMP-2), initially described as an extracellular zinc proteinase, but whose intracellular targets have been described over the last decades.

Myosin light chain 1 and 2 (MLC1, 2) are important components of the myosin molecule also know as essential and regulatory light chains, respectively. MLC2 (regulatory light chain) modulates the interaction between myosin and actin, hence regulating force of contraction (Morano 1999). Complimentary, MLC1 (essential light chain) is believed to play an important role in the stability of the myosin-actin complex (Hernandez, Jones et al. 2007). We have shown that both MLC1 and 2 are post-translational modified during ischemia and reperfusion and that these modifications lead to increase degradation by MMP-2 (Doroszko, Polewicz et al. 2009; Doroszko, Polewicz et al. 2010; Polewicz, Cadete et al. 2011). Nitration and nitrosylation of tyrosine and cysteine residues, respectively, potentiates the degradation of myosin light chains by MMP-2. Importantly, it has been shown that MLC2 is lost from the myofilament during myocardial infarction (Akiyama, Akopian et al. 1997).

The involvement of kinases in the pathology of I/R is a common denominator between the several factors involved in the development of I/R injury. Adaptive and mal-adaptive mechanisms activated in response to the ischemic insult have been associated with the activation of kinase-mediated pathways. AMP-activated protein kinase (AMPK) has been associated with the development of metabolic uncoupling during I/R contributing to a slower restoration of intracellular pH (Stanley, Recchia et al. 2005). Activation of the Na+/H+ exchanger in I/R is mediated by extracellular-signal-regulated kinase (ERK), ultimately leading to calcium overload and poor contractile function during reperfusion (Fliegel 2009). Calcium buildup can activate the calcium/calmodulin-dependent protein kinase (CaM kinase) that, in turn, can activate myosin light chain kinase (MLCK). MLCK has been described to phosphorylate MLC2 increasing force of contraction. Concomitant with this activation of MLCK is the activation of the Rho/Rho

kinase pathway. Rho is part of a family of small GTP-binding proteins implicated in several cardiovascular pathologies. Activation of Rho/Rho kinase pathway during I/R can lead to inhibition of myosin light chain phosphatase (MLCP). The activation of MLCK together with inhibition of MLCP will result in the disruption of the tight balanced MLC2 phosphorylation/dephosphorylation and can result in hypercontractility and consequent worsening of contractile function.

1.2. Hypothesis

The evidence present in the literature provides strong evidence of the importance of post-translational modifications in the homeostasis of contractile proteins and regulation of cardiac contractility. Based on these observations we hypothesized that post-translational phosphorylation of contractile proteins, such as MLC1, is a key event in physiology and development of I/R injury.

1.3. Objectives

1. Identify ischemia/reperfusion-induced post-translational phosphorylation of contractile proteins, such as MLC1 in the isolated perfused rat heart;
2. Determine the role of phosphorylation of MLC1 during ischemia/reperfusion and its implications to contractile function recovery
3. Develop pharmacological strategies to protect against the degradation of contractile function and the reduction of I/R injury.

The evidence already gathered by our laboratory, together with the work proposed here will support the introduction of a "novel concept". Current interest resides in the regulation of pathological enzymatic activity by prevention of its activation, reduction in its activity or protein levels directly. One of the common events leading to enzyme activation, as described above, is phosphorylation and many compounds that inhibit kinase activity have been developed and are currently available for research. However, little attention has been given to the substrates on which these enzymes act. The evidence we have gathered and reported so far indicate that peroxynitrite-induced posttranslational modification of contractile proteins is an important factor in their degradation by proteolytic enzymes. These observations, together with the data generated by this project will allow for the establishment of post-translational modifications of substrates as modulators of enzymatic activity.

Although our observations have been made at the level of the contractile machinery of the heart, it is expected that this level of enzymatic activity regulation will be present in many, if not all, tissues and be an important part not only in pathological events but also in physiological protein turnover regulation.

1.4. Posttranslational modifications of myosin light chains determine the protein fate

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1.4.1. Preface

The following manuscript was published as a chapter in the book "Proteomics: Human Diseases and Protein Functions", 239-254, ISBN 978-953-307-832-8, Intech, 2012.

This manuscript performs a literature review of the current knowledge on posttranslational modifications of cardiac contractile proteins and introduces a new paradigm for understanding the development of cardiac contractile dysfunction following ischemia/reperfusion. This manuscript revises crucial background information for the understanding of this thesis, and parts of this manuscript are a consequence of the data gathered and the manuscripts published as integrative part of my PhD.

1.4.2. Introduction

The advances in proteomics over the last decade have made it possible for a more detailed study of protein posttranslational modifications. Posttranslational modification of proteins is an important signaling mechanism regulating vital pathways ranging from transcription to translation, in metabolism, cell survival, and cell death. Posttranslational modification of proteins has commonly been associated with the loss/gain of function and signal transduction with the concept of phosphorylation being the hallmark. However, many other posttranslational modifications of proteins have been detected and their implication to overall cellular homeostasis remains to be elucidated.

The cardiovascular system, in particular the heart due to its high metabolic rates, sensitivity to oxidative stress and necessity to adapt quickly to new environments, is an ideal candidate to the study of posttranslational modifications in physiology and pathology. Cardiac contractile function relies significantly on the integrity of its contractile apparatus, with the myosin light chains being important contractile elements. We have recently described the role of nitration and nitrosylation of ventricular myosin light chains (MLCs) on its degradation by the proteolytic enzyme matrix metalloproteinase-2 (MMP-2) (Doroszko, Polewicz et al. 2009; Doroszko, Polewicz et al. 2010; Polewicz, Cadete et al. 2010). Using distinct experimental models of oxidative stress, such as hypoxia-reoxygenation or ischemia/reperfusion, we have detected pathological nitration and nitrosylation of MLC induced by oxidative stress. According to our findings, nitration and nitrosylation of MLCs is associated with an increased affinity for MMP-2 and a consequent increase in degradation of these proteins that is associated with a worsening in cardiac contractile function during either reoxygenation or reperfusion.

Since contractile dysfunction is a predictor of patient outcome (Antman, Anbe et al. 2004), it is crucial to understand the mechanisms behind the development of contractile dysfunction. Moreover, the identification of mechanisms that lead to contractile dysfunction can help and result in the development of new therapeutic approaches aiming at preventing and/or treating contractile dysfunction following oxidative stress.

This review will focus on the current knowledge of posttranslational modification of myosin light chain, a cardiac contractile protein, and how these modifications contribute to

protection or pathogenesis in the setting of cardiac injury and contractile dysfunction triggered by oxidative stress. Moreover, this review will deal with the importance of posttranslational modifications of proteins and its determination of protein fate.

1.4.3. Proteomics

The term "PROTEOME" (PROTEin complement to the genOME), introduced in 1994, has attracted great attention, as approximately 30,000 human genes correspond to several million different gene products (proteins, peptides). The genome is intrinsically static and basically the same in every cell type, while the proteome is highly dynamic, differs between cell types, and does all the work. Proteins are the most common diagnostic and therapeutic targets in medicine, and the search for the proteome may lead to the discovery of new diagnostic and therapeutic targets. Classical proteomics, or what is now referred as "expression profiling", is a process in which total cellular or tissue proteins are separated on 2D gels and the visible protein spots are identified by peptide mass fingerprinting (Dunn 2000; Pandey and Mann 2000). This approach has been used to generate extensive proteomics online databases containing protein data obtained from the hearts of animals with cardiovascular disease states (Evans, Wheeler et al. 1997; Scheler, Li et al. 1999; Arrell, Neverova et al. 2001; Arrell, Neverova et al. 2001).

The field of proteomics has its roots in the marriage between 2D electrophoresis and mass spectrometry. In most cases, 2-dimensional electrophoresis is used to separate individual proteins and their modified forms, which are then identified and further characterized/analyzed by mass spectrometry. To date, proteomics has identified changes in more than 40 proteins in heart diseases such as dilated cardiomyopathy, varying degrees of I/R injury, and heart failure (Corbett, Why et al. 1998; Foster and Van Eyk 1999; Arrell, Neverova et al. 2001; Jiang, Tsubakihara et al. 2001; Jager, Jungblut et al. 2002; Schwertz, Langin et al. 2002).

Proteomics is an ideal approach to elucidate PTMs associated with kinase activity. Positive and negative modulation of heart contractility by short-term phosphorylation reactions at multiple sites in MLC2, TnI, TnT, α-tropomyosin, and myosin binding protein-C, have been known for almost a decade (Schaub, Hefti et al. 1998). An example of this modification is the discovery of novel phosphorylation of MLC1 in preconditioned cardiomyocytes (Arrell,

Neverova et al. 2001). However, the role of this PTM is not known. Phosphorylation of MLC1 was also detected in congestive heart failure (CHF) and this was associated with a decreased sensitivity to 8-Br-cGMP-mediated smooth muscle relaxation (Karim, Rhee et al. 2004). Similarly, three different PTMs were found in functionally important N-terminal sites of MLC2, two occurred in normal hearts (phosphorylation and deamidation) and one (n-terminal truncation) was associated with I/R injury (White, Cordwell et al. 2003). We have found the same PTMs in MLC1 in our model IR with the exception that phosphorylation and deamidation were associated with truncated forms of MLC1. Thus, the use of the proteomics approach to investigate mechanisms underlying heart disease should result in the generation of new therapeutic strategies and the establishment of precise and sensitive diagnostic markers. A schematic representation of a proteomic workflow is given in figure 1.1.

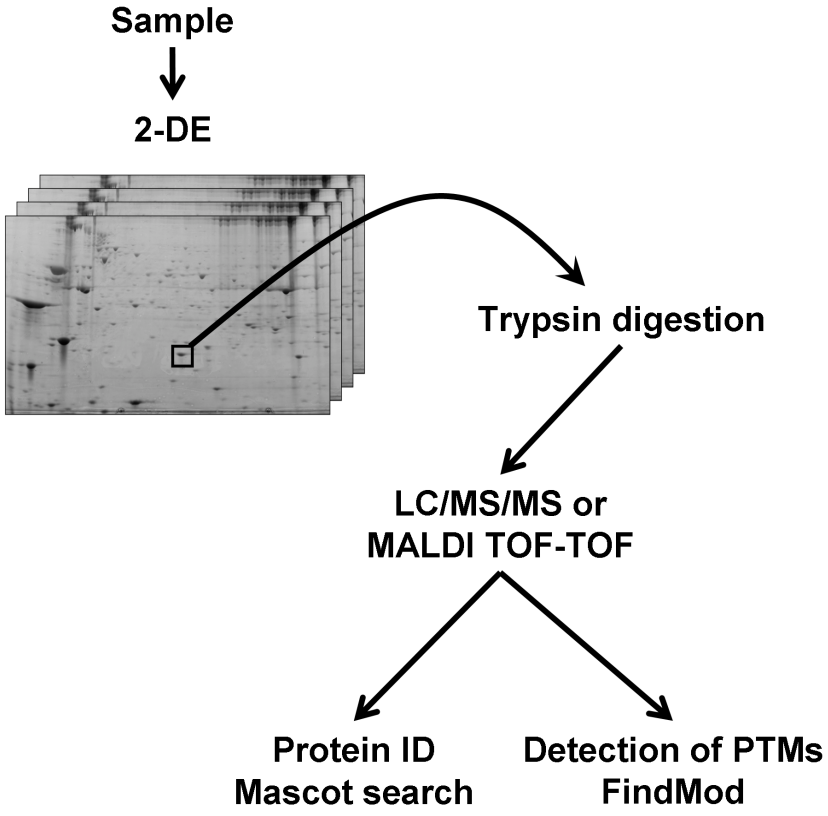

Figure 1.1 – Schematic representation of a proteomic method workflow. Samples are loaded and separated using 2-dimensional eletrophoresis (2-DE). Following 2-DE, protein spots of interest are identified and subjected to in-gel tryptic digestion followed by a mass spectrometry protocol

(typically LC/MS/MS or MALDI TOF-TOF). Data generated from mass spectrometry can be used to identify the protein using the Mascot search database or, after protein identification, for detection of posttranslational modifications (PTMs) using the ExPASy-FindMod tool (http://web.expasy.org/findmod/findmod_masses.html).

1.4.4. Methodology used in the study of myosin light chains posttranslational modifications

Although new advances have been made recently in the development of new technology for protein separation, the proteomic method relies significantly on 2-dimensional electrophoresis (2-DE) for protein separation for further analysis by mass spectrometry. One of the early limitations of the use of 2-DE for sample generation for mass spectrometry analysis was reproducibility. The problem was generated by the fact that gradient gels are difficult to cast consistently and only 2 gels could be run simultaneously. Recent technological advances gave rise to commercially available pre-cast gels (Criterion pre-cast gels, BioRad, Hercules, CA, USA) and the development of dodeca electrophoresis systems allowing for the simultaneous run of up to 12 gels (Criterion Dodeca Cell, BioRad, Hercules, CA, USA). These advances were very important in the achievement of reproducibility of sample generation by 2-DE.

The majority of the results here described in terms of the study of posttranslational modifications of myosin light chain 1 and 2 were obtained using the following methodology as described by:

Protein samples for 2-DE were prepared 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, Hercules, CA, USA]) at room temperature. Samples were sonicated for 2X5 seconds and centrifuged (10 minutes at 10,000g) to remove insoluble particles. Protein content of the heart extract in rehydration buffer was measured with the BioRad Bradford protein assay.

Protein samples (400 µg) were applied to each of 11 cm immobilized linear pH gradient (5-8) strips (IPG, BioRad, Hercules, CA, USA), with rehydration for 16–18 h at 20ºC. Isoelectrofocusing was performed using the BioRad Protean IEF cell 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). Following isoelectrofocusing the strips were equilibrated according to the manufacturer's instructions. The second dimension of 2-DE was performed 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, Hercules, CA, USA). Because of this limitation for 2-DE analysis we used 4 hearts from each group. All the gels were stained in the same bath and next scanned with a calibrated densitometer GS-800 (BioRad, Hercules, CA, USA). Quantitative analysis of MLC1 and MLC2 spot intensities from 2-DE were measured with PDQuest 7.1 measurement software (BioRad, Hercules, CA, USA).

MLC1 and MLC2 protein spots were manually excised from the 2-DE gel. These spots were then processed using a MassPrep Station (Waters, Milford, MA, USA) using the methods supplied by the manufacturer. 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. Next, the gel was washed twice with water. The protein extraction was performed overnight at room temperature with 50 µL of a mixture of formic acid, water, and isopropanol (1:3:2, vol:vol). The resulting solution was 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 (Waters, Milford, MA, USA) coupled with Q-TOF-2 mass spectrometer (Waters, Milford, MA, USA). 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. Posttranslational modifications were determined using the ExPASy-FindMod tool (http://web.expasy.org/findmod/findmod_masses.html).

1.4.5. Cardiac contractile proteins

The heart is the central organ for the circulatory system and is responsible for providing an efficient flow of blood to the whole body in order to meet the metabolic demands of the organism by delivering oxygen and nutrients and, at the same time, removing metabolic waste. Often seen as a pump, the heart relies on the integrity of its contractile machinery in order to efficiently perform its function. The basic unit of contraction is the sarcomere. The sarcomere is constituted of thick and thin filaments that, during contraction, slide over each other leading to the shortening of the sarcomere and contraction. The thick filament is mainly constituted of myosin while the thin filament is mainly constituted of actin, tropomyosin, and troponins (Figure 1.2). The interaction between thin and thick filaments, the crucial component for the generation of a contractile force, occurs between actin and the myosin head.

1.4.6. Myosins

Myosin is a large complex molecule. It consists of two heavy chains, an α-helical tail, and four myosin light chains (Rayment, Rypniewski et al. 1993; Dominguez, Freyzon et al. 1998; Craig and Woodhead 2006). The heavy chains (myosin heavy chain, MHC) have the ATPase activity necessary to trigger sliding between filaments and the consequent contraction. The two light chains (myosin light chain 1 and 2, MLC1 and MLC2) confer stability to the myosin head and also have actin binding motifs. MLC1 is also referred to as the essential light chain (ELC) and is present in the hinge of the myosin head for stability purposes. MLC2 is also referred as regulatory light chain (RLC) and together with MLC1 forms the hinge region between the globular head and the α-helical tail of myosin.

The essential light chains (ELC) are expressed by three different genes (MYL1, 3 and 4) which give rise to four isoforms of ELC/MLC (Hernandez, Jones et al. 2007). The nomenclature adopted depends on the tissue expressed (ELC_a and ELC_v for atrium and ventricular ELC/MLC, respectively) or whether it is full or short MLC (MLC1 and MLC3 for long and short MLCs, respectively) (Hernandez, Jones et al. 2007). The nomenclature for myosin light chains is not

always obvious and for this manuscript we will refer to MLC1 as the full length myosin light chain present in the sarcomeres of the ventricle.

It has been described that the amino terminus of MLC1 interacts with the carboxy terminus of actin during contraction (Henry, Winstanley et al. 1985; Trayer, Trayer et al. 1987; Milligan, Whittaker et al. 1990; VanBuren, Waller et al. 1994; Morano, Ritter et al. 1995; Efimova, Stepkowski et al. 1998; Nieznanska, Nieznanski et al. 1998; Andreev and Borejdo 1999; Timson, Trayer et al. 1999; Miyanishi, Ishikawa et al. 2002; Nieznanska, Nieznanski et al. 2002; Nieznanski, Nieznanska et al. 2003). This interaction of MLC1 with actin suggests an important role of MLC1 in the regulation of contraction. Indeed, selective removal of MLC1 from the myosin molecule resulted in a reduction of ~50% of the force generated (VanBuren, Waller et al. 1994).

The regulatory light chain (RLC), referred as MLC2 in this review, is involved, as the name suggests, in the regulation of contraction. In the heart, two isoforms are found: a ventricular specific ($MLC2_v$) and an atrium specific ($MLC2_a$) isoform (Collins 2006). MLC2, together with MLC1, contributes to the mechanical stability of the hinge of the head region of the myosin molecule. MLC2 has been better studied and characterized due to the fact that it can be phosphorylated. MLC2 phosphorylation under basal conditions has been demonstrated to regulate Ca^{2+}-dependent contraction (High and Stull 1980; Stull, Manning et al. 1980; Sweeney and Stull 1986; Mizuno, Isotani et al. 2008).

In order for proper sarcomeric contraction, the myosin structure has to be stable and fine tuned. It is the role of the light chains, present in the hinge of the head region, to assure stability of the head region and fine tune contraction by regulating the interaction between MHC and actin.

1.4.7. Posttranslational modifications

Virtually all proteins are subjected to posttranslational modifications. In this text, posttranslational modification will refer to the addition of a chemical group to amino acid residue which has a biological functional. Mass spectrometry can be used to determine peptide masses belonging to the native protein. According to the mass of each peptide one can infer about the

presence or absence of a posttranslational modification that has a unique mass signature. A useful tool in determining posttranslational modifications by using peptide masses is ExPASy-FindMod tool (available at http://web.expasy.org/findmod/findmod_masses.html). Up to date the available information from ExPASy-FindMod tool, shows seventy one groups of posttranslational modifications that can be detected from analysis of peptide mass fingerprints. Of these, phosphorylation is by far the most studied and well know, mainly due to the identification of enzymes mediating phosphorylation of protein residues: protein kinases. Phosphorylation is commonly associated with signal transduction, the hallmark of signaling cascades mediated by kinases. Other posttranslational modifications have recently gained more attention, mainly due to the fact that they are associated with oxidative stress. Protein nitration and nitrosylation are common events occurring in cells subjected to oxidative stress. Contrary to phosphorylation, no enzyme has been described to mediate nitration and nitrosylation and these modifications are often seen as a non-enzymatic posttranslational modification dependent on the presence, identity and concentration of reactive nitrogen species. The role of protein nitration on cellular signal transduction pathways has been reviewed by Yakovlev and Mikkelsen (Yakovlev and Mikkelsen 2010). The authors conclude that the gathered evidence supports the notion of protein nitration being a specific reaction. Though not entirely clear, it appears that nitration of protein residues by reactive nitrogen species is dependent on the tertiary structure of the protein and in particular the chemical environment of the tyrosin residues.

Due to the number of possible posttranslational modifications currently identified and the fact that the same posttranslational modification can occur in different amino acids, it is clear that the study of posttranslational modifications of protein under physiological and pathological conditions is a difficult task. Moreover, posttranslational modifications are not isolated reactions. A protein molecule present in physiological or pathological conditions may have more than one posttranslational modification. Also, the same protein can exhibit different types of posttranslational modifications at one time. Hence, the study of posttranslational modifications of proteins is difficult but also of high importance due to the nature of physiological and pathological consequences these modifications often cause. Also of importance is the fact that the study of posttranslational modification of cardiac contractile proteins can result in the identification of disease-specific markers of heart injury, hence contributing to the development of more sensitive and specific biomarkers of heart injury.

1.4.8. Biomarkers of heart injury

A biomarker is defined as a reproducibly detectable molecular feature, usually present in an accessible bodily fluid or tissue that is correlated with a disease state. Cardiac enzymes have long been used as front-line diagnostic tools in the detection of myocardial injury caused by myocardial ischemia. However, the most commonly used enzymes (such as creatine kinase (CK) and its myocardial fraction CK myocardial band (MB), aspartic aminotransferase, and lactate dehydrogenase) are limited in their ability to detect myocardial injury by short diagnosis windows, have limited sensitivities, and lack specificity because of their presence in skeletal muscle. Similarly, myoglobin also lacks specificity because its release from skeletal muscle cannot be distinguished from its release from the heart muscle (Christenson and Azzazy 1998). Thus, there is a need to develop novel biomarkers in order to more effectively treat and diagnose myocardial infarction (MI). Using the proteomics approach a time-dependent increase of TnI in the serum from patients with MI was reported (Labugger, Organ et al. 2000). This new finding led to the suggestion that MLC1, as a contractile protein, could be considered as a new protein biomarker in I/R injury of the heart (Sato, Kita et al. 2004; Lee and Vasan 2005). The list of biomarkers in cardiovascular diseases will grow, particularly when the proteomics approach is used. This method has already identified 177 different proteins (including their different molecular forms) with the potential to be good candidates as biomarkers (Anderson 2005) in cardiovascular disease such as stroke.

1.4.9. MLCs in heart injury

Muscles contract when filaments containing a molecular motor, myosin, pull against another set of filaments containing mainly actin. The source of energy for this directional movement is provided by the hydrolysis of ATP, which is catalyzed by myosin. Muscle myosin is a hexamer consisting of two heavy chains (MHC), two regulatory (or phoshorylatable) light chains (known as MLC2 or RLC) and two essential chains (known as MLC1, and alkali or ELC). The myosin heavy chain is an elongated molecule where more then 90% of the protein is a coiled coil tail formed by the two heavy chains. However, the N-terminus of MHC is globular and

contains ATPase activity, the actin binding site, and MLC1 and MLC2 binding sites (Rayment, Holden et al. 1993; Rayment, Rypniewski et al. 1993). The light chains from cardiac and skeletal muscles are not directly involved in the regulation of contraction. However, both MLC1 and MLC2 can exert a subtle modulatory effect.

The precise molecular basis for myocardial stunning remains unknown, but protein damage within the myofilament is a likely mechanism. It is almost certain that stunning is a multifactoral process. One potential target is ventricular MLC2, which via changes in its phosphorylation status, modulates contractile force generation arising from actin-myosin MHC interaction (the structure, function and malfunction of MLC2 have been reviewed by Szczesna, (Szczesna 2003)). Three years ago an Australian group, using an experimental protocol similar to ours, found changes in phosphorylation of MLC2 and showed how these changes are correlated with the function of stunned myocardium (White, Cordwell et al. 2003). In another model, involving pharmacologically preconditioned isolated cardiomyocytes, altered phosphorylation of MLC1 was also found, but the role of this modification is not yet known (Arrell, Neverova et al. 2001).

Not only does heart injury induce chemical modification of MLCs, but during acute congestive heart failure entire MLC molecules, or their degradation products, are released into the circulation (Hansen, Stanton et al. 2002; Goto, Takase et al. 2003). Van Eyk and colleagues have shown that the release of degradation products of MLC1 to coronary effluent is positively correlated with the duration of ischemia (Van Eyk, Powers et al. 1998). And White and co-workers found that both to MLC1 and MLC2 are released into the effluent of ischemic hearts (White, Cordwell et al. 2003). There was no evidence as to what proteolytic enzyme could be responsible for MLC degradation or what molecular mechanism could account for the release of their products into the circulation. Our work on the degradation of MLC1 in I/R heart shows that MMP-2 is responsible (at least in part) for the degradation of this protein (Sawicki, Leon et al. 2005; Doroszko, Polewicz et al. 2009; Polewicz, Cadete et al. 2010). Although, the mechanism of release is still unknown, it could result from a loss of cell membrane integrity. Despite the many unanswered questions about the molecular basis of I/R injury in the heart, cardiac MLC1 is becoming a very important candidate as a biomarker of heart injury.

1.4.10. Phosphorylation

Phosphorylation is a posttranslational modification that consists of the addition of a phosphate group to serine (Ser), threonine (Thr) or tyrosine (Tyr). The addition of the phosphate group to these amino acids is catalyzed by kinases. The currently described mechanism of phosphorylation is that it essentially works as a switch, turning the function the phosphorylated protein on or off. Other consequences of protein phosphorylation may involve subcellular localization of proteins, protein-protein interaction, and proteolytic degradation. In fact, our ongoing studies on role of posttranslational modifications in the development of cardiac contractile dysfunction implies that the phosphorylation of MLC1 during ischemia/reperfusion results in its increase degradation, possibly by MMP-2, contributing to ischemia/reperfusion injury.

Phosphorylation of MLC1 has been demonstrated previously (Arrell, Neverova et al. 2001) but it has been associated with stability of the myosin head. The authors reported phosphorylation of rat/human Thr 69/64 and Ser 200/194 or 195. Our unpublished data demonstrates that phosphorylation of MLC1 has direct implications in its degradation by MMP-2. We observed *in vitro* phosphorylation (by myosin light chain kinase) of human recombinant MLC1 at Thr127, Thr129 or Tyr 130, as well as Ser179 and Tyr186. In MLC1 from isolated rat hearts subjected to ischemia/reperfusion we observed six phosphorylated residues: Thr69, Thr77 or Tyr78, Thr132, Thr134 or Tyr135, Thr164, Ser184 and Tyr190. Our data suggests a physiological role for MLC1 phosphorylation of Thr69 and Thr132, Thr134 or Tyr135, since these phosphorylations are present in aerobic control hearts, with the remaining four phosphorylations being induced by ischemia/reperfusion (Table 1.1). The observed phosphorylations of MLC1 induced by ischemia/reperfusion resulted in an increased degradation of MLC1. In an unpublished *in vitro* study we observed that when MLC1 was phosphorylated by the myosin light chain kinase (MLCK), the affinity of MMP-2 for MLC1 was increased and this increase in affinity resulted in an increase in the degradation of MLC1. Taken together, these observations suggest a role for protein phosphorylation in the induction of proteolytic degradation, namely by MMP-2.

To our knowledge these are the first observations concerning phosphorylation of a target protein contributing to the direct proteolytic degradation of that protein. Since it is well known

that during several distinct disease processes the activation of phosphorylation cascades occur we speculate that besides up- and down-regulation of protein activity, phosphorylation is responsible for signaling protein degradation contributing directly to the progression of the disease process.

1.4.11. Nitration and S-nitrosylation

Protein tyrosine nitration has been implicated in many pathological conditions and diseases such as inflammation, chronic hypoxia, myocardial infarction and diabetes among others (MacMillan-Crow, Crow et al. 1996; Giasson, Duda et al. 2000; Blantz and Munger 2002; Reynolds, Berry et al. 2005; Pacher, Beckman et al. 2007; Reynolds, Berry et al. 2007; Donnini, Monti et al. 2008; Naito, Takagi et al. 2008; Reyes, Reynolds et al. 2008; Upmacis 2008; Jones, Ying et al. 2009; Koeck, Corbett et al. 2009; Pieper, Ionova et al. 2009; Smith 2009; Brindicci, Kharitonov et al. 2010; Kang, Ross et al. 2010; Pavlides, Tsirigos et al. 2010; Zhang, Chen et al. 2010). However, a physiological role for protein tyrosine nitration should not be excluded. Not all the tyrosine residues in a protein are targets for nitration either *in vitro* or *in vivo*. Moreover, the observed nitrations of tyrosine very seldom coincide between *in vitro* and *in vivo* studies. Of importance is the fact that nitration of tyrosine residues is a selective process that appears to be under tight control, even though the exact mechanisms for the regulation of tyrosine nitration remain unknown. Nitration of protein tyrosine residues (formation of nitrotyrosine) has been suggested to facilitate proteolysis of the nitrated protein (Yakovlev and Mikkelsen 2010). We have recently shown that the contractile proteins MLC1 and MLC2 (part of the thick filament of the sarcomere) are subjected to tyrosine nitration and cysteine s-nitrosylation in cardiac models of oxidative stress (Doroszko, Polewicz et al. 2009; Doroszko, Polewicz et al. 2010; Polewicz, Cadete et al. 2010). Using an *in vivo* model of neonatal asphyxia in piglets we have shown that both MLC1 and MLC2 are significantly decreased following hypoxia-reoxygenation (Doroszko, Polewicz et al. 2009; Doroszko, Polewicz et al. 2010). Mass spectrometry analysis for nitration and nitrosylation revealed that MLC1 is S-notrosylated at Cys 138 and nitrated at Tyr 141. Interestingly, these residues are located at the positions P3 and P1' of the cleavage site for MMP-2 and hypoxia-reoxygenation was associated with an increase in MMP-2 activity. Also, MLC2 from hearts subjected to hypoxia-reoxygenation was nitrated at Tyr 118 and Tyr 152, while no

nitration was observed for the control group (Table 1.1). These data suggest a pathological role for MLC2 tyrosine nitration associated with hypoxia-reoxygenation. Using human recombinant mutant MLC2, in which the tyrosine residue is replaced with phenylalanine, (Y152F) the *in vitro* incubation with peroxynitrite as a nitrating agent resulted in the prevention of MLC2 degradation by MMP-2, with no nitration observed at position 152. These observations indicate that although MLC2 has two nitration sites, it is Tyr 152 that mediates the signaling of degradation by MMP-2. MLC1 was also studied in a model of isolated adult rat cardiomyocytes subjected to simulated ischemia. Mass spectrometry analysis revealed nitration of Tyr 190, consistent with what was observed in piglet hearts. However, the Cys in the P3 position of the MMP-2 cleavage site was not S-nitrosylated as observed in MLC1 from piglet hearts. Moreover, MLC1 from rat cardiomyocytes was also nitrated at Tyr 78 and S-nitrosylated at Cys 81. *In vitro* human recombinant MLC1 was nitrated by peroxynitrite (used as a nitrating agent) at Tyr 73 (corresponding to rat MLC1 Tyr 78) Tyr 185 (corresponding to rat MLC1 Tyr 190), Tyr 140 and S-nytrosilated at Cys 76 (corresponding to rat Cys 81) and Cys 67. *In vitro* nitrated and S-nitrosylated MLC1 was more susceptible to degradation by MMP-2.

These data support the concept of highly regulated nitration and S-nitrosylation of proteins previously suggested, even though the exact mechanism remains unknown. Moreover, not only these processes are highly specific, they are also tightly associated with pathophysiological consequences. In this case, nitration and S-nitrosylation of protein residues is associated with an increase in its degradation by the proteolytic enzyme MMP-2 both *in vitro* and *in vivo*.

Table 1.1 – Identification of MLC1 and MLC2 protein residues subjected to posttranslational modification leading to protein degradation.

	Identified posttranslational modified residues	
Posttranslational Modification	MLC1	MLC2
Phosphorylation	*in vitro* (human recombinant)	
	Thr127/Thr129/Tyr130, Ser179, Tyr186	
	ex vivo (rat heart)	
	Thr69, Thr77/Tyr78, Thr132/Thr134/Tyr135, Thr164, Ser184, Tyr190	
Tyr nitration	*in vitro* (human recombinant)	
	Tyr73, Tyr130, Tyr185	Tyr152
	in vivo (piglet heart)	
	Tyr141	Tyr118, Tyr152
	ex vivo (rat heart)	
	Tyr78, Tyr190	
Cys S-nitrosylation	*in vitro* (human recombinant)	
	Cys67, Cys76	
	in vivo (piglet heart)	
	Cys138	
	ex vivo (rat heart)	
	Cys81	

1.4.12. Conclusion

With the development of proteomics technology over the last two decades, more and more information about protein posttranslational modifications has been gathered. The difficulty of studying posttranslational modification of proteins and their physiological and pathological consequences lies on the fact that often (if not always) a protein will exhibit more than one type of posttranslational modification at any given time or more than one posttranslational modification of the same type.

Classically, enzymatic production of a certain product, from a given substrate, was limited by the enzyme activity. Also, posttranslational modification of the enzyme, such as phosphorylation, is a valid process to increase enzyme activity. We propose a new paradigm in the regulation of enzymatic activity by modification of proteins previously resistant to degradation. Here we have described the role of nitrosylation, nitration and phosphorylation of cardiac contractile proteins, as substrates for enzymatic reaction, in models of oxidative stress which result in their increased degradation by a proteolytic enzyme (MMP-2) both *in vitro* and *in vivo* (Figure 1.2).

It has been described that posttranslational modification of MMP-2 triggered by oxidative stress can activate the enzyme (Viappiani, Nicolescu et al. 2009). Although this may be the case in the *in vivo* and *ex vivo* models, the same observations were made in *in vitro* experiments inwhich MMP-2 is not posttranslational modified. This new paradigm, that posttranslational modification determine fate of proteins, is an important advance in the understanding of the molecular mechanisms by which oxidative stress can trigger cardiac contractile dysfunction, in pathological processes such as ischemia/reperfusion and hypoxia-reoxygenation, activation of MLCK and phosphorylation of MLC1. These posttranslational modifications increase the affinity of MMP-2 for MLC1 and MLC2. MMP-2 degrades MLC1 and MLC2 leading to cardiac contractile dysfunction.

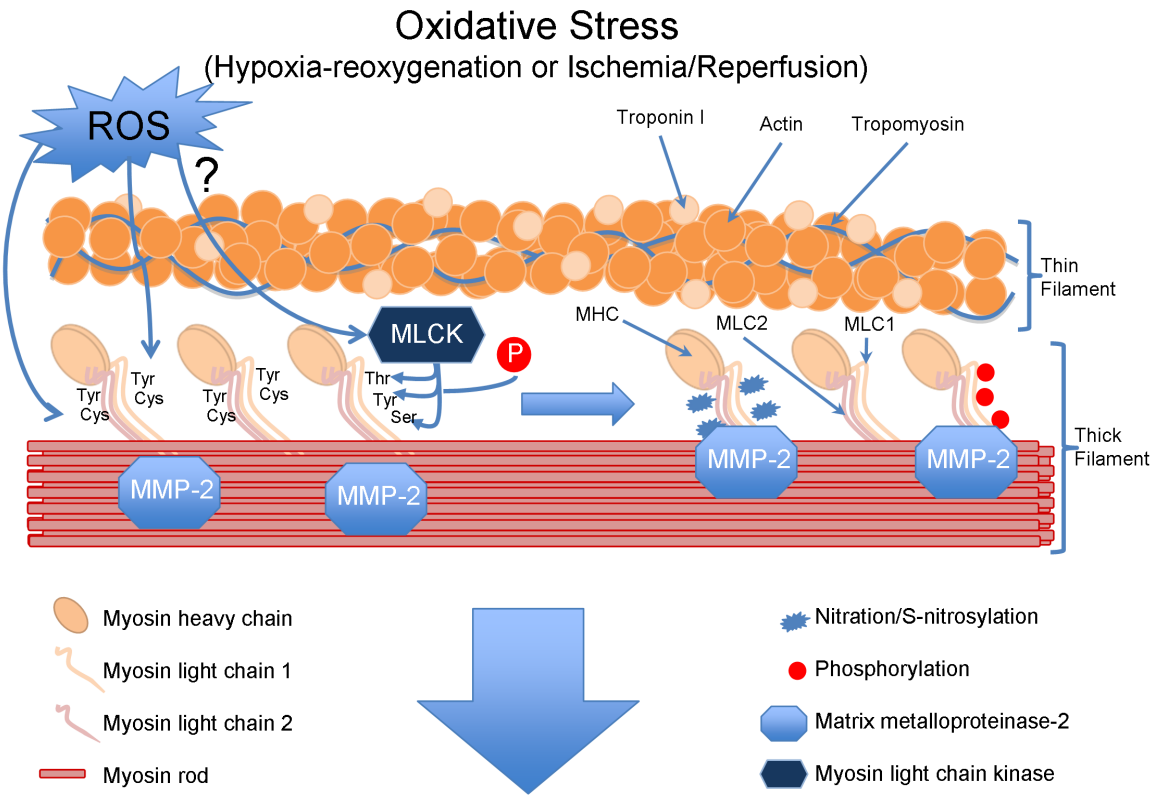
Oxidative Stress
(Hypoxia-reoxygenation or Ischemia/Reperfusion)
ROS
?
Troponin I
Actin
Tropomyosin
Thin Filament
MHC
MLCK
P
MLC2
MLC1
Tyr
Cys
Thr
Tyr
Ser
MMP-2
Thick Filament
Myosin heavy chain
Myosin light chain 1
Myosin light chain 2
Myosin rod
Nitration/S-nitrosylation
Phosphorylation
Matrix metalloproteinase-2
Myosin light chain kinase
Contractile Dysfunction due to
degradation of MLC1 and MLC2

Figure 1.2 – Cartoon representation of our proposed model for regulation of contractile protein fate by posttranslational modifications. Reactive oxygen species (ROS) generated during ischemia/reperfusion or hypoxya-reoxygenation can lead to the direct nitration/S-nitrosylation of tyrosine and cysteine residues of MLC1 and MLC2. Also, ROS can lead to the phosphorylation of MLC1 and MLC2.

1.4.13. Acknowledgements

We would like to thank Steve Arcand for the editorial contribution to this work. Also Jolanta Sawicka for the contribution in gathering the unpublished, ongoing data on phosphorylation of MLC1.

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1.5. Relationship between protein posttranslational modifications and protein structure

As previously described, oxidative stress triggers the posttranslational modification of cardiac contractile proteins, namely MLC1. These posttranslational modifications are associated with a decrease in cardiac contractile function (Doroszko, Polewicz et al. 2009; Doroszko, Polewicz et al. 2010; Polewicz, Cadete et al. 2010). Posttranslational modifications in the vicinity or within know cleavage sites for proteolytic enzymes can result in the stimulation or inhibition of the activity of the proteolytic enzyme. Modifications in other regions of the protein can result in conformational changes of the protein, interfering with its function.

MLC1 is a cardiac contractile protein implicated in the stability of the myosin motor head and force development during contraction (VanBuren, Waller et al. 1994; Komiyama, Soldati et al. 1996; Hernandez, Jones et al. 2007). The tertiary structure for myosin has been reported (Rayment, Rypniewski et al. 1993). MLC1 lies at the base of the myosin head domain providing structural support to the heavy chains and allowing proper contraction (Figure 1.3).

The previous chapter describes the posttranslational modifications of MLC1 in response to oxidative stress. The data we have gathered suggests a clear relationship between phosphorylation of MLC1 residues in the vicinity, or within the cleavage site for MMP-2, and increased degradation of MLC1 by MMP-2. The phosphorylation of Ser184, in the P6 position of the MMP-2 cleavage site, and Tyr190, in the P'1 position of the MMP-2 cleavage site, change the affinity of MMP-2 for MLC1 resulting in a stimulation of MLC1 degradation by MMP-2 (Figure 1.4).

Besides the posttranslational phosphorylation in the vicinity, or within the cleavage site for MMP-2, we have identified other phosphorylation sites within the MLC1 molecule (Figure 1.5). A possible PKC-dependent phosphorylation of MLC1 on T132/T134/Y135 was visible in rat hearts perfused under both aerobic and I/R conditions, indicating a possible regulatory role for this phosphorylation in protein function. Also of possible physiological importance is the MLCK-induced phosphorylation of T69 of MLC1 which has been identified in cardiac MLC1from both aerobic and I/R rat hearts. Lastly, the phosphorylation of T77/Y78, which is induced by ischemia, can possibly be involved in the pathological consequences of ischemia on MLC1. It is plausible that this phosphorylation of T77/Y78, due to its 3D proximity to the MMP-2 cleavage site, can either create alosteric changes in the MLC1 tertiary structure that

potentiate/facilitate further phosphorylation by MLCK in the vicinity, or within, the MMP-2 cleavage site, or potentiate/facilitate MMP-2 proteolytic activity on the phosphorylated MLC1.

Other posttranslational modifications have been identified and were previously summarized in table 1.1.

MYOSIN SUBFRAGMENT-1, ALPHA CARBON COORDINATES ONLY FOR THE TWO LIGHT CHAINS

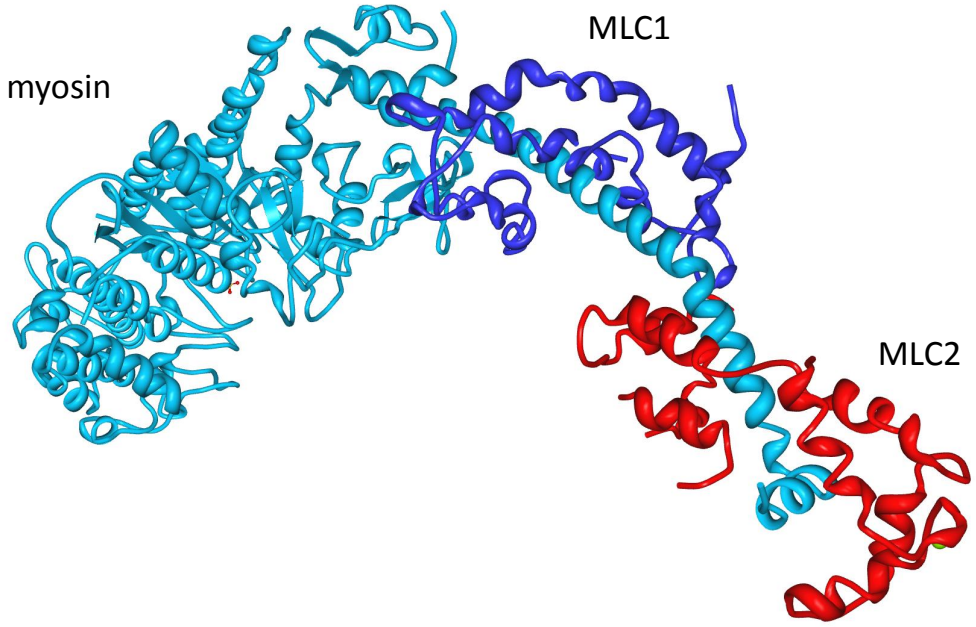


Figure 1.3 – Three-dimensional structure of myosin in complex with myosin light chains 1 and 2. Image of 2MYS (Rayment, I., Rypniewski, W.R., Schmidt-Base, K., Smith, R., Tomchick, D.R., Benning, M.M., Winkelmann, D.A., Wesenberg, G., Holden, H.M., (1993) Three-dimensional structure of myosin subfragment-1: a molecular motor. Science 261: 50-58) created with Protein Workshop (J.L. Moreland, A. Gramada, O.V. Buzko, Q. Zhang, P.E. Bourne (2005) The Molecular Biology Toolkit (MBT): a modular platform for developing molecular visualization applications. BMC Bioinformatics 6:21).

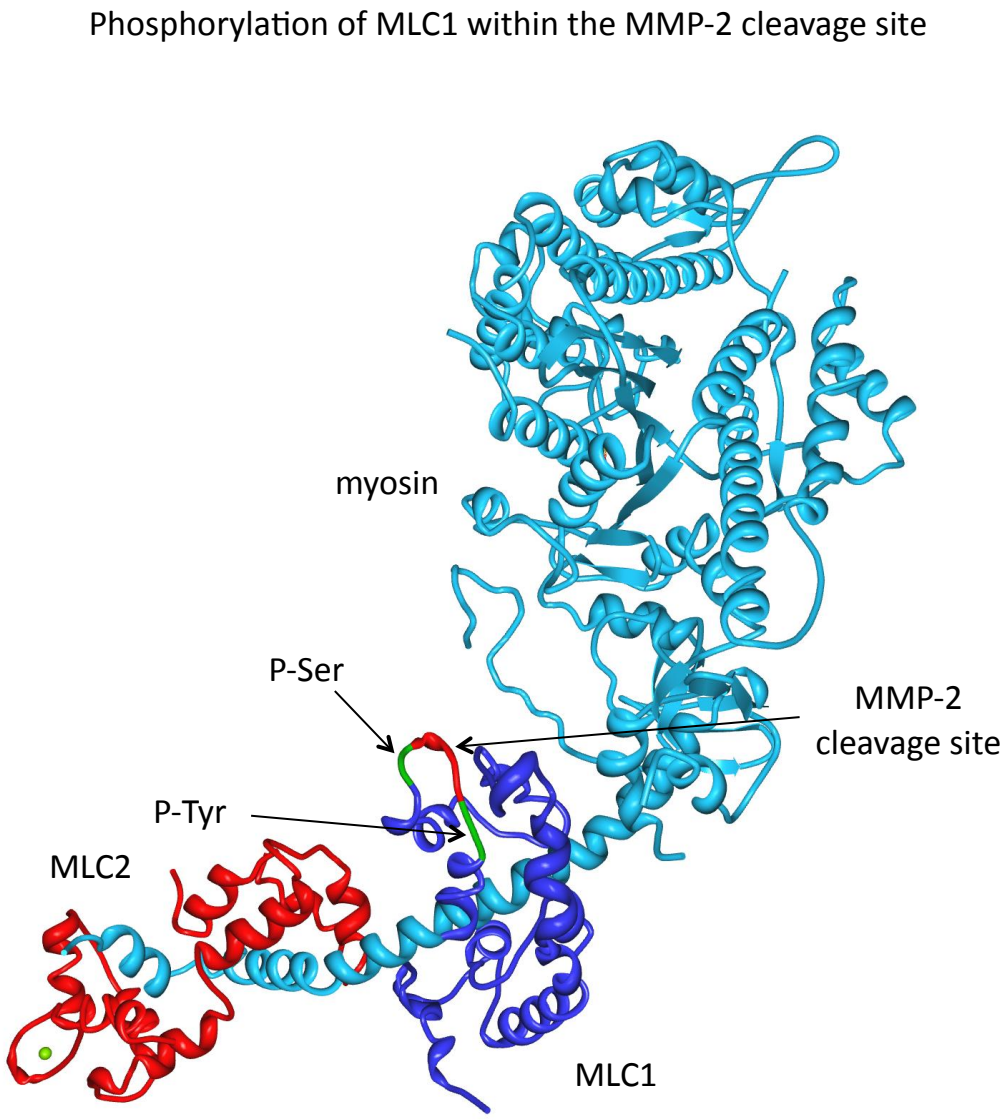


Figure 1.4 – Three-dimensional structure of myosin in complex with myosin light chains 1 and 2 showing phosphorylation of MLC1 within the MMP-2 cleavage site. Image of 2MYS (Rayment, I., Rypniewski, W.R., Schmidt-Base, K., Smith, R., Tomchick, D.R., Benning, M.M., Winkelmann, D.A., Wesenberg, G., Holden, H.M., (1993) Three-dimensional structure of myosin subfragment-1: a molecular motor. Science 261: 50-58) created with Protein Workshop (J.L. Moreland, A. Gramada, O.V. Buzko, Q. Zhang, P.E. Bourne (2005) The Molecular Biology Toolkit (MBT): a modular platform for developing molecular visualization applications. BMC Bioinformatics 6:21).

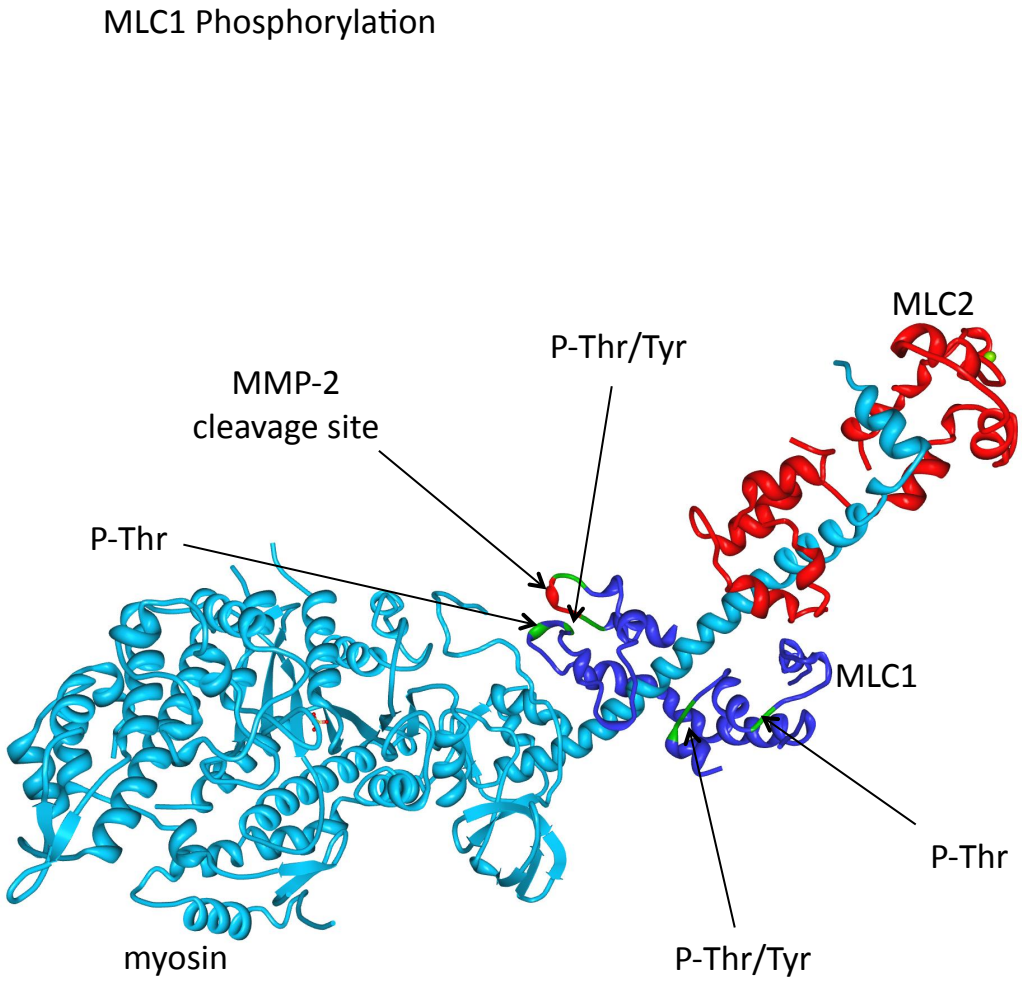


Figure 1.5 – Three-dimensional structure of myosin in complex with myosin light chains 1 and 2 depicting phosphorylation of MLC1. Image of 2MYS (Rayment, I., Rypniewski, W.R., Schmidt-

Base, K., Smith, R., Tomchick, D.R., Benning, M.M., Winkelmann, D.A., Wesenberg, G., Holden, H.M., (1993) Three-dimensional structure of myosin subfragment-1: a molecular motor. Science 261: 50-58) created with Protein Workshop (J.L. Moreland, A. Gramada, O.V. Buzko, Q. Zhang, P.E. Bourne (2005) The Molecular Biology Toolkit (MBT): a modular platform for developing molecular visualization applications. BMC Bioinformatics 6:21).

2. Ischemia/reperfusion-induced myosin light chain 1 phosphorylation increases its degradation by matrix metalloproteinase-2

Virgilio J. J. Cadete; Jolanta Sawicka; Jagdip Jaswal; Gary D. Lopaschuk; Richard Schulz; Danuta Szczesna-Cordary; Grzegorz Sawicki

2.1. Preface

The manuscript here presented has published in *FEBS Journal (**Cadete et al.,2012**, FEBS J, 279(13):2444-54)* . This manuscript addresses objectives 1 and 2 of my thesis:

1. Identify ischemia/reperfusion-induced posttranslational phosphorylation of contractile proteins, such as MLC1, in the isolated perfused rat heart;
2. Determine if phosphorylation of myosin light chain 1 during I/R contributes to the worsening of contractile function recovery.

In this manuscript, we determined that an important cardiac contractile protein – myosin light chain 1 (MLC1) – is phosphorylated in response to ischemia/reperfusion (I/R). Moreover, the phosphorylation of MLC1 results in the degradation by the proteolytic enzyme matrix metalloproteinase-2 (MMP-2). We also identify the kinase responsible for the phosphorylation of MLC1 – myosin light chain kinase (MLCK). Using a commercially available specific inhibitor of MLCK (ML-7) we were able to prevent I/R-induced phosphorylation of MLC1, its degradation by MMP-2, which resulted in the preservation of contractile function during reperfusion following ischemia.

2.2. Abstract

Degradation of myosin light chain 1 (MLC1) by matrix metalloproteinase-2 (MMP-2) during myocardial ischemia/reperfusion (I/R) injury has been established. However, the exact mechanisms controlling this process remain unknown. I/R increases the phosphorylation of MLC1, but the consequences of this modification are not known. We hypothesized that phosphorylation of MLC1 plays an important role in its degradation by MMP-2. To examine this, isolated perfused rat hearts were subjected to 20 min global ischemia followed by 30 min of aerobic reperfusion. I/R increased phosphorylation of MLC1 (as measured by mass spectrometry). If hearts were subjected to I/R in the presence of ML-7 (a myosin light chain kinase (MLCK) inhibitor) or doxycycline (a MMP inhibitor) an improved recovery of contractile function was seen compared to aerobic hearts and MLC1 was protected from degradation. Enzyme kinetic studies revealed an increased affinity of MMP-2 for the phosphorylated form of MLC1 compared to non-phosphorylated MLC1. We conclude that MLC1 phosphorylation is important mechanism controlling the intracellular action of MMP-2 and promoting the degradation of MLC1. These results further support previous findings implicating posttranslational modifications of contractile proteins as a key factor in the pathology of cardiac dysfunction during and following ischemia.

2.3. Introduction

Myosin light chain 1 (MLC1), also known as myosin essential light chain is an integral structural component of the actomyosin cross-bridge, but it also plays a role in force development and muscle contraction (for review see (Morano, Ritter et al. 1995; Timson 2003; Hernandez, Jones et al. 2007)). The functional importance of MLC1 is further implicated by the recent identification of several missense mutations in the human ventricular MLC1 isoform, which are associated with hypertrophic cardiomyopathy (Hernandez, Jones et al. 2007). However, the mechanism underlying the role of MLC1 in heart disease is not known.

Matrix metalloproteinase-2 (MMP-2) is a metalloproteinase initially described to be involved in both physiological and pathological remodeling/degradation of extracellular matrix components (Woessner 1998). Over a decade ago studies also revealed a novel action of MMP-2 at the cellular level, with a time frame of action of minutes rather than days (Sawicki, Salas et al. 1997; Sawicki, Sanders et al. 1998).

The novel actions of MMP-2 include the intracellular degradation of heart contractile proteins, such as troponin I (Wang, Schulze et al. 2002), titin (Ali, Cho et al. 2010) and MLC1 (Sawicki, Leon et al. 2005; Doroszko, Polewicz et al. 2009; Polewicz, Cadete et al. 2011), during ischemia reperfusion (I/R) injury. This intracellular substrate cleavage action of MMPs, and their role in biology and pathology, have been recently reviewed by Cauwe and Opdenakker (Cauwe and Opdenakker 2010).

Although cardiac MLC1 phosphorylation has been previously described (Arrell, Neverova et al. 2001), the functional implications of this modification remain unknown. Our previous studies showed that post-translational modification of MLC1 during hypoxia-reoxygenation (I/R) resulted in a decrease of MLC1 level and was also associated with an increase in MMP-2 activity (Doroszko, Polewicz et al. 2009). Based on these observations we hypothesize that phosphorylation of cardiac MLC1 triggered by I/R may lead to an increase in its degradation by MMP-2, and that this degradation may underlie contractile dysfunction of I/R hearts.

In this study we show that increased phosphorylation of the MLC1 during I/R in isolated perfused rat hearts is associated with a decrease in cardiac function. Furthermore, inhibition of this phosphorylation, secondary to inhibiting myosin light chain kinase (MLCK), results in an

improved mechanical function of the myocardium during reperfusion. Moreover, we show that the *in vitro* phosphorylation of MLC1 increases the affinity of MMP-2 for MLC1. As a result, establishing the molecular mechanisms responsible for the degradation of contractile proteins in the development of heart injury, triggered by I/R or other conditions that cause oxidative stress, may lead to the development of new therapeutic strategies aimed at preserving contractile function of the heart.

2.4. Materials and Methods

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

2.4.1. Heart preparations

Male Sprague-Dawley rats (300g to 350g) 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, Paris, France) and maintained at 37°C. Hearts were perfused in the Langendorff mode at a constant pressure of 60 mmHg 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% O_2/5% CO_2 (pH 7.4).

A water-filled latex balloon connected to a pressure transducer was inserted through an incision in the left atrium into the left ventricle through the mitral valve. The volume was adjusted to achieve an end diastolic pressure of 10 mmHg. Coronary flow, heart rate and left ventricular pressure were monitored using an EMKA recording system with IOX2 software (EMKA Technologies, Paris, France). Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace. The rate pressure product (RPP) was calculated as the product of heart rate and LVDP. Stock solutions (140x) of various reagents were infused into the heart via a side-port proximal to the aortic cannula at the rate 140 times lower than coronary flow (CF), usually 0.1 mL min^{-1} with a Gilson mini pump Minipuls 3 (Gilson, Middleton, WI, USA).

2.4.2. Ischemia/reperfusion protocol

Control hearts (aerobic control, n=12) were perfused aerobically for 75 minutes. Ischemic hearts (I/R, n=9), after 25 min of aerobic perfusion, were subjected to 20 minutes global no-flow

ischemia (by closing of the aortic inflow line), followed by 30 minutes of aerobic reperfusion. In two separate groups of I/R hearts (n=6 each) either ML-7 (1-5 μM [Merck, Mississauga, Canada]), myosin light chain kinase (MLCK) inhibitor or doxycycline (Doxy, 1-30 μM [Sigma, Taufkirchen, Germany]), an inhibitor of MMP-2, were infused 10 min before of onset of ischemia and for the first 10 min of reperfusion. Water was used as vehicle for Doxy while ethanol (50% (v/v) was the vehicle for ML-7. The maximal concentration of ethanol infused during the heart was less than or equal to 0.025% (v/v). The scheme of the experimental protocol is shown in Figure 2.1. At the end of perfusion the hearts were freeze clamped in liquid nitrogen and used for biochemical studies.

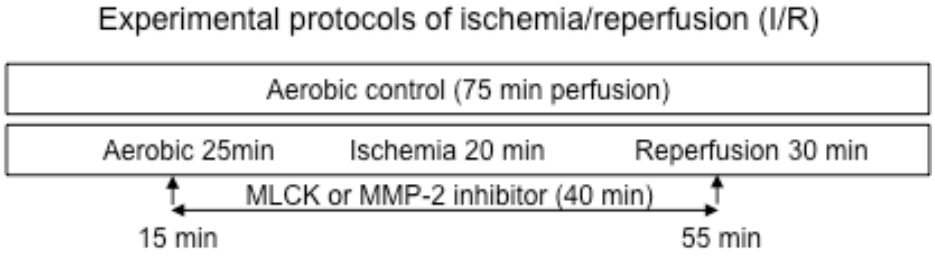


Figure 2.1 – Schematic representation of the perfusion protocol of isolated Langendorff hearts. When present, ML7, an inhibitor of MLC kinase or doxycycline, an inhibitor of MMP were administered 10 min before the onset of ischemia, during ischemia and for the first 10 min of reperfusion.

2.4.3. Preparation of heart protein extracts

Protein samples for 2-dimensional gel electrophoresis (2-DE) 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, Hercules, CA, USA]) at room temperature. Samples were sonicated twice for 5 seconds and centrifuged for 10 minutes at 10,000*g* to remove any insoluble particles. Protein content of the heart extract in rehydration buffer was measured with the BioRad Bradford protein assay.

For other biochemical studies, frozen heart tissue powder was homogenized on ice in 150mM 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.

2.4.4. Two-Dimensional gel electrophoresis (2-DE)

Protein samples (0.4 mg) were applied to each of 11 cm immobilized linear pH gradient (5-8) strips (IPG, BioRad, Hercules, CA, USA), with rehydration for 16–18 h at 20ºC. For isoelectrofocusing, 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 isoelectrofocusing, the strips were equilibrated according to the manufacturer's instructions. The 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, Hercules, CA, USA). Because of this limitation for 2-DE analysis we used 4 hearts from each group. All the gels were stained in the same bath and next scanned with a calibrated densitometer GS-800 (BioRad, Hercules, CA, USA). Quantitative analysis of MLC1 and MLC2 spot intensities from 2-DE were measured with PDQuest 7.1 measurement software (BioRad, Hercules, CA, USA).

2.4.5. Mass Spectrometry

MLC1 and MLC2 protein spots were manually excised from the 2-DE gel. These spots were then processed using a MassPrep Station (Waters, Milford, MA, USA) using the methods supplied by the manufacturer. 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. Next, the gel was washed twice with water. The protein extraction was performed overnight at room temperature with 50 µL of a mixture of formic acid, water, and isopropanol

(1:3:2, vol:vol). The resulting solution was then subjected to trypsin digestion and mass spectrometry analysis. 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 (Waters, Milford, MA, USA) coupled with Q-TOF-2 mass spectrometer (Waters, Milford, MA, USA). 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. MLCK activity assay

Frozen ventricular tissue was homogenized in a solution containing 20 mM Tris-HCl (pH 7.4 at 4ºC), 50 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 0.25 mM sucrose, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and 1 mM dithiothrietol. Protein contents of the homogenates were determined using the Bradford protein assay. MLCK activity was assessed using 0.3 mg of total ventricular protein, incubated in a solution containing 40 mM HEPES, 80 mM NaCl, 8% (w/v) glycerol, 0.2 mM EDTA, 500 mM $CaCl_2$ 0.01 % triton X-100, 0.8 mM DTT, 10 mM $MgCl_2$, 200 mM [γ^{32}P]ATP, and 200 mM Ziptide (KKLNRTLSFAEPG), in the absence or presence of 20 mM EGTA. Reactions were incubated for 10 min at 30°C, and terminated with addition of H_3PO_4 (3% v/v final concentration). An aliquot of each reaction was spotted on P81-phosphocellulose, which was washed in 1% H_3PO_4 (5-6x, 10 min each wash), and subsequently acetone (2x, 5 min each wash). The incorporation of γ^{32}P into the Ziptide substrate was determined by scintillation counting.

2.4.7. 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 1ml DEAE cellulose column. The PKC enriched fraction (9µg 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 100V 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.

2.4.8. Cloning, expression and purification of human cardiac MLC1

The cDNA clone for the human ventricular myosin essential light chains (MLC1), a product of the *MYL3* gene, was isolated as previously described (Muthu, Wang et al. 2011) by the method of two-tube RT-PCR using the Omniscript RT kit (Qiagen, Valencia, CA, USA) using total adult human heart RNA purchased from Stratagene (Agilent, Santa Clara, CA, USA), Oligo (dT)15 (Promega, Madison, WI, USA) and specific primers designed based on the published nucleotide sequence from NCBI: NG_007555. Restriction sites for NcoI and BamHI were inserted to facilitate ligation into the pET 3D plasmid (Merck KGaA, Darmstadt, Germany). PCR was performed according to the Omniscript manual and a resulting PCR product was gel purified using a Qiaex II kit (Qiagen, Valencia, CA, USA). The eluted DNA was then digested with Nco I and BamHI and ligated into similarly digested pET 3d plasmid using T4 DNA Ligase (NEBiolabs, Ipswich, MA, USA). Subcloning efficiency DH5 α competent cells (Invitrogen, Eugene, OR, USA) were transformed and plated onto LB-CB plates. Resultant colonies were screened for the MLC1 insert and the positive colonies were selected. The isolated plasmid DNA was sent for sequencing (Cardiovascular Facility, University of Miami, Miami, Florida USA) and the clones were confirmed to have the correct MLC1 sequence (Swiss-Prot: P08590). Confirmed DNA was used to transform BL21 (DE3) Codon Plus competent cells (Agilent, Santa Clara, CA, USA) for MLC1 wild-type protein expression. MLC1 protein was expressed in 8l of enriched media consisting of 30g of peptone/l, 20g of select yeast extract/l,

and M9 minimal salts 10g/l with 20 μg/ml ampicillin and purified using column chromatography S-Sepharose followed by DEAE-Sephacel.

2.4.9. In vitro phosporylation of MLC1

MLC1 (7.5 μg) was incubated with 25 ng of the active form of myosin light chain kinase (MLCK) (SignalChem, Richmond, ON, Canada) and 1.4 μg of ATP (Sigma, St Louis, MO, USA) for 20 min at 37° C in 40 mM MOPS-NaOH reaction buffer with pH 7.0, containing 0.5 mM $CaCl_2$ and 1 μM calmodulin (Upstate, Temecula, CA, USA). The total volume of reaction mixture was 30 μl. *In vitro* phosphorylation of MLC1 was verified by mass spectrometry. In some experiments MLCK was incubated for 5 min with 10 μM of ML-7 (inhibitor of MLCK) before ATP was added to the reaction mixture.

2.4.10. Degradation of phosphorylated MLC1 by MMP-2

Phosphorylated or non-phosphorylated MLC1 (7.5 μg) was incubated at 37^{o}C for 60 minutes with 0.2 μg of 64 kDa MMP-2 (Calbiochem, Merck KGaA, Darmstadt, Germany) in 50 mM Tris-HCl buffer, pH 7.6 and containing 5 mM $CaCl_2$ and 150 mM NaCl, total volume 60 μL) (Sawicki, Leon et al. 2005). The reaction mixture was analyzed by 15% SDS-PAGE under reducing conditions and visualized by immunoblot analysis with anti-MLC1 IgG (Abcam, Cambridge, MA, USA).

2.4.11. Kinetic analysis of MMP-2 activity in presence of phoshorylated and non-phosphorylated MLC1 (competition assay)

The hydrolysis of OmniMMP fluorogenic substrate (Enzo Life Sciences, PA, USA, 0-25 μM) by MMP-2 was measured at 37˚C in a continuous plate reader-based protocol (Sariahmetoglu, Crawford et al. 2007). Briefly, the sample containing MMP-2 (0.2 nM), substrate and either phosphorylated or non-phosphorylated MLC1 (0-25 μM,) was measured

every 30 seconds for 1 hour (λ_{ex} 328 nm, λ_{em} 393 nm). The rate of product formation was determined through linear regression of the fluorescence-time data by the plate reader software. Linear reaction rates for control data was fitted to the Michaelis-Menten equation in order to obtain K_M and V_{max} values. Linear reaction rates from assays containing MLC1 were fitted to an equation for linear competitive inhibition in order to determine K_M, V_{max} and K_i values for the MLC1 samples.

2.4.12. Immunoblot analysis

Recombinant MLC1, its degradation products, MLC1, MLC2 and phospho-MLC2 content in the heart extracts were determined by immunoblotting. Equal volumes of MLC samples from the *in vitro* experiments or 20µg of protein from each heart extract were analyzed by SDS-PAGE using 15% gels. After electrophoresis protein was transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). MLC1, MLC2 and phospho-MLC2 were identified using mouse monoclonal anti-MLC1, rabbit polyclonal anti-MLC2 and rabbit polyclonal anti-phospo-MLC2 antibodies (Abcam, Cambridge, MA, USA).

Co-localization of myocardial MLC1 with myocardial MLCK or MLCP was analyzed by separating protein complexes in non-reducing and non-denaturing conditions on native mini-PROTEAN TGX pre-cast gels (BioRad, Hercules, CA, USA), followed by dual-immunoblot detection. MLC1 was detected with mouse monoclonal anti-MLC1 antibody (Abcam, Cambridge, MA, USA) using goat anti-mouse IgG tagged with Alexa fluor 488 (Invitrogen, Eugene, OR, USA) as secondary antibody. MLCK was detected with rabbit monoclonal antibody (Abcam, Cambridge, MA, USA) with goat anti rabbit IgG tagged with Alexa fluor 647 (Invitrogen, Eugene, OR, USA) as a secondary antibody.

Band densities were measured with VersaDoc 5000 and Quantity One software (BioRad, Hercules, CA, USA).

2.4.13. PKC activity assay

Protein kinase C (PKC) activity was determined using a non-radioactive PKC kinase activity assay (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol. Briefly, 2 μg of protein from crude heart extracts were loaded onto PKC substrate pre-coated wells. Activity of PKC over the substrate is determined using a phospho-specific antibody followed by horseradish peroxidase-coupled secondary. The assay was developed with tetramethylbenzidine and color developed according to the extent of substrate phosphorylation by PKC in the crude extracts. Color development was stopped with an acid stop solution and the plate read at 450 nm for quantitative measurements.

2.4.14. Statistical Analysis

Protein spot intensity was measured using PDQuest software (BioRad, Hercules, CA, USA) and evaluated by the Kruskal Walis and Mann-Whitney U tests. ANOVA or Kruskal-Wallis tests were used in functional studies (followed by Tukey's post-hoc test) and an unpaired t-test was used in immunoblot analysis. Data are expressed as mean±SEM. A $p < 0.05$ was considered statistically significant.

2.5. Results

2.5.1. MLC2 in IR hearts

In the heart, myosin regulatory light chain (MLC2) is the major target of MLCK. Therefore, it was important to assess the level of MLC2 as well as its phosphorylation in the hearts subjected to I/R. MLC2 levels, as well as its phosphorylation status, were measured in control hearts, in the hearts subjected to I/R, and in I/R hearts perfused with MLCK inhibitor, ML-7. As shown in Fig. 2.2a and 2.2b, no changes in MLC2 protein levels measured either by 2-dimensional electrophoresis (2-DE) or immunobloting were observed in any of the analyzed heart groups. Also, as analyzed by mass spectrometry (data not shown), there were no visible changes in the MLC2 phosphorylation in any of the experimental groups.

Although ML-7 is considered a selective inhibitor of MLCK, there are reports showing that ML-7 inhibits other kinases (Bain, McLauchlan et al. 2003). According to the supplier information the K_i of ML-7 is approximately 70 times higher for protein kinase A (21 μM) and 140 times higher for protein kinase C (42 μM) than for MLCK (0.3 μM). To rule out potential effects of ML-7 on PKC, we measured PKC activity in the 3 experimental groups. PKC activity was significantly increased in response to I/R. Five micromolar of ML-7 did not affect PKC activity in hearts subjected to I/R (Fig. 2.3a). We then confirmed the inhibitory effect of ML-7 on MLCK activity (Fig. 2.3b). While I/R did not change MLCK activity, administration of 5 μM of ML-7 to I/R hearts significantly reduced MLCK activity by more than 60 % (Fig. 2.3b).

Because we did not observe any changes in MLC2 protein levels or its phosphorylation status in any of the experimental groups, and because of ML-7 concentration used in the assay (5 μM in *ex vivo* conditions, that is below the K_i for other kinases), we assume that in our experimental model MLCK was acting on MLC1 as a main target.

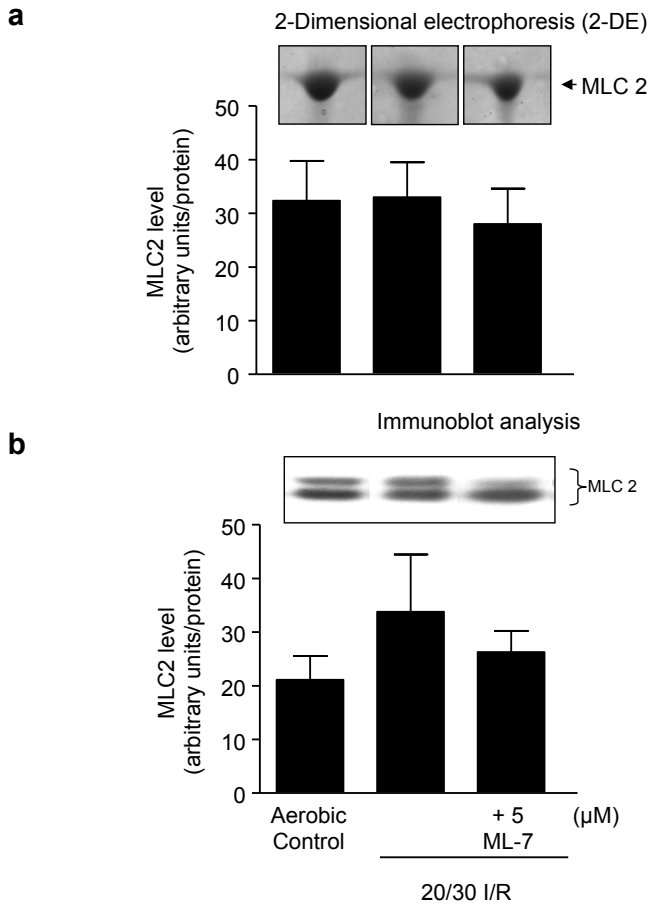


Figure 2.2 – Densitometric analysis of the levels of cardiac MLC2 determined by 2-DE (a) and by immunoblotting (b) in heart homogenates (n=4/group for 2-DE and n=3/group for immunoblotting).

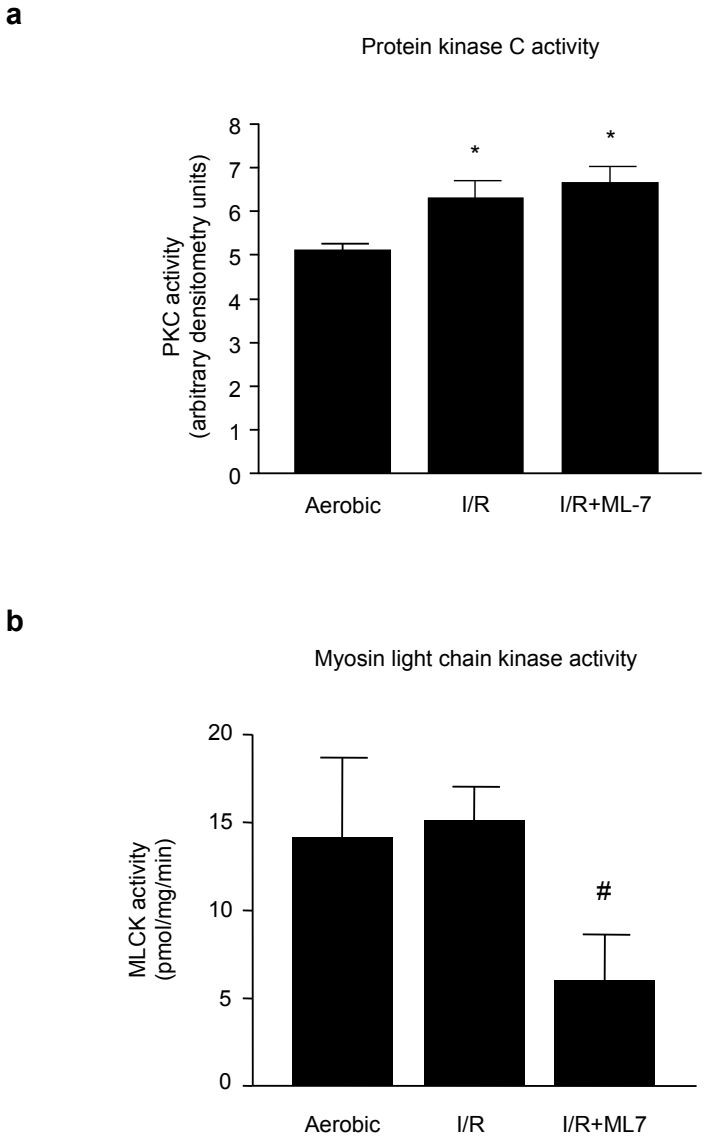


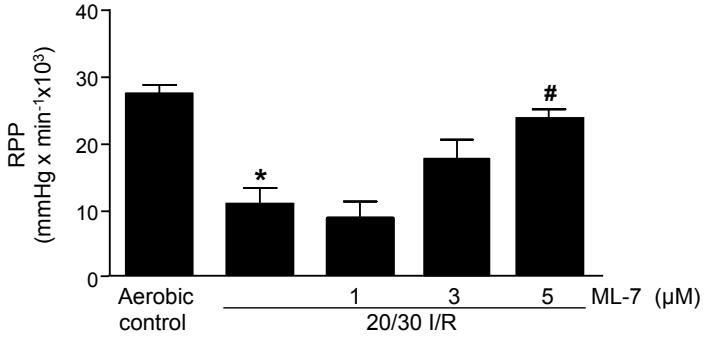
Figure 2.3 – Effect of 5 μM of ML-7 on protein kinases in isolated rat hearts. (a) Protein kinase C activity in isolated perfused rat hearts under aerobic conditions or subjected to I/R in the presence or absence of the myosin light chain kinase inhibitor ML-7. (n=4). (b) Myosin light chain kinase activity in isolated perfused rat hearts under aerobic conditions or subjected to I/R in the presence or absence of the myosin light chain kinase inhibitor ML-7 (n=4-6). *$p < 0.05$ *vs.* aerobic control; $^{\#}p < 0.05$ *vs.* I/R.

2.5.2. Inhibition of MLCK-dependent phosphorylation of myocardial MLC1 during I/R

Heart function, assessed by rate pressure product (RPP), was significantly decreased in hearts reperfused following ischemia, in comparison to hearts perfused aerobically for 75 minutes (10.6±2.4 *vs.* 27.4±1.3 bpm*mmHg*10^3; respectively; $p<0.05$) (Fig. 2.4a). Pharmacological inhibition of MLCK by ML-7 resulted in an increased functional recovery of hearts during reperfusion. The protection seen by ML-7 occurred in a concentration-dependent manner. Using 5 μM ML-7 resulted in an increased recovery of contractile function (Fig. 2.4a) and, in addition, it also prevented the degradation of MLC1 protein during I/R (Fig. 2.4b). A protective effect of ML-7 against MLC1 degradation was confirmed by 2-DE (Fig. 2.4c). This was accompanied by the decreased formation of the truncated form of MLC1 in hearts treated with ML-7 in comparison to MLC1 from I/R hearts (Fig. 2.4c).

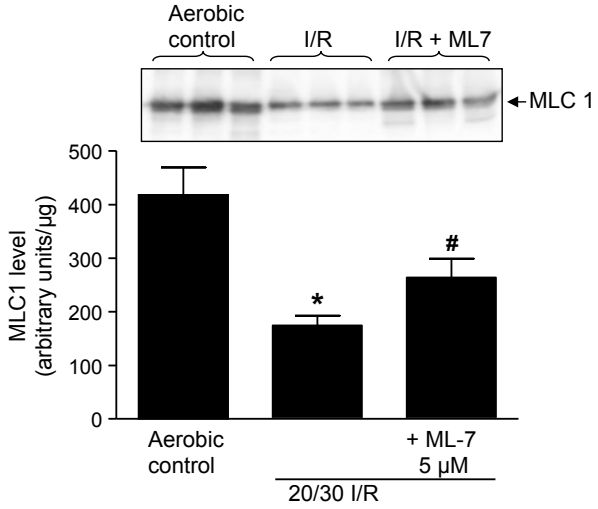
a

Mechanical function of the I/R heart with ML-7 a MLC kinase inhibitor



b

MLC1 level in the I/R heart with ML-7 a MLC kinase inhibitor



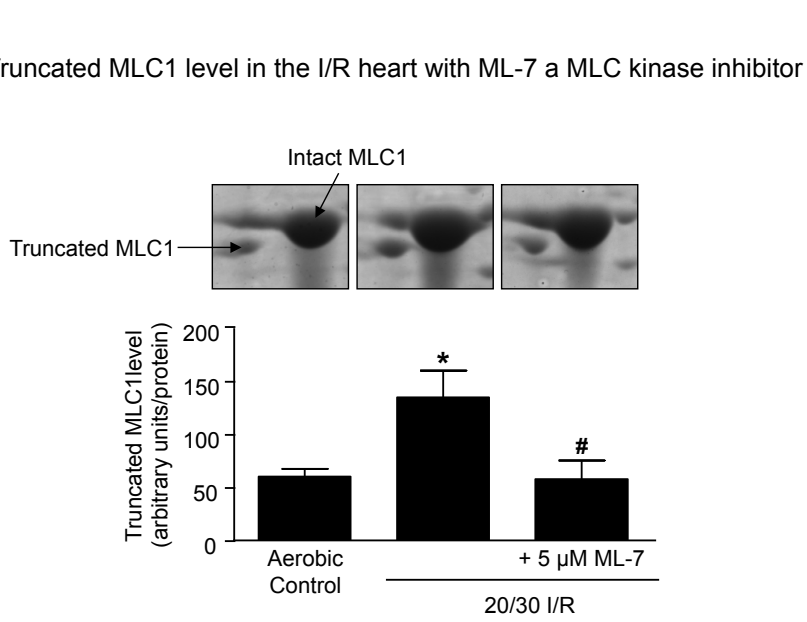


Figure 2.4 – Protection of cardiac mechanical function and MLC1 levels by ML-7. (a) Mechanical function (determined as RPP) of hearts submitted to I/R perfused in the presence or absence of ML-7 (MLC kinase inhibitor) (n=6–9/group). (b) Densitometric analysis of the levels of MLC1 in heart homogenates analyzed by immunoblotting (n=3/group). (c) Densitometric analysis of the levels of truncated MLC1 as determined by 2-DE (n=4/group). RPP – rate pressure product; MLC1 – Myosin light chain 1; I/R – ischemia/reperfusion. *$p < 0.05$ *vs.* aerobic control; $^{\#}p < 0.05$ *vs.* I/R.

2.5.3. Analysis of in vitro and ex vivo MLC1 phosphorylation

Since rat MLC1 protein was not commercially available we used a recombinant human cardiac MLC1 (hMLC1) in our *in vitro* studies. Phosphorylation status of hMLC1 phosphorylated *in vitro* with MLCK was compared with the MLC1 from *ex vivo* I/R heart (Fig. 2.5). *In vitro* incubation of hMLC1 with MLCK resulted in phosphorylation of either threonine 127 (T127) or T129 or tyrosine 130 (Y130) (T127/T129/Y130). Because these amino acids are

localized in the same tryptic peptide it is difficult to determine, by analysis of the tryptic peptides, which residue is phosphorylated. In addition, phosphorylation of serine 179 (S179) and Y185 was detected. Inhibition of MLCK by ML-7 abolished phosphorylation of S179 and Y186, but did not inhibit phosphorylation of T127/T129/Y130 site (Table 2.1).

MLC1 from rat hearts subjected to I/R was phosphorylated at either T69 and T77, or Y78 (T77/Y78). As mentioned above, and similarly to human MLC1, the exact identification of which amino acid is phosphorylated is difficult. Also, T132/T134/Y135, T164, S184, and Y190 were identified as phosphorylation sites (Fig. 2.5 and Table 2.2).

The two phosphorylated amino acids located at the C-terminus [S184 (rat MLC1) and S179 (hMLC1) together with Y190 (rat MLC1) and Y185 (hMLC1)] were found to be located very close (S in P6 position) or next to (Y in P'1 position), the known MMP-2 cleavage site. The cleavage site for MMP-2 is localized between asparagine N189 (P1) and tyrosine Y190 (P'1) in rat MLC1, and N184 (P1) and Y185 (P'1) in hMLC1 (see inset in Fig. 2.5).

Table 2.1 – *In vitro* phosphorylation of human recombinant MLC1 with MLC kinase (MLCK)

Identified phosphorylation sites in MLC1		
Control	+ MLCK*	
		+ ML-7** (5μM)
n/d	T 127 or T 129 or Y 130	T 127 or T 129 or Y 130
n/d	S 179	n/d
n/d	Y 186	n/d

and its inhibition by ML-7, an inhibitor of MLCK.

T – Threonine; Y – Tyrosine; S – Serine

n/d – not detected

*Human recombinant MLC1 (7.5 μg) was incubated with MLCK (25 ng) and ATP (1.4 μg) for 20 min, as described in the Methods section.

**ML-7 (5 μg) was added to the reaction mixture 5 min before ATP.

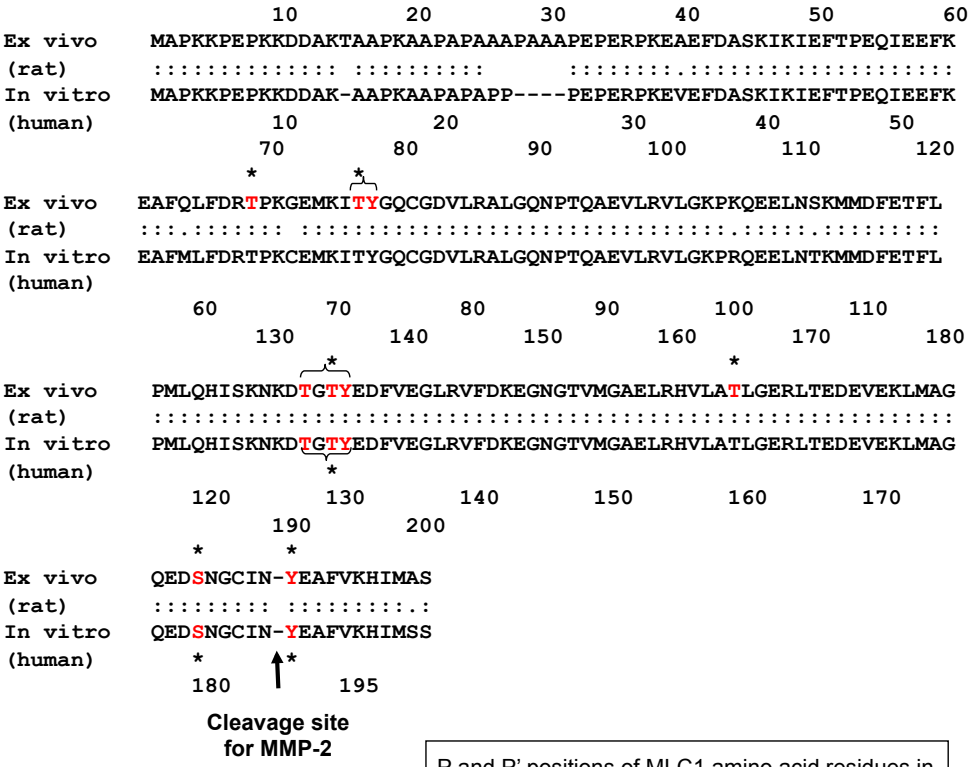
Table 2.2 – Phosphorylation status of MLC1 from rat I/R hearts perfused with ML-7 an inhibitor of MLC kinase (MLCK)

Identified phosphorylation sites in myocardial MLC1		
Aerobic heart	I/R heart (20/30)	
		+ ML-7 (5 μM)
T 69	T 69	n/d
n/d	T 77 or Y 78	T 77 or Y 78
T 132	T 132	T 132
or T 134	or T 134	or T 134
or Y 135	or Y 135	or Y 135
n/d	T 164	n/d
n/d	S 184	n/d
n/d	Y 190	n/d

T – Threonine; Y – Tyrosine; S – Serine

n/d – not detected

Comparison of MLC1 phosphorylation from rat I/R hearts (Ex vivo) to human recombinant MLC1 phosphorylated with MLC kinase (*In vitro)*



P and P' positions of MLC1 amino acid residues in MMP-2 cleavage site

	184						190					
Rat MLC1	S	N	G	C	I	N	-Y	E	A	F	V	K
Human MLC1	S	N	G	C	I	N	-Y	E	A	F	V	K
	P6						P1	P'1				P'6
	179						185					

Figure 2.5 – Comparative analysis of MLC1 sequences from rat I/R hearts and human recombinant MLC1 phosphorylated *in vitro* with myosin light chain kinase and respective phosphorylation sites as determined by mass spectrometry (phosphorylated amino acid residues are shown in red). Homology between rat and human MLC1 is 93.5% identity between rat (200 aminoacids) and human (195 aminoacids). Insert in box shows P and P' positions of MMP-2 cleavage site within the C-terminal of rat and human MLC1.MMP-2 – matrix metallopreoteinase-2; MLC1 – myosin light chain 1.

* - phosphorylated residues.

: – represents full homology between amino acids

. – represents partial homology between amino acids

2.5.4. Effect of phosphorylated MLC1 on MMP-2 kinetic

We also determined what effect MLC1 phosphorylation had on the affinity of MLC1 for MMP-2, and the consequent degradation by MMP-2. Kinetic analysis of the MMP-2 reaction with OmniMMP substrate, in presence of MLCK-phosphorylated hMLC1 revealed a higher affinity of phospho-MLC1 to MMP-2 (decreased V_{max} for OmniMMP substrate) compared to non-phosphorylated MLC1 (V_{max} not changed) (fig 2.6).

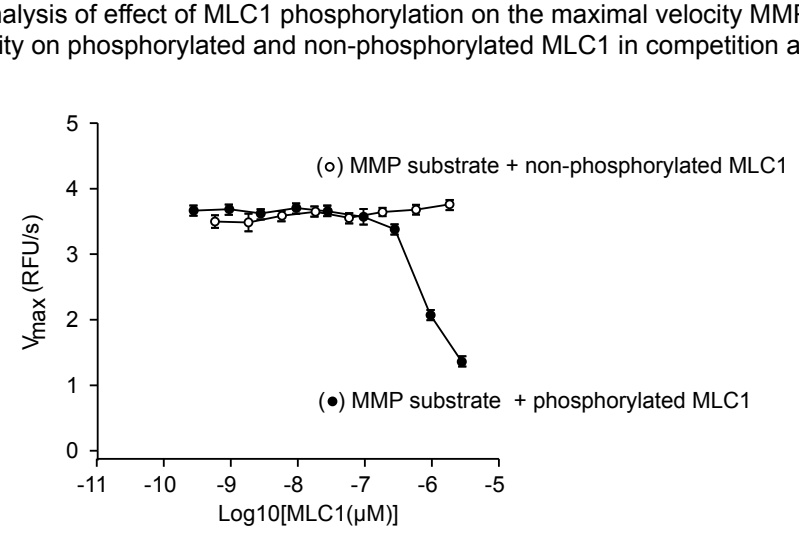


Figure 2.6 – Effect of MLC1 phosphorylation on the maximal velocity of MMP-2 activity (V_{max}) in a competition assay (n=3-4).

2.5.5. Co-localization of MLC1 with MLCK in I/R hearts

Potential co-localization of MLC1 with MLCK was evaluated by separating protein complexes from heart homogenates under non–reducing and non-denaturing conditions (using native gels), followed by dual-immunoblotting (Fig. 2.7). Under non-reducing conditions, two distinct bands of MLC1 (red) were seen in aerobic control, I/R and I/R+ML-7 heart homogenates (Fig. 2.7a, middle panel). Blotting for MLCK revealed only one band (green) (Fig. 2.7a, top panel). Overlap of both blots revealed co-localization of MLCK with MLC1 (yellow) (Fig. 2.7a, bottom panel). Quantitative analysis of the blots showed a slightly increased co-localization of MLC1 with MLCK in I/R hearts compared to aerobic controls, but the difference was not statistically significant ($p=0.0661$, Fig. 2.7b). Administration of ML-7 significantly reduced the co-localization of MLC1 with MLCK in comparison to non-treated hearts subjected to I/R (Fig. 2.7b).

2.5.6. Protection of contractile function and MLC1 protein levels by doxycycline

Apart from its antibacterial function, doxycylcine has also been shown to be an inhibitor of MMPs (Golub, Lee et al. 1998). It was also shown that doxycylcine mediated inhibition of MMP-2 improves cardiac functional recovery following I/R (Cheung, Sawicki et al. 2000; Wang, Schulze et al. 2002; Sawicki, Leon et al. 2005; Fert-Bober, Leon et al. 2008). We therefore used doxycyclin in our studies to inhibit the activity of MMP-2. We found that mechanical function of I/R hearts was protected by doxycycline in a concentration-dependent manner (Fig. 2.8a). Administration of 30 μM doxycycline resulted in full recovery of contractile function during reperfusion (Fig. 2.8a) and also prevented the degradation of MLC1 protein during I/R (Fig. 2.8b).

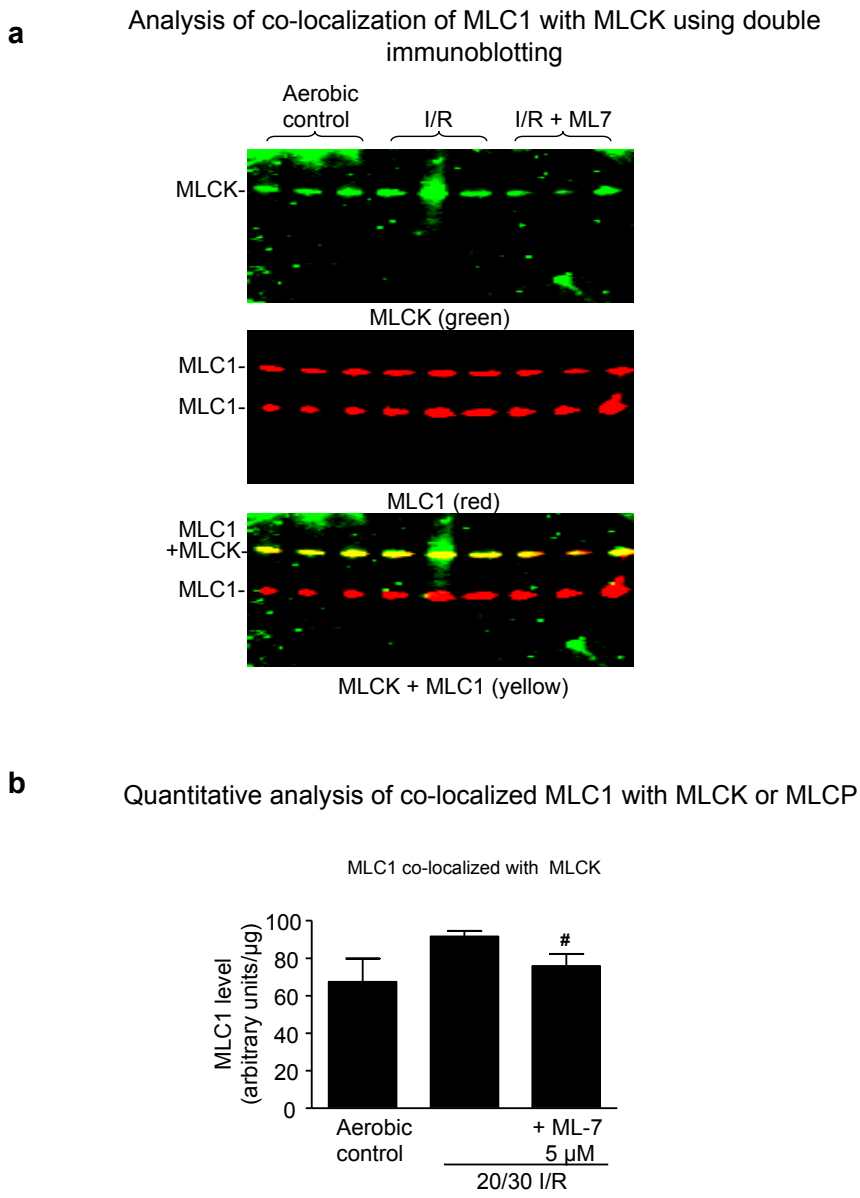


Figure 2.7 – Co-localization of MLC1 with MLCK and MLCP. (a) Analysis of co-localization of MLC1 with MLCK and MLCP by dual immunoblotting. (b) Quantitative analysis of MLC1 levels co-localized with MLCK – myosin light chain 1; MLCK – myosin light chain kinase; I/R – ischemia/reperfusion. *$p < 0.05$ *vs.* aerobic control; n=3/group.

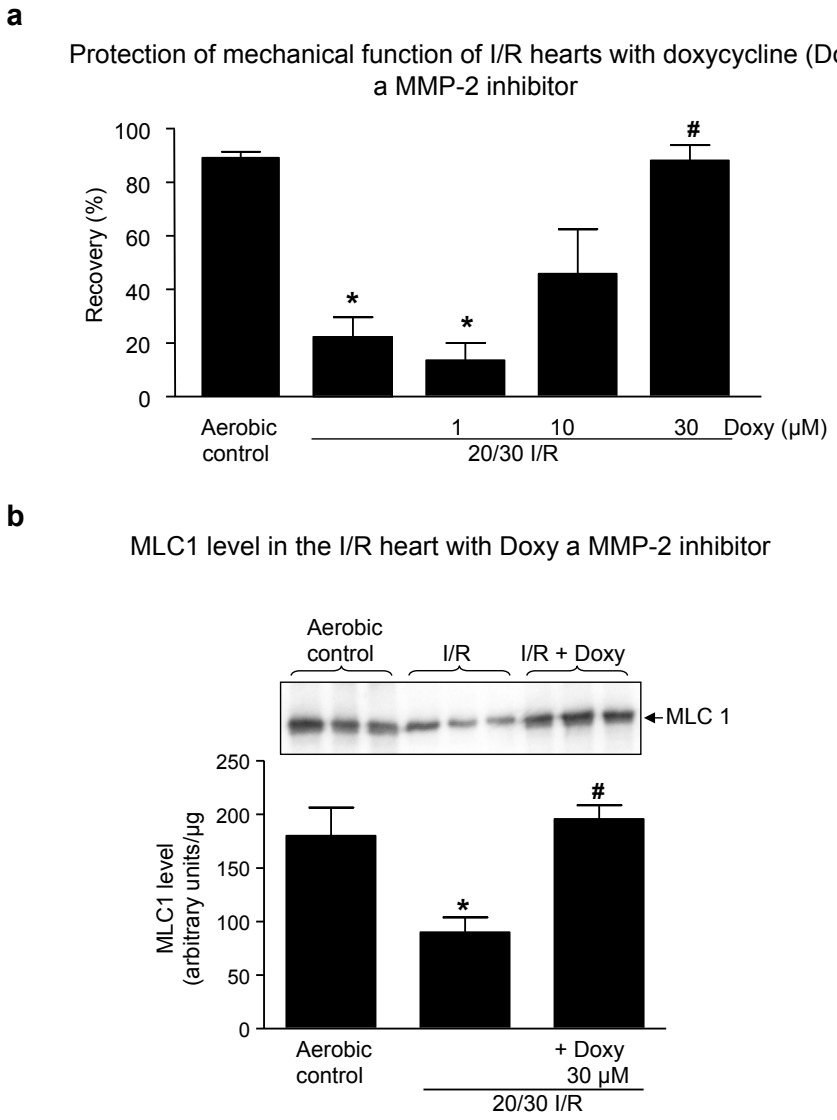


Figure 2.8 – Protection of cardiac mechanical function and MLC1 levels by doxycycline (Doxy), an inhibitor of MMP-2. (a) Mechanical function (determined as RPP) of hearts subjected to I/R and perfused in the presence or absence of Doxy (n=6–9/group). (b) Densitometric analysis of the levels of MLC1 in heart homogenates determined by immunoblotting (n=3/group). RPP – rate pressure product; MLC1 – Myosin light chain 1; I/R – ischemia/reperfusion. *$p < 0.05$ *vs.* aerobic control; $^{\#}p < 0.05$ *vs.* I/R.

2.6. Discussion

Cardiac contraction has been well studied and many of the molecular elements involved in the physiology of contraction identified. Myosin light chain 1 is an important contractile protein involved in the structural and functional stability of the contractile apparatus. Here we show that MLC1 phosphorylation plays an important role in the pathology of cardiac contractile dysfunction following ischemia-reperfusion. MLC1 is phosphorylated during I/R, possibly by MLCK, and this phosphorylation results in increased degradation of MLC1 by MMP-2 that is associated with the development of contractile dysfunction. Our data show that phosphorylation of MLC1 increases the affinity of MMP-2 for MLC1. This was evident by a decrease in V_{max} in a competition assay with the phosphorylated MLC1 compared to the non-phosphorylated counterpart. In addition we show that either inhibition of MLC1 phosphorylation or inhibition of MMP-2 activity in I/R hearts results in the protection of contractile function during reperfusion, as well as the preservation of MLC1 protein levels. Moreover, our results from the *in vitro* study confirm what has been previously suggested (Van Eyk, Powers et al. 1998; Arrell, Neverova et al. 2001) that in addition to MLC2, MLC1 can also be a suitable substrate for phosphorylation by MLCK.

Mass spectrometry analysis of human recombinant MLC1 (hMLC1) phosphorylated *in vitro* revealed three phosphorylation sites located on the C-terminus of hMLC1. Since these phosphorylation sites are located in the P6 and P'1 positions of the known MMP-2 cleavage site (Sawicki, Leon et al. 2005), it is likely that phosphorylation of these residues results in a conformational change of MLC1 exposing the cleavage site of MLC1 for MMP-2. This is supported by our data showing an increased affinity of phosphorylated MLC1 to MMP-2.

Since we observed no changes in the phosphorylation status of MLC2 with I/R in the presence or absence of ML-7, and we used concentrations of ML-7 below the k_i for other kinases, we can infer that the beneficial effects of MLCK inhibition are due to the inhibition of MLC1 phosphorylation and consequent preservation of its protein levels. Inhibition of MLCK with ML-7 prevented the phosphorylation of amino acid residues from P6 and P'1 positions but failed to inhibit phosphorylation of T127/T129/Y130. Indeed, mass spectrometry analysis of MLC1 from perfused rat hearts revealed four phosphorylation sites that can be attributed to MLCK, two of which are in the vicinity of the MMP-2 cleavage site and are I/R-dependent.

Interestingly, we also found two phosphorylation sites on MLC1 in aerobically perfused hearts and of these two only one (T69) appears to be MLCK-dependent. This suggests that other kinases apart of MLCK may be involved in the process of MLC1 phosphorylation. The analysis of the T127/T129/Y130 phosphorylation site of human MLC1 and T132/T134/Y135 phosphorylation site of rat MLC1 for kinase consensuses sequence indicate AMPK (AMP-activated protein kinase) and PKC as two other potential mediators. Indeed, activation of these kinases has been reported in I/R (Lopaschuk 2008; Palaniyandi, Sun et al. 2009). Phosphorylation of T69 and T132/T134/Y135 is observed in MLC1 from aerobically perfused hearts (controls). This suggests that phosphorylation of these sites may be important in physiological conditions.

Recently, a cardiac specific MLCK has been identified in human heart failure and described as the protein kinase responsible for phosphorylating MLC2 and regulating cardiac contractility (Seguchi, Takashima et al. 2007; Chan, Takeda et al. 2008). Here we report a slightly increased co-localization of MLCK with MLC1, triggered by I/R, with no changes in MLCK activity. To date, changes in cardiac MLCK activity have not been described in response to I/R. Our results confirm that MLCK activity does not change in response to I/R but that 5 μM ML-7 administration significantly reduce MLCK activity.

Although ML-7 has been suggested to be a specific MLCK inhibitor, it has been reported to affect the activity of other kinases, similar to what occurs with the vast majority of protein kinase inhibitors (for review see (Bain, McLauchlan et al. 2003)). Inhibition of PKC by ML-7 has been shown *in vitro* at high concentrations (Bain, McLauchlan et al. 2003). We have measured the activity of PKC in all our groups. PKC activity was significantly increased with I/R but unaffected by 5 μM ML-7, which inhibits MLCK activity. PKC has been described to potentially phosphorylate MLC1 (and MLC-2) (Venema, Raynor et al. 1993) and increase its activity in response to I/R (Shintani-Ishida and Yoshida 2011). Our data show a potential phosphorylation site on MLC1 under PKC regulation (T127/T129/Y130 site of human MLC1 and T132/T134/Y135 site of rat MLC1). This site is phosphorylated under both physiological (aerobic) and pathological (I/R) conditions and is unaffected by ML-7 administration. This indicates that phosphorylation of MLC1 by PKC is independent of I/R and may regulate MLC1 structure and/or function.

MLCK has been described as being the enzyme responsible for phosphorylating MLC2 at S15 of the N-terminus, and this phosphorylation is considered responsible for the regulatory character of MLC2 in skeletal and cardiac muscle contraction (High and Stull 1980; Stull, Manning et al. 1980; Moore and Stull 1984; Sweeney and Stull 1986). However, results from our *in vitro* studies demonstrate that MLC1 is also a substrate for MLCK. Studies of protein-protein interactions, where samples were run under non-reducing conditions to determine localization of free proteins or co-localized with protein complexes, showed that in isolated perfused heart homogenates MLC1 is present in two protein bands. However, only one of these bands corresponds to a complex of MLC1 with MLCK. These observations suggest the existence of two pools of intracellular MLC1: one associated with, and under regulation of, MLCK and another independent of MLCK. Also, the second MLC1 band might correspond to free MLC1. Another possible explanation is that the second band of MLC1, detected by immunoblotting under non-reducing and non-denaturing conditions, corresponds to degraded MLC1. Interestingly we observed a slightly increased co-localization of MLC1 and MLCK triggered by I/R, with no changes in MLCK activity, with this effect being inhibited by ML-7. This suggests that part of the mechanism of cardioprotection by ML-7 involves the inhibition of complex formation of MLCK with MLC1, in addition to inhibition of MLCK activity.

Isolated perfused rat hearts, subjected to I/R in the presence of doxycycline show a significant increase in contractile function recovery during reperfusion, in comparison to I/R alone. Moreover, this protection in cardiac contractile function is associated with a preservation of MLC1 levels, similar to what was observed with ML-7 administration.

In summary these results together with our previous studies show an important role of post-translational modifications triggered by I/R, such as phosphorylation, on contractile protein MLC1, and its degradation by MMP-2 and association of this process with contractile dysfunction.

2.7. Acknowledgments

We would like to thank Dr. Olga M. Hernandez for the cloning, expression and purification of human cardiac MLC1.

This project was funded by grants from Canadian Institutes of Health Research and the Saskatchewan Health Research Foundation. GS is an investigator supported by the Heart and Stroke Foundation of Canada. DSC is supported by the National Institutes of Health Grants HL071778 and HL090786.

3. Synergistic effect of inhibitors of MLC1 phosphorylation and MMP-2 activity on protection of heart from ischemia/reperfusion injury

Virgilio J. J. Cadete; Jolanta Sawicka; Adrian Doroszko; Grzegorz Sawicki

3.1. Preface

The manuscript here presented will be submitted to the *British Journal of Pharmacology*. This manuscript addresses objective 3 of my thesis:

3. Develop pharmacological strategies to protect against the degradation of contractile function and the reduction of I/R injury.

In this manuscrip, building from our previous work identifying MLC1 phosphorylation and degradation as an important mechanism in the development of ischemia/reperfusion (I/R) injury, we develop a new therapeutic paradigm by using subthreshold concentration of drugs that prevent MLC1 phosphorylation (ML-7 and Y-27632) and degradation (doxycycline). The use of a combination of these three drugs at subthreshold concentrations resulted in a full protection of cardiac contractile function following I/R and prevention of MLC1 degradation. Also, because each drug is used at a concentration at which it has no protective effect we determined that the mechanism by which these drugs interact, in conferring cardioprotection, is synergy.

3.2. Abstract

Phosphorylation of myosin light chain 1 (MLC1) and the degradation of phophorylated MLC1 during myocardial ischemia/reperfusion (I/R) injury has been established. Also it has been shown that matrix metalloproteinase-2 (MMP-2) degrades MLC1 and that this degradation is increased when MLC1 is phosphorylated. We hypothesize that simultaneous inhibition of phosphorylation of MLC1 and MMP-2 action will protect hearts from I/R injury. Because, phosphorylation of MLC1 and MMP-2 activity is important for normal heart function, instead of using high (protective) doses of drugs we used a mixture of subtreshold doses of inhibitors of MLC1 phosphorylation and MMP-2 activity.

Isolated rat hearts were subjected to 20 min ischemia and 30 min reperfusion. The recovery of cardiac function was improved in a concentration-dependent manner by the MLC kinase inhibitor ML-7 (1-5μM), and by the MLC phosphatase activator Y-27632 (0.05-1μM). Co-administration of both drugs in subthreshold doses (1μM of ML-7 and 0.05μM of Y-27632) showed a synergistic effect in protecting cardiac contractility and MLC1 levels in I/R hearts. Doxycycline (Doxy, MMPs inhibitor) improved cardiac recovery in a concentration-dependent manner (1-30μM). Combination of subthreshold doses of ML-7 and Y-27632 with subthreshold concentration of Doxy (1μmM) showed better protection than the mixture of ML-7 and Y-27632.

The results of this study show a new direction for pharmacological prevention of reperfusion injury resulting, for instance, from coronary revascularization. An additional benefit of using low doses of drugs will be to reduce the cardiac cytotoxicity seen at high doses of the currently available MMP-2 and kinase inhibiting drugs.

Key words: myosin light chain, phosphorylation, matrix metalloproteinase, MLC kinase, MLC phosphatase

3.3. Introduction

Cardiac ischemia, followed by reperfusion, often results in the development of cardiac contractile dysfunction limiting the recovery prognosis of patients. Currently, the goal of pharmacological therapy in the course of ischemic heart disease is to improve the oxygen supply/demand ratio for the heart. There are two general strategies to do it: 1) to restore normal coronary blood flow, and 2) to decrease myocardial oxygen consumption. For the pharmacological prevention of cardiovascular disease, current clinical practice involves a multidrug regimen, consisting of aspirin, a β blocker, an angiotensin-converting-enzyme inhibitor, and a statin. In some cases, the β blocker is replaced by a calcium-channel blocker. However, to this existing strategy, new directions in pharmacological prevention or treatment of ischemic heart diseases are needed.

Understanding the molecular mechanisms involved in the development of cardiac contractile dysfunction is essential to the development of new strategies whose aims are to protect the heart muscle from ischemia/reperfusion (I/R) injury.

Myosin light chain 1 (MLC1) is a cardiac contractile protein involved in the stabilization of the myosin head (Hernandez, Jones et al. 2007). Also known as an essential light chain, MLC1 plays an important role in the regulation of force development. We have previously shown that MLC1 is susceptible of posttranslational modification induced by oxidative stress (Doroszko, Polewicz et al. 2009; Polewicz, Cadete et al. 2011). MLC1 can be nitrated, S-nitrosylated, and phosphorylated and these posttranslational modifications are associated with an increase in the affinity between MLC1 and the proteolytic enzyme matrix metalloproteinase (MMP-2). This increase in affinity results in an increased degradation of MLC1 and is also associated with the development of cardiac contractile dysfunction in several models of cardiac oxidative stress such as *in vivo* hypoxya-reoxygenation (Doroszko, Polewicz et al. 2009), *ex vivo* isolated heart I/R, and *in vitro* isolated cardiomyocytes simulated ischemia (Polewicz, Cadete et al. 2011).

The pharmacological regulation of the phosphorylation status of a certain protein can be achieved by the inhibition of the kinase responsible for the particular phosphorylation or by the stimulation of the corresponding phosphatase. Phosphorylation of MLCs has been attributed mainly to MLCK (myosin light chain kinase) while dephosphorylation of only MLC2 (regulatory

light chain) has been attributed to the myosin light chain phosphatase. MLCK can be specifically inhibited by ML-7 (Bain, McLauchlan et al. 2003) while MLCP activity can be increased indirectly by the use of the Rho Kinase pathway inhibitor Y-27632 (Cadete, Sawicka et al. 2010).

Most pharmacological approaches that target the protection of the heart against I/R injury are based on the use of single particular drugs at full protective dose, targeting only a single molecular mechanism involved in the development of contractile dysfunction. However, most of the molecular mechanisms involved in the pathology of contractile dysfunction are also important in the normal physiological function of the heart. We hypothesized that the use of subthreshold doses of inhibitors targeting different pathways involved in the phosphorylation of MLC1 and its degradation can act synergistically in eliminating the pathological components activity of these pathways, without interfering with their physiological roles.

3.4. Material and methods

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

3.4.1. Heart preparations

Male Sprague-Dawley rats (300g to 350g) 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, Paris, France) and maintained at 37°C. Hearts were perfused in the Langendorff mode at a constant pressure of 60 mmHg 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% O_2/5% CO_2 (pH 7.4).

A water-filled latex balloon connected to a pressure transducer was inserted through an incision in the left atrium into the left ventricle through the mitral valve. The volume was adjusted to achieve an end diastolic pressure of 10 mmHg. Coronary flow, heart rate, and left ventricular pressure were monitored using an EMKA recording system with IOX2 software (EMKA Technologies, Paris, France). Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace. The rate pressure product (RPP) was calculated as the product of heart rate and LVDP. Stock solutions (140x) of various reagents were infused into the heart via a side-port proximal to the aortic cannula at the rate 140 times lower than coronary flow (CF), usually 0.1 mL min^{-1} with a Gilson mini pump Minipuls 3 (Gilson, Middleton, WI, USA).

3.4.2. Ischemia/reperfusion protocol

Control hearts (aerobic control, n=12) were perfused aerobically for 75 minutes. Ischemic hearts (I/R, n=9), after 25 min at aerobic perfusion, were subjected to 20 minutes global no- flow

ischemia by closing the aortic inflow line, followed by 30 minutes of aerobic reperfusion. In three separate groups of I/R hearts (n=6 each) either ML-7 (1-5 μM [Sigma, St Louis, MO, USA]), myosin light chain kinase (MLCK) inhibitor, Y-27632 (0.05-1 μM [Sigma, St Louis, MO, USA]), an activator of myosin light chain phosphates (MLCP), or doxycycline (Doxy, 1-30 μM [Sigma, Taufkirchen, Germany]), an inhibitor of MMP-2, were infused 10 min before of onset of ischemia and for the first 10 min of reperfusion. To study possible synergistic/additive effects of these drugs, different combinations of subthreshold concentrations of ML-7 (1 μM), Y-27632 (0.05 μM), and Doxy (1 μM) were infused to the I/R hearts. Water was used as vehicle for Y-27632 and Doxy while ethanol (50% (v/v) was the vehicle for ML-7. The maximal concentration of ethanol infused during the heart was less than or equal to 0.025% (v/v). The scheme of the experimental protocol is shown in Figure 1. At the end of perfusion the hearts were freeze clamped in liquid nitrogen and used for biochemical studies.

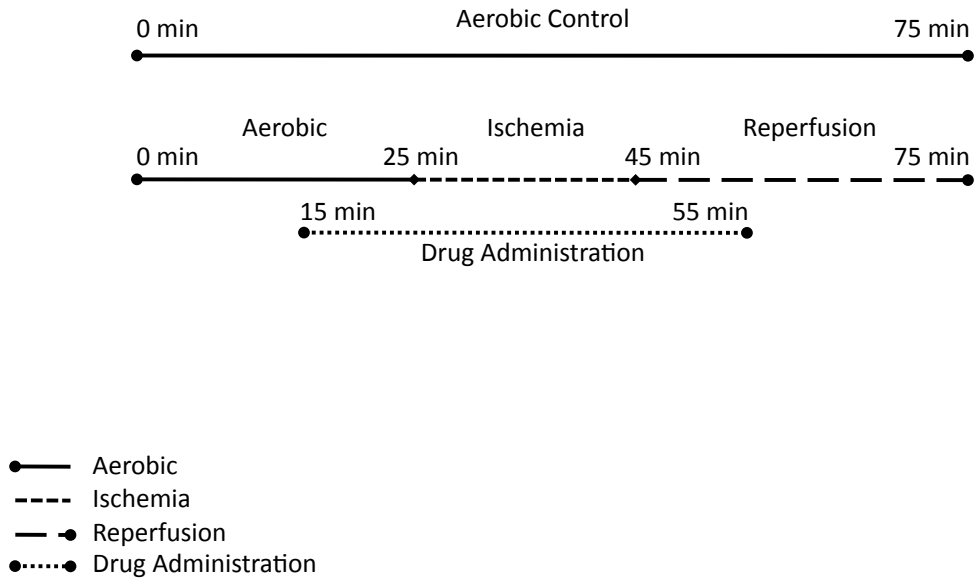


Figure 3.1 – Schematic representation of the perfusion protocols used. Control hearts (Aerobic control) were perfused aerobically for 75 min. In ischemia/reperfusion protocols, after 25 min of aerobic perfusion (Aerobic) hearts were subjected to 20 min of global no-flow ischemia (Ischemia) followed by 30 min of reperfusion (Reperfusion). Infusion of the drugs started 10 min

before the onset of ischemia and was stopped after the first 10 min of reperfusion (Drug Administration).

3.4.3. Preparation of heart protein extracts

Frozen heart tissue powder was homogenized on ice in 150mM 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.

3.4.4. Immunoblot analysis

MLC1 content in heart extracts was determined by immunoblotting. Twenty micrograms of protein from each heart extract were analyzed by SDS-PAGE using 15% gels. After electrophoresis protein was transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). MLC1was identified using mouse monoclonal anti-MLC1 (Abcam, Cambridge, MA, USA).

Co-localization of myocardial MLC1 with myocardial MLCK or MLCP was analyzed by separating protein complexes in non-reducing and non-denaturing conditions on native mini-PROTEAN TGX pre-cast gels (BioRad, Hercules, CA, USA), followed by dual-immunoblot detection. MLC1 was detected with mouse monoclonal anti-MLC1 antibody (Abcam, Cambridge, MA, USA) using goat anti-mouse IgG tagged with Alexa fluor 488 (Invitrogen, Eugene, OR, USA) as secondary antibody. MLCK and MLCP were detected with rabbit monoclonal antibody (Abcam, Cambridge, MA, USA) and rabbit polyclonal antibody (Affinity BioReagents, Golden, CO, USA) respectively, with goat anti rabbit IgG tagged with Alexa fluor 647 (Invitrogen, Eugene, OR, USA) as a secondary antibody.

Band densities were measured with VersaDoc 5000 and Quantity One software (BioRad, Hercules, CA, USA).

3.4.5. Statistical Analysis

ANOVA or Kruskal-Wallis tests were used in functional studies (followed by Tukey's post-hoc test) and an unpaired t-test was used in immunoblot analysis. Data are expressed as mean±SEM. A $p < 0.05$ was considered statistically significant.

3.5. Results

3.5.1. Co-localization of MLC1 with MLCK and MLCP in I/R hearts

Co-localization of MLC1 with myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) was evaluated by separating protein complexes from heart homogenates under non–reducing and non-denaturing conditions, using native gels (see methods), followed by dual-immunoblotting (Fig. 3.2). Under these conditions two distinct bands for MLC1 (red color) were visible (middle panel), while for MLCK or MLCP only one band (green color) was seen (top panel). Overlap of both blots revealed co-localization (yellow color) of either MLCK or MLCP with MLC1 (bottom panel).

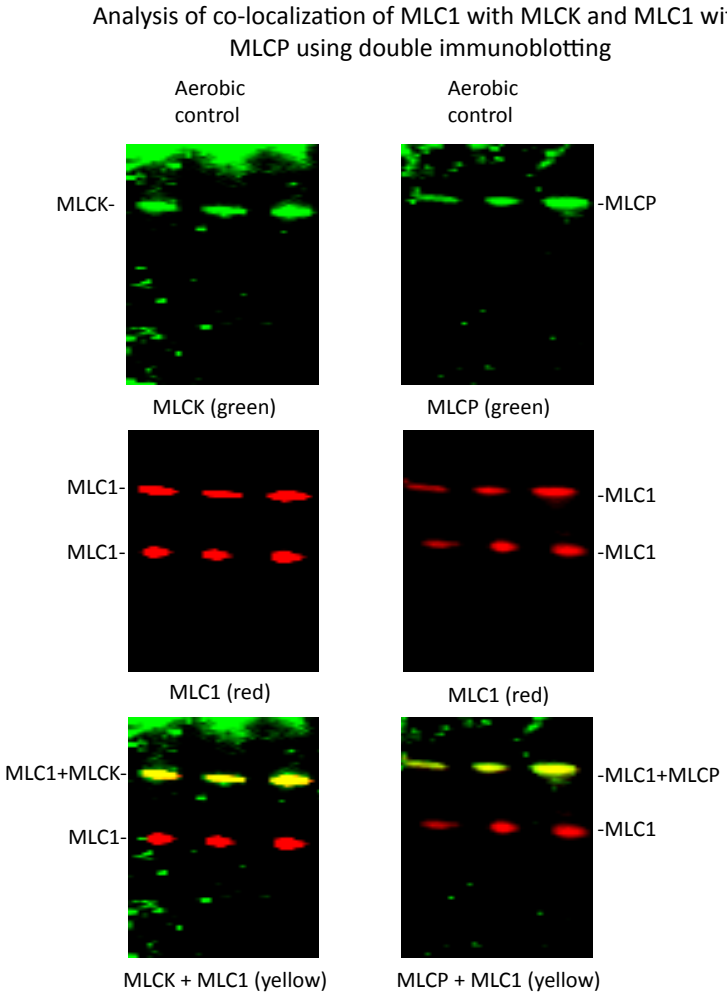


Figure 3.2 – Co-localization of MLC1 with MLCK and MLCP in rat hearts. Co-localization of MLC1 with MLCK or MLCP was determined by using double immunoblotting under non-reducing and non-denaturing complexes to preserve protein-protein interactions. Co-localization is determined by overlap of the dual immunoblots and represented by a change in color (yellow) when both proteins are present together. MLCK – myosin light chain kinase; MLCP – myosin light chain phosphatase;

3.5.2. Inhibition of phosphorylation of myocardial MLC1 during I/R

The mechanical heart function was significantly decreased in hearts subjected to ischemia followed by reperfusion, in comparison to hearts perfused aerobically for 75 minutes (Fig. 3.3A and B).

Pharmacological inhibition of MLCK by ML-7 resulted in increased contractile recovery during reperfusion in a concentration-dependent manner (Fig. 3.3A). Five micromolar of ML-7 resulted in an almost full recovery of mechanical function of I/R heart whereas 1 μM is a subtreshold concentration and did not improve of the heart contractility (Fig. 3.3A).

In order to assess whether the activation of MLCP (leading to the reduction of MLC1 phosphorylation) could have a protective effect on the mechanical function in I/R hearts, we used Y-27632, an activator of MLCP through inhibition of the Rho-kinase pathway. Y-27632 protected cardiac contractility in a concentration-dependent manner (Fig. 3.3B). One micromolar of Y-27632 resulted in the full recovery of cardiac mechanical function, whereas 0.05 μM represents the subtreshold concentration and did not improve mechanical function of I/R hearts (Fig. 3.3B).

3.5.3. Synergistic effect of MLCK inhibitor and MLCP activator on mechanical function of the heart after I/R

Potential synergistic/additive effects of ML-7 and Y-27632 were determined by using subthreshold concentrations of both drugs and evaluated in terms of their protection of

mechanical function and preservation of MLC1 levels in hearts subjected to I/R (Fig. 3.4). Co-administration of Y-27632 (0.05 μM) and ML-7 (1 μM) at subthreshold doses, that have no protective effect when administered alone, resulted in approximately 60% recovery of cardiac function during reperfusion (Fig. 3.4A). Furthermore, co-administration of these two drugs at subthreshold concentrations protected MLC1 from degradation in I/R hearts (Fig. 3.4B).

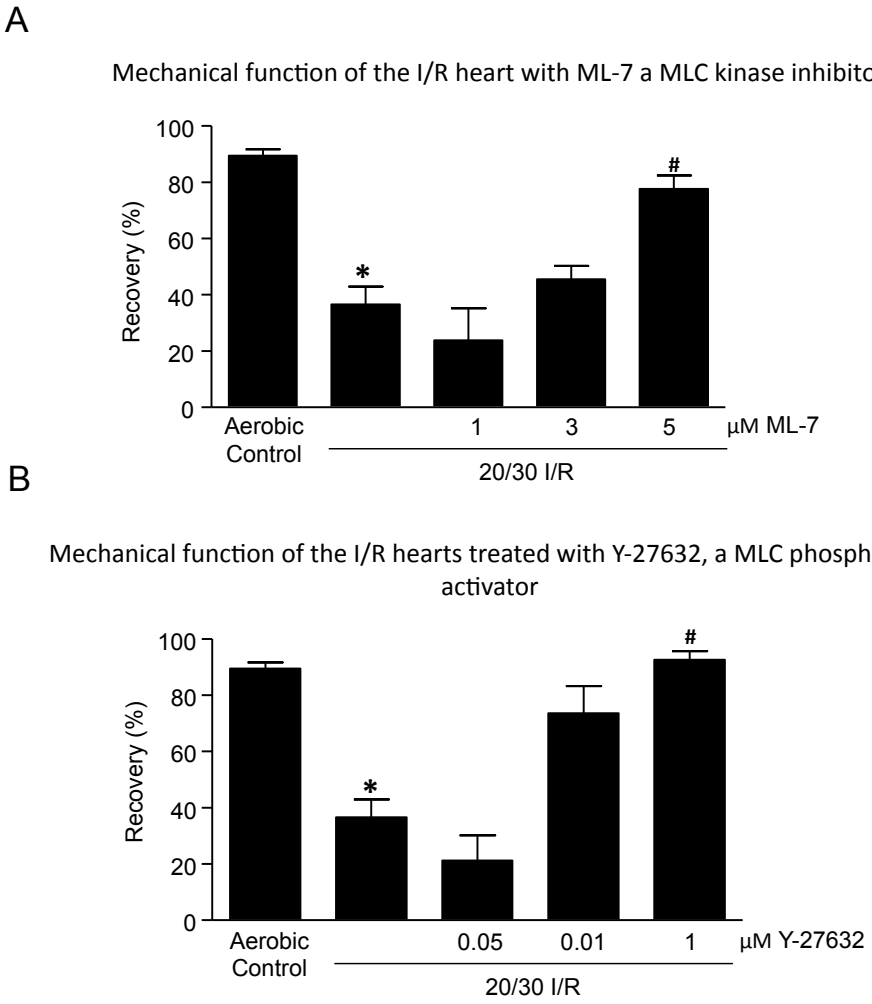


Figure 3.3 – Cardioprotective effect of inhibition of MLC1 phosphorylation with ML-7 an inhibitor of MLCK (A) and with Y-27632 non-direct activator of MLCP (B). Isolated rat hearts were subjected to 20 min of ischemia followed by 30 min of reperfusion in the presence or absence of ML-7 or Y-27632. MLCK – myosin light chain kinase; MLCP – myosin light chain phosphatase; I/R – ischemia/reperfusion. Percent recovery was calculated based on RPP during

the first 25 min of the perfusion protocol. * p<0.05 vs. Aerobic control; #p<0.05 vs. I/R; n=6-9/group.

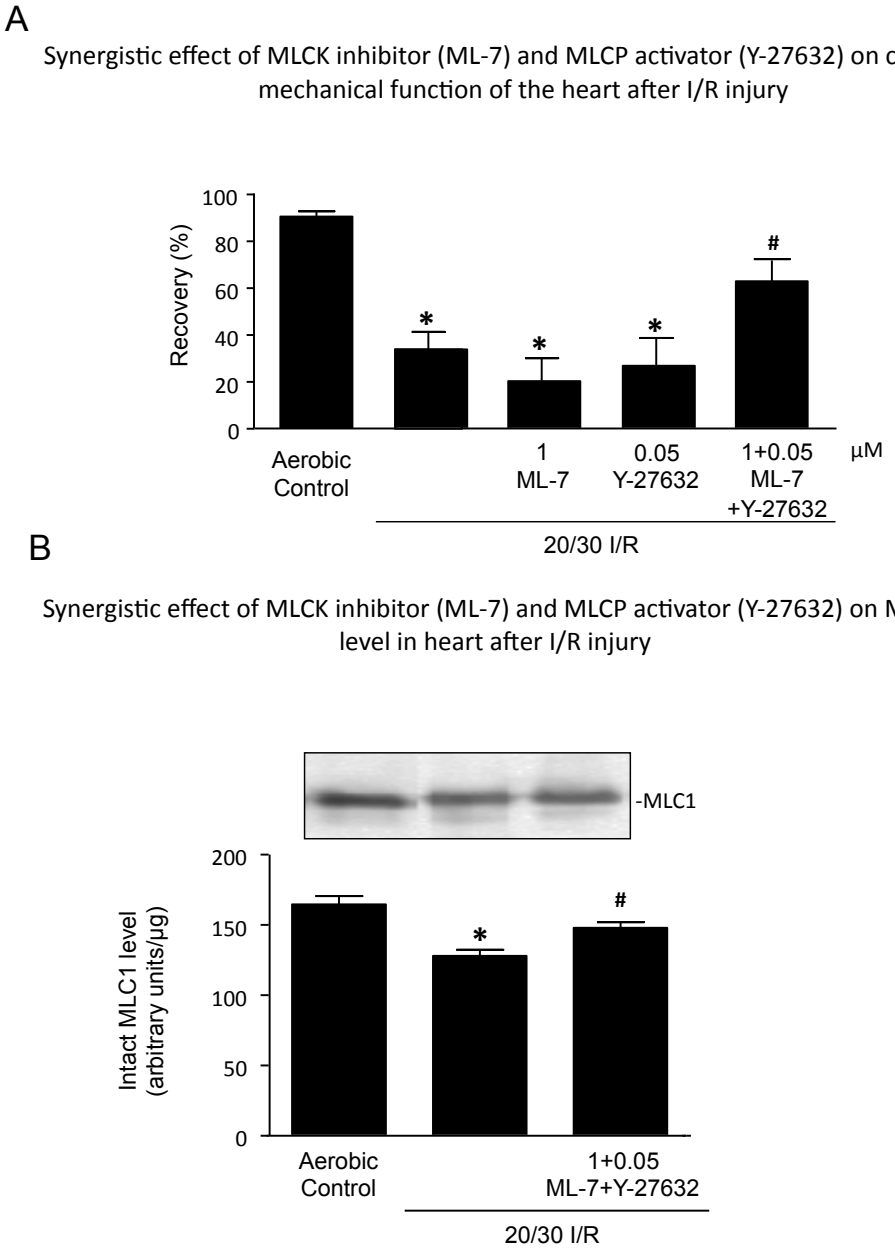


Figure 3.4 – Synergistic effect of subthreshold concentrations of inhibitors of MLC1 phosphorylation on contractile function recovery (A) and MLC1 protein levels (B). MLC1 – Myosin light chain; MLCK – myosin light chain kinase; MLCP – myosin light chain phosphatase; I/R – ischemia/reperfusion. Percent recovery was calculated based on RPP during

the first 25 min of the perfusion protocol. * $p<0.05$ vs. Aerobic control; #$p<0.05$ vs. I/R; n=6-9/group in functional study, n=3/group in immunoblot analysis.

3.5.4. Synergistic effect of multi drug administration (Doxycycline, ML-7 and Y-27632) on cardiac contractile function after I/R

Because doxycycline (Doxy) is known for its inhibition of MMPs (Cheung, Sawicki et al. 2000; Fert-Bober, Leon et al. 2008) improving cardiac function recovery following I/R in a concentration-dependent manner (Fig. 3.5), we used this drug in our studies as an inhibitor of MMP-2. One micromole of Doxy did not protect cardiac contractility, being a subthreshold concentration, whereas 30 μM show more than 70% protection.

Administration of a mixture containing subthreshold doses of Doxy (1 μM) and ML-7 (1 μM) showed a protective effect on cardiac contractility of approximately 60% (Fig. 3.6A). Similarly, administration of a mixture of subthreshold concentrations of Doxy (1 μM) and Y-26732 (0.05 μM) showed protection of cardiac contractility of also ~50% (Fig. 3.6B).

Administration of a combination of subthreshold concentrations of Doxy, ML-7 and Y-27632 to the hearts, before the onset of ischemia, resulted in a recovery of cardiac function during reperfusion of over 85% (Fig. 3.6C). The addition of Doxy, an inhibitor of MMP-2, to the mixture of ML-7 and Y-27632 showed further improvement of cardiac contractility in comparison to the combination of two drugs inhibiting MLC1 phosphorylation (Fig. 3.6C). The protection of contractile function by combining subthreshold concentrations of these three drugs was associated with a preservation of MLC1 protein levels (Fig. 3.6D).

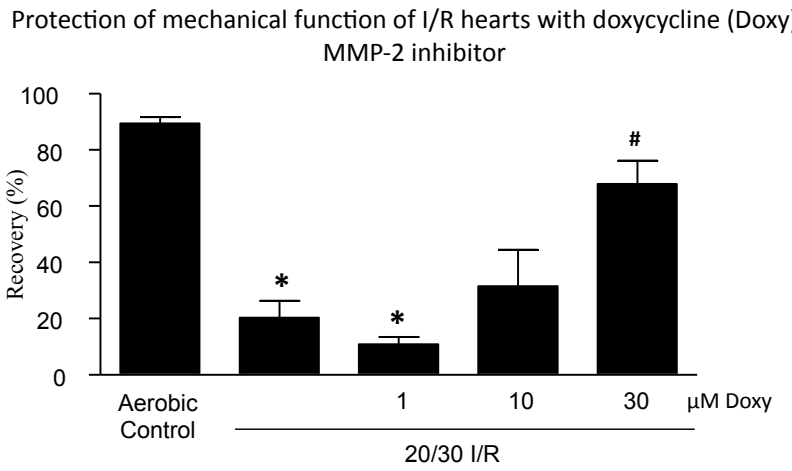
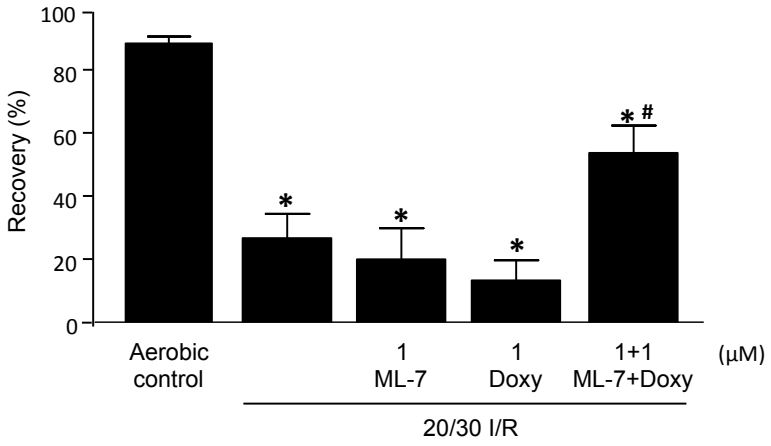


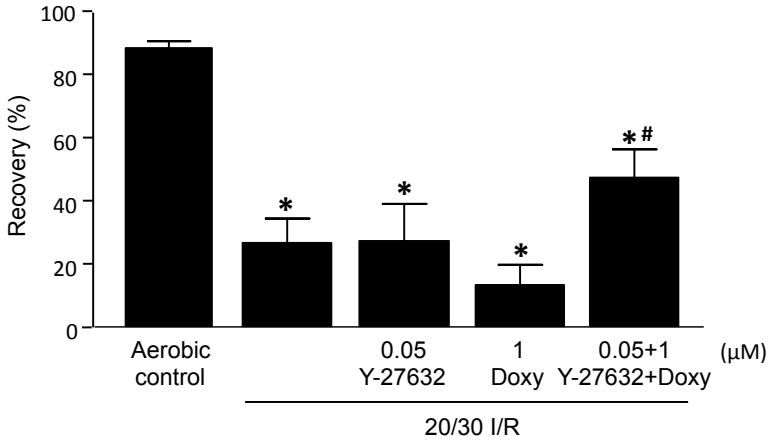
Figure 3.5 – Concentration-response histogram of doxycycline (Doxy) on contractile function recovery. I/R – ischemia/reperfusion. Percent recovery was calculated based on RPP during the first 25 min of the perfusion protocol. * $p<0.05$ vs. Aerobic control; #$p<0.05$ vs. I/R; n=6-9/group.

Protection of mechanical function of I/R hearts with mixture of subthreshold concentrations of ML-7, Y-27632 and doxycycline (Doxy)

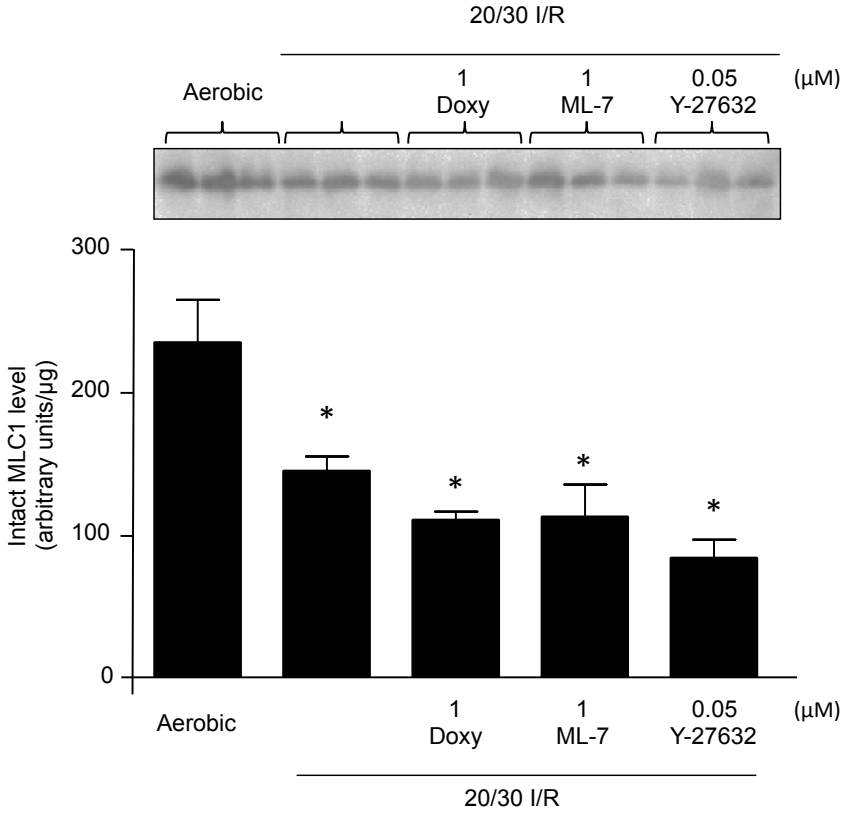
A



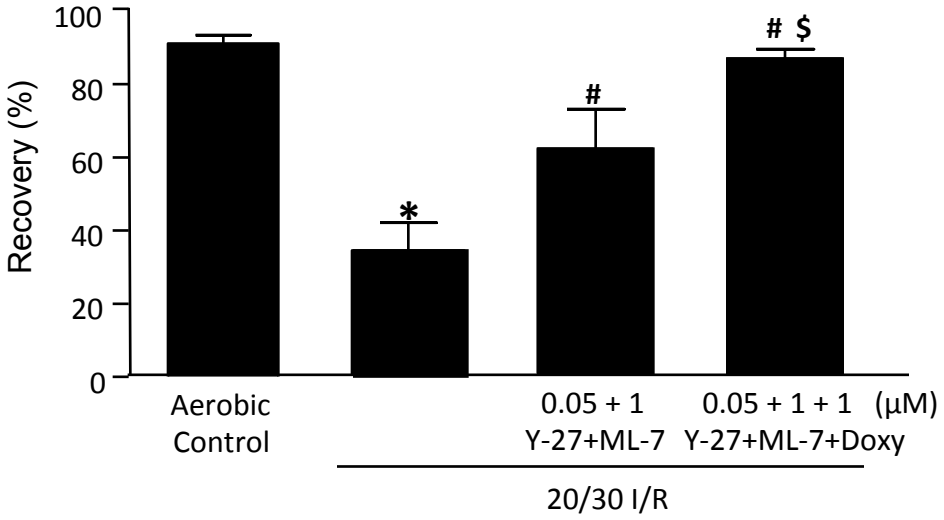
B



C


20/30 I/R
Aerobic
1
Doxy
1
ML-7
0.05
Y-27632
(μM)
300
200
100
0
Intact MLC1 level
(arbitrary units/μg)
*
*
*
*
Aerobic
1
Doxy
1
ML-7
0.05
Y-27632
(μM)
20/30 I/R

D



E

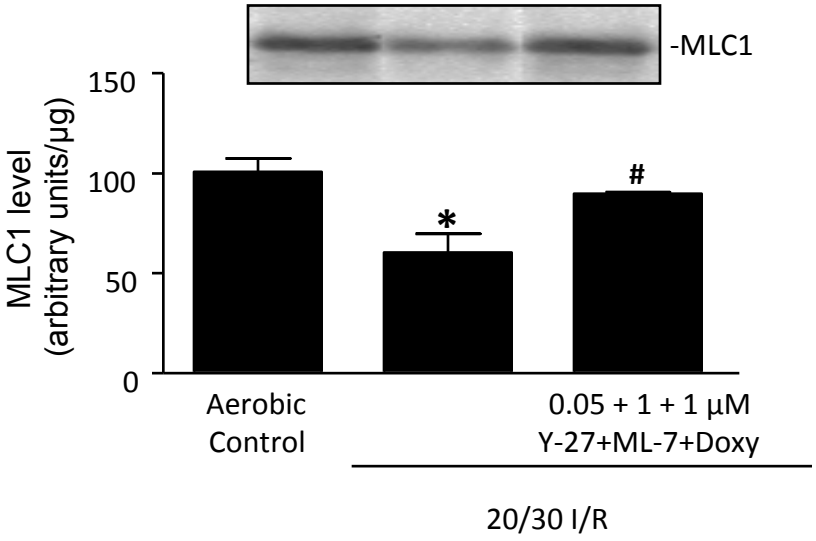


Figure 3.6 – Synergistic effect of multidrug administration on recovery of the mechanical function of I/R hearts and MLC1 protein levels. (A) Synergystic effect between inhibition of MLCK and MMP-2 with subthreshold concentrations of ML-7 and Doxy on the protection of contractile function recovery. (B) Synergystic effect between stimulation of MLCP and

inhibition of MMP-2 with subthreshold concentrations of Y-27632 and Doxy on the protection of contractile function recovery. (C) MLC1 protein levels measured by immunoblotting in hearts subjected to I/R in the presence or absence of subthreshold concentrations of ML-7, Y-27632 and Doxy. (D) Comparison of synergystic effects of inhibition of MLC1 phosphorylation (with subthreshold concentrations of ML-7 and Y-27632) and inhibition of MLC1 phosphorylation together with MMP-2 activity (with additional subthreshold concentration Doxy) on the protection of contractile function recovery. (E) Synergystic effect between inhibition of MLC1 phosphorylation (with ML-7 and Y-27632) and MMP-2 activity with subthreshold concentrations of ML-7, Y-27632 and Doxy on the protection of MLC1 protein levels (n=3). MLC1 – myosin light chain 1; MLCK – myosin light chain kinase; MLCP – myosin light chain phosphatase; I/R – ischemia/reperfusion. Percent recovery was calculated based on RPP during the first 25 min of the perfusion protocol. * $p<0.05$ vs. Aerobic control; #$p<0.05$ vs. I/R; n=6-9/group.

3.6. Discussion

The development of new therapeutic strategies aiming at preventing and treating ischemia/reperfusion (I/R) injury are needed. Here we establish a new, valid therapeutic strategy for the prevention of the development of I/R injury. The use of subthreshold concentrations of drugs that prevent MLC1 phosphorylation and degradation showed a cardioprotective effect due to a synergistic action between the three compounds used. Since these drugs are used at subthreshold concentrations toxicity and drug-drug interactions are less likely. Hence, this approach can prove to be a valid strategy for prevention and protection of the heart against I/R injury.

It has been previously shown that MLC1 (myosin light chain 1) is phosphorylated in response to oxidative stress (Arrell, Neverova et al. 2001; Arrell, Elliott et al. 2006). We have recently shown that, in a model of I/R, MLC1 phosphorylation is MLCK dependent and results in increased degradation of MLC1 by MMP-2. The loss of MLC1 has been related with a deterioration of cardiac contractile function associated with I/R injury. Since phosphorylation of MLC1 is involved in the pathology of I/R injury, inhibition of MLC1 phosphorylation is a viable target for protection of the heart against I/R injury. Inhibition of phosphorylation can be achieved by inhibition of the kinase or stimulation of the phosphatase activity. MLCK has been shown to phosphorylate MLC1 both *in vitro* and *ex vivo*, though its primary target is MLC2. Myosin light chain phosphatase (MLCP) is the enzyme responsible for counteracting the actions of MLCK in terms of MLC2 phosphorylation status. Here we show that in heart protein extracts both MLCK and MLCP co-localize with MLC1. Moreover, selective inhibition of MLCK with ML-7 or indirect stimulation of MLCP with the Rho-kinase inhibitor Y-27632 (at full protective doses) can result in almost full recovery of cardiac contractile function. In our concentration-response studies we identified the subthreshold concentration for ML-7 being 1 μM and for Y-27632 being 0.05 μM. At these doses no improvement of protection of cardiac contractile function recovery is observed. However, when both drugs are administered to hearts undergoing an I/R protocol a significant improvement of cardiac contractile function recovery is observed. Since these concentrations, when given alone, show no protective effect on cardiac contractile function recovery, we can conclude that the mechanism by which ML-7 and Y-27632 confers cardioprotection at subthreshold doses is by a synergistic interaction, rather than an additive

effect, since single doses show zero effect. This protection of contractile function was associated with a preservation of MLC1 levels.

MLC1 has been previously shown to be a substrate for the matrix metalloproteinase-2 (MMP-2) and that posttranslational modification of MLC1 leads to increased affinity for, and degradation by, MMP-2 in I/R (Sawicki, Leon et al. 2005; Doroszko, Polewicz et al. 2009; Polewicz, Cadete et al. 2011). Since inhibition of MLC1 phosphorylation with subthreshold concentrations of ML-7 and Y-27632 showed an improvement in contractile function during reperfusion but did not confer full protection, we decided to inhibit MLC1 degradation at the level of MMP-2. Full protective concentration (30 μM) of doxycycline (Doxy, a know inhibitor of MMPs) resulted in a recovery of ~65% following I/R. We identified the subthreshold concentration for Doxy to be 1 μM, the concentration at which no protection of cardiac contractile function recovery is observed. When given together with either ML-7 or Y-27632 (inhibitors of MLC1 phosphorylation) at subthreshold doses, doxy acted synergistically with either ML-7 or Y-27632 to confer a protection of cardiac function of ~50%. Since full protection was not observed we administered subthresold concentrations of ML-7, Y-27632 and doxy to hearts undergoing I/R. When the three drugs were present at subthreshold concentrations a full recovery of cardiac contractile function was observed. These three drugs acted synergistically to protect cardiac contractile function from I/R injury and this protection was mediated by a preservation of cardiac MLC1 levels. The mechanism of action involved is synergy, rather than a net effect resulting from the addition of the effects of individual drug administration (additive effect).

Our findings presented here support the notion that specific pathways involved in the pathology of I/R injury also have important physiological roles. Hence, the inhibition of the pathological fraction of the enzymatic activity is preferrable over full pathway inhibition. Our data support this concept in which it shows that the use of inhibitors of MLCK, MMP-2 and activators of MLCP at subthreshold concentrations can fully protect cardiac contractile function against I/R injury.

Synergy between drugs aiming at protecting the heart from I/R has been shown previously (Huang, Zhong et al. 2011) between isofluorane preconditioning and propofol postconditioning. Also, potential synergy between lipid-lowering and blood-pressure-lowering regimens has been suggested (Sever, Dahlof et al. 2006). To the best of our knowledge this is the first report

showing that targeting molecular mechanisms involved in the development of I/R injury and contractile dysfunction, with subthreshold concentrations of drugs, is a viable therapeutic approach to prevent I/R injury. Moreover, we show that preventing the phosphorylation (with ML-7 and Y-27632) and degradation of MLC1 (with Doxy) during I/R can be achieved by the use of a combination of subthreshold concentrations and results in full protection of cardiac contractile function and MLC1 protein levels. Our results suggest that the use of subthreshold concentrations of inhibitors of enzymes with both a physiological and a pathological roles acts by eliminating the pathological component of the enzyme activity without interfering with its normal physiological actions.

In summary, this study confirms the involvement of MLC1 phosphorylation and consequent degradation in the development of I/R injury. Moreover we establish a new viable therapeutic approach aiming at preventing the development of cardiac I/R injury.

3.7. Funding sources

This project was funded by grants from Canadian Institutes of Health Research (CIHR) and the Saskatchewan Health Research Foundation (SHRF). GS is an investigator supported by the Heart and Stroke Foundation of Canada and by CIHR/SHRF.

3.8. Disclosures

No conflict of interests is declared.

4. Effect of the Rho kinase inhibitor Y-27632 on the proteome of hearts with ischemia-reperfusion injury

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Preface

This manuscript has been publishe in the journal *Proteomics* (2010 Dec;10(24):4377-85).

From the results gathered addressing the objectives proposed for my PhD project we observed the highest protective effects against I/R injury with the use of the Rho kinase inhibitor Y-27632 (indirect MLCP activator). We hypothesized that these significant protective effects of Y-27632 were independent of MLC1 protection against degradation.

We show here that Y-27632 potentially protects the heart against I/R injury by modulating energy producing enzymes, such as lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase, improving energy production and contractile function, secondary to protection of MLC1 from I/R-induced degradation.

These results are relevant since it demonstrates that Rho kinase inhibitors have the potential to be metabolic modulators with a promising application in heart injury treatment.

4.1. Abstract

Growing attention has been given to the role of the Rho kinase pathway in the development of heart disease and ischemia/reperfusion (I/R) injury. Y-27632 is a Rho kinase inhibitor demonstrated to protect against I/R injury, but the exact mechanism by which it does so remains to be elucidated. The goal of this project was to determine new targets by which Y-27632 can protect the heart against I/R injury. Isolated rat hearts were perfused under aerobic conditions or subjected to I/R in the presence or absence of Y-27632. Administration of Y-27632 (1 μM) before ischemia and during the first 10 minutes of reperfusion resulted in complete recovery of cardiac function. Two-dimensional electrophoresis followed by mass spectrometry identified 4 proteins whose levels were affected by Y-27632 treatment. Lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were significantly increased in the Y-27632 treated group, while creatine kinase was normalized to control levels. In addition, we found increased level of two different molecular fragments of ATP synthase, which were normalized by Y-27632. This increase suggests that during ischemia ATP synthase is subjected to degradation. The changes in metabolic enzymes' levels and their regulation by Y-27632 suggest that the cardioprotective effect of Y-27632 involves increased energy production.

4.2. Introduction

Myocardial infarction (MI) and congestive heart failure are still among the most common reasons of mortality and morbidity (Alpert, Thygesen et al. 2008; Roger 2009). Over the last two decades, coronary reperfusion therapy has become a well established therapeutic strategy for the management of MI (Van de Werf, Bax et al. 2008). However, restoration of blood flow to previously ischemic myocardium has pathological consequences resulting from ischemia/reperfusion (I/R) injury.

The cellular mechanisms involved in the pathogenesis of myocardial I/R injury are complex and involve the interactions of various cells. Recently, growing attention has been given to the role of the Rho/Rho kinase (Rho/ROCK, or simply ROCK) pathway in the development of cardiovascular disease and irreversible injury (Shimokawa and Takeshita 2005; Zhou and Liao 2009). Rho is a family of small guanosine triphosphate (GTP) binding proteins that can initiate a cascade of kinase-mediated signaling, following activation (Amano, Chihara et al. 1997; Majumdar, Seasholtz et al. 1998; Yasui, Amano et al. 1998). The ROCK pathway has been described to be involved in the pathology of a number of cardiovascular disorders (Shimokawa and Takeshita 2005). Moreover, ROCK activation results in the inhibition of protein kinase B/Akt (Wolfrum, Dendorfer et al. 2004) (a pro-survival pathway) and can lead to cell death in heart tissue subjected to ischemia/reperfusion (I/R) (Wolfrum, Dendorfer et al. 2004).

The beneficial effects of ROCK inhibitors in I/R have been associated with its effects on the contractile machinery of the heart, namely phosphorylation-dephosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), respectively Although this mechanism of regulation of contractile function is well described and accepted, the ROCK pathway regulates at least several other pathways involved in cell survival and metabolism.

Y-27632 is a ROCK inhibitor that has been shown to protect the heart against irreversible reperfusion injury (Hamid, Bower et al. 2007), alongside with beneficial effects in hypertension (Uehata, Ishizaki et al. 1997) and heart failure (Kobayashi, Horinaka et al. 2002). Y-27632 inhibits ROCK by more than 85% but has also been reported to inhibit the activity of other kinases to similar and lesser extents, such as protein kinase C related protein kinase (PRK), mitogen activated protein kinase (MAPK) associated protein kinase 1b (MAPKAP-K1b),

mitogen- and stress-activated protein kinase 1 (MSK1) and phosphorylase kinase (PHK) (Davies, Reddy et al. 2000).

I/R injury causes an inflammatory response that triggers stress-dependent signaling processes and can result in apoptosis and cell death. There are a number of chemical mediators and pathways involved in I/R response. Up to date, the treatment of MI is mainly focused on urgent reperfusion of previously ischemic myocardium (Antman, Anbe et al. 2004). However, present therapeutic schemes do not include treatment aiming at the reduction of damage of the ischemic heart during reperfusion. Thus, from a therapeutic point of view, it would be important to focus on the active mediators of I/R injury and to manipulate these in order to improve cell function and survival.

Taking into account the limited information about the effectors of the Rho/ROCK pathway and the wide spectra of action for ROCK inhibitors action in cardiovascular diseases we hypothesized that the beneficial effects of Y-27632 are well beyond modulation of contraction via phosphorylation or dephosphorylation of MLC 2. The objective of this study is to identify possible molecular targets involved in the mechanism of cardioprotection by Y-27632.

4.3. Materials and Methods

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

4.3.1. Heart perfusion and I/R protocol

Male Sprague-Dawley rats (weighing 250-300 g) were anesthetized with pentobarbital (160 mg/kg). Hearts were excised and perfused *via* the aorta using the Langendorff method at constant pressure (60 mmHg) with Krebs-Henseleit buffer at 37°C as previously described (Cheung, Sawicki et al. 2000). Hemodynamic function was monitored throughout the entire protocol. Left ventricular developed pressure was calculated as the difference between systolic and diastolic pressures of 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. Y-27632, when present, was infused for 10 min before the onset of ischemia and during the first 10 min of reperfusion at concentrations of 0.05, 0.1 or 1 μM (n=6/group). The hearts were subsequently frozen for biochemical studies.

4.3.2. Rho Kinase (ROCK) inhibitor

Y-27632 (Sigma Aldrich, St Louis, MO, USA), R-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, is a highly potent cell permeable, selective Rho-associated protein kinase inhibitor (non selective for both isoforms, ROCK I and ROCK II).

4.3.3. Preparation of heart extracts for 2-dimensional electrophoresis

Protein samples for 2-dimensional electrophoresis (2-DE) 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, Hercules, CA, USA]) at room temperature. Samples were sonicated twice for 5 s and centrifuged for 10 min at 10 000*g* at 4ºC to remove any insoluble particles. Protein content of the heart extract in rehydration buffer was measured with the BioRad (Hercules, CA, USA) protein assay. For other biochemical studies, frozen heart tissue powder was homogenized on ice in 150mM 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°C until further use.

4.3.4. Two-Dimensional PAGE

Heart extract protein (400 µg) was applied to 11-cm immobilized pH gradient (5-8) strips (IPG, BioRad, Hercules, CA, USA) and equilibrated for 16 to 18 hours at 20°C in rehydration buffer. For isoelectrofocusing, the BioRad Protean isoelectrofocusing cell was used as previously described (Sawicki, Leon et al. 2005; Fert-Bober, Basran et al. 2008). Next, 2-DE was carried out using Criterion precast gradient gels with 8% to 16% acrylamide (BioRad, Hercules, CA, USA). To minimize variations in resolving proteins during the 2-DE run, 12 gels were run simultaneously using a Criterion Dodeca Cell (BioRad, Hercules, CA, USA). The reproducibility of 2-DE and quality of protein loading was previously verified by us (Sawicki, Dakour et al. 2003; Sawicki and Jugdutt 2004; Sawicki, Leon et al. 2005). After separation, proteins were detected with Coomassie Brilliant Blue R-250 (BioRad, Hercules, CA, USA). All the gels were stained in the same bath. Developed gels were scanned with a calibrated GS-800 densitometer (BioRad, Hercules, CA, USA). Quantitative analysis of spot intensity from 2-DE was measured with PDQuest 7.1 software (BioRad, Hercules, CA, USA), and intensities of the separate bands from SDS-PAGE were analyzed and expressed in arbitrary units with Quantity One 4.4 measurement software (BioRad, Hercules, CA, USA). 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 4 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. Equal protein loading was additionally verified by measurement of actin level in 2-D gels (figure 3C).

4.3.5. Mass Spectrometry (MS)

Protein spots whose levels were changed by I/R and further affected by Y-27632 (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 µl of 50% acetonitrile with 50 mM ammonium bicarbonate at 37°C for 30 min. Next, the gel was washed twice with water. The protein extraction was performed overnight at room temperature with 50 µL of a mixture of formic acid:water:isopropanol (1:3:2, v:v:v). The resulting solution was then subjected to trypsin digestion and mass spectrometry analysis. For electrospray, quadruple time-of-flight analysis, 1 µl of the solution was used. LC/MS/MS was performed on a CapLC high-performance liquid chromatography unit (Waters, Milford, MA, USA) coupled with a quadruple time-of-flight-2 mass spectrometer (Micromass from Waters, Milford, MA, USA). 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 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 (Perkins, Pappin et al. 1999) was used to justify accuracy of protein identification which is incorporated in the Mascot search engine.

4.3.6. Immunoblot analysis

Protein (30 µg) from heart homogenate was separated using 12% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was identified using mouse monoclonal anti-GAPDH antibody

(abcam, Cambridge, MA, USA), L-lactate dehydrogenase (LDH) was identified using goat polyclonal anti-LDH antibody (abcam, Cambridge, MA, USA). Creatine kinase (CK) was identified with rabbit polyclonal anti-CK antibody (abcam, Cambridge, MA, USA). Band densities were measured using Versa Doc 5000 and Quantity One 4.6 software (Bio-Rad, Hercules, CA, USA). Equal protein loading was additionally verified by measurement of actin level with mouse monoclonal anti-actin antibody (Millipore, Billerica, MA, USA) (Figure 5D).

4.3.7. 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 mass spectrometry. ANOVA and Kruskal-Wallis test was used in functional studies. Data are expressed as the mean±SEM.

4.4. Results

4.4.1. Protection of cardiac mechanical function by Y-27632

Ischemia followed by reperfusion resulted in a significant decrease of heart mechanical function (measured as RPP) and of coronary flow (figure 4.1).

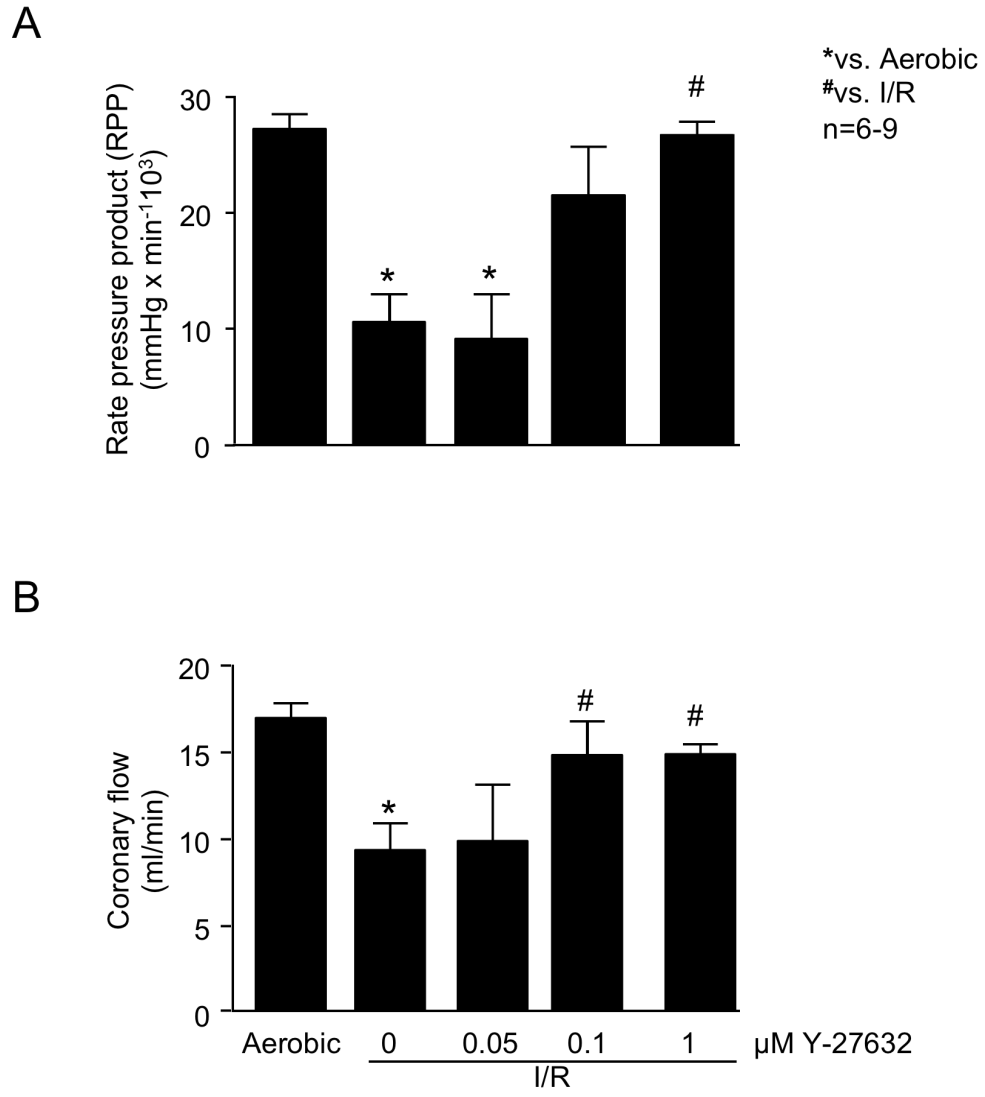


Figure 4.1 – Hemodynamic parameters of retrograde rat heart perfusions. A) Mechanical function presented as rate pressure product (RPP). B) Coronary flow throughout the perfusion protocol for each of the groups. * $p<0.05$ vs Aerobic, # $p<0.05$ vs I/R

The administration of 0.05 μM of Y-27632 did not prevent the decrease of neither cardiac contractile dysfunction nor coronary flow due to I/R. Treatment with 0.1 μM of Y-27632 prevented the deterioration of cardiac coronary flow (figure 4.1B) while 1 μM of Y-27632 significantly improved RPP and coronary flow, in comparison to I/R (figure 4.1 A and B).

4.4.2. Effects of Y-27632 treatment on the proteome of aerobically perfused hearts

Protein extracts from hearts perfused aerobically without (control) or with Y-27632 were separated by 2-DE. Analysis of the protein spot intensity variation (using an arbitrary threshold) identified two spots with significantly reduced intensity in the group treated with Y-27632, in comparison to control (figure 2). Protein spot SSP 4206 was identified by mass spectrometry as being peroxiredoxin 3 while SSP 4207 was determined to be a fragment of the alpha subunit of ATP synthase (Table 4.1).

Table 4.1 – Identification of protein spots from aerobic plus Y-27632 protocol

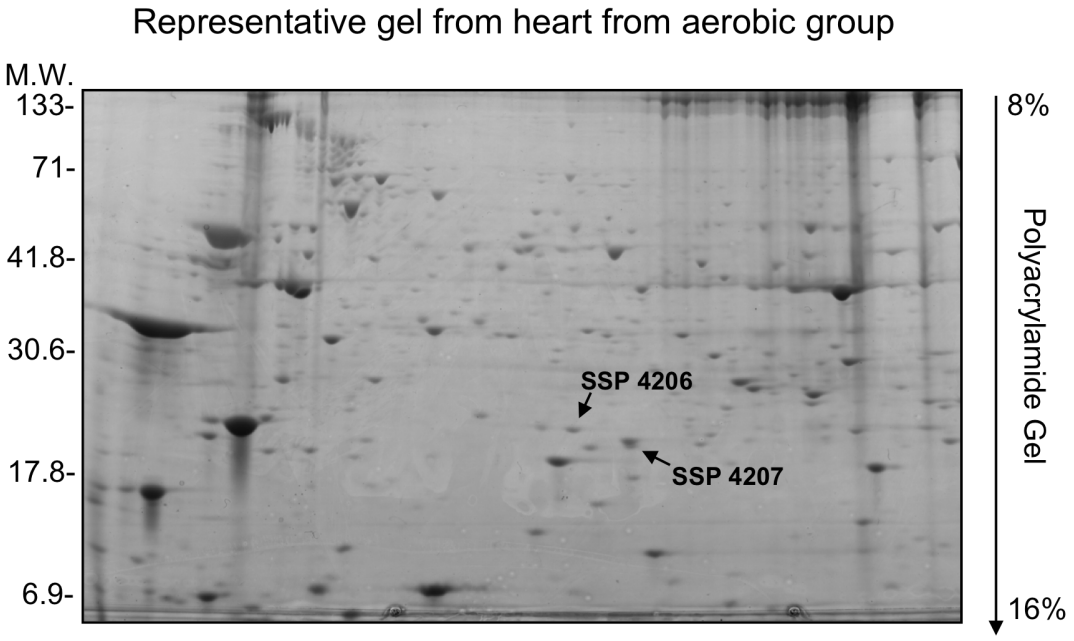
Protein Spot (SSP*)	Mowse Score**	Queries Matched	Sequence Coverage (%)	Molecular Weight (kDa)	Protein Identification
4206 ⇓***	306	18	38	28.5	Peroxiredoxin 3
4207 ⇓	909	21	24	58.9	ATP synthase alpha subunit, fragment

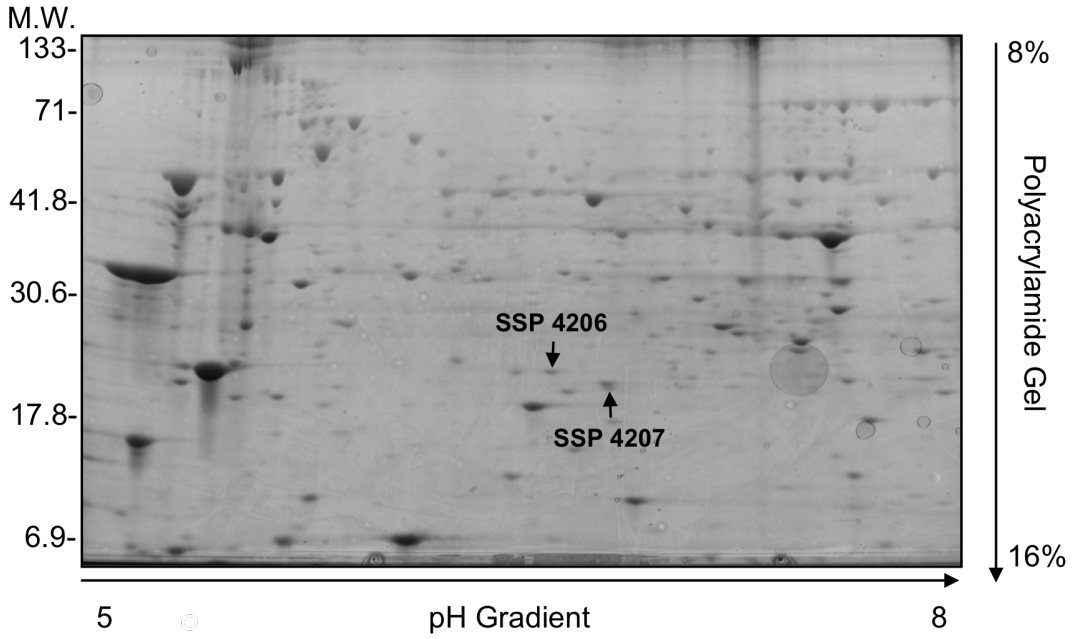
* SSP – Standard Spot Number

** -10log(P), where P is the probability that the observed match is a random event. Individual ions scores >40 indicate identity or extensive homology ($p<0.05$)

*** the arrow (⇓) indicates decreased protein level (vs. control)

A


Representative gel from heart from aerobic group
M.W.
133-
71-
41.8-
30.6-
17.8-
6.9-
SSP 4206
SSP 4207
8%
Polyacrylamide Gel
16%


Representative gel from heart from aerobic group with Y-27632
M.W.
133-
71-
41.8-
30.6-
17.8-
6.9-
SSP 4206
SSP 4207
8%
Polyacrylamide Gel
16%
5
pH Gradient
8

B

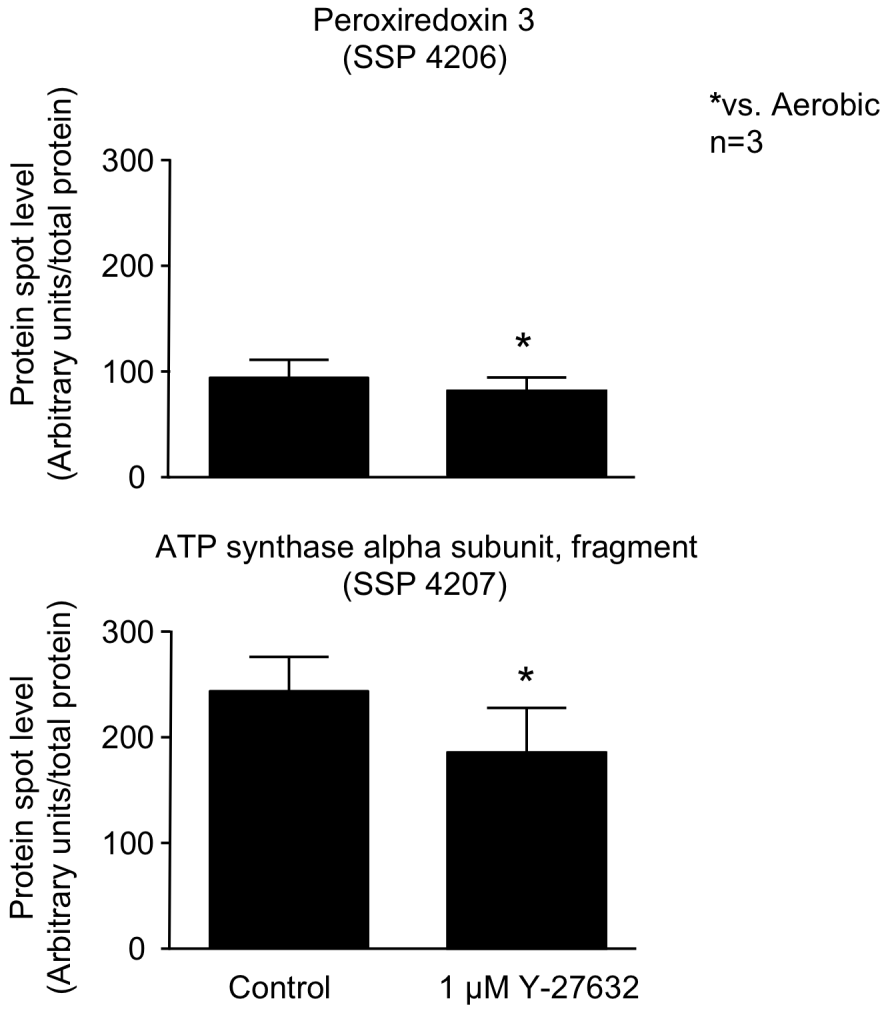


Figure 4.2 – Two-dimensional electrophoresis (2-DE) of heart homogenates. A) Representative gels of 2-DE of protein extracts from aerobically perfused hearts in the absence (top gel) or presence (bottom gel) of Y-27632. The arrows indicate the protein spots that, according to an arbitrary threshold, were significantly decreased in the Y-27632 treated group. B) Histogram representing the densitometric analysis of protein spots identified from 2-DE gels. * $p<0.05$ vs Control.

4.4.3. Effects of Y-27632 treatment on the proteome of hearts subjected to I/R

Heart protein extracts from aerobic control, I/R and I/R + 1 μM of Y-27632 were analyzed by 2-DE. Using an arbitrary threshold for variation, five protein spots were identified as being significantly different from non-treated I/R hearts (figure 4.3 A). Mass spectrometry was used to identify these protein spots (table 4.2). All the five protein spots corresponded to enzymes involved in metabolic pathways and energy production. The proteins involved in metabolic pathways, namely carbohydrate metabolism were L-lactate dehydrogenase (LDH) (SSP 4502) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (SSP 8501), both revealed a similar profile of change being significantly increased in I/R hearts treated with 1 μM of Y-27632, in comparison to control (figure 4.3 B). Two distinct protein fragments, with molecular mass approximately 20 KDa, of the alpha subunit of ATP synthase were identified (SSP 6301 and 6309), and both were normalized to control levels by Y-27632 administration. Also, the normalization of the protein levels by Y-27632 was observed for the fifth protein spot SSP 7402 which was identified as a fragment of the sarcomeric mitochondrial creatine kinase (CK) (figure 4.3B).

4.4.4. Identification of two different fragments of alpha subunits of ATP synthase

The analysis of SSP 6301 and 6309 protein spots with Mascot MS/MS ions search engine identified these protein spots as fragments of alpha subunit of ATP synthase. The matching of the identified peptides to whole sequence of alpha subunit of ATP synthase showed that these proteins have a different primary structure (figure 4.4). The SSP 6301 protein spot represents the N-terminal part of the alpha subunit whereas the protein spot SSP 6309 seems to be composed of approximately 100 amino acids from the N-terminal and approximately 150 amino acids from C-terminal parts of this subunit of ATP synthase.

4.4.5. Immunoblot analysis of identified protein

Immunoblot analysis for glyceraldehydes 3-phosphate dehydrogenase (GAPDH), L-lactate dehydrogenase (LDH) and creatine kinase (CK) showed that Y-27632 further increases

levels of GAPDH and LDH, and normalizes the level of CK (figure 5A, B and C). The obtained results by immunoblot analysis are similar to these obtained from 2-DE (figure 4.3B).

Table 4.2 – Identification of protein spots from I/R protocol

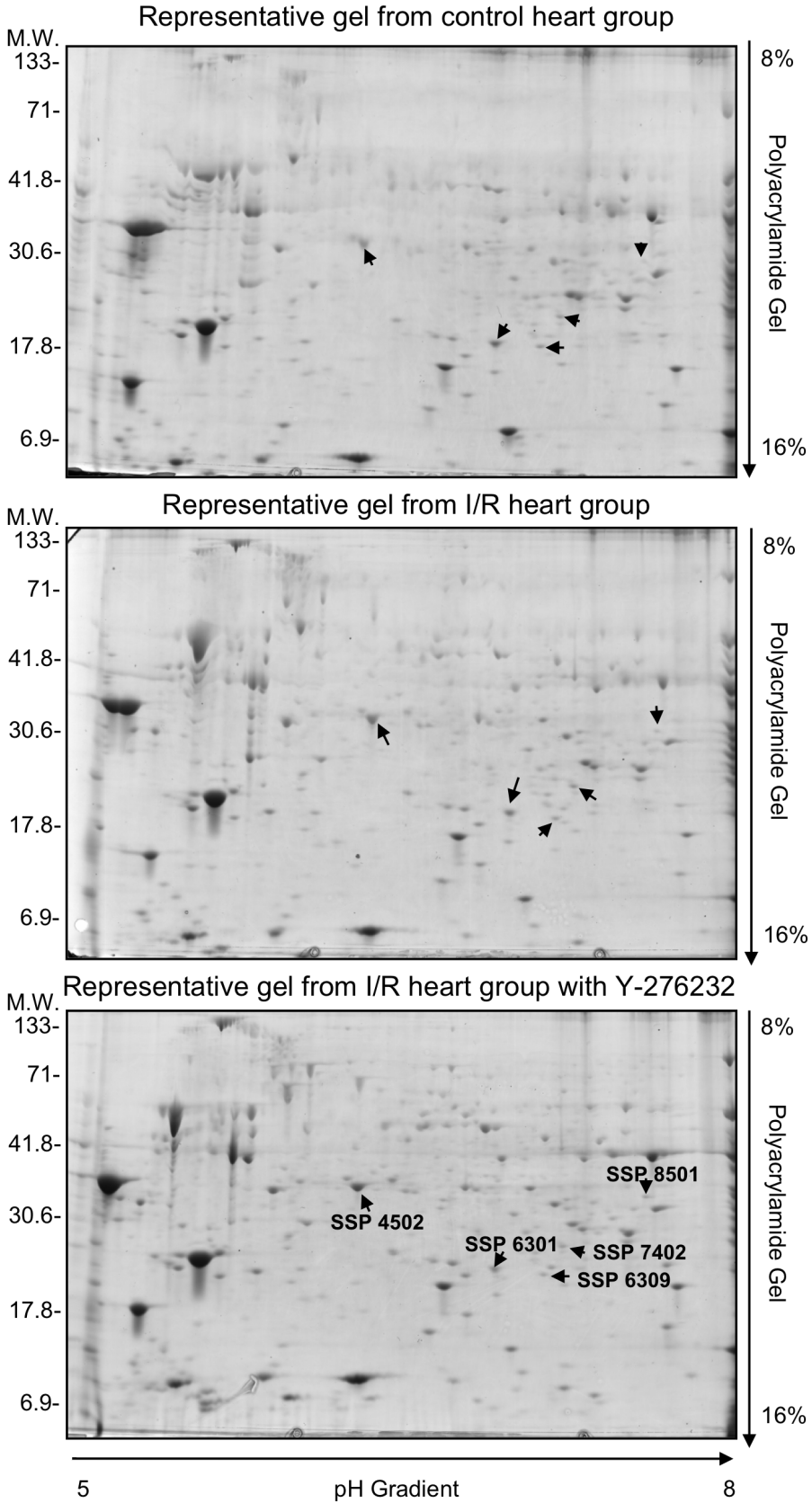
Protein Spot (SSP*)	Mowse Score**	Queries Matched	Sequence Coverage (%)	Molecular Weight (kDa)	Protein Identification
4206 ⇑***	908	27	54	36.9	L-Lactate dehydrogenase B
6301 ⇓	909	21	24	58.9	ATP synthase alpha subunit, fragment
6309 ⇓	1105	21	30	47.4	ATP synthase alpha subunit, fragment
7402 ⇓	166	18	5	43.2	Sarcomeric mitochondrial creatine kinase, fragment
8501 ⇑	560	18	49	36.1	Glyceraldehyde 3-phophate-dehydrogenase

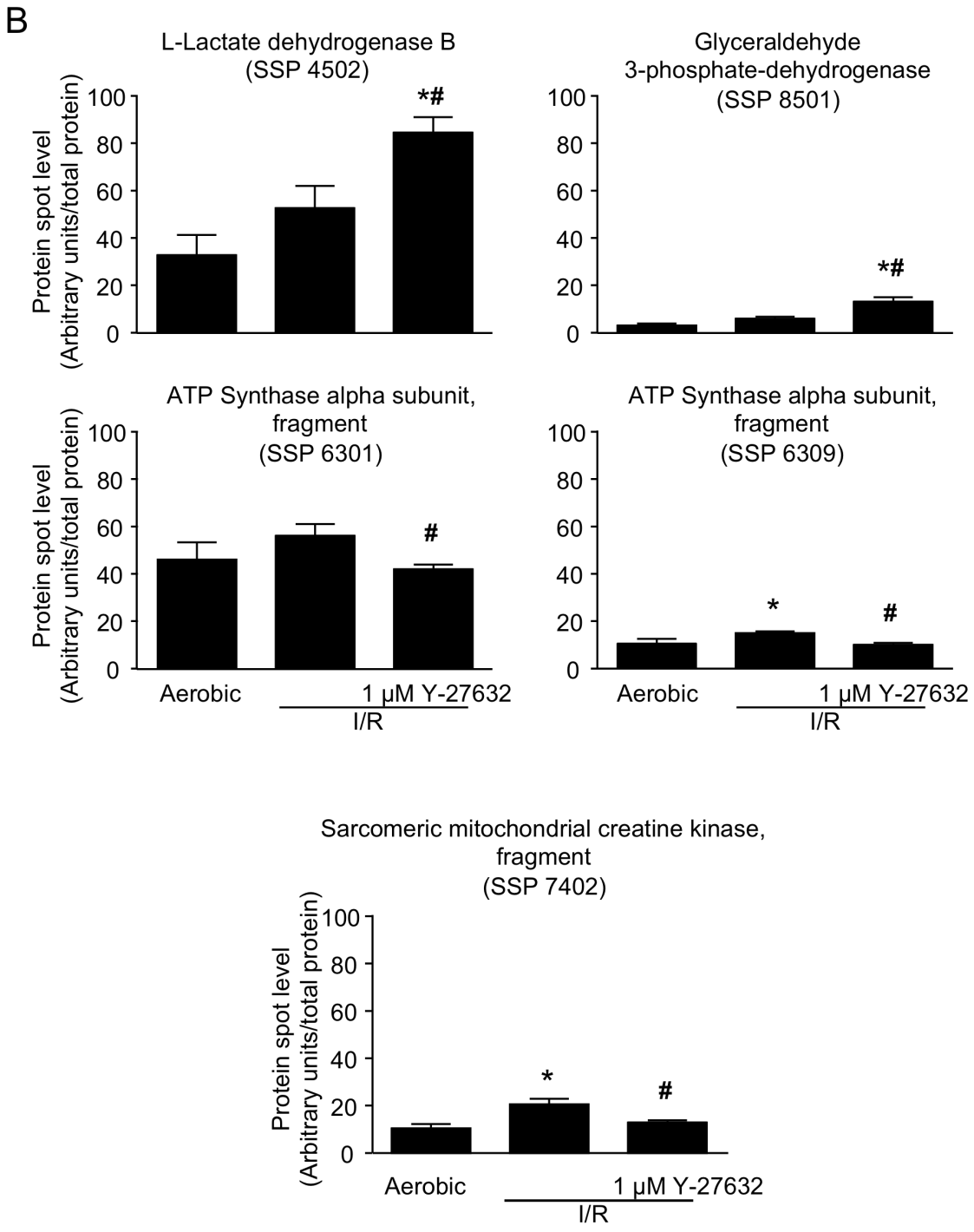
* SSP – Standard Spot Number

** -10log(P), where P is the probability that the observed match is a random event. Individual ions scores >40 indicate identity or extensive homology ($p<0.05$)

*** the arrows (⇑,⇓) indicate changes in protein levels (vs. control)

A


Representative gel from control heart group
M.W.
133-
71-
41.8-
30.6-
17.8-
6.9-
8%
Polyacrylamide Gel
16%
Representative gel from I/R heart group
M.W.
133-
71-
41.8-
30.6-
17.8-
6.9-
8%
Polyacrylamide Gel
16%
Representative gel from I/R heart group with Y-276232
M.W.
133-
71-
41.8-
30.6-
17.8-
6.9-
SSP 8501
SSP 4502
SSP 6301
SSP 7402
SSP 6309
8%
Polyacrylamide Gel
16%
5
pH Gradient
8


B
L-Lactate dehydrogenase B
(SSP 4502)
Protein spot level
(Arbitrary units/total protein)
100
80
60
40
20
0
*#
Glyceraldehyde
3-phosphate-dehydrogenase
(SSP 8501)
*#
ATP Synthase alpha subunit,
fragment
(SSP 6301)
#
ATP Synthase alpha subunit,
fragment
(SSP 6309)
*
#
Aerobic
1 μM Y-27632
I/R
Sarcomeric mitochondrial creatine kinase,
fragment
(SSP 7402)
*
#

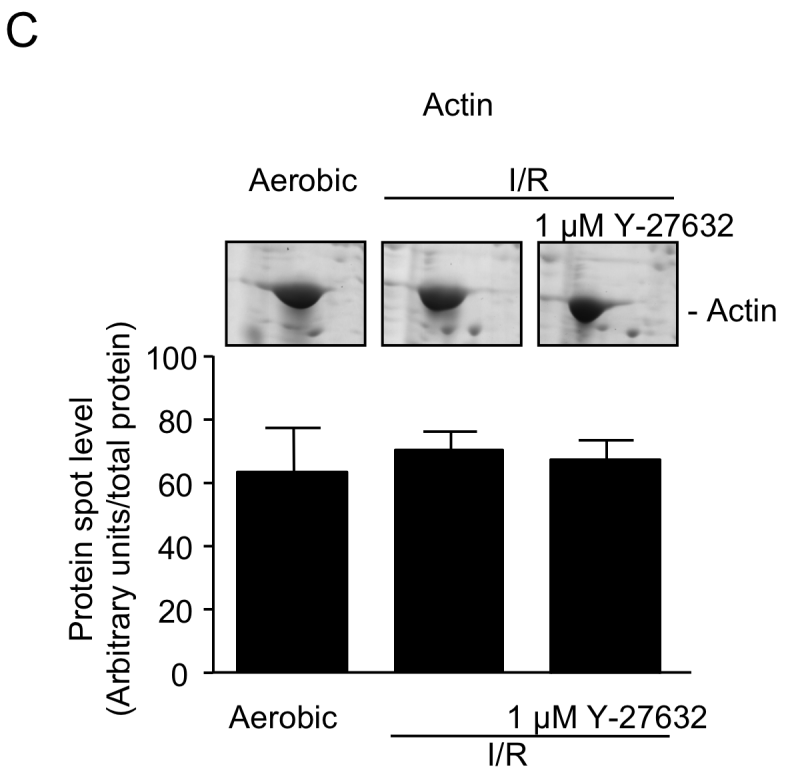


Figure 4.3 – Effect of Y-27632 treatment on the cardiac proteome. A) Representative 2-DE gels from aerobic, I/R and I/R + Y-27632 hearts. Arrows indicate the protein spots changed by I/R and affected by Y-27632. B) Densitometric analysis of affected protein spots. C) Densitometric analysis of actin as a control of equal protein loading. * $p<0.05$ vs Aerobic, # $p<0.05$ vs I/R.

SSP6301

```
  1 RRALPRRAGL VSKNALGSSF VGTRNLHASN TRLQKTGTAE MSSILEERIL
 51 GADTSVDLEE TGRVLSIGDG IARVHGLRNV QAEEMVEFSS GLKGMSLNLE
101 PDNVGVVVFG NDKLIKEGDI VKRTGAIVDV PVGDELLGRV VDALGNAIDG
151 KGPVGSKIRR RVGLKAPGII PRISVREPMQ TGIKAVDSLV PIGRGQRELI
201 IGDRQTGKTS IAIDTIINQK RFNDGTDEKK KLYCIYVAIG QKRSTVAQLV
251 KRLTDADAMK YTIVVSATAS DAAPLQYLAP YSGCSMGEYF RDNGKHALII
301 YDDLSKQAVA YRQMSLLLRR PPGREAYPGD VFYLHSRLLE RAAKMNDSFG
351 GGSLTALPVI ETQAGDVSAY IPTNVISITD GQIFLETELF YKGIRPAINV
401 GLSVSRVGSA AQTRAMKQVA GTMKLELAQY REVAAFAQFG SDLDAATQQL
451 LSRGVRLTEL LKQGQYSPMA IEEQVAVIYA GVRGYLDKLE PSKITKFESA
501 FLSHVVSQHQ SLLGNIRSDG KISEQSDAKL KEIVTNFLAG FEP
```

SSP6309

```
  1 RRALPRRAGL VSKNALGSSF VGTRNLHASN TRLQKTGTAE MSSILEERIL
 51 GADTSVDLEE TGRVLSIGDG IARVHGLRNV QAEEMVEFSS GLKGMSLNLE
101 PDNVGVVVFG NDKLIKEGDI VKRTGAIVDV PVGDELLGRV VDALGNAIDG
151 KGPVGSKIRR RVGLKAPGII PRISVREPMQ TGIKAVDSLV PIGRGQRELI
201 IGDRQTGKTS IAIDTIINQK RFNDGTDEKK KLYCIYVAIG QKRSTVAQLV
251 KRLTDADAMK YTIVVSATAS DAAPLQYLAP YSGCSMGEYF RDNGKHALII
301 YDDLSKQAVA YRQMSLLLRR PPGREAYPGD VFYLHSRLLE RAAKMNDSFG
351 GGSLTALPVI ETQAGDVSAY IPTNVISITD GQIFLETELF YKGIRPAINV
401 GLSVSRVGSA AQTRAMKQVA GTMKLELAQY REVAAFAQFG SDLDAATQQL
451 LSRGVRLTEL LKQGQYSPMA IEEQVAVIYA GVRGYLDKLE PSKITKFESA
501 FLSHVVSQHQ SLLGNIRSDG KISEQSDAKL KEIVTNFLAG FEP
```

Figure 4.4 – ATP synthase alpha subunit fragments sequence coverage. The two fragments identified correspond to very distinct regions of the native protein. Identified residues are highlighted in bold red.

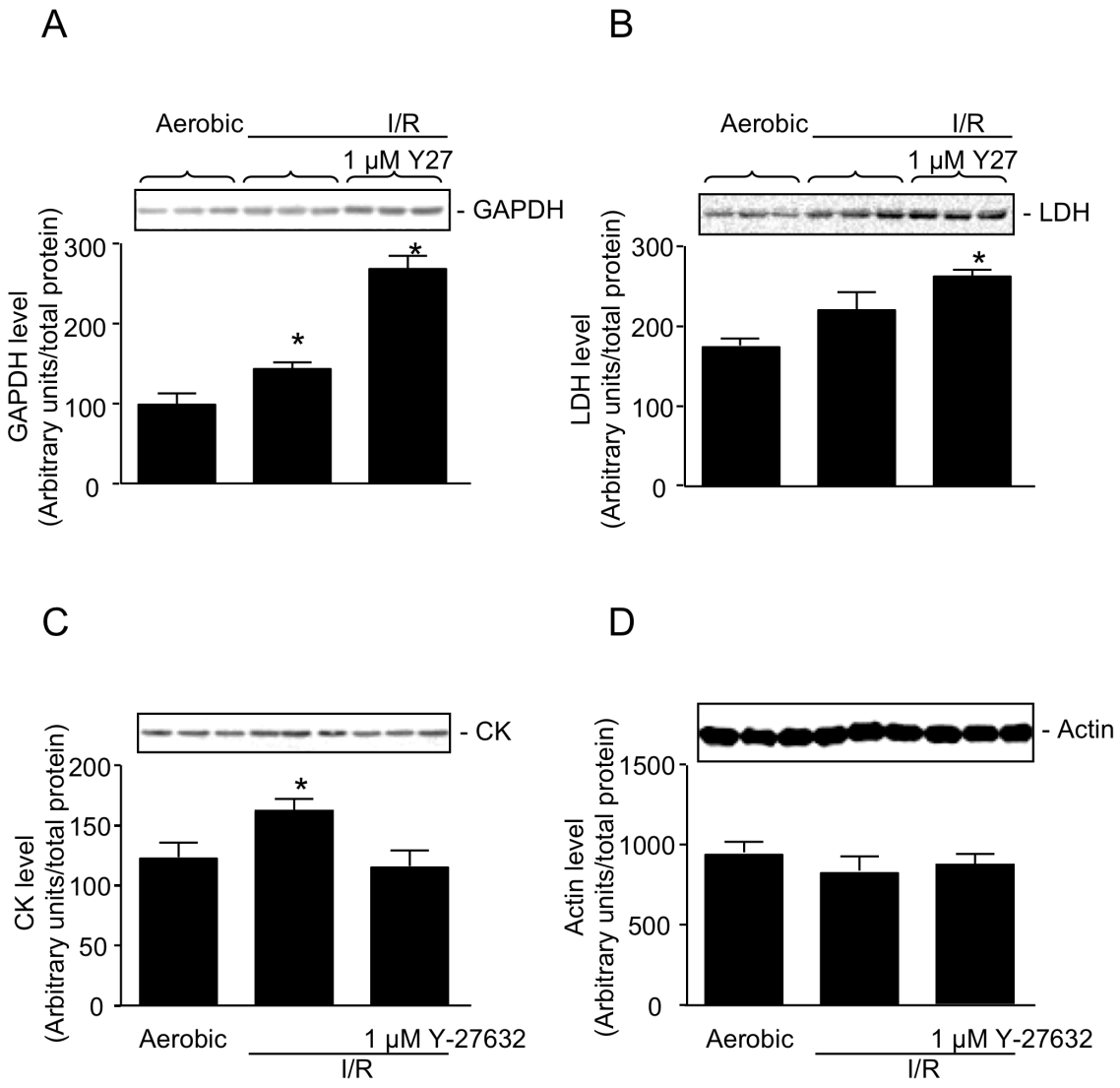


Figure 4.5 – Quantitative analysis of affected proteins by immunoblotting: (A) GAPDH – glyceraldehyde-3-phosphate-dehydrogenase; (B) LDH – L-lactate dehydrogenase; (C) CK – sarcomeric mitochondrial creatine kinase; (D) Actin, as a control of equal protein loading . n=3/group; * $p<0.05$ vs Aerobic, # $p<0.05$ vs I/R.

4.5. Discussion

Ischemia/reperfusion (I/R) injury can seriously compromise function of heart during reperfusion and long-term outcome. The intracellular signaling pathways that mediate stress responses of cardiomyocytes and determine cell death or survival have not been fully investigated. However, recently it has been demonstrated that myocardial I/R injury, with the contribution of reactive oxygen species (ROS), activates several protein kinases (Armstrong 2004; Bao, Hu et al. 2004; Dyck and Lopaschuk 2006; Scolletta and Biagioli 2010). Protein kinase activation potentially regulates the onset of myocardial cell injury. The primary protein kinase pathways that are potentially activated by myocardial I/R include the mitogen activated protein kinases (MAPKs): ERK 1/2, JNK 1/2, p38 and the cell survival kinase, Akt (Armstrong 2004). The phosphorylation and de-phosphorylation of transcriptional regulators mediated by specific protein kinases and protein phosphatases is the most common mechanism of controlling gene expression (Hill and Treisman 1995; Whitmarsh and Davis 2000). However, the exact mechanisms by which these signaling cascades promote cardio-protection or cardiac damage still remain unclear. The cardioprotective effects of inhibition of the ROCK pathway by Y-27632 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 (Kobayashi, Horinaka et al. 2002).

Here we implicate the ROCK pathway in the regulation of the levels of, at least, five proteins involved mainly in energy production and that inhibition of this pathway can contribute to optimization of energy production during reperfusion. In our experimental model glucose is the only exogenous substrate provided for energy production (component of the buffer used for perfusion). Hence, these hearts rely mainly on glucose metabolism for energy production to meet the energetic demands of contractile work. During ischemia, the absence of oxygen to support aerobic metabolism results in a switch in the metabolic profile of the heart towards the anaerobic energy-producing pathway of glycolysis (Stanley, Recchia et al. 2005). Glycolysis metabolizes glucose (exogenous or derived from glycogen) to pyruvate with the production of ATP. Under aerobic conditions pyruvate enters the mitochondria to feed the tricarboxylic acid (TCA) cycle and generate reducing equivalents ($NADH/H^+$ and $FADH_2$) that are used in the oxidative phosphorylation pathway to produce ATP. Under anaerobic conditions, such as ischemia, the pyruvate produced by the glycolytic pathway cannot enter the TCA cycle and is converted to

lactate by lactate dehydrogenase (LDH) in the cytoplasm. LDH catalyzes the conversion of pyruvate to lactate and *vice versa*. Under aerobic conditions lactate can be converted to pyruvate and oxidized in the TCA cycle for energy production. The observed increase in LDH protein levels in our study suggest that Y-27632 treatment may increase the rates of conversion of lactate to pyruvate providing the heart with a supplemental energy producing pathway during reperfusion.

Simultaneously, Y-27632 treatment of hearts subjected to I/R resulted in increased levels of glyceraldehydes 3-phosphate dehydrogenase (GAPDH). GAPDH is the enzyme involved in the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in the glycolytic pathway. This step of glycolysis is one of the indirect energy producing steps since it generates reducing equivalents (NADH/H+) that can be utilized by the oxidative phosphorylation pathway in the mitochondria to produce ATP. Increased levels of GAPDH can be seen as an indicator of increased glycolytic flow, which coupled with oxidation of pyruvate would result in higher energy production and consequently contribute for the observed increase in contractile function recovery observed in hearts treated with Y-27632.

Two protein fragments of the alpha subunit of ATP synthase were identified and normalized by Y-27632 treatment. The alpha subunit of ATP synthase is a component of F1 and is believed to contain a regulatory substrate-binding site while the beta subunit contains the catalytic binding site. Changes in alpha and beta subunit protein levels have been previously implicated in the development of hypertension in old spontaneous hypertensive rats (Jullig, Hickey et al. 2008). Interestingly, the two fragments found in our experimental model are distinct, although they present similar variation profile towards a normalization of its levels to aerobic control. One of the fragments (SSP 6301) corresponds to a 24 % of the full sequence of alpha subunit of ATP synthase and refers to the amino terminal [N-terminal], and SSP 6309 covers 30% of the full sequence of this protein. The primary structure of the SSP 6309 is very interesting, 10% of identified peptides corresponds to N-terminal part of the protein and 20% covers C-terminal part of the protein sequence. This phenomenon can be explained by alternative splicing and/or post-translational modification of the full size protein by proteolytic cleavage. The normalization of these fragments by Y-27632 treatment indicates that in I/R injury there is a modulation of the structure of the alpha subunit of ATP synthase that could result in altered ATP production by the oxidative phosphorylation. This modification and/or alteration of the structure

of the F1 functional domain could result in impaired mitochondrial ATP production contributing to the pathology of I/R injury.

The protective effects of Y-27632 against development of I/R injury can also be observed by the normalization of the protein levels of sarcolemal mitochondrial creatine kinase (CK) after I/R. CK catalyzes both the forward and reverse reactions of the generation of phospho-creatine (PCr) or generation of ATP and creatine (Cr) from PCr. Hydrolysis of PCr is usually seen as an energetic reserve and a protective mechanism during periods of energy deprival. Also, CK levels in blood are used as indicators of myocardial infarction (Friess and Stark 2009). The normalization of the sarcolemal mitochondrial CK levels by Y-27632 indicates not only a reduction of myocardial infarction and injury but also an improved energetic status of the cardiomyocyte.

We conclude that the cardioprotective effect of inhibition of the ROCK 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.

This study provides new insights into the molecular mechanisms of a broad variety of heart pathologies related to I/R injury. Kinase inhibitors hold promise as novel strategies reducing the impact of cardiovascular disease. Additionally, we provide new evidence, which could lead to the discovery of potential drug targets for the development of new therapeutic strategies for treatment of MI.

4.6. Acknowledgements

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4.7. Conflict of Interest

None to be declared.

5. Discussion

The prevalence of cardiovascular diseases as one of the major burdens to society justifies the need of better understanding of the molecular mechanisms involved in the development of cardiac injury. Our studies establish a novel mechanism by which ischemia/reperfusion (I/R) induce the development of cardiac contractile dysfunction (I/R injury). Based on these findings we have developed a therapeutic strategy of potential clinical application for the prevention and protection of I/R injury and cardiac contractile dysfunction.

Myosin light chain 1 (MLC1), also known as myosin essential light chain, is a cardiac contractile protein involved in the stability of the myosin head domain and the myosin-actin complex stability (Hernandez, Jones et al. 2007). Despite its apparent important role, MLC1 has been overshadowed by the regulatory functions of contraction of MLC2 (myosin regulatory light chain). MLC1 was reported to be phosphorylated in models of oxidative stress (Arrell, Neverova et al. 2001; Arrell, Elliott et al. 2006) but the implications and function of this posttranslational modification remained to be elucidated. Here we show that I/R triggers phosphorylation of MLC1 by the myosin light chain kinase (MLCK) and this phosphorylation signals MLC1 for degradation by the matrix metalloproteinase-2 (MMP-2). Phosphorylation of MLC1 causes an increase in the affinity of MMP-2 for MLC1 and consequent increased degradation by MMP-2. Interestingly, our *in vitro* studies show that, under basal, non-phosphorylative conditions MMP-2 degrades MLC1. Upon phosphorylation by MLCK the proteolytic degradation of MLC1 by MMP-2 is significantly increased.

Mass spectrometry analysis of MLC1 protein from rat hearts perfused under aerobic conditions detected the presence of two phosphorylation sites (T69 and T132/T134/Y135), with one of these sites (T69) being present in a consensus sequence for MLCK. These phosphorylated residues were also observed under I/R conditions. However, inhibition of MLCK using the specific inhibitor ML-7 inhibited the phosphorylation of T69 previously observed, confirming that this is most likely a MLCK phosphorylation site. The observation of MLCK-dependent phosphorylation of MLC1 under aerobic conditions together with a basal activity of MMP-2 over MLC1, *in vitro*, indicates a physiological role for MLCK and MMP-2 in the physiological turnover of MLC1.

The identification of MLCK-dependent phosphorylation of MLC1, and consequent degradation by MMP-2, under both physiological and I/R conditions lead us to develop a pharmacological approach aiming at modulating MLC1 phosphorylation and degradation. Several compounds are commercially available for the inhibition of MLCK (ML-7, ML-9, among others) and MMP-2 (doxycycline) activities. These compounds, when given at full protective doses, confer a certain degree of protection of cardiac contractile function against I/R injury. However, neither function nor MLC1 protein levels are fully protected with administration of any of the inhibitors (with the exception of Y-27632 which confers full protection to only contractile function).

Another approach to regulate the phosphorylation status of a determined protein is by an increase in the phosphatase activity. Myosin light chain phosphatase (MLCP), as with MLCK, has been described as the protein phosphatase responsible for the dephosphorylation of MLC2 (Morano 1999; Morano 2003). MLCP activity can be indirectly stimulated by the use of Rho kinase pathway inhibitors such as Y-27632 or the clinically approved fasudil (Kobayashi, Horinaka et al. 2002).

I/R-induced MLC1 phosphorylation is MLCK-dependent and leads to an increased degradation of MLC1 by MMP-2. MLC1 degradation by MMP-2 is likely a key factor in the development of I/R-induced cardiac contractile dysfunction. Here we show that phosphorylation of MLC1 is involved in the pathology of I/R injury. Hence, inhibition of MLC1 phosphorylation is a viable target for protection of the heart against I/R injury. Inhibition of phosphorylation can be achieved by either inhibiting the activity of the kinase or stimulating the activity of the phosphatase. By inhibiting MLCK (responsible for MLC1 phosphorylation) and stimulating MLCP (responsible for MLC1 dephosphorylation) we show that selective inhibition of MLCK with ML-7 or indirect stimulation of MLCP with the Rho-kinase inhibitor Y-27632 (at full protective doses) can result in an increased recovery of cardiac contractile function. Concentration-response studies determined that the subthreshold concentration for ML-7 being 1 μM and for Y-27632 being 0.05 μM. When both drugs are administered to hearts subjected to an I/R protocol, a significant improvement of cardiac contractile function recovery is observed. Since these subthreshold concentrations, given alone, show no protection on cardiac contractile function, we can conclude that the mechanism of cardioprotection by ML-7 and Y-27632, at subthreshold doses, is synergy, rather than an addition. The protection of contractile function was

associated with a protection of MLC1 against degradation by MMP-2. The administration of a combination of inhibitors, at subthreshold concentrations, before the onset of ischemia, throughout ischemia and during early reperfusion, resulted in the full protection of cardiac contractile function from I/R. This protection was associated with a preservation of MLC1 protein levels. The mechanism of action involved is synergy, rather than a net effect resulting from the addition of the effects of individual drug administration (additive effect).

Our findings presented here support the notion that specific pathways involved in the pathology of I/R injury also have important physiological roles. Hence, the inhibition of the pathological fraction of the enzymatic activity is preferrable over full pathway inhibition. Our data support this concept in which it shows that the use of inhibitors of MLCK, MMP-2 and activators of MLCP at subthreshold concentrations can fully protect cardiac contractile function against I/R injury.

We show that preventing the phosphorylation (with ML-7 and Y-27632) and degradation of MLC1 (with Doxy) during I/R can be achieved by the use of a combination of subthreshold concentrations and results in full protection of cardiac contractile function and MLC1 protein levels. Our results suggest that the use of subthreshold concentrations of inhibitors of enzymes with both a physiological and a pathological roles acts by eliminating the pathological component of the enzyme activity without interfering with its normal physiological actions.

Of the three inhibitors used, Y-27632 (Rho kinase inhibitor) was the only one to demonstrate a full protection of cardiac contractility following I/R, in comparison to aerobic contractile function. This full protection of cardiac contractile function was not accompanied with a full protection of MLC1 from degradation. This observation led us to hypothesize that administration of Y-27632 to rat hearts undergoing I/R injury can have other molecular targets capable of conferring protection against cardiac contractile dysfunction. Our proteomics approach revealed mainly changes in protein levels of enzymes associated with energy metabolism. I/R-induced changes of cardiac energy metabolism have been extensively studied [for review see (Stanley, Recchia et al. 2005)] and considered to be a relevant factor in the development of I/R injury. Our data shows that Y-27632, besides its regulation of contraction by regulating the phosphorylation status of MLC1 and MLC2, also has a protective effect by increasing the protein levels of enzymes associated with energy production, hence increasing

ATP production in the heart. Further studies are needed to elucidate about metabolic modulation actions of Y-27632.

My PhD project had three main objectives:

1. Identify ischemia/reperfusion-induced post-translational phosphorylation of contractile proteins, such as MLC1, in the isolated perfused rat heart;
2. Determine the role of phosphorylation of MLC1 during ischemia/reperfusion and its implications to contractile function recovery;
3. Develop pharmacological strategies to protect against the degradation of contractile function and the reduction of I/R injury.

We have determined that the cardiac contractile proteins MLC1 and MLC2 are subjected to posttranslational modifications in response to I/R. Phosphorylation of MLC1 by MLCK was observed in a model of isolated heart perfusion. The phosphorylation of MLC1 was show to mark the protein for degradation by MMP-2 with a consequent worsening of cardiac contractile function. The use of drugs aiming at preventing MLC1 phosphorylation (ML-7 and Y-27632) and degradation (Doxy) can effectively protect the heart against I/R-induced contractile dysfunction.

6. Implications and Future Directions

The work here presented contributes significantly to the establishment of a new scientific paradigm concerning the understanding of the molecular mechanisms contributing to cardiac contractile dysfunction following ischemia/reperfusion. Moreover, this work also establishes a viable therapeutic approach to treat cardiac contractile dysfunction and, possibly, can be applied to other medical fields for multi-factorial pathologies.

Although our studies clearly show the importance of MLC1 in contraction and contractile dysfunction induced by I/R, further detailed investigation is needed to elucidate the proper mechanism and timeframe of MLC1 posttranslational modifications and consequences of these modifications. We have shown previously that MLC1 is subjected to nitration and nitrosylation in response to oxidative stress, such as I/R. It remains to be determined the effect of nitration and nitrosylation on MLC1 phosphorylation, and vice-versa. It is likely that posttranslational modifications of proteins are connected in a determining fashion, i.e. the nature and location of the first posttranslational modification determines nature and location of subsequent modifications and, ultimately, protein fate. Further studies modulating the order and location of posttranslational modifications of MLC1, by using reactive nitrogen oxide species (RNOS) scavengers to prevent nitration and nitrosylation, together with phosphorylation inhibitors, should be performed to determine the interaction between posttranslational modifications. Also, the possible physiological role of phosphorylation on MLC1 turnover should be confirmed.

The new therapeutic paradigm established here needs to be confirmed in pre-clinical studies. *In vivo* studies in rodents treated with prophylactic drug combinations, followed by induction of I/R injury will help validate the findings from our study and can prove the clinical validity and applications of synergy at subthreshold concentrations.

Our study on the proteomic effects of the Rho kinase inhibitor Y-27632 in I/R revealed a metabolic modulatory component to Y-27632 mechanism of cardioprotection. These findings indicate that besides modulation of contraction, Y-27632 can also modulate energy production. These characteristics make Rho kinase inhibitors (if this dual effect is seen with other members of this family, such as the clinically approaved fasudil) a very important tool in the prevention

and treatment of I/R injury. It is important to perform detailed studies measuring the metabolic modulation effects of Y-27632 on cardiac energy metabolism.

Our results, of scientific and clinical relevance, still lie years away from clinical application since independent validation is required and pre-clinical studies mandatory.

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