OXIDATIVE STRESS-INDUCED, PEROXYNITRITE-DEPENDENT, MODIFICATIONS OF MYOSIN LIGHT CHAIN 1 LEAD TO ITS INCREASED DEGRADATION BY MATRIX METALLOPROTEINASE-2

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ABSTRACT

Damage to cardiac contractile proteins such as myosin light chain 1 (MLC1), during oxidative stress is mediated by reactive oxygen species such as peroxynitrite ($ONOO^-$), resulting in impairment of cardiac systolic function. The purpose of this study is to investigate the effects of the increased level of $ONOO^-$ on MLC1 degradation by the proteolytic enzyme matrix metalloproteinase-2 (MMP-2) during oxidative stress which ultimately decreases cardiac function.

In the present study two distinct models were utilized to demonstrate the mechanism by which MLC1 is modified by $ONOO^-$ and how these post-translational modifications lead to its increased degradation by MMP-2. In a model of newborn hypoxia-reoxygenation in piglets we demonstrated that $ONOO^-$-induced nitration and nitrosylation of tyrosine and cysteine residues of MLC1 increase its degradation by MMP-2. Furthermore, we found nitration of a tyrosine residue located adjacent to the cleavage site for MMP-2. We verified these results by using a model of isolated rat heart myocytes to determine that the same mechanism responsible for cardiac dysfunction in newborn piglets occurs in isolated myocytes and that the MMP-2 involved in degradation of MLC1 is located within the myocytes. Moreover, we were able to determine that this mechanism occurs during ischemia itself before the onset of reperfusion.

Furthermore, we have found that pharmacological intervention aimed at inhibition of MLC1 nitration/nitrosylation during ischemia by the $ONOO^-$ scavenger FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]), or inhition of MMP-2 activity with phenanthroline, provides an effective protection of cardiomyocyte contractility. The work presented here provides new evidence on the mechanisms of regulation of contractile proteins during the development of contractile dysfunction.

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ABBREVIATIONS

2-DE	2-dimensional electrophoresis
ANOVA	analysis of variance
ATP	adenine triphosphate
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
cDNA	complementary deoxyribonucleic acid
CO	cardiac output
DOC	deoxycholate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
eNOS	endothelial nitric oxide synthase
FeTPPS	5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]
H_2O_2	hydrogen peroxide
HPLC	high pressure liquid chromatography
H-R	hypoxia-reoxygenation
HR	heart rate
I/R	ischemia/reperfusion
IEF	isoelectrofocusing
IgG	immunoglobulin G
IPG	immobilized pH gradient

MALDI-TOF	matrix assisted laser desorption/ionization time of flight
MAP	mean arterial pressure
MHC	myosin heavy chain
MLC	myosin light chain
MLC1-WT	wild type myosin light chain 1
MMP	matrix metalloproteinase
MS	mass spectrometry
MT-MMP	membrane-type metalloproteinase
NCBI	national center for biotechnology information
NO	nitric oxide
NO_x^-	nitrite/nitrate
$^{\cdot}OH$	hydroxyl radical
$ONOO^-$	peroxynitrite
$O_2^{\cdot -}$	superoxide
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PTM	post-translational modification
Q-TOF	quadrupole time of flight
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid

ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SV	stroke volume
TEMED	N,N,N’,N’-tetra-methyl-ethylenediamine
TIMP	tissue inhibitor of metalloproteinase
TnI	troponin I
VM	ventricular myocyte

CHAPTER 1

INTRODUCTION

1.1 Cardiovascular disease

Cardiovascular disease encompasses any injury which occurs to the heart, coronary blood vessels or any other part of the vascular system. The likelihood of developing cardiovascular disease increases with a variety of risk factors such as smoking, obesity, diabetes, a sedentary lifestyle and high blood pressure and it has been estimated that 9 out of 10 Canadians possess at least one of these risk factors [1]. Although the rate of heart disease in Canada has been steadily declining since the 1950's, cardiovascular disease continues to account for more deaths than any other medical condition [2].

Cardiovascular disease can manifest itself in many forms including cardiac myopathy, cardiac hypertrophy, coronary artery disease, hypertension, atrial fibrillation, atherosclerosis and others. These may subsequently result in myocardial infarction, stroke or congestive heart failure. One factor contributing to the pathology of these conditions is the excessive generation of reactive oxygen species (ROS). For example, during a myocardial infarction, the blockage of a blood vessel results in inadequate blood supply to the heart and therefore results in the development of ischemia during which profound myocardial damage may occur at both microscopic and macroscopic levels [3]. During ischemia ion pumps do not function properly because ATP is progressively depleted which is accompanied by an increase in intracellular calcium (Ca^{2+}) concentration (calcium overload) which further drives the depletion of ATP. Intracellular Ca^{2+} continues to rise during ischemia and into reperfusion which results in an accumulation of Ca^{2+}. During reperfusion the restoration of blood supply to the heart leads to reintroduction of oxygen which results in generation of ATP as well as the production of ROS. The excessive accumulation of Ca^{2+} as well as the increase in ROS production leads to opening

of the mitochondrial permeability transition pore which compromises cellular energetics which may result in a rupture of the plasma membrane and cell death [4].

Congestive heart failure is a complex condition which involves a variety of factors. It has been shown that increased oxidative stress, as well as decreased action of nitric oxide (NO), are important factors associated with the development of heart failure since administration of antioxidants has been shown to improve endothelium-dependent vasodilation in subjects with chronic heart failure [5]. One study observed an increase in ROS production with the progression of heart failure and concluded that oxidative stress plays an adverse role in patients with heart failure [6]. Therefore, it can be concluded that increased oxidative stress may be a target for development of therapeutic strategies to improve endothelial function and prognosis of patients with heart failure.

Cardiovascular disease may be manifested in many forms and although the pathophysiology is complex, an important factor which contributes to the development of cardiovascular disease is oxidative stress which results from increased production of reactive oxygen species and leads to cellular damage.

1.2 Oxidative stress

Reactive oxygen species (ROS) are toxic compounds which are formed during a variety of normal as well as pathological biochemical reactions. In normal physiological conditions, ROS are involved in cell signalling and redox regulation of signal transduction pathways [7]. Since ROS contain unpaired electrons they can be reactive and unstable and therefore excessive

production of ROS can induce oxidative damage to proteins, DNA and lipids [8]. This ROS-induced 'oxidative stress' results in cell damage which eventually leads to cell death.

Although ROS are produced as a result of normal cellular metabolism, an imbalance between the formation and the neutralization of pro-oxidants results in their increased levels and therefore to cellular damage. Several examples of ROS are hydroxyl radical ($^{\cdot}OH$), nitric oxide (NO), superoxide anion (O_2^-) and peroxynitrite ($ONOO^-$). Peroxynitrite is a potent oxidant and markers of its formation can be found in many disease states including brain injury [9-11], heart [12-14], lung [15-16], liver disease [17-18] and inflammation [19-22].

Reactive oxygen species are produced in all cell types in the body with the majority of these compounds being produced in the mitochondria [7]. Increased ROS production in cardiovascular cells such as cardiac myocytes has been implicated in the initiation and progression of heart injury and disease [23-25], and has been associated with the development of contractile dysfunction, resulting in part from cell death, following myocardial infarction and pressure overload [26-28]. However, the precise role of ROS in the pathophysiology of cardiac injury is not well understood.

1.3 Patho-physiological roles of reactive oxygen species in the heart

Reactive oxygen species such as superoxide anion and the hydroxyl radical play an important role in cardiovascular disease. One source of ROS production is the mitochondria. During hypoxia-reoxygenation, increased levels of ROS depress electron transport within the mitochondria which further promotes the production of ROS [24] and as a result an ongoing

cycle develops which is characterized by a high level of ROS production and a dysfunctional electron transport which induces mitochondrial death and the release of cytochrome c. Cytochrome c in turn activates apoptosis which, together with protease-induced cell cleavage, results in cellular death [4].

During cardiac ischemia and reperfusion, an increase in production of oxygen-derived free radicals within the heart results in ischemia/reperfusion (I/R) injury which is characterized by protein denaturation, inactivation of homeostatic enzymes and lipid peroxidation [3] which subsequently leads to cellular death and to the development of cardiac dysfunction [29]. However, it is currently unknown whether oxidative stress-induced damage begins during ischemia itself and continues during reperfusion or whether the majority of the damage occurs during reperfusion.

There is a balance in the production of the important vasodilator nitric oxide (NO) during physiological conditions [30]. The production of cardiac NO from L-arginine is mediated by the enzyme endothelial nitric oxide synthase (eNOS) [31] and is important for maintaining a variety of physiological functions in the heart such as modulating contractile function via vasorelaxation, providing an antioxidant environment in the heart [32] and inhibiting platelet aggregation [33]. In addition to its important roles in the regulation of cardiac muscle function and myocardial energetics [34], NO has been shown to react with the superoxide anion (O_2^-) at a very rapid rate to form $ONOO^-$, a reactive oxygen species which contributes to cardiac injury.

1.3.1 Peroxynitrite

The peroxynitrite anion ($ONOO^-$) is an oxidant formed when O_2^- reacts with NO. Under physiological conditions $ONOO^-$ is generated at very low levels with minimal oxidative damage [35]. However, under pathological conditions an increase in NO production is accompanied by an increase in O_2^- with the net effect being an increase in $ONOO^-$ which may then induce cell damage, tyrosine nitration and oxidation of cysteine to disulfides or to various sulphur oxides [33]. The damage induced by $ONOO^-$ includes structural damage, enzyme dysfunction, ion channel and transporter malfunction [36]. An increase in $ONOO^-$ generation was observed by Guzik *et al* in human blood vessels [37] and has also been implicated in the development of a variety of pathological processes such as atherosclerosis [38], hypertension [39-40] and ischemic brain injury [41]. Peroxynitrite alters numerous biological compounds such as proteins, lipids and nucleic acids. Furthermore, by reacting with crucial mitochondrial components, such as cytochrome c [42-44] and mitochondrial aconitase [45-46], $ONOO^-$ causes cellular damage which results in an imminent cell death [35]. Peroxynitrite can further induce cell damage indirectly by reacting with carbon dioxide to produce carbonate and nitrogen dioxide radicals, which also react with biomolecules [47].

Studies have shown that there is an increase in $ONOO^-$ generation during ischemia-reperfusion (I/R) and that this increase is associated with the mechanical dysfunction of the heart seen in cardiac injury [48-50]. Despite the evidence that $ONOO^-$ contributes to I/R injury, its precise role remains unknown [48, 51-52]. Therefore, it is important to identify potential targets of $ONOO^-$ as well as the sites where this oxidant induces its damage in order to minimize cardiac injury during oxidative stress. The proteolytic enzymes matrix metalloproteinases (MMPs) have been shown to be activated by $ONOO^-$ [53-54]. Furthermore, generation of $ONOO^-$ is correlated

with an increase in matrix metalloproteinase-2 (MMP-2) activity [53]. This indicates a potential involvement of MMP-2 in heart injury during oxidative stress, however, the interaction between the two has not been studied.

1.4 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of endopeptidases. Most MMPs contain zinc within their active site which is necessary for performing catalytic functions [55]. Matrix metalloproteinases are involved in a variety of physiological processes such as remodelling of bone, connective tissue and extracellular matrix [56-58] as well as in embryonic development [59]. Matrix metalloproteinases play both a physiological and pathological role in inflammation. Their physiological role involves mediating inflammatory signals involving chemokines and cytokines as well as dampening inflammation meaning that tight regulation is required in order to maintain an appropriate inflammatory response [60]. However, MMPs are also involved in a variety of inflammatory disease states such as pulmonary fibrosis, chronic obstructive pulmonary disease, cystic fibrosis and pulmonary arterial hypertension [61]. Matrix metalloproteinases have also been implicated in a variety of other pathological states such as tumour cell invasion, metastasis and stroke [62], among others. Increased MMP activity has been further noted during heart transplantation and is mediated by immunologic mechanisms indicating a potential role of MMPs in transplant rejection [63].

One of the most widely studied MMPs is matrix metalloproteinase-2 (MMP-2) which is also referred to as gelatinase A (type IV collagenase) since it degrades gelatin and collagen type IV which is a major component of the basement membrane in a variety of tissues. The 72 kDa

form of MMP-2 is a pro-enzyme which contains a propeptide domain which must be removed for activation since the propeptide sequence contains a conserved cysteine which chelates the active zinc site. The propeptide sequence of the pro-enzyme must be cleaved to the 64 kDa active form [58, 64].

1.4.1 Physiological and pathological roles of matrix metalloproteinase-2

MMP-2 is best known for its role in turnover of the extracellular matrix by digestion of gelatin and collagen. Its other physiological functions include bone formation [65], wound healing [66] and angiogenesis [67], all of which require the synthesis of new matrix components. MMP-2 also plays an important role in follicular development, luteal formation and ovulation [68-69]. Despite its importance in a variety of physiological processes, it is also implicated in many pathological states. Evidence points towards its involvement in regulation of human platelet aggregation [64] indicating its pro-thrombotic role. Moreover, increased MMP-2 activity has been implicated in the formation and development of atherosclerotic plaques [70]. MMP-2 activity has also been shown to be involved in autoimmune disorders such as rheumatoid arthritis [71] and multiple sclerosis [72] as well as in the progression of tumour cell invasion and metastasis [73-74].

Until recently, MMP-2 was thought to be mainly associated with extracellular actions, but a growing body of evidence suggests important intracellular roles of this proteolytic enzyme. One of these intracellular functions is the detrimental role of MMP-2 in cardiovascular disease. MMP-2 has been shown to contribute to acute cardiac dysfunction by degradation of intracellular proteins, such as troponin I [75], myosin light chain 1 [76] and alpha-actinin [77], which are

important components of the cardiac contractile machinery. MMP-2-induced cardiac dysfunction can be attenuated with the use of MMP-2 inhibitors such as doxycycline and o-phenanthroline [78]. Furthermore, these compounds have also been shown to reduce endothelial damage since MMP-2 is involved in the damage to endothelial integrity in hearts subjected to ischemia reperfusion [79].

1.4.2 Regulation of matrix metalloproteinase-2 activity

Matrix metalloproteinase activity is regulated at multiple levels including activation, phosphorylation of its residues, transcription, localization and inhibition of activity by tissue inhibitors of metalloproteinases (TIMPs) [80-84]. MMPs are expressed as latent enzymes (pro-enzymes) which are activated by proteolytic cleavage of the N-terminal propeptide by a membrane-type MMP (MT-MMP) [85-89]. The proteolytic removal of the propeptide region disrupts the binding of a key cysteine thiol residue with the active zinc site [58]. The disruption of this cysteine-zinc bond can be also induced by oxidizing agents like $ONOO^-$ via S-glutathiolation [90] which will in turn modulate the activity of MMP-2.

Phosphorylation and dephosphorylation of MMP-2 residues may be involved in its regulation. This indicates that certain kinases, such as protein kinase C (PKC), and phosphatases, such as alkaline phosphatase [80, 91], may be involved in the direct activation and deactivation of MMP-2. Increased MMP-2 activity has been observed in endothelial cells as well as in isolated cardiac myocytes following induction by angiotensin II indicating a neurohormonal influence on MMP-2 activation [92-93]. Stimulation of angiotensin II receptors activates

downstream Src-family tyrosine kinase and phosphoinositide 3-kinase (PI3K)-dependent mechanisms which results in activation of MMP-2 [93].

MMP regulation is also mediated by inhibitors of proteinases such as TIMPs which are endogenous MMP inhibitors. More specifically, MMP-2 is inhibited by, and its level is directly affected by, TIMP-4 which inhibits MT1-MMP and therefore prevents the cleavage of the propeptide domain of MMP-2 and as a result MMP-2 remains inactive [94]. A study by Donnini *et al* [95], has shown that $ONOO^-$ is capable of inhibiting TIMP-4 which may have potential implications on cardiac function since this would result in increased activity of MMP-2. Furthermore, an imbalance between TIMP-4 and MMP-2 level has been observed during ischemia reperfusion which contributes to cardiac injury during oxidative stress [96].

Another potential, but not very well studied, mechanism of MMP activity includes modification of its substrates. The concept that post-translational modifications (PTMs) render normally proteolysis-resistant proteins more susceptible to these enzymes has not been well studied and is under investigation. $ONOO^-$ can react with cellular proteins thereby making these proteins potential candidates for degradation by proteolytic enzymes [46]. Using an *in vivo* dog model it has been found that myocardial infarction-induced myocardial dysfunction is associated with NO, O_2^- and $ONOO^-$ related PTMs of the δ-subunit of ATP synthase [97]. Furthermore, the protein alpha-actinin is degraded by MMP-2 which is a $ONOO^-$-dependent process resulting in cardiac contractile dysfunction. Other cardiac contractile proteins degraded by MMP-2 include troponin I [98] and myosin light chain 1 [76]. Post-translational modifications of these crucial cardiac contractile proteins play important roles in their degradation and thus promote the development of contractile dysfunction.

1.5 Contractile proteins in the heart

It is well known that the heart is composed of cardiac striated myocytes, smooth muscle and endothelial cells. Cardiac myocytes are the most abundant cell type in the heart and constitute the underlying mechanism responsible for cardiac contraction. The fundamental unit of the myocyte's contractile machinery is the sarcomere which is further composed of thick and thin filaments. Whereas the thick filament contains myosin, the thin filament is composed of actin and other proteins such as tropomyosin and troponin. Muscles contract when the filaments containing myosin pull against the filaments containing actin [99].

The myosin molecule is further composed of three domains: the motor domain, a lever arm domain and a tail region. The motor domain contains a catalytic site (myosin ATPase), which uses ATP as a source of energy, and an actin binding site [100-104]. The myosin motor domain is connected to the lever arm domain which contains two motifs that form the attachment site for myosin light chain [100, 102, 105], which can alter myosin ATPase activity when phosphorylated [99].

There are two types of myosin light chain: a regulatory light chain (MLC2) and two essential light chains (MLC1 and MLC3). The essential light chains modulate the rate of contraction by increasing or decreasing the attachment and detachment rates of myosin-actin cross-bridges [106-108]. Along with the light chains myosin also consists of two heavy chains (MHC). Although the importance of MLC1 in structural stability of the myosin lever arm domain has been documented, the specific role of MLC1 in force development and regulation of muscle contraction have yet to be resolved [108-110]. Previous studies suggest that MLC1 provides the fine tuning of the myosin motor function. Haase *et al,* [111] found that the

expression of NH_2-terminal MLC1 peptide in a transgenic rat is associated with an improvement in the intrinsic contractile state of isolated perfused hearts. Both MLC1 and MLC2 appear to be involved in force development during muscle contraction [109, 112-113].

1.5.1 Role of myosin light chain in heart injury

The precise molecular basis for oxidative stress induced contractile dysfunction is currently unknown however evidence points towards protein damage within the myofilaments. This type of damage may be a result of post-translational modifications of cardiac contractile proteins. One model, using isolated cardiomyocytes, showed increased ischemia-induced phosphorylation of MLC1 [114]. Furthermore, the regulatory protein myosin light chain 2 (MLC2) is phosphorylated during myocardial stunning [115]. Subsequently, modifications of MLCs, such as MLC1 and MLC2, result in the degradation of these proteins as was shown using a model of acute congestive heart failure in which MLC degradation products were released into the circulation [116]. Moreover, the release of MLC1 and MLC2 degradation products into coronary effluent is positively correlated with the duration of ischemia [117-118]. Although the mechanism behind the degradation and release of contractile proteins has not been elucidated, one enzyme partly responsible is MMP-2 [79] which has been shown to degrade MLC1 during I/R [76]. Therefore, MMP-2 is implicated in I/R-induced contractile dysfunction.

1.6 Objectives and hypothesis

Oxidative stress triggers post-translational modifications of contractile proteins rendering these proteins substrates for MMP-dependent degradation. Furthermore, oxidative stress increases MMP activity which contributes to impairment of myocardial contractile function by degradation of these contractile proteins which, under physiological conditions, are proteolysis-resistant.

The main objective of this thesis is to analyze the oxidative stress induced post-translational modifications of MLC1 which lead to its MMP-2-induced degradation and to the development of cardiac contractile dysfunction. This will be investigated in two experimental models of oxidative stress. In Chapter 2, the model used is an *in vivo* model of hypoxia-reoxygenation using newborn piglets. In Chapter 3 the experimental model is the isolated rat cardiac myocyte subjected to ischemia in order to determine whether the observed mechanism is intracellular meaning that it is observed in myocytes and is not associated with the myocardium.

My hypothesis is that during oxidative stress there is an increase in the generation of ROS, such as $ONOO^-$, and that these ROS induce post-translational modifications of the contractile protein MLC1 which leads to its subsequent degradation by MMP-2 and to the development of systolic dysfunction. As a result, inhibition of contractile protein modifications, together with inhibition of MMP-2 activity, will provide cardiac protection during oxidative stress.

CHAPTER 2

CARDIAC DYSFUNCTION IN AN ANIMAL MODEL OF NEONATAL ASPHYXIA IS ASSOCIATED WITH INCREASED DEGRADATION OF MLC1 BY MMP-2

Doroszko A, Polewicz D, Sawicka J, Richardson JS, Cheung PY, Sawicki G. *Cardiac dysfunction in an animal model of neonatal asphyxia is associated with increased degradation of MLC1 by MMP-2*. Basic Res Cardiol, 2009. **104**(6): p. 669-79.

2.1 Preface

The contents of this chapter have been published in the peer-reviewed journal "Basic Research in Cardiology". A piglet heart model was used to analyze the relationship between $ONOO^-$, MLC1 and MMP-2 during oxidative stress and the effect this relationship has on oxidative stress-induced cardiac dysfunction. Therefore, the manuscript presented in this chapter supports the hypothesis of my thesis that during oxidative stress $ONOO^-$ modifies MLC1 which then leads to its increased degradation by MMP-2.

All work in this manuscript was performed in a team setting. I performed the biochemical experiments, data collection, data analysis and manuscript preparation.

Copyright permission has been granted from "Basic Research in Cardiology" for including this manuscript in my thesis.

2.2 Introduction

Neonatal asphyxia results in hypoxic injury to organs throughout the body but generally the most serious effects are seen in the central nervous system and the cardiovascular system. Asphyxia, the third most common cause of neonatal death, is estimated to be responsible for almost one quarter of the 4 million neonatal deaths reported annually worldwide [119]. Although more than three-quarters of newborns with birth asphyxia will survive if correct resuscitative procedures are followed [119], despite improvements in fetal monitoring and neonatal intensive care medicine, organ damage occurring around the time of the hypoxia remains an important cause of morbidity, and mortality, both in the short term and throughout the lives of the survivors. Therefore, it is important to develop new therapeutic strategies to reduce or prevent

the hypoxia-related organ damage. This would not only increase short term survival rates, it would also improve the long-term prognosis of people who had survived a period of anoxia due to neonatal asphyxia or other causes.

In cases of neonatal asphyxia, the goal of resuscitation efforts is to restore normal oxygen levels in the blood and thereby in the hypoxic tissues. However, when a hypoxic tissue is re-oxygenated, there is an acute increase in oxidative stress which triggers a cascade of pathophysiological events that result in damage to that tissue. Contributing to the pathogenesis of cardiovascular hypoxia-reoxygenation (H-R) injury, are an increase in the production of peroxynitrite ($ONOO^-$) [48, 120], and an increase in the activity of matrix metalloproteinases (MMPs) including MMP-2 [62, 78, 121]. $ONOO^-$ is a potent free radical, and markers of the formation and activity of $ONOO^-$ are found in many disorders including heart injury [13-14]. MMP-2 is a proteolytic enzyme that plays important intracellular and extracellular roles in a variety of physiological processes [62]. Excessive MMP-2 activity has been implicated in the pathogenesis of cardiac ischemia/reperfusion (I/R) injury, and other acute and chronic cardiovascular disorders [122-124]. The role of $ONOO^-$ in I/R injury and preconditioning was extensively reviewed by Ferdinandy and Schulz [30]. MMP-2 activity is elevated following cardiac I/R, and MMP-2 blockers reduce the acute cardiac dysfunction typically seen when heart tissue is exposed to I/R injury [78]. MMP-2 has been shown to degrade two intracellular myocardial proteins: the contractile machinery regulating protein troponin I (TnI) [75], and a component of the cardiac contractile protein myosin called myosin light chain 1 (MLC1) [76]. These important discoveries indicate that the development of pharmacological agents capable of selectively regulating MMP-2 may provide important new strategies in the treatment of oxidative stress-induced injury to the heart and other tissues.

Although *in vitro* studies suggest that MMP-2 is activated by $ONOO^-$ [53, 90], it has not yet been demonstrated how this comes about, nor has the interaction of these two agents under *in vivo* conditions been determined. In the present study, we used the *in vivo* neonatal pig asphyxiation model of H-R injury to investigate the relationship between MMP-2, MLC1, and $ONOO^-$. We also determined $ONOO^-$ induced modifications of the cardiac MLC1 molecule from animals exposed to H-R injury.

2.3 Methods

2.3.1 Animals

Twelve mixed breed piglets 1 to 3 days of age, weighing 1.4 to 2.3 kg, were used. This investigation conforms to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (1996).

The instrumentation and protocol have been described previously [125-126]. Briefly, animals were anesthetized by inhalational halothane (2-5%) and then switched to intravenous fentanyl (0.005-0.015 mg/kg/h), midazolam (0.1-0.2 mg/kg/h) and pancuronium (0.05-0.1 mg/kg/h) after starting mechanical ventilation. Femoral arterial and venous catheters (5F, Argyl®, Sherwood Medical Co., St. Louis, MO) were placed and positioned in the abdominal aorta and right atrium, respectively. Piglets were monitored for heart rate (HR), mean arterial pressure (MAP) and cardiac output (CO) with a Hewlett Packard 78833B monitor (Hewlett Packard Co., Palo Alto, CA). Inspired oxygen concentration was measured using an Ohmeda 5100 oxygen monitor (Ohmeda Medical, Laurel, MD) and maintained at 0.21-0.24 to keep fraction of volume for oxygen saturation between 90% and 96%, which was continuously

monitored with a pulse oximeter (Nellcor, Hayward, CA). Maintenance fluids during experimentation consisted of 10% dextrose in water at 10 ml/kg/h and 0.9% NaCl at 2 ml/kg/h. Piglet temperature was maintained at 38.5°C to 39.5°C using an overhead warmer and a heating pad.

After endotracheal intubation via tracheotomy, pressure-controlled assisted ventilation was commenced (Sechrist infant ventilator model IV-100, Sechrist Industries Inc., Anaheim, CA) with pressures of 18/4 cm H_2O at a rate of 15 to 20 breaths/min. A left anterior thoracotomy in the third intercostal space was performed. Following the ligation of Ductus Arteriosus, a 6-mm transonic flow probe (6SB906, Transonic Systems Inc., Ithica, NY) was placed around the main pulmonary artery for continuous measurement of blood flow, as a surrogate of cardiac output (CO). Arterial blood gas analysis and co-oximetry were determined during the experimental period using ABL500 and OSM3 analyzers (Radiometer, Copenhagen, Denmark).

2.3.2 Experimental protocol

After preparation, piglets were allowed to recover from the surgical procedure until baseline hemodynamic measures were stable (change less than 10% over 20 min). Then, the animals were separated into two groups (n=6/group): 1) an operated normoxic group with no period of H-R, and 2) a H-R group where animals were exposed to alveolar normocapnic hypoxia at inspired oxygen concentrations of 10 to 15% for 120 min. At the end of hypoxia, the animals were in cardiogenic shock with decreased CO (<50% of normoxic baseline), severe hypotension and metabolic acidosis. The hypoxic period was followed by reoxygenation with 100% oxygen for 60 min and then 21% oxygen for 180 min (Figure 2.1). At the end of the

experiment, animals were euthanized with a bolus of 100 mg/kg pentobarbital, and the muscle of the left ventricle of the heart was harvested and freeze-clamped in liquid nitrogen for subsequent proteomic analysis.

2.3.3 Measurement of hemodynamic parameters

Heart rate (HR), mean arterial pressure (MAP) and cardiac output (CO) were continuously monitored and recorded throughout the experiment. Analogue outputs of the pressure amplifiers and flow monitors were digitized by a DT 2801-A analogue to digital converter board (Data Translation, Ontario, Canada). Software was custom written using the Assist programming environment. Stroke volume (SV) was calculated by dividing the CO by the HR. The hemodynamic parameters were analyzed at baseline, in the 60^{th} and 120^{th} min of hypoxia, in the 10^{th} and 60^{th} min of reoxygenation with 100% oxygen, and in the 60^{th}, 120^{th} and 180^{th} min of reoxygenation with 21% oxygen.

2.3.4 Preparation of heart extracts

Protein samples for 2-dimensional electrophoresis (2-DE) were prepared at room temperature by mixing frozen (-80°C), powdered heart tissue (40 to 60 mg wet weight) with 200 µl rehydration buffer (8 mM urea, 4% CHAPS, 10 mM DTT, 0.2% Bio-Lytes 3/10 [BioRad]). Samples were sonicated twice for 5 s and centrifuged for 10 min at 10000*g* at temperature 4°C to remove any insoluble particles. Protein content of the heart extract in rehydration buffer was measured with the BioRad protein assay in which dye binds to protein and differential colour

change occurs based on the amount of protein. Protein content based on absorbance was measured using the BioRad Model 680 Microplate Reader using a 595 nm filter. The efficient solubilization of contractile proteins using this method was verified to be suitable. For other biochemical studies, frozen heart tissue powder was homogenized on ice in 50 mM Tris-HCl (pH 7.4) containing 3.1 mM sucrose, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin and 0.1% Triton X-100. Homogenates were centrifuged at 10000*g* at 4°C for 10 min, and the supernatant was collected and stored at -80°C until use.

2.3.5 Immunoblotting

MLC1 protein content in the myocardium was determined by immunoblot. We separated 20 μg of protein from each heart extract using 15% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The separating gel used for SDS-PAGE contains 30% acrylamide (BioRad), 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate (BioRad) and N,N,N',N'-tetra-methyl-ethylenediamine (TEMED) [BioRad] while the stacking gel contains 30% acrylamide (BioRad), Tris/SDS pH 6.8, 10% ammonium persulfate (BioRad) and TEMED (BioRad). Electrophoresis was performed using the BioRad PowerPac Basic 300 at 150 V for approximately 30 min. Next, protein was transferred to a polyvinylidene difluoride membrane using the Semi Dry Transfer Cell (Bio-Rad) at 25 V for 30 min at room temperature. The membrane was then blocked overnight in 5% milk/TTBS buffer. MLC1 was identified using polyclonal anti-MLC1 antibody (from Santa Cruz Biotechnology Inc.). Band densities were measured using GS-800 calibrated densitometer and Quantity One measurement software 4.6 (BioRad).

2.3.6 Measurement of MMP-2 by zymography

Gelatin zymography was performed as described previously [78-79]. Briefly, heart extract preparations (30 µg of protein) were applied to 8% polyacrylamide gel copolymerized with 2 mg/ml gelatin. After electrophoresis, gels were rinsed 3 times for 20 min each in 2.5% Triton X-100 to remove SDS. The gels were then washed twice in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM NaCl, and 0.05% NaN_3) for 20 min each at room temperature and then incubated in incubation buffer at 37°C for 24 h. The gels were stained in 0.05% Coomassie Brilliant Blue G in a mixture of methanol: acetic acid: water (2.5: 1: 6.5, v:v) and destained in aqueous 4% methanol: 8% acetic acid (v:v). Developed gels were scanned with GS-800 densitometer (BioRad) and the MMP-2 activity was measured using Quantity One measurement software 4.6.

2.3.7 Immunoprecipitation

We incubated 300 µg heart extract proteins with 12 µg rabbit anti–MMP-2 IgG or 0.5 µg goat anti-MLC1 IgG in a total volume of 500 µl RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM PMSF) overnight at 4°C. This buffer was chosen because of its known high stringency to avoid unspecific binding. As a negative control, unrelated IgG was used instead of anti–MMP-2 or anti-MLC1 IgG. We added 100 µl of a slurry of protein A–Sepharose beads and incubated the mixture overnight at 4°C. The mixture was washed 3 times with 0.5 ml of RIPA buffer at 4°C

and 20 µl of sample buffer was added. The immunoprecipitates were analyzed by zymography for MMP-2 activity or by immunoblotting for MLC1 level.

2.3.8 2-dimensional electrophoresis (2-DE)

2-DE was performed as described [79]. After preparing the tissue extract, 100 µg protein was applied to each of 11 cm immobilized linear pH gradients (5 to 8) strips (IPG, BioRad), with rehydration for 16–18 h at 20ºC. For isoelectrofocusing (IEF), the BioRad Protean IEF cell was used with the following conditions at 20ºC with fast voltage ramping: Step 1: 15 min with end voltage at 250 V; Step 2: 150 min with end voltage at 8000 V; Step 3: 35 000 V-hours (approximately 260 min). After IEF, the strips were equilibrated according to the manufacturer's instructions. Second dimension of 2-DE was then carried out with Criterion pre-cast gels (8 to 16%) (BioRad). To minimize variations in resolving proteins during the 2-DE run, all gels were run simultaneously using a Criterion Dodeca Cell (BioRad). All the gels were stained in the same staining bath with the Silver Staining Plus kit (BioRad).

2.3.9 Image analysis

Developed gels were scanned using a GS-800 calibrated densitometer (BioRad). The intensity of spots from 2-DE gels was measured using PDQuest 7.1 software (BioRad).

2.3.10 Mass spectrometry (MS)

MLC1 protein spots were manually excised from the 2-DE gel. These spots were then processed using a MassPrep Station from Micromass using the methods supplied by the manufacturer. Briefly, the excised gel fragment containing the protein spot was first destained, reduced, alkylated, digested with trypsin and extracted. Analysis of the trypsin digest of MLC1 for $ONOO^-$ dependent modifications was performed on Q-TOF Ultima Global (Waters). 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 NCBI nr and Swiss-Prot databases with *Mammalia* specified. We used the Mascot (*www.matrixscience.com*) search engine to search the protein database.

2.3.11 Measurement of nitrite and nitrate $(NO_x)^-$ concentration

We measured $(NO_x)^-$ as a marker of induction of $ONOO^-$ biosynthesis. Myocyte homogenates were diluted 1:1 with deionized water and then deproteinized by centrifugal ultrafiltration (Ultrafree-MC micro-centrifuge tubes UFC3, Millipore Corporation, Bedford, MA). Ultrafiltrates were analyzed for total nitrate and nitrite content according to the method of Green [16]. Briefly, all nitrates were first reduced to nitrites online by passing the sample through a high-pressure liquid chromatography column packed with copper-coated cadmium. These nitrites were then reacted online with Griess reagent to produce an "azo" compound that was detected at 546 nm by means of a visible light detector. The concentrations of nitrite in each sample were quantified through generation of a standard curve using sodium nitrite.

2.3.12 Statistical analysis

The protein spot levels were analyzed using PDQuest measurement software and evaluated by t-test and/or Mann-Whitney test. The proteins of interest were identified by mass spectrometry. ANOVA or Kruskal-Wallis test was used in functional studies. Correlation was performed with Pearson Moment or Spearman test as appropriate. Data are expressed as the mean±SEM.

2.4 Results

2.4.1 Cardiac function

A comparison of hemodynamic parameters between the H-R and normoxic groups, as well as within the groups, is presented in Figure 2.1, and characterized below:

1. Stroke volume (SV). Along with the decreased CO, 2 h of hypoxia resulted in a significant decrease in SV due to the deterioration of the global contractile function of the left ventricle. However, upon reoxygenation, SV partially recovered but was still significantly lower than that of the normoxic group ($P<0.05$).

2. Heart rate (HR). Hypoxia initially induced tachycardia which subsequently disappeared in the second hour of hypoxia. After reoxygenation, heart rate was significantly higher than that of normoxic piglets over the last hour of the experimental period.

3. Cardiac output (CO). CO decreased during the hypoxic period in the H-R group reaching a significant difference from the normoxic group after 2 h of hypoxia. Upon resuscitation, CO

partially recovered but remained significantly lower than the normoxic group throughout reoxygenation ($P<0.05$).

4. Mean arterial pressure (MAP). Significantly reduced MAP in H-R subjects was observed during hypoxia ($P<0.05$). Upon reoxygenation, MAP partially recovered but remained significantly lower than that in the normoxic group throughout the reoxygenation period.

During normocapnic alveolar hypoxia (33±1 mmHg) and severe metabolic acidosis (data not shown) developed (7.05±0.03). Upon reoxygenation, the arterial pH of H-R piglets gradually normalized and was not significantly different from that of the normoxic group at the end of the experiment (7.32±0.02 vs 7.37±0.03, respectively).

2.4.2 Analysis of MLC1 level and MMP-2 activity

Immunoblot analysis of MLC1 levels revealed a significant decrease of MLC1 levels in the H-R group as compared to the normoxia group (28.28±5.70AU *vs* 45.75±2.82AU, respectively; p=0.0262) (Figure 2.2A). Myocardial MMP-2 activity measured by zymography was significantly higher in the H-R group as compared to the normoxic control (9.0±2AU *vs* 3.7±1AU) (Figure 2.2B).

2.4.3 Co-localization of MMP-2 with MLC1

Immunoprecipitation of myocardial MLC1 with anti-MMP-2 IgG and immunoprecipitation of myocardial MMP-2 with anti-MLC1 IgG from pig heart homogenates were performed and followed by immunoblot analysis for MLC1 level and zymography for

MMP-2 activity. Figure 2.3 reveals the co-localization of MLC1 with MMP-2 in normoxic and in H-R hearts. The quantitative comparison of normoxic *vs* H-R immunoprecipitates showed that the MLC1 level was approximately 50% higher and the MMP-2 level was approximately 25% higher in H-R hearts (206.2 AU vs. 319.1 AU and 28.7 AU vs. 35.3 AU respectively). The comparison of masses of MLC1 co-localized with MMP-2 with standard MLC1 (Figure 2.3, immunoblot) reveals a decrease of MLC1 mass by approximately 1000 Da.

2.4.4 Correlation of MLC1 level and MMP-2 activity with hemodynamic parameters

Stroke volume (SV), used as an indicator of systolic function of left ventricle, was decreased by approximately 30% in the H-R group. In the normoxic group (Figure 2.4A) there was a negative correlation between MLC1 level and stroke volume (SV) (r^2=0.97; p=0.0003). Also, a positive correlation of SV with MMP-2 activity (r^2=0.81; p=0.0383) was observed. When the relationship between MLC1 level and MMP-2 activity was analyzed (Figure 2.4B), a negative correlation was indicated (r^2=0.97; p=0.0003).

In the H-R group (Figure 2.5), in contrast to the normoxic group, there were no significant correlations between SV and MLC1 level or MMP-2 activity (Figure 2.5A). However, similar to the normoxic group but less profound, a negative correlation between MLC1 level and MMP-2 activity was observed (r^2=0.66; p=0.0497) (Figure 2.5B).

2.4.5 Analysis of MLC1 modifications and $(NO_x)^-$ levels using 2-DE followed by MS

2-DE was performed in order to separate the cardiac MLC1 (Figure 2.6A) and to use this purified protein for analysis of peroxynitrite-dependent modifications. Similarly to the results from immunoblotting (Figure 2.2A), the MLC1 level measured by 2-DE in the heart from H-R animals was lower in comparison to the normoxic group (70.6±21.9 *vs* 111.7±17.3, respectively; p=0.0049) (Figure 2.6B).

In order to assess the biosynthesis of $ONOO^-$, we measured $(NO_x)^-$ as a marker of its production. In hearts from the H-R group, the $(NO_x)^-$ level was approximately 2-fold higher than that in the control group (8.74±1.72 vs. 3.41±0.85 pmol/mg protein, respectively; P<0.005) (Figure 2.7A). The Q-TOF MS analysis of excised MLC1 protein spots from 2DE gels showed the S-nitrosylation of cysteine (C) 138 and the nitration of tyrosine (Y) 141 (Figure 2.7B). Both modifications are localized in direct proximity to the MMP-2 cleavage site which is located between asparagine (N) 140 and tyrosine (Y) 141 (Figure 2.7B). Modified amino acid residues were present in the hearts from only H-R rats.

2.5 Discussion

This is the first study to demonstrate not only the tight correlations between cardiac MMP-2 activity, cardiac MLC1 levels and heart systolic function in physiological conditions and to show that these correlations are disrupted in H-R animals, but also that H-R leads to increased degradation of myocardial MLC1 which is associated with increased myocardial MMP-2 activity and increased myocardial $ONOO^-$ production. Following H-R, the increased biosynthesis of $ONOO^-$ resulted in post-translational modifications of MLC1 protein including nitration and

nitrosylation. These modifications occurred adjacent to the site where MMP-2 cleaves MLC1 which suggests that they enhance the susceptibility of MLC1 to degradation by MMP-2.

The etiology of myocardial dysfunction during H-R is multifactorial. The myocardium undergoes many structural and biochemical changes during oxidative stress induced by H-R or by ischemia/reperfusion (I/R) [4, 30, 127-128]. The majority of these changes are due to the oxygen paradox, i.e., that reoxygenation initiates an inflammatory signalling cascade due to the increased generation of reactive oxygen species (ROS) that results in increased intracellular calcium influx and the development of metabolic acidosis [76, 129-131]. There is evidence that ROS action particularly that related to $ONOO^-$ is associated with increased MMP-2 activity in the extracellular space as well as within the cardiomyocyte [53-54, 90, 131]. The novel aspects of MMP-2 action in heart injury have been reviewed by Schulz [62] and the role of MMPs in cardiac diseases has been reviewed by Spinale [121]. It has been shown that MMP-2 degrades MLC1 during I/R injury of the heart [76]. Also it has been shown that the proteolysis of MLC1 leads to contractile dysfunction resulting from a decrease in the stability of the myosin neck region [132]. In addition, MLC1 degradation products have been observed in the heart after myocardial infarction in humans [133] and dogs [134].

In this study we did not treat H-R animals with MMP-2 inhibitors due to the limited number of experimental animals and the lack of selective, non-toxic MMP-2 inhibitors. However, based on the results of studies conducted by us and by others, we are convinced that the increased MLC1 degradation during H-R is MMP-2 dependent, and that the degradation, at least in part, might form the molecular basis of cardiac contractile dysfunction associated with oxidative stress following hypoxia or ischemia. The use of MMP inhibitors in cardiovascular

disorders is reviewed by Dorman and colleagues [135]. However, the question "how is the action of MMP-2 regulated inside the cardiomyocyte?" still remains unanswered.

MLC1 is a sarcomeric protein that appears to play an important role in cardiac muscle contraction; however its precise role has not yet been established [100]. MLC1 important for the structural stability of the α-helical lever arm domain of the MHC head. Although, the functional significance of the myosin lever arm has been well documented, the specific role of MLC1 in force development and the regulation of muscle contraction have yet to be resolved [109-110]. An alteration in sarcomeric structure could severely affect the contractile performance of the heart.

In this study we detected an increased level of nitrates $(NO_x)^-$ in H-R hearts which indirectly reflects an increased production of $ONOO^-$. Since the endogenous level of $(NO_x)^-$ also depends on dietary factors, the results have to be interpreted with caution. In the experimental setting used in our studies, animals from both groups were fed identically and thus the more than two-fold increase in $(NO_x)^-$ levels observed in the H-R piglets cannot be dependent on the diet. Because it has been shown that $ONOO^-$ increases MMP-2 activity under *in vitro* conditions [53, 90], it is reasonable to explain the increased activity of myocardial MMP-2 in the present study as being due to the action of myocardial $ONOO^-$. Furthermore, this study shows for the first time that nitration and S-nitrosylation of MLC1 occurs within H-R hearts. These modifications are most likely due to the enhanced $ONOO^-$ production and may increase the susceptibility of MLC1 to the proteolytic action of MMP-2. This speculation is based on evidence that the modified amino acid residue nitrated tyrosine (Y141) is located to adjacent to, and S-nitrosylated cysteine (C138) is located in close proximity, of a known cleavage site for MMP-2 in the MLC1 molecule [76] and thus these modifications may change the kinetic catalysis of MMP-2. Other

interesting observations from this study are the strong correlations between MLC1 levels with MMP-2 activity, and between MLC1 and MMP-2 with systolic function of the hearts in the normoxic group. These observations are the first to suggest that the modulation of MLC1 level by MMP-2 might be a part of the system regulating contractile function of cardiac muscle under physiological conditions. For instance, MMP-2 could be responsible for the normal physiological turnover of cardiac contractile proteins such as MLC1. These correlations were not observed in the H-R group.

Kanski and colleagues showed that MLC1 can be nitrated in the heart [136] or in the skeletal muscles [137] due to biological aging. The exact role of $ONOO^-$ on protein turnover is not known, however protein nitration increases its degradation by the proteolytic enzymes [46] and there is an accelerated degradation of mildly oxidized proteins during normal cellular function (reviewed in [138]). However, extensively oxidized proteins are poor substrates for the proteolytic enzymes and may accumulate in cells [139]. Thus our study, along with other studies showing that $ONOO^-$ activates MMP-2 [53, 90], give new information on the possible role of $ONOO^-$ in MLC1 degradation by MMP-2 during oxidative stress.

Hemodynamic changes (e.g., hypotension and cardiac dysfunction [140-142]) appear immediately in our piglets (Figure 2.1) and in 21-75% of human neonates [143] with severe hypoxia, although the myocardium of neonates might be more resistant to oxidative stress than the myocardium of adults. Evidence of hypoxic myocardial injury or necrosis in newborns leading to death [144-145] has also been shown. While the current guidelines on neonatal resuscitation continue to support the use of 100% oxygen, oxygen toxicity should be avoided [146]. Our experimental protocol of reoxygenation seems prolonged but mimics the clinical scenario when asphyxiated neonates are managed in community hospitals until the neonatal

transport team arrives 60-90 min later. Our results in normoxic conditions suggest that the actions of MMP-2 on MLC1 may play a normal physiological role in the regulation of the MLC1 level. Since the increased degradation of MLC1 by MMP-2 was also shown in ischemia-reperfusion injury [76], increased MLC1 degradation may be a common feature of oxidative stress. $ONOO^-$-dependent activation of MMP-2 as well as nitration and nitrosylation of its substrate, MLC1, appear to be important pathophysiological mechanisms leading to depletion of MLC1 during oxidative stress [147]. Therefore, in addition to antioxidants and ONOO- scavengers, pharmacological treatment with MMP-2 inhibitors and/or inhibitors of nitration/nitrosylation of MLC1 could, by reducing MLC1 degradation, lower heart injury during the resuscitation of asphyxiated newborns, and thus improve their long-term prognosis. The attenuation of I/R injury by MMP-2 inhibition with doxycycline in isolated rat hearts has been previously described [78]. Indeed a role for MMP inhibitors in the treatment of cardiovascular disease was suggested in a recent review [135]. Further studies are required to examine the effectiveness of this therapy *in vivo* as well as clinically when tetracycline agents are relatively contraindicated in the pediatric population because of the possible adverse osteogenic and cerebral effects.

In conclusion, in this study we have shown for the first time that the contractile dysfunction of the neonatal heart following H-R is associated with decreased MLC1 levels. Also, on the basis of previous studies by us and by others, we speculate that the decrease of MLC1 level is caused by proteolytic action of MMP-2. Since the ability to maintain cardiovascular function during hypoxia is an important determinant of immediate survival and future pathological outcomes, understanding the cellular mechanisms responsible for the deleterious

responses to hypoxia is a key step in reducing and ultimately preventing hypoxic/ischemic injury to cardiac and other tissues.

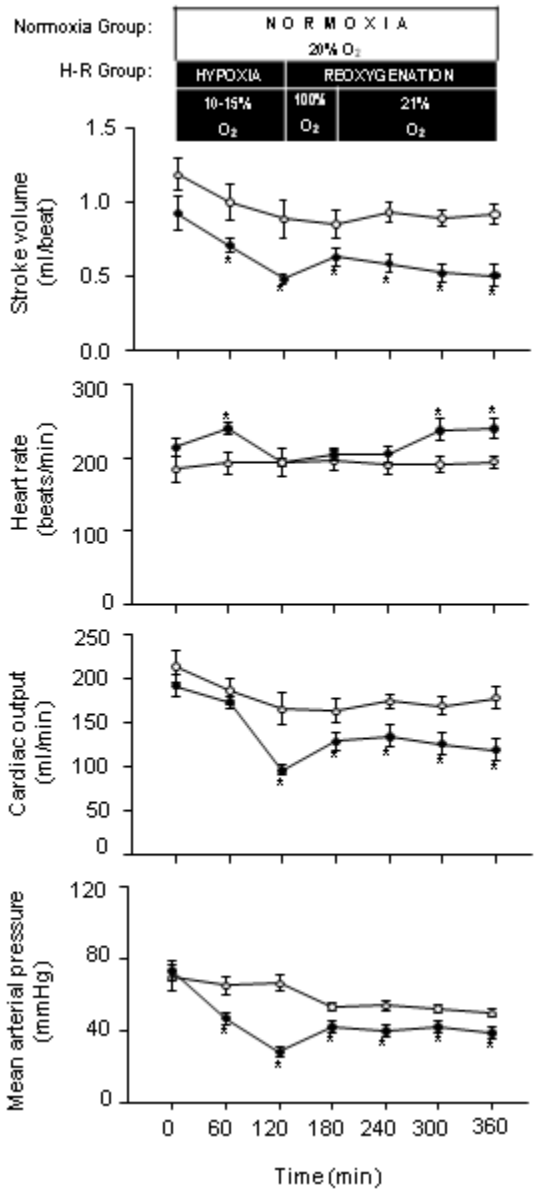

Figure 2.1 Hemodynamic data for newborn piglets (n=6, closed symbols) subjected to 2 h of hypoxia and 4 h of reoxygenation (closed bar). Open symbols and open bar represent normoxic (control) piglets (n=6). The following hemodynamic parameters were analyzed: stroke volume (SV), heart rate (HR), cardiac output (CO) and mean arterial pressure (MAP).

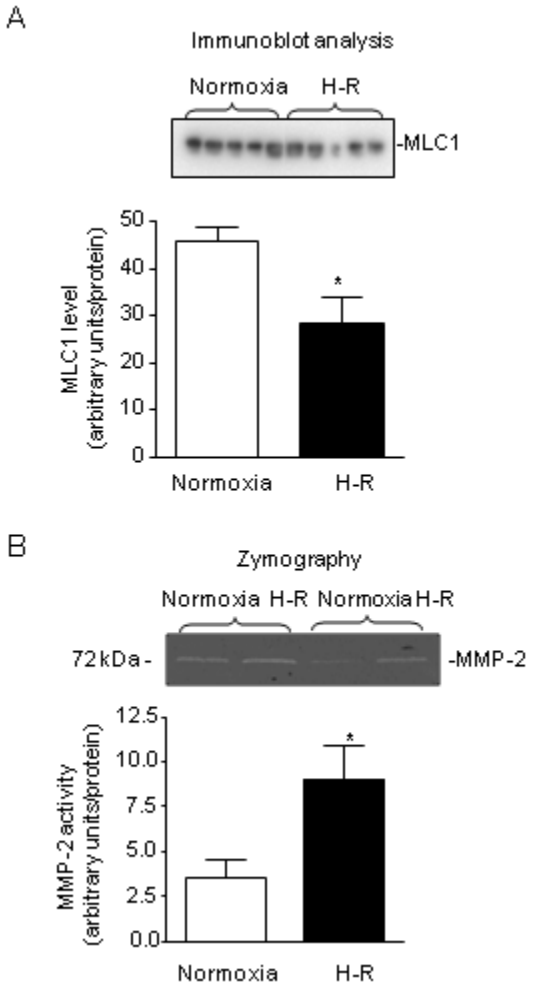

Figure 2.2 Quantitative analysis of MLC1 level by immunoblot, and MMP-2 activity by zymography, from normoxic heart tissue samples (n=6) and those subjected to H-R (n=6). A. Upper panel shows representative immunoblot. **p*<0.05 *vs* control. B. Quantitative analysis of MMP-2 activity in control (n=5) and H-R hearts (n=5). **p*<0.05 *vs* control

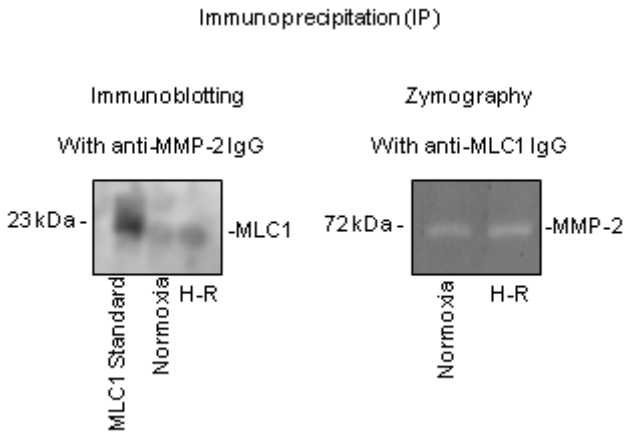

Figure 2.3 Immunoprecipitation with anti-MMP-2 IgG or anti-MLC1 IgG.

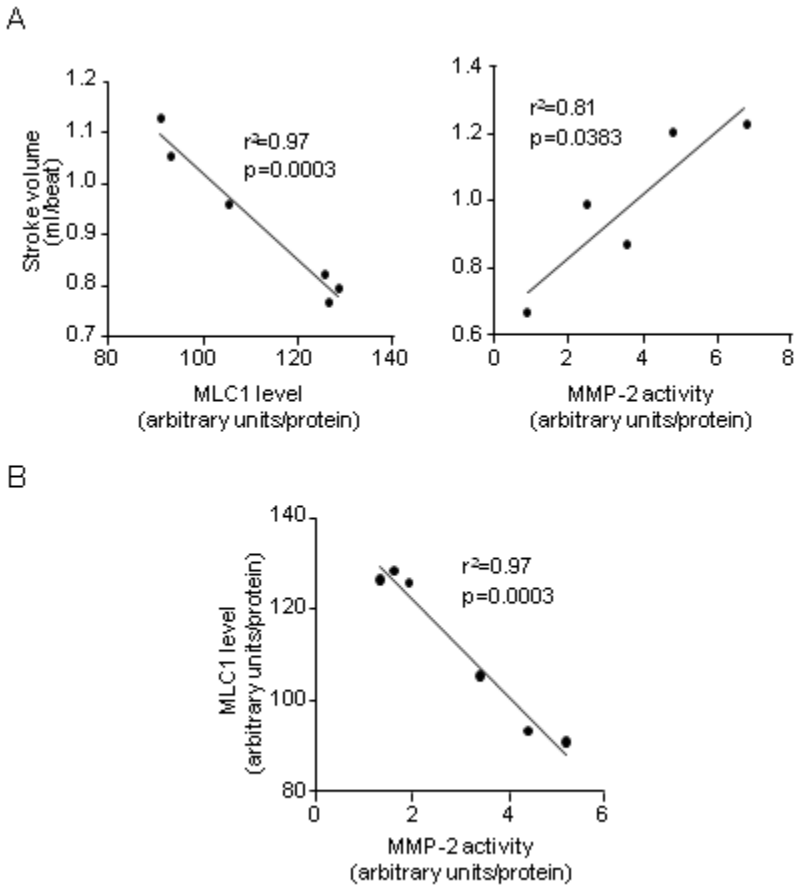

Figure 2.4 A. Correlation of cardiac function (represented by stroke volume) in normoxic piglets with MLC1 level and with MMP-2 activity. B. Correlation between MLC1 level and MMP-2 activity in normoxic hearts.

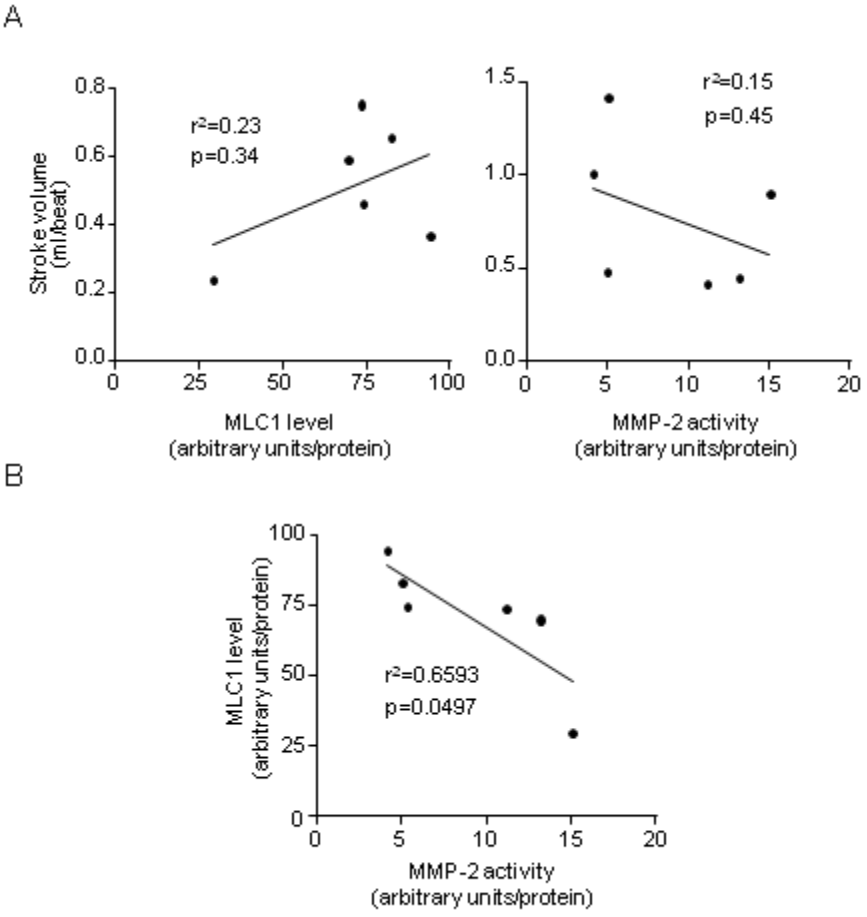

Figure 2.5 A. Correlation of cardiac function (stroke volume) with MLC1 level and with MMP-2 activity in piglets subjected to 2 hours of hypoxia followed by 4 hours of reoxygenation. B. Correlation of MLC1 level with MMP-2 activity in H-R hearts.

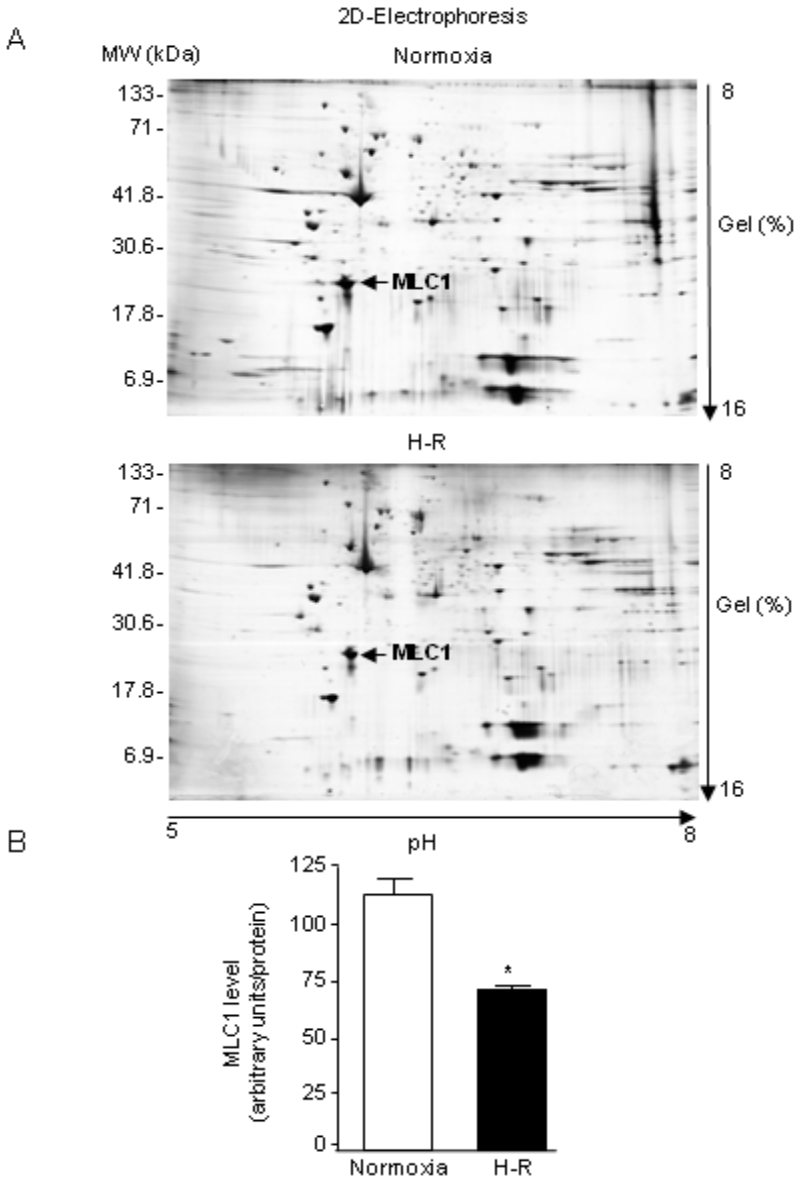

Figure 2.6 A. Representative gels of 2D electrophoresis of heart proteins from the normoxic group (n=6) and from the H-R group (n=6) are shown. Arrows point to the MLC1 spot. B. Quantitative analysis of MLC1 levels in heart tissue from normoxic and H-R hearts (n=6 each). *$p < 0.05$ vs. control.

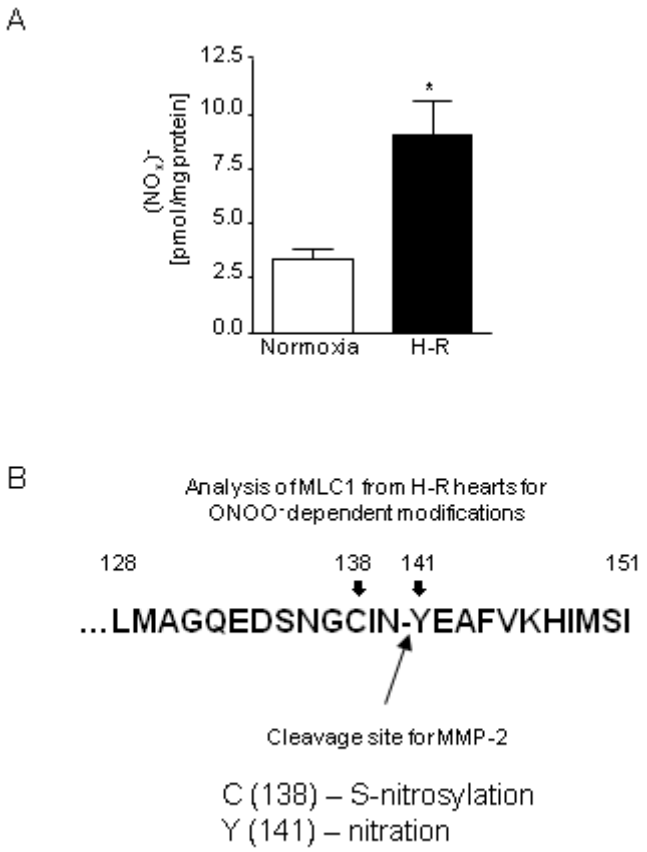

Figure 2.7 A. Comparison of $(NO_x)^-$ level between normoxic and H-R groups. **p*<0.05**.** B. Results from mass spectrometry (MS) analysis for nitration and nitrosylation of cardiac MLC1. The modified amino acid residues are indicated by the arrows.

CHAPTER 3

ISCHEMIA INDUCED PEROXYNITRITE DEPENDENT MODIFICATIONS OF CARDIOMYOCYTE MLC1 INCREASES ITS DEGRADATION BY MMP-2 LEADING TO CONTRACTILE DYSFUNCTION

Polewicz D, Cadete JJ, Doroszko A, Hunter BE, Sawicka J, Szczesna-Cordary D, Light PE, Sawicki G. *Ischemia induced peroxynitrite dependent modifications of cardiomyocyte MLC1 increases its degradation by MMP-2 leading to contractile dysfunction*. J Cell Mol Med, 2010. In press.

3.1 Preface

The contents of this chapter have been published in the "Journal of Cellular and Molecular Medicine". In Chapter 2, a whole heart model was used to assess the relationship between $ONOO^-$, MLC1 and MMP-2 during oxidative stress. Next, we wanted to study this relationship at the cellular level. Therefore in this chapter I present an experimental model of isolated rat cardiac myocytes which was used to assess the extent of damage to the cardiac contractile apparatus during ischemia itself. Furthermore, by using isolated myocytes we were able to analyze the role of intracellular MMP-2 on contractile dysfunction in absence of extracellular MMP-2. The manuscript presented in this chapter supports the hypothesis of my thesis, as well as the manuscript presented in Chapter 2, that during oxidative stress $ONOO^-$ modifies MLC1 which then leads to its increased degradation by MMP-2.

The majority of contents of the manuscript (including isolation of myocytes, measurement of contractility/viability, biochemical experiments, data analysis and manuscript preparation) were performed equally by my colleague Virgilio Cadete and myself and we share equal co-authorship of this manuscript.

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3.2 Introduction

Ischemia/reperfusion (I/R) injury consists of a complex series of processes ranging from metabolic to morphological and contractile adaptations in response to ischemia and/or reperfusion. However, since most observations are made after reperfusion, the weight of each

phenomena to the development of lethal injury remains a matter of controversy (reviewed in [148]).

The role of peroxynitrite ($ONOO^-$) in cardiac I/R injury has been extensively studied and there is evidence showing a burst in $ONOO^-$ generation at the beginning of reperfusion [48]. Even though an increase in generation of nitric oxide (NO) and superoxide anion (O_2^-), substrates for $ONOO^-$ synthesis, has been shown during ischemia [149-150], there is no evidence for increased $ONOO^-$ generation before beginning of the reperfusion phase. Peroxynitrite is a highly reactive oxidant which is generated from the coupling between nitric oxide and superoxide. Its detrimental action on the development of cardiac I/R injury, as well as its negative effects on cardiac systolic function, has been well established [30, 51, 151-154]. Nonetheless, the exact mechanisms by which $ONOO^-$ modulates I/R injury and systolic dysfunction remain to be explained.

The nitration of cardiac proteins, including myofilament components such as troponin T and I, alpha actin, and MLC1, during I/R has been previously described [117, 136, 155-158]; however, the mechanism and molecular consequences of this modification remain unknown. Recent studies by Kanski and colleagues have shown that myosin light chain 1 (MLC1), a protein of the muscle contractile machinery, can be nitrated in cardiac [136] and skeletal muscle [147] due to biological aging, but the consequence of this modification has not been established. In addition, it has been well documented that myocardial MLC1 is degraded by matrix metalloproteinase-2 (MMP-2) during I/R injury [76] and hypoxia/reoxygenation (H/R) [159]. MMP-2 is expressed ubiquitously in the heart, including the cardiac myocyte, and has been implicated in a variety of cardiovascular diseases [75, 121, 160]. MMP-2 exhibits both extracellular activity (degrading and remodelling of extracellular matrix) and intracellular

activity, namely the degradation of myofibrillar proteins [62, 123, 161]. Our most recent study on H/R, using an animal model of neonatal asphyxia, showed that the degradation of MLC1 by MMP-2 is associated with post-translational modifications of MLC1 such as tyrosine nitration and cysteine S-nitrosylation [159]. This study suggested that nitration and/or nitrosylation might play an important regulatory role in controlling intracellular proteolytic action of MMP-2 in MLC1 degradation.

Based on our previous work we hypothesize that ischemia induces $ONOO^-$- dependent MLC1 nitration/nitrosylation and that these modifications, together with increased intracellular MMP-2 activity, lead to increased degradation of MLC1 within the cardiomyocyte.

3.3 Methods

3.3.1 Preparation of cardiomyocytes and simulated ischemia protocol

3.3.1.1 Myocyte isolation

Adult male Sprague-Dawley rats were anaesthetized with sodium pentobarbital (150 mg/kg i.p.) and hearts were removed. Right ventricular myocytes were obtained by enzymatic dissociation as previously described [162-163]. First, the isolated heart was hung by the aorta onto a steel catheter of a perfusion system and was perfused at 37°C at 10 ml/min using three separate solutions. The heart was first perfused for 5 min using Solution A (120 mM NaCl, 5 mM KCl, 1.7 mM NaAc, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 20 mM $NaHCO_3$, 5.4 mM glucose and 1 mM taurine) which contains 100 µl of 1 M $CaCl_2$ per 100 ml of solution. Next, the heart was perfused for 8 min in Solution B which is the same as Solution A however it contains 0.5 µl of 1 M $CaCl_2$ per 100 ml of solution. The heart was then perfused for 12 min in Solution C (120 mM

NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.4 mM glucose, 1 mM taurine and 5.5 mM HEPES) which contains 4 µl $CaCl_2$ as well as 16.7 mg collagenase and 1.3 mg protease per 100 ml of solution. The heart was then removed from the canula and placed in a weight boat with Solution F which is the same as Solution C, however it also contains 3 µl of 1 M $CaCl_2$ and 150 mg of bovine serum albumin (Sigma) per 50 ml of solution. The right ventricle was then removed, cut into small pieces and placed into a flask containing 5 ml of Solution G which is the same as Solution F, however it also contains 15 mg collagenase and 1 mg protease per 15 ml of solution. Right ventricular myocytes (VMs) were used as we have found that they are a more suitable cell type to use for the experiments we performed for two reasons. First, it is much easier to obtain a consistently high yield of viable/live right VMs compared to left VMs. The enzymatic dissociation of VMs from the thicker left ventricle results in a much higher proportion of dead cells, over-digested cells or "groups" of under-digested cells. Second, right VMs are more homogeneous in their properties compared to left VMs that are isolated from the endo, mid and epi layers of the left ventricle [164].

Next, the flask was placed in a shaker water bath which was pre-heated to 37°C for 20 min after which the supernatant was removed and spun down at 2000*g* for 90 s in the IEC CL31R Multispeed Centrifuge (Mandel). The supernatant was discarded and the pellet resuspended in 3 ml of Solution G and then placed back into the shaker for 10 min. This digestion process was repeated two more times (at 5 min each) for a total of four digestions which we found to be desirable for isolation of cardiac myocytes. After isolation the cells were suspended in Solution F and enriched with 5% CO_2, 95% O_2 for 20 min to allow the cells to stabilize.

3.3.1.2 Simulated ischemia

Ischemia was induced by covering the cell pellets with a thin layer of mineral oil. This method is well established as a biological model of ischemia using isolated cardiomyocytes and is reviewed in [165]. At 15, 60 and 120 min of incubation (n=4 in each group) mineral oil was removed and the cells were rapidly frozen in liquid nitrogen. The control group was kept exposed to atmospheric air for 60 min at 37°C and then frozen. The viability of cardiomyocytes after ischemia was assessed by trypan blue exclusion test [165-167] which is based on the principle that live cells contain intact membranes and dead cells do not meaning that dye, such as trypan blue, will penetrate into dead cells staining them blue. First, 500 µl of cell suspension was spun down, the supernatant discarded and the pellet resuspended in 100 µl of Solution F. Next, 25 µl of trypan blue was added (1:5) and incubated at room temperature for 5 min after which a drop was placed onto a chamber of a hemocytometer (Exacta) and covered with a cover-slip. The hemocytometer was then placed on the Leitz Diavert inverted microscope and the both live and dead cells were counted in four 1 mm corner squares. The percentage of live cells was then calculated to determine viability.

Experiments in which FeTPPS, a scavenger of $ONOO^-$ [168] (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]) (Frontier Scientific), was present were done as follows: after isolation and stabilization, cardiomyocytes were incubated with FeTPPS (100 µM) for 10 min. As a negative control for FeTPPS, 100 µM of TPPS was used. After incubation, the cells were divided into equal suspension volumes and either subjected to ischemia as described above or maintained in aerobic conditions for control groups.

Similar to the experiments performed with FeTPPS, 1,10-phenanthroline monohydrate

(Sigma) was added to myocyte suspensions 10 min before the onset of ischemia at a final concentration of 100 μM. Since phenanthroline has poor solubility in water, a stock solution of 0.2 M was prepared in 100% ethanol and then diluted so that the final concentration of ethanol in the myocyte suspension was 5 x 10^{-4} % (v/v) and of phenanthroline 100 μM.

For assessment of the effect of ischemia on cardiomyocyte contractility, a 100 μl fraction of the cardiomyocyte suspension was placed on a glass coverslip mounted on an inverted microscope (Nikon Japan). After a stabilization period the chamber was perfused with oxygenated buffer at a constant temperature of 37˚C. Pacing was induced at 1 Hz and an amplitude of 5 V (IonOptix MyoPacer). Contractile function was measured using a side-mounted IonOptix MyoCam and the IonWizard 6.0 software which uses edge detection to record cell length and analyze cellular contractility. Peak shortening was recorded to determine the maximal percentage of shortening, maximal velocity of shortening was recorded to determine the velocity at which the myocyte contracts and the maximal velocity of relenthening was recorded to determine the velocity at which the myocytes relaxes to its resting length. An average of 3-5 cells per fraction was analyzed for 10 min for determination of contractile function.

3.3.1.3 Preparation of myocyte extracts

Protein samples for 2-dimensional electrophoresis (2-DE) were prepared by mixing frozen cardiomyocytes (30 mg wet weight) with 120 μl of rehydration buffer (8 M urea, 4% CHAPS, 10 mM DTT, 0.2% Bio-Lytes 3/10 (BioRad)) at room temperature. Samples were sonicated twice for 5 s and centrifuged for 10 min at 10000*g* at room temperature to remove any insoluble particles. Protein content of the heart extract in rehydration buffer was measured using the Bradford protein assay (BioRad).

For other biochemical studies frozen cells were homogenized on ice in 50 mM Tris-HCl (pH 7.4) containing 3.1 mM sucrose, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, and 0.1% Triton X-100. Homogenates were then centrifuged at 10000*g* at 4°C for 10 min and the supernatant was collected and stored at -80°C until further use.

3.3.2 Preparation of recombinant human cardiac MLC1

The cDNA clone for the human ventricular myosin light chain 1 (MLC1) (NCBI # NM_000258) was isolated with a two-tube RT-PCR method and Omniscript RT kit (Qiagen) using total adult human heart RNA (Stratagene), Oligo dT_{15} (Promega) and MLC1 specific primers. The sequence of MLC1 wild type (MLC1-WT) clone was verified and confirmed. MLC1-WT DNA was used to transform BL21 (DE3) Codon Plus competent cells (Stratagene). The MLC1 protein was expressed in 8 L of enriched media consisting of 30 g of peptone/L, 20 g of select yeast extract/L, and 10 g/L of M9 minimal salts with 20 μg/ml of ampicillin and purified using column chromatography (S-Sepharose, DEAE-Sephacel). The fractions of protein purity 97-99% were pooled and stored frozen at -80°C until needed.

3.3.3 2-dimensional electrophoresis (2-DE)

Protein (100 μg) was applied to each of 11 cm immobilized linear pH gradient (5-8) IPG strips (BioRad), with rehydration for 16–18 hours at 20ºC. For isoelectrofocusing (IEF), the BioRad Protean IEF cell was used with the following conditions at 20ºC with fast voltage ramping: Step 1: 15 min with end voltage at 250 V; Step 2: 150 min with end voltage at 8000 V;

Step 3: 35 000 V-hours (approximately 260 min). After IEF, the strips were equilibrated according to the manufacturer's instructions. Second dimension of 2-DE was then carried out with Criterion pre-cast gels (8-16%) (BioRad). After separation, proteins were stained with Silver Stain Plus kit (BioRad). To minimize variation in staining all gels were stained in the same bath. The reproducibility of 2-DE and quality of protein loading has been previously verified by us [76, 169-170]. Developed gels were scanned using a GS-800 calibrated densitometer (BioRad). Quantitative analysis of MLC1 protein spots' intensities from 2-DE gels were measured using PDQuest 7.1 software (BioRad).

3.3.4 Mass spectrometry (MS)

MLC1 protein spots were manually excised from the 2-DE gel. The spots containing rat cardiac MLC1 protein (2-DE) and preparations of human cardiac MLC1 protein from the *in vitro* study were processed using a MassPrep Station from Micromass using the methods supplied by the manufacturer. Briefly, the excised gel fragment containing the protein spot was first destained, reduced, alkylated, digested with trypsin and extracted. Mass analysis of the trypsin digest of MLC1 was performed on MALDI-TOF Voyager DE-Pro from Applied Biosystems and ESI Q-TOF Ultima Global (Waters). A mass deviation of 0.5 was tolerated and one missed cleavage site was allowed. Resulting values from mass spectrometry analysis for monoisotopic peaks were used to search the NCBInr and Swiss-Prot databases for *Rattus norvegicus* or *Homo sapiens*. The Mascot (*www.matrixscience.com*) search engine to identify the protein and to detect protein nitration and nitrosylation. The Mowse scoring algorithm [171] was used to justify accuracy of MLC1 protein identification which is incorporated in the Mascot search engine.

3.3.5 *In vitro* nitration/nitrosylation and degradation of MLC1 by MMP-2

Because rat cardiac MLC1 (accession number: P16409) is commercially unavailable, we used recombinant human cardiac MLC1 (accession number: P08590) for *in vitro* degradation of MLC1 by MMP-2. Using the LALIGN peptide comparison program (*www.ch.embnet.org/software/LALIGN_form.html*), the primary sequence of rat cardiac MLC1 was compared to human cardiac MLC1 showing 93.5% identity.

Recombinant human cardiac MLC1 was used for assessment of the $ONOO^-$ effect on *in vitro* degradation of MLC1 by MMP-2. The MLC1 (12 μg) was pre-incubated with $ONOO^-$ (0.01 mM, 0.1 mM and 1 mM) for 30 min at room temperature. This was followed by incubation with 200 ng of MMP-2 (Calbiochem) in 50 mM Tris-HCl buffer containing 5 mM $CaCl_2$ and 150 mM NaCl (total volume 40 μl) at 37ºC for 60 min, which corresponds to 60 min of ischemia. The reaction mixtures were analyzed by 12% SDS-PAGE under reducing conditions in which the separating gel contains contains 30% acrylamide (BioRad), 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate (BioRad) and N,N,N',N'-tetra-methyl-ethylenediamine (TEMED) [BioRad] while the stacking gel contains 30% acrylamide (BioRad), Tris/SDS pH 6.8, H_2O, 10% ammonium persulfate (BioRad) and TEMED (BioRad). The protein bands within the gel were then visualized by staining with Coomassie Brilliant Blue G-250 for 1-2 h at room temperature and destaining in aqueous 4% methanol: 8% acetic acid (v:v). Developed gels were scanned using a GS-800 calibrated densitometer (BioRad). The degradation of MLC1 was calculated using Quantity One 4.6 software (BioRad).

3.3.6 Examination of experimental peptide mass fingerprinting for nitration and nitrosylation

As an additional method for detection of protein modification, the FindMod tool (2007) was used to find $ONOO^-$ related post-translational modifications (PTMs) in the experimental tryptic peptides of MLC1 (*http://au.expasy.org/tools/findmod/*). Two types of PTMs were considered: nitration of tyrosine (Y) and S-nitrosylation of cysteine (C) with the assumption that up to 3 PTMs can exist in one tryptic peptide.

3.3.7 Measurement of MMP-2 activity

Gelatin zymography was performed as described [78]. Briefly, homogenates from myocyte preparations containing 30 μg of protein were applied to 8% polyacrylamide gel (30% acrylamide, Tris-HCl (ph 8.8), 10% ammonium persulfate and TEMED) copolymerized with 2 mg/ml gelatin. Electrophoresis was performed using the BioRad PowerPac Basic 300 at 150 V for approximately 30 min. After electrophoresis gels were rinsed three times for 20 min in 2.5% Triton X-100 to remove SDS. The gels were then washed twice in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM NaCl and 0.05% NaN_3) for 20 min at room temperature and incubated in incubation buffer at 37°C for 24 h. The gels were stained using 0.05% Coomassie Brilliant Blue G-250 in a mixture of methanol:acetic acid:water (2.5:1:6.5, v:v:v) and destained in aqueous solution of 4% methanol:8% acetic acid (v:v). Developed gels were scanned with GS-800 calibrated densitometer and MMP-2 activity was measured using Quantity One 4.6 software.

3.3.8 Measurement of nitrotyrosine levels

Nitrotyrosine level, although not a specific marker of $ONOO^-$-dependent tyrosine nitrosylation, has been the hallmark for the evaluation of the effects of $ONOO^-$ on protein modification. Nitrotyrosine and tyrosine concentrations in cardiomyocyte homogenates were determined by high pressure liquid chromatography (HPLC) as previously described [172-173]. Briefly, isolated adult cardiomyocytes were sonicated in 400 μl of sodium acetate (10 mM, pH 6.5) and vortexed for 1 h. Samples were then centrifuged at 12000*g* for 10 min and a 50 μl aliquot was removed and used for a protein assay according to the Bradford method which is based on the principle that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change [174] which was then analyzed using the BioRad Model 680 Microplate Reader. Proteolysis was performed by adding 150 μl of the supernatant to 25 μl of sodium acetate buffer and 50 μl of pronase (1 mg/ml in acetate buffer) followed by heating at 50°C for 18 h. After digestion, samples were dried in a Speed Vac system and the extract was dissolved in acetonitrile. Derivatization with 4-fluoro-7-nitrobenzeno-2-oxa-1,3-diazole was performed. A 10 μl aliquot was used for HPLC quantification as described [172].

3.3.9 Immunoblot analysis

Rat cardiac MLC1 and nitrotyrosine level was determined by immunoblot analysis. Protein (30 μg) from isolated cardiomyocyte homogenate was separated using 12% SDS-PAGE [175]. The separating gel used for SDS-PAGE contains H_2O, 30% acrylamide (BioRad), 1.5 M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate (BioRad) and N,N,N',N'-tetra-methyl-

ethylenediamine (TEMED) [BioRad] while the stacking gel contains 30% acrylamide (BioRad), Tris/SDS pH 6.8, H_2O, 10% ammonium persulfate (BioRad) and TEMED (BioRad). Electrophoresis was performed using the BioRad PowerPac Basic 300 at 150 V for approximately 30 min. Next, protein was transferred to a polyvinylidene difluoride membrane using the Semi Dry Transfer Cell (Bio-Rad) at 25 V for 30 min at room temperature. The membrane was then blocked overnight in 5% milk/TTBS buffer. MLC1 was identified using polyclonal anti-MLC1 antibody (Santa Cruz Biotechnology). Nitrotyrosine was identified using rabbit polyclonal anti-nitrotyrosine antibody (Cell Signaling Technology). Band densities were measured using GS-800 calibrated densitometer and Quantity One 4.6 software.

3.3.10 Immunoprecipitation

Immunoprecipitation of MMP-2 and rat cardiac MLC-1 was performed as previously described [159]. Briefly, 300 μg of protein from isolated cardiomyocyte homogenates were incubated with 12 μg of rabbit anti-MMP-2 IgG overnight at 4°C. Unrelated IgG was used as an internal negative control. The immunoprecipitates were analyzed by immunoblot for MLC1 level.

3.3.11 Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the differences between the groups. Post-hoc analysis was performed using the Tukey-Kramer multiple comparisons test. Analysis of spot densities of the 2-DE gels was carried out by the Mann-Whitney U-test and Student's t-test which are incorporated in the PDQuest measurement software. A p value of less

than 0.05 indicated statistically significant differences. Data are expressed as mean ± SEM.

3.4 Results

3.4.1 Effect of duration of ischemia on cardiomyocyte viability and contractility

After isolation, cardiomyocytes were subjected to 15, 60 or 120 min of ischemia. In order to determine the times of ischemia for this study we evaluated cell viability at the end of the different ischemic times. After 15 min of ischemia, cell viability and contractility were similar to control (Figure 3.1). Sixty minutes of ischemia significantly reduced cell viability but to a lesser extent than 120 min of ischemia (Figure 3.1A). To further validate our model, we measured contractility of isolated rat cardiomyocytes. The goal of this experiment was to determine the contractile status of isolated cardiomyocytes after different times of ischemia. As seen in panel B (Figure 3.1), peak shortening did not change after 15 min of ischemia but was significantly reduced after 60 min of ischemia. After 120 min of ischemia peak shortening was further reduced.

Since very low viability and severe impairment of contractility at 120 min of ischemia were observed, we decided in our study to analyze cardiomyocytes from 15 and 60 min ischemia groups. Fifteen minutes represents a mild ischemia with no significant cell death and 60 min represents an acute ischemic episode.

3.4.2 Peroxynitrite ($ONOO^-$) formation during ischemia in cardiomyocytes

The production of $ONOO^-$ was determined by measuring the levels of nitrotyrosine by HPLC and by immunoblotting in cardiomyocytes subjected to simulated ischemia and compared to the aerobic (control) myocytes. Analysis of the nitrotyrosine to tyrosine ratio showed a two-fold increase in nitration of tyrosine during 60 min of ischemia in comparison to the control group (Figure 3.2A). Immunoblot analysis (Figure 3.2B) showed association of increased levels of nitrotyrosines with increasing duration of ischemia. Increased nitration of tyrosine in the protein band corresponding to MLC1 standard (panel a) in both ischemic groups vs. the control group (panel b) was observed.

3.4.3 Analysis of MLC1 level and MMP-2 activity in cardiomyocytes

Two-dimensional polyacrylamide gel electrophoresis analysis of the MLC1 level in cardiomyocyte homogenates is presented in Figure 3.3A. As shown, the MLC1 level was significantly decreased in cells subjected to 60 min of ischemia. Similarly, a significant reduction of the MLC1 level was observed by immunoblot analysis (Figure 3.3B).

The specific activities of the 72kDa and 62kDa isoforms of MMP-2 in myocyte homogenates from control and ischemic groups are illustrated in Figure 3.4A. A significant, progressive increase in total MMP-2 activity was observed after 15 and 60 min of ischemia compared to control.

Immunoprecipitation of MMP-2 protein with anti-MLC1 IgG followed by immunoblotting for MLC1 demonstrated that MLC1 co-localizes with MMP-2 and that the

amount of the MMP-2-MLC1 complex (enzyme-substrate) increases with duration of ischemia (Figure 3.4B).

3.4.4 Effect of $ONOO^-$ on the degradation of recombinant human cardiac MLC1 by MMP-2 *in vitro*

After 60 min of incubation, degradation of human cardiac recombinant MLC1 was observed in all samples containing MMP-2, independent of pre-incubation of MLC1 with $ONOO^-$. Quantitative analysis showed that the degradation of MLC1 was significantly potentiated by pre-incubation with increasing concentrations of $ONOO^-$ (Figure 3.5A). A negative correlation (r=-0.999) between $ONOO^-$ concentration and the MLC1 level was observed (Figure 3.5B).

3.4.5 Mass spectrometry analysis for nitrations/nitrosylations within MLC1 peptide obtained from cardiomyocytes

In both, the 15 and 60 min ischemic groups nitration of tyrosines 78 (Y78) and 190 (Y190) of rat cardiomyocyte MLC1 were observed. Additionally, nitrosylation of cysteine 81 (C81) was observed in MLC1 but only in myocytes subjected to 60 min of simulated ischemia (Figure 3.6).

MS analysis of $ONOO^-$ treated recombinant human cardiac MLC1 from *in vitro* studies revealed that the tyrosine residues 73 (Y73) and 185 (Y185) were nitrated. In addition, tyrosine 130 (Y130) was nitrated in human MLC1 protein. Human cardiac MLC1 was nitrosylated at cysteine 67 (C67) and cysteine 76 (C76) corresponding to C81 of rat cardiac MLC1. We did not

observe the nitrosylation of rat cysteine corresponding to human C67 due to its substitution by glycine 72 (G72) (Figure 3.6).

3.4.6 Cardiomyocyte viability and function after simulated ischemia

After 15 min of ischemia, cell viability was similar to control, and after 60 min of ischemia cell viability significantly reduced (data not shown). To further validate our model we measured contractility of isolated rat cardiomyocytes. The goal of this experiment was to determine the contractility of isolated cardiomyocytes after ischemia. As seen in Figure 3.7 three parameters were analyzed in order to determine mechanical function of isolated myocytes: peak shortening, maximal velocity of shortening and maximal velocity of relengthening. As indicated, the three parameters did not change significantly after 15 min of ischemia but were significantly reduced after 60 min of ischemia (Figure 3.7).

3.4.7 Effect of treatment with selective $ONOO^-$ scavenger (FeTPPS) and MMP-2 inhibitor (phenanthroline) on cardiomyocyte contractile function.

Pre-treatment of isolated cardiomyocytes with 100 μM of FeTPPS, a scavenger of $ONOO^-$, prevented impairment of contractile function, as assessed by peak shortening, maximal velocity of shortening and maximal velocity of relengthening when compared to control (Figure 3.7).

Similarly, pre-treatment of isolated cardiomyocytes with 100 μM of the MMP-2 inhibitor phenanthroline resulted in a significant increase in peak shortening and maximal velocity of shortening after 60 min of ischemia, in comparison to ischemia alone (Figure 3.8). Since the

vehicle for phenanthroline was ethanol, aerobic cardiomyocytes were treated with 5 x 10-4 % (v/v) ethanol and function was assessed. Ethanol pre-treatment for 10 min did not affect function in comparison to non-treated cardiomyocytes, both aerobic or those subjected to 60 min ischemia.

3.4.8 Effect of treatment with selective $ONOO^-$ scavenger (FeTPPS) and MMP-2 inhibitor (phenanthroline) on MLC1 level

Immunoblot analysis showed that pre-treatment of isolated rat cardiomyocytes with 100 μM of FeTPPS (Figure 3.9) or with 100 μM of the MMP-2 inhibitor phenanthroline (Figure 3.10) resulted in protection of MLC1 against ischemic-induced degradation.

3.5 Discussion

This is the first study to demonstrate the importance of the $ONOO^-$-MLC1-MMP-2 axis in heart injury during ischemia. We show that during ischemia there is an increase in the generation of $ONOO^-$ which induces MLC1 post-translational modifications, such as nitration and nitrosylation, and that these modifications are responsible for increased degradation of MLC1 and impaired myocyte contractility.

The results from our study indicate a novel mechanism of intracellular regulation of MLC1 degradation during ischemia, which is based on $ONOO^-$-dependent changes in its susceptibility to MMP-2 dependent degradation. Analysis of tyrosine nitration by immunoblotting as well as by HPLC has shown an increase in $ONOO^-$ production in cells subjected to simulated ischemia compared to control aerobic cells. Mass spectrometry analysis of

MLC1 from the *in vivo* study showed nitration of tyrosines 78 and 190, as well as nitrosylation of cysteine 81 in the rat cardiac MLC1 protein, which were also present in homologous amino acid residues within human cardiac MLC1 used in the *in vitro* study. Because tyrosine 190 is located adjacent to the cleavage site for MMP-2 (Figure 3.6), the modification of this amino acid residue by $ONOO^-$ may explain the increased MLC1 degradation by MMP-2.

The role of other modifications remains unclear; however, modifications of tyrosine 78 and cysteine 81 might indicate a new proteolytic site for MMP-2 action within the MLC1 molecule. In contrast to results from studies on hypoxia-reoxygenation [159] we did not detect nitrosylation of cysteine 187 of rat MLC1, which corresponds to cysteine 138 of porcine skeletal MLC1, located in close proximity to the cleavage site for MMP-2. The lack of nitrosylation of cysteine 187 in rat cardiac MLC1 was most likely related to experimental model differences or conformational changes in the MLC1 molecule that could be species specific.

Our previous reports [76, 159-160, 176] suggest that studying post-translational modifications of MLC1 (including $ONOO^-$- related modifications) and its subsequent degradation by MMP-2 may provide new information regarding the mechanisms of ischemia-induced MMP-2-dependent degradation of intracellular proteins. Of importance is the concept of $ONOO^-$ scavenging by incubation of cardiomyocytes with agents neutralizing $ONOO^-$ deleterious actions such as FeTPPS. Pre-treatment with FeTPPS protected MLC1 against ischemia induced degradation by MMP-2. Therefore the use of a specific $ONOO^-$ scavenger in clinical practise may provide better cardiac protection against ischemic injury. This data is consistent with another study in which FeTPPS conferred protection of cardiac function against cytokine-induced injury [168]. Furthermore, phenanthroline, which is an inhibitor of MMP-2,

also protected contractile function and this protection was associated with preservation of the protein levels of intact MLC1.

These results may lead to the development of new therapeutic strategies for the treatment of ischemic injury. Future pharmacological targets may include the inhibition of nitration and nitrosylation of MLC1 and possible other protein targets together with inhibition of MMP-2 action. Inhibition of both targets might contribute to better synergistic cardiac protection following myocardial infarction.

In summary we conclude that: (a) ischemia is associated with increased generation of peroxynitrite, (b) peroxynitrite causes nitration and nitrosylation of MLC1 which enhances its degradation by MMP-2 during ischemia and (c) administration of a peroxynitrite scavenger or an MMP-2 inhibitor results in better recovery of viability and contractile function after ischemia.

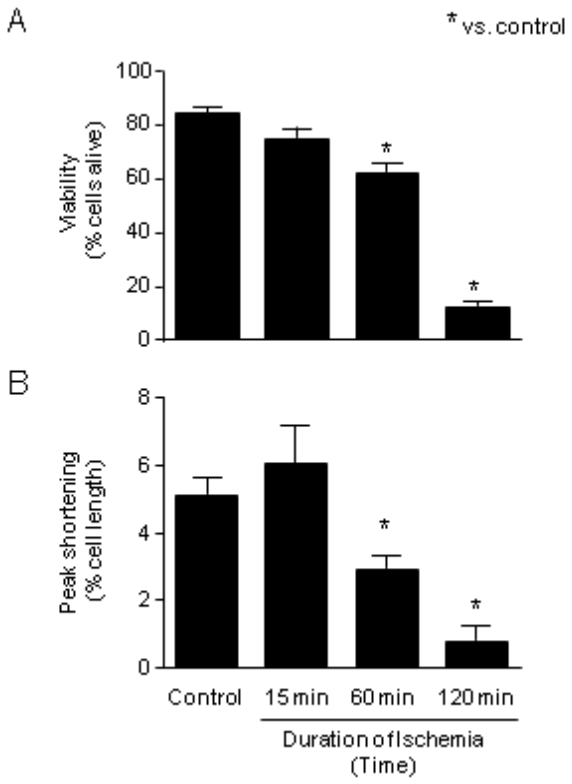

Figure 3.1 Effect of duration of ischemia on cardiomyocyte viability and contractility. A. Cell viability after simulated ischemia. Viability was defined as the percentage of live cells. B. Cell contractility (peak shortening) was measured with stimulation at 1 Hz, 5V. Results are presented as % of total cell length. Data are means ±SEM, n=4, *p<0.05.

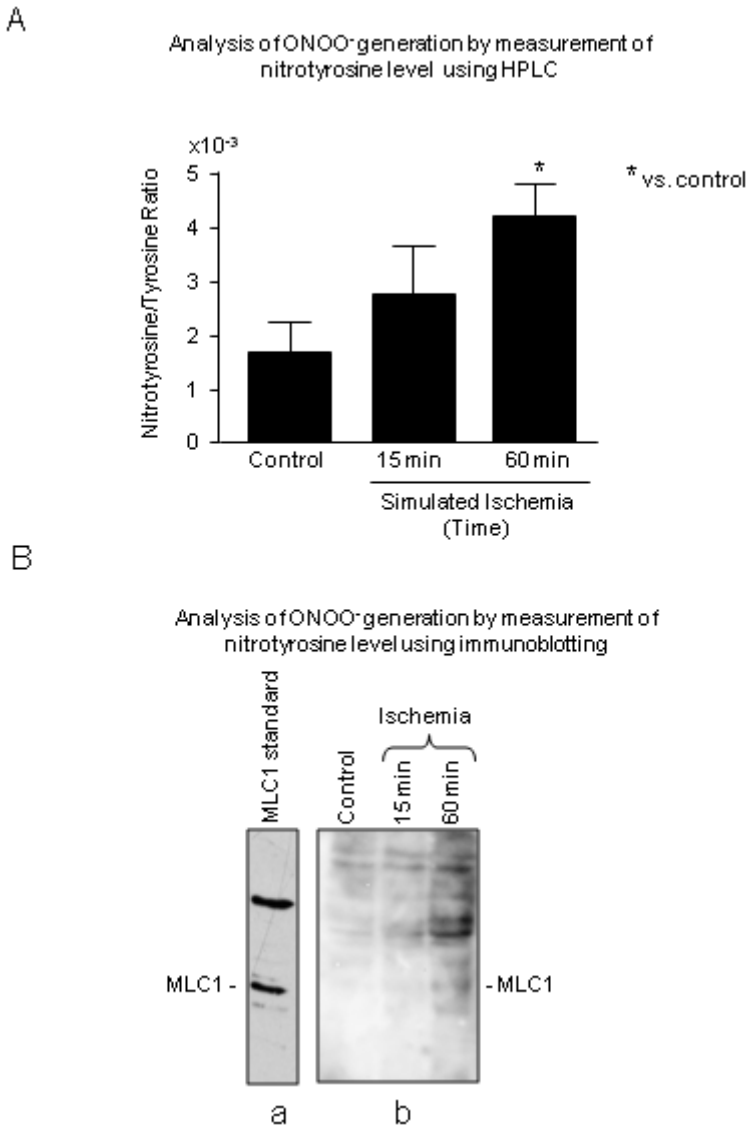

Figure 3.2 Nitrotyrosine levels in isolated cardiomyocytes subjected to 15 and 60 min of ischemia. A. Nitrotyrosine level analysis by HPLC. The $ONOO^-$ production level is shown as a ratio of nitrotyrosine to tyrosine. B. Detection of $ONOO^-$ production by measurement of nitrotyrosine (marker $ONOO^-$ production) using immunoblot analysis with anti-nitrotyrosine antibody. Data are expressed as mean±SEM, n=4/group; *$p<0.05$.

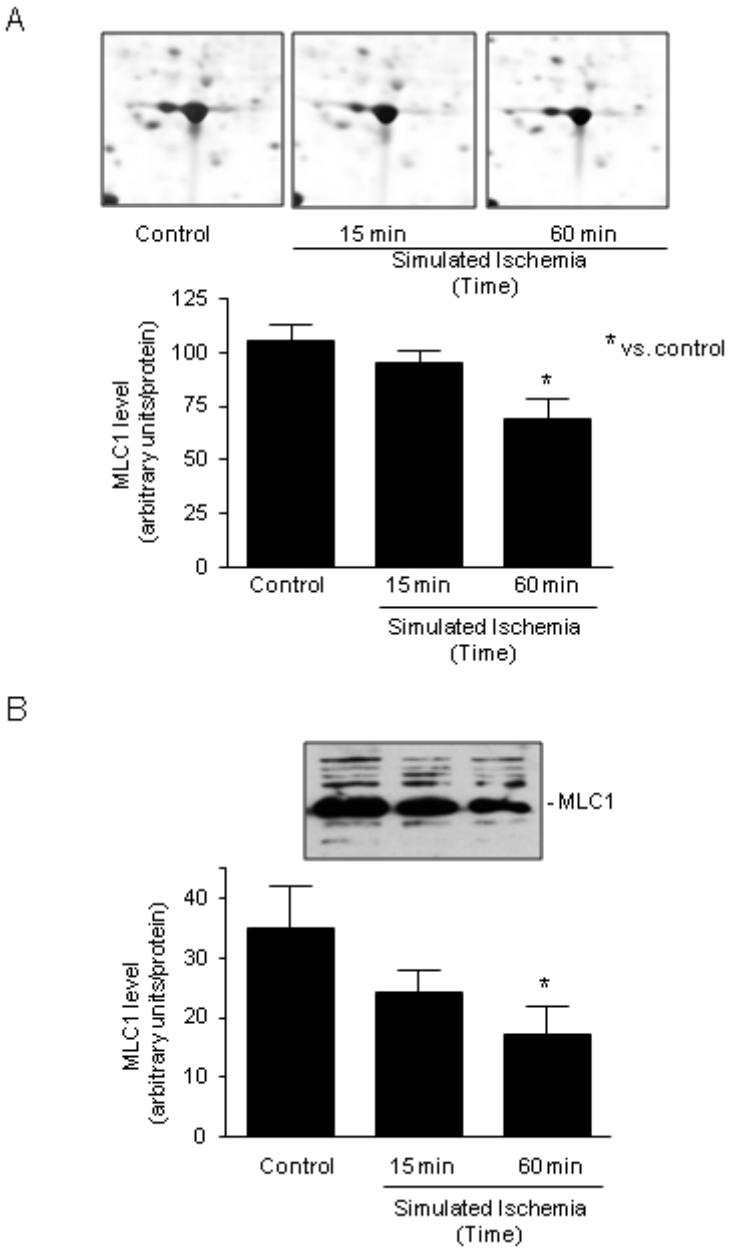

Figure 3.3 MLC1 levels during 15 and 60 min of simulated ischemia. A. Upper panel shows representative enlarged 2-dimensional electrophoresis gel fragments of the MLC1 region from control, 15 and 60 min ischemia groups. Lower panel shows the densitometric analysis of intensities of MLC1 protein spots. B. Immunoblot analysis of MLC1 level in control group and those groups subjected to 15 and 60 min of simulated ischemia. The insert shows a representative blot. Data are expressed as mean±SEM, n=4/group; *$p<0.05$.

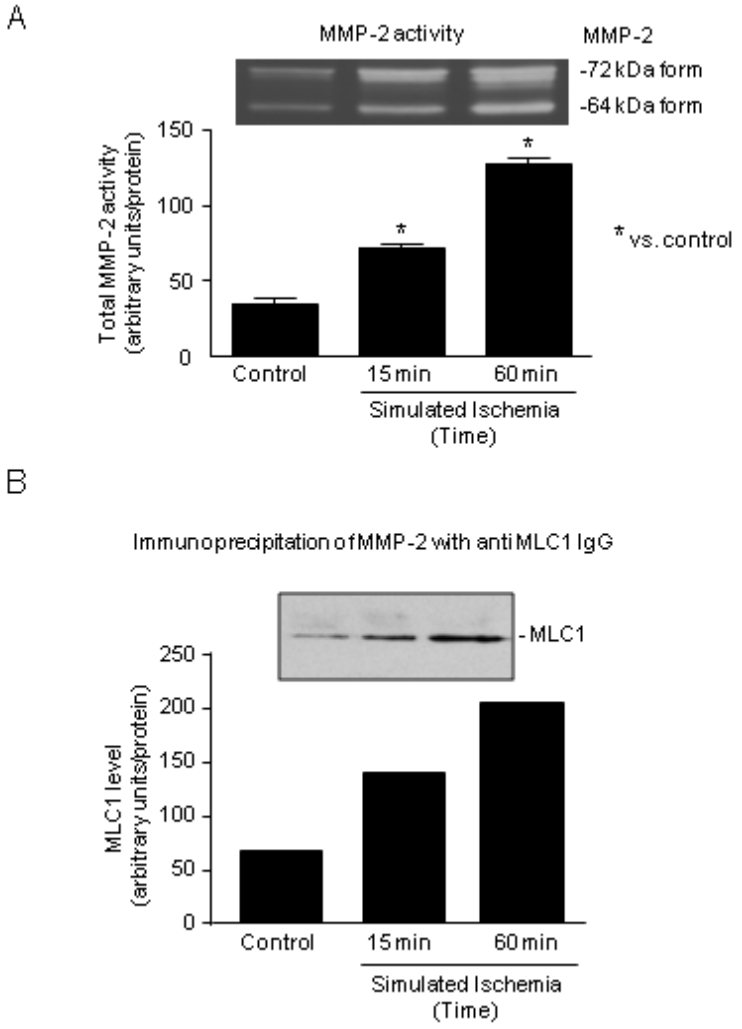

Figure 3.4 MMP-2 activity and co-localization of MMP-2 and MLC1 in isolated rat cardiomyocytes subjected to simulated ischemia. A. Analysis of total MMP-2 activity by zymography from control cardiomyocytes and those subjected to 15 and 60 min of simulated ischemia. The insert shows a representative zymogram. Data are expressed as mean±SEM, n=4/group; *$p<0.05$. B. Detection and assessment of MLC1 level co-localized with MMP-2 by immunoprecipitation.

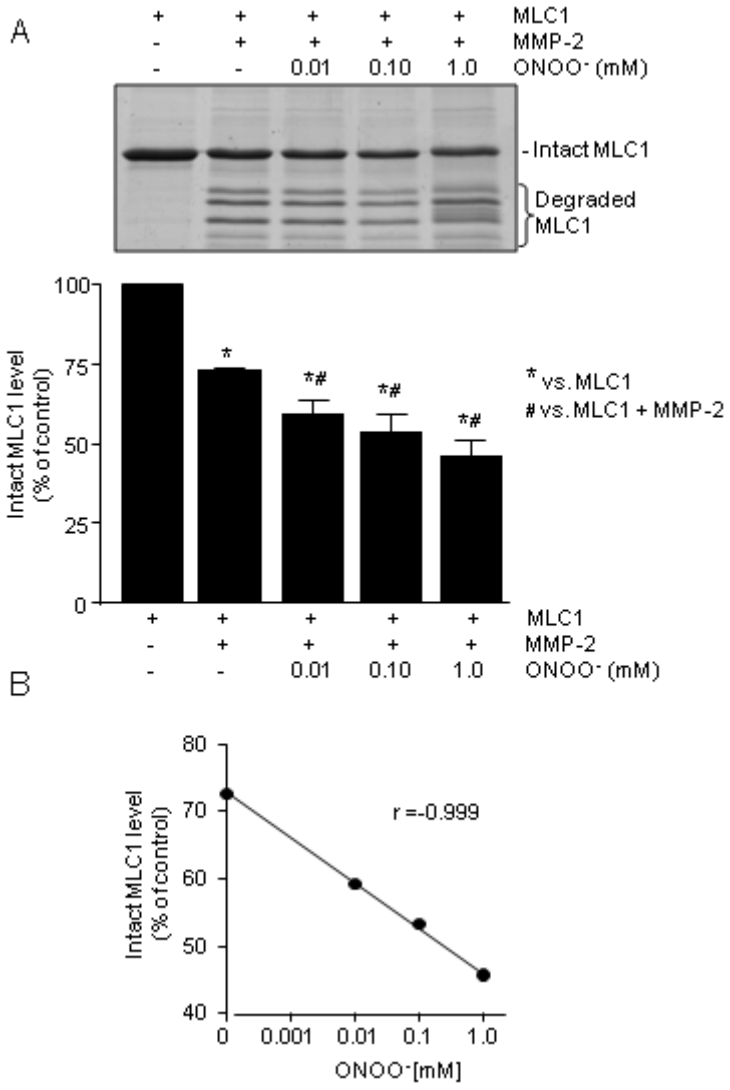

Figure 3.5 The effect of peroxynitrite ($ONOO^-$) on i*n vitro* degradation of recombinant human cardiac MLC1 by MMP-2. A. SDS-PAGE analysis of *in vitro* degradation of $ONOO^-$ treated MLC1 by MMP-2. The insert shows a representative SDS-PAGE. Data are expressed as mean±SEM, n=4/group; *$p<0.05$. B. Correlation between intact MLC1 level and $ONOO^-$ concentration

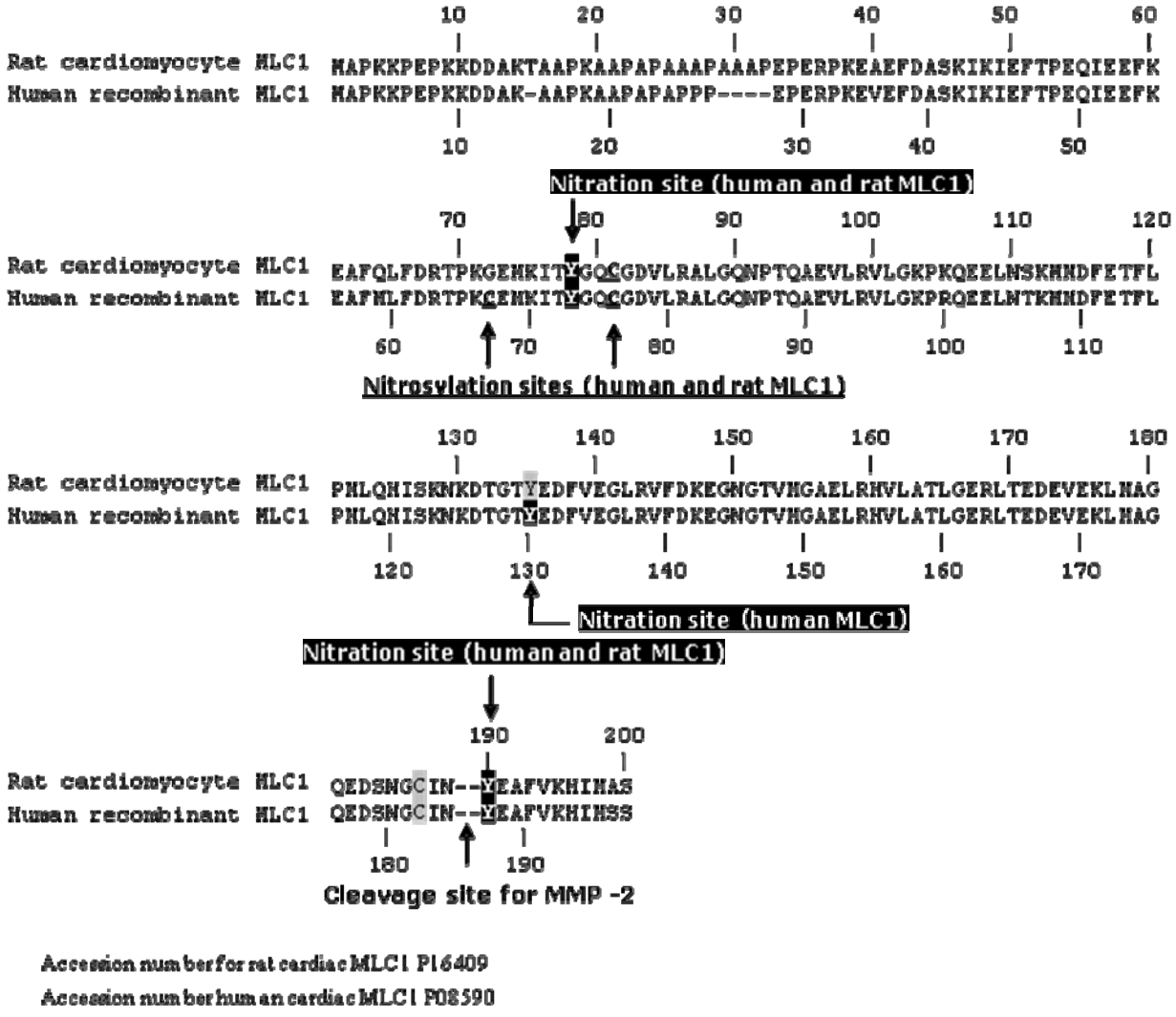

Figure 3.6 Mass spectrometry analysis for nitration and nitrosylation of human (P08590) and rat (P16409) cardiac MLC1. The nitrated amino acid residues are labelled in black boxes and nitrosylated amino acid residues are underlined. Both types of post-translational modifications (PTMs) are indicated by the arrows. Non-modified tyrosine and cysteine residues are also shown and are highlighted in gray. The cleavage site for MMP-2 located between asparagine 189 (N189) and tyrosine 190 (Y190) is indicated.

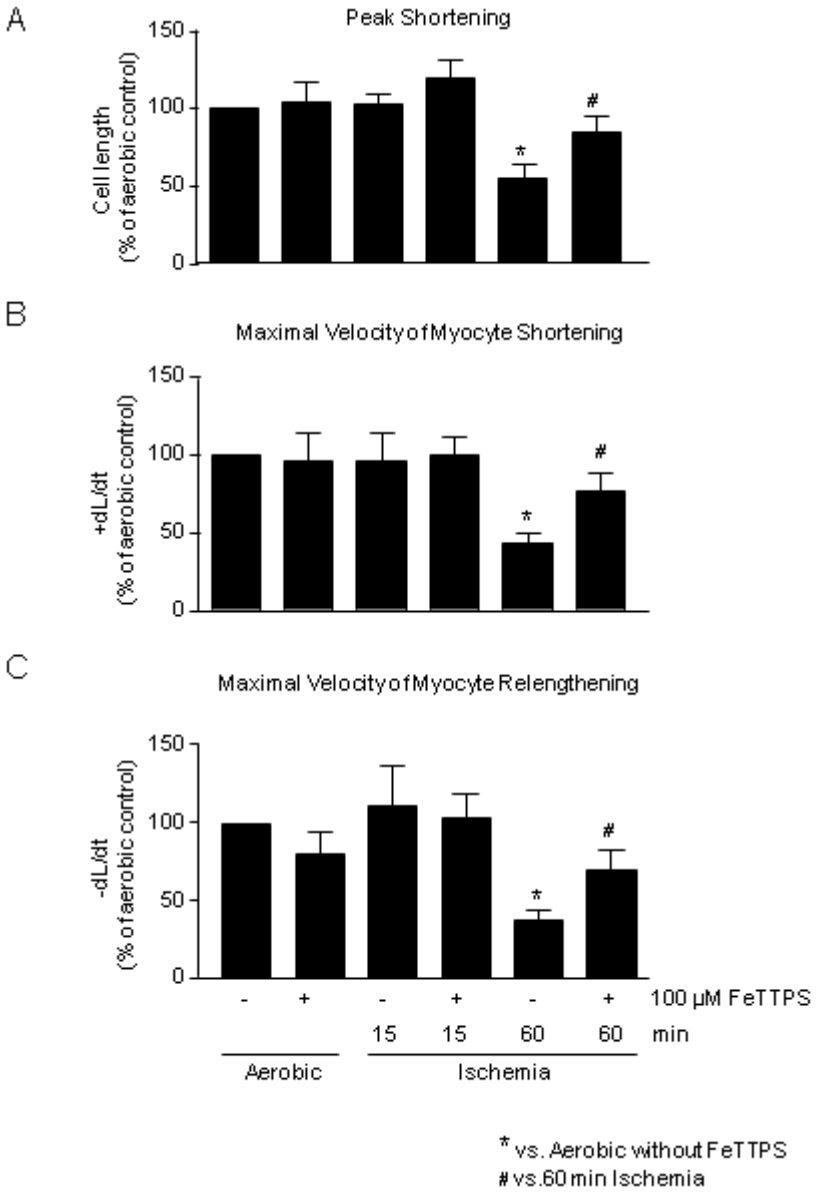

Figure 3.7 Evaluation of contractile function of isolated rat cardiomyocytes subjected to simulated ischemia. Freshly isolated rat cardiomyocytes were either maintained in aerobic conditions or subjected to 15 or 60 min of simulated ischemia. In parallel, groups were pre-treated with 100 µM FeTPPS, a scavenger of peroxynitrite, for 10 min prior to the onset of ischemia. Results are presented as percentage of aerobic control values. A. Function expressed as peak shortening. B. Function expressed as maximal velocity of myocyte shortening. C. Function expressed as maximal velocity of myocyte relengthening. Data are expressed as mean±SEM, n=4/group; *$p<0.05$ vs aerobic control, #$p<0.05$ vs. 60 min ischemia.

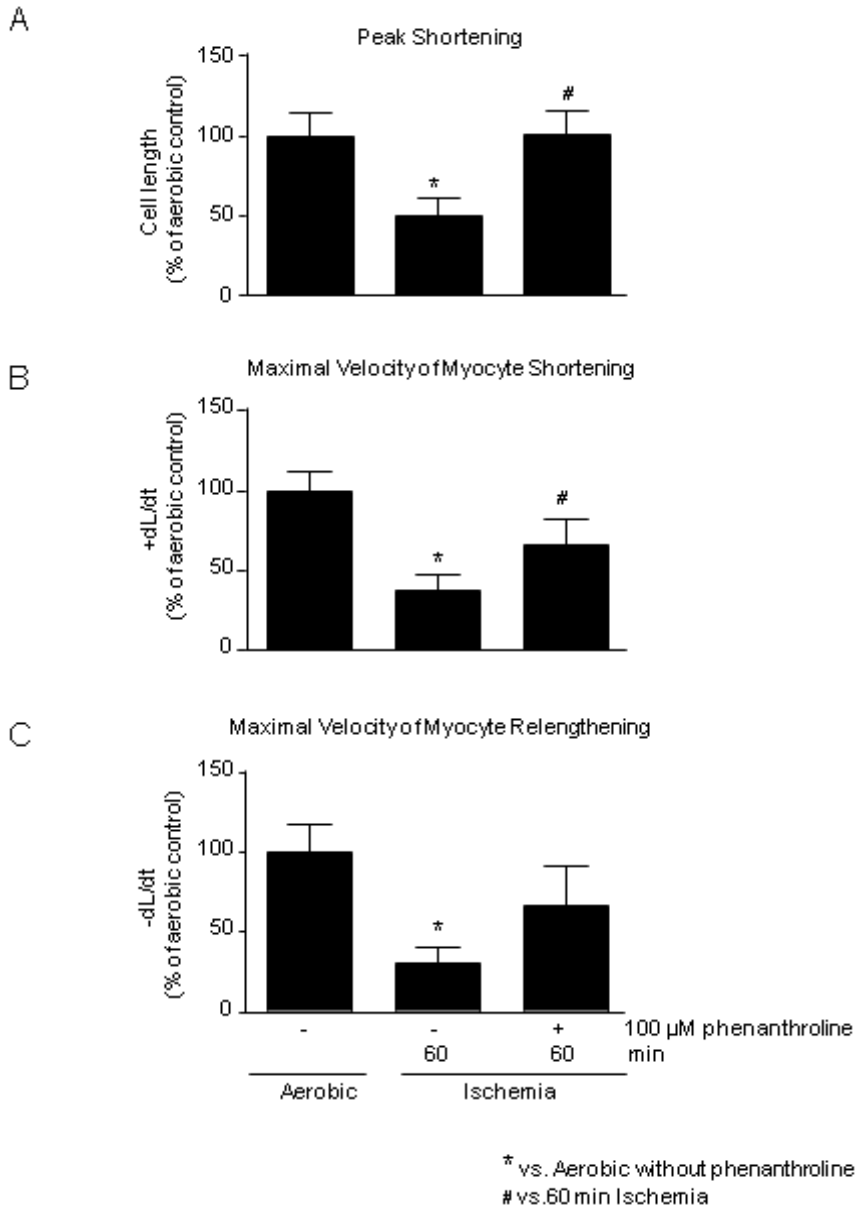

Figure 3.8 Evaluation of contractile function of isolated rat cardiomyocytes subjected to simulated ischemia with phenanthroline treatment. Freshly isolated rat cardiomyocytes were either maintained in aerobic conditions or subjected to 60 min of simulated ischemia. In parallel, groups were pre-treated with 100 µM phenanthroline, an MMP-2 inhibitor, for 10 min prior to the onset of ischemia. Results are presented as percentage of aerobic control values. A. Function expressed as peak shortening. B. Function expressed as maximal velocity of myocyte shortening. C. Function expressed as maximal velocity of myocyte relengthening. Data are expressed as mean±SEM, n=4/group; *$p<0.05$ vs aerobic control, #$p<0.05$ vs. 60 min of ischemia.

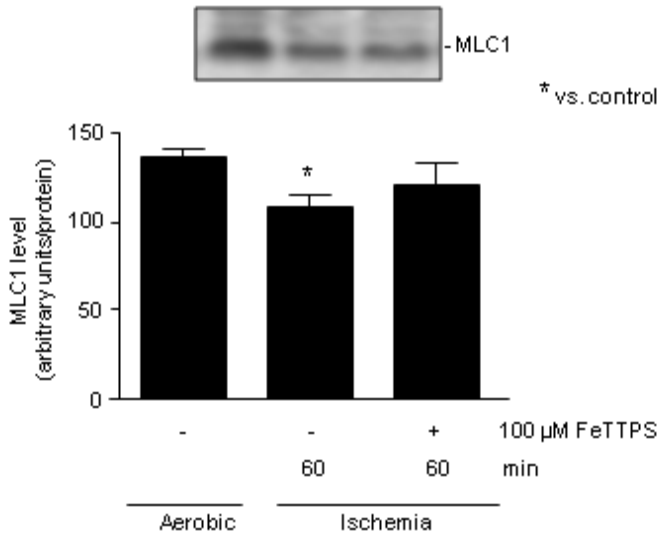

Figure 3.9 Protection of MLC1 degradation by peroxynitrite scavenger FeTPPS. Cardiomyocytes subjected to 60 min of ischemia in the presence or absence of FeTPPS pre-treatment were used for immunoblot analysis of MLC1 levels. Data are expressed as mean±SEM, n=4/group; *$p<0.05$ vs aerobic control.

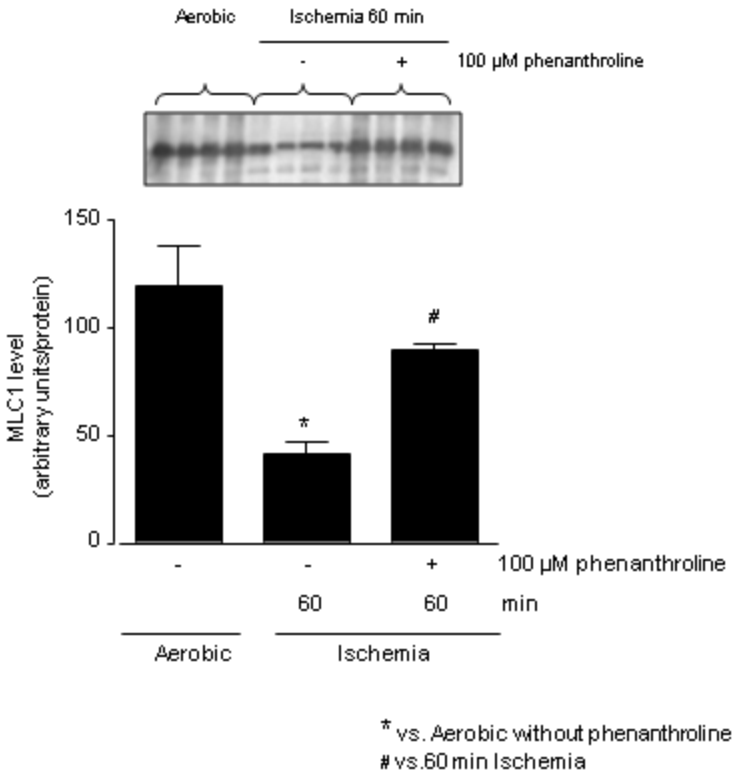

Figure 3.10 Protection of MLC1 degradation by the MMP-2 inhibitor phenanthroline. Cardiomyocytes subjected to 60 min of ischemia, in the presence or absence of phenanthroline pre-treatment, were used for immunoblot analysis of MLC1 levels. Data are expressed as mean±SEM, n=4/group; *$p<0.05$ vs aerobic control.

CHAPTER 4

CONCLUSIONS, LIMITATIONS AND

FUTURE DIRECTIONS

4.1 Conclusions

In the proposed research two experimental models, a piglet heart and isolated rat cardiac myocytes, were utilized in order to analyze potential oxidative stress-induced modifications to the crucial contractile protein myosin light chain 1 (MLC1) which leads to its degradation by the proteolytic enzyme matrix metalloproteinase-2 (MMP-2). This results in a dysfunctional contractile apparatus within the cardiac sarcomeres and leads to the subsequent development of cardiac dysfunction.

Since oxidative stress has been implicated in a variety of cellular dysfunctions which can lead to cell death, a model of hypoxia-reoxygenation (H/R) was used to assess the extent of reactive oxygen species production. An increase in generation of $ONOO^-$ has been observed in many systems including astrocytes which has been implicated in Alzheimer's disease [177], in skeletal muscles of patients with type 1 diabetes [178] and in the eye which is associated with ocular inflammation [179]. $ONOO^-$ has also been implicated in cardiac dysfunction following I/R injury [152, 180] leading us to hypothesize that $ONOO^-$ is involved in the degradation of contractile proteins such as MLC1. We have found that during H/R, there is in fact a substantial increase in $ONOO^-$ production which is associated with decreased levels of MLC1 and an increase in MMP-2 activity. Furthermore, mass spectrometry analysis revealed that $ONOO^-$-induced modifications of the MLC1 proteins were present including nitration of a tyrosine located adjacent to the cleavage site for MMP-2.

Next, using isolated myocytes, we wanted to determine the extent of damage which occurs during ischemia which may be useful for procedures such as angiography, since most studies focus on ischemia/reperfusion. This is useful in order to determine at which point during

oxidative stress damage to contractile proteins occurs. Both viability and contractility of isolated cardiac myocytes were reduced following 60 min of ischemia indicating that cardiac damage does in fact begin during ischemia and progressively develops during reperfusion. We observed an increase in the nitrotyrosine/tyrosine ratio during ischemia which corresponds to an increase in $ONOO^-$ generation. Furthermore, a decrease in MLC1 protein levels was observed which was associated with an increase in MMP-2 activity. Since this experiment was performed on isolated cardiac myocytes (i.e. in the absence of other cellular sources and extracellular targets for MMP-2) we can conclude that the generation of MMP-2 is attributed to the cardiomyocytes and that MMP-2 acts endogenously within the cardiac myocytes. This is furthermore supported by a study which has shown that MMP-2 is localized within the sarcomere of cardiomyocytes [75]. As with the H-R model, mass spectrometry allowed us to study modifications of MLC1 which may potentially be responsible for the degradation of this contractile protein. We found several modifications with the most important one being the nitration of a tyrosine adjacent to the cleavage site for MMP-2. Thus, MLC1 modification and degradation leading to cardiac contractile dysfunction does in fact begin during ischemia.

The $ONOO^-$ scavenger FeTPPS, which catalyzes the decomposition of this reactive compound, has been shown to confer cardioprotection during ischemia/reperfusion by minimizing ischemic injury [181]. In the present study we have found that FeTPPS improves viability and contractility of isolated cardiac myocytes by protecting these cells from $ONOO^-$-induced contractile injury. Since an increase in MMP-2 activity within isolated cardiac myocytes was associated with degradation of MLC1 during ischemia, we speculated that the use of an MMP-2 inhibitor would attenuate ischemia-induced contractile dysfunction. For this reason we used 1,10-phenanthroline monohydrate which chelates divalent metals such as zinc which is

present within the active site of MMP-2 and is necessary for its catalytic activity. Phenanthroline prevented the increase in MMP-2 activity which in turned minimized the degradation of MLC1 and therefore prevented ischemia induced contractile injury.

In summary, oxidative stress has been widely implicated in cardiac injury due to a rapid generation of increased levels of reactive oxygen species such as $ONOO^-$. In the present project, two experimental models, a whole heart as well as isolated myocytes, were utilized to determine that $ONOO^-$ levels increase substantially during oxidative stress. This increase is associated with degradation of the crucial contractile protein MLC1 as well as with an increase in activity of the proteolytic enzyme MMP-2. Furthermore, mass spectrometry analysis revealed $ONOO^-$- induced modifications of MLC1, particularly nitration of a tyrosine adjacent to the cleavage site for MMP-2. These results led to the conclusion that during oxidative stress (hypoxia-reoxygenation and ischemia) MLC1 is modified by $ONOO^-$ which increases its degradation by MMP-2 and results in cardiac contractile dysfunction. Pharmacological inhibition of MMP-2 activity (phenanthroline) as well as reduction of $ONOO^-$-induced damage (FeTPPS) may lead to the development of novel strategies for the treatment of pathologies during periods of oxidative stress.

4.2 Limitations

Although the data presented in this thesis are compelling, several limitations must be acknowledged. First and foremost, the two models utilized in this thesis were a heart model and an isolated cardiac myocyte model creating an environment very different from that observed in *in vivo* conditions, therefore the results from these *ex vivo* experiments should be interpreted with

caution when extending to *in vivo* conditions. *In vivo* experiments should be performed to verify these *ex vivo* results. Furthermore, the perfusion conditions were not identical to those observed in an *in vivo* model meaning that the buffer did not properly represent blood. Also, the pH, temperature, concentration of gases, etc. are not identical to that observed in an *in vivo* environment.

Peroxynitrite has a half-life of approximately 1.9 seconds [182] and is photosensitive meaning that it rapidly decomposes, therefore it is very difficult to measure its intracellular levels. For this reason nitrotyrosine levels are measured as a marker to analyze the effects of $ONOO^-$ on protein modifications. Measuring nitrotyrosine levels is thought to be a reliable index to analyze $ONOO^-$ formation [183-184], however by using this method we are speculating that these levels correspond to the concentration of $ONOO^-$. Furthermore, since $ONOO^-$ has a short half-life it is not possible to accurately determine the concentration that actually enters cells as well as the concentration that is being used in *in vitro* experiments. Also, FeTPPS is known as a selective $ONOO^-$ scavenger however we cannot exclude the possibility that it scavenges a number of other reactive oxygen species or cellular components.

Gelatin zymography is a sensitive method of analyzing MMP-2 activity, however this method detects only MMP-2 and MMP-9, therefore it is not possible to analyze the contribution of other MMPs which may also play roles in cardiac injury. Furthermore, with zymography we measure total activity of MMP-2 without taking into account TIMPs which are present in tissues and will significantly affect levels of MMP-2 activity.

4.3 Future Directions

The data presented in this thesis provide a solid base for future studies. In future experiments, an *in vivo* ischemia-reperfusion model (coronary occlusion followed by reperfusion) could be utilized to verify the results presented in this thesis as the experiments would be conducted under physiological conditions.

Up to date it has not been determined whether MMPs other than MMP-2 degrade MLC1 during oxidative stress. A deeper analysis into whether MMPs or other enzymes play a role in MLC1 degradation would be beneficial in order to better understand the mechanism of contractile dysfunction during oxidative stress and to find potential pharmacological targets to minimize cardiac injury. As this thesis indicates, post-translational modifications, nitration and nitrosylation, increase MMP-2 dependent MLC1 degradation. Identifying other post-translational modifications which MLC1 undergoes during oxidative stress will better contribute to our understanding of other potential ROS and mechanisms implicated in MLC1 degradation and development of cardiac contractile dysfunction.

Lastly, in this thesis I have presented data indicating that pretreatment of cardiac myocytes with the $ONOO^-$ scavenger FeTPPS and with the MMP-2 inhibitor phenanthroline protect MLC1 against oxidative stress-induced degradation, however no synergism experiments were conducted. Future experiments using specific $ONOO^-$ scavengers along with specific MMP-2 inhibitors will allow us to analyze potential synergistic effects of these classes of drugs which would contribute to better cardiac recovery following a period of oxidative stress. Furthermore, other intracellular targets, such as inhibition of nitration/nitrosylation of MLC1, may be identified to confer protection to contractile proteins such as MLC1.

CHAPTER 5

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