ROLE OF OXIDATIVE STRESS IN ISCHEMIA-REPERFUSION INDUCED ALTERATIONS IN MYOFIBRILLAR ATPASE ACTIVITIES IN THE HEART

BY

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A thesis submitted to the Faculty of Graduate Studies

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ABSTRACT

Although ischemia-reperfusion (IR) in the heart has been shown to produce myofibrillar remodeling and depress Ca^{2+}-sensitivity of myofilaments, the mechanisms for these alterations are not clearly understood. Since the generation of reactive species and oxidants is known to produce cardiac dysfunction in IR hearts, it is likely that changes in myofibrils occur as a consequence of oxidative stress due to IR in the myocardium. In this study, therefore, isolated rat hearts were subjected to global ischemia for 30 min followed by a 30 min period of reperfusion upon treatments with or without superoxide dismutase (SOD) plus catalase, an oxyradical scavenging mixture, or N-acetylcysteine (NAC), an antioxidant. Because IR is also known to produce intracellular Ca^{2+}-overload and subsequent proteolysis, experiments were carried out upon treating the hearts with leupeptin, an inhibitor of Ca^{2+}-dependent protease. Alterations in cardiac function were monitored by measuring left ventricular developed pressure (LVDP), rate of pressure development (+dP/dt), rate of pressure decay (-dP/dt) and left ventricular end diastolic pressure (LVEDP) whereas changes in myofibrils were determined by measuring myofibrillar ATPase activities and gene expression for α- and β-myosin heavy chain (MHC) as well as myosin light chain 1 (MLC1).

IR was found to induce cardiac dysfunction as reflected by depressed LVDP, +dP/dt and -dP/dt as well as elevated LVEDP. These changes in cardiac function were simulated upon perfusing the heart with xanthine (X) plus xanthine oxidase (XO), an oxyradical generating system, and H_2O_2, an oxidant. Depression in myofibrillar Ca^{2+}-stimulated ATPase activity in the IR heart was not associated with any changes in

myofibrillar Mg^{2+} ATPase. Although myofibrillar Ca^{2+}-stimulated ATPase activity was depressed, Mg^{2+} ATPase activity was increased in hearts perfused with X and XO. The mRNA levels for α-MHC, β-MHC and MLC1 were decreased in IR hearts. Alterations in cardiac function, myofibrillar Ca^{2+}-ATPase as well as gene expression for α-MHC, β-MHC and MCL1 in IR hearts were attenuated by pretreatments with SOD plus catalase mixture, NAC and leupeptin. These results suggest that oxidative stress and associated intracellular Ca^{2+}-overload play an important role in myofibrillar remodeling in hearts due to IR injury.

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LIST OF ABBREVIATIONS

ALC	atrial light chain
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
cDNA	complementary DNA
CON	control hearts
CT	control with treatment
DNA	deoxyribonucleic acid
+dP/dt	rate of pressure development
-dP/dt	rate of pressure decay
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid
IR	ischemia-reperfusion
IRT	IR hearts with treatment
K-H	Krebs Henseleit solution
LDL	low density lipoprotein
LVDP	left ventricular developed pressure
LVEDP	left ventricular end diastolic pressure
MDA	malondialdehyde
MHC	myosin heavy chain

MLC	myosin light chain
NAC	N-acetylcysteine
MLCK	myosin light chain kinase
mRNA	messenger RNA
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
SL	sarcolemma
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
SSC	standard saline citrate
TCA	trichloroacetic acid
VLC	ventricular light chain
X	xanthine
XO	xanthine oxidase

I. LITERATURE REVIEW

Ischemia-reperfusion (IR), a clinically relevant problem, is associated with thrombolysis, coronary bypass surgery and angioplasty, where blood flow is restored following myocardial ischemia (1-5). Restoration of blood flow to the jeopardized myocardium is crucial for its salvage from the IR injury, which is generally reduced if reperfusion is carried out within 3 hours from the onset of regional myocardial ischemia. On the other hand, the ischemic heart may be subjected to injurious insult, which is termed "myocardial IR injury", if reperfusion is not carried out within a certain time period. The IR injury includes a series of events like arrhythmias, microvascular damage, myocardial stunning, ultrastructural damage and cell death, which may occur either together or separately. There are two hypotheses which explain the mechanisms of IR injury: (a) oxidative stress and (b) intracellular calcium overload; however, it is not clear whether these two events occur simultaneously or one precedes the other (3, 5). Oxidative stress results from increased production of reactive oxygen species (ROS) that subsequently act on various subcellular organelles such as sarcolemma (SL), sarcoplasmic reticulum (SR), mitochondria and myofibrils to produce cardiac dysfunction (1-5). Defects at the level of SL, SR and mitochondria are considered to be associated with the development of intracellular Ca^{2+}-overload whereas defects in myofibrils are believed to explain contractile abnormalities of the IR hearts (3, 5-7). Changes in subcellular activities due to oxidation of functional groups of different proteins by oxidative stress and proteolysis by intracellular Ca^{2+}-overload have been proposed to explain the acute adverse effects of IR (1). On the other hand, the chronic effects of IR on cardiac function are considered to be due to the actions of both oxidative stress and

intracellular Ca^{2+}-overload on cardiac gene expression and subcellular remodeling (1). While a considerable amount of work on the role of oxidative stress and intracellular Ca^{2+}-overload in IR injury has been the subject of several detailed reviews (1-7, 13), it is planned to describe some of the salient features of these mechanisms in this section. Furthermore, in view of the extensive information available on SL, SR and mitochondrial abnormalities and the relative lack of information regarding myofibrillar defects in IR hearts (1-5), it is intended to focus this review on changes in myofibrillar assembly due to IR.

A. Oxidative stress in ischemia reperfusion injury:

Oxidative stress is a crucial pathophysiological mechanism for the occurrence of IR injury. Hack et al (8) have shown that oxidative stress in IR murine hearts is associated with impaired contractile function, cell damage, impaired endothelial-dependent dilation in response to ADP and decreased glutathione redox status. Similarly, Molyneux et al (9) have demonstrated the role of oxidative stress in IR injury in isolated buffer-perfused rat hearts indicating that oxidative stress causes increased malondialdehyde (MDA) levels in the coronary effluent, luminal membrane blebs and capillary constriction; these effects were prevented upon treatment of the hearts with a combination of some antioxidant vitamins. Further, the role of oxidative stress in IR is supported by the observations that vanadate and selenium as well as a mixture of superoxide dismutase (SOD) plus catalase, which are found to possess antioxidant actions, produce beneficial effects on the IR hearts (10-12). It should be pointed out that there are different antioxidant defense mechanisms in the living organism, which

maintain the cell survival against oxidative stress. Table 1 shows a list of some endogenous antioxidants and their sites of action in cardiomyocytes. During IR, these defense mechanisms are impaired and thus lead to deleterious effects of oxidative stress. There are various sources of ROS production such as mitochondrial electron transport system, xanthine oxidase reaction, neutrophil activation, arachidonic acid metabolism and autoxidation of catecholamines which participate in the development of oxidative stress (13) and some of these are discussed below:

(i). Mitochondrial electron transport system:

Mitochondria are the major physiological source of ROS within most cells. The electron transport system of mitochondria is involved in oxidative phosphorylation leading to the production of ATP as an energy source for the cells. Molecular oxygen is the final acceptor of electrons during electron transport. Degradation of ATP pool during ischemia as well as reoxygenation during reperfusion causes electron leakage from the mitochondrial membrane and leads to the formation of excess ROS. In fact, mitochondria are both targets and sources of damage during IR (14, 15). Cardiac ischemia damages the mitochondrial transport chain (16) and decreases the rate of oxidative phosphorylation (17). Chen et al (18) have shown that treatment of the heart with amobarbital, an inhibitor at the rotenone site of complex I in the mitochondrial electron transport system, reduces myocardial injury following reperfusion

(ii). Xanthine oxidase reaction:

Xanthine oxidase is a widely reported source of free radicals in the IR tissue (19). It is localized in vascular endothelial cells and exists in the dehydrogenase form in vivo, which uses NAD^+ as an electron acceptor during the oxidation of hypoxanthine to

Table 1. Major endogenous antioxidants and their actions in cardiomyocytes.

Antioxidants	**Action**
Superoxide dismutase(SOD): Cu SOD, Zn SOD, and MnSOD	Catalyzes the dismutation of O_2^- to O_2 and H_2O_2
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$
Glutathione peroxidase	$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$
Glutathione	Cellular reductant
Coenzyme Q10	Redox active electron carrier
Vitamin E	Inhibits lipid peroxidation
β-Carotene	Inhibits oxidation of LDL
Vitamin C	Acts as a reducing agent or a cofactor for vitamin E

H_2O_2, hydrogen peroxide; O_2^-, superoxide radical; GSH, reduced glutathione; GSSG, oxidized glutathione; LDL, low density lipoprotein.

xanthine and xanthine to urate. Studies on isolated intestine model have shown that ischemia promotes conversion of xanthine dehydrogenase to xanthine oxidase (20). Both hypoxanthine and xanthine are substrates for xanthine oxidase. Saugstad and Aasen (21) have observed increased plasma levels of hypoxanthine during hypoxia or ischemia which upon reperfusion or reoxygenation would result in the production of superoxide radicals by xanthine oxidase. Since rats, dogs, mice and guinea pigs have beenshown to have high xanthine oxidase activity while humans, rabbits and pigs have low myocardial xanthine oxidase activity (22), these differences in concentrations of the enzyme may explain variation in the effects of IR in different experimental models and highlight the controversies regarding the role of xanthine oxidase in IR injury.

(iii). Activated neutrophils:

Neutrophils have been implicated as a primary mechanism underlying IR injury in vivo as they possess membrane bound NADPH-oxidase, which generates superoxide radicals (23) in response to proinflammatory cytokines, platelet activating factor (PAF) and other stimuli. The role of neutrophils in myocardial IR injury was first demonstrated by histologic studies showing a direct correlation between the duration of ischemia as well as infarct size and the extent of neutrophil accumulation within the myocardial tissue(24). Simpson et al (25) have shown that induction of neutropenia and inhibition of neutrophil adhesion result in a cardioprotective effect, thus indicating the importance of neutrophils in contributing to the development of myocardial reperfusion injury.

B. Intracellular Ca^{2+}-overload in ischemia-reperfusion:

Ca^{2+} is one of the most important cations which are needed for cellular integrity, regulation of metabolism, cell growth and proliferation (6, 7). It plays a vital role in actin-myosin cross bridge cycling that leads to the contraction of the muscle. A large gradient of Ca^{2+} exists across the sarcolemmal membrane. The concentration of Ca^{2+} is about 1.25 mM in the extracellular space and it ranges from 10^{-7} M to 10^{-5} M in the intracellular space. The amount of Ca^{2+} entering or leaving the cell during contraction or relaxation must be the same or the cell would gain or lose Ca^{2+} and in fact, Ca^{2+}-homeostasis in cardiomyocytes is primarily maintained by SL and SR associated proteins. Ca^{2+}-induced Ca^{2+}-release occurs in cardiomyocytes during excitation contaction coupling. A small amount of Ca^{2+} entering the cell from the extracellular space through L-type Ca^{2+}-channels acts as a trigger for the release of more Ca^{2+} from the SR Ca^{2+}-stores. This allows the Ca^{2+} to bind with troponin C of the contractile apparatus and initiates the cardiac contraction. Decrease in intracellular Ca^{2+} due to SL Ca^{2+}-efflux and SR Ca^{2+}-pump mechanisms during diastole allows Ca^{2+} to dissociate from troponin C and thus, results in relaxation of cardiac muscle. It is pointed out that intracellular Ca^{2+}-overload in IR occurs as a result of ROS acting on different SL proteins. The beneficial effects of Ca^{2+}-antagonists (26, 27) and Na^{+}-H^{+} inhibitors (28, 29) support the role of intracellular Ca^{2+}-overload during IR. It has been shown that the function of SL Na^{+}-K^{+} pump fails in IR and this could be one of the possible mechanisms for the occurrence of intracellular Ca^{2+}-overload through the activation of Na^{+}-Ca^{2+}-exchanger (30, 31). Some studies have shown that cytosolic Ca^{2+}-concentration rises even during ischemia but none of the effects of intracellular Ca^{2+}-overload are seen during ischemia alone. On the other hand,

impaired myofilament responsiveness to Ca^{2+}, most probably due to proteolytic degradation of the various contractile and regulating proteins, occur during reperfusion (32, 33).

C. Myofibrillar assembly

Myofibrils are the contractile machinery in the cardiac cell. More than half of the volume of cardiomyocytes is made up of myofibrillar proteins. The major function of these myofibrils is the cyclic contraction and relaxation process that is highly regulated by the complex interaction between actin and myosin filaments in conjunction with the regulatory proteins of the myofibrils and different membrane proteins of the heart (34-36). Various components of the contractile machinery of the heart include actin (thin filament), myosin (thick filament), troponins, tropomyosin, titin, myosin-binding protein and desmin. Table 2 shows a list of different myofibrillar proteins and their molecular weights; myosin forms the major proportion of all these proteins in cardiomyocytes. In a longitudinal section, myofibrils are arranged into sarcomeres. A sarcomere is demarcated by two dark lines called Z-lines and is called the contractile unit of cardiac muscle. The average length of a sarcomere is 2 μm; the Z line has a passive role in transmitting the force generated by the myofilaments and its associated proteins have an important role in signal transduction mechanisms. On either side of the Z-line, there is a light band called I-band which consists of thin filaments primarily of actin. The region between two I-bands in a sarcomere is known as *A* band, which contains thick filaments composed primarily of myosin. The thin filaments extend from the Z-line towards the center of the sarcomere and overlap a portion of thick filaments. This overlapping portion is

Table 2. Different myofibrillar proteins and their structural properties

Protein	**Location**	**Molecular weight (kDa)**
Contractile proteins		
Myosin	Thick filament	460
Actin	Thin filament	42
Regulatory proteins		
Tropomyosin	Thin filament	67
Troponin I	Thin filament	24
Troponin C	Thin filament	18
Troponin T	Thin filament	38
Tropomodulin	Thin filament	43
Structural proteins		
Titin	From Z to M lines	2.7×10^3
C-protein	Thick filament	140
α-actinin	Z lines	400
β-actinin	Thin filament	400
M-line proteins	M lines	~ 750

represented by a dark area at the end of *A* band. The light area present at the center of sarcomere is called H-band, which represents the portion of *A* band that contains only myosin filaments. A dark line at the center of sarcomere, M-line, includes proteins that are critical for the organization and alignment of the thick filaments in the sarcomere.

(i) **Organization of thick filaments:**

A thick filament is composed of about 300 myosin molecules which are arranged in a tail-to-tail fashion. Each myosin molecule, consisting of a rigid tail and a bilobed head, is a large protein with a molecular weight of approximately 480 kDa. The myosin head is a complex globular structure consisting of 6 different polypeptides with a pair of heavy chains (~ 200 kDa), 2 pairs of light chains (~ 20 kDa), a domain that binds with actin and a site that exhibits ATPase activity. Atria and ventricles consist of several myosin heavy chain (MHC) isoforms that differ in structure and intrinsic ATPase activity. The α-MHC is an αα homodimer called V_1, which has high ATPase activity and promotes faster shortening velocity of cardiac myofibers (37, 38). The β-MHC is a ββ homodimer called V_3 and works more economically because it is able to produce high cross bridge force in spite of low ATPase activity (39). The abundance of these isoforms varies in atria and ventricles among different species and also during the developmental stages of an animal. α-MHC is more predominant in the ventricles of small mammals like rat and mice, whereas β-MHC is predominantly present in the ventricles of humans. In all mammals, β-MHC is referred to as fetal isoform in the ventricles as it is more predominat during the developmental stages and soon after birth it is replaced by α-MHC isoform. It should also be noted that there are two types of myosin light chains present along the neck of each myosin head - a pair of essential light chains (MLC_1) and a pair of

regulatory light chains (MLC_2) which together form 4 light chains per bilobed myosin head. MLC_1 is an integral part of the myosin head and functions as a lever arm between thick and thin filaments and generates a large-scale of force (40, 41). MLC_2 increases Ca^{2+}-responsiveness of the myofilaments upon phosphorylation by myosin light chain kinase (MLCK) (42). There are different isoforms of MLCs in human atria and ventricles. Three of these isoforms are regulatory chains (VLC_2, VLC_2^* and ALC_2) and other two are essential light chains (ALC_1 and VLC_1). ALC_1 is normally found in the developing heart and skeletal muscle and is therefore referred to as embryonic MLC.

(ii). Organization of thin filaments:

Thin filaments are formed by the aggregation of actin molecules, ~ 45kDa (G-actin or globular actin) into two stranded helical filament called F-actin or filamentous actin (43). The important properties of actin include activation of myosin ATPase and reversible binding to myosin. This allows the reconstitution of actomyosin and liberation of chemical energy from ATP that is needed for muscle contraction. Thin filament forms a complex of actin-tropomyosin and troponin complex in a 7:1:1 ratio .Tropomyosin is a filamentous molecule that is made up of 2 α helical peptide chains. Each tropomyosin spans 7 actin monomers and lies in the grooves formed by the two strands of an actin filament. Tropomyosin regulates the interactions between actin and myosin in a Ca^{2+}-dependent manner. During muscle relaxation, when the cytoplasmic level of Ca^{2+} is low, tropomyosin blocks the myosin-binding site on actin. Upon initiation of an action potential, Ca^{2+} is released into the myofilament from the SR and binds to troponin C. This causes a conformational change in tropomyosin and exposure of myosin binding sites on actin, thereby initiating cross-bridge cycling. The troponin complex comprises

three subunits, namely, troponin T (TnT), troponin C (TnC) and troponin I (TnI). They are present along with tropomyosin in the groove of actin in a stoichiometric ratio of 1:1 :1. Troponin T is the tropomyosin binding subunit that is essential for the transduction of the Ca^{2+}-binding signal and triggers muscle contraction. There are four different isoforms of cardiac troponin T – cTnT1, cTnT2, cTnT3 and cTnT4, which are developmentally regulated with the predominance of cTnT1 in fetal life and cTnT3 in adults. N-terminus of cTnT is essential for maximal activation of cardiac myofilaments (44) and different isoforms differ in the N-terminal hypervariable region. Troponin C is the Ca^{2+}-binding molecule of the troponin complex; Ca^{2+}-binding sites on this molecule are critical for the activation of muscle contraction. Inhibitory troponin (troponin I) is the key regulatory protein in cardiac muscle that regulates the Ca^{2+}-sensitivity of force. In the rat myocardium, there are two isoforms of troponin I (TnI), slow skeletal and cardiac isoforms. During myocardial development, there occurs a transition in TnI isoform expression from the slow skeletal isoform (ss TnI) in embryonic or fetal myocardium to the cardiac isoform (cTnI) expressed in adult hearts. A specific 30-amino acid sequence at N-terminal region of cTnI has two cyclic AMP-dependent protein kinase (PKA) phosphorylation sites (45). Changes in the phosphorylation states of cTnI cause conformational alterations in the troponin complex leading to altered Ca^{2+}-affinity of TnC and relaxation (46).

(iii). Titin and Myosin-binding protein C:

Titin is a large cytoskeletal protein that tethers the thick myosin filaments to the Z-line. It spans half of the sarcomere from Z-line to M-line and is critical in organizing the assembly of myofibrillar proteins (47). There are two isoforms of titin in the heart,

N2BA and N2B.Their ratio determines the active and passive mechanical properties of the sarcomere. On the other hand, myosin-binding protein C (MyBPC) is a sarcomeric protein (also called C-protein) which has a role both in the structural assembly and stability of the sarcomere, as well as in the modulation of contraction. Three isoforms of human MyBPC have been identified – fast skeletal, slow skeletal and cardiac isoforms (48). The genes encoding these isoforms are located on different chromosomes and are therefore not the product of alternative splicing. MyBPC has sites for myosin, actin, and titin and cardiac isoform has three phosphorylation sites (49). The state of phosphorylation of MyBPC determines the packing of myosin filament and their heads; the extent of phosphorylation correlates with an increased ability of myosin heads to interact with the thin filament.

(iv). Interaction between thick and thin filaments and cross-bridge cycling:

Activation of the contractile apparatus is initiated upon a transient increase in the cytosolic Ca^{2+}-concentration. Under normal physiological conditions, Ca^{2+}-entry during the plateau phase of the cardiac action potential is not sufficient to directly activate the myofilaments, but instead serves as a trigger to release Ca^{2+} from SR (Ca^{2+}-induced Ca^{2+}-release). The Ca^{2+} released from the SR store binds to TnC which then facilitates the movement of the associated tropomyosin towards the cleft of the actin filament (43). This movement of tropomyosin exposes the myosin-binding site on the actin filament, allowing the interaction between thin and thick filaments and thereby forms a cross-bridge and generates tension. Binding of myosin to the actin filament causes further shift in tropomyosin. In a relaxed state of the muscle, a tropomyosin molecule extends over seven actin molecules. Strong binding of myosin to actin results in the movement of an

adjacent tropomyosin molecule, exposing myosin binding sites on as many as 14 actin molecules. Once myosin is bound to actin, it undergoes ATP-dependent conformational changes and results in the movement of the actin filaments towards the center of the sarcomere. This shortens the length of the sarcomere and thereby contracts the muscle fiber. During diastole, cross-bridges are either blocked from interacting with actin or they are in a weak binding-unbinding state with actin without generating force (50-52). In systole, activation of the thin filament allows for a strong-binding cross-bridge state and this is associated with force generation and high rate of ATP hrdrolysis. Activation of thin filament is controlled by several thin filament regulatory proteins, tropomyosin, TnI, TnT and TnC.

D. Myofibrillar alterations under pathophysiological conditions:

Myofibrils, which are composed of the contractile proteins of the myocardium, determine the ability of the heart to pump properly. The contraction and relaxation of the cardiac muscle has been visualized in terms of binding and removal of Ca^{2+} from TnC which serves as a Ca^{2+}-receptor whereas tropomyosin transmits conformational changes from troponin to the contractile elements leading to the movements of actin and myosin. The energy required for contraction is provided by the hydrolysis of ATP by actomyosin ATPase (35, 36). Thus, any derangements in the organization of the contractile apparatus as well as in myofibrillar ATPase and Ca^{2+}-binding activities can lead to the development of dysfunction of cardiac contraction and relaxation processes. Many studies have implicated alterations in myofibrils at cellular, molecular and gene level in different cardiac disease models employing rats, mice and rabbits. Myofibrillar remodeling occurs

as a consequence of proteolysis, oxidation and phosphorylation of some functional groups in both contractile and regulatory proteins in failing hearts with different etiologies (35, 36).

(i). Thick filament remodeling:

The thick filament protein myosin has a site that exhibits ATPase activity and it is the myofibrillar ATPase activity which determines the force of contraction of the cardiac muscle; a decrease in myofibrillar ATPase activity is considered to indicate heart failure (53). The two isoforms of cardiac MHC differ in their structure and magnitude of ATPase activity. α-MHC has a high ATPase activity where as β-MHC has low ATPase activity. A small change in the composition of these MHC isoforms has been shown to have a profound effect on the myocardial function. Several investigators have reported an increase in β-MHC and a decrease α-MHC isoform in the hypertrophied ventricles in rats (54, 55) and humans (56) and have found a close correlation between the expression of their mRNA levels and the degree of cardiac hypertrophy (54, 57). However, during MHC expression, transcriptional activity does not necessarily reflect mRNA or protein levels. Although the mRNA level for α-MHC is directly correlated with its nascent pre-mRNA, the β-MHC pre-mRNA and mRNA data are paradoxical. The regulation of α-MHC takes place mainly at the transcriptional level but the regulation of β-MHC is more complex and involves post transcriptional factors. Alterations in the myofibrillar ATPase activity as well as MHC isoform protein content and gene expression were observed in failing rat hearts following myocardial infarction (58). These changes were partially prevented by enalapril (an angiotensin converting enzyme inhibitor), losartan (an angiotensin II receptor type I antagonist) suggesting the beneficial effects of blockade of

rennin-angiotensin system in heart failure with partial prevention of myofibrillar remodeling (58). Alterations in MHC isoform protein and gene expression in infarcted hearts were also prevented by treatment with imidapril (59), an angiotensin converting enzyme inhibitor. Myofibrillar remodeling associated with decreased protein contents of MLC (regulatory light chain), MLCK and phosphorylation of MLC has been implicated in diabetic cardiomyopathy (60, 61).

(ii). Thin filament remodeling:

Various studies have shown the involvement of thin filament remodeling in humans as well as experimental models of heart failure; this has been indicated by altered Ca^{2+}-sensitivity and altered maximal activation (36). In myocardial infarction model of rat, Ca^{2+}-sensitivity of noninfarcted myocardium was reduced (62). In contrast, both dog pacing heart failure model and human failing tissue demonstrated increased protein kinase A (PKA) activity (63). Some investigators found a differential response to protein kinase C (PKC) stimulation in failing human myocardium in comparision to controls, suggesting a thin filament basal modulation of myocardial performance (64). It is pointed out that the human heart predominantly expresses α-cardiac and α-skeletal actin isoforms. There is a large shift in actin isoform expression form α-cardiac to α-skeletal isoform in the developing human heart. An increased expression of α-skeletal actin mRNA has been reported in rat myocardial infarction model (65). However, no changes in the actin isoform expression levels were observed in end-stage human cardiomyopathy compared to control subjects. It has also been reported that increased phosphorylation of TnI contributes toward the depression in cardiac myofibrillar ATPase activity in the failing heart (63).

(iii). Myofibrillar remodeling in ischemic heart disease:

Myocardial ischemia often causes decreased cardiac function. Studies employing various animal models of ischemia-reperfusion have shown that both myofilament regulatory and structural proteins are vulnerable to cleavage and loss (36). Ischemia, as well as myocardial stunning result in a change in the myofilament Ca^{2+}-responsiveness (32, 66, 67) and this decreased Ca^{2+}-responsiveness of myofilaments is considered to be due to changes in the myofibrillar content or structural alterations as a result of changes in the gene expression (68). It was suggested that degradation of myofibrillar proteins occurs rapidly after reperfusion of ischemic myocardium (69) and it has been shown that ROS during ischemia-reperfusion is involved in the modification of myofibrils by direct oxidation of myofibrillar proteins (70). Degradation of TnI has been reported by some investigators in the ischemic myocardium (33, 71, 72). However, no degradation of cTnI was identified in ischemic hearts by other investigators (73, 74). Van Eyk et al (75) have demonstrated the loss of protein content of α-actin, a main component of Z line, from ischemic-reperfused rat hearts and correlated these changes with alterations in myofilament sensitivity to Ca^{2+} and maximum force generation. Varying degrees of changes in both contractile and regulatory proteins of myofilaments have been shown to occur as a consequence of protein oxidation and/or proteolysis due to global myocardial ischemia, myocardial stunning and IR (76-85). Thus, it appears that IR may induce remodeling of myofibrils and this may explain the depressed Ca^{2+}-sensitivity of myofilaments and contractile dysfunction of the IR heart.

II. STATEMENT OF THE PROBLEM AND HYPOTHESIS TO BE TESTED

From the foregoing discussion, it is evident that contractile abnormalities in the IR heart are associated with a wide variety of defects in subcellular organelles such as SL, SR, mitochondria and myofibrils. Although changes in SL, SR and mitochondria in IR hearts have been shown to be due to oxidative stress (10, 86-92), very little information is available in the literature regarding the mechanisms of myofibrillar remodeling upon subjecting the heart to IR injury. The present study was therefore undertaken to test the hypothesis that IR induces alterations in myofibrillar ATPase activities and gene expression of MHC proteins as a consequence of oxidative stress in the heart. This hypothesis is based on the observations that ROS and oxidants, which account for the occurrence of oxidative stress in the IR heart (93-95), have been reported to depress myofibrillar ATPase and creatine kinase activities as well as myofilament Ca^{2+}-sensitivity (96-98). Accordingly, isolated rat hearts will be subjected to 30 min of global ischemia and 30 min of reperfusion for inducing irreversible IR injury. IR hearts will be treated with or without SOD plus catalase mixture (for scavenging ROS) and N-actelylcysteine (NAC, an antioxidant) for preventing oxidative stress. Since the development of intracellular Ca^{2+}-overload and subsequent proteolysis (2, 3, 5, 13) are considered critical for inducing IR injury, some experiments will also be carried out in IR hearts with or without leupeptin (an inhibitor of Ca^{2+}-dependent protease) treatment. Alterations in cardiac function, myofibrillar ATPase and gene expression for some myofibrillar proteins (α-MHC and β-MHC as well as MLC1) will be monitored.

III. MATERIALS AND METHODS

A. Isolated rat heart preparation:

All animal protocols were approved by the University of Manitoba Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (250-300 g) were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (9 mg/kg). The hearts were rapidly excised, mounted on the Langendorff apparatus and perfused with Krebs-Henseleit (K-H) buffer gassed with a mixture of 95% O_2 and 5% CO_2 at a constant flow of 10 ml/min. The temperature and pH of the solution were maintained at 37° C and pH 7.4, respectively. The composition of K-H buffer (in mM) was 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.25 $CaCl_2$ and 11 glucose. The hearts were electrically stimulated at 300 beats /min using a Phipps and Bird stimulator (Richmond, VA). A water-filled latex balloon inserted into the left ventricle was connected to pressure transducer (model 1050 BP, BIOPAC Systems; Goleta, CA) and left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), rate of pressure development (+dP/dt) and rate of pressure decay (-dP/dt) were measured. LVEDP was adjusted to 10-12 mm Hg at the beginning of the experiment by inflating the balloon and the difference between LVSP and LVEDP was taken as the left ventricular developed pressure. All these data were recorded using an analog-to-digital interface (MP-100, Biopac Systems) and Acknowledge 3.5.3 software. The procedures for heart perfusion, induction of global ischemia and recording of cardiac function were similar to those used earlier (88, 99, 100). The hearts were divided into four different groups - control, control with treatment, IR and IR with treatment; all the hearts were initially stabilized for a period of 20 min. Control hearts

were perfused for 60 min after 20 min of stabilization period. Hearts in IR group were subjected to 30 min of global ischemia and 30 min of reperfusion after 20 min of initial stabilization; global ischemia was induced by stopping the coronary flow. In another group of experiments, both controls and IR hearts were treated with an antioxidant mixture containing SOD (5×10^4 U/l) plus catalase (7.5×10^4 U/l) for 10 min after 10 min of stabilization as well as during the last 30 min of reperfusion (88). Similarly, for other two sets of experiments, both control and IR hearts were also treated with another antioxidant NAC (100 μM) (100) or a protease inhibitor, leupeptin (25 μM) (99). The experimental protocol used in this study is depicted in Figure 1. At the end of the experiment, left ventricles were separated quickly, frozen in liquid nitrogen and stored at -80°C until use for biochemical analysis. In order to test if changes in cardiac function in IR hearts are due to the occurrence of oxidative stress, stabilized hearts were perfused for 30 min with a mixture of xanthine (X, 2 mM) and xanthine oxidase (XO, 0.06 U/l), a mixture which is known to generate ROS, or H_2O_2 (100 μM), a well known oxidant.

B. Isolation of myofibrils:

Myofibrils were isolated according to the method described elsewhere (101). Left ventricle was washed in ice cold 0.9% NaCl to get rid of the blood, and connective tissue, and the visible vasculature was dissected. The tissue was homogenized twice with sucrose/imidazole buffer (pH 7.0) containing 0.3 M sucrose and 10 mM imidazole in a blender for 30 sec. The homogenate was filtered using a gauze and centrifuged in Beckman centifuge at 12,000 rpm for 20 min at 4°C.The supernatant was discarded and the pellet was resuspended in a solution (pH 7.0) containing 60 mM KCl, 30 mM

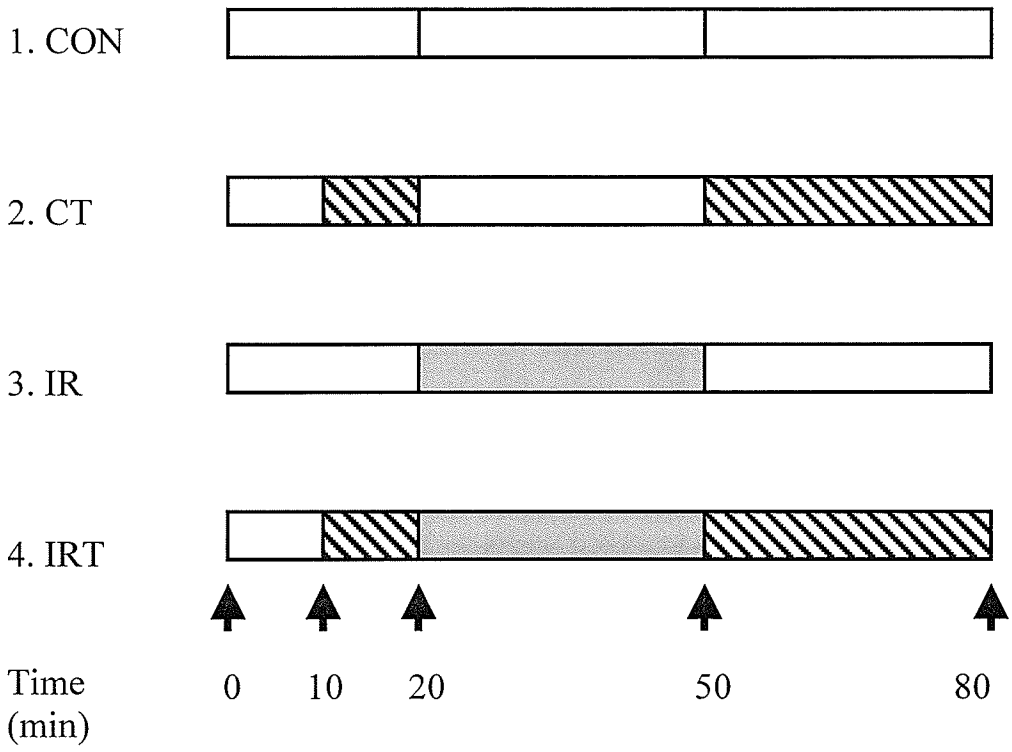

Figure 1. Experimental protocol for perfusing isolated rat hearts under different conditions. Panels 1 and 2 show control hearts with and without treatment. Panels 3 and 4 show IR hearts with and without treatment. Global ischemia ▭ was induced by stopping the coronary flow for 30 min and hearts were reperfused for a period of 30 min. The duration of treatment in each experiment is indicated ▨ . CON, Control; CT, Control with treatment; IR, Ischemic reperfused hearts; IRT, ischemic-reperfused hearts with treatment.

imidazole, 2 mM $MgCl_2$ (standard buffer solution). During the resuspension, care was taken to leave behind any coarse bottom layer of debris. The suspension was then homogenized (15X) using a hand held Potter-Elvejan homogenizer and then centrifuged at 2500 rpm for 15 min. The supernatant was discarded and the pellet was again resuspended; this step was repeated four times. The preparation thus obtained consisted of crude myofibrils; these were suspended again in the standard buffer solution containing 2 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) and centrifuged at 2500 rpm for 15 min. Purification of the myofibrils was achieved by suspension of the pellet in the standard buffer solution containing 1% Triton X-100 to eliminate contamination by mitochondrial, SL and SR membranes; this step was repeated once again. Finally, the myofibrils were washed twice in the standard buffer solution to remove any traces of Triton X–100 and the purified myofibrillar preparation was suspended in 1-2 ml of suspension medium (pH 7.0) containing 0.1 M KCl and 20 mM Tris-HCl. Care was taken to avoid over dilution of the homogenate in all the above steps by limiting the volume of the added buffers to not more than 3-4 ml. Concentration of the myofibrillar protein was estimated as described above (88, 99); the protein concentration was adjusted to 5 mg/ml with the suspension buffer.

C. Measurement of myofibrillar ATPase activities:

Myofibrillar ATPase activities were measured on the same day of myofibrillar isolation (102). Myofibrils at a concentration of 5mg/ml were used for the measurement of Mg^{2+} ATPase and Ca^{2+}-stimulated ATPase activities. The Mg^{2+} ATPase activity was determined by incubating 100 µl of the myofibrillar suspension in a medium containing

200 mM imidazole, 30 mM $MgCl_2$, 50 mM NaN_3, 500 mM KCl, and 10 mM EGTA for 5 min at 30°C. Blank tubes contained all reagents except myofibrillar protein suspension. The reaction was initiated by the addition of 22.6 mM Na_2ATP (pH 7.0) in all the tubes and carried out at 30°C for 5 min; the final volume in each tube was 1 ml. The reaction was terminated by the addition of 1 ml of 12% trichloroacetic acid (TCA) and the samples were centrifuged at 3000 rpm for 10 min. Phosphate in the protein-free supernatant was determined according to the method described previously in our laboratory (102). Total ATPase activity was determined by a method similar to that for Mg^{2+} ATPase activity except that EGTA in the reaction medium was replaced by 10 μM Ca^{2+}. The Ca^{2+}-stimulated ATPase activity was taken as the difference between the values obtained for total ATPase and Mg^{2+} ATPase activities.

D. RNA isolation and Northern blot analysis:

Total RNA was extracted from the left ventricles of control, IR and treatment groups by TRIzol extraction method or acid guanidium thiocyanate-phenol-chloroform method (Invitrogen, Burlington, ON) according to the manufacturer's instructions. Briefly, 100 mg of the heart tissue was ground into powder in liquid nitrogen and homogenized in the presence of TRIzol at 15,000 rpm using polytron homogenizer. The samples were centrifuged at 9,500 rpm for 10 min at 4°C. The supernatant was collected in a separate tube and phase separation (aqueous phase, interphase and organic phase) was achieved by the addition of chloroform. The aqueous phase containing RNA was then transferred to a fresh tube and RNA was precipitated using isopropyl alcohol. Finally, the RNA precipitated was washed in 75% ethanol, vacuum dried and dissolved in

DEPC water. RNA samples obtained were stored at -80°C until further analysis. RNA concentration was estimated at 260 and 280 nm by a spectrophotometer. Distilled water was used as a blank. Samples of 20 μg RNA were prepared for agarose gel electrophoresis for use in northern blot analysis. A 1.2% formaldehyde agarose gel containing 1X MOPS (20 mM MOPS and 1 mM EDTA, pH 8.0), 3.7% formaldehyde and ethidium bromide (0.03 μg/ml) was used to run the RNA samples. 20 μg of RNA sample was mixed with loading buffer containing 67% formamide, 8% formaldehyde, 30 mM MOPS, 1.5 mM EDTA, 6.7% glycerol (autoclaved), 0.1% SDS, 2% dye (10 mg bromophenol blue and 10 mg xylene cyanole in 100 μl sterile distilled water). Electrophoresis was carried out overnight at 23 V in a buffer of 1X MOPS. After the electrophoresis was completed, the gel was visualized under UV light and photographed. RNA separated was transferred on to Nitran membrane in 10X SSC (standard saline citrate, pH 7.0) containing 1.5 M NaCl and 165 mM sodium citrate through capillary action. The Northern blot set up consisting of filter papers dipped in the transfer buffer, a gel above it followed by the membrane and a stack of filter papers and a weight above it was properly leveled for efficient transfer of RNA and left overnight. After transferring, the membrane was dried with face up for 30 min and crosslinked under UV radiation (UV Stratalinker 2400, Stratagene, Cedar, TX, USA). The membrane was blocked in a solution containing 0.9% SDS, 45% formamide, 16% Denharts (1g Ficoll Type 400, 1g polyvinyl pyrrolidone, 1g chicken egg fraction V or BSA to a final volume of 100 ml with double distilled water), 9 mM EDTA, fish DNA (0.45mg/ml) and 22% 4X RNA solution (3M NaCl, 0.6M Tris, 0.18M NaH_2PO_4, 0.24M Na_2HPO_4, 9 mM $Na_4P_2O_7$, pH 7.5) at 42°C overnight. The membrane was probed for α-MHC, β-MHC and MLC1. The

probes used for the detection of MHC isoforms were oligonucleotide probes and their sequences are as follows:

α MHC:

5' GGG ATA GCA ACA GCG AGG CTC TTT CTG CTG GAC AGG TTA 3'

β MHC:

5' GCC TTG CTC CGG TGT TCA TTC ATC TGG TCC TCC AGG GTC CGG CAC ATC TTC 3'

These oligonucleotide probes are labeled by forward labeling reaction using T4 polynucleotide kinase and 5X forward reaction buffer (Invitrogen, Burlington, ON). 2 µl of oligonucleotide probe (α or β-MHC, 10 pmol) was incubated with 9 µl of sterile H_2O, 4 µl of 5X forward reaction buffer, 5 µl of γ ^{32}P ATP and 2 µl of T4 polynucleotide kinase (10 U/µl) in a 1.5 ml Eppendorf tube at 37°C for 2 hr. The reaction was stopped by heat inactivation at 75°C for 10 min. The labeled probe was passed through Bio-Spin 6 Column (Bio-Rad) for purification according to the manufacturer's instructions. The purified oligonucleotide probe was added to the membrane in blocking solution and incubated overnight at 42°C and 65 rpm. After probing, the membrane was washed with a solution containing 1 X SSC and 1% SDS to remove unbound probe and covered with a plastic wrap. The membrane was then exposed to Bio-max maximum sensitivity film and developed. The results of autoradiographs were analyzed by Imaging densitometer. The expression of MLC1 was detected similar to that of MHC isoforms except that labeling of the MLC1 probe (a cDNA probe MYL1 obtained from American Type Culture Collection, Clone hA5-13) was done by random primer DNA labeling system using Klenow fragment. At the end the blot was stripped with boiling double distilled water

and probed for 18S RNA (oligonucleotide probe), which served as a loading control. The densitometric values obtained for MHC isoforms and MLC1 were normalized to those of 18S to account for the differences in loading. These methods for RNA extraction and Northern blot preparations were similar to those described elsewhere (88, 89).

E. Statistical analysis

All the data were analyzed using Microcal Origin software and are expressed as mean ± standard error (S.E.) The differences between two groups were analyzed using Student's t-test whereas the data from more than two groups were evaluated by one-way ANOVA followed by the Newman-Keuls test. A P value < 0.05 was taken to represent a significant difference.

IV. RESULTS

A. Cardiac performance:

Cardiac performance of the isolated heart was assessed by measuring LVDP, LVEDP, +dP/dt and -dP/dt parameters. Hearts subjected to 30 min of global ischemia and 30 min of reperfusion showed a depression in LVDP, +dP/dt and -dP/dt and a marked increase in LVEDP (Table 3). To investigate the role of oxidative stress in causing cardiac dysfunction due to IR, rat hearts were pretreated with a well known antioxidant enzymatic mixture, SOD plus catalase. This treatment improved the contractile function of IR hearts, as the depression in LVDP, +dP/dt and -dP/dt as well as elevation in LVEDP were markedly attenuated (Table 3). In order to confirm that the beneficial effects produced by SOD plus catalase were due to reduction in oxidative stress, hearts were pretreated with another antioxidant, NAC. Treatment of IR hearts with NAC showed improved recovery of contractile function because changes in LVDP, +dP/dt and -dP/dt as well as LVEDP were prevented significantly (Table 4). It can also been seen from data in Tables 3 and 4 that SOD plus catalase mixture as well as NAC did not affect the cardiac function in control hearts. On the other hand, hearts perfused with X plus XO or H_2O_2 showed marked depression in LVDP, +dP/dt or -dP/dt and elevation in LVEDP (Table 5) which alterations were similar to the changes seen in untreated IR hearts. For assessing the role of Ca^{2+}-overload and subsequent proteolysis in IR hearts, rat hearts were treated with a protease inhibitor, leupeptin. A recovery in contractile function of IR hearts, as indicated by improvement in LVDP, +dP/dt and -dP/dt and reduction in LVEDP, was seen with leupeptin treatment (Table 6). Leupeptin did not affect the cardiac function in the control groups (Table 6).

Table 3. Cardiac performance and myofibrillar ATPase activities in control and ischemic reperfused rat hearts with and without SOD plus catalase.

	CON	CT	IR	IRT
A. Cardiac performance				
LVDP (mm Hg)	105 ± 20.3	91 ± 3.6	36.4 ± 12.1*	71.5 ± 9.5 #
LVEDP(mm Hg)	6.1 ± 1.0	7.6 ± 1.7	72.5 ± 10.5*	33.8 ± 5.5 #
+dP/dt (mm Hg/sec)	5640 ± 100	5641± 151	1372 ± 589 *	3562 ± 817 #
-dP/dt (mm Hg/sec)	3942 ± 829	3906 ± 69	985 ±123 *	2594 ±166 #
B. Myofibrillar ATPase activities (μ mol Pi/mg/hr)				
Mg^{2+} ATPase activity	3.5 ± 0.5	3.1 ± 0.1	4.0 ± 0.2	3.1 ± 0.3
Ca^{2+}-stimulated ATPase activity	13.3 ± 0.3	12.8 ± 0.2	10.7 ± 0.4 *	12.9 ± 0.2 #

Data are expressed as mean ± S.E; n = 4; *P < 0.05 in comparison to control; #P < 0.05 in comparison to IR; CON, Control; CT, Control with SOD plus catalase (5 X 10^4 U/L & 7.5 X 10^4 U/L respectively) treatment; IR, ischemic reperfused hearts; IRT, ischemic reperfused hearts with SOD plus catalase treatment; LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; +dP/dt, rate of pressure development; and -dP/dt, rate of pressure decay.

Table 4. Cardiac performance and myofibrillar ATPase activities in control and ischemia-reperfused rat hearts with and without N-acetylcysteine (NAC).

	CON	CT	IR	IRT
A. Cardiac performance				
LVDP (mm Hg)	100 ± 9.8	131 ± 11.1	40.2 ± 13*	117 ± 14.4 #
LVEDP (mm Hg)	5.0 ± 1.0	4.8 ± 0.4	70.1 ± 11*	40.3 ± 4.9 #
+dP/dt (mm Hg/sec)	5500 ± 100	6243 ± 407	1225 ± 98 *	5218 ± 809 #
-dP/dt (mm Hg/sec)	4140 ± 720	4727 ±169	845 ± 66*	3413 ± 315 #
B. Myofibrillar ATPase activities (μ mol Pi/mg/hr)				
Mg^{2+} ATPase activity	3.6 ± 0.7	3.9 ± 0.2	4.2 ± 0.4	3.1 ± 0.1
Ca^{2+}-stimulated ATPase activity	13.1 ± 0.2	12.9 ± 0.04	10.0 ± 0.3 *	13.9 ± 0.1 #

Data are expressed as mean ± S.E; n = 4; *P < 0.05 in comparison to control; #P < 0.05 in comparison to IR; CON, Control; CT, Control with NAC (100μM) treatment; IR, ischemic reperfused hearts; IRT, ischemic reperfused hearts with NAC treatment; LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; +dP/dt, rate of pressure development; and -dP/dt, rate of pressure decay.

Table 5. Effect of xanthine plus xanthine oxidase or hydrogen peroxide perfusion on cardiac performance and myofibrillar ATPase activities in rat hearts.

	CON	X + XO	H_2O_2
A. Cardiac performance			
LVDP (mm Hg)	115 ± 10.1	31 ± 2.8 *	34.6 ± 7.6 *
LVEDP (mm Hg)	5.3 ± 0.5	79 ± 4.0 *	64.9 ± 5.0*
+dP/dt (mm Hg/sec)	5500 ± 80	109 ± 5.0 *	329 ± 26.2*
-dP/dt (mm Hg/sec)	4012 ± 800	261 ±19.2 *	273 ± 10.4*
B. Myofibrillar ATPase activities (μ mol Pi/mg/hr)			
Mg^{2+} ATPase activity	3.6 ± 0.1	10.7 ± 0.2 *	ND
Ca^{2+}-stimulated ATPase activity	12.7 ± 0.1	6.9 ± 0.2 *	ND

Data are expressed as mean ± S.E; n = 4;*P < 0.05 in comparison to control; CON, control; X + XO, hearts perfused with xanthine plus xanthine oxidase (2 mM and 0.06 U/L, respectively) for 30 min; H_2O_2, hearts perfused with hydrogen peroxide (100μM) for 30 min; LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; +dP/dt, rate of pressure development; -dP/dt, rate of pressure decay; and ND, not determined.

Table 6. Cardiac performance and myofibrillar ATPase activities in control and ischemic reperfused rat hearts with and without leupeptin.

	CON	CT	IR	IRT
A. Cardiac performance				
LVDP (mm Hg)	95 ± 5.5	129 ± 11.1	35 ± 12*	98 ± 11.6 #
LVEDP (mm Hg)	6.0 ± 1.0	3.6 ± 0.4	70 ± 9.9*	31 ± 5.9 #
+dP/dt (mm Hg/sec)	5440 ± 95	5045 ± 334.6	1242 ± 415 *	3737 ± 509 #
-dP/dt (mm Hg/sec)	4122 ± 800	3859 ± 307	905 ±115 *	3097 ± 410 #
B.Myofibrillar ATPase activities (μ mol Pi/mg/hr)				
Mg^{2+} ATPase activity	3.4 ± 0.4	3.4 ± 0.1	4.1 ± 0.3	3.3 ± 0.1
Ca^{2+} -stimulated activity	13.5 ± 0.3	13.5 ± 0.4	10.5 ± 0.2*	13.6 ± 0.3 #

Data are expressed as mean ± S.E; n = 4; *P < 0.05 in comparison to control; #P < 0.05 in comparison to IR; CON, Control; CT, Control with leupeptin (25μM) treatment; IR, ischemic reperfused hearts; IRT, ischemic reperfused hearts with leupeptin treatment; LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; +dP/dt, rate of pressure development; and -dP/dt, rate of pressure decay.

B. Myofibrillar ATPase activities:

Myofibrillar ATPase activities were measured in control, IR and different treatment groups. IR hearts exhibited a lower Ca^{2+}-stimulated ATPase activity in comparison to control groups but no change in Mg^{2+} ATPase activity was evident (Tables 3, 4 and 6). Treatment of IR hearts with SOD plus catalase prevented the depression in Ca^{2+}-stimulated activities (Table 3). Likewise, NAC and leupeptin treatments attenuated the changes in Ca^{2+}-stimulated activities without affecting the Mg^{2+} ATPase activity (Tables 4 and 6). Treatment of control hearts with SOD plus catalase, NAC or leupeptin did not affect the myofibrillar ATPase activities (Tables 3, 4 and 6). It can be seen from Table 5 that perfusion of the heart with X plus XO depressed the Ca^{2+}-stimulated ATPase activity but increased the Mg^{2+} ATPase activity significantly.

C. Gene expression of MHC isoforms and MLC1:

In order to study the effects of IR as well as different treatments on cardiac gene expression for both α-MHC, β-MHC and MLC1, mRNA from various groups were evaluated by the Northern blot analysis. The results in Fig. 2-4 indicate that the mRNA levels for α-MHC and β-MHC were decreased by 40 to 60% and that for MLC1 were decreased by 30 to 40% in IR hearts in comparison to the control group. Furthermore, treatments of IR hearts with SOD plus CAT, NAC and leupeptin attenuated the observed changes in MRNA levels for α-MHC, β-MHC or MLC1 significantly (Fig. 2-4). However, SOD plus CAT, NAC or leupeptin did not produce any significant effect on gene expression in control hearts (Fig. 2-4).

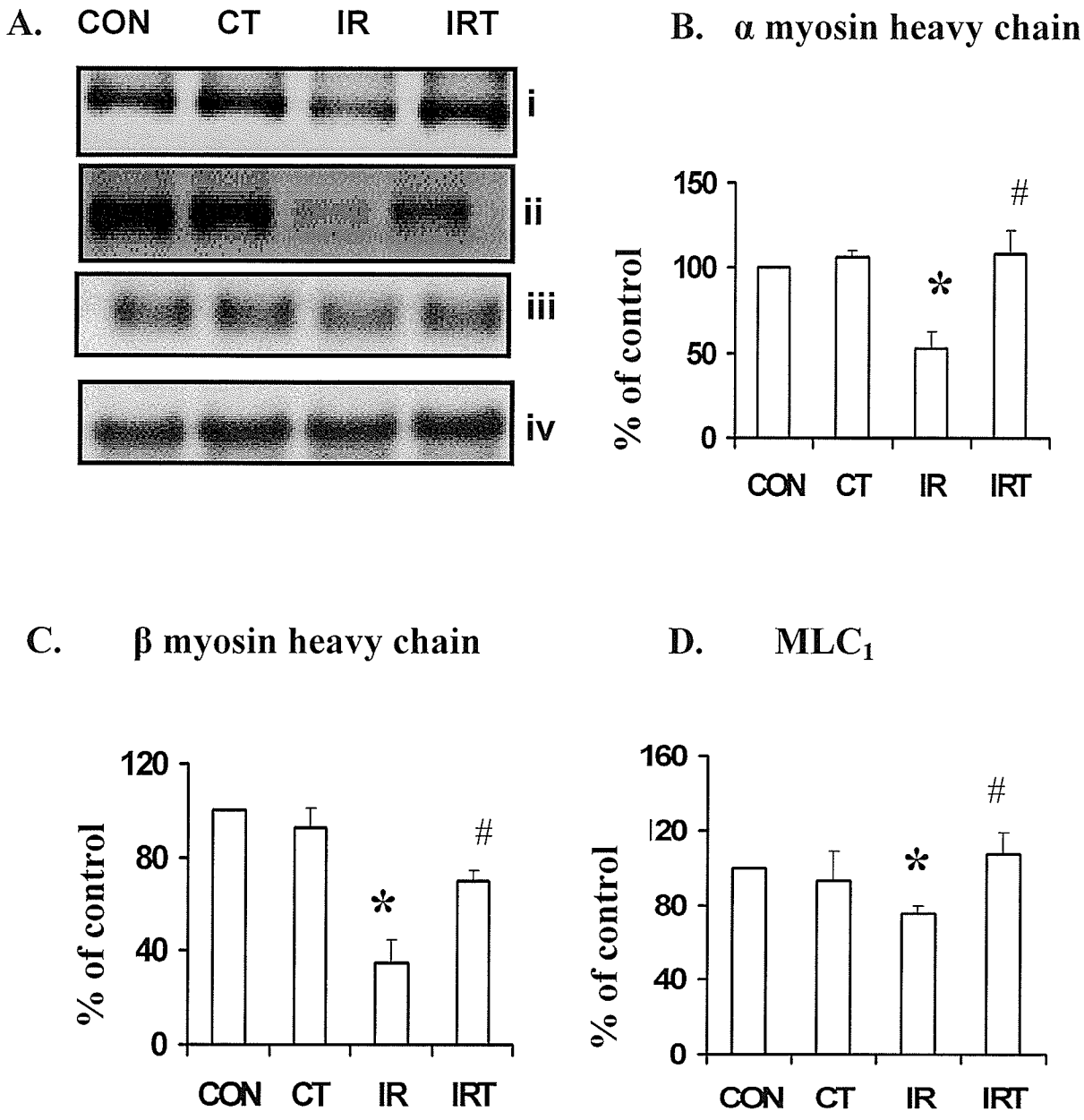

Figure 2. Effect of SOD plus catalase on the gene expression of myosin heavy chain isoforms and myosin light chain 1. Panel A shows Northern blot analysis of (i) α myosin heavy chain; (ii) β myosin heavy chain; (iii) myosin light chain (MLC1) in control and IR hearts treated with and without SOD plus Catalase; (iv) mRNA blot of 18S ribosomal RNA showing equal loading in all the groups. Panels B, C, D: mRNA expression of α myosin heavy chain, β myosin heavy chain and MLC1 respectively in comparison to control. Data are expressed as mean ± S.E; n = 5; *P < 0.05 in comparison to control; # P < 0.05 in comparison to IR.

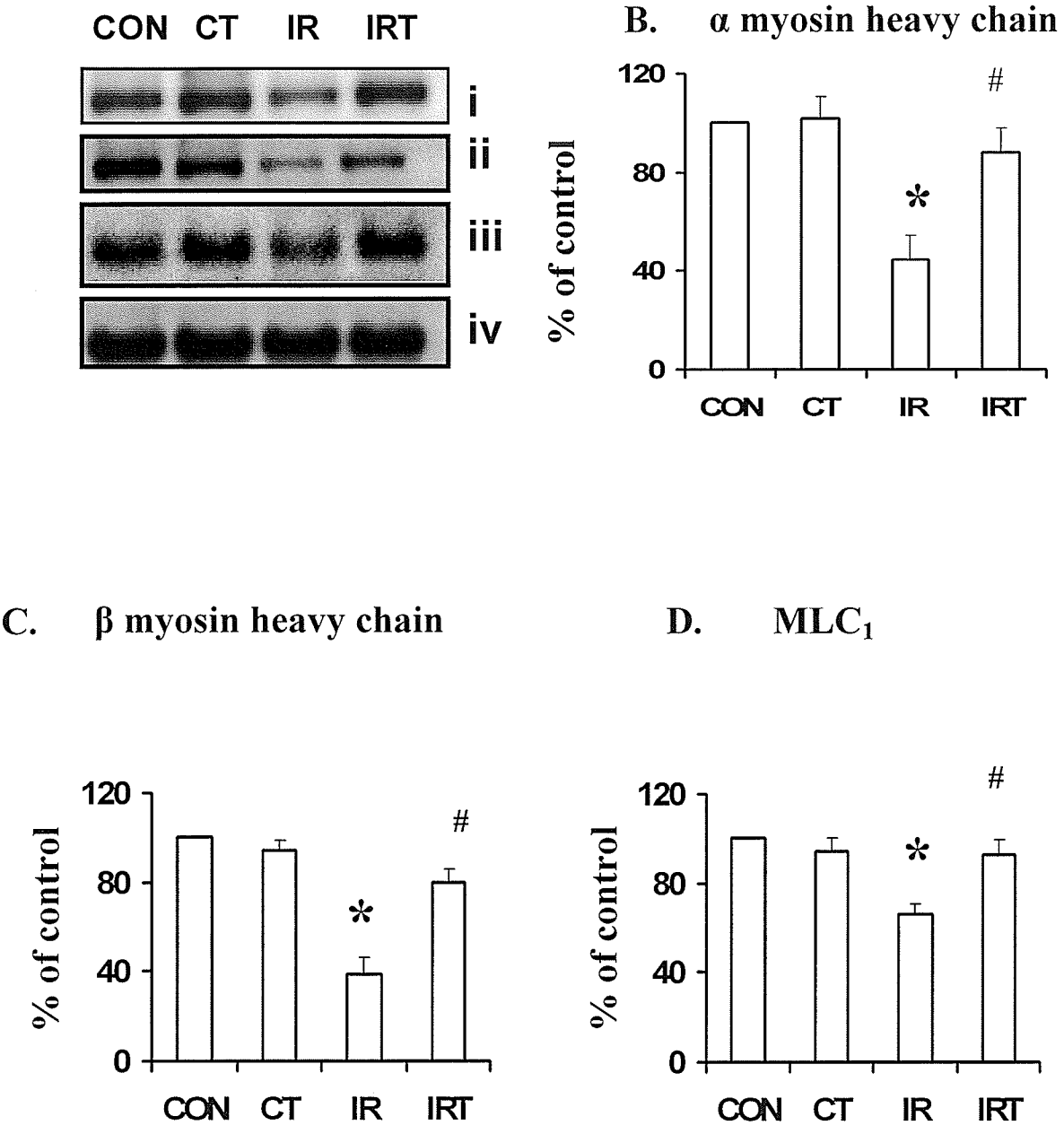

Figure 3. Effect of NAC on the gene expression of myosin heavy chain isoforms and myosin light chain 1. Panel A shows Northern blot analysis of (i) α myosin heavy chain; (ii)β myosin heavy chain; (iii) myosin light chain (MLC1) in control and IR hearts treated with and without NAC; (iv) mRNA blot of 18S ribosomal RNA showing equal loading in all the groups. Panels B, C, D: mRNA expression of α myosin heavy chain, β myosin heavy chain and MLC1 respectively in comparison to control. Data are expressed as mean ± S.E; n = 5; *P < 0.05 in comparison to control; # P < 0.05 in comparison to IR.

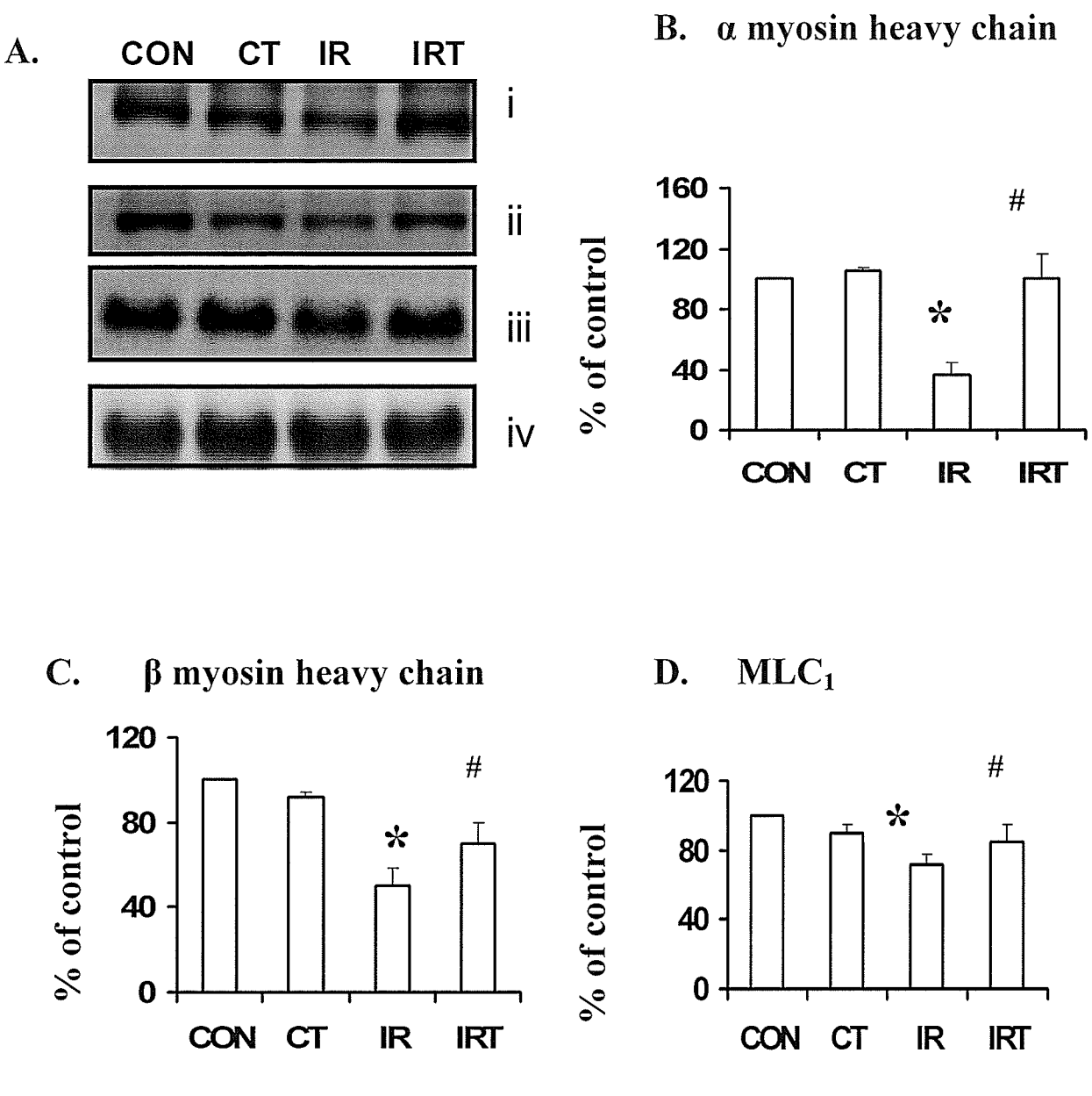

Figure 4. Effect of leupeptin on the gene expression of myosin heavy chain isoforms. Panel A shows Northern blot analysis of (i) α myosin heavy chain isoform (ii) β myosin heavy chain (iii) myosin light chain (MLC1) in control and IR hearts treated with and without leupeptin; (iv) mRNA blot of 18S ribosomal RNA showing equal loading in all the groups. Panels B, C, D: mRNA expression of α myosin heavy chain, β myosin heavy chain and MLC1 respectively in comparison to control. Data are expressed as mean ± S.E; n = 5; *P < 0.05 in comparison to control; # P < 0.05 in comparison to IR.

V. DISCUSSION

In this study, we have observed a depression in the LVDP, +dP/dt and -dP/dt as well as an elevation in the LVEDP upon subjecting the isolated hearts to IR injury. Such contractile abnormalities in IR hearts are in agreement with our previous observations indicating cardiac dysfunction due to IR (87-92). Furthermore, it was observed that myofibrillar Ca^{2+}-stimulated ATPase activity was decreased in IR hearts. This change may be of some specific nature because myofibrillar Mg^{2+}-ATPase activity was not altered under the experimental conditions employed in this study. Since the magnitude of cardiac contractile force is linearly related to myofibrillar ATPase activity (35, 36), the observed decrease in myofibrillar Ca^{2+}-stimulated ATPase activity in the IR hearts may contribute to the depressed cardiac function in IR hearts. Depression in myofibrillar Ca^{2+}-stimulated ATPase has also been reported in heart failure due to myocardial infarction (58, 59), diabetic cardiomyopathy (102) and other etiologies (36). Whether the observed depression in myofibrillar Ca^{2+}-stimulated ATPase activity due to IR is due to any changes in α-MHC and MLC1 protein content or some subtle changes in some functional groups, which determine the activity of α-MHC and MLC1 (35, 36), cannot be ruled out. Since IR has been reported to produce varying degree of changes in some proteins which are known to regulate myofibrillar ATPase activity (33, 70-74), it is also likely that the depression in myofibrillar Ca^{2+}-ATPase activity in IR hearts may be due to a decrease in the sensitivity of these regulatory proteins to Ca^{2+}. In fact, IR has been shown to depress Ca^{2+}-sensitivity of myofilaments from the myocardium (72, 75). Although mRNA levels for α-MHC, β-MHC and MLC1 were depressed in IR hearts, these changes in gene expression may not alter the myofibrillar ATPase activity because

of the short duration of reperfusion used in this study. As IR has also been found to adversely affect other subcellular organelles (87, 91), the observed change in myofibrillar Ca^{2+}-ATPase activity should be considered to be one of the multiple defects which may account for cardiac dysfunction in IR hearts.

The results described in this study indicate that both cardiac dysfunction and depression in myofibrillar Ca^{2+}-ATPase activity in IR hearts were attenuated upon treatment with SOD plus catalase, a ROS scavenging mixture, as well as NAC, an antioxidant. These results suggest that the observed changes in cardiac function and myofibrillar ATPase activity in the IR heart may be due to the development of oxidative stress. This view is consistent with the finding that there occurs oxidative stress due to an excessive generation of ROS in the IR hearts (93-95, 103, 104). Furthermore, alterations in different subcellular organelles such as SR, SL and mitochondria due to IR were prevented by SOD plus catalase (87-91). It should also be noted that the IR-induced changes in cardiac function were simulated in hearts perfused with X plus XO, an oxyradical generating system as well as H_2O_2, an oxidant. In addition, perfusion of the heart with X plus XO depressed the myofibrillar Ca^{2+}-stimulated ATPase activity as seen in the IR hearts. Although myofibrillar Mg^{2+} ATPase activity in hearts perfused with X plus XO was increased unlike the IR hearts, the reason for such a change is not clear at present. Nonetheless, Ca^{2+}-stimulated ATPase activity of myofibrils was found to be depressed upon incubation with X plus XO and H_2O_2 under in vitro conditions (98). It should also be pointed out that IR-induced changes in gene expression were attenuated by treatment of IR hearts with SOD plus catalase or NAC. On the basis of these observations, it is likely that IR-induced changes in myofibrillar Ca^{2+}-stimulated ATPase

activity as well as gene expression for α-MHC, β-MHC and MLC1 occur as a result of the development of oxidative stress in the IR hearts.

In addition to oxidative stress, the occurrence of intracellular Ca^{2+}-overload has been suggested to explain defects in cardiac function as well as SL and SR activities in the IR hearts (2, 3, 5, 6, 100). Although IR has been considered to cause the development of intracellular Ca^{2+}-overload by activating Na^{+}- Ca^{2+}-exchanger as a consequence of stimulation of Na^{+}-H^{+}-exchanger and depression of Na^{+}-K^{+} ATPase (5, 6), oxidative stress has also been shown to produce intracellular Ca^{2+}-overload in the myocardium as a consequence of changes in membrane permeability (5) and Ca^{2+}-handling abnormalities in cardiomyocytes (100). Thus, some of the effects of oxidative stress, including cardiac dysfunction and changes in gene expression in IR hearts, can be attributed to intracellular Ca^{2+}-overload. In this regard, it is pointed out that intracellular Ca^{2+}-overload has been reported to promote proteolysis of different subcellular organelles and various factors involved in gene expression in the heart (2, 5, 6, 7). Therefore, the observed beneficial effects of leupeptin, a protease (calpain) inhibitor, in preventing IR-induced changes in cardiac function, myofibrillar Ca^{2+}-stimulated ATPase activity as well as gene expression for α-MHC, β-MHC and MLC1 in the IR heart are likely due to attenuation of alterations induced by intracellular Ca^{2+}-overload. This contention is consistent with a previous study in which leupeptin has been demonstrated to attenuate IR-induced changes in cardiac function, SR Ca^{2+}-uptake, SR Ca^{2+}-release, SR protein content and SR gene expression (99). Several other investigators have shown the occurrence of proteolytic changes in different regulatory proteins of cardiac myofilaments due to IR (33, 70-74). Thus, further studies are required for making any meaningful conclusion regarding

proteolytic changes in myofibrils in hearts subjected to IR.

VI. CONCLUSIONS

This study examined the effect of oxidative stress and intracellular Ca^{2+}-overload due to IR in mediating alterations in cardiac performance, myofibrillar ATPase activities as well as gene expression for MHC isoforms and MLC1 in isolated rat hearts perfused with or without SOD plus catalase, NAC and leupeptin treatments. Our results suggest the following:

1. Acute depression of cardiac performance in IR hearts may be associated with defects in myofibrillar function as Ca^{2+}-stimulated ATPase activity was decreased upon subjecting the heart to IR.

2. Although IR was observed to depress the levels of mRNA for α-MHC, β-MHC and MLC1, these changes in gene expression may not account for the observed alterations in myofibrillar ATPase activity because of the short duration of IR used here but may play a critical role in delayed recovery of cardiac function (chronic effects) in IR hearts.

3. In view of our observations that changes in cardiac function in hearts perfused with X plus XO or H_2O_2 were similar to those for IR hearts and these alterations as well as changes in myofibrillar ATPase and gene expression due to IR were prevented by SOD plus catalase and NAC treatments, it is evident that oxidative stress plays a critical role in myofibrillar remodeling due to IR injury.

4. Attenuation of cardiac dysfunction as well as changes in myofibrillar ATPase activity and gene expression in IR hearts by leupeptin indicate the importance of intracellular Ca^{2+}-overload and associated proteolysis in myofibrillar remodeling in hearts due to IR injury.

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