Mechanisms of Cardiac Dysfunction and Changes in Sarcolemmal Na^+-K^+-ATPase Activity in Hearts Subjected to Ischemia Reperfusion Injury

By

Raja Balraj Singh

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology, Faculty of Medicine
University of Manitoba
Winnipeg

ACKNOWLEDGEMENTS

Dr. Naranjan. S. Dhalla personifies deep commitment to the advancement of our knowledge in various aspects of heart disease. His passion for understanding minute details of cardiac disease is reflected in his over a thousand refereed journal articles, book chapters and countless students whose life he has shaped. He is an institution in himself. Working under his supervision has been a deeply humbling experience. His personality and commitment to science has influenced my career in numerous ways not limited to the science alone. Thank you Dr. Dhalla for all your support and giving me this opportunity to work under your tutelage.

My gratitude to my committee members, Drs. Pawan Singal, Davinder Jassal and Darren Freed, who could spare time from their busy schedules and provide input that helped me immensely during the pursuit of my research. Thanks is also due to the department head, Dr. Janice Dodd, for her support.

A word of remembrance for our dear friend Ken Dhalla, who is deeply missed. He along with Dr. Vijayan Elimban and Donald Chapman were very helpful in the laboratory and their assistance is much appreciated. The help and support of my colleagues and other staff especially Ms. Eva Little and Ms. Gail McIndles is noteworthy.

It is said that success has many fathers, failure has none. The only ones standing besides you when the chips are down are your family and friends. I am indebted to the support and constant encouragement I have received from my wife, Dr. Punam Chohan. It would not have been possible for me to accomplish this task without her support and affection. A special thanks to my children, Yuvraj Singh and Sabbena Singh. Thank you for understanding when I missed any of your games or performances or did not have time to play with you. A constant source of inspiration in my life has been my father, Dr. S. S. Bhatti and my mother Mrs. Narinder Kaur. Thank you for all your support and words of wisdom over the years. I also appreciate the support of my brother, Dr. Rajbir S. Bhatti and his family over the years. Thanks are also due to my in-laws, Commandant Santokh Singh and Mrs. Sukhwinder Kaur for their prayers and the deep affection of my brothers-in-law and their families.

ABSTRACT

Impaired cardiac function and depressed subcellular activities including sarcolemmal (SL) Na^+-K^+-ATPase and sarcoplasmic reticulum (SR) Ca^{2+}-uptake have been observed in hearts subjected to ischemia reperfusion (I/R) injury. Furthermore, different cellular and biochemical abnormalities namely defects in endothelial function, development of intracellular Ca^{2+} overload, occurrence of oxidative stress and activation of protease such as calpain and metalloproteinases (MMP) have been identified in ischemic heart disease. However, neither the relationship among these changes nor the exact mechanisms of cardiac dysfunction during development of I/R injury are fully understood. This study therefore, tested the hypothesis that both endothelial defect and oxidative stress play a critical role in the activation of calpain, inhibition of SL Na^+-K^+-ATPase, and induction of cardiac dysfunction in hearts subjected to I/R injury. Unless indicated, all experiments in this study were carried out in isolated rat hearts perfused at a constant flow (CF) and I/R was induced by subjecting the heart to 30 min global ischemia followed by 60 min reperfusion.

Since hearts perfused at CF and constant pressure (CP) show difference in their contractile response to I/R, some experiments were carried out to examine mechanisms responsible for the I/R-induced alterations in CF-perfused and CP-perfused hearts. Rats hearts, perfused at CF (10ml/min) or CP (80 mm Hg), were

subjected to I/R injury and changes in cardiac function as well as SL Na^+-K^+-ATPase activity and endothelial function were monitored; alteration in SR Ca^{2+}-uptake were also measured for the purpose of comparison. The I/R induced depression in cardiac function and both SL Na^+-K^+-ATPase and SR Ca^{2+}-uptake activities were greater in hearts perfused at CF than in hearts perfused at CP. In hearts perfused at CF, I/R-induced increase in calpain activity and decrease in endothelial nitric oxide synthase (eNOS) protein content in the heart as well as decrease in nitric oxide (NO) concentration of the perfusate were greater than in hearts perfused at CP. These changes in contractile activity and biochemical parameters due to I/R in hearts perfused at CF were attenuated by treatment with L-arginine, a substrate for NO synthase, while those in hearts perfused at CP were augmented by treatment with L-NAME, an inhibitor of NO synthase. The results indicate that the I/R-induced differences in contractile responses and alteration in subcellular organelles between hearts perfused at CF and CP may partly be attributed to greater endothelial dysfunction in CF-perfused hearts than that in CP-perfused hearts. It is suggested that the I/R induced depression in endothelial function may activate calpain and thus reduce subcellular activities and impair cardiac performance.

In view of the observation that both I/R and oxidative stress promote the occurrence of intracellular Ca^{2+} -overload and increase in Calpain activity, this study was undertaken to investigate if the activation of calpain in I/R hearts is

associated with alteration in SL Na^+-K^+-ATPase activity and its isoform content. For this purpose, isolated rat hearts treated with and without two different calpain inhibitors (leupeptin and MDL28170) were subjected to I/R injury. The I/R-induced depression in cardiac function as well as the Na^+-K^+-ATPase activity and protein content of Na^+-K^+-ATPase isoforms were associated with an increase in calpain activity, which was prevented by treatment of hearts with leupeptin. Incubation of SL membranes with calpain decreased the Na^+-K^+-ATPase activity and protein content of its isoforms; these changes were also attenuated by leupeptin. The I/R induced alteration in cardiac function, SL Na^+-K^+-ATPase and calpain activity were Ca^{2+}-dependent and were prevented by MDL28170, a specific inhibitor of calpain. The I/R-induced translocation of calpain isoforms (I and II) from the cytosol to SL membrane and changes in the distribution of calpastatin were also attenuated by treatment with calpain inhibitors. These results suggest that depressions in cardiac function and SL Na^+-K^+-ATPase activity in the I/R hearts may be due to changes in the activity as well as translocation of calpain.

We further tested whether the activation of calpain during I/R is mediated through oxidative stress; changes in the activity of MMP were monitored for the purpose of comparison. Depression of cardiac function and Na^+-K^+-ATPase activity in the I/R hearts were associated with increased calpain and MMP activities. These alterations due to I/R were simulated in hearts perfused with a hypoxic medium (in the absence and presence of glucose) as well as oxidant, H_2O_2, and an oxyradical

generating system, xanthine plus xanthine oxidase. The I/R-induced changes were attenuated by ischemic preconditioning, which is known to reduce oxidative stress in the I/R myocardium. Perfusion of hearts with an antioxidant, N-acetylcysteine and mercaptopropionylglycine, also ameliorated the I/R-induced alterations. Inhibition of MMP activity in hearts treated with doxycycline improved the I/R-induced depression in cardiac function and SL Na^+-K^+-ATPase activity without affecting the calpain activation. On the other hand, inhibition of calpain activity upon treatment with leupeptin or MDL 28170 reduced the MMP activity significantly in addition to attenuating the I/R-induced depression in SL Na^+-K^+-ATPase activity. These results suggests that I/R-induced depression in SL Na^+-K^+-ATPase activity and cardiac function may be a consequence of increased activities of both calpain and MMP due to oxidative stress in heart.

TABLE OF CONTENTS

LIST OF FIGURES

PAGE

LIST OF TABLES

PAGE

I. REVIEW OF LITERATURE

1. Background: Ischemic heart disease

Cardiovascular disease (CVD) consists of a spectrum of diseases that affect the heart and blood vessels (arteries and veins) and is often referred to as ischemic heart disease (IHD) or coronary artery disease (CAD). IHD consists of myocardial ischemia, angina (chest pain), stroke, myocardial infarction (MI) and eventually heart failure (HF). Although the occurrence of MI is predominantly due to atherosclerosis, other factors such as thrombosis and coronary spasm are also considered important in this regard. One of the major concerns regarding CVD is the high mortality rate associated with this disease; it kills more people than other diseases including cancer. The mortality due to CVD was 34.3% of all 2, 426, 264 deaths in United States (Llyod-Jones et al. 2010). Deaths in males and blacks were greater than in females and whites (306.6 per 100, 000 for white males; 422.8 per 100, 000 for black males; 215.5 per 100, 000 for white females; and 298.2 per 100, 000 for black females). There was a steady increase in mortality from IHD in the last century; however deaths from CVD have declined steadily by about 29% between 1996 and 2006 (Stamler J. 1985). The current mortality rate due to CVD translates into approximately 2300 deaths each day, an average of 1 death every 38 seconds in the United States (Llyod-Jones et al. 2010).

While early studies on male and female death rates from IHD showed women in better health, current estimates have revealed women are equally predisposed to IHD (Anderson TW. 1973). Infact, CVD kills more women than even breast cancer (Stamler J. 1985). There is also growing incidence of IHD in lower socio-economic groups (Davies et al. 2010). It is estimated that every 25 seconds, an American has a coronary event and every minute someone will die from CAD (Lloyd-Jones et al. 2010). CAD resulted in the death of 425,425 persons (all ages) in the United States in 2006 (Keenan et al. 2011). Similarly, every year about 800, 000 people experience a new or recurrent stroke; about 600, 000 of these have first attacks while 200, 000 have recurrent attacks (Lloyd-Jones et al. 2010). The 2006 mortality data indicated that 1 in every 18 deaths was due to stroke, which translates into approximately 137, 119 deaths (Keenan et al. 2011); a stroke every 40 seconds. HF is another common CVD that eventually sets in as the heart is unable to meet nutritional requirement of the body; 1 in 8.6 deaths were recorded to be due to due to HF in the United States (Lloyd-Jones et al. 2010).

Although significant resources are allocated for treatment of IHD, the morbidity of those afflicted with the disease has become an enormous economic burden. It has been observed that the number of procedures and operations for CVD during 1996-2000 increased (33%) from 5, 444, 000 to 7, 235, 000 annually (Lloyd-Jones et al. 2010). The total direct and indirect health cost of CVD and stroke in the United States for 2010 was estimated to be $503.2 billion (Lloyd-Jones et al.

2010). This included direct costs such as physicians and other professionals, hospital and nursing home services, prescribed medications and home care, as well as indirect costs such as lost productivity resulting from morbidity and mortality. Average cost of taking care of a patient post-stroke was around $ 15, 000. On the other hand, total direct and indirect cost of all cancer and benign neoplasms in 2008 was $212 billion. CVD costs were more than that for any other diagnostic group.

Numerous studies in the past 50 years have investigated the underlying causes of CVD and have pointed out unhealthy lifestyle and socioeconomic conditions as the major problems. Consumption of trans and saturated fats, sugars and other high calorie foods as well as lack of regular exercise have been implicated in IHD becoming an epidemic (Blessey. 1985). It has been observed that the average calorific intake of men and women has increased by about 500 calories per day over and above their daily need. It is also pointed out that IHD and CVD risk factors are more prevalent in less educated compared to more educated and aware people (Strand et al. 2004 and Toft et al. 2007). There is an alarming trend of increase in IHD in native and indigenous populations of North America (Shah et al. 2000). While the major cause of IHD is the development of atherosclerosis in coronary arteries, it has been reported that every individual is born with some degree of plaque formation (Tonstad et al. 2000). Since lifestyles influence further progression of disease, diagnosis of atherosclerosis invariably occurs when CAD is at an advanced stage. It is thus evident that modification of risk factors such as

diet, exercise, smoking and alcohol is the best strategy to prevent the disease outcome.

In view of the fact that IHD is a complex problem involving atherosclerosis, thrombosis, vascular abnormalities, cardiomyocyte dysfunction, cell damage and scar formation, extensive research has been carried out to understand its pathophysiology. Since endothelium is known to regulate both vascular and cardiomyocyte functions, a great deal of information on the state of endothelial function in IHD needs to be acquired. Furthermore, there are real gaps in our knowledge concerning the mechanisms of cardiac abnormalities due to IHD under acute and chronic conditions. This study was therefore undertaken to address some the mechanisms of cardiac dysfunction under acute ischemic situations. In particular it planned to focus on the mechanisms of I/R injury with respect to cardiac function.

2. Mechanisms of ischemia reperfusion injury

Early reperfusion of the ischemic myocardium is critical for the reduction of infarct size (Darsee et al. 1981) and the salvage of viable myocardium in the ischemic zone in patients as well as animal models that do not have significant collateral formation (Pohl et al. 2001). Paradoxically, the restoration of coronary flow to the ischemic heart, if not instituted within a certain period of ischemic insult, has been associated with a depression in cardiac contractile function (Braunwald et al. 1985), and is generally referred to as ischemic/reperfusion (I/R) injury (Braunwald et al. 1985, Ambrosio et al. 1999 and Bolli et al. 1999). In fact,

reperfusion of the ischemic heart has been shown to produce changes in subcellular organelles such as the sarcolemma (SL) (Dhalla et al. 1988, Dhalla et al. 2007, Ostadal et al. 2004 and Ostadal et al. 2003), sarcoplasmic reticulum (SR) (Singh et al. 2004a and Temsah et al. 1999), myofibrils (Dhalla et al. 2007 and Gao et al. 1997) and mitochondria (Makazan et al. 2007). A wide variety of mechanisms (Barry 1987) including the occurrence of oxidative stress (Suzuki et al. 1987 and Singh et al. 2004b), development of intracellular Ca^{2+}-overload (Bolli et al. 1999) and activation of proteases (Singh et al. 2004a, Singh et al. 2004b and Yoshida et al. 1995) have been suggested to explain subcellular alterations and cardiac dysfunction as a consequence of I/R injury. While some studies have indicated that genesis of the intracellular Ca^{2+}-overload due to I/R injury is attributed to abnormalities in cation channels in the SL and SR membranes (Tani et al. 1989 and Tani et al 1990), others have suggested that the intracellular Ca^{2+}-overload results in the activation of various proteases including calpain (Singh et al. 2004a and Singh et al. 2004b) and contributes to the SL and SR dysfunction (Singh et al. 2004a, 2004b and Singh et al. 2008). In addition, the activation of calpain has been shown to induce abnormalities in mitochondrial function (Makazan et al. 2007 and Chen et al. 2002) and contractile proteins (Gao et al. 1997). Although calpain is considered to play a role in normal physiologic processes at the basal Ca^{2+} levels (Wu et al. 2007), an increase in the intracellular concentration of free Ca^{2+} has been reported to be associated with pathologic activation of calpain (Tani et al. 1989). In fact, both SR Ca^{2+}-handling proteins

(Singh et al. 2004a and Yoshida et al. 1995) and nitric oxide regulating synthase (Chohan et al. 2006) have been shown to be the target of the calpain activation in the heart. Since both oxidative stress and Ca^{2+} overload are known to explain cardiac dysfunction in IHD, these mechanisms for the occurrence of I/R injury are discussed below:

a. Oxidative stress and generation of reactive oxygen species

Various studies (Zweier et al. 1989 and Bolli et al. 1988) have shown that the reactive oxygen species (ROS) are produced in the heart during I/R. ROS target cellular membranes, cation channels, contractile proteins and many other subcellular organelles and this may cause contractile abnormalities. By administration of agents such as mercaptopropionylglycine (MPG) a minute before reperfusion, it has been shown that ROS are produced during the first minute of reperfusion and peak between four to ten minutes (Zweier et al. 1989 and Bolli et al. 1988). It may be noted that about 2% of all oxygen consumed during oxidative phosphorylation is converted to ROS (Boveris et al. 1976 and Boveris et al. 1984), which then cause severe cellular damage if not detoxified through various antioxidant mechanisms (Melov et al. 1999, Melov et al 1998). As discussed below, superoxide anion (O_2^-) can be formed by mitochondrial electron transport chain complexes, phagocytic and nonphagocytic NAD(P)H oxidases (Pagano et al. 1995, Griendling et al. 1994), xanthine oxidase (XO) (Kuppusamy et al. 1989), cyclooxygenase (COX), lipooxygenase (LOX) and cytochrome P_{450}

mono-oxygenase in cardiomyocytes, fibroblasts and endothelial cells during normol physiologic conditions and I/R (Kim et al. 1998).

Most cells in the vasculature produce ROS that acts as a signaling molecule to regulate key events like cell growth (Suzuki et al. 1999). NAD(P)H oxidases are a major source of O_2^- in cardiomyocytes and vasculature cell types and are believed to be essential for normal physiological responses like growth, migration and extracellular matrix remodeling. NAD(P)H oxidases found in neutrophils are called phagocytic oxidases and those in vasculature and cardiac cells are called the non-phagocytic types. The phagocytic NAD(P)H are membrane bound electron transport chain oxidases that produce small amounts of O_2^- while the non-phagocytic oxidases produce 10-100 times more O_2^- than the former but in short bursts (Griendling et al. 2000). In addition mitochondria are known to produce most of the ROS under normal physiologic conditions through the respiratory chain (Chance et al. 1979). Through oxidative phosphorylation in the respiratory chain, mitochondria produce 80-90% of high-energy phosphates (adenosine triphosphate; ATP) in mammalian tissues; ATP production is coupled with generation of water. During this process, enzymes and coenzymes deliver electrons from reduced nicotinamide adenine dinucleotide ($NADH^+$) – reduced flavin adenine dinucleotide ($FADH^+$) pathways to molecular oxygen. This molecular oxygen is reduced to water by cytochrome oxidase. Majority (98%) of these electrons are tightly coupled to ATP production as their high energy is

converted to ATP but about 1-2% results in the O_2^- formation (Boveris et al. 1976, Cadenzas et al. 2000, Raha et al. 2000, Sugioka et al. 1988).

Under normal physiologic conditions, natural defenses of the cell (mitochondrial manganese, cytosolic copper and zinc superoxide dismutases; Mn SOD and Cu/Zn SOD), glutathione peroxidase (GSH-PO) and phospholipid hydroperoxide (Boveris et al. 1976, Cadenzas et al. 2000, Raha et al. 2000, Sugioka et al. 1988, Augustin et al. 1997) clear O_2^- to form hydrogen peroxide (H_2O_2), which provides water and molecular oxygen due to the action of catalase and or glutathione peroxidases. H_2O_2 can also act as a second messenger to regulate various enzyme (phospholipase A_2, C and D, Src Kinase, p38 MAPK, cJNK and Akt/PKB) activities for cell growth, calcium release or apoptosis (Griendling et al. 2000). On the other hand, during stress such as ischemia, H_2O_2 forms a highly reactive hydroxyl radical via the Fenton and or the Haber Weiss pathways (Fenton. 1894, Haber et al. 1934). O_2^- forms peroxynitrite ($ONOO^-$), a highly toxic ROS, through a reaction with nitric oxide (NO) (Beckman et al. 1990). NO and O_2^- are produced by the same cell types during ischemia (vascular endothelium, nutrophils and myocytes). Peroxynitrite causes cellular damage through lipid peroxidation (Radi et al. 1991, Lin et al. 1997, Van der Vliet et al. 1991), DNA fragmentation (apoptosis) (Lin et al. 1997), and depletion of antioxidants (glutathione and cysteine). Through its reaction with carbon dioxide (CO_2) it forms carbonium (CO_3^-), which reacts with NO_2^- to form nitronium ion (NO_3^-) that causes nitration of proteins resulting in organ damage (Ma et al. 1997). During reperfusion of the

ischemic heart, NO production (and release) by endothelial NO synthase (eNOS) and inducible NOS (iNOS) (Dinerman et al. 1993) coincides with the O_2^- production that facilitates peroxynitrite formation (Liu et al. 1997, Yasmin et al. 1997). Thus in addition to oxyradical production, excessive formation of peroxynitrite and other oxidants during I/R injury may promote the occurrence of oxidative stress in the myocardium.

b. Intracellular calcium overload and calcium homeostasis

In order to understand the role of intracellular Ca^{2+} overload in cardiac dysfunction during I/R, it is important to describe some salient features of Ca^{2+} homeostasis in cardiomyocytes. A large (10,000 fold) gradient of Ca^{2+} exists across the SL membrane. Ca^{2+}concentration in the extracellular spaces is approximately 1.25 mM while that in the intracellular space ranges between 10^{-7}M (diastole) and 10^{-5} M (systole) respectively. There is tight regulation of the amount of Ca^{2+} that enters or leaves the cell during cardiac contraction and relaxation. It must be the same or the cell would gain or lose Ca^{2+} causing cardiomyocyte dysfunction (Bers. 2002). It is pointed out that the cell requires Ca^{2+} ion for cellular integrity, regulation of metabolism, cell growth and proliferation. Furthermore, Ca^{2+} is responsible for cardiac electrical activity as well as for the activation of myofilaments leading to contraction. Disturbance of this Ca^{2+} homeostasis in cardiomyocytes during I/R leads to contractile abnormalities and arrhythmias.

Excitation-contraction (EC) coupling is the process where electrical excitation of the myocyte eventually results in contraction. Ca^{2+} acts as a second messenger, which is involved in cardiac electrical activity and direct activation of the myofilaments (Bers. 2002). A small amount of Ca^{2+} enters the cell through L-type Ca^{2+}-channels during depolarization of SL from the extracellular space (primary source of Ca^{2+}). This small amount of Ca^{2+} causes the release of a large amount of Ca^{2+} from the SR Ca^{2+} stores through the Ca^{2+}-release channels or ryanodine receptor (RyR) (Fabiato et al. 1977, Fabiato. 1983). This increase in $[Ca^{2+}]$ in the cytosol causes Ca^{2+} to bind to troponin C (remove inhibition), causing activation of the contractile apparatus. However, during diastole, for relaxation to occur, $[Ca^{2+}]$ in the cytosol must decrease. This occurs due to uptake of Ca^{2+} by SR Ca^{2+}-pump ATPase, exchange of Ca^{2+} for Na^{+} via the Na^{+}-Ca^{2+}-exchange (NCX), SL Ca^{2+}-pump ATPase and mitochondrial Ca^{2+}-uptake causing Ca^{2+} to dissociate from troponin (Bers. 2002, Wolska et al. 1993, Bassani et al. 1994, Negretti et al. 1993). Ca^{2+} release from SR causes an exponential increase in intracellular Ca^{2+} levels (Bassani et al. 1994). RyR is phosphorylated by Ca^{2+}calmodulin dependent protein kinase (CaMK at Ser-2809) and by cAMP-dependent protein kinase (PKA) for its actions. It is pointed out that RyR has three isoforms, RyR1 (skeletal muscle), RyR2 (cardiac tissue) and RyR3 (ubiquitous), which are encoded by three different genes (Marks et al. 1987). Furthermore, RyR complexes (functional) with other proteins such as CQS, junctin, triadin and FK506 (Zhang et al. 1997)

and plays an important role in T-lymphocyte activation and fertilization (Marks. 1997) besides EC coupling in the myocardium.

Due to the presence of Ca^{2+}-pump ATPase (SERCA; a Ca^{2+} and Mg^{2+}-dependent ATPase protein) (Komuro et al. 1989), SR sequesters Ca^{2+} from the cytosol to cause relaxation of the myofilaments and make Ca^{2+} available for the action potential and restoring the gradient across the SR. Encoded by three highly homologous genes: SERCA1, SERCA2, and SERCA3, it occurs as SERCA 1a, 1b (adult and neonatal fast-twitch skeletal muscles) and 2a (normal or stressed myocardium and slow-twitch skeletal muscle (Arai et al. 1994, Zarain-Herzberg et al. 1990, Anger et al. 1994). SERCA2b (smooth muscle and non-muscle tissues) and SERCA3 (non-muscle isoform) are mainly expressed in epithelial and endothelial cells (Lytton et al. 1989, Anger et al. 1993). SERCA2a activity is regulated by direct phosphorylation by CaMK at Ser-38 and phosphalamban (PLB), a small (52 amino acid) SR protein that regulates SR Ca^{2+} transport (Toyofuku et al. 1994). It has been proposed that in a dephosphorylated state PLB decrease the affinity of SERCA2a for Ca^{2+} (MacLennan et al. 1997). PLB phosphorylation by PKA and CaMK II facilitates uptake of Ca^{2+} and cardiac relaxation by removing its inhibitory effect on SERCA2a. PLB regulation of SERCA2a has been proposed to act through; (i) direct physical interaction between SERCA2a and PLB (Kimura et al. 1997), (ii) changes in SR membrane potential by phosphorylation of PLB, (iii) changes in the lipid motion that affect the membrane fluidity (Chiesi et al. 1989, Cornea et al. 1997) and (iv) by a Ca^{2+}

leak mediated by PLB through pores formed by its pentameric structure (108). Encoded by one gene in all species, PLB is a highly conserved protein expressed only in one form in the cardiac and skeletal muscle (Simmerman et al. 1998).

Calsequestrin (CQS) stores Ca^{2+} in the SR lumen (Mitchel et al. 1988) to be released by the next wave of depolarization. Between the two isoforms of CQS only one isoform is expressed in the developing, adult and aging cardiac tissue (Mitchel et al. 1988, Yano et al. 1994). CQS forms a functional complex with RyR and other SR proteins (FKBP, junctin and triadin), which facilitate Ca^{2+}-release (Zhang et al. 1997). On the other hand, a delicate balance between phosphorylation and dephosphorylation of SR Ca^{2+}-handling proteins maintains Ca^{2+}-homeostasis in cardiomyocytes and therefore cardiac contraction and relaxation process. An endogenous CaMK phosphorylates RyR, SERCA2a, and PLB (Baltas et al. 1995) while PKA phosphorylates RyR and PLB (Yano et al. 1994, Baltas et al. 1995, Le Peuch et al. 1979, Mithel et al. 1988, Maclenna et al. 1971, Cala et al. 1991, Kranias et al. 1985, Xu et al. 1993). As discussed above, SERCA2a affinity for Ca^{2+} is inhibited by the dephosphorylated form of PLB during systole (Suzuki et al. 1986) while phosphorylation of PLB removes its inhibitory effect on SERCA2a and causes increased affinity and uptake of Ca^{2+} by SERCA2a causing relaxation during diastole (Davis et al. 1983, Talosi et al. 1993, Lompre et al. 1994). Similarly, RyR phosphorylation by CaMK and PKA increases its open state thereby increasing Ca^{2+} release and promoting contraction (Takasago et al. 1991). Under normal physiologic conditions, these

phosphorylated proteins are dephosphorylated by an endogenous phosphatase (both CaMK and PKA phosphorylated substrates) in a non-discriminatory manner (Kranias et al. 1986). Hence, protein content and phosphorylation of SR proteins RyR, SERCA2a, PLB and CQS is critical for Ca^{2+} homeostasis as I/R injury has been shown to disturb this delicate balance causing intracellular Ca^{2+} overload and contractile dysfunction. It is now widely accepted that development of intracellular Ca^{2+} overload due to I/R results in cardiac dysfunction, cell damage and contracture. Intracellular Ca^{2+} overload induced severe tissue damage and contracture were also seen when hearts perfused with Ca^{2+} free buffer were reperfused with a buffer containing Ca^{2+} (Zimmerman et al. 1967). In I/R-induced Ca^{2+} overload, a combination of factors that start with acidosis (accumulation of H^+ ions and lactate) due to oxygen deficiency during ischemia result in decreased levels of ATP and the sodium-hydrogen exchanger (NHX) gets activated causing increased influx of Na^+. Upon reperfusion however, the sodium-calcium exchanger (NCX) also becomes activated (as the acidosis is reversed), and exchanges Ca^{2+} for Na^+ inside the cell causing excessive amount of Ca^{2+} accumulation inside the cell (Grinwald. 1982). Subsequently, it was shown that I/R caused disruption of the Na^+ - K^+ ATPase pump that accentuates the development of intracellular Ca^{2+} overload (Grinwald. 1992, Marban et al. 1990). Results from our and other laboratories have shown that SR and SL Ca^{2+} cycling and regulating proteins are targets for proteolytic action of Ca^{2+} activated proteases such as calpain (Singh et al. 2004a, Yoshida et al. 1993, Yoshida et al.

1990). Recent studies have noted a transient but significant increase in intracellular Ca^{2+} that could cause activation of calpains (Carlos et al. 2010). Thus it appears that cardiac dysfunction elcited by I/R may be a consequence of the proteolytic action as well as depletion of the myocardial energy stores due to the development of intracellular Ca^{2+} overload.

3. Role of proteases in ischemia reperfusion injury

Different types of enzymes with proteolytic activity have been identified in the myocardium (calpain, MMP, cathepsins, ubiquitin-proteosome) (Yoshida et al. 1995, Ali et al. 2009, Mitch et al. 1996, Goldberg. 2005). Calpain is a cytosolic calcium-dependent cysteine protease involved in a variety of Ca^{2+} -regulated cellular processes such as signal transduction, cytoskeletal remodeling, cell proliferation and differentiation, apoptosis, membrane fusion, platelet activation, cell cycle regulation, insulin secretion and sex determination (Saido et al. 1994, Sorimachi et al. 1997). Several types of calpain exist, but two ubiquitous isoforms that differ in their Ca^{2+} sensitivity are common. These are μ-calpain and m-calpain (named due to their micromolor and millimolar Ca^{2+} requirement). It exists in the cytosol as an inactive enzyme and translocates to the cell membrane in response to increases in the cellular Ca^{2+} level that occur transiently during ischemia and I/R, where it is further activated by the presence of Ca^{2+} and phopholipids. Calpain activation occurs by autolysis and is triggered by the association of Ca^{2+} to at least 3 different sites on the enzyme. Under normal physiology, calpain activity is tightly regulated by Ca^{2+}, because disorder of calpain activity causes excessive

degradation or accumulation of coexisting cellular proteins resulting in serious cellular damage and pathological conditions (Saido et al. 1994, Sorimachi et al. 1997, Carafoli et al. 1998, Sorimachi et al. 2001, Huang et al. 2001). It is pointed out that calpastatin is a highly selective endogenous inhibitor of calpain, which is a heat-stable protein and is resistant to many denaturing agents such as urea, SDS, and tricholoracetic acid (Goll et al. 2003, Wendt et al. 2004).

Earlier studies have shown that calpain causes proteolytic modification of SR Ca^{2+} handling proteins such as SERCA, RyR, PLB (Singh et al. 2004a) and the SL proteins NCX, NHX and Na^{+} - K^{+} -ATPase during I/R (Samanta et al. 2010, Inserte et al. 2005, Inserte et al. 2006, Singh et al. 2010, Zolotarjova et al. 1994). This contributes in part to the genesis of intracellular Ca^{2+} overload. Calpain has been shown to degrade the membrane cytoskeleton proteins like fodrin (calspectin or non-erythroid spectrin) and ankyrins that maintain cell membrane integrity (Yoshida et al. 1995, Yoshida. 2000). Some studies have found degradation of α-fodrin and calpastatin during ischemia and I/R (Yoshida et al. 1995). Besides fodrin other cytoskeletal proteins such as desmin and α-actinin are also targeted by calpain. The reduction in degradation of these proteins seen with leupeptin (Leu) and calpain inhibitor I has been shown to correlate with improved cardiac contractility suggesting an association between calpain degradation of the cytoskeletal proteins and cardiac contractility (Matsumura et al. 1996). It has been claimed that fodrin is probably the only cytoskeletal protein to be degraded in I/R induced proteolysis as no evidence for the proteolysis of other earlier reported

proteins was seen (Tusji et al. 2001). While some studies have claimed the Ca^{2+} release channel of the SR (Rardon et al. 1990) and Ca^{2+} pump ATPase (Yoshida et al. 1990) to be possible targets for Calpain, the identity of the proteins mentioned is not clear. L-type Ca^{2+} channel (Kameyama et al. 1998) and RyR from the skeletal muscle have also been shown to be degraded by calpain (Rardon et al. 1990). Intracellular Ca^{2+} overload in patients with atrial fibrillation was found to be associated with calpain mediated proteolytic degradation of atrial proteins and this alteration in the structural integrity of the sarcomere was implicated in its pathogenesis (Goette et al. 2002).

Various studies have reported that the Ca^{2+} sensitivity of myofilaments is reduced in I/R (Carozza et al. 1992, Kusuoka et al. 1987). Ca^{2+} responsiveness of skinned muscle fibres decreases when exposed to calpain I (Gao et al. 1996), which makes calpain activation during I/R responsible for altering the myofilament structure and hence response to Ca^{2+}. Calpain induced degradation of contractile protein troponin T (TnT) results in persistence of immunoreactivity of the TnT in the serum of patients recovering from acute MI. TnT has emerged as one of the current diagnostic tools for MI (Gao et al. 1997, Katus et al. 1991, Gorza et al. 1996). Cross-linked proteins have been suggested to be responsible for the persistent immunoreactivity seen in MI patients. Uncontrolled Ca^{2+} dependent calpain activation has been implicated in several diseases such as stroke and Alzheimer's disease besides I/R. In addition, calpains have been reported to be involved in a wide range of pathological states associated with genetic mutations

in calpain. These include: type II diabetes which results from a mutation in calpain 10 (CAPN10), cataracts, Duchenne's muscular dystrophy, Parkinson's disease and Alzheimer's disease (Zatz et al. 2000, Tidball et al. 2000).

Another family of proteolytic enzymes found in the myocardium and implicated in I/R injury are the metalloproteinases (MMP). These exist in an inactive zymogen state under normal conditions and are activated by proteolysis, nitrosylation, glutathiolation, phosphorylation and oxidative stress (Ali et al. 2009, Van Hart et al. 1990, Viappiani et al. 2009). MMP-2 is located intracellularly in cardiac myocytes. It colocalized with troponin I within myofilaments and the nucleus in an inactive state. Furin activates it subsequent to ischemia and it is responsible for causing ECM degradation and increasing fibroblast migration. Significant amounts of MMP-2 exist in cardiomyocytes, which is activated by oxidative stress mediated through peroxynitrite (Ali et al. 2009). MMP activation can also occur through limited proteolysis by trypsin and elastase (Strongin et al. 1995). MMP activation is dependent upon two key promoters, Furin and membrane Type1-MMP (MT-MMP). Furin (a subtilisin/kexin-like proprotein convertase), which is a serine endopeptidase located in the trans golgi apparatus region of cells including cardiocytes. It is crucial for the activation of MMPs especially MT1 and MMP-2 responsible for fibroblast migration. Its expression is increased in ischemia. Similarly, MT-MMP and MMP-14 are proteases which are activated by Furin and located in the golgi apparatus, once activated these translocate to the membrane (Roghi et al. 2010). M-MMP activates MMPs such as MMP-2 causing increased

ECM and vascular endothelial basement membrane degradation. TGF beta 1 increases the MT1 and MMP-2 activity in cardiac fibroblasts thereby increasing their migration (Stawowy et al. 2004, Schram et al. 2011).

Two types of cell death occurs; necrotic and apoptotic. Necrosis (or non - apoptotic cell death) (Nicholson et al. 1997) is a rapid, irreversible process that occurs when cells are severely damaged and involves ATP depletion, loss of ion gradients and membrane rupture (Searle et al. 1982). Apoptosis on the other hand is a programmed, highly organized, energy dependent mechanism whereby a cell commits suicide without causing damage to the surrounding tissue with maintenance of ATP and DNA fragmentation (Kaufmann et al. 2001). Apoptosis occurs in cardiac tissue during ischemia and/or reperfusion and is mediated by a family of cysteine-dependent aspartate specific proteases called caspases (Haunstetter et al. 1998). This is believed to occur due to activation of plasma membrane death receptors and/or translocation of Bcl-2 homologous proteins to mitochondria, mitochondrial permeability transition followed by cytochrome c release, caspase activation and contractile dysfunction. Besides apoptosis, cardiomyocytes also undergo caspase-independent 'autophagic' death (Knaapen et al. 2001, Kitanaka et al. 1999). Apoptosis plays a pivotal role in the development of septal, valvular and other vascular structures and various congenital cardiac diseases (Rothenberg et al. 2003). It also causes fatal arrhythmias, heart blocks and sudden death in young patients with partially destroyed or completely absent AV nodes, sinus nodes or internodal pathways (James et al. 1996). Apoptosis is

also involved in NO synthase (NOS3 or eNOS) deficiency causing heart failure and congenital septal defects (Feng et al. 2002). Further, apoptosis occurs in chronic heart failure (CHF) with associated atrial fibrillation (due to atrial structural remodeling) through angiotensin II dependent and independent pathways (Cardin et al. 2003). Thus it is evident that apoptosis may play an important role in the pathophysiology of I/R-induced cardiac dysfunction.

II. STATEMENT OF THE PROBLEM AND HYPOTHESIS

Earlier studies have shown that cardiac dysfunction due to I/R is associated with depression in activities of subcellular organelles such as SL, SR, myofibrils and mitochondria. Various mechanisms including the occurrence of intracellular Ca^{2+} overload, development of oxidative stress and activation of proteases (calpain and MMPs) have been suggested to explain I/R induced cardiac dysfunction and alterations in subcellular activities. However, the exact relationship among these mechanisms and cardiac abnormalities due to I/R injury is not fully understood. Although endothelial dysfunction has also been identified to occur in IHD, the exact role of changes in endothelial function in I/R induced impairment of cardiac performance and depression in subcellular activites is far from clear. Therefore, in this study we examined the status of endothelium and involvement of endothelial alterations in I/R induced changes in cardiac function as well as SL Na^+-K^+ ATPase and SR Ca^{2+}-uptake activities. Furthermore, we have investigated the role of calpain activation in inducing cardiac dysfunction and depressing SL Na^+-K^+ ATPase and SR Ca^{2+}-uptake activity due to I/R injury. We also studied the role of oxidative stress in activation of calpain and MMPs as well as depression of cardiac function and SL Na^+-K^+ ATPase activity upon exposing the heart to I/R injury. This investigation was undertaken to test the hypothesis that both endothelial dysfunction and oxidative stress play a critical role in I/R-induced activation of calpain, depression of SL Na^+-K^+ ATPase activity and impairment of cardiac function. The following three sets of experiments were carried out to gain some

information in this regard.

1. I/R-induced changes in cardiac function, subcellular activities and endothelial dysfunction

Although cardiac dysfunction due to I/R has been shown to occur in isolated rat heart preparations perfused either at CF or at CP (Dhalla et al. 1988, Singh et al. 2004a, Singh et al. 2004b, Chohan et al. 2006, Hearse et al. 2000), the depression in cardiac function due to I/R in CF hearts was found to be greater then that in CP-perfused hearts. Furthermore, it is now well known that the activities of SL Na^+-K^+ ATPase and SR Ca^{2+}-uptake are depressed in hearts subjected to I/R injury. Since endothelial function with respect to the production of NO was observed to be defective in IHD (Adams. 2006, Anderson. 2003), this set of experiments was carried out to test the hypothesis that differences in the I/R-induced changes in cardiac contractile parameters and subcellular activities between hearts perfused at CF and CP are due to corresponding alterations in endothelial function. Since the duration of ischemia has been shown to determine the extent of I/R-induced injury (Dhalla et al. 1988, Saini et al. 2005), some experiments will be conducted to investigate if the I/R-induced changes in cardiac performance in hearts perfused at CF and CP are dependent upon the duration of ischemia. The I/R-induced alterations in SR Ca^{2+}-uptake and SL Na^+-K^+ ATPase activities as well as the activity of calpain in both CF-perfused and CP-perfused hearts will be examined to study if subcellular changes in these preparations are associated with the activation of proteolysis. It should be pointed out that a marked increase in

calpain activity has been shown to occur in I/R hearts (Singh et al. 2004a). The role of endothelium in eliciting I/R-induced changes in cardiac function and subcellular activities will be studied upon treatment of CF-perfused hearts and CP-perfused hearts with L-arginine (LA), a substrate for NO synthase (Chohan et al. 2006) and L-NAME (LN), a NO synthase (eNOS) inhibitor (Chohan et al. 2006), respectively. Furthermore, the status of NO production will be monitored by measuring the concentration of nitrate/nitrite in perfusate as well as protein content for eNOS in the myocardium. In addition, the CF-perfused and CP-perfused hearts will be used for studying changes in coronary pressure due to some agents, which are known to affect the coronary vessels through their actions on the endothelium (Djuric et al. 2007, Hearse et al. 1992, Lin et al. 2000).

2. Ischemia reperfusion-induced changes in sarcolemmal Na^+-K^+ ATPase and activation of calpain in the heart

It has been reported that the I/R induced depression in cardiac function is associated with a decrease in the SL Na^+-K^+ ATPase activity whereas oxidative stress is considered to contribute to alterations in the Na^+-K^+ ATPase isoforms (Ostadal et al. 2003, Kim et al. 1987). However, it remains to be examined if the Na^+-K^+ ATPase isoforms are targets for the action of calpain during the development of I/R injury. It is believed that an increase in the intracellular concentration of Ca^{2+} during I/R is sufficient to cause the activation of calpain isoforms 1 and 2 in the heart (Aono et al. 2001). Calpain translocation has also been observed in cancer cells upon activation by Ca^{2+} (Tagliarino et al. 2003),

however, no information regarding the translocation of calpain in cardiac cells due to I/R is available in the literature. Since both I/R and oxidative stress have been reported to promote the occurrence of intracellular Ca^{2+}-overload and activate calpain, this study will be undertaken to investigate if the activation of calpain in I/R hearts is associated with alterations in the SL Na^{+}-K^{+} ATPase activity and its isoform content. The direct effect of calpain on Na^{+}-K^{+} ATPase will be tested by incubating the isolated SL membranes with calpain in the absence and presence of calpain inhibitor, leupeptin. Dependency of the I/R-induced alterations in cardiac function, SL Na^{+}-K^{+} ATPase activity and calpain activity on Ca^{2+} will be determined by perfusing the hearts with media containing different concentrations of Ca^{2+}. The role of calpain in eliciting I/R-induced changes in cardiac function and SL Na^{+}-K^{+} ATPase will be further established by using MDL28170 (MDL), a specific inhibitor of calpain (Li et al. 1998, Inserte et al. 2006). The translocation of calpain will be examined by confocal microscopy of cardiomyocytes obtained from hearts subjected to I/R in the absence or presence of protease inhibitors.

3. **Activation of proteolytic enzymes, depression in sarcolemmal Na^{+}-K^{+} -ATPase and oxidative stress in ischemia-reperfused heart**

The development of intracellular Ca^{2+}-overload in the I/R heart has been reported to activate calpain, degrade different subcellular proteins, depress the activities of various subcellular organelles and result in the impairment of cardiac function (Singh et al. 2004a, Chohan et al. 2006). On the other hand, the occurrence of oxidative stress in the I/R heart has been shown to increase the activity of MMPs,

degrade different proteins in the extracellular matrix and produce cardiac dysfunction (Muller et al. 2011, Schulz. 2007, Spinale. 2007, Spinale. 2010). Various studies have also identified the localization of MMP-2 in cardiomyocytes and the activity of this proteolytic enzyme has been shown to increase in hearts subjected to I/R as well as oxidative stress (Ali et al, 2009, Ali et al. 2010, Schulz. 2007, Sung et al. 2007, Wang et al. 2002). In fact, different subcellular proteins have been reported to be the targets of MMP-2 and thus the activation of intracellular MMP has also been suggested to explain subcellular defects and cardiac dysfunction due to I/R (Sawicki et al. 2005, Shulze et al. 2003, Spinale. 2010). It is pointed out that, unlike the activation of MMP due to oxidative stress, such information with respect to the activation of calpain is not available. Furthermore, although the I/R-induced depression in SL Na^+-K^+-ATPase activity is associated with activation of calpain (Singh et al. 2010), the relationship between the I/R-induced MMP activation and SL Na^+-K^+-ATPase inhibition has not been examined previously. This study, therefore, will be undertaken to test if the activation of proteolytic enzymes, calpain and MMP, due to I/R is mediated through oxidative stress in hearts under different experimental conditions, which simulate or ameliorate the effects of I/R in the heart. Lack of oxygen and substrate during myocardial ischemia, experimental perfusion with H_2O_2 (an oxidant) and xanthine plus xanthine oxidase (X+XO; an oxyradical generating mixture) are known to produce oxidative stress (Makazan et al. 2007, Saini et al. 2005). We plan to study if I/R-induced effects on the heart are simulated by hypoxia-

reoxygenation as well as reperfusion of the heart exposed to H_2O_2 or X+XO. In order to further establish the role of oxidative stress in eliciting I/R-induced alterations, the effects of I/R will be investigated in heart subjected to ischemic preconditioning, which has been shown to reduce oxidative stress and intracellular Ca^{2+}-overload (Saini et al. 2005). In addition, hearts treated with antioxidants such as N-acetylcysteine (NAC) and mercaptopropionylglyceine (MPG) (Makazan et al. 2007) will be used for examining the I/R-induced changes in cardiac function as well as SL Na^+-K^+-ATPase and proteolytic enzyme activities. The significance of increases in calpain and MMP activities in I/R hearts for inducing depression in cardiac function and SL Na^+-K^+-ATPase activity will also be examined by employing hearts perfused with doxycycline (Dox), a MMP inhibitor (Sawicki et al. 2005), as well as Leu and MDL, calpain inhibitors (Li et al. 1998, Singh et al. 2010). This study will elucidate the role of protease activation in depressing subcellular activities and development of cardiac dysfunction in the pathogenesis of cardiac dysfunction due to I/R injury.

HYPOTHESIS

1. Hearts subjected to I/R produce defects in endothelium and oxidative stress, which play a critical role in the activation of calpain, inhibition of SL Na^+-K^+-ATPase activity, resulting in cardiac dysfunction.

2. I/R induced activation of calpain is associated with alterations in SL Na^+-K^+-ATPase isoform protein content and activity.

3. Oxidative stress mediates activation of calpain during I/R. Together, oxidative stress and calpain are instrumental in the activation of MMPs.

III. MATERIALS AND METHODS

The experimental protocol for all experiments was approved by the Animal Care and Ethics Committee of the University of Manitoba and conforms to the Guidelines of the Canadian Council on Animal Care.

1. Experimental design

By employing an isolated rat heart as an experimental model, we have examined: a) the role of endothelium in I/R induced cardiac dysfunction and subcellular enzyme activities; b) the effect of calpain activation on ischemia reperfusion induced alterations in Na^+-K^+-ATPase activity; c) the effects of oxidative stress on I/R induced alterations in protease activity, cardiac function and Na^+-K^+-ATPase activity. Various methods used in this study are as follow.

2. Perfusion protocol and measurement of cardiac performance

Male Sprague-Dawley rats weighing 225-275 g were anaesthetized with a mixture of ketamine (90 mg/kg) and xylazine (9 mg/kg). Hearts were rapidly excised and cannulated to the Langendorff apparatus for retrograde perfusion with a modified Krebs-Henseleit (K-H) buffer as described earlier (Chohan et al. 2006, Singh et al. 2004a). Left ventricular developed pressure (LVDP), left ventricular end diastolic pressure (LVEDP) and the rate of ventricular pressure development (+dP/dt) and rate of ventricular pressure decay (-dP/dt) were measured with a water filled latex balloon inserted into the left ventricle and attached to a pressure transducer. Data were recorded using model MP100 analogue to digital interface and processed using the AcqKnowledge 3.5.3 software for Windows (BIOPAC Systems Inc.,

Goleta, CA).

Hearts were perfused at a constant flow (CF) of 10 ml/min or at a constant pressure (CP) of 80 mm Hg and stabilized with K-H buffer for 20 min; these parameters of perfusion in CF-perfused and CP-perfused hearts were selected to match the coronary flow in both experimental preparations. All experimental hearts were exposed to 30 min global no-flow ischemia, unless otherwise indicated in the text, followed by 60 min of reperfusion. In all experiments, control hearts perfused at CF or CP for corresponding duration were not subjected to I/R.

3. L-arginine and L-NAME Treatment

Hearts perfused at CF and exposed to 30 min ischemia and 60 min reperfusion were treated with and without L-arginine (LA; 3mM), a substrate for NO synthase, (Chohan et al. 2006), or a combination of LA and a NOS inhibitor, L-NAME (LN; 200 μM) (Chohan et al. 2006). On the other hand, hearts perfused at CP and exposed to 30 min ischemia and 60 min reperfusion were treated with and without LN (200 μM) or LA (3mM). Treatments with these agents were carried out for 10 min before inducing ischemia as well as for 20 min during reperfusion (Chohan et al. 2006). It should be mentioned that these treatments did not alter the control coronary pressure (76.1 ± 4.7 mm Hg) in CF-perfused hearts or control coronary flow (12.5 ± 1.1 ml/min) in CP-perfused hearts.

4. Calpain inhibitor (Leupeptin and MDL) treatment

Hearts were perfused at a CF of 10 ml/min and stabilized with K-H buffer for 20 min before being distributed to various groups. All experimental hearts were exposed to 30 min global no-flow ischemia, followed by 60 min of reperfusion except controls. Hearts perfused and exposed to 30 min ischemia and 60 min reperfusion were treated with and without calpain inhibitors, leupeptin and MDL (Calbiochem) (Singh et al. 2004a; Inserte et al. 2006). Treatments with these agents were carried out for 10 min before inducing ischemia as well as for 20 min during reperfusion. Different concentrations of leupeptin were used to determine the optimal concentration (25 μmol/l) whereas a 10 μmol/l concentration of MDL28170 was selected on the basis of that employed by other investigators (Inserte et al. 2006).

5. Oxygen lack and substrate depletion perfusion protocol

Hearts were perfused at a CF of 10 ml/min and stabilized with K-H buffer for 20 min before being distributed to various groups: (a) Control hearts were perfused for 60 min with oxygenated medium; (b) I/R hearts were exposed to 30 min global no-flow ischemia followed by 30 min reperfusion; (c) Hearts in the oxygen-lack and substrate-depletion group were exposed to hypoxia in the presence and absence of glucose; these hearts were then reperfused with the KH-buffer (Temsah et al. 2002); (d) To simulate I/R-induced changes in cardiac performance, hearts were perfused with H_2O_2 (100 μM) or xanthine (X) (2 mM; Sigma-Aldrich) plus

xanthine oxidase (XO) (60 mU/ml; Boeringer-Mannheim Canada, Laval, Quebec) mixture for 30 min and then reperfused for 30 min (Temsah et al. 1999); (e) In another set of experiments, hearts were treated with N-acetylcysteine (NAC) (100μM) and mercaptopropionylglycine (MPG) (300μM; both from Sigma-Aldrich) for 10 min before inducing ischemia and 20 min at the beginning of reperfusion (Makazan et al. 2007).

6. Ischemic Preconditioning protocol

Hearts were subjected to ischemic preconditioning (IPC) by subjecting them to three cycles of 5 min ischemia and 5 min reperfusion; the IPC hearts were then subjected to 30 min of global ischemia and 30 min of reperfusion (Elmoselhi et al. 2003).

7. MMP inhibitor (Doxycycline) treatment

To investigate the activation of MMP, I/R hearts were treated with and without an MMP inhibitor, doxycycline (Dox) (100 μM), for 10 min before and 20 min after ischemia (Cheung et al. 2000).

All hearts were removed and freeze-clamped using liquid nitrogen and stored at -70^{o}C for biochemical analysis.

8. Isolation of sarcolemmal membrane

SL membranes were isolated from perfused hearts by a method as described earlier (Dixon et al. 1992, Singh et al. 2008, Pitts. 1979). Briefly, hearts were homogenized and centrifuged at 12,000 x g for 30 min and the supernatant was

collected and centrifuged at 100,000 x g for 60 min. The resultant pellet was suspended in MOPS-KCl buffer and layered onto a solution containing Tris-HCl, sodium pyrophosphate, KCl and sucrose, and centrifuged at 100,000 x g. This yielded three layers; the middle white layer was carefully suctioned out and centrifuged at 100, 000 x g for 30 min. The resultant pellet containing purified SL was suspended in 250 mM sucrose - 10 mM histidine buffer and frozen in liquid nitrogen and stored at –80^{o}C till further use.

9. Isolation of sarcoplasmic reticulum vesicles

In another set of experiments, SR vesicles were isolated using an established technique (Chohan et al. 2006, Singh et al. 2004a). Briefly, ventricular tissue was homogenized and centrifuged for 20 min at 10,000 x g. The supernatant was centrifuged for 45 min at 43,666 x g. The cytosolic fraction from the resultant supernatant was frozen in liquid nitrogen prior to storage at -80^{o}C for determining the calpain activity. The pellet was resuspended in a buffer containing 0.6 M KCl and 20 mM Tris-HCl (pH 6.8) and centrifuged for 45 min at 43,666 x g. The final pellet representing SR fraction was suspended in a buffer containing 250 mM sucrose and 10 mM histidine (pH 7.0) and frozen in liquid nitrogen prior to storage at -80^{o}C.

All buffers used for the isolation of SL and SR fractions contained a cocktail of protease inhibitors to prevent protein degradation during the isolation procedure (Chohan et al. 2006, Singh et al. 2004a).

10. Measurement of SL Na^+-K^+-ATPase Activity

Na^+-K^+-ATPase activity was measured in SL preparations by a technique described earlier (Dixon et al. 1992, Singh et al. 2008, Singh and Dhalla. 2010). SL was incubated at 37°C in assay tubes containing 1.0 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra acetic acid (EGTA), 5 mM NaN_3, 6 mM $MgCl_2$, 100 mM NaCl and 10 mM KCl in a total volume of 0.5ml. Reaction was started by the addition of 25 μl of 80 mM Tris-ATP, pH 7.4 and terminated after 10 min with 0.5 ml ice cold 12% trichloroacetic acid. The tubes were centrifuged at 3000 rpm for 10 min and the supernatant was used for Pi assay. Na^+-K^+-ATPase activity was calculated as the difference between activities with and without Na^+ plus K^+.

11. Measurement of SR Ca^{2+}-Uptake

SR Ca^{2+}-uptake was measured by a procedure described earlier (Chohan et al. 2006, Singh et al. 2004a). The reaction mixture contained (in mmol/L): 50 Tris-Maleate (pH 6.8), 5 NaN_3, 5 ATP, 5 $MgCl_2$, 120 KCl, 5 potassium oxalate, 0.1 EGTA, 0.1 $^{45}CaCl_2$ (20 mCi/L) and 25 μmol/L ruthenium red. Ruthenium red, a blocker of the SR Ca^{2+}-release channel was added to inhibit SR Ca^{2+}-release. The reaction was initiated by adding SR vesicles (20 μg protein) to the reaction mixture at 37^0C and terminated after 1 min by filtration. Ca^{2+}-uptake was initiated by adding SR vesicles (20 μg protein) to the reaction mixture at 37°C and terminated after 1 min by filtration; the filters were washed, dried and counted in a beta scintillation counter (Beckman Coulter Inc., Fullerton, USA).

12. Determination of NO formation

The concentration of nitrate/nitrite, an index of NO formation, was determined in the coronary perfusate by modifying a previously described method (Chohan et al. 2006). Coronary perfusate from the CF-perfused and CP-perfused hearts during the 60 min period of reperfusion was collected and its volume in both cases was adjusted to 750 ml with K-H buffer but no correction was made for these adjustments for reporting the concentration of nitrate/nitrite. The assay detects NO photometrically by measuring its oxidation products, nitrate and nitrite; nitrate is converted to nitrite, which reacts with sulfanilamide to form a red diazo dye that is read at 550 nm.

13. Measurement of coronary perfusion pressure and coronary flow

Coronary perfusion pressure was measured in CF-perfused hearts to examine the endothelial function/responsiveness in the presence and absence of acetylcholine (ACH; Sigma-Aldrich) and U46619 (a thrombaxane A2 receptor agonist from Sigma-Aldrich). Both ACH (1 μM) and U46619 (10 nM) were infused in the perfusion stream for a period of 10 min (Djuric et al. 2007, Garcia et al. 2005, Lin et al. 2000, Waldron et al. 1999). In order to measure coronary perfusion pressure in CP-perfused preparations, the hearts were switched to CF system for 3 min before infusing ACH or U46619. For the measurement of coronary perfusion pressure, a pressure transducer was connected to the aortic cannula and the pressure was recorded using model MP100 analogue to digital interface and

processed using the AcqKnowledge 3.5.3 software for Windows (BIOPAC Systems Inc., Goleta, CA). Coronary flow was measured by collecting the perfusate coming out of the coronary sinus.

14. Isolation of cardiomyocytes

Cardiomyocytes were isolated using a technique described elsewhere (Saini et al. 2005). Briefly, control and experimental hearts were switched to a perfusion medium containing 0.04% collagenase, 0.1% BSA, and 50 μM $CaCl_2$ at the end of 60 min of reperfusion. Hearts were removed from the canula and the ventricles were cut into small pieces and digested in a fresh collagenase solution containing 1% BSA gassed with a mixture of 95% O_2-5% CO_2 in a shaking water bath at 37°C. The cells from three to four harvests were combined and resuspended for 5 min in buffers containing gradually increasing concentrations of Ca^{2+} (250, 500, and 750 μM) to a final concentration of 1 mM. Cell viability in all experimental groups was checked with trypan blue (Sigma-Aldrich, Oakville, ON, Canada) exclusion method and the cells were counted in the Neubauer chamber. The final cell suspension had about 75- 80% viable cardiomyocytes.

15. Estimation of calpain activity

Calpain activity was measured in the cytosolic fraction obtained from above hearts as described earlier (Singh et al. 2004a) using a kit from Biovision. The reaction was carried out in a 96 well plate and the samples were read in a fluorescence microplate reader at 400 nm excitation and 505 nm emission (Molecular Devices, Sunnyvale, CA). The results were expressed in relative fluorescent

units (RFU). It is pointed out that no protease inhibitor was used in the buffer when the cystolic fraction was obtained for the determination of calpain activity.

16. Estimation of MMP activity

MMP activity was measured in the cytosolic fraction using a fluorgenic assay kit from Calbiochem. MMP in the sample cleave a flurogenic substrate [(Gly-Pro-Hyp*)5-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly↓Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-(Gly-Pro-Hyp)5-NH2]. *Hyp, 4-hydroxy-L-proline], which is selective for MMP-2 and -9, causing an increase in fluorescence. This increase is measured at an excitation wavelength of 320 nm and an emission wavelength of 405 nm using a microplate reader from Molecular Devices. All samples were prepared in an activation buffer and incubated for 6 hr after addition of the working solution, all of which were provided in the kit. It should be mentioned that the activity of calpain as measured here represent the activity of different isoforms of calpain whereas that for MMP represent the activities of both extracellular and intracellular MMP isoforms (Müller and Dhalla 2010).

17. Confocal microscopy imaging for calpain 1, 2 and calpastatin

Cardiomyocytes were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 3.7 mg/ml $NaHCO_3$ and 100 μg/ml gentamicin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cells were fixed onto coverslips with 4% paraformaldehyde after being washed three times with phosphate buffered saline (PBS). After brief permeablization with

Triton X, the coverslip contained cells were incubated with primary antibodies against calpain 1, 2 and calpastatin overnight at 4 °C. The cells were then treated with fluorescence-conjugated secondary antibodies [Alexa Fluo] for 90 min. After washing the cells three times with PBS, the coverslips were mounted to the slides and viewed by confocal microscope (Nikon Eclipse TE2000-U) (Saini et al. 2007).

18. SDS-PAGE and Western blot assay

a. Protein content of Na^+-K^+-ATPase isoforms

Protein content of Na^+-K^+-ATPase isoforms was determined by separating SL membranes (20 μg of total protein/lane) on a 10–12% SDS-PAGE gel and were electroblotted to polyvinylidene difluoride membranes (PVDF). The Na^+-K^+-ATPase isoforms were detected using the following primary antibodies: monoclonal anti-α_1 mouse IgG (0.05 μg/ml); polyclonal anti-α_2 rabbit IgG (1: 1,000); monoclonal anti-β_1 mouse IgG (0.8 μg/ml); polyclonal anti-β_2 rabbit IgG (1:1,000) (Upstate Biotechnology; Lake Placid, NY). Secondary antibodies consisted of biotinylated anti-mouse IgG (1:3,000) for α_1, β_1, and biotinylated anti-rabbit IgG (1:3,000) for α_2, and β_2 subunits (Upstate Biotechnology). Membranes were incubated for 1 hr with strepdavidin-conjugated horseradish peroxidase (1:5,000) and then processed for chemiluminescence (ECL Kit) on hyperfilm-ECL (Amersham Life Science). An imaging densitometer (model GS-670, Bio-Rad; Hercules, CA) was used to scan the bands, which were quantified using the Image Analysis Software (version 1.3). A rat brain purified microsomal preparation (Upstate Biotechnology) was used as a positive control to identify different Na^+-

K^+-ATPase isoforms. A 5-25% gradient gel (stained with Coomassie brilliant blue) was used to examine the protein content in SL samples incubated with calpain in the presence and absence of leupeptin. To determine relative densities of proteins, blot radiograms were scanned and the scan value for control in each group was taken as 100% and others were expressed as % of control. PVDF membranes were subjected to Coomassie brilliant blue to demonstrate equal protein loading after the radiograms were obtained.

b. Determination of eNOS Protein Content

eNOS protein content was determined in SL membrane by Western Immunoblotting (6). SL preparation (20-25 μg) was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in milk-TBS overnight and probed with eNOS antibody (Affinity BioReagents Inc. Golden, CO) in a dilution of 1:1500. The antibody detects eNOS at ~140kDa. A molecular weight marker from Sigma-Aldrich Canada, Oakville, ON was used to determine the band corresponding to eNOS. Equal protein loading was checked in every experiment by staining the membrane with Ponceau red before immunoblotting as well as with Coomassie Brilliant Blue at the end of the experiment. It should be mentioned that since iNOS, unlike eNOS, is mainly expressed in vascular smooth muscle and thus changes in iNOS content were not measured while studying alterations in endothelial function due to I/R injury.

19. Statistical Analysis

The data were expressed as mean ± S.E.M. and the differences between two groups were evaluated statistically by the Student's t-test. Differences among more than two groups were evaluated by one-way Analysis of Variance (ANOVA) followed by the Newman-Keuls test using Microsoft Origin. The data for the I/R-induced changes in CF-perfused and CP-perfused heart were also analyzed by 2-way ANOVA test for the purpose of comparison. A level of $P<0.05$ was considered the threshold for statistical significance between the control and experimental groups.

IV. RESULTS

1. Involvement of endothelium in ischemia reperfusion induced cardiac dysfunction and subcellular enzyme activities

a. Cardiac performance in CF-perfused and CP-perfused Hearts

In order to examine if the recovery of cardiac function in I/R hearts was dependent upon the duration of ischemia, hearts perfused at CF were subjected to ischemia for 15 and 30 min whereas hearts perfused at CP were subjected to 30 and 60 min of ischemia before initiating reperfusion for 60 min. The results in Table 1 indicate that values for the recovery of LVDP, +dP/dt and –dP/dt were progressively depressed whereas those for LVEDP were progressively increased upon reperfusion in 15 and 30 min ischemic CF-perfused hearts as well as in 30 and 60 min ischemic CP-perfused hearts. Furthermore, data in Table 1 show that I/R-induced depressions in LVDP, +dP/dt and –dP/dt as well as increase in LVEDP in 15 min and 30 min ischemic hearts perfused at CF were comparable to the respective values for these parameters in 30 min and 60 min ischemic hearts perfused at CP, respectively. Thus, it is apparent that hearts perfused at CP required longer duration of ischemic insult for inducing cardiac dysfunction similar to that in hearts perfused at CF. For all other experiments, hearts perfused at CF and CP were made ischemic for 30 min before inducing reperfusion to study differences in the effects of I/R in these preparations.

Another set of experiments was undertaken to investigate the role of NO

Table 1. Cardiac performance of hearts subjected to varying duration of ischemia followed by reperfusion for 60 min.

	Constant Flow Perfusion (CF)			Constant Pressure Perfusion (CP)		
	Control	15 min ischemia	30 min ischemia	Control	30 min ischemia	60 min ischemia
LVDP (mm Hg)	116 ± 7.2	73.2 ± 5.0	20.1 ± 3.2*	98 ± 6.7	66.2 ± 5.1	21.9 ± 2.7#
LVEDP (mm Hg)	4.4 ± 0.13	24.1 ± 7.1	94.9 ± 8.0*	4.5 ± 0.22	40 ± 7.2	89.4 ± 8.0#
+dP/dt (mm Hg/sec)	2456 ± 540	1386 ± 111	200 ± 69*	1960 ± 386	1486 ± 110	145 ± 67#
-dP/dt (mm Hg/sec)	1804 ± 412	1208 ± 140	178 ± 55*	1426 ± 307	1436 ± 119	195 ± 56#

LVDP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, +dP/dt: rate of pressure development and -dP/dt: rate of pressure decay. Since the control values at 15 min and 30 min for CF-perfused as well as at 30 min and 60 min for CP-perfused hearts were overlapping, the respective values were grouped together. Values are mean ± SEM of 5 hearts for each group. *$P<0.05$ in comparison to 15 min ischemia values in CF-perfused hearts; #$P<0.05$ in comparison to 30 min ischemia values in CP-perfused hearts. Statistical evaluation of data for 15 min and 30 min ischemia groups in CF-perfused and CP-perfused hearts as well as for 30 min and 60 min ischemia groups in CF-perfused and CP-perfused hearts did not show any significant ($P>0.05$) differences, respectively.

production in determining the differential effects of I/R in CF-perfused and CP-perfused hearts. The data in Figure 1 indicate that the I/R-induced depressions in LVDP, +dP/dt and -dP/dt as well as increase in LVEDP were significantly attenuated by treatment of CF-perfused hearts with 3 mM LA, which is known to serve as a substrate for NO synthase in the myocardium (Chohan et al. 2006). This beneficial effect of LA was not apparent when the hearts were treated with LA in the presence of 200 μM LN (Figure 1); this indicated that the effects of LA in improving the recovery of cardiac function are associated with the generation of NO. On the other hand, it can be seen from Figure 2 that the I/R-induced depressions in LVDP, +dP/dt and –dP/dt as well as increase in LVEDP were augmented by treatment of CP-perfused hearts with 200 μM LN, which is known to inhibit the production of NO (Chohan et al. 2006). Comparison of data in Figures. 1 and 2 indicated that the I/R-induced changes in cardiac function in CF-perfused hearts were greater than those in CP-perfused hearts ($P<0.05$). Furthermore, treatment of CP-perfused hearts (n=3) with 3 mM LA was found to prevent the I/R-induced depressions in LVDP, +dP/dt and –dP/dt as well as increase in LVEDP by 85 to 95% (data not shown).

b. SL Na^+-K^+ ATPase, SR Ca^{2+}-uptake and calpain activities

To examine the effects of I/R on subcellular function in hearts perfused at CF and CP, SL Na^+-K^+ ATPase and SR Ca^{2+}-uptake activities were determined and the results are shown in Figure 3. Although I/R was found to decrease SL Na^+-K^+ ATPase and SR Ca^{2+}-uptake activities in both CF-perfused and CP-perfused

Figure 1. Cardiac performance of the isolated rat hearts exposed to 30 min of ischemia and 60 min of reperfusion at constant flow

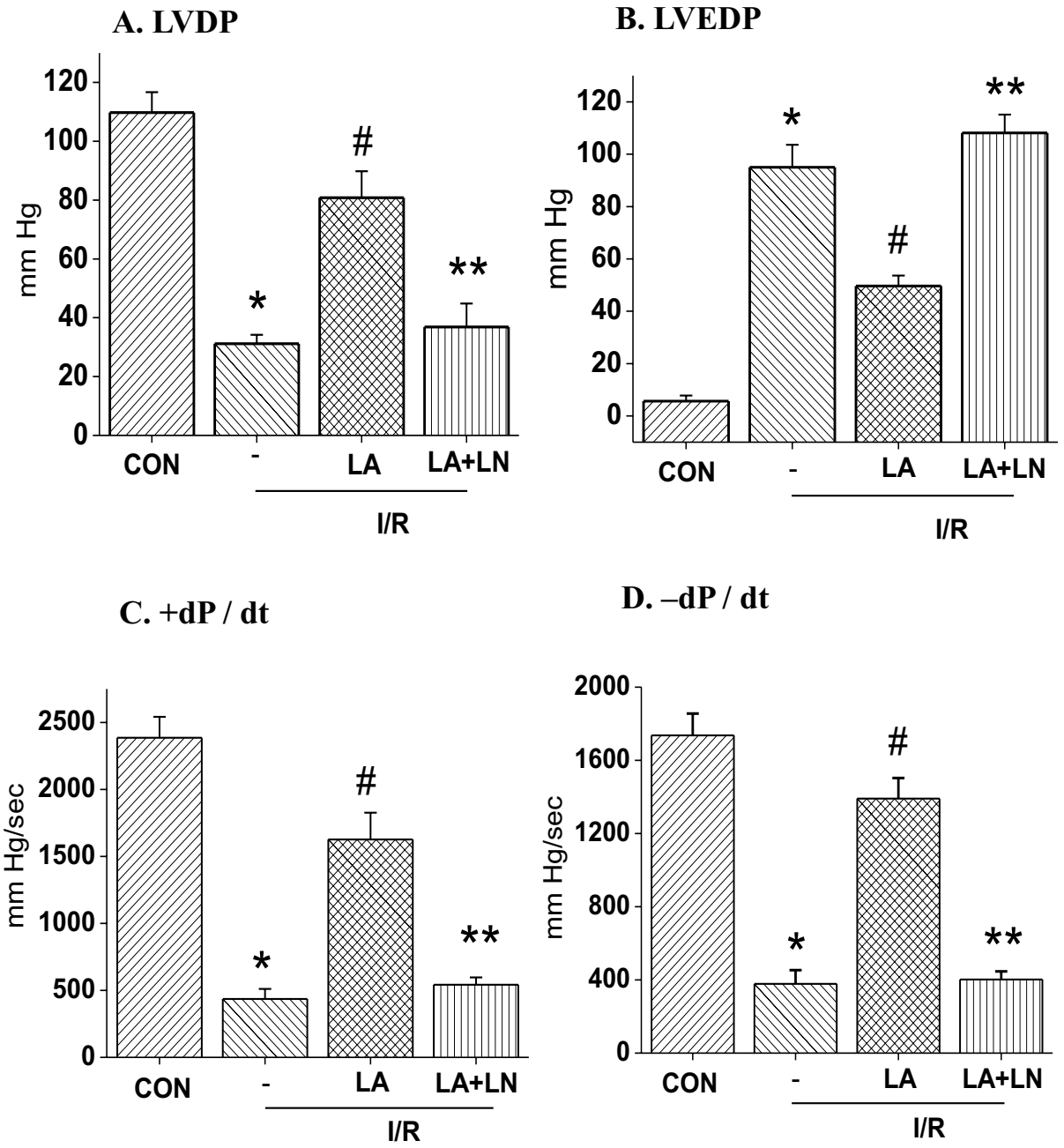


Figure 2. Cardiac performance of isolated rat hearts exposed to 30 min of ischemia and 60 min of reperfusion at constant pressure

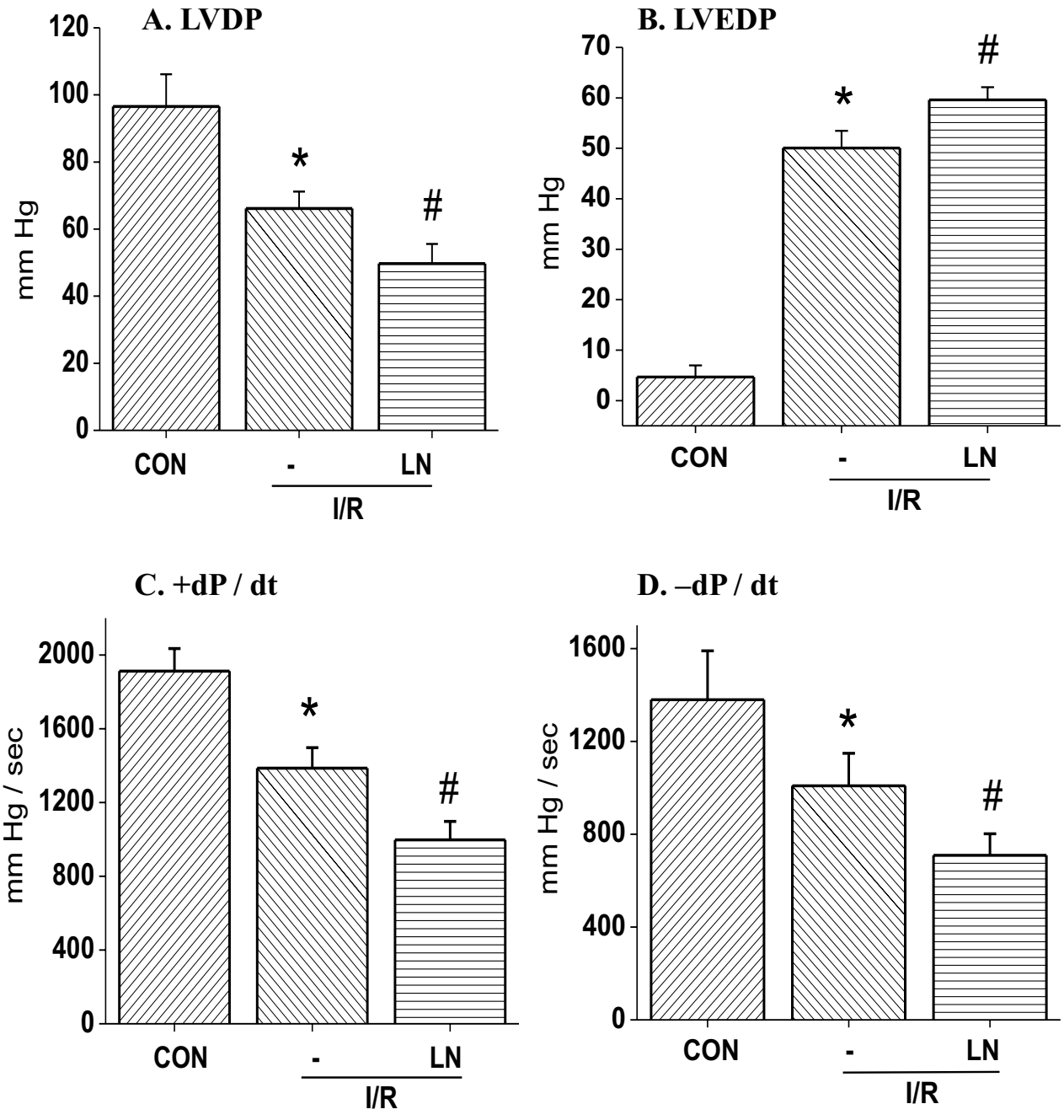
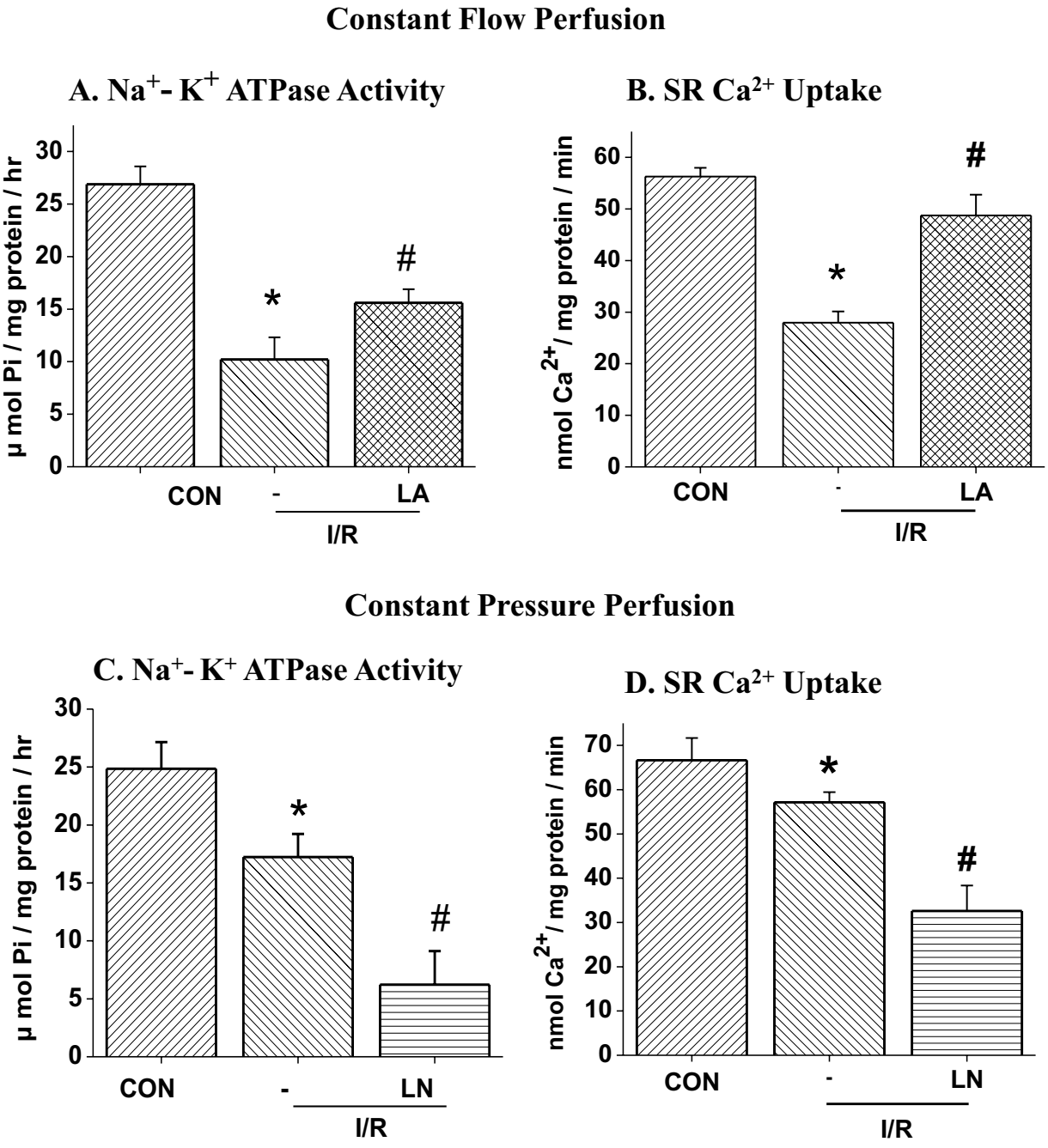


Figure 3. Sarcolemmal Na^+-K^+ ATPase activity and sarcoplasmic reticulum Ca^{2+}- uptake activities in isolated hearts subjected to 30 min of ischemia and 60 min of reperfusion at constant flow and constant pressure



hearts, statistical analysis of results in Figure 3 revealed that the I/R-induced depressions in both SL Na^+-K^+ ATPase and SR Ca^{2+}-uptake activities in CF-perfused hearts were greater than those in CP-perfused hearts ($P<0.05$). Furthermore, the treatment of CF-perfused hearts with 3 mM LA attenuated the I/R-induced depressions whereas the treatment of CP-perfused hearts with 200 μM LN augmented the I/R-induced depressions in both SL Na^+-K^+ ATPase and SR Ca^{2+}-uptake activities (Figure 3).

In order to determine if the differences in I/R induced changes in subcellular activities in CF-perfused and CP-perfused hearts are associated with differential alterations in the proteolysis in these preparations, the activity of a proteolytic enzyme, calpain, was measured in the cytosolic fraction. The results in Table 2 show that I/R induced an increase in calpain activity in both CF-perfused and CP-perfused hearts; however, the increase in calpain activity due to I/R in CF-perfused hearts was greater than that in CP-perfused hearts ($P<0.05$). Treatment of CF-perfused hearts with 3 mM LA attenuated whereas treatment of CP-perfused hearts with 200 μM LN augmented the I/R-induced increase in calpain activity (Table 2).

c. Formation of NO and estimation of eNOS Protein Content

In a separate set of experiments, the status of endothelial function in CF-perfused and CP-perfused hearts was examined by monitoring the concentration of nitrate/nitrite, an index of NO formation, in the coronary perfusate as well as protein content for eNOS in the myocardium. It is pointed out that the total

Table 2. Cytosolic calpain activity in hearts subjected to 30 min ischemia and reperfused for 60 min.

	Experimental Groups	Calpain Activity (Relative Fluorescent Units)
A.	Constant Flow Perfusion (CF)	
	Control	100 ± 6
	I/R	396 ± 5*
	I/R + LA	200 ± 13#
B.	Constant Pressure Perfusion (CP)	
	Control	83 ± 4
	I/R	236 ± 17 *
	I/R +LN	387 ± 13 #

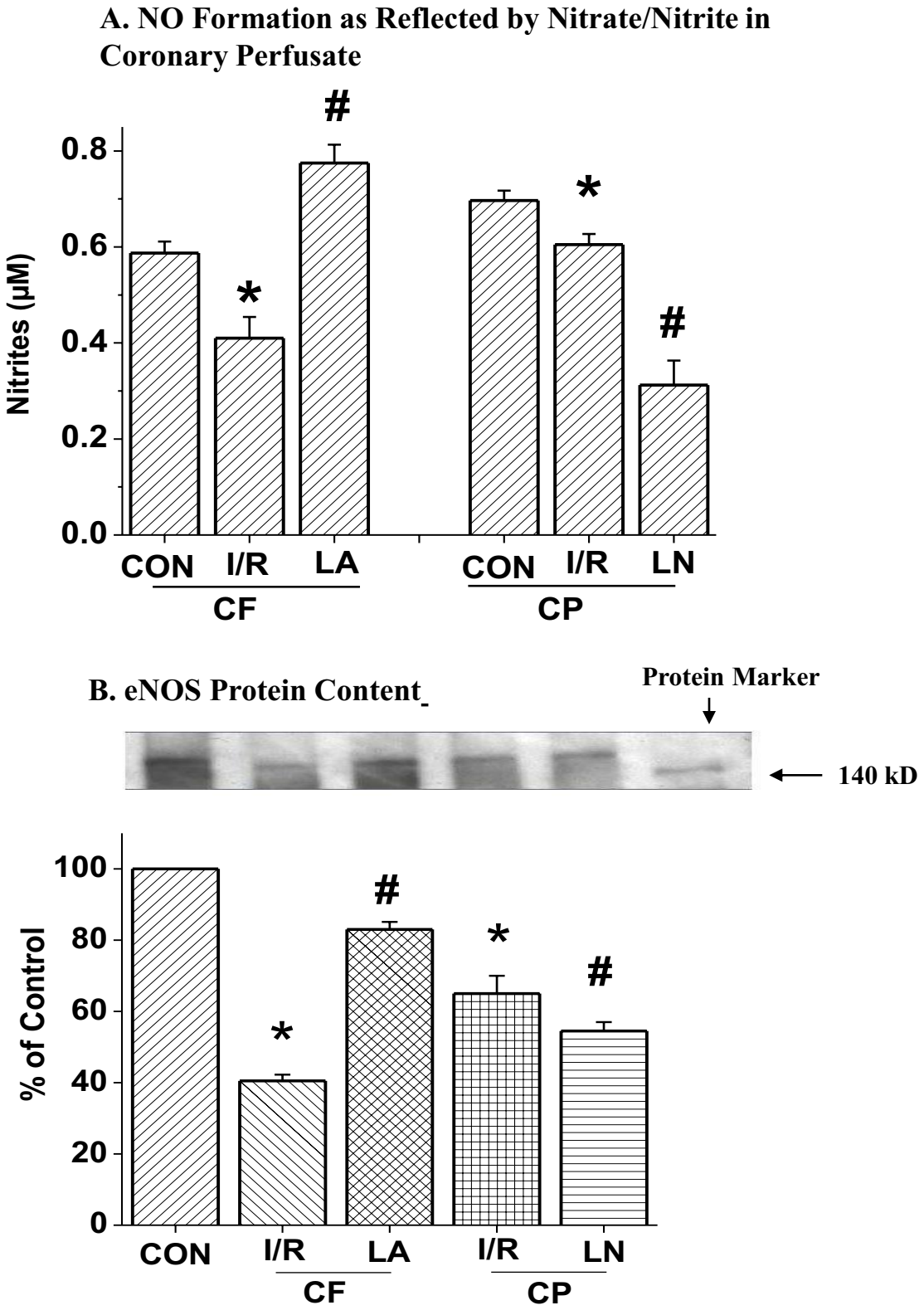
I/R: ischemia reperfusion, LA: L-arginine (3 mM), LN: L-NAME (200 μM). All values are mean ± SEM of 4 hearts in each group, * $P<0.05$ in comparison to respective control and # $P<0.05$ in comparison to respective IR.

volume of perfusate in CF-perfused and CP-perfused heart was about 600 ml and 735 ml, respectively during the 60 min of reperfusion and thus this volume in both cases was adjusted to an equal amount of 750 ml before monitoring the concentration of nitrate/nitrite. The data in Figure 4 reveal that I/R decreased the concentration of nitrate/nitrite in both CF-perfused and CP-perfused hearts; however, the depression in CF-perfused hearts was greater than that in CP-perfused hearts ($P<0.05$). Furthermore, treatment of CF-perfused hearts with 3 mM LA not only prevented the I/R-induced decrease in nitrate/nitrite levels but in fact also increased its level in the perfusate above the control levels. Treatment of CP-perfused hearts with 200 μM LN augmented the I/R-induced depression in the nitrate/nitrite level in the perfusate (Figure 4). It can also be seen from Figure 4 that I/R decreased the eNOS protein content in both CF-perfused and CP-perfused hearts; however, the I/R-induced depression in eNOS protein content in CF-perfused hearts was significantly greater ($P<0.05$) than that in CP-perfused hearts ($P<0.05$). The I/R-induced decrease in eNOS protein content was attenuated by treatment of CF-perfused hearts with 3 mM LA. On the other hand, the I/R-induced eNOS protein content slightly but significantly decreased in CP-perfused hearts upon treatment with 200 μM LN (Figure 4).

d. Characteristics of coronary flow or pressure in CF-perfused and CP-perfused Hearts

To gain further information regarding the endothelial function in CF-perfused and CP-perfused hearts, coronary pressure and coronary flow were measured in hearts

Figure 4. Concentration of nitrate/nitrite in perfusate and eNOS protein content from I/R hearts perfused at constant flow or constant pressure



perfused at CF and CP. The results in Table 3 show that the coronary perfusion pressure in CF-perfused ischemic hearts was markedly increased upon initiating reperfusion and thereafter it gradually decreased towards the control level during the 60 min period. On the other hand, the coronary flow in CP-perfused ischemic hearts was increased during the first 5 min of starting reperfusion but thereafter started declining towards the control levels during the reperfusion period.

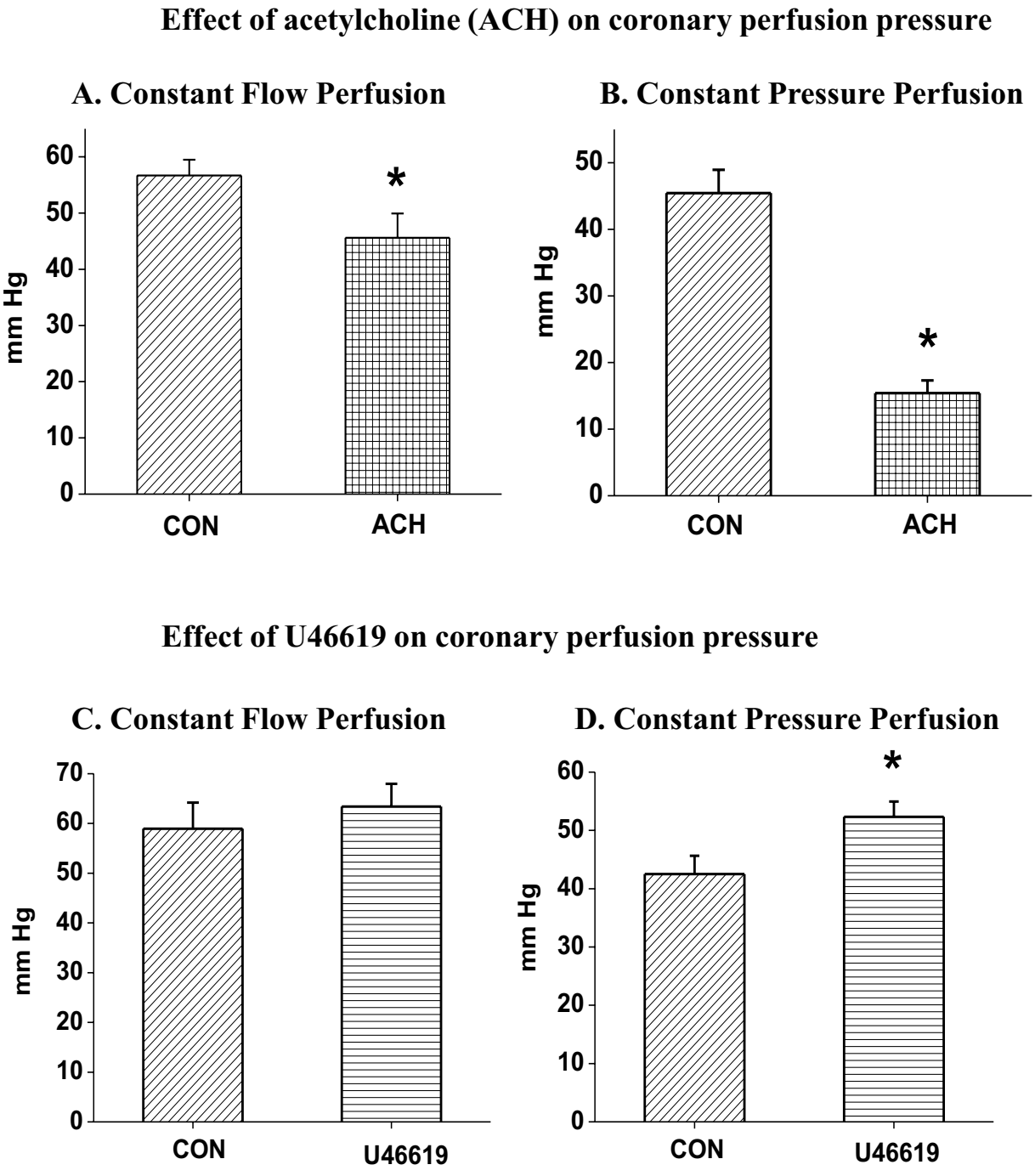
At the end of coronary pressure and coronary flow measurements in CF-perfused and CP-perfused hearts, respectively, responses of CF-perfused and CP-perfused hearts to 1 μM ACH and 10 nM U-46619 infusions were examined. For this purpose, CF-perfused hearts were futher perfused in the same mode for 3 min whereas the CP-perfused hearts were switched to CF perfusion mode and the changes in coronary perfusion pressure was monitored to determine changes due to ACH and U46619 infusion. It should be noted from the results shown in Figure 5 that infusion of ACH decreased the coronary perfusion pressure in both CF-perfused and CP-perfused hearts; however, the ACH-induced decrease in coronary perfusion pressure was greater in CP-perfused hearts than that in CF-perfused hearts ($P<0.05$). On the other hand, infusion of U46619 did not increase the coronary perfusion pressure in CF-perfused hearts significantly ($P < 0.05$) whereas this agent produced an increase in coronary perfusion pressure in CP-perfused hearts significantly (Figure 5).

Table 3. Changes in perfusion pressure and coronary flow in I/R hearts perfused at constant flow and constant pressure.

Time (min)	Constant Flow Perfusion (CF)	Constant Pressure Perfusion (CP)
	Coronary Pressure (mm Hg)	Coronary Flow (ml/min)
Before ischemia	76.1 ± 4.7	12.5 ± 1.1
During ischemia	1.1 ± 0.1	0
Reperfusion at (min)		
1	51.0 ± 1.2	15.2 ± 0.1
2	41.5 ± 0.9	16.3 ± 0.3
5	45.9 ± 1.2	20.2 ± 0.7
10	48.4 ± 1.3	18.4 ± 1.0
20	51.6 ± 1.1	13.3 ± 0.6
30	54.0 ± 1.2	11.2 ± 0.4
60	57.0 ± 1.3	9.9 ± 0.1

Isolated hearts perfused at constant flow (CF) or constant pressure (CP) were made ischemic for 30 min followed by reperfusion for 60 min. All values are mean ± SEM for n=4-5 hearts in each group

Figure 5. Coronary perfusion pressure in hearts perfused at constant flow and constant pressure and infused with 1 μM acetylcholine or 10 nM U46619 (a thromboxane A2 mimetic)



2. Effects of calpain activation on ischemia reperfusion induced alterations in cardiac function and Na^+-K^+-ATPase activity

a. Effect of leupeptin on I/R-induced alterations in the heart

In one set of experiments, the effects of different concentrations of leupeptin was studied on the I/R-induced changes in cardiac performance, SL Na^+-K^+ ATPase and calpain activity. The results in Table 4 indicate that the cardiac performance, as represented by LVDP, +dP/dt and –dP/dt, as well as the SL Na^+-K^+ ATPase activity were depressed whereas both LVEDP and calpain activity were markedly increased in the I/R hearts. These I/R-induced changes were unaffected by a treatment with 10 μmol/l leupeptin but significantly attenuated by treatments with 20 or 50 μmol/l leupeptin (Table 4). In view of these observations, 25 μmol/l leupeptin was used in subsequent experiments. Another set of experiments was undertaken to test if leupeptin affected the heart perfused with control medium or hearts subjected to I/R. The results in Figure. 6 and 7 show that while the I/R-induced alterations in cardiac performance, SL Na^+-K^+ ATPase and calpain activity were fully or partially prevented by leupeptin, this protease inhibitor did not affect any of these parameters in control hearts. It can be seen from Figure 7 that neither I/R nor leupeptin treatment affected the SL Mg^{2+} ATPase activity.

In order to test if the observed alteration in the SL Na^+-K^+ ATPase activity due to I/R was associated corresponding changes in the α- and β-isoforms of the enzyme, protein content of different Na^+-K^+ ATPase subunits were determined in control

Table 4. Contractile parameters, SL Na^+-K^+-ATPase activity and calpain activity of I/R hearts treated with and without different concentrations of leupeptin (10, 20 and 50 µmol/l).

Parameters	CON	I/R	I/R+10	I/R + 20	I/R + 50
LVDP	108 ± 3.1	44.6 ± 4.6*	42.3 ± 4.3	85.3 ± 5.0#	96.7 ± 3.2#
LVEDP	4.2 ± 1.5	73.4 ± 5.3*	64.1 ± 3.3	23.7 ± 1.5#	25.7 ± 1.5#
+dP/dt	1794 ± 32.6	754 ± 88.3*	676 ± 53. 5	1604 ± 120#	1694 ± 120#
-dP/dt	2625 ± 94.6	806 ± 123*	754 ± 44. 9	1985 ± 122#	2220 ± 132#
Na^+-K^+- ATPase Activity (µmol/Pi/mg/hr)	23.1 ± 3.1	12.5 ± 3.4*	12.9 ± 1.1	16.8 ± 1.9#	19.8 ± 2.9#
Calpain Activity (RFU)	55.7 ± 13.6	567 ± 45.3*	499 ± 44.5	207 ± 47.4#	238 ± 37.7#

Left ventricular developed pressure (LVDP), Left ventricular end diastolic pressure (LVEDP), Rate of left ventricular pressure development (+dP/dt), rate of left ventricular pressure decay (-dP/dt). RFU=relative fluorescent units. Each value is a mean ± SEM from 5 to 6 experiments in each group. *P<0.05 in comparison to the control #P<0.05 in comparison to I/R.

Figure 6. Cardiac performance of isolated rat hearts exposed to 30 min of ischemia and 60 min of reperfusion and treated with or without calpain inhibitor leupeptin.

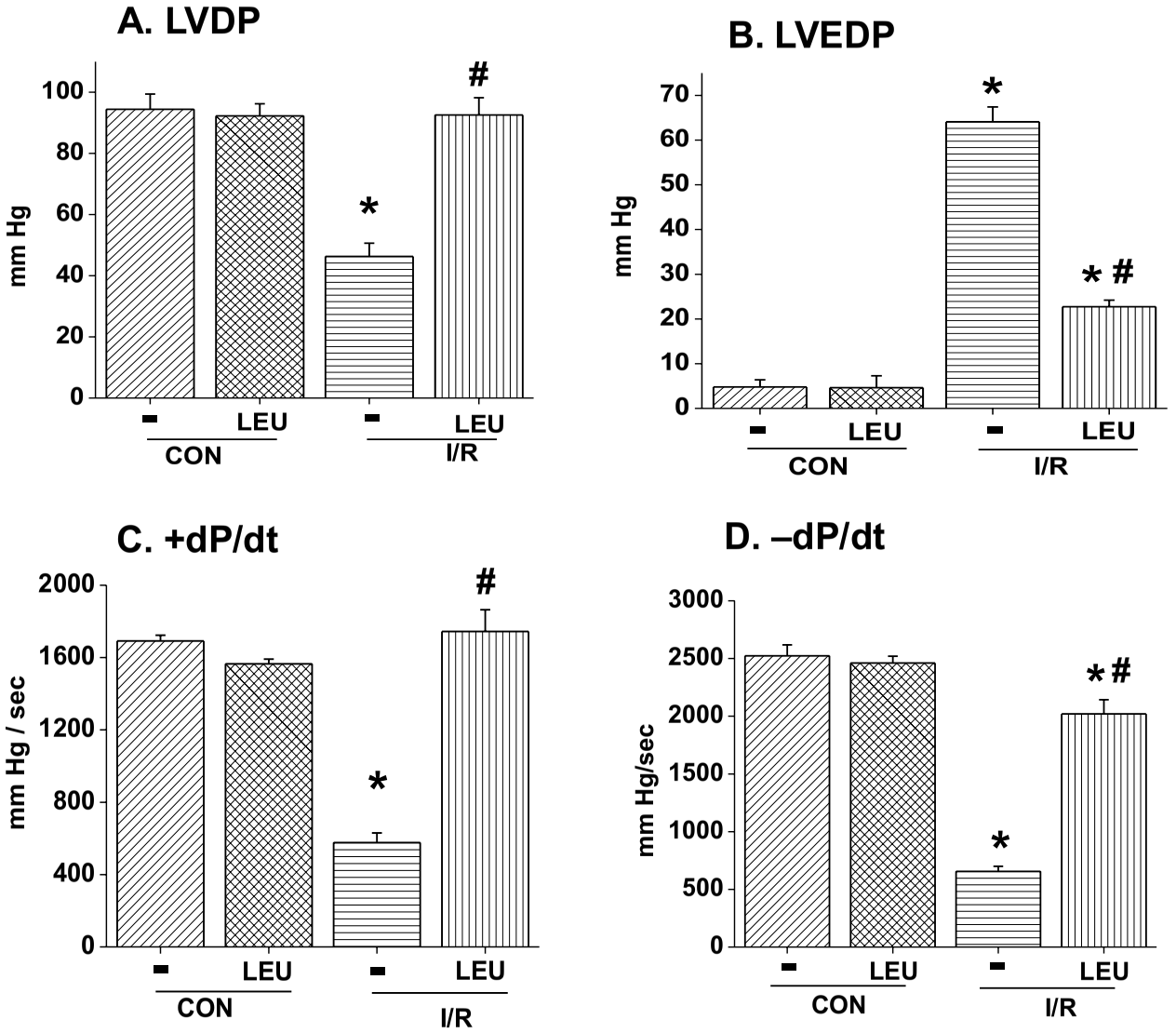
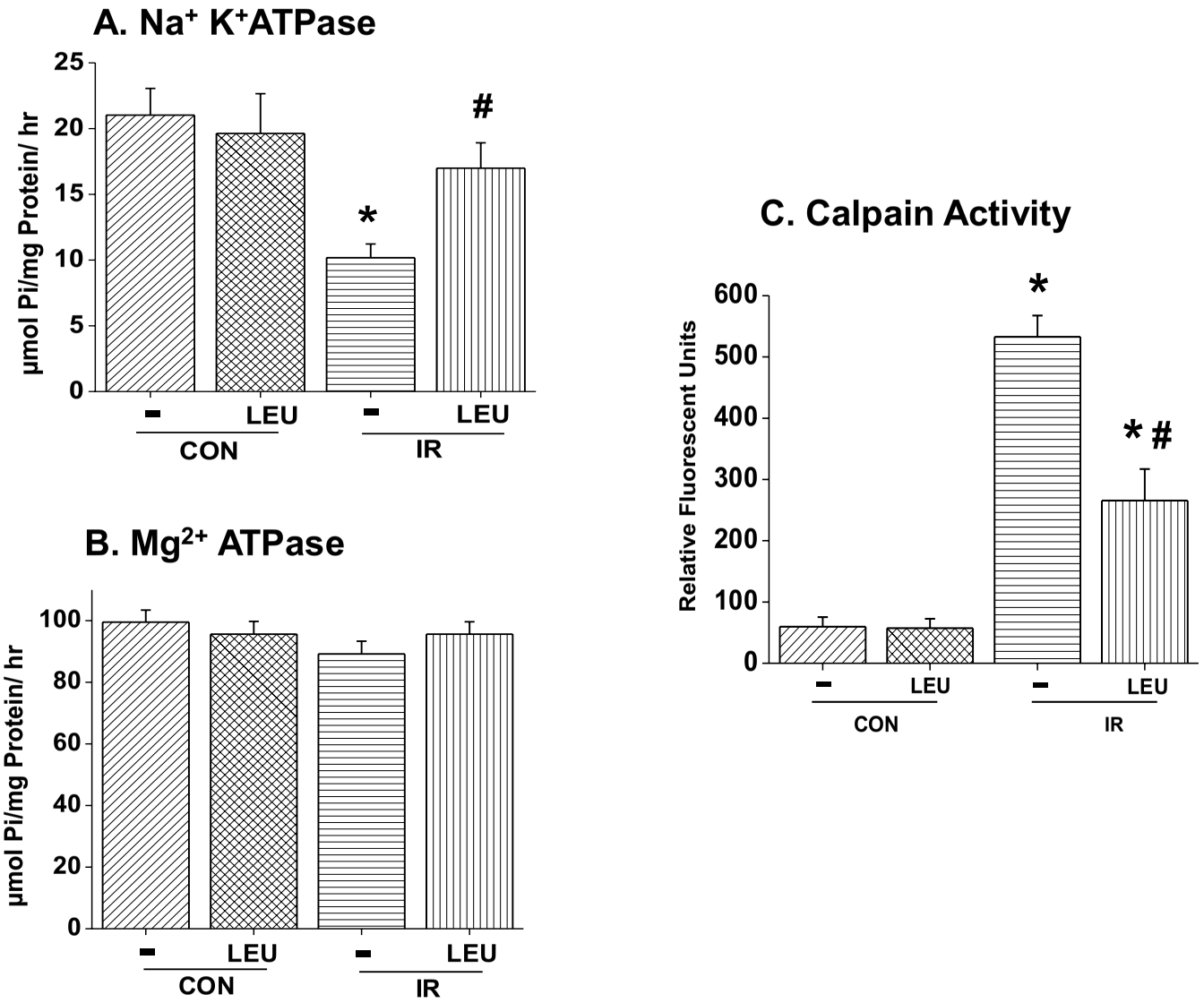


Figure 7. SL Na^+-K^+ ATPase, Mg^{2+} ATPase activities and cytosolic calpain activity in isolated rat hearts subjected to 30 min of ischemia and 60 min of reperfusion treated with or without calpain inhibitor leupeptin.



and I/R hearts with or without leupeptin treatment. The results in Figure 8 reveal that protein content for α_1-, α_2-, β_1- and β_2-isoforms was decreased in I/R hearts and these alterations were attenuated by treatment of I/R hearts with leupeptin. It should be noted that leupeptin treatment did not affect the protein content of α- or β-subunits in control hearts (Figure 8).

To show if calpain exerted a direct effect in depressing the SL Na^+-K^+ ATPase activity, SL preparations from the unperfused hearts were incubated with calpain in the absence and presence of leupeptin. From the results in Figure 9, it is evident that the SL Na^+-K^+ ATPase activity was decreased by calpain whereas no change in the activity of SL Mg^{2+}-ATPase was detected. Furthermore, separation of various protein bands by gradient gel revealed a depression in both α- and β-subunits of Na^+-K^+ ATPase in calpain-treated preparations. These calpain-induced alterations in Na^+-K^+ ATPase activity and subunits were attenuated by the presence of leupeptin.

b. Ca^{2+}-dependency of I/R-induced alterations in the heart

Since the I/R-induced changes in cardiac performance are known to be dependent on the concentration of extracellular Ca^{2+}, the Ca^{2+}-dependency of I/R-induced alterations in SL Na^+-K^+ ATPase and calpain activities was also examined. The data in Table 5 indicate that the I/R-induced depressions in LVDP, +dP/dt, -dP/dt and SL Na^+-K^+ ATPase activity were more dramatic in hearts perfused with high concentration (2.5 mM) than those with low concentration (0.25 mM) of Ca^{2+}. Likewise, the magnitude of increases in LVEDP and calpain activity due to I/R

Figure 8. SL Na^+-K^+ ATPase isoforms protein content from hearts exposed to 30 min of ischemia and 60 min reperfusion and treated with or without leupeptin.

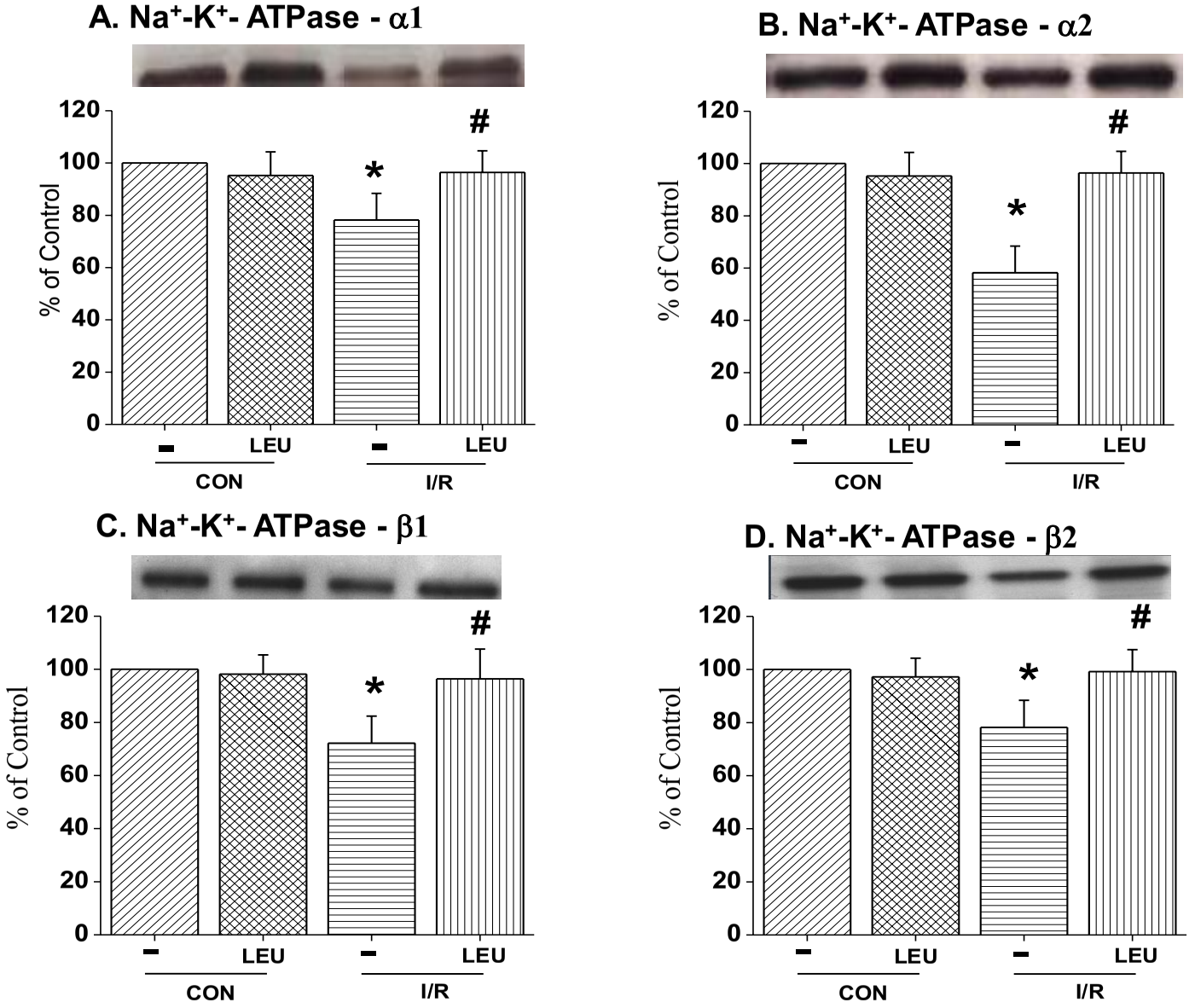


Figure 9. Na^+-K^+ ATPase and Mg^{2+} ATPase activities of SL preparations from unperfused hearts incubated with or without calpain in the presence or absence of leupeptin.

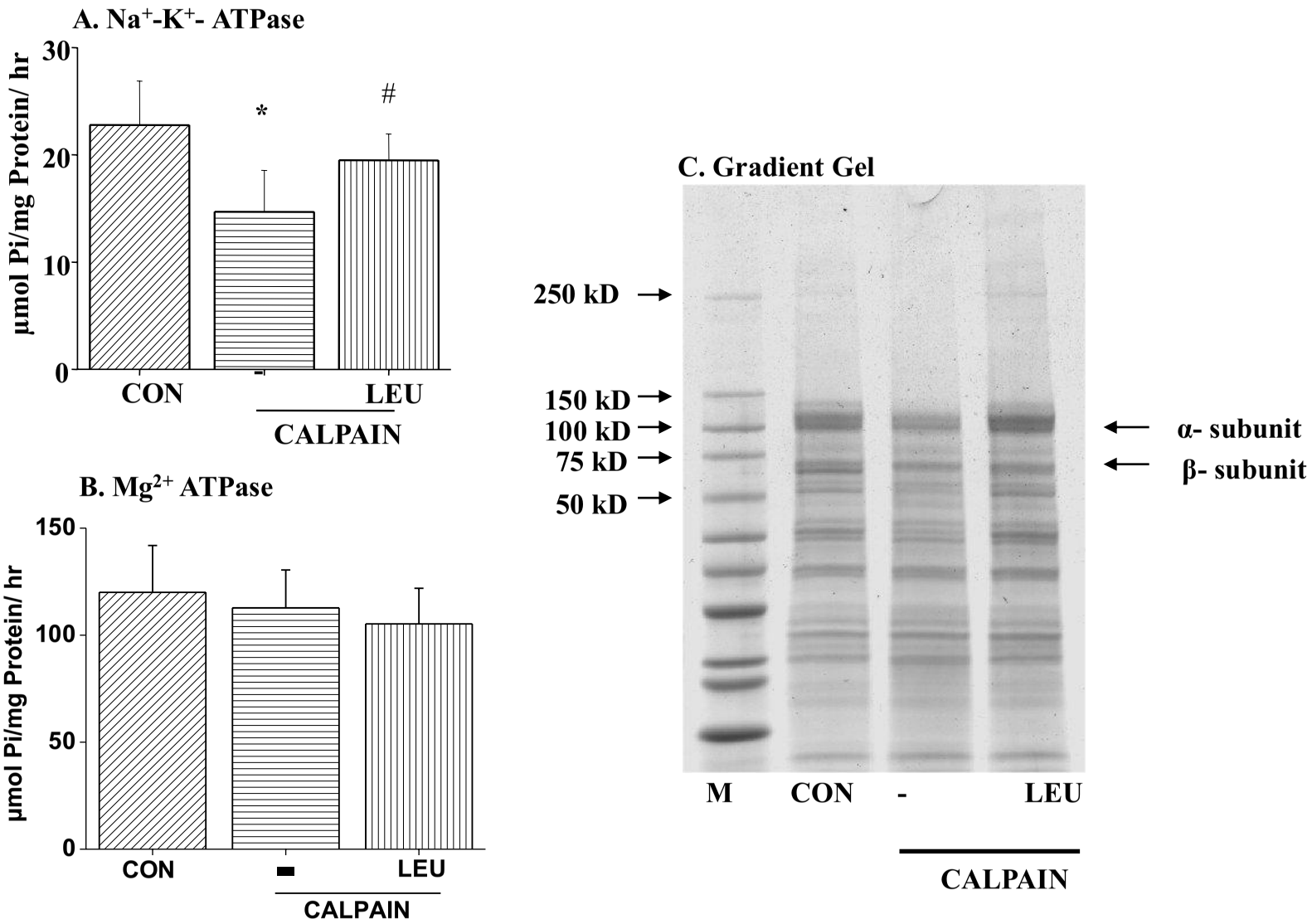


Table 5. Contractile parameters of hearts perfused with Kreb's Hensleit Buffer containing two different concentrations of Ca^{2+} (0.25 mM and 2.5 mM) and subjected to I/R.

Parameters	0.25 mM Ca^{2+}		2.5 mM Ca^{2+}	
	CON	IR	CON	IR
LVDP	18.9 ± 2.8	13.8 ± 2.9*	140 ± 9	38.9 ± 6.7*
LVEDP	12.2 ± 0.6	22.9 ± 2.6*	4.5 ± 0.8	66.5 ± 7.9*
+dP/dt	747 ± 172	490 ± 148*	7151 ± 452	626 ± 84.5*
-dP/dt	590 ± 119	338 ± 119*	5664 ± 216	652 ± 52.7*
Na^{+}-K^{+}- ATPase Activity (μmol Pi/mg/hr)	6.7 ± 0.3	5.4 ± 0.5	20.3 ± 1.2	9.5 ± 0.3*
Calpain Activity (RFU)	103 ± 34	287 ± 25*	162 ± 24	491 ± 10*

Left ventricular developed pressure (LVDP), Left ventricular end diastolic pressure (LVEDP), Rate of left ventricular pressure development (+dP/dt), rate of left ventricular pressure decay (-dP/dt). CON=control, IR=Ischemia reperfusion, RFU=Relative Fluorescent units. n=4 for each group. Data expressed as Mean ± SE. *P<0.05 in comparison to the control at 0.25 mM Ca^{2+}. **P<0.05 in comparison to the control at 2.5 mM Ca^{2+}.

was greater in hearts perfused with 2.5 mM Ca^{2+} than those in hearts perfused with 0.25 mM Ca^{2+}.

c. Effect of MDL on I/R-induced changes in the heart

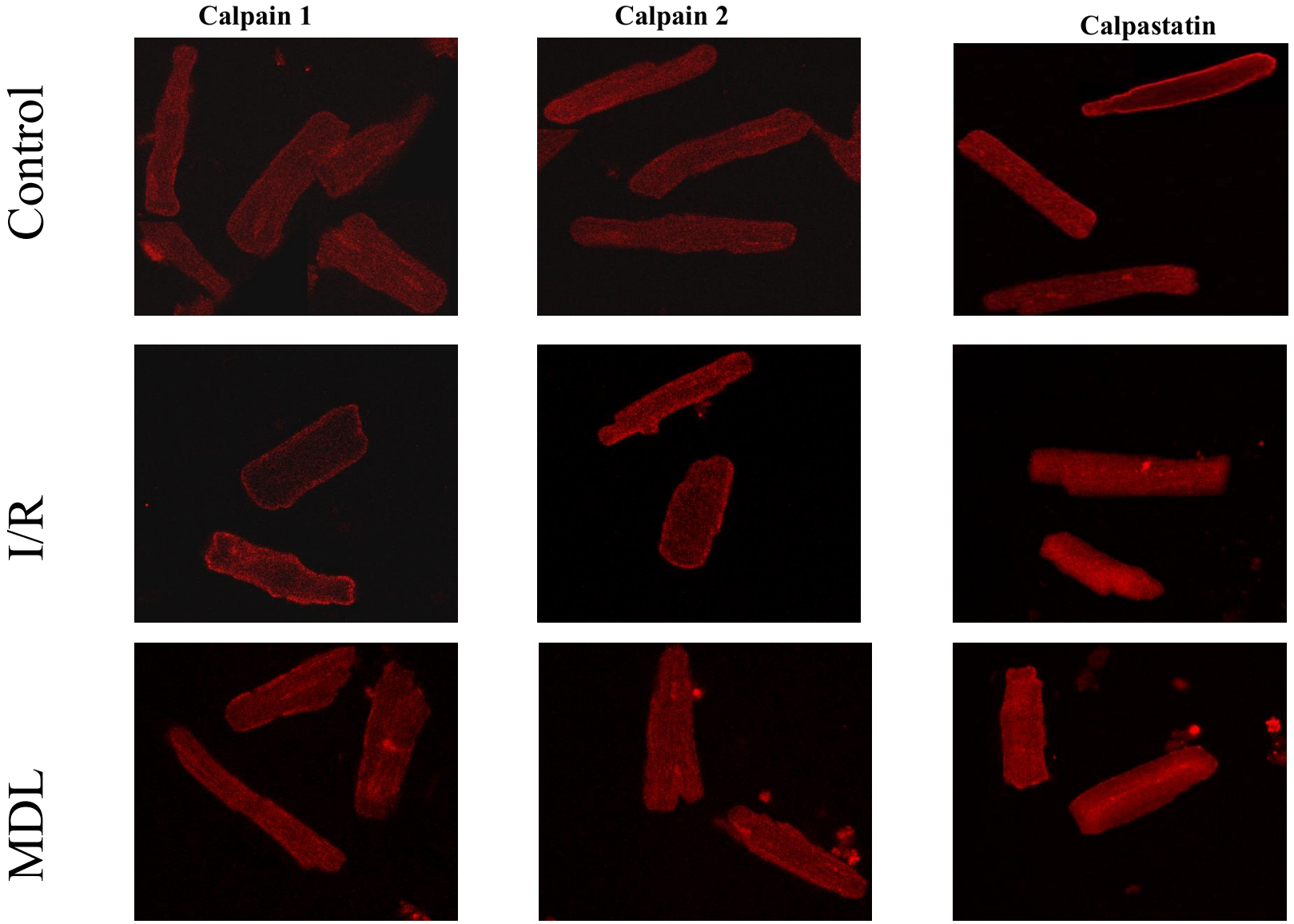
In order to further show that the activation of calpain due to I/R is associated with depressed SL Na^{+}-K^{+} ATPase and contractile function, the hearts perfused with control medium as well as I/R hearts were treated with or without MDL (10 μmol/l), a specific inhibitor of calpain. The results in Table 6 indicate that MDL had no effect on control hearts but attenuated the I/R-induced depressions in LVDP, +dP/dt and –dP/dt as well as SL Na^{+}-K^{+} ATPase activity. This agent was also found to prevent the activation of calpain and increase in LVEDP due to I/R (Table 6). Cardiomyocytes from control as well as I/R hearts treated with or without MDL were examined by confocal microscopy to investigate changes in the localization of calpain 1 and 2 isoforms (Sorimachi and Suzuki. 2001) as well as of the calpastatin, an endogenous inhibitor of calpain due to I/R. Both calpain 1 and 2 isoforms were distributed in the nucleus and cytosol whereas calpastatin was present in the cytosol predominantly with some cell showing membranous and nuclear localization in cardiomyocytes from three control hearts (Figure 10, upper panel). On the other hand, both calpain 1 and 2 isoforms exhibited tendency to translocate to the SL membrane and calpastatin showed more diffuse cystolic localization in cardiomyocytes preparations obtained from three I/R hearts (Figure 10, middle panel). Treatments of three I/R hearts with MDL (10 μmol/l) restored the distribution of calpain 1 and 2 isoforms as well as calpastatin toward their

Table 6. Contractile parameters, SL Na^+-K^+- ATPase activity and calpain activity of I/R hearts treated with and without calpain specific inhibitor MDL28170 (T).

Parameters	CON	CON + T	I/R	I/R + T
LVDP	109 ± 2.3	110 ± 3.6	49.6 ± 5.6*	96.7 ± 4.6#
LVEDP	4.9 ± 3.5	4.6 ± 2.1	69.4 ± 6.1*	15.5 ± 6.4#
+dP/dt	2115 ± 96.8	2005 ± 103	673 ± 78.3*	1899 ± 109#
-dP/dt	1978 ± 106	2001 ± 98.5	701 ± 113*	1865 ± 105#
Na^+-K^+- ATPase Activity (µmol Pi/mg/hr)	24.5 ± 3.6	23.2 ± 4.1	11.5 ± 3.4*	21.3 ± 2.1#
Calpain Activity (RFU)	56.1 ± 6.1	54.3 ± 2.4	510 ± 35.6*	105 ± 45.6#

Left ventricular developed pressure (LVDP), Left ventricular end diastolic pressure (LVEDP), Rate of left ventricular pressure development (+dP/dt), rate of left ventricular pressure decay (-dP/dt). RFU= relative fluorescent units. Each value is a ± SEM of 5 to 6 experiments for each group. The concentration of MDL28170 was 10 µmol/l. *P<0.05 in comparison to the CON. #P<0.05 in comparison to I/R.

Figure 10. Expression of calpain 1, calpain 2 and calpastatin in cardiomyocytes isolated from hearts subjected to 30 min ischemia and 60 min reperfusion in the absence or presence of a calpain inhibitor, MDL 28170.



normal pattern (Figure 10, lower panel). Treatment of two I/R hearts with 25 μmol/l leupeptin also showed results similar to those for the MDL treatment (data not shown).

3. Alterations in ischemia reperfusion induced protease activities, cardiac function and Na^+-K^+-ATPase activity due to oxidative stress

a. Simulation of I/R-induced changes by hypoxia and oxidant or oxyradicals

Reperfusion of the 30 min ischemic hearts showed a depression in cardiac function, as reflected by decreased LVDP, +dP/dt and –dP/dt as well as increased LVEDP (Table 7). These alterations due to I/R were associated with depressed SL Na^+-K^+-ATPase activity without any changes in Mg^{2+}-ATPase activity as well as increased MMP and calpain activities (Figure 11). Likewise, reperfusion of the 30 min hypoxic and substrate-depleted hearts resulted in depressed cardiac function and SL Na^+-K^+-ATPase activity as well as increased MMP and calpain activities without any changes in Mg^{2+}-ATPase activity (Table 7 and Figure 11). Furthermore, hearts perfused for 30 min with H_2O_2 or X+XO were reperfused with normal medium and the results are shown in Table 8 and Figure 12. The data revealed that H_2O_2 or X+XO treated reperfused hearts showed cardiac dysfunction, depressed SL Na^+-K^+-ATPase as well as increased MMP and calpain activities without any changes in Mg^{2+}-ATPase activity. These observations indicated that the I/R-induced alterations in the heart are similar to those produced upon reperfusion of hearts subjected to hypoxia in the absence or presence of glucose as well as H_2O_2 or X+XO.

Table 7. Comparison of the effects of 30 min reperfusion with normal medium following 30 min of hypoxia in the presence or absence of glucose with I/R on cardiac performance in isolated rat hearts.

Group	**Control**	**I/R**	**Hypoxia with glucose**	**Hypoxia without glucose**
LVDP (mm Hg)	105 ± 7	52 ± 5*	73 ± 12*	62 ± 4*
LVEDP (mm Hg)	4 ± 2	53 ± 5*	30 ± 10*	56 ±10*
+dP/dt	5432 ± 203	1345 ± 96*	1435 ± 187*	1476 ± 133*
-dP/dt	4569 ± 106	1337 ± 109*	2305 ± 205*	1263 ± 150*

Values are means ± SE of 8 hearts for cardiac function in each group. I/R; 30 min ischemia followed by 30 min reperfusion. LVDP; left ventricular developed pressure, LVEDP; left ventricular end diastolic pressure, +dP/dt; rate of pressure development, –dP/dt; rate of pressure decay. * $P < 0.05$ compared to Control.

Figure 11. Comparison of the effects of hypoxic perfusion in the absence or presence of glucose on SL Na^+-K^+-ATPase and Mg^{2+}-ATPase activities as well as MMP and calpain activities in the heart.

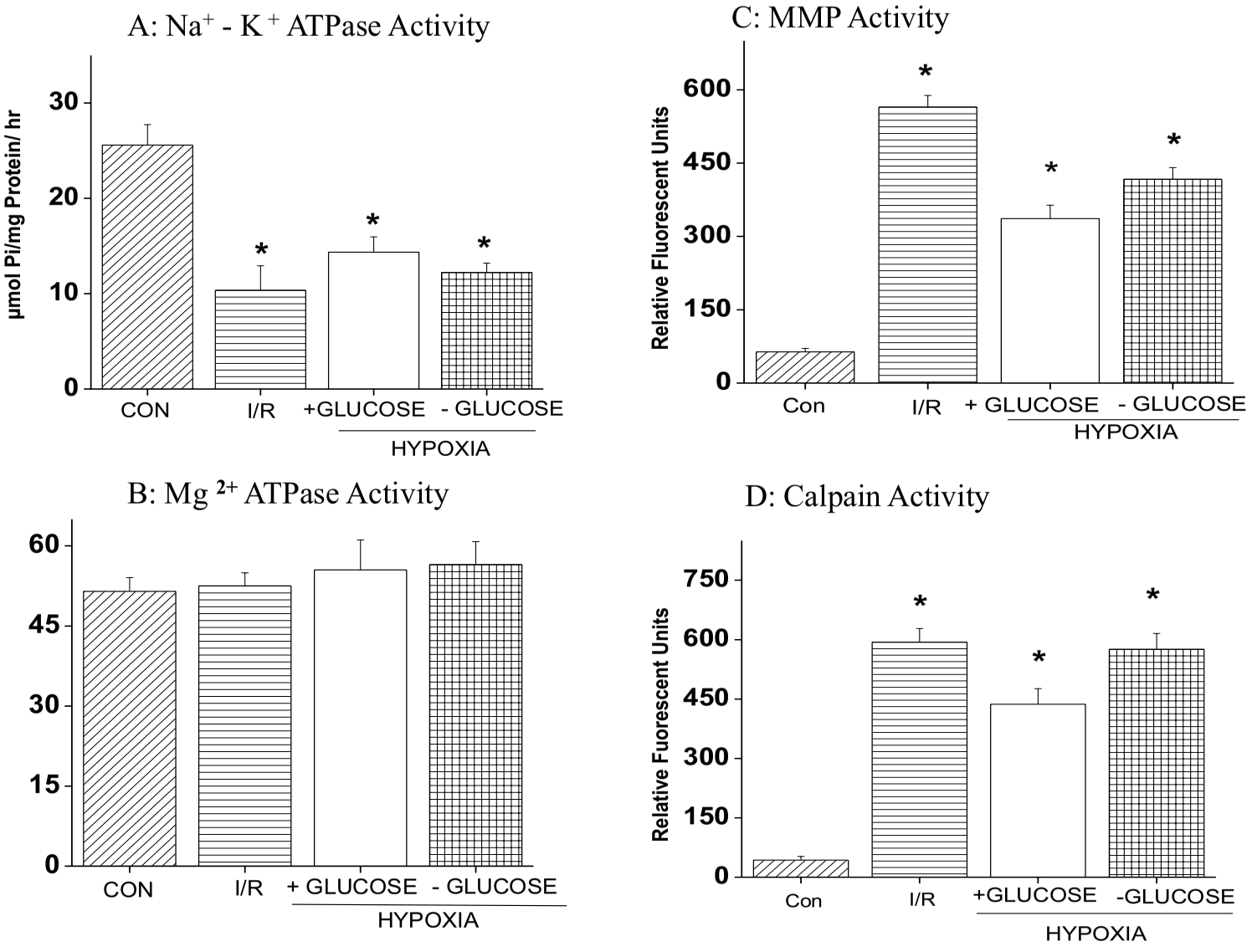
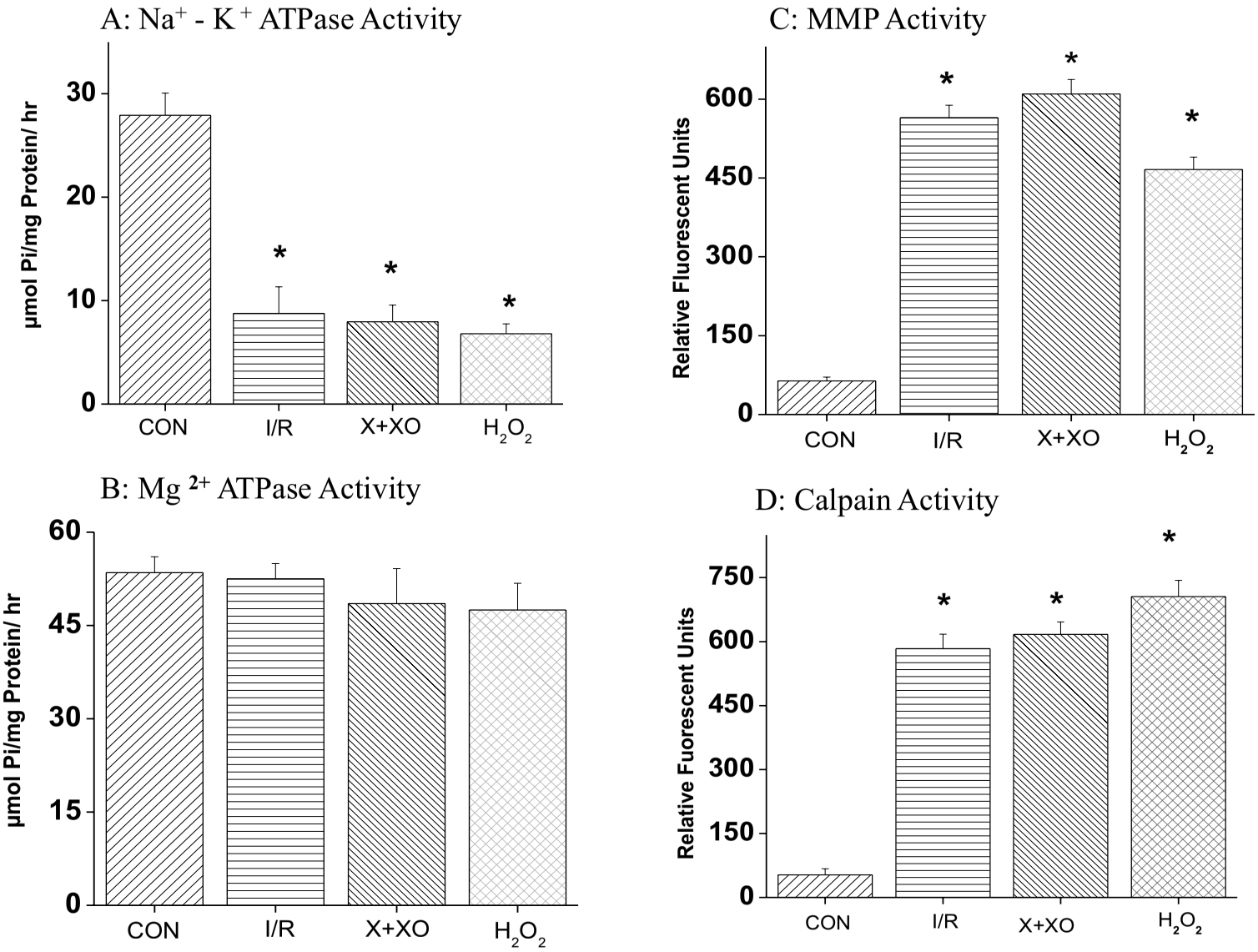


Table 8. Comparison of the effects of 30 min reperfusion with normal medium following 30 min perfusion with H_2O_2 or xanthine plus xanthine oxidase (X+XO) with I/R on cardiac performance in isolated rat hearts.

Group	**Control**	**I/R**	**H_2O_2**	**X+XO**
LVDP (mm Hg)	107 ± 4	54 ± 3*	43 ± 2*	56 ± 3*
LVEDP (mm Hg)	3 ± 1	50 ± 2*	79 ± 7*	72 ±5*
+dP/dt	5460 ± 145	1633 ± 60*	1115 ± 148*	1552 ± 183*
-dP/dt	4491 ± 140	930 ± 105*	772 ± 129*	1150 ± 195*

Values are means ± SE of 8 hearts for cardiac function in each group. I/R; 30 min ischemia followed by 30 min reperfusion. LVDP; left ventricular developed pressure, LVEDP; left ventricular end diastolic pressure, +dP/dt; rate of pressure development, –dP/dt; rate of pressure decay. * $P < 0.05$ compared to Control.

Figure 12. Comparison of the effects of perfusion with H_2O_2 or xanthine + xanthine oxidase on SL Na^+-K^+-ATPase and Mg^{2+}-ATPase activities as well as MMP and calpain activities in the heart.



b. Modification of I/R effects by preconditioning or antioxidant treatments

Preconditioning of the control heart was found to decrease +dP/dt and –dP/dt and increase both calpain and MMP activities as well as LVEDP significantly without any changes in LVDP, as well as SL Na^+-K^+-ATPase and Mg^{2+}-ATPase activities (Table 9 and Figure 13). In spite of these small alterations in the control hearts, preconditioning was found to attenuate the I/R-induced changes in cardiac function, SL Na^+-K^+-ATPase and proteolytic enzyme activities (Table 9 and Figure 13.). Likewise, treatment of hearts with well known anti-oxidants, NAC and MPG, was observed to depress the I/R-induced cardiac dysfunction, reduction in the SL Na^+-K^+-ATPase activity and increases in both calpain and MMP activities (Table 10 and Figure 14). The data in Figure 14 also indicate a slight but significant ($P<0.05$) increase in SL Mg^{2+}-ATPase activity in the MPG-treated hearts due to I/R; however, the exact reason for this MPG effect is not clear at present. It is also pointed out that treatment of control hearts with NAC or MPG did not show any effect on cardiac function, SL Na^+-K^+-ATPase and Mg^{2+}-ATPase activities as well as proteolytic enzyme activities (data not shown).

c. **Effects of MMP or calpain inhibitors on I/R-induced changes**

Perfusion of the control heart with Dox, an inhibitor of MMP, was found to exert no effects on cardiac function, SL Na^+-K^+-ATPase or proteolytic enzyme activities (Table 11 and Figure 15). However, the I/R-induced depression in cardiac function and SL Na^+-K^+-ATPase activity were attenuated without any changes in Mg^{2+}-ATPase activity by Dox treatment (Table 11 and Figure 15). The data in

Table 9. Cardiac performance of isolated rat hearts subjected to I/R with or without ischemic preconditioning (IPC).

Group	**Control**	**IPC**	**I/R**	**I/R+IPC**
LVDP (mm Hg)	105 ± 3	90 ± 6	57 ± 2*	86 ± 5$^{\#}$
LVEDP (mm Hg)	4 ± 1	14 ± 4*	52 ± 4*	18 ±3$^{\#}$
+dP/dt	4871±105	3954±168*	1494±73*	4171±152$^{\#}$
-dP/dt	3924±119	2992±189*	1248±82*	3754±139$^{\#}$

Values are means ± SE of 8 hearts for cardiac function in each group. I/R; 30 min ischemia followed by 30 min reperfusion, IPC; three cycles of 5 min ischemia and 5 min reperfusion, LVDP; left ventricular developed pressure, LVEDP; left ventricular end diastolic pressure, +dP/dt; rate of pressure development, –dP/dt; rate of pressure decay. * $P < 0.05$ compared to Control and $^{\#}P < 0.05$ compared to I/R.

Figure 13. Effects of ischemic preconditioning on I/R-induced changes in SL Na^+-K^+-ATPase and Mg^{2+}-ATPase activities as well as MMP and calpain activities in hearts.

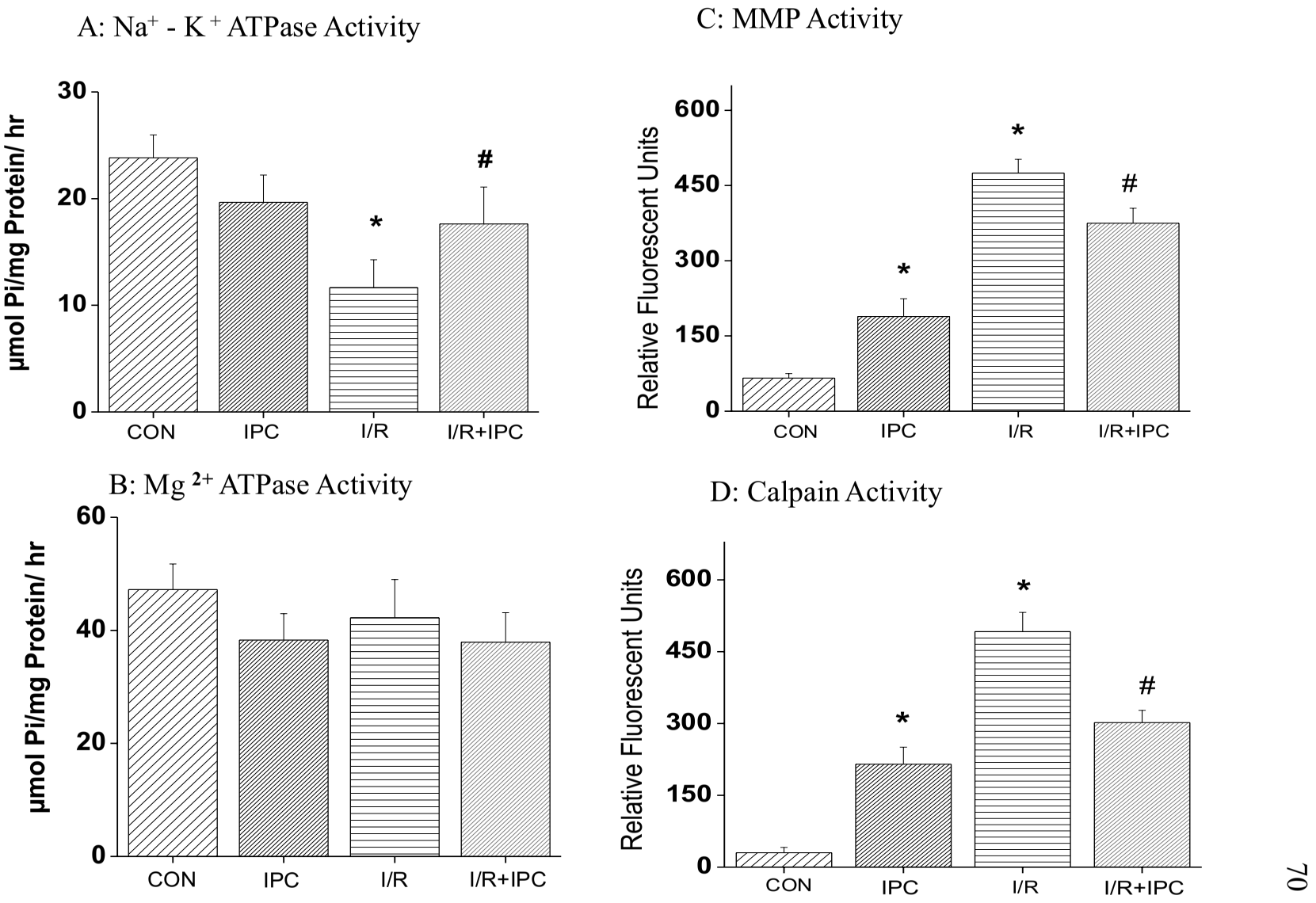


Table 10. Cardiac performance of isolated rat hearts subjected to I/R and treated with or without treatment with NAC and MPG.

Group	**Control**	**I/R**	**NAC+I/R**	**MPG+I/R**
LVDP (mm Hg)	120 ± 5	47 ± 7*	110 ± 9#	123 ± 11#
LVEDP (mm Hg)	4 ± 2	47 ± 6*	12 ± 7#	9 ± 3#
+dP/dt	6020 ± 137	1843 ± 101*	5249 ± 128#	5432 ± 193#
-dP/dt	4991 ± 180	1010 ± 125*	3790 ± 149#	4129 ± 175#

Values are means ± SE of 8 hearts for cardiac function in each group. I/R; 30 min ischemia followed by 30 min reperfusion, LVDP; left ventricular developed pressure, LVEDP; left ventricular end diastolic pressure, +dP/dt; rate of pressure development, –dP/dt; rate of pressure decay. Treatment with NAC (100μM) and MPG (300μM) were carried out 10 min before and 20 min after inducing I/R. * $P < 0.05$ compared to Control, #$P<0.05$ compared to I/R.

Figure 14. Effects of N-acetylcysteine and merceptopropionylglycine on I/R-induced changes in SL Na^+-K^+-ATPase and Mg^{2+}-ATPase activities as well as MMP and calpain activities.

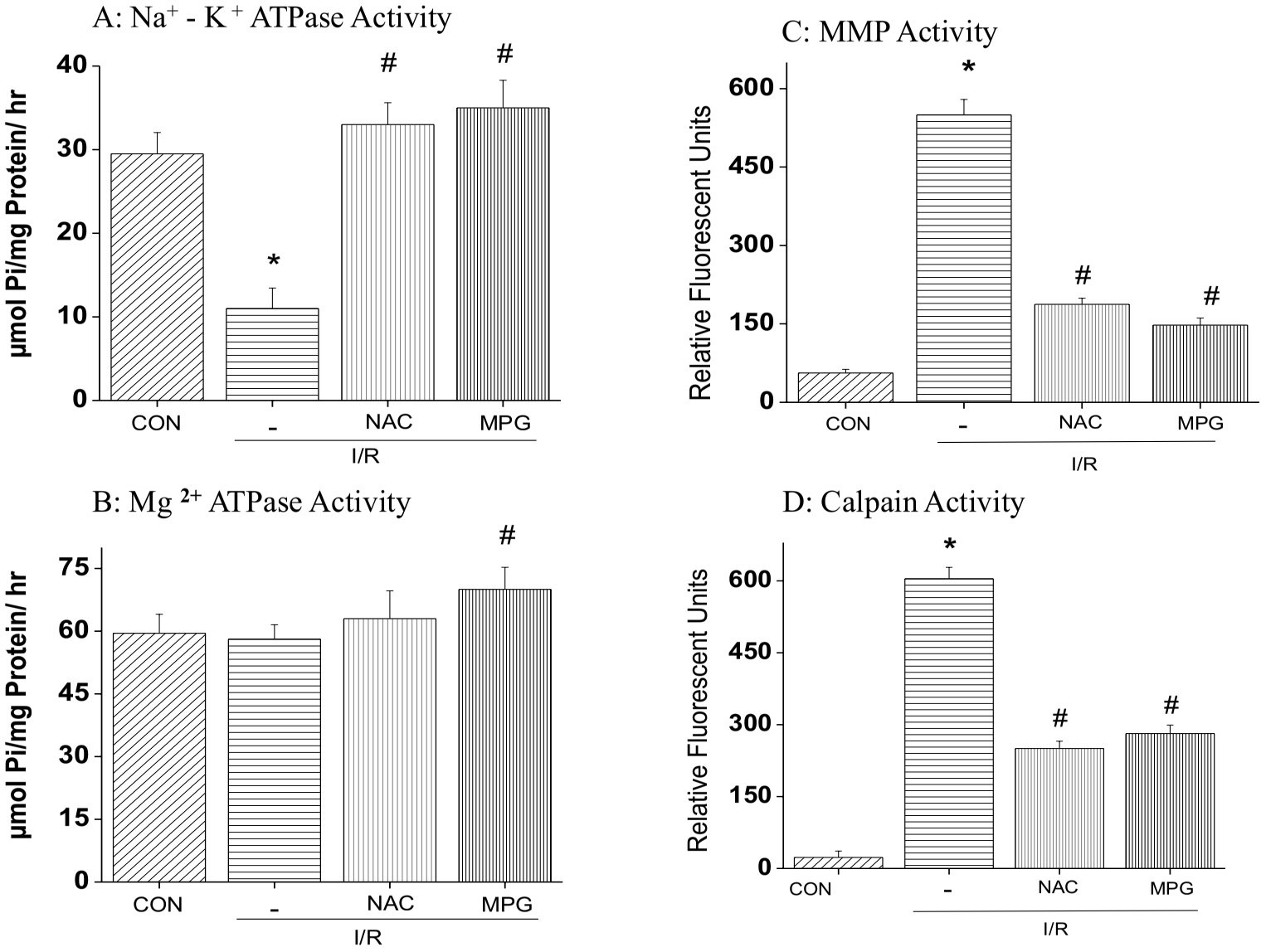


Table 11. Cardiac performance of isolated rat hearts subjected to I/R and treated with or without MMP inhibitor Dox (100 μM).

Group	Control	Control+Dox	I/R	I/R+Dox
LVDP (mm Hg)	110 ± 5	105 ± 7	56 ± 7*	77± 5#
LVEDP (mm Hg)	4 ± 1	4 ± 2	49 ± 8*	17 ±3#
+dP/dt	4436 ± 235	4259 ± 269	1615 ± 138*	3389 ± 197#
-dP/dt	3921 ± 195	4081 ± 233	1572 ± 239*	2990 ± 167#

Values are means ± SE of 8 hearts for cardiac function in each group. I/R; 30 min ischemia followed by 30 min reperfusion. LVDP; left ventricular developed pressure, LVEDP; left ventricular end diastolic pressure. Hearts were treated with Dox 10 min before and 20 min after inducing I/R. * $P < 0.05$ compared to Control, #P<0.05 compared to I/R.

Figure 15. Effects of doxycycline on I/R-induced changes in SL Na^{+}-K^{+}-ATPase and Mg^{2+}-ATPase activities as well as MMP and calpain activities.

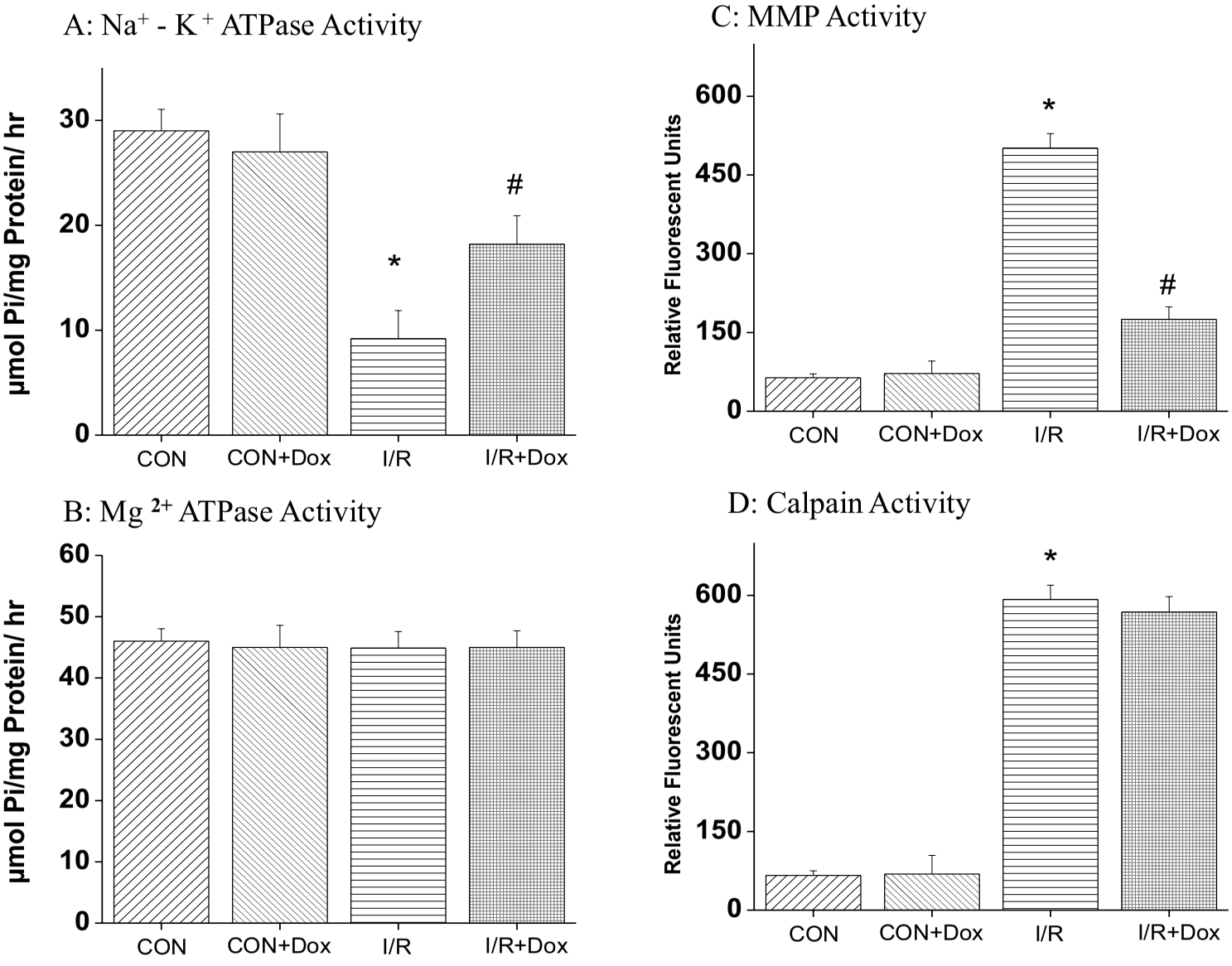


Figure 15 also reveal that the I/R-induced increase in MMP activity, unlike the increase in calpain activity, was depressed in the Dox-treated hearts. In another experiment, perfusion of hearts with calpain inhibitors, Leu and MDL, depressed the I/R-induced cardiac dysfunction and SL Na^+-K^+-ATPase activity without any changes in Mg^{2+}-ATPase activity (Figure 16 and Table 12). Furthermore, treatment with either Leu or MDL depressed the I/R-induced increase in calpain activity and significantly reduced the I/R-induced increase in MMP activity (Figure 14). It should also be mentioned that perfusion of control hearts with Leu or MDL did not affect cardiac function, SL Na^+-K^+-ATPase and Mg^{2+}-ATPase activities as well as proteolytic enzyme activities (data not shown).

In order to determine the impact of changes in proteolytic enzyme activities in depressing cardiac function and SL Na^+-K^+-ATPase activity due to I/R injury, the data on alterations in MMP and calpain activities were analyzed in comparison to their respective control values in each experiment in this study. The results in Figure 11 reveal that MMP activity was 8.8, 5.3 and 6.5 fold whereas that of calpain was 13.8, 10.1 and 13.4 fold when hearts were subjected to I/R, hypoxia-reoxygenation in the presence of glucose and hypoxia-reoxygenation in the absence of glucose, respectively. Analysis of the data in Figure 12 indicate that MMP activity was 8.1, 8.8 and 6.8 fold whereas that of calpain was 11.1, 11.6 and 13.3 fold upon subjecting the hearts to I/R, X+XO-reperfusion and H_20_2-reperfusion, respectively. The hearts which underwent IPC also showed that MMP activity was 2.6 fold whereas calpain activity was 5.2 fold (Figure 13).

Upon exposure of these hearts to I/R, MMP activity was 6.6 and 5.3 fold, and calpain activity was 12.0 and 7.4 fold in control and IPC hearts, respectively. In comparison to the I/R-induced changes in proteolytic enzyme activities in control hearts, IPC was found to depress the I/R-induced activation of MMP and calpain by 20 and 38%, respectively (Figure 13). I/R-induced activities of MMP in control, NAC-treated and MPG-treated hearts were found to be 9.8, 3.3 and 2.6 fold whereas those for calpain were 24.0, 10.0 and 11.2 fold, respectively (Figure 14). These results indicate that I/R-induced activation of MMP was depressed by 66 and 73% whereas that of calpain was depressed by 58 and 53% by NAC and MPG, respectively (Figure 14). When the hearts were treated with Dox, MMP activity was 1.1 fold whereas calpain activity was 1.0 fold (Figure 15). However, when these hearts were exposed to I/R, the I/R-induced activities for MMP and calpain were 7.5 and 2.5 fold, and 8.9 and 8.5 fold in control and Dox-treated hearts, respectively. This indicates that the I/R-induced activity of MMP was depressed by 65% whereas that for calpain was depressed 4% by Dox (Figure 15). On the other hand, I/R-induced MMP activities were 7.5, 5.4 and 5.0 fold whereas those for calpain were 14.9, 8.5 and 6.5 fold in control, Leu-treated and MDL-treated hearts, respectively (Figure 16). These results indicate that I/R-induced MMP activities were depressed 22 and 33% whereas those for calpain activities were depressed 43 and 56% by Leu and MDL, respectively (Figure 16).

Figure 16. Effects of leupeptin and MDL 28170 treatment on I/R-induced changes in SL Na^+-K^+-ATPase and Mg^{2+}-ATPase activities as well as MMP and calpain activities.

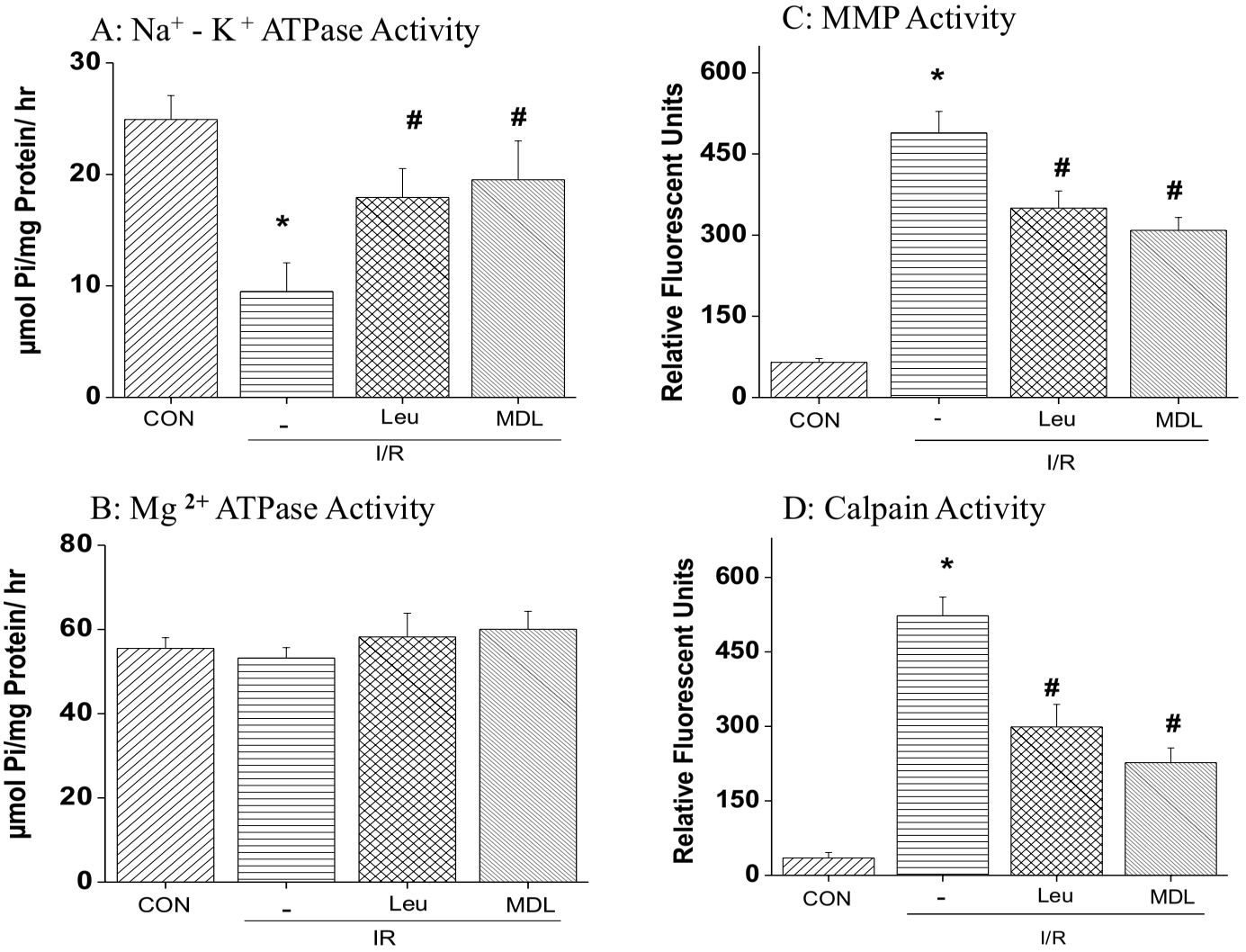


Table 12. Cardiac performance of isolated rat hearts subjected to I/R and treated with or without calpain inhibitors, leupeptin (leu; 25 μM) and MDL 28170 (MDL; 10 μM).

Group	**Control**	**I/R**	**I/R+Leu**	**I/R+MDL**
LVDP (mm Hg)	100 ± 5	56 ± 3*	91 ± 9#	96 ± 7#
LVEDP (mm Hg)	5 ± 2	58 ± 5*	18 ± 3#	15 ±4#
+dP/dt	5511 ± 127	1802 ± 103*	5046 ± 113#	5302 ± 113#
-dP/dt	4875 ± 167	1101 ± 113*	4025 ± 117#	4011 ± 105#

Values are means ± SE of 8 hearts for cardiac function in each group. I/R; 30 min ischemia followed by 30 min reperfusion. LVDP; left ventricular developed pressure, LVEDP; left ventricular end diastolic pressure, +dP/dt; rate of pressure development, –dP/dt; rate of pressure decay. Hearts were treated with Leu or MDL 10 min before and 20 min after inducing I/R. * $P < 0.05$ compared to Control, #$P<0.05$ compared to I/R.

V. DISCUSSION

1. Role of endothelium in ischemia reperfusion induced cardiac dysfunction and subcellular enzyme activities

In this study we have shown that I/R produced cardiac dysfunction as reflected by depressed LVDP, +dP/dt and -dP/dt as well as increased LVEDP in isolated rat hearts. Although I/R induced impaired recovery of cardiac function is in agreement with our previous reports (Saini et al. 2005, Takeda et al. 2005, Takeda et al. 2003, Temsah et al. 1999), the observed alterations in all parameters of cardiac performance due to I/R were of greater magnitude in hearts perfused at CF than those at CP. These contractile changes in CF-perfused and CP-perfused I/R hearts were found to be dependent upon the duration of ischemia because 15 min and 30 min of ischemia in CF-perfused hearts produced changes comparable to those seen when the CP-perfused hearts were exposed to 30 min and 60 min of ischemia, respectively. Since oxidative stress in I/R hearts is considered to play a critical role in depressing cardiac function (Dhalla et al. 2007), it is likely that these differences in cardiac function due to I/R between CF-perfused and CP-perfused hearts may be due to differences in the magnitude of oxidative stress in these preparations. Although I/R was found to depress the activities of both SL Na^+-K^+ ATPase and SR Ca^{2+}-uptake, which observations are in agreement with our previous work (Singh et al. 2004a, Temsah et al. 1999), the I/R-induced depressions in these membrane activities in CF-perfused hearts were greater than those in CP-perfused hearts. Since the depressed SL Na^+-K^+ ATPase and SR

Ca^{2+}-uptake activity in the I/R hearts are considered to induce Ca^{2+}-handling defects in cardiomyocytes and cardiac dysfunction (Dhalla et al. 2007, Singh et al. 2004a, Temsah et al. 1999), the observed differences with respect to these biochemical parameters in CF-perfused and CP-perfused hearts may explain the I/R-induced differential changes in cardiac function in these preparations. In view of the fact that the I/R-induced increase in calpain activity (Hussain et al. 2005, Nangle et al. 2006, Stalker et al. 2005) in CF-perfused hearts was much higher than that in CP-perfused hearts, it is evident that the greater depressions in both SL Na^{+}-K^{+} ATPase and SR Ca^{2+}-uptake activities in CF-perfused hearts in comparison to CP-perfused hearts may be due to differences in degree of proteolysis in these preparations.

The results in this study indicate that endothelial function with respect to NO formation in I/R hearts may be impaired and this defect may be of a greater magnitude in CF-perfused hearts than that in CP-perfused hearts. This view is based on our observations that the concentration of nitrate/nitrite in the perfusate as well as protein content for eNOS in the myocardium were lower in CF-perfused hearts in comparison to those in CP-perfused hearts. In fact, the concentration of nitrate/nitrite in the effulent as well as the volume of perfusate from control hearts perfused at CF were also lower than those from control hearts perfused at CP. It should be pointed out that endothelium is now well known to control the coronary flow as well as cardiac function through the release of several substances including NO (Chohan et al. 2006, Jones and Bolli. 2006, Kincaid et al. 2005, Lin

et al. 2000, Waldron et al. 1999). Furthermore, coronary flow in the heart is determined by coronary perfusion pressure (Djuric et al. 2007), which remained below the control level, while the coronary flow is maintained during reperfusion of CF-perfused hearts. On the other hand, coronary flow is increased above the control level during early periods while the coronary pressure is maintained during reperfusion of CP-perfused hearts. Thus, it is likely that the CP-perfused and CF-perfused preparations are subjected to varying degrees of shear and stress and differences in the characteristics of coronary flow and coronary pressure may explain the differences in their responses to I/R. In this regard, it should be noted that shear and stress have been shown to cause endothelial dysfunction due to the development of intracellular Ca^{2+}-overload in the endothelial cells (Hong et al. 2006, Kwan et al. 2003, Shen et al. 1992, Tran et al. 2000). However, this may not serve as a mechanism for observed changes because shear stress is likely to be higher in CP-perfused hearts due to greater blood flow but the endothelial dysfunction was found to be less in comparison to the CF-perfused hearts. Since we did not measure shear stress in both CF-perfused and CP-perfused heart preparations, some caution should be exercised while explaining differential response of these preparations in terms of differences in the degree of shear stress. Nonetheless, differences in the degree of defects in the endothelium in CF-perfused and CP-perfused hearts were evident from the differences in responses of these preparations to ACH, which is known to produce coronary vasodilation (Waldron et al. 1999), and U46619, a thromboxane A2 mimetic, which is known

to produce coronary constriction (Djuric et al. 2007, Garcia et al. 2005Lin et al. 2000, Wang et al. 2005). This point is substantiated by our observations that the depression in coronary perfusion pressure due to ACH in control CP-perfused hearts was greater than that in control CF-perfused hearts. On the other hand, the increase in coronary perfusion pressure due to U46619 was more in CP-perfused hearts in comparison to that in CF-perfused hearts.

This study has revealed that the I/R-induced changes in cardiac function (as seen by depressed LVDP, +dP/dt and -dP/dt as well as elevated LVEDP) were attenuated in both CF-perfused and CP-perfused hearts upon treatment with LA whereas these alterations were augmented in CP-perfused hearts upon treatment with LN. Furthermore, the beneficial effects of LA on I/R induced changes in cardiac performance in CF-perfused hearts were ameliorated by LN. These observations suggest the possibility of substrate deficiency for the conversion of endogenous L-arginine to NO under conditions of I/R. Depressions in the SL Na^+-K^+ ATPase and SR Ca^{2+}-uptake activities as well as increase in calpain activity due to I/R were also attenuated in CF-perfused hearts by treatment with LA and augmented in CP-perfused hearts with LN. In view of the role of LA as a substrate for NOS and LN as an inhibitor of eNOS (Chohan et al. 2006), it is apparent that the observed differences for changes in cardiac function, subcellular activities and calpain activity due to I/R in CF-perfused and CP-perfused hearts may be a consequence of differential alterations in the endothelium with respect to NO production. These results therefore indicate that differences in the I/R induced

changes in cardiac function as well as subcellular activities in CF-perfused and CP-perfused hearts may partly be due to differential alterations in endothelial function with respect to NO production. Whether these differences between CF-perfused and CP-perfused hearts are also mediated by differential development of intracellular Ca^{2+}-overload, which is known to be a major mechanism of I/R injury, remain to be investigated.

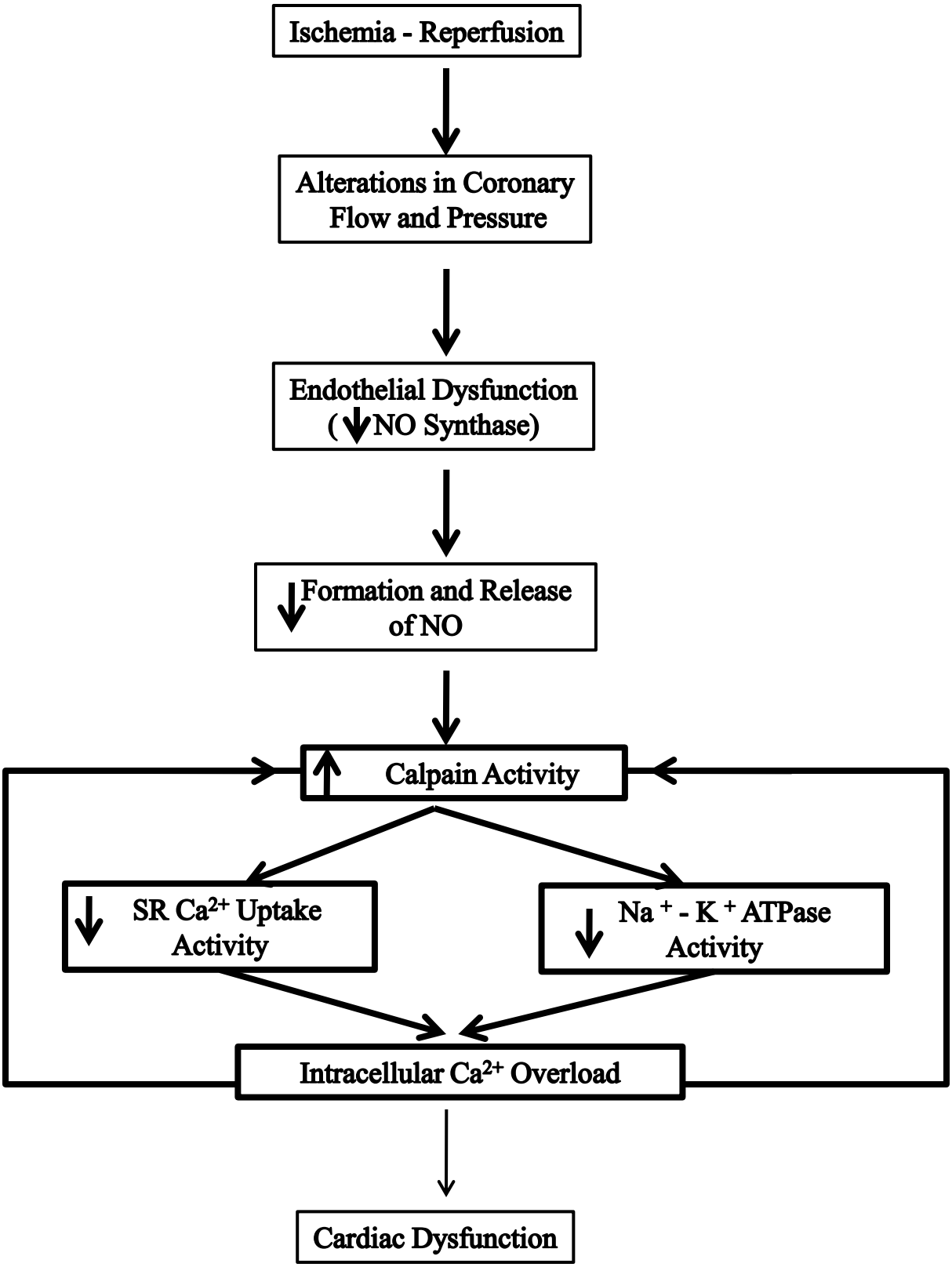
The results described in this study indicate that alterations in cardiac performance due to I/R injury in both CF-perfused and CP-perfused hearts were dependent upon the time of ischemic insult. These I/R-induced changes in cardiac performance were not only greater in CF-perfused hearts in comparison to those in CP-perfused hearts but were also associated with corresponding alterations in SL Na^{+}-K^{+} ATPase, SR Ca^{2+}-uptake and calpain activities. There was also a greater decrease in nitrate/nitrite concentration in perfusate as well as eNOS protein content in CF-perfused hearts in comparison to that in CP-perfused hearts. The beneficial effects of LA and adverse actions of LN indicate that I/R-induced changes in contractile, subcellular and biochemical activities may be due to alterations in endothelial function and availability of substrate for NO bioavailability. In addition, the difference with respect to endothelial function between CF-perfused and CP-perfused preparations was apparent upon studying responses to ACH or U46619. Since maintaining the coronary flow in the CF-perfused hearts by using a pump was found to produce greater damage to endothelial function in comparison to the CP-perfused hearts, it would be prudent

to exercise some caution for the use of pump which is commonly employed during cardiac surgery involving a heart-lung bypass machine or during organ transplant procedure. Furthermore, agents such as LA, which was found to prevent I/R-induced injury to endothelium, should be used in the perfusion fluid during cardiac bypass surgery for improving recovery of the heart. A scheme representing the sequence of events involving endothelial dysfunction and changes in subcellular activities in the pathophysiology of I/R-induced cardiac dysfunction is depicted in Figure 17.

2. Role of calpain activation in ischemia reperfusion induced alterations in cardiac function and SL Na^+-K^+ ATPase activity

By employing isolated rat hearts in this study, we have demonstrated that cardiac performance and SL Na^+-K^+ ATPase were depressed while calpain activity was increased due to I/R. These observations are consistent with earlier reports showing cardiac dysfunction (Saini et al. 2005), depressed SL Na^+-K^+ ATPase activity (Ostadal et al. 2004) and dramatic increase in calpain activity (Singh et al. 2004a) in hearts subjected to I/R injury. Since both oxidative stress and intracellular Ca^{2+}-overload are known to induce abnormalities in the I/R hearts (Dhalla et al. 1988, Dhalla et al. 2007), it is likely that both these mechanisms are involved in inducing the observed changes in SL Na^+-K^+ ATPase and calpain activities due to I/R injury. In fact, different oxyradicals and oxidants have been shown to decrease the SL Na^+-K^+ ATPase activity directly by inactivating some functional groups of the enzyme as well as by promoting lipid peroxidation in the

Figure 17. Proposed sequence of events involving endothelial dysfunction and depressed subcellular activities in I/R-induced cardiac dysfunction.



SL membranes (Kaneko et al. 1989a, Kaneko et al. 1989b). In addition, both oxidative stress and I/R may increase the activities of different proteases including calpain through the development of intracellular Ca^{2+}-overload and thus may degrade SL Na^+-K^+ ATPase upon proteolysis (Dhalla et al. 2007, Singh et al. 2008). Since Na^+-K^+ ATPase is known to maintain the concentrations of Na^+ and K^+ directly and of Ca^{2+} concentration indirectly in the cell (Dhalla et al. 2007, Tani and Neely, 1989), the observed depression in SL Na^+-K^+ ATPase activity may contribute to contractile dysfunction in the I/R heart. However, the contribution of abnormalities in myofibrils and SR, which are also known to occur due to I/R (Maddika et al. 2009, Singh et al. 2004a), cannot be overlooked while explaining cardiac dysfunction in the I/R hearts.

The observed depression in SL Na^+-K^+ ATPase activity in the I/R hearts may be due to a decrease in protein content of the enzyme. This view is supported by our observation that protein content for both α_1 and α_2 as well as β_1 and β_2 isoforms of Na^+-K^+ ATPase was decreased in the I/R hearts (Figure 8). The I/R-induced activation of calpain has also been reported to be associated with depressions in the activities of SR Ca^{2+}-cycling proteins (Singh et al. 2004a). This depressant effect on SL Na^+-K^+ ATPase of I/R may be due to the activation of calpain activity in the I/R heart because incubation of the SL preparations with calpain was found to produce similar changes (Figure 7 and 8). The activation of calpain by I/R was also observed to be associated with translocation of both calpain 1 and 2 isoforms from cytosole to the SL membrane as well as changes in the distribution of

calpasatin, an endogenous inhibitor of calpain (Pontremoli et al. 1992), in the cardiomyocytes. Since the protein content for calpain 1 and 2 isoforms did not change whereas that of calpastatin decreased in the I/R (Singh et al. 2004a), it appears that the increased calpain activity in the I/R hearts may partly be due to decreased protein content for calapstatin.

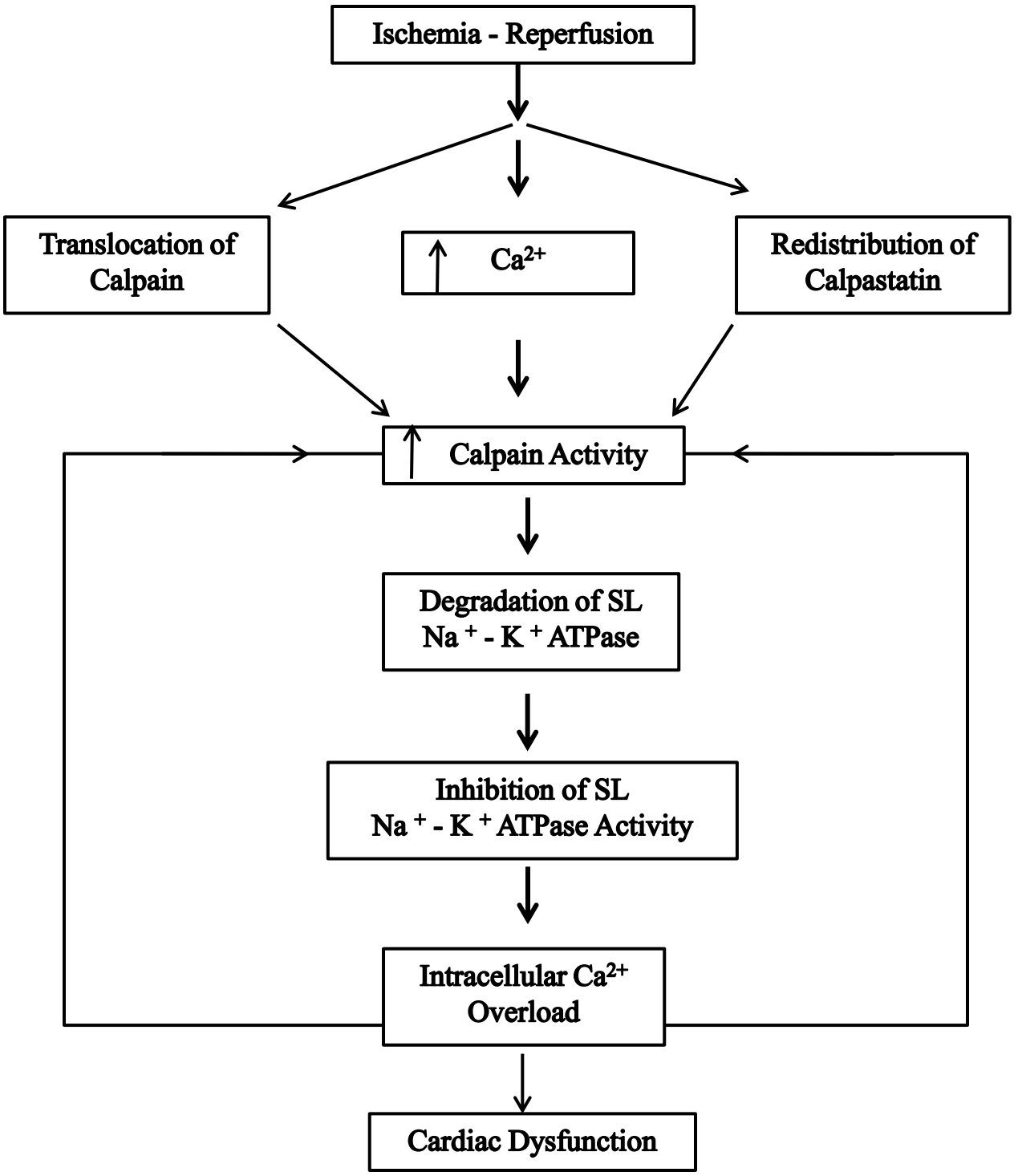
Treatment of hearts with leupeptin was observed to attenuate the I/R-induced changes in cardiac function, SL Na^+-K^+ ATPase activity and protein content for SL Na^+-K^+ ATPase isoforms. The effects of leupeptin appear to be due to the inhibition of calpain activity because the I/R-induced activation of calpain was depressed by treatment with leupeptin. The calpain-induced depressions in Na^+-K^+ ATPase activity and Na^+-K^+ ATPase isoforms as seen by incubating SL membranes with calpain were also attenuated by leupeptin. Furthermore, MDL, a specific inhibitor of calpain (Inserte et al. 2006, Li et al. 1998), produced effects on I/R-induced alterations in cardiac function, SL Na^+-K^+ ATPase activity and calpain activity, which were similar to those seen with leupeptin. Both MDL and leupeptin were also found to attenuate the I/R-induced changes in the distribution of calpain 1 and 2 isoforms as well as calpastatin in cardiomyocytes. It is pointed out that leupeptin has also been reported to mitigate the I/R-induced alterations in cardiac function, activities and content of SR Ca^{2+}-cycling proteins as well as calpain activity in the heart (Singh et al. 2004a). In addition, leupeptin was observed to attenuate the I/R-induced changes in myofibrillar ATPase activity (Maddika et al. 2009). Thus the observations reported in this study as well as

those indicated above suggest that the activation of calpain may play a critical role in inducing subcellular changes including the depression of SL Na^+-K^+ ATPase during the development of cardiac dysfunction due to I/R injury. It is therefore likely that inhibitors of calpain as well as other proteases may prove beneficial for preventing the development of cardiac dysfunction upon reperfusion of the ischemic myocardium as well as the treatment of ischemic heart disease. A scheme representing the sequence of events involving increased calpain activity and depressed SL Na^+-K^+ ATPase activity in I/R-induced changes in cardiac function is shown in Figure 18.

3. Role of oxidative stress on ischemia reperfusion induced alterations in protease activity, cardiac function and Na^+-K^+-ATPase activity

In this study, we have shown that the depression in cardiac function, as reflected by decreased LVDP, +dP/dt and –dP/dt as well as increased LVEDP, was associated with increases in the activities of both calpain and MMP in hearts upon subjecting to I/R injury. These observations are consistent with earlier reports showing depressed cardiac function as well as increased calpain activity (Singh et al. 2004, Chohan et al. 2006, Singh and Dhalla 2010, Khalil et al. 2005) and MMP activity (Sawaicki et al. 2005, Lalu et al. 2005) due to I/R. Since the I/R-induced alterations in cardiac function were attenuated by Leu and MDL, inhibitors of calpain (Singh et al. 2004a, Singh and Dhalla 2010, Urthaler et al. 1997), as well as Dox, an inhibitor of MMP (Sawicki et al. 2005), it is likely that the I/R-induced cardiac dysfunction may be due to the activation of both calpain and MMP. The

Fig 18. Proposed sequence of events involving increased Calpain activity and depressed SL Na^+-K^+- ATPase in I/R –induced cardiac dysfunction.



view is supported by several observations indicating the involvement of calpain activation (Inserte et al. 2005, Urthaler et al. 1997, Pedrozo et al. 2009) as well as the activation of both extracellular and intracellular MMP (Schulz 2007, Chow et al. 2007, Kandasamy et al. 2010, Spanikova et al. 2010) in I/R-induced functional and structural abnormalities in the heart. However, it should be noted that treatment of the heart with Dox was found to attenuate I/R-induced cardiac dysfunction as well as the activation of MMP without affecting the elevated levels of calpain. On the other hand, attenuation of I/R-induced cardiac dysfunction by Leu and MDL was associated with a marked depression in the activation of calpain and a significant reduction in the MMP activity in I/R hearts. These observations seem to indicate that the I/R-induced activation of MMP may partly be due to the activation of calpain. Other proteolytic enzymes, cathepsins, have also been reported to be involved in the activation of extracellular MMP (Felbor et al. 2000, Maciewiez and Etherington 1988). Furthermore, calpastatin, a known endogenous inhibitor of calpain, has been indicated to inhibit the MMP-2 activity (Kandasamy et al. 2010). Accordingly, it is suggested that the I/R-induced cardiac dysfunction due to calpain may partly be elicited through the activation of MMP in the heart.

The increased activities of both calpain and MMP in the heart due to I/R may be explained as a consequence of several mechanisms. The imbalance of calpain and its endogenous inhibitor, calpastatin, has been reported to increase the calpain activity in the I/R heart (Sorimachi et al. 1997). Likewise, the increased MMP

activity in the I/R heart has been shown to be due to an imbalance of MMP and their endogenous tissue inhibitor of metalloproteinase (TIMP) (Schulze et al. 2003). While the occurrence of intracellular Ca^{2+}-overload due to I/R has been suggested to account for the activation calpain (Dhalla et al. 2007, Singh et al. 2004a, Singh and Dhalla 2010), generation of oxidative stress has been indicated to explain the increase in MMP-2 activity in the I/R heart (Schulz 2007, Kandasamy et al. 2010, Leon et al. 2008). Since oxidative stress has been shown to produce intracellular Ca^{2+}-overload in the myocardium (Dhalla et al. 2000, Dhalla et al. 2007, Saini and Dhalla 2005, Saini et al. 2005), the I/R-induced oxidative stress can also be seen to increase the calpain activity indirectly. In this study, we have observed that the I/R-induced increases in both calpain and MMP activities were simulated upon reperfusion of hearts subjected to hypoxic insult in the presence or absence of glucose. Furthermore, these proteolytic enzyme activities were also increased upon reperfusing the hearts exposed to H_2O_2 or X+XO. In this regard, it is pointed out that I/R, hypoxia, H_2O_2 and X+XO have been reported to generate oxidative stress and produce intracellular Ca^{2+}-overload in the myocardium (Saini and Dhalla 2005, Makazan et al. 2006, Dhalla et al. 2007). The direct or indirect involvement of oxidative stress in the I/R-induced activation of both calpain and MMP is further attested by our observations that the increases in the activities of both these proteolytic enzymes were attenuated by treatment of hearts with antioxidants, namely NAC and MPG (Makazan et al. 2006, Dhalla et al. 2000). In addition, ischemic preconditioning, which has been

reported to attenuate oxidative stress and intracellular Ca^{2+}-overload (Saini and Dhalla 2005, Inserte et al. 2006) was found to reduce the I/R-induced activation of both calpain and MMP. Taken together, the results reported in this study support the concept that oxidative stress directly or indirectly plays a critical role in increasing the activities of proteolytic enzymes in the heart due to I/R injury.

Earlier studies have revealed that the I/R-induced depression in SL Na^+-K^+-ATPase activity is elicited by oxidative stress in the myocardium (Ostadal et al. 2004, Singh and Dhalla 2010, Dhalla et al. 2007). The results described here lend further support to this viewpoint because the I/R-induced changes in Na^+-K^+-ATPase activity were simulated by perfusing the hearts with interventions such as hypoxia, H_2O_2 and X+XO, which are known to generate oxidative stress (Saini and Dhalla 2005, Dhalla et al. 2007, Dhalla et al. 2004). Furthermore, antioxidants, NAC and MPG, as well as preconditioning, which are known to prevent the effects or reduce the intensity of oxidative stress (Makazan et al. 2006, Saini et al. 2004, Saini and Dhalla 2005) were observed to attenuate the I/R-induced inhibition of SL Na^+-K^+-ATPase activity. It may be noted that I/R-induced oxidative stress has also been reported to decrease the SL Na^+-K^+-ATPase activity by inactivation of the SH-groups in the enzyme (Dhalla et al. 2004, Dhalla et al. 2007). Since the treatments of hearts with Leu, MDL or Dox were found to reduce the I/R-induced depression in SL Na^+-K^+-ATPase activity, it is likely that activation of both calpain and MMP may be involved in depressing SL Na^+-K^+-ATPase activity due to I/R. Although Leu has been reported to prevent the I/R-

induced changes in SL Na^+-K^+-ATPase subunits as a consequence of degradation of the enzyme due to activation of calpain (Singh and Dhalla 2010), no such information regarding Dox-induced inhibition of MMP activation is available in the literature. An association of depression in SL Na^+-K^+-ATPase activity with degradation of the enzyme and its subunits by the activation of both calpain and intracellular MMP appears likely in hearts subjected to I/R. In this regard, calpain activation as well as MMP-2 activation have been reported to affect other subcellular proteins including that of the SR, mitochondria, myofibrils, as well as cytoskeletal and signal transduction system (Singh et al. 2004, Pedrozo et al. 2010, Chen et al. 2002, Urthaler 2007, Sung et al. 2007, Chow et al. 2007, Sawicki et al. 2005, Müller and Dhalla 2010). Thus it appears that the observed depression in SL Na^+-K^+-ATPase may be due to both oxidation of SH-groups and degradation of different subunits of the enzyme in the I/R heart.

From the results described in this study it is evident that oxidative stress generated due to I/R injury may result in the development of intracellular Ca^{2+}-overload and activation of calpain activity in the heart. Oxidative stress can also be seen to activate the intracellular MMP-2 activity directly in the myocardium. The resultant increase in the activities of both calpain and intracellular MMP-2 would then degrade subcellular proteins including Na^+-K^+-ATPase and depress their activities. Oxidative stress may also depress the activity of SL Na^+-K^+-ATPase directly by inactivating its functional groups in the I/R hearts. These events leading to cardiac dysfunction in hearts subjected to I/R injury are depicted in

Figure 19. It is pointed out that the present scheme showing the impairment of cardiac function due to I/R is not intended to de-emphasize the role of activation of extracellular MMP and subsequent degradation of different extracellular matrix proteins.

Although it is difficult to differentiate the role of calpain activation from that of MMP activation on subcellular alterations and contractile dysfunction in hearts subjected to I/R, it appears that calpain activation in comparison to MMP activation may be a more important determinant of cardiac abnormalities shown in this study. This view is supported by our observations that the I/R-induced activation of calpain was greater than that of MMP. Likewise, higher activation of calpain in comparison to that of MMP was also seen in hearts subjected to hypoxia in absence or presence of glucose in the perfusion medium. Furthermore, hearts exposed to X+XO-reperfusion or H_20_2-reperfusion showed higher activities of calpain than the respective values for MMP activities. In addition, hearts subjected to IPC also showed a greater activation of calpain in comparison to that of MMP as well as a greater depression of the I/R-induced activation of calpain than that of MMP. It is noteworthy that the depression of the I/R-induced increase of calpain activity in hearts treated with calpain inhibitors, Leu and MDL, was greater than that of MMP activity. On the other hand, depression of the I/R-induced increase in MMP activity was more than that of the calpain activity upon treatment of hearts with antioxidants, NAC or MPG. In addition, a greater depression of the I/R-induced increase in MMP activity was seen in comparison to

that in calpain activity in hearts treated with DOX, an inhibitor of MMP. Since the inhibitors of MMP and calpain as well as antioxidants, NAC and MPG, are generally considered to be of nonspecific nature, some caution should be exercised in the interpretation of data reported in this study. Nonetheless, the present results are consistent with the view that the I/R-induced depression in SL Na^+-K^+-ATPase and cardiac function may be mediated through both calpain-dependent and MMP-dependent pathways. Furthermore, the data also seem to support the concept that I/R-induced activation of calpain is mediated by the combination of oxidative stress and intracellular Ca^{2+}-overload whereas MMP is activated by calpain and oxidative stress.

4. General mechanisms of the I/R-induced changes in the heart

From the foregoing discussion and the results described in this study, the following three major observations in myocardial I/R injury can be made:

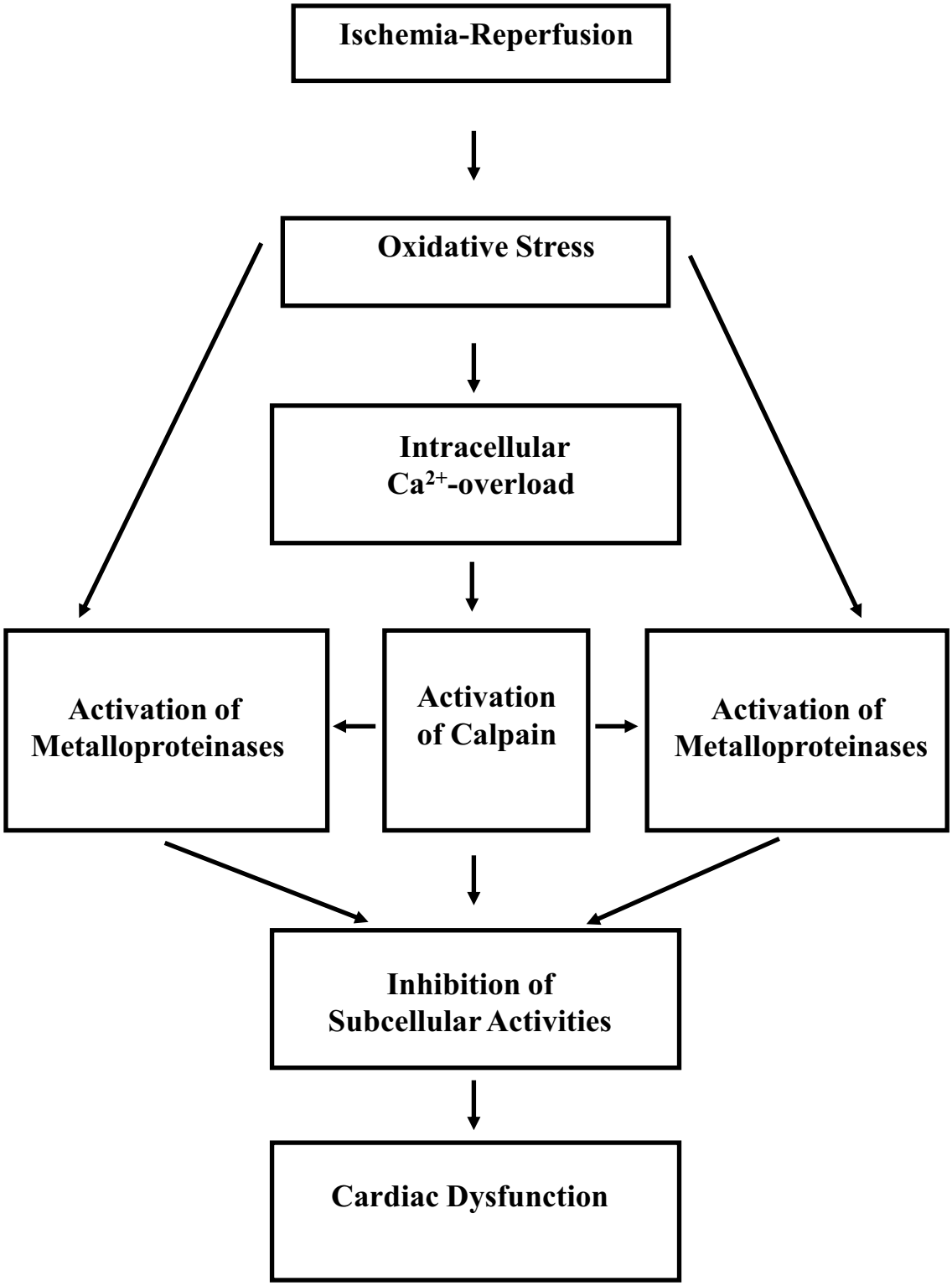
(a) The endothelium becomes defective with respect to NO formation and release. This change may induce activation of calpain, depression of SL Na^+-K^+-ATPase activity and impairment of cardiac performance due to I/R injury to the heart.

(b) The I/R induced activation of calpain due to interacellular Ca^{2+} overload degrades different subunits of SL Na^+-K^+-ATPase, depresses the activity of the enzyme and results in cardiac dysfunction.

(c) I/R-induced oxidative stress not only contributes to activation of calpain but also increases the activity of MMP markedly and thus the combined proteolytic activity may induce depression in SL Na^+-K^+-ATPase activity and cardiac dysfunction.

These findings lend support to our view that both endothelial dysfunction and development of oxidative stress due to I/R injury are intimately involved in the increase of proteolytic activity as well as depression of subcellular functions and defects in cardiac performance.

Figure 19. Role of oxidative stress-induced proteolysis of subcellular proteins due to activation of calpain and MMP in cardiac dysfunction as a consequence of I/R injury.



The involvement of defective endothelial function with respect to reduced formation of and release of NO in inducing activation of calpain, depression of SL Na^+-K^+-ATPase and cardiac dysfunction due to I/R injury is evident from the observation that these changes were attenuated by the treatment of hearts with NO donors such as L-arginine. Since the I/R-induced depression in SR Ca^{2+} uptake activity was also attenuated by L-arginine, it is apparent that functions of different subcellular organelles in the cardiomyocytes are regulated by endothelium. Furthermore, the role of endothelial dysfunction is also evident from the observation that greater reduction in I/R-induced formation of NO in hearts perfused at CF was associated with greater degree of I/R-induced cardiac abnormalities in comparison to those changes seen in hearts perfused at CP. Treatment of hearts perfused at CP with L-NAME, an inhibitor of NO production, was found to augment I/R-induced alterations. These observations in hearts perfused at CF and CP not only indicate the occurrence of endothelial dysfunction but also support the role of endothelial defects play in the development of I/R-induced cardiac abnormalities.

In this study, we have observed that activation of calpain was associated with depressions in SL Na^+-K^+-ATPase activity and cardiac function upon subjecting the hearts to I/R injury. Since I/R injury was also observed to reduce different subunits of SL Na^+-K^+-ATPase, it is likely that the observed inhibition of this anzyme may be due to its degradationby calpain. This view is consistent with our earlier observation that I/R-induced depression in SR Ca^{2+} uptake was associated

with reduction in SR Ca^{2+} cycling protein content. The role of calpain in depressing SL Na^{+}-K^{+}-ATPase activity and inducing cardiac dysfunction due to I/R injury is evident from our observation that treatment of hearts with Leu and MDL attenuates all I/R-induced changes. It is pointed that in addition to activation of calpain, I/R was observed to increase MMP activity in the heart. In fact activities of both calpain and MMP were increased under conditions which simulated the effects of I/R on SL Na^{+}-K^{+}-ATPase and cardiac function. The importance of MMP activation due to I/R injury is apparent because I/R-induced changes in SL Na^{+}-K^{+}-ATPase and cardiac function were ameliorated by treatment of hearts with Dox, an inhibitor of MMP. Furthermore, I/R-induced activation of both, calpain and MMP, as well as depressions in SL Na^{+}-K^{+}-ATPase and cardiac function were attenuated by treatment with NAC, MPG and preconditioning. Since treatment with NAC, MPG and preconditioning are known to attenuate I/R-induced oxidative stress, these experiments provide support to the view that oxidative stress plays an important role in inducing all the observed changes in hearts exposed to I/R injury.

Since I/R-induced activation of calpain and depressions in SL Na^{+}-K^{+}-ATPase activity and cardiac function were attenuated by interventions such as L-arginine, Leu and MDL, it seems likely that various NO donors and calpain inhibitors may serve as cardioprotective agents. Similarly, in view of the observation that I/R-induced depression in SL Na^{+}-K^{+}-ATPase activity and cardiac dysfunction were reduced by Dox treatment, it appears that different MMP inhibitors may be useful

in protecting the heart against I/R injury. Since I/R-induced activation of both calpain and MMP, depression of SL Na^+-K^+-ATPase activity and cardiac performance were attenuated by NAC and MPG, these observations support the view that different antioxidants may be valuable tools of cardioprotection. It must be pointed that all agents used in this study only extended partially beneficial effects against I/R injury, which may be due to insufficient concentration of these agents employed. Alternatively, a combination therapy by using several agents acting at different sites may prove valuable for achieving full recovery of ischemic hearts.

VI. CONCLUSIONS

By employing isolated rat hearts as an experimental model of I/R injury, we have examined different mechanisms of cardiac dysfunction. The experiments in this study have led us to make the following conclusions:

1. I/R injury was observed to induce a defect in endothelial function with respect to reduced formation and release of NO.
2. I/R-induced endothelial defects may activate calpain, depress subcellular activities and induce cardiac dysfunction.
3. I/R-induced activation of calpain was Ca^{2+} dependent and this may degrade SL Na^{+}-K^{+}-ATPase and thus depress the activity of Na^{+}-K^{+}-ATPase.
4. I/R-induced activation of both, calpain and MMP, may degrade subcellular proteins and produce cardiac dysfunction.
5. Various interventions such as NO donors like L-arginine, protease inhibitors like Leu and MDL as well as antioxidants like NAC and MPG, may prove useful for improving cardiac function in IHD.

VII. REFERENCES

Adams JA. Endothelium and cardiopulmonary resuscitation. Crit Care Med 2006; 34: S458-65.

Ali M, Shulz R. Activation of MMP_2 as a key event in oxidative stress injury to the heart. Front in Biosc 2009; 14: 699-716.

Ali, M.A., Cho, W.J., Hudson, B., Kassiri, Z., Granzier, H., and Shulz, R. Titin is a target of matrix metalloproteinase-2: Implications in myocardial ischemia/reperfusion injury. Circulation 2010; 122(20): 2039-47.

Ambrosio, G., and Tritto, I. Reperfusion injury: experimental evidence and clinical implications. Am. Heart J. 1999; 138: S69–S75.

Anderson TJ. Nitric oxide, atherosclerosis and the clinical relevance of endothelial dysfunction. Heart Fail Rev 2003; 8: 71-86.

Anderson TW. The changing pattern of ischemic heart disease. Can Med Assoc J. 1973;108(12):1500-4.

Anger M, Samuel JL, Marotte F, Wuytack F, Rappaport L, Lompre AM. The sarco(endo)plasmic reticulum Ca^{2+}-ATPase mRNA isoform, SERCA 3, is expressed in endothelial and epithelial cells in various organs. FEBS Lett. 1993;334(1):45-8.

Anger M, Samuel JL, Marotte F, Wuytack F, Rappaport L, Lompre AM. In situ mRNA distribution of sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoforms during ontogeny in the rat. J Mol Cell Cardiol. 1994;26(4):539-50.

Aono, Y., Ariyoshi., H. Tsuji, Y., Ueda, A., Tokunaga, M., Sakon, M., and Monden, M. Localized activation of m-calpain in human umbilical vein endothelial cells upon hypoxia. Thromb. Res. 2001;102: 353-361.

Arai M, Matsui H, Periasamy M. Sarcoplasmic reticulum gene expression in cardiac hypertrophy and heart failure. Circ Res. 1994;74(4):555-64.

Augustin W, Wiswedel I, Noack H, Reinheckel T, Reichelt O. Role of endogenous and exogenous antioxidants in the defence against functional damage and lipid peroxidation in rat liver mitochondria.Mol Cell Biochem. 1997;174:199-205.

Baltas LG, Karczewski P, Krause EG. The cardiac sarcoplasmic reticulum phospholamban kinase is a distinct delta-CaM kinase isozyme. FEBS Lett. 1995;373(1):71-5.

Barry WH. Mechanisms of myocardial cell injury during ischemia and reperfusion. J Card Surg 1987; 2: 375-383.

Bassani RA, Bassani JW, Bers DM Relaxation in ferret ventricular myocytes: unusual interplay among calcium transport systems. J Physiol. 1994;476(2):295-308

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A. 1990;87:1620-4.

Bittar EE ,Rivett AJ (Eds) :Intacellular protein degradation. Advances Cell Mol Biol 1998; 27, 1- 301.

Blessey R. Epidemiology, risk factors, and pathophysiology of ischemic heart disease. Phys Ther. 1985;65(12):1796-1805.

Bolli R, Cannon RO, Speir E, Goldstein RE, Epstein SE. Role of cellular proteinases in acute myocardial infarction. I. Proteolysis in nonischemic and ischemic rat myocardium and the effects of antipain, leupeptin, pepstatin and chymostatin administered in vivo. J Am Coll Cardiol. 1983; 2(4): 671-80.

Bolli R, Patel BS, Jeroudi MO, Lai EK, McCay PB Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap alpha-phenyl N-tert-butyl-nitrone. J Clin Invest. 1988 ;82:476-85.

Bolli R, Jeroudi MO, Patel BS, Aruoma OI, Halliwell B, Lai EK, McCay PB. Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury.Circ Res. 1989;65:607-22.

Bolli R.. Basic and clinical aspects of myocardial stunning. Prog. Cardiovasc. Dis. 1998; 40: 477–516.

Bolli R, Marbán E. Molecular and cellular mechanisms of myocardial stunning. Physiol Rev 1999;79: 609-634.

Boveris A, Cadenas E, Stoppani AO. Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochem J. 1976;156:435-444

Boveris A. Determination of the production of the superoxide radicals and hydrogen peroxide in mitochondria. Methods Enzymol. 1984;105:429-435.

Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? J Clin Invest 1985;76: 1713-1719.

Cadenas E and Davies KJ. Mitochonrial free readical generation, oxidative stress and aging. Free Radical Biol Med. 2000; 29: 222-30.

Carafoli E, Molinari M: Calpain: a protease in search of a function? Biochem Biophys Res Commun 1998; 247, 193 –203.

Carlos A. Valverde, Dmytro Kornyeyev, Marcela Ferreiro, Azade´ D. Petrosky, Alicia Mattiazzi, and Ariel L. Escobar. Transient Ca^{2+} depletion of sarcoplasmic reticulum at the onset of reperfusion. Cardiovascular Res. 2010. 85, 671-680

Cala SE and Jones LR. Phosphorylation of cardiac and skeletal muscle calsequestrin isoforms by casein kinase II. Demonstration of a cluster of unique rapidly phosphorylated sites in cardiac calsequestrin. J Biol Chem. 1991;266(1):391-8.

Caulfield JB, Borg TK. The collagen network of the heart. Lab Invest. 1979;40: 364-372.

Cardin S, Li D, Thorin-Trescases N, Leung TK, Thorin E, Nattel S. Evolution of the atrial fibrillation substrate in experimental congestive heart failure: angiotensin-dependent and -independent pathways. Cardiovasc Res. 2003;60(2):315-25.

Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev 1979;59:527-605.

Chen, M., Won, D.J., Krajewski, S., and Gottlieb, R.A. Calpain and mitochondria in ischemia/reperfusion injury. J. Biol. Chem 2002; 277:29181-86.

Cheung, P.Y., Sawicki, G., Wozniak, M., Wang, W., Radomski, M.W., and Schulz, R.. Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. Circulation 2000; 101(15): 1833-39.

Chiesi M, Schwaller R. Involvement of electrostatic phenomena in phospholamban-induced stimulation of Ca^{2+} uptake into cardiac sarcoplasmic reticulum. FEBS Lett. 1989;244(1):241-4

Chohan PK, Singh RB, Dhalla NS, Netticadan T. L-arginine administration recovers sarcoplasmic reticulum function in ischemic reperfused hearts by preventing calpain activation. Cardiovasc Res 2006; 69: 152-163.

Chow, A.K. Cena, J., and Schulz, R. Acute actions and novel targets of matrix metalloproteinases in the heart and vasculature. Br. J. Pharmacol. 2007;152(2): 189-205.

Cornea RL, Jones LR, Autry JM, Thomas DD. Mutation and phosphorylation change the oligomeric structure of phospholamban in lipid bilayers. Biochemistry. 1997; 36(10):2960-7.

Carrozza JP Jr, Bentivegna LA, Williams CP, Kuntz RE, Grossman W, Morgan JP. Decreased myofilament responsiveness in myocardial stunning follows transient calcium overload during ischemia and reperfusion. Circ Res. 1992;71(6):1334-40.

Dancu MB, Tarbell JM. Coronary endothelium expresses a pathologic gene pattern compared to aortic endothelium: Correlation of asynchronous hemodynamics and pathology in vivo. Atherosclerosis 2007;192: 9-14.

Darsee JR, Braunwald E. Time course of regional function after coronary occlusions of 1- to 120-min duration. Am J Physiol 1981;240: H399-H407.

Davis BA, Schwartz A, Samaha FJ, Kranias EG. Regulation of cardiac sarcoplasmic reticulum calcium transport by calcium-calmodulin-dependent phosphorylation. J Biol Chem. 1983;258(22):13587-91.

Davies CA, Leyland AH. Trends and inequalities in short-term acute myocardial infarction case fatality in Scotland, 1988-2004. Popul Health Metr. 2010;8:33.

Dhalla NS, Saini HK, Tappia PS, Sethi R, Mengi SA, Gupta SK. Potential role and mechanisms of subcellular remodeling in cardiac dysfunction due to ischemic heart disease. J Cardiovasc Med 2007;8: 238-250.

Dhalla, N. S., Elmoselhi, A. B., Hata, T., Makino, N. Status of myocardial antioxidants in ischemia-reperfusion injury. Cardiovasc. Res. 2000; 47: 446-456.

Dhalla NS, Panagia V, Singal PK, Makino N, Dixon IM, Eyolfson DA. Alterations in heart membrane calcium transport during the development of ischemia-reperfusion injury. J Mol Cell Cardiol 1988; 20: S3-S13.

Dixon IMC, Hata T, Dhalla NS. Sarcolemmal Na^+-K^+-ATPase activity in congestive heart failure due to the myocardial infarction. Am J Physiol Cell Physiol 1992; 262: C664–C671.

Dinerman JL, Lowenstein CJ, Snyder SH. Molecular mechanisms of nitric oxide regulation. Potential relevance to cardiovascular disease. Circ Res. 1993;73:217-22.

Djuric D, Vusanovic A, Jakovljevic V. The effects of folic acid and nitric oxide synthase inhibition on coronary flow and oxidative stress markers in isolated rat heart. Mol Cell Biochem 2007;300: 177-183.

Donald M. Bers. Cardiac excitation contraction coupling. Nature 2002; 415, 198-205.

Donato, M., D'Annunzio, V., Buchholz, B., Miksztowicz, V, Carrion, C.L., Valdez, L.B., et al.. Role of matrix metalloproteinase-2 in the cardioprotective effect of ischaemic postconditioning. Exp. Physiol. 2010; 95(2): 274-281.

Fabiato A, Fabiato F. Calcium release from the sarcoplasmic reticulum. Circ Res 1977;40(2):119-29.

Fabiato A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum.Am J Physiol. 1983; 245(1):C1-14.

Felbor, U., Dreier, L., Bryant, R.A., Ploegh, H.L., Olsen, B.R., and Mothes, W. Secreted cathepsin L generates endostatin from collagen XVIII. EMBO. J. 2000;19(6): 1187-94.

Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, Yee SP. Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. Circulation. 2002;106(7):873-9.

Fenton H. Oxidation of tartaric acid in the presence of iron. J Chem Soc. 1894:899.

Gao WD, Atar D, Liu Y, Perez NG, Murphy AM, Marban E. Role of troponin I proteolysis in the pathogenesis of stunned myocardium. Circ Res 1997;80: 393-399.

Goette A, Arndt M, Rocken C, Staack T, Bechtloff R, Reinhold D, Huth C, Ansorge S, Klein HU, Lendeckel U Calpains and cytokines in fibrillating human atria. Am J Physiol Heart Circ Physiol 2002 ;283(1):H264-72.

Gao WD, Liu Y, Mellgren R, Marban E. Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium. A consequence of Ca^{2+}-dependent proteolysis? Circ Res. 1996;78(3):455-65.

Garcia-Villalon AL, Amezquita YM, Monge L, Fernandez N, Climent B, Sanchez A, Dieguez G. Effect of ischemia duration and nitric oxide on coronary vasoconstriction after ischemia-reperfusion. Eur J Pharmacol 2005;509: 165-170.

Goldberg AL: Nobel committee tags ubiquitin for distinction. Neuron 2005; 45: 339–344.

Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. Physiol Rev.2003;83(3):731-801.

Gorza L, Menabo R, Vitadello M, Bergamini CM, Di Lisa F. Cardiomyocyte troponin T immunoreactivity is modified by cross-linking resulting from intracellular calcium overload. Circulation. 1996;93(10):1896-904.

Grinwald PM. Calcium uptake during post-ischemic reperfusion in the isolated rat heart: influence of extracellular sodium. J Mol Cell Cardiol. 1982;14:359-65.

Grinwald PM. Sodium pump failure in hypoxia and reoxygenation. J Mol Cell Cardiol. 1992;24:1393-8.

Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res. 1994;74:1141-8.

Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res. 2000; 86:494-501.

Haber F and Weiss J. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. London, Ser. A. 1934; 147, 332–351.

Haunstetter A, Izumo S. Apoptosis: basic mechanisms and implications for cardiovascular disease. Circ Res. 1998; 82(11): 1111-29.

Hearse DJ, Bolli R. Reperfusion induced injury: manifestations, mechanisms, and clinical relevance. Cardiovasc Res 1992; 26: 101-108.

Hearse DJ, Sutherland FJ. Experimental models for the study of cardiovascular function and disease. Pharmacol Res 2000; 41: 597-603.

Hong D, Jaron D, Buerk DG, Barbee KA. Heterogeneous response of microvascular endothelial cells to shear stress. Am J Physiol Heart Circ Physiol 2006, 290: H2498-H2508.

Hussain AM, Zhang QX, Murray AG. Endothelial cell calpain activity facilitates lymphocyte diapedesis. Am J Transplant 2005; 5: 2640-2648.

Huang Y, Wang KKW: The calpain family and human disease. Trend Mol Med 2001; 7, 355 –362.

Inserte, J., Garcia-Dorado, D., Hernando, V., and Soler-Soler J. Calpain-mediated impairment of Na+-K+ ATPase activity during early reperfusion contributes to cell death after myocardial ischemia. Circ. Res. 2005; 97(5): 465-473.

Inserte, J., Garcia-Dorado, D., Hernando, V., Barba, I., and Soler-Soler, J. Ischemic preconditioning prevents calpain-mediated impairment of Na+/K+-ATPase activity during early reperfusion. Cardiovasc. Res. 2006; 70:364-373.

James TN, St Martin E, Willis PW 3rd, Lohr TO. Apoptosis as a possible cause of gradual development of complete heart block and fatal arrhythmias associated with absence of the AV node, sinus node, and internodal pathways. Circulation. 1996; 93(7): 1424-38.

Jennings, R.B., and Reimer, K.A. The cell biology of acute myocardial ischemia. Ann. Rev. Med. 1991; 42: 225-246.

Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol 2006; 40: 16-23.

Kameyama M, Kameyama A, Takano E, Maki M. Run-down of the cardiac L-type Ca2+ channel: partial restoration of channel activity in cell-free patches by calpastatin. Pflugers Arch. 1998;435(3):344-9.

Kaneko, M., Beamish, R.E., and Dhalla, N.S. Depression of heart sarcolemmal Ca2+-pump activity by oxygen free radicals. Am. J. Heart Circ. Physiol. 1989; 256: H368-374.

Kaneko, M., Elimban, V., and Dhalla, N.S. Mechanism for depression of heart sarcolemmal Ca2+-pump by oxygen-free radicals. Am. J. Physiol. Heart Circ. Physiol. 1989b; 257: H804-H811.

Kato K, Chapman DC, Rupp H, Lukas A, Dhalla NS. Alterations of heart function and Na^{+}-K^{+}-ATPase activity by etomoxir in diabetic rats. J Appl Physiol 1999; 86: 812-818.

Kincaid K, Ward M, Nair U, Hainsworth R, Drinkhill M. The coronary baroreflex in humans. J Extra Corpor Technol 2005; 37: 306-310.

Kim, M.S., and Akera, T. O_2 free radicals: cause of ischemia–reperfusion injury to cardiac Na+–K+-ATPase. Am. J. Physiol. 1987; 252: H252–57.

Kandasamy, A.D., Chow, A.K., Ali, M.A., and Schulz, R. Matrix metalloproteinase-2 and myocardial oxidative stress injury: beyond the matrix. Cardiovasc. Res. 2010; 85(3): 413-23.

Katus A.H, Remppis A, Kuebler W et al. Diagnostic efficency of troponin T measurements in acute myocardial infarction. Circulation 1991;83:902-12.

Katunuma N, Matsui A, Inubushi T, Murata E, Kakegawa H, Ohba Y, Turk D, Turk V, Tada Y, Asao T. Structure-based development of pyridoxal propionate derivatives as specific inhibitors of cathepsin K in vitro and in vivo. Biochem Biophys Res Commun 2000; 267(3):850-4.

Kaufmann SH, Hengartner MO. Programmed cell death: alive and well in the new millennium. Trends Cell Biol. 2001;11(12): 526-34.

Keenan NL, Shaw KM; Centers for Disease Control and Prevention (CDC). Coronary heart disease and stroke deaths - United States, 2006. MMWR Surveill Summ. 2011;60 Suppl:62-6.

Ken-ichi Yoshida. Myocardial ischemia-reperfusion injury and proteolysis of fodrin, ankyrin, and calpastatin.Methods Mol Biol. 2000;144:267-75

Khalil, P.N., Neuhof, C., Huss, R., Pollhammer, M., Khalil, M.N. Neuhof, H., et al. calpain inhibition reduces infarct size and improves global hemodynamics and left ventricular contractility in porcine myocardial ischemia/reperfusion model. Europ. J. Pharmacol. 2005;528(1-3): 124-131.

Kim KS, Takeda K, Sethi R, Pracyk JB, Tanaka K, Zhou YF, Yu ZX, Ferrans VJ, Bruder JT, Kovesdi I, Irani K, Goldschmidt-Clermont P, Finkel T. Protection from reoxygenation injury by inhibition of rac1. J Clin Invest. 1998;101:1821-6.

Kirshenbaum, L. A., de Moissac, D. The bcl-2 gene product prevents programmed cell death of ventricular myocytes. Circulation. 1997; 96: 1580-85.

Kimura Y, Kurzydlowski K, Tada M, MacLennan DH. Phospholamban inhibitory function is activated by depolymerization. J Biol Chem. 1997; 272(24):15061-4.

Kirschke H, Langer J, Riemann S, Weideranders B, Ansorge S and Bobley P. Lysosomal cysteine proteinases. In: Protein degradation in health and disease, Ciba foundation 75, Excerpta Medica, Elsevier, North Holland 1980; 15 – 35.

Kitanaka, C., Kuchino, Y. Caspase-independent programmed cell death with necrotic morphology. Cell Death Differ. 1999; 6, 508-515.

Knaapen, M. W., Davies, M. J., De Bie, M., Haven, A. J., Martinet, W., Kockx, M. M. Apoptotic versus autophagic cell death in heart failure. Cardiovasc. Res. 2001;51, 304-312.

Kranias EG. Regulation of calcium transport by protein phosphatase activity associated with cardiac sarcoplasmic reticulum. J Biol Chem. 1985; 260(20):11006-10

Kranias EG and Di Salvo J. A phospholamban protein phosphatase activity associated with cardiac sarcoplasmic reticulum. J Biol Chem. 1986; 261(22):10029-32.

Komuro I, Kurabayashi M, Shibazaki Y, Takaku F, Yazaki Y. Molecular cloning and characterization of a Ca^{2+} + Mg^{2+}-dependent adenosine triphosphatase from rat

cardiac sarcoplasmic reticulum. Regulation of its expression by pressure overload and developmental stage. J Clin Invest. 1989; 83(4):1102-8

Kuppusamy P, Zweier JL. Characterization of free radical generation by xanthine oxidase. Evidence for hydroxyl radical generation J Biol Chem 1989; 264:9880-4.

Kusuoka H, Porterfield JK, Weisman HF, Weisfeldt ML, Marban E . Pathophysiology and pathogenesis of stunned myocardium. Depressed Ca^{2+} activation of contraction as a consequence of reperfusion-induced cellular calcium overload in ferret hearts.J Clin Invest. 1987; 79(3):950-61.

Kwan HY, Leung PC, Huang Y, Yao X. Depletion of intracellular Ca^{2+} stores sensitizes the flow-induced Ca^{2+} influx in rat endothelial cells. Circ Res 2003; 92: 286-292.

Lalu, M.M., Pasini, E., Schulze, C.J., Ferrari-Vivaldi, M., Ferrari-Vivaldi, G., Bachetti, T., et al. Ischaemia-reperfusion injury activates matrix metalloproteinases in the human heart. Eur. Heart J. 2005; 26(1): 27-35.

Le Peuch CJ, Haiech J, Demaille JG. Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cyclic adenosine monophosphate dependent and calcium--calmodulin-dependent phosphorylations. Biochemistry. 1979;18(23):5150

Lin S, Fagan KA, Li KX, Shaul PW, Cooper DMF, Rodman DM. Sustained endothelial nitric-oxide synthase activation requires capacitative Ca^{2+} entry. J Biol Chem 2000; 275: 17979-85.

Li, P.A., Howlett, W., He, Q.P., Miyashita, H., Siddiqui, M., and Shuaib A. Postischemic treatment with calpain inhibitor MDL 28170 ameliorates brain damage in gerbil model of global ischemia. Neurosci. Lett. 1998; 247(1): 17-20.

Li, C. Y., Lee, J. S., Ko, Y. G., Kim, J. I., Seo, J. S. Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. J. Biol. Chem. 2000; 275: 25665-71.

Lin KT, Xue JY, Sun FF, Wong PY. Reactive oxygen species participate in peroxynitrite-induced apoptosis in HL-60 cells. Biochem Biophys Res Commun. 1997;230:115-9.

Liu P, Hock CE, Nagele R, Wong PY. Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats. Am J Physiol. 1997; 272:H2327-36.

Lloyd-Jones D, Adams RJ, Brown TM, et al. American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Executive summary: heart disease and stroke statistics—2010 update: a report from the American Heart Association. Circulation. 2010; 121(7):948-54. Erratum in: Circulation. 2010 30; 121(12):e259.

Lompre AM, Anger M, Levitsky D. Sarco(endo)plasmic reticulum calcium pumps in the cardiovascular system: function and gene expression. J Mol Cell Cardiol. 1994; 26(9):1109-21.

Lytton J, Zarain-Herzberg A, Periasamy M, MacLennan DH. Molecular cloning of the mammalian smooth muscle sarco(endo)plasmic reticulum Ca^{2+} -ATPase. J Biol Chem. 1989; 264(12):7059-65.

Maddika, S.,Elimban, V., Chapman D., and Dhalla, N.S.. Role of oxidative stress in ischemia-reperfusion-induced alterations in myofibrillar ATPase activities and gene expression in the heart. Can. J. Physiol. Pharmacol. 2009; 87: 120-129.

McDonough, A.A., Geering, K., and Farley, R.A. The sodium pump needs its beta subunit. FASEB J. 1990; 4: 1598–1605.

Matsumura Y, Kusuoka H, Inoue M, Hori M, Kamada T. Protective effect of the protease inhibitor leupeptin against myocardial stunning. J Cardiovasc Pharmacol. 1993; 22(1): 135-42.

Matsumura Y, Saeki E, Inoue M, Hori M, Kamada T, Kusuoka H. Inhomogeneous disappearance of myofilament-related cytoskeletal proteins in stunned myocardium of guinea pig.Circ Res. 1996; 79(3):447-54.

Ma XL, Lopez BL, Liu GL, Christopher TA, Ischiropoulos H. Peroxynitrite aggravates myocardial reperfusion injury in the isolated perfused rat heart. Cardiovasc Res. 1997; 36:195-204.

MacLennan DH, Toyofuku T, Kimura Y. Sites of regulatory interaction between calcium ATPases and phospholamban. Basic Res Cardiol. 1997; 92 Suppl 1:11-5.

MacLennan DH, Wong PT. Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. Proc Natl Acad Sci U S A. 1971; 68(6):1231-5

Marban E, Kitakaze M, Koretsune Y, Yue DT, Chacko VP, Pike MM. Quantification of $[Ca^{2+}]i$ in perfused hearts. Critical evaluation of the 5F-BAPTA and nuclear magnetic resonance method as applied to the study of ischemia and reperfusion. Circ Res. 1990; 66:1255-67.

Marks, A. R. Intracellular calcium-release channels: regulators of cell life and death. Am J Physiol. 1997;272:H597-605.

Marks AR, Tempst P, Hwang KS, Taubman MB, Inui M, Chadwick C, Fleischer S, Nadal-Ginard B. Molecular cloning and characterization of the ryanodine receptor/junctional channel complex cDNA from skeletal muscle. Proc Natl Acad Sci U S A. 1987; 86(22):8683-37.

Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC. Mitochondrial disease in superoxide dismutase2 mutant mice. Proc. Natl. Acad Sci USA. 1999; 96:846-51.

Melov S, Schneider JA, Day BJ, Hinerfeld D, Coskun P, Mirra SS, Crapo JD, Wallace DC. A novel neurological phenotype in mice lacking mitochondrial manganese sueroxide dismutase. Natl Genet. 1998; 18:159-163.

Mitch WE, Goldberg AL: Mechanisms of muscle wasting: The role of the ubiquitin-proteasome system. N Engl J Med 1996; 335: 1897–1905.

Mitchell RD, Simmerman HK, Jones LR. Ca^{2+} binding effects on protein conformation and protein interactions of canine cardiac calsequestrin. J Biol Chem. 1988; 263(3):1376-81.

Müller, A.L., and Dhalla, N.S. Role of various proteases in cardiac remodeling and progression of heart failure. Heart Fail. Rev. 2011; DOI: 10.1007/s10741-011-9269-8.

Nangle MR, Cotter MA, Cameron NE. The calpain inhibitor, A-705253, corrects penile nitrergic nerve dysfunction in diabetic mice. Eur J Pharmacol 2006; 538: 148-153.

Negretti N, O'Neill SC, Eisner DA. The relative contributions of different intracellular and sarcolemmal systems to relaxation in rat ventricular myocytes. Cardiovasc Res. 1993; 27(10):1826-30

Nicholson DW, Thornberry NA. Caspases: killer proteases. Trends Biochem Sci. 1997; 22: 299–306.

Ostadal P, Elmoselhi AB, Zdobnicka I, Lukas A, Chapman D and Dhalla NS. Ischemia-reperfusion alters gene expression of Na^+-K^+-ATPase isoforms in rat heart. Biochem Biophys Res Commun 2003; 306: 457-62.

Ostadal P, Elmoselhi AB, Zdobnicka I, Lukas A, Elimban V, Dhalla NS. Role of oxidative stress in ischemia-reperfusion-induced changes in Na^+-K^+-ATPase isoform expression in rat heart. Antioxid Redox Signal 2004; 6: 914-23.

Pagano PJ, Ito Y, Tornheim K, Gallop PM, Tauber AI, Cohen RA. An NADPH oxidase superoxide-generating system in the rabbit aorta. Am J Physiol. 1995; 268:H2274-80.

Pedrozo Z., Sanchez, G., Torrealba N., Valenzuela, R., Fernandez, C., Hildago, C., et al. Calpains and proteosomes mediate degradation of ryanodine receptors in a model of cardiac ischemic reperfusion. Biochem. Biophys. Acta. 2011; 1802(3): 356-62.

Piper HM, Meuter K Schafer C. Cellular mechanisms of ischemia-reperfusion injury. Ann Thorac Surg 2003; 75: S644–48.

Pitts, BJ. Stoichiometry of sodium-calcium exchange in cardiac sarcolemmal vesicles. Coupling to the sodium pump. J Biol Chem 1979; 245: 6232-35.

Plank MJ, David T. The role of endothelial calcium and nitric oxide in the localisation of atherosclerosis. Math Biosci 2007; 207: 26-39.

Pohl T, Seiler C, Billinger M, Herren E, Wustmann K, Mehta H, Windecker S, Eberli FR, Meier B. Frequency distribution of collateral flow and factors influencing collateral channel development. Functional collateral channel measurement in 450 patients with coronary artery disease. J Am Coll Cardiol 2001; 38: 1872-78.

Pontremoli, S., Viotti, P.L., Michetti, M., Salamino, F., Sparatore, B., and Melloni, E. Modulation of inhibitory efficiency of rat skeletal muscle calpastatin by phosphorylation. Biochem. Biophys. Res. Commun. 1992; 187: 751–759.

Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys. 1991; 288:481-87.

Raha S and Robinson BH. Mitochondria, oxygen free radicals, disease and aging. Trends Biochem Sci. 2000; 25: 502-508.

Rardon DP, Cefali DC, Mitchell RD, Seiler SM, Hathaway DR, Jones LR. Digestion of cardiac and skeletal muscle junctional sarcoplasmic reticulum vesicles with calpain II. Effects on the Ca^{2+} release channel. Circ Res. 1990; 67(1):84-96.

Roghi C, Jones L, Gratian M, English WR, Murphy G. Golgi reassembly stacking protein 55 interacts with membrane-type (MT) 1-matrix metalloprotease (MMP) and furin

and plays a role in the activation of the MT1-MMP zymogen. FEBS J. 2010; 277(15):3158-75.

Rothenberg F, Fisher SA, Watanabe M. Sculpting the cardiac outflow tract. Birth Defects Res Part C Embryo Today. 2003; 69(1): 38-45.

Saido TC, Sorimachi H, Suzuki K: calpain: new perspectives in molecular diversity and physiological, pathological involvement. FASEB J 1994; 8, 814–22.

Saini HK and Dhalla NS. Defective calcium handling in cardiomyocytes isolated from hearts subjected to ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2005; 288(5): H2260-70.

Saini, H.K., Elimban, V., and Dhalla, N.S. Attenuation of extracellular ATP response in cardiomyocytes isolated from hearts subjected to ischemia-reperfusion. Am. J. Physiol. Heart Circ. Physiol. 2005; 289(2): H614-23.

Saini, H.K., Machackova, J., and Dhalla, N.S. Role of reactive oxygen species in ischemic preconditioning of subcellular organelles in the heart. Antioxid. Redox Signal. 2004; 6(2): 393-404.

Saini, H.K., and Dhalla, N.S.. Sarcolemmal cation channels and exchangers modify the increase in intracellular calcium in cardiomyocytes on inhibiting Na+-K+-ATPase. Am. J. Physiol. Heart Circ. Physiol. 2007; 293: H169-81.

Samanta K, Kar P, Chakraborti T, Chakraborti S. Calcium-dependent cleavage of the Na^{+}/Ca^{2+} exchanger by m-calpain in isolated endoplasmic reticulum. J Biochem. 2010;147(2):225-35.

Sawicki, G., Leon, H., Sawicka, J., Sariahmetogiu, M., Schulze, C.J., Scott, P.G., et al. Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury: a new intracellular target for matrix metalloproteinase-2. Circulation 2005; 112(4): 544-52.

Schram K, Ganguly R, No EK, Fang X, Thong FS, Sweeney G. Regulation of MT1-MMP and MMP-2 by leptin in cardiac fibroblasts involves Rho/ROCK- dependent actin cytoskeletal reorganization and leads to enhanced cell migration. Endocrinology. 2011;152(5):2037-47.

Searle, J., Kerr, J. F., Bishop, C. J. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol Ann. 1982; 17: 229-259.

Shah BR, Hux JE, Zinman B. Increasing rates of ischemic heart disease in the native population of Ontario, Canada. Arch Intern Med. 2000; 160(12): 1862-6.

Shen J, Luscinskas FW, Connolly A, Dewey CF, Gimbrone MA. Fluid shear stress modulates cytosolic free calcium in vascular endothelial cells. Am J Physiol Cell Physiol 1992; 262: C384-90.

Shimomura H, Terasaki F, Hayashi T, Kitaura Y, Isomura T, Suma H.. Autophagic degeneration as a possible mechanism of myocardial cell death in dilated cardiomyopathy. Japanese Circulation Journal 2001; 65(11): 965-68.

Shulz, R. Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches. Annu. Rev. Pharmacol. Toxicol. 2007; 47: 211-42.

Shulze, C.J., Wang, W., Saurez-Pinzon, W.L., Sawicka, J., Sawicka, G., and Shulz, R. Imbalance between tissue inhibitor of metalloproteinase-4 and matrix

metalloproteinases during acute myocardial ischemia-reperfusion injury. Circulation 2003; 107(19): 2487-92.

Simmerman HK, Jones LR Phospholamban: protein structure, mechanism of action, and role in cardiac function. Physiol Rev. 1998; 78(4):921-47.

Singh RB, Chohan PK, Dhalla NS, Netticadan T. The sarcoplasmic reticulum proteins are targets for calpain action in the ischemic-reperfused heart. J Mol Cell Cardiol 2004a; 37: 101-10.

Singh RB, Dandekar SP, Elimban V, Gupta SK, Dhalla NS. Role of proteases in the pathophysiology of cardiac disease. Mol Cell Biochem 2004b ; 263: 241-56.

Singh, R.B., Elimban, V., and Dhalla, N.S. Differences in ischemia-reperfusion-induced endothelial changes in hearts perfused at constant flow and constant pressure. J. Appl. Physiol. 2008; 105:1779-87.

Singh, R.B., and Dhalla, N.S. Ischemia reperfusion-induced changes in sarcolemmal Na+-K+ ATPase are due to the activation of calpain in the heart. Can. J. Physiol. Pharmacol. 2010; 88(3): 388-97.

Skrzypiec-Spring M, Grotthus B, Szelag A, Schulz R. Isolated heart perfusion according to Langendorff - still viable in the new millennium. J Pharmacol Toxicol Methods 2007; 55: 113-126.

Sorimachi, Y., Harada, K., Saido, T.C., Ono, T., Kawashima, S., and Yoshida, K. Downregulation of calpastatin in rat heart after brief ischemia and reperfusion. J. Biochem. 1997; 122(4):743-48.

Spanikova, A., Ivanova, M., Matejikova, J., Ravingerova, T., and Barancik, M. Influence of ischemia/reperfusion and modulation of P13K/Akt kinase pathway in matrix metalloproteinase-2 in rat hearts. Gen. Physiol. Biophys. 2010; 29(1): 31-40.

Spinale, F.G. Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function. Physiol. Rev. 2007; 87(4): 1285-42.

Spinale, F.G. Amplified bioactive signaling and proteolytic enzymes following ischemia reperfusion and aging: Remodeling pathways that are not like fine wine. Circulation 2010; 12(4): 322-24.

Stawowy P, Margeta C, Kallisch H, Seidah NG, Chrétien M, Fleck E, Graf K. Regulation of matrix metalloproteinase MT1-MMP/MMP-2 in cardiac fibroblasts by TGF-beta1 involves furin-convertase. Cardiovasc Res. 2004;63(1):87-97.

Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. J Biol Chem. 1995; 270(10):5331-8.

Sung, M.M., Shulz, C.G., Wang, W., Sawicki, G., Bautista-Lopez, N.L., and Shulz, R. Matrix metalloproteinase-2 degrades the cytoskeletal protein alpha-actinin in peroxynitrite mediated myocardial injury. J. Mol. Cell. Cardiol. 2007; 43(4): 429-36.

Sorimachi H, Ishiura S, Suzuki K: Structure and physiological function of calpains. Biochem J 1997; 328, 721 –32

Sorimachi H, Suzuki K: The structure of calpain. J Biochem 2001; 129, 653 –664.

Stalker TJ, Gong Y, Scalia R. The calcium-dependent protease calpain causes endothelial dysfunction in type 2 diabetes. Diabetes 2005; 54: 1132-1140.

Stamler J. The marked decline in coronary heart disease mortality rates in the United States, 1968-1981; summary of findings and possible explanations. Cardiology. 1985; 72(1-2):11-22.

Strand BH, Tverdal A. Can cardiovascular risk factors and lifestyle explain the educational inequalities in mortality from ischaemic heart disease and from other heart diseases? 26 year follow up of 50,000 Norwegian men and women. J Epidemiol Community Health. 2004 ; 58(8):705-9.

Sugioka K, Nakano M, Totsune-Nakano H, Minakami H, Tero-Kubota S, Ikegami Y. Mechanism of O^2- generation in reduction and oxidation cycle of ubiquinones in a model of mitochondrial electron transport system. Biochem Biophys Acta. 1988; 936: 377-85.

Suzuki YJ, Ford GD. Redox regulation of signal transduction in cardiac and smooth muscle. J Mol Cell Cardiol. 1999; 31:345-53.

Suzuki T and Wang JH. Stimulation of bovine cardiac sarcoplasmic reticulum Ca^{2+} pump and blocking of phospholamban phosphorylation and dephosphorylation by a phospholamban monoclonal antibody.J Biol Chem. 1986; 261(15):7018-23.

Suzuki S, Kaneko K, Chapman DC, Dhalla NS. Alterations in cardiac contractile proteins due to oxygen free radicals. Biochim Biophys Acta 1991; 1074: 95-100.

Tagliarino, C., Pink, J.J., Reinicke, K.E., Simmers, S.M., Wuerzberger-Davis S.M., and Boothman, D.A. Mu-calpain activation in beta-lapachone-mediated apoptosis. Cancer Biol. Ther. 2003; 2:141-52.

Takeda S, Mochizuki S, Saini HK, Elimban V and Dhalla NS. Modification of alterations in cardiac function and sarcoplasmic reticulum by vanadate in ischemic-reperfused rat hearts. J Appl Physiol 2005; 99: 999-1005.

Takeda S, Prajapati D, Mochizuki S, Elimban V, Dhalla NS. Attenuation of ischemia-reperfusion induced changes in cardiac performance and sarcoplasmic reticulum function by vanadate. Exp Clin Cardiol 2003; 8: 134–138.

Takasago T, Imagawa T, Furukawa K, Ogurusu T, Shigekawa M. Regulation of the cardiac ryanodine receptor by protein kinase-dependent phosphorylation. J Biochem (Tokyo). 1991; 109(1):163-70.

Talosi L, Edes I, Kranias EG. Intracellular mechanisms mediating reversal of beta-adrenergic stimulation in intact beating hearts. Am J Physiol. 1993; 264(3 Pt 2):H791-97.

Tani, M., and Neely, J.R. Role of intracellular Na+ in Ca2+ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H^+–Na^+ and Na^+-Ca^{2+} exchange. Circ. Res. 1989; 65: 1045–56.

Tani, M. Mechanisms of Ca2+ overload in reperfused ischemic myocardium. Annu. Rev. Physiol. 1990; 52:543-59.

Temsah, R.M., Netticadan, T., Chapman, D., Takeda, S., Mochizuki, S., and Dhalla, N.S. Alterations in sarcoplasmic reticulum function and gene expression in ischemic-

reperfused rat heart. Am. J. Physiol. Heart Circ. Physiol. 1999; 277(2 Pt 2): H584-94.

Temsah, R.M., Netticadan, T., Kawabata, K., and Dhalla, N.S. Lack of both oxygen and glucose contributes to I/R-induced changes in cardiac SR function. Am. J. Physiol. Cell. Physiol. 2002; 283(4): C1306-12.

Tidball JG, Spencer MJ. Calpains and muscular dystrophies. Int J Biochem Cell Biol. 2000; 32(1):1-5.

Toft UN, Kristoffersen LH, Aadahl M, von Huth Smith L, Pisinger C, Jørgensen T. Diet and exercise intervention in a general population--mediators of participation and adherence: the Inter99 study. Eur J Public Health. 2007;17(5):455-63.

Tonstad S, Joakimsen O, Leren TP, Ose L. Does maternal or paternal heredity affect carotid atherosclerosis in children with familial hypercholesterolaemia? Acta Paediatr. 2000; 89(12):1490-92.

Toyofuku T, Curotto Kurzydlowski K, Narayanan N, MacLennan DH. Identification of Ser38 as the site in cardiac sarcoplasmic reticulum Ca^{2+}-ATPase that is phosphorylated by Ca^{2+} calmodulin-dependent protein kinase. J Biol Chem. 1994; 269(42):26492-96.

Tran QK, Ohashi K, Watanabe H. Calcium signalling in endothelial cells, Cardiovasc Res 2000; 48: 13-22.

Tsuji T, Ohga Y, Yoshikawa Y, Sakata S, Abe T, Tabayashi N, Kobayashi S, Kohzuki H, Yoshida KI, Suga H, Kitamura S, Taniguchi S, Takaki M. Rat cardiac contractile

dysfunction induced by Ca2+ overload: possible link to the proteolysis of alpha-fodrin. Am J Physiol Heart Circ Physiol. 2001; 281(3):H1286-94.

Urthaler, F., Wolkowicz, P.E., Digerness, S.B., Harris, K.D., and Walker, A.A. MDL-28170, a membrane permeant calpain inhibitor, attenuates stunning and PKC proteolysis in reperfused ferret hearts. Cardiovasc. Res. 1997; 35(1): 60-67.

Van der Vliet A, Smith D, O'Neill CA, Kaur H, Darley-Usmar V, Cross CE, Halliwell B. Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion. Biochem J. 1994; 303:295-301.

Van Hart HE, Birkedal-Hansen H. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci USA. 1990; 87: 5578-82.

Viappiani S, Nicolescu AC, Holt A, Sawicki G, Crawford BD, León H, van Mulligen T, Schulz R.. activation and modulation of 72 kDa matrix metalloproteinase – 2 by peroxynitrite and glutathione. Biochem Pharmacol. 2009; 77: 826-34.

Waldron GJ, Ding H, Lovren F, Kubes P, Triggle CR. Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase. Br J Pharmacol 1999; 128: 653-58.

Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., Reed, J. C. Ca2+ induced apoptosis through calcineurin dephosphorylation of BAD. Science. 1999, 284, 339-43.

Wang W, Viappiani S, Sawicka J, Schulz R. Inhibition of endogenous nitric oxide in the heart enhances matrix metalloproteinase-2 release. Brit J Pharmacol 2005; 145: 43-49.

Wang, W., Schulze, C.J., Suarez-Pinzon, W.L., Dyck, J.R., Sawicki, G., and Schulz R. 2002. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. Circulation, 106(12): 1543-1549.

Wendt A, Thompson VF, Goll DE. Interaction of calpastatin with calpain: a review. Biol Chem. 2004; 385(6):465-72.

Wolska BM, Lewartowski B The role of sarcoplasmic reticulum and Na^{+}- Ca^{2+} exchange in the Ca^{2+} extrusion from the resting myocytes of guinea-pig heart: comparison with rat. J Mol Cell Cardiol. 1993; 25(1):75-91

Wu, H.Y., Tomizawa, K., and Matsui, H. calpain-calcineurin signaling in the pathogenesis of calcium-dependent disorder. Acta Med. Okayama. 2007; 61:123-137.

Xu A, Hawkins C, Narayanan N. Phosphorylation and activation of the Ca^{2+} pumping ATPase of cardiac sarcoplasmic reticulum by Ca2+/calmodulin-dependent protein kinase.J Biol Chem. 1993; 268(12):8394-97.

Yasmin W, Strynadka KD, Schulz R.Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. Cardiovasc Res. 1997; 33:422-32.

Yano K, Zarain-Herzberg A. Sarcoplasmic reticulum calsequestrins: structural and functional properties. Mol Cell Biochem. 1994; 135(1):61-70.

Yoshida K, Sorimachi Y, Fujiwara M, Hironaka K. calpain is implicated in rat myocardial injury after ischemia or reperfusion. Jpn Circ J 1995, 59: 40-48.

Yoshida K, Yamasaki Y, Kawashima S. calpain activity alters in rat myocardial subfractions after ischemia or reperfusion. Biochim Biophys Acta. 1993;1182(2):215-20.

Yoshida Y, Shiga T, Imai S. Degradation of sarcoplasmic reticulum calcium-pumping ATPase in ischemic-reperfused myocardium: role of calcium-activated neutral protease. Basic Res Cardiol 1990; 85: 495-507.

Yoshida K, Inui M, Harada K, Saido TC, Sorimachi Y, Ishihara T, Kawashima S, Sobue K. Reperfusion of rat heart after brief ischemia induces proteolysis of calspectin (nonerythroid spectrin or fodrin) by calpain. Circ Res. 1995; 77(3): 603-10.

Yoshida K, Harada K. Proteolysis of erythrocyte-type and brain-type ankyrins in rat heart after postischemic reperfusion. J Biochem. 1997;122(2):279-85.

Yoshikawa F, Iwasaki H, Michikawa T, Furuichi T, Mikoshiba K.. Cooperative formation of the ligand-binding site of the inositol 1,4, 5-trisphosphate receptor by two separable domains. J Biol Chem 1999; 274: 328-34.

Zahler R., Brines, M., Kashgarian, M., Benz, E.J. Jr., and Gilmore-Hebert, M. The cardiac conduction system in the rat expresses the alpha 2 and alpha 3 isoforms of the Na+ K+-ATPase. Proc. Natl. Acad. Sci. USA 1992, 89: 99–103.

Zarain-Herzberg A, MacLennan DH, Periasamy M. Characterization of rabbit cardiac sarco(endo)plasmic reticulum Ca2(+)-ATPase gene. J Biol Chem. 1990; 265(8):4670-77.

Zatz M, Starling A. Calpains and disease. N Engl J Med. 2005; 352(23):2413-23.

Zhang L, Kelley J, Schmeisser G, Kobayashi YM, Jones LR. Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane. J Biol Chem. 1997; 272(37): 23389-97.

Zimmerman ANE, Daems W, Hulsmann WC, Snijder J, Wisse E, Durrier D. Morphological changes of heart muscle caused by successive perfusion with calcium-free and calcium-containing solutions (calcium paradox). Cardiovasc Res 1967;1:201–9.

Zolotarjova N, Ho C, Mellgren RL, Askari A, Huang WH. Different sensitivities of native and oxidized forms of Na+/K(+)-ATPase to intracellular proteinases. Biochim Biophys Acta 1994; 1192: 125-31.

Zweier JL, Kuppusamy P, Williams R, Rayburn BK, Smith D, Weisfeldt ML, Flaherty JT. Measurement and characterization of postischemic free radical generation in the isolated perfused heart. J Biol Chem. 1989; 264:18890-95.