ATTENUATION OF ABNORMALITIES IN SARCOPLASMIC RETICULUM OF THE ISCHEMIC REPERFUSED HEART BY LEUPEPTIN

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Raja Balraj Singh

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

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DEDICATED TO

The people who keep me going

Punam, Yuvraj, Papa and Mama

who were always there for me

when I needed them

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Abstract

The high incidence of morbidity and mortality associated with acute myocardial ischemia (AMI) led to substantial research for ways to salvage the dying myocardium. Early restoration of flow to the dying myocardium by different interventional therapies has significantly reduced the mortality associated with acute coronary syndrome (ACS) but could not improve morbidity. Earlier studies indicate post ischemic reperfusion and not ischemia alone, as the cause of myocardial dysfunction. Myocardial stunning, ventricular arrhythmias and cell death are prominent sequels of ischemia reperfusion (IR) injury and are postulated to result from calcium overload and free radical injury that are mutually nonexclusive. The exact mechanisms by which calcium overload and free radicals mediate myocardial injury are still speculative. A possible downstream mechanism hypothesized by which calcium overload causes myocardial dysfunction subsequent to IR is the activation of calcium dependent proteases like the cysteine protease calpain. Calpain has been implicated by various studies in degradation of intracellular cytoskeletal and contractile proteins like ankyrin, fodrin, troponin and myosin as a possible cause for IR induced cardiac dysfunction. We studied the effects of calpain on the sarcoplasmic reticulum dysfunction observed during IR as a possible mechanism for the depression observed in cardiac contractile function. A protease inhibitor, leupeptin, was used to inhibit calpain activity during IR and examine the

beneficial effects on cardiac contractility. Our results indicate that leupeptin attenuates SR dysfunction by reducing the degradation of various SR proteins involved in calcium homeostasis during contraction and relaxation thereby improving the cardiac function in IR. The calpain activity observed during IR was significantly higher than that observed with ischemia alone and leupeptin successfully attenuated the calpain activity during IR. Calpain mediated proteolytic damage to the SR proteins involved in calcium homeostasis may be another mechanism by which calcium overload causes cardiac dysfunction during IR.

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List of Abbreviations

ANOVA.....analysis of variance

ATP.....adenosine triphosphate

BSA.....bovine serum albumin

Ca^{2+}.....calcium

$[Ca^{2+}]_I$.....concentration of intracellular calcium

CANP.....calcium-activated neutral protease

CON.....control hearts

CQS.....Calsequestrin

+dP/dt.....rate of pressure development

-dP/dt .....rate of pressure decay

IR.....Ischemia Reperfusion

ECM.....extracellular matrix

H^+.....hydrogen ion / proton

IR+Rx .....ischemia-reperfusion hearts treated with leupeptin

K^+ .....potassium ion

KH..... .....Krebs Henseleit

LVDP.....left ventricular developed pressure

LVEDP .....left ventricular end diastolic pressure

Mg^{2+}.....magnesium

Na^+.....sodium

$[Na^+]_I$ .....concentration of intracellular sodium ion

Pi.....inorganic phosphate

PLB.....phospholamban

Rx.....hearts treated with leupeptin

ROS.....reactive oxygen species

RyR.....ryanodine receptor

SERCA2.....sarcoplasmic reticulum Ca^{2+} ATPase

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I. Introduction

Restoration of flow is critical to salvage the ischemic myocardium but reperfusion has been shown to depress the cardiac function, which recovers gradually over a period of time (1). The post ischemic cardiac depression associated with the restoration of flow has been termed as myocardial stunning (2) and is considered to be a complex syndrome rather than a single entity. Tennant and Wiggers (3) and later Jennings et al (4) demonstrated that the post ischemic dysfunction was a sequel to reperfusion. Myocardial stunning can be defined as the cardiac abnormality that persists in a previously ischemic myocardium on restoration of a normal or near normal blood flow and when no reversible damage has been done to the myocardium (5). It is clearly differentiated from myocardial hibernation, which is reflected by a persistent contractile dysfunction in a viable myocardial tissue with reduced coronary flow. Furthermore, hibernation is an adaptive condition of the myocardium as has been shown by Diamond et al (6) and popularized by Rahimtoola (7). Nevertheless, both share a common denominator of being reversible in nature. In fact both, hibernation and stunning are milder forms of myocardial infarction and it remains to be seen if they share any common mechanisms with infarction.

The exact mechanism of stunning is speculative though different hypotheses have been forwarded to explain the cardiac dysfunction and ventricular arrhythmias observed in IR. Two decades of research aimed at elucidating the cause of stunning has resulted in the emergence of the oxyradical and calcium overload hypotheses as

two significant pathogenic mechanisms that explain most (if not all) of the cardiac abnormalities observed in IR injury. It has become obvious that the two are not mutually exclusive. Steenbergen et al (8) showed that IR caused an increase in the cytosolic free calcium that was not immediately lethal but could activate degradative enzymes. These were later shown to be calcium activated neutral proteases (CANP) (9, 10). Similarly Ferrari et al (11) have shown that IR injury is associated with the generation of reactive oxygen species (ROS) and administeration of dimercapto-propanolol and other antioxidants could prevent this.

The genesis of Ca^{2+} overload can briefly be summarized as to begin with ischemia and is accentuated by reperfusion. Decreased tissue perfusion decreases the cytosolic pH due to ATP depletion and inorganic phosphate accumulation (12). Oxygen deficiency during ischemia shifts the metabolism from aerobic to anaerobic producing lactic acid that further accentuates the cytosolic acidosis. Fiolet et al (13) have shown that the mean free energy of ATP hydrolysis decreases with time as ischemia continues and this affects the transsarcolemmal ionic gradient especially the Na^{+}-K^{+} gradient. The Na^{+}-H^{+} exchanger (NHX) gets activated and removes H^{+} from the cytosol for Na+ ions thereby increasing the intracellular [Na^{+}]. The sodium calcium exchanger (NCX), in turn, gets activated albeit in the reverse mode and brings in Ca^{2+} for Na^{+} causing an increase in the intracellular Ca^{2+} (14, 15). The functioning of the membrane bound enzymes can also be impaired by the oxidation of the membrane phospholipids and

accumulation of fatty acid metabolites and incorporation of the long chain fatty acids in the membranes (16). Reperfusion washes out the H^+ and increases the pH causing a reversal of the acidosis (17). The NHX is activated upon reperfusion (18) resulting in an increase in $[Na^+]i$, leading to subsequent activation of the NCX (19, 15) which further increases $[Ca^{2+}]i$. The Ca^{2+} overload upon reperfusion is also attributed in part to the inability of intracellular organelles to maintain Ca^{2+} homeostasis (20.). The genesis of the Ca^{2+} overload is discussed in detail elsewhere.

The mechanisms by which IR induced Ca^{2+} overload is hypothesized to mediate cardiac dysfunction includes activation of Ca^{2+}- dependent proteases resulting in proteolytic damage to important proteins involved in cardiac contractility, phospholipases and altered sensitivity of the contractile myofilaments to Ca^{2+}. Most sequels of Ca^{2+} overload seem to narrow down to the activation of the Ca^{2+} dependent proteases especially calpain, which has been implicated in the proteolytic degradation/modification of various cardiac proteins. Some of the important proteins degraded/modulated by calpains are the cytoskeletal proteins like fodrin (21), desmin and α-actinin (22) responsible for cellular integrity and contractile proteins (Figure. 1) such as troponin T and I (23). Calpain may also target some of the proteins involved in Ca^{2+} cycling such as the L-type Ca^{2+} channels (24), Ryanodine receptor (25) or the Ca^{2+} pump ATPase (26). Ca^{2+} overload has been shown to occur in patients of atrial fibrillation (AF) and proteolytic degradation / modulation of the atrial proteins by calpains has been

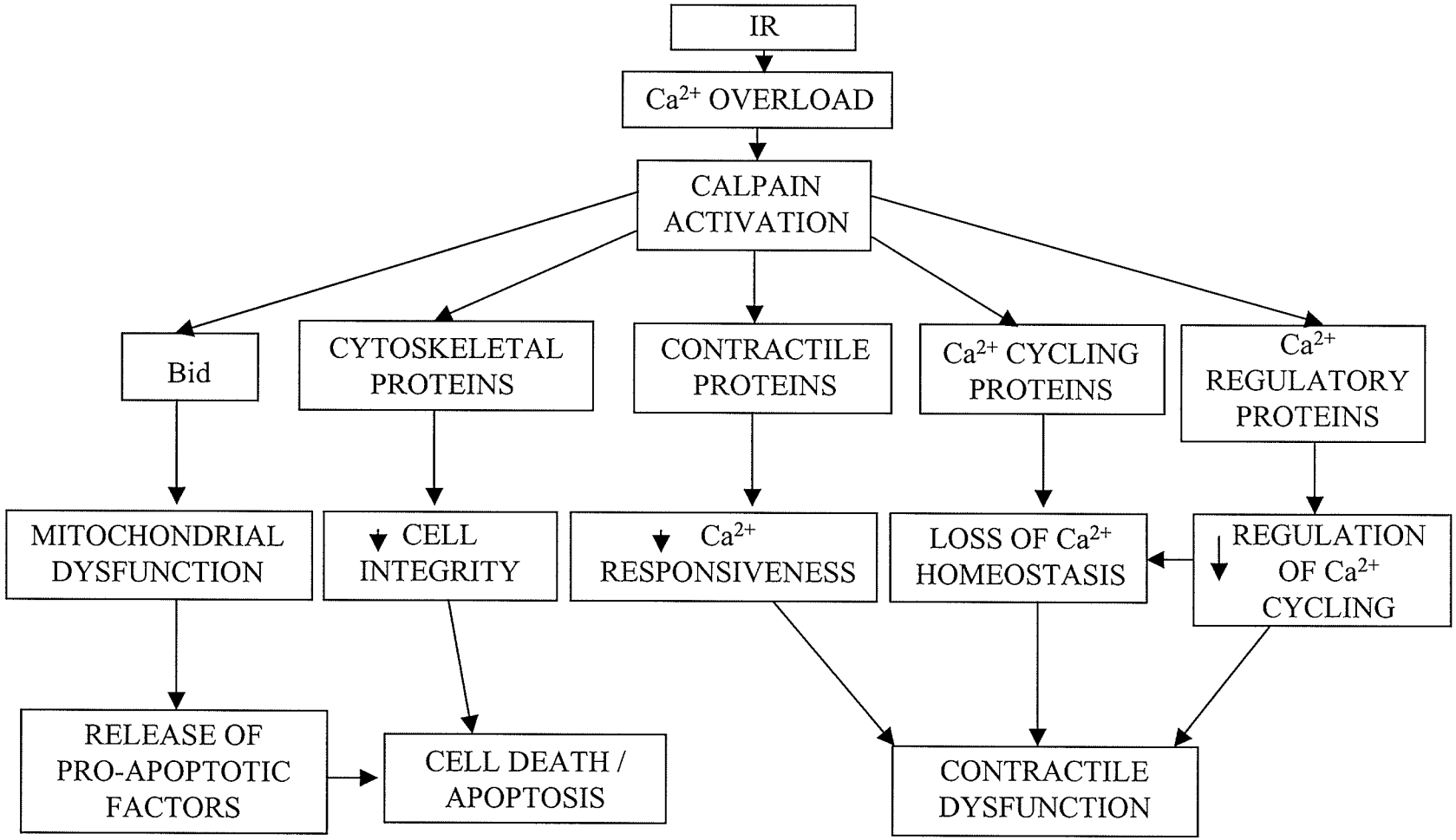


Figure. 1. Schematic representation of I/R induced Ca^{2+} overload causing activation of proteases (calpain) resulting in proteolysis of different cellular proteins. This leads to cardiac contractile dysfunction or cell death. (Bid = bis is death; pro-apoptotic BH3-only Bcl-2 family member).

implicated in the pathogenesis of AF. Alterations in the structural integrity of the sarcomere have been postulated to cause prolonged mechanical dysfunction in patients of AF even after successful cardio version of AF (27). Altered Ca^{2+} sensitivity of myofilaments is another phenomena observed by some studies in IR (28, 29). Goa et al (30) have shown that the Ca^{2+} responsiveness of skinned muscle fibres decreases when exposed to Calpain I by studying their steady state force Ca^{2+} relationship thus holding calpain activation during IR to be responsible for altering the myofilament structure and hence its response to Ca^{2+}.

Ventricular arrhythmia is the other pathological manifestation of IR that occurs within seconds of the reflow. It varies from ventricular premature beats to severe ventricular fibrillation and many mechanisms have been proposed to explain their origin. The oxidant stress as a consequence of excess production of free radicals causes damage to the lipid membranes and alters membrane permeability to different substances especially cations. Some studies have indicated that mechanisms responsible for free radical production increase the susceptibility of heart to arrhythmias (31) but this would vary from one animal model to another depending upon different factors such as host antioxidant defense mechanisms (32). Stimulation of the adrenergic receptors and increase in the cyclic adenosine monophosphate (cAMP) levels as well as abnormalities in cationic homeostasis are some of the other causes of IR induced arrhythmias forwarded. Avkiran et al (17) have shown that the acidosis caused by ischemia and subsequent reperfusion

induced washout of H^+ causes degeneration of ventricular tachyarythmias into fatal fibrillations that could be prevented by inhibiting this washout of H^+.

II. Review of Literature

A. Pathophysiology of Mocardial Ischemia Reperfusion Injury

(a). Oxidative stress

Zweier et al (33) and Bolli et al (34) have separately shown the production of reactive oxygen species (ROS) during IR and its association with contractile dysfunction. It has been shown that IR injury can be attenuated by administering mercaptopropionyl glycine (MPG) a minute before reperfusion, indicating that the ROS are produced during the first minute of reperfusion and peak between four to ten minutes (34); this finding was later confirmed directly by using the spin trap method (35). Under physiological conditions different scavenging mechanisms effectively clear the free radicals but under conditions of stress, the free radical production far outweighs the scavenging capabilities of the cardiomyocyte. ROS have been shown to attack the cellular membranes and contractile proteins, which could explain in part the contractile dysfunction seen with IR. Some of the sources for generation of ROS due to IR are discussed below.

1. Mitochondria

Mitochondria are the main source of free radicals being produced even under normal physiologic conditions (36). Mitochondria produce majority (80-90%) of the high-energy phosphates (adenosine tri phosphate; ATP) in mammalian tissues by the process of oxidative phosphorylation involving the respiratory chain. The mitochondrial respiratory chain (or the electron transport chain) consists of enzymes and coenzymes that convey high-energy electrons from the reduced

nicotinamide adenine dinucleotide ($NADH^+$) – reduced flavin adenine dinucleotide ($FADH^+$) pathway to molecular oxygen. Cytochrome oxidase reduces molecular oxygen (in complex IV) to form water thus coupling ATP production with the generation of water. The high- energy of electrons is converted to ATP and about 98 % of the electrons are tightly coupled to the ATP production while 1-2% results in the superoxide anion (O_2^-) formation (37). Some investigators have indicated this to happen at the level of the NADH-ubiquinone-complex or complex I (and some at complex II) (37), while others have indicated this to happen mainly at complex III (38-40). But these free radicals are neutralized by the natural defenses of the cell that include the mitochondrial (manganese), cytosolic (copper and zinc) superoxide dismutases (Mn SOD and CuZn SOD), glutathione peroxidase (GSH-PO) and phospholipid hydroperoxide glutathione peroxidase (36, 41). Various SOD enzymes scavenge O_2^- to form H_2O_2, which though not a free radical, acts as a second messenger and can regulate the activity of enzymes essential for calcium release, cell growth or apoptosis; these enzymes include phospholipase A_2, C and D, Src Kinase, p38 mitogen activated protein kinases (MAPK) c-Jun kinase (cJNK) and survival kinase (Akt/PKB) (42). H_2O_2 forms water and molecular oxygen under the enzymatic action of catalase and or glutathione peroxidases. It forms the highly reactive hydroxyl radical under conditions of stress via the Fenton (43) and or the Haber Weiss (44) pathways.

2. NAD(P)H oxidases

The neutrophil NAD(P)H oxidases are commonly known as the phagocytic oxidases while those found in the vasculature or the cardiac cells are called the non-phagocytic types. Virtually every cell type in the vasculature has been shown to produce ROS that regulates key cellular events like cell growth, acting as a signaling molecule (45). In the vascular cells and cardiomyocytes, NAD(P)H oxidases are the major source of superoxide production in response to various stimuli like cytokines and growth factors and are believed to be essential for normal physiological responses like growth, migration and matrix remodeling. NAD(P)H oxidases in the cardiovascular system are poorly understood though it is believed that nonphagocytic cell oxidases are similar to the nuetrophilic NAD(P)H oxidases. The phagocytic NAD(P)H oxidase is a membrane bound electron transport chain that mediates the flow of electrons from the substrates to molecular oxygen forming superoxide. For NADH (46,47) and NAD(P)H (48), there is controversy regarding the preferred substrate for superoxide generation. Though similar, the non-phagocytic oxidases produce superoxide chronically and are always in an active state compared to the phagocytic oxidases that are activated by the association of cytosolic agonists with cytochrome. The latter produce 10-100 times more superoxide than the former but in short bursts (42). The exact downstream effectors for the oxidase produced ROS are poorly understood but p38 MAPK, JNK and members of the stress activated kinase family have been postulated (46).

3. Superoxide and peroxynitrite

It has been suggested that about 0.4-2% of all the oxygen consumed during oxidative phosphorylation is converted to ROS (37,49). Though small, this is enough to cause severe pathologies in the living organisms if not detoxified by the antioxidant mechanisms. Melov et al (50,51) have showed that transgenic mice deficient in mitochondrial SOD have a maximum life span of 8 days only. Apart from the mitochondrial electron transport chain complexes, O_2^- can be formed by the phagocytic and nonphagocytic NAD(P)H oxidases (46-48), xanthine oxidase (XO) (52), cyclooxygenase (COX), lipooxygenase (LOX) and cytochrome P_{450} mono-oxygenase; these enzymatic reactions occur in cardiac myocytes, fibroblasts and endothelial cells during normoxia, anoxia and upon reoxygenation (53). Cardiac myocytes and fibroblasts differ in their response to superoxide anion. Fibroblasts are more resilient and proliferate secreting the transforming growth factor (TGF-β) (an important stimulus for fibrosis) as compared to the apoptosis and the activation of various kinases seen in cardiac myocytes (54). In an attempt to unify the oxyradical and calcium overload hypotheses, many authors have implicated O_2^- in damage to the sarcoplasmic reticulum (SR) by modifying the uptake and release of Ca^{2+} causing cytosolic Ca^{2+} overload (55,56). To this end, it was shown that ROS from the xanthine-XO system depressed the SR Ca^{2+} uptake in homogenates and isolated SR vesicles, respectively (57,58). This would imply an abnormal regulation of Ca^{2+} by SR Ca^{2+} ATPase (SERCA) or the SR ryanodine sensitive Ca^{2+} release channel (RyRC). Okabe et al (59,60) have reported that ROS

depress the SR Ca^{2+} uptake and enhances the release from the Ca^{2+} release channel. More recently, they have shown that O_2^- modulates the gating characteristics of the SR Ca^{2+} release channel or RyRC, and this is brought about by a degradation of calmodulin (the calcium binding protein) by the O_2^-. This modulation of the RyRC causes enhanced release and subsequent accumulation of Ca^{2+} in the cytosol (61). Recent work from our laboratory has shown preservation of SR function and gene expression for SR Ca^{2+} pump (SERCA2A) and ryanodine sensitive Ca^{2+} release channel (RyRC) (62) as well as preservation of SR protein phosphorylation (calcium\calmodulin dependent kinase, CaMK, and exogenous cAMP dependent protein kinase, PKA) (63) by treatment of IR hearts with SOD and catalase indicating a role for ROS in causing SR dysfunction.

Superoxide readily forms highly toxic **peroxynitrite** ($ONOO^-$) via a biradical reaction with nitric oxide (NO) (64). Peroxynitrite is not a free radical itself as it shares two unpaired electrons on superoxide and nitric oxide that form a new bond (65,66). NO is produced by the same cell types that produce the superoxide anion under conditions of stress (vascular endothelium, nutrophils and myocytes). Peroxynitrite has been suggested to have both cardiotoxic and cardioprotective effects. It induces cellular injury by causing lipid peroxidation (67), DNA fragmentation as seen in apoptosis (68), damage to proteins and lipids in the plasma (69), depletion of antioxidants like glutathione and cysteine as well as nitration of proteins causing organ dysfunction (70). Most of its effects are mediated by its intermediate $ONOOH^-$. It reacts with carbon dioxide (CO_2) to form

CO_3^- and NO_2^- to eventually form the nitronium ion (NO_3^-) that facilitates protein nitration and alters organ function (71). Absence of CO_2 would explain the inability of peroxynitrite to cause profound damage under in-vitro conditions. In IR, activated neutrophils and endothelium produce NO via the endothelial NO synthase (eNOS) and the inducible NOS (iNOS) (72). The release of NO coincides with the superoxide production during the early phase of reperfusion and thus facilitates peroxynitrite formation (73). Yasmin et al (74) have shown that peroxynitrite formation peaks in the early seconds of reperfusion and this coincides with the formation of both NO and superoxide. Soszynski et al (75) have shown membrane sulfhydryl group oxidation of the red blood cells without hemolysis at concentrations of 0.1-2mM while Kondo et al (76) have shown hemolysis to occur at lower concentrations of 100-500μM suggesting a possible dose dependent effect. Peroxynitrite aids white blood cells to kill infectious and cancerous cells due to its cytocidal oxidant properties (77) and it has also been shown to cause apoptosis in white cells themselves (68,77). Thiols (glutathione) neutralize the effects of peroxynitrites and the protection is believed to be due to depression in the degradation of the myocardial enzyme aconitase, which is a part of the mitochondrial respiratory chain (78).

(b). Calcium overload hypothesis

Calcium (Ca^{2+}) ion is essential for cellular integrity, regulation of metabolism, cell growth and proliferation (79). It is needed for activation of myofilaments leading to contraction and for cardiac electrical activity. A large

gradient of Ca^{2+} (about 10,000 fold) exists across the sarcolemmal (SL) membrane. In the extracellular space the concentration of ionized calcium $[Ca^{2+}]_i$ is about 1.25 mM whereas in the intracellular space it ranges from 10^{-7} M to 10^{-5} M between diastole and systole respectively. The amount of Ca^{2+} entering or leaving a cell during contraction or relaxation must be the same or the cell would gain or lose Ca^{2+}. IR injury disturbs this delicate Ca^{2+} homeostasis maintained by the SL and the SR. Ca^{2+} homeostasis, therefore, becomes integral to the proper functioning of the myocyte and imbalances lead to contractile dysfunction and arrhythmias as seen in different pathological conditions.

1) Ca^{2+} homeostasis during excitation contraction coupling

Excitation contraction coupling involves excitation of the myocyte and eventually ends with its contraction after passing through a series of steps. SL depolarization during action potential results in the entry of a relatively small amount of Ca^{2+} from the extracellular space (primary source of Ca^{2+}) via L-type Ca^{2+}-channels. Ca^{2+} entry into the cell triggers the release of a large amount of Ca^{2+} from the SR Ca^{2+}-release channels; a phenomena known as Ca^{2+}-induced-Ca^{2+}-release (80). This increase in $[Ca^{2+}]$ due to Ca^{2+} entry into the cell and subsequent release of more Ca^{2+} from the SR allows Ca^{2+} to bind to troponin C thereby removing the inhibition and turning on the contractile machinery. Decrease in $[Ca^{2+}]$ during diastole allows for Ca^{2+} to dissociate from troponin. This is achieved by the activity of the (i) SR Ca^{2+}-pump ATPase (81), (ii) exchange of Ca^{2+} for Na^{+} via the Na^{+}-Ca^{2+}-exchange (NCX) (82, 83)., (iii) SL Ca^{2+}-pump ATPase and (iv)

mitochondrial Ca^{2+}-uptake. SR Ca^{2+}-pump ATPase activity is higher in rat ventricular myocardium than rabbit due to a greater concentration of the pump molecules and is responsible for 92% of Ca^{2+} uptake while 7% of Ca^{2+} is removed by the NCX. In human, rabbit, cat, ferret and guinea pig the SR Ca^{2+}-pump ATPase removes 70-75% of Ca^{2+}, whereas 25-30% is removed by NCX (84.). The mitochondria and SL Ca^{2+}-pump ATPase have been considered of minor importance. However, slow and cumulative changes in the mitochondrial $[Ca^{2+}]$ can activate important dehydrogenases causing an increased production of NADH and ATP to match any increased energy demands (85).

2) SR Ca^{2+}-transport mechanisms

Ultrastructural studies of mammalian ventricular myocardial cells have revealed that the SR is composed of at least three distinct structures (86): a) network SR which represents the major region of the SR surrounding the myofibrils (87), b) peripheral- and interior-junctional SR that are closely apposed to the SL and T-tubules, respectively. They are composed of cisternae and longitudinal regions where the former is connected by junctional processes called "feet" (87, 88), and c) corbular SR that is confined to the I-band of the sarcomere. Both the junctional and corbular SR are extensions of the network SR. The SR membranes are composed of several proteins that are of functional significance among which are: ryanodine receptors (RyR), Ca^{2+}-pump ATPase (SERCA2a), phospholamban (PLB) and calsequestrin (CQS).

a) SR Ca^{2+}-release channel (RyR)

Ryanodine receptor (RyR) is the Ca^{2+} release channel of SR and is responsible for increase in the intracellular Ca^{2+} levels in response to a small amount of Ca^{2+} entering the cell with each beat from the sarcolemmal L type Ca^{2+} channels (80). The channel is so named due to its capability to bind to ryanodine; a highly toxic alkaloid plant that induces different affects depending on the dose applied (89). It is specific in its expression to some cell types unlike the inositol 1, 4, 5- triphosphate (IP_3) gated Ca^{2+} channels that are ubiquitously present. It has three isoforms namely RyR1, found predominantly in the skeletal muscle, RyR2 that localizes to the cardiac tissue and RyR3, which is more ubiquitous than the other two suggesting RyR involvement in Ca^{2+} homeostasis in a wider selection of cells than earlier thought of. The three different isoforms of RyR (RyR1, RyR2 and RyR3) are encoded by three distinct genes (90). RyR2, the isoform expressed in cardiac tissue SR is the largest protein identified in the SR (kDa 565) (91). It consists of over 5,000 amino acids and has a higher ryanodine affinity than the skeletal muscle receptors (92) and is composed of four monomers that form a tetrameric structure. RyR forms a functional complex due to its association with several other proteins like CQS, junctin, triadin and FK506 (93). The Ca^{2+} release channels have diverse functions apart from triggering EC coupling that include T-lymphocyte activation and fertilization (94). RyR is phosphorylated by Ca^{2+}/calmodulin dependent protein kinase (CaMK) at Ser-2809 and by cAMP-dependent protein kinase. In cardiomyocytes the close anatomic proximity between the L-type Ca^{2+}-channels on

the T-tubules and RyR of the SR allows for a small amount of Ca^{2+} entering the cell to induce a large Ca^{2+}-release from the SR via RyR (80). In skeletal muscle fibers, RyR activation and subsequent Ca^{2+}-release depends upon the depolarization of the transverse tubules.

b) SR Ca^{2+}-pump ATPase (SERCA2a)

The sarco(endo)plasmic reticulum Ca^{2+}-pump ATPase (SERCA) activity determines the amount of Ca^{2+}-sequestered to be available for release in the next wave of excitation and is responsible for the restoration of Ca^{2+}gradient across the SR between cytoplasm and the SR lumen. It is therefore an essential protein for the determination of rate and extent of relaxation and the rate and amplitude of contraction. SERCA is encoded by three highly homologous genes: SERCA1, SERCA2, and SERCA3 (95). SERCA1a and 1b isoforms are expressed in adult and neonatal fast-twitch skeletal muscles, respectively (96.). SERCA2a is the cardiac and slow-twitch skeletal isoform (97) whereas SERCA2b is expressed in smooth muscle and non-muscle tissues (98). SERCA3 is a non-muscle isoform and it is mainly expressed in epithelial and endothelial cells (99). It is now well established that SERCA2a is the only isoform that is expressed in normal or stressed myocardium (100). The SERCA pump (105kDa) is a Ca^{2+} and Mg^{2+}-dependent ATPase protein (101) localized mainly in the longitudinal portion of the SR (102) and constitutes 35-40% of the SR proteins (103). Upon hydrolysis of one molecule of ATP, Ca^{2+}-pump transports two Ca^{2+}- ions against a high ionic gradient ranging between 100 nM-10 μM in the cytosol and 1 mM in the SR (104).

SERCA2a is also known to undergo direct phosphorylation by CaMK at Ser-38 providing a potential regulatory mechanism for Ca^{2+} removal (105).

c) Phospholamban (PLB)

Phospholamban (PLB) is a 52 amino acid SR protein that regulates the activity of SERCA2a and therefore SR Ca^{2+} transport. The exact mechanism by which PLB acts or mediates its effects is unknown. PLB in a dephosphorylated state is proposed to interact with SERCA2a and decrease its affinity for Ca^{2+} (106). PLB phosphorylation during β- adrenergic stimulation by cAMP dependent protein kinase A (PKA) at the Ser 16 residue or by Ca^{2+} - calmodulin dependent protein kinase II (CaMK II) at the Thr 17 residue (107) removes its inhibitory effect on SERCA2a facilitating Ca^{2+} uptake and cardiac relaxation. PLB has a pentameric assembly, which some studies have suggested forms a Ca^{2+} selective channel in the lipid bilayer (108). The different mechanisms postulated thus far by which PLB regulates the SERCA2a activity include; (i) a possible direct physical interaction between SERCA2a and PLB (109), (ii) changes in the SR- membrane potential can be obtained by phosphorylation of PLB (110), (iii) changes in the lipid motion that affect the membrane fluidity (111) or (iv) by a Ca^{2+} leak mediated by PLB through pores formed by its pentameric structure (108).

PLB is highly conserved since it is encoded by one gene in all species and is expressed in one form in the cardiac and skeletal muscle (112). Cardiac PLB is an integral part of the SR proteins (kDa 27) and it comprises of two low molecular weight forms, the pentameric and the monomeric form (113). The two forms are in

dynamic equilibrium and it is suggested that the pentameric form may be an inactive reservoir for the active monomeric form (109). Immunohistochemical studies showed that PLB is localized in the SR. The highest distribution is in the longitudinal region of the SR where it is co-localized with SERCA2a. This anatomical proximity indicates the functional correlation between both proteins. PLB phosphorylation in vivo has been postulated to play a key role in mediating a lusitropic effect (cardiac relaxation) and the inotropic effect (rate of contraction) of β-adrenergic system (114).

d) Calsequestrin (CQS)

Calsequestrin (CQS) is characterized as a high-capacity moderate affinity Ca^{2+}-binding protein, which stores Ca^{2+} in the SR lumen (115) 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 (116) CQS is composed of 396 amino acid residues (kDa 55) and is anchored to the junctional SR (the cisternae part) in close proximity with RyR (117). CQS, RyR and other SR proteins (FKBP, junctin and triadin) are hypothesized to form a functional complex for the coordination of Ca^{2+}-release (93). CQS is a known preferred substrate for casein kinase II phosphorylation at Ser-378 both in vivo and in vitro (118), but the functional consequence of this phosphorylation is not yet understood.

3) Role of SR protein phosphorylation in Ca^{2+}homeostasis

Ca^{2+} movement during cardiac contraction and relaxation is controlled by phosphorylation and dephosphorylation of the proteins involved in Ca^{2+}-cycling.

The balance between phosphorylation-dephosphorylation is important in modulating cellular responses to different stimuli by regulating Ca^{2+}-homeostasis in cardiomyocytes. SR function and Ca^{2+} transport is regulated by an endogenous CaMK (119) and PKA (120) mediated phosphorylation of SR proteins. CaMK phosphorylates RyR, SERCA2a, and PLB whereas PKA phosphorylates RyR and PLB (114,121). SERCA2a affinity for Ca^{2+} is inhibited by its protein-protein interaction on its cytoplasmic and transmembrane domains by the dephosphorylated form of PLB during diastole (122). The phosphorylation and nucleotide domains of SERCA are essential for PLB interaction while the 1A cytoplasmic domian of PLB is essential for its functional association with SERCA (123). Under physiological conditions PLB phosphorylation is mediated upon CaMK activation and also by increased levels of cAMP due to the activation of the β-adrenergic system (124). PLB phosphorylation by PKA (123,125) and CaMK (126) relieves this inhibition resulting in increased SERCA2a affinity for Ca^{2+} (127), enhanced SR Ca^{2+}-uptake (128) and improved cardiac relaxation (129, 130). SR releases its load of Ca^{2+} with the next wave of depolarization (130). SERCA2a is directly phosphorylated by CaMK (131), which increases ATP hydrolysis and thus stimulates Ca^{2+}-transport into the SR lumen by enhancing Vmax (121,129). RyR phosphorylation by CaMK increases its open state thereby increasing the Ca^{2+} release (132). RyR is also phosphorylated by PKA, at a level 4 times less than that of CaMK phosphorylation (132,133), though much is not known about the functional significance of this phosphorylation. The phosphorylated proteins are

later dephosphorylated by an endogenous phosphatase that reverses the phosphorylation effects (134) The endogenous SR phosphatase has the capability for dephosphorylation of both CaMK and PKA phosphorylated substrates in a non-discriminatory fashion (134).

It is clear from the above discussion that Ca^{2+} homeostasis in the myocyte is maintained by the interplay of membrane proteins like RyR, SERCA2a, PLB and CQS and their phosphorylated - dephosphorylated state controlled by CaMK and PKA. IR injury affects some of these Ca^{2+} regulating mechanisms resulting in calcium overload and contractile dysfunction. The genesis of Ca^{2+}overload and decreased myofilaments response to Ca^{2+} is discussed below.

(1) Genesis of calcium overload

Oxygen deficiency during ischemia causes a shift in myocardial metabolism resulting in decreased levels of ATP and accumulation of hydrogen ions and lactate. This acidosis causes activation of the sodium-hydrogen exchanger (NHX) resulting in sodium (Na^+) overload. Until reperfusion begins, Na^+ accumulates and the acidosis prevents the activity of the sodium-calcium exchanger (NCX). Reperfusion of the myocardium causes reversal of the acidosis and activates the NCX. This pushes Ca^{2+} into the cardiomyocytes in exchange for Na^+ resulting in the Ca^{2+} overload. This was a simple hypothesis forwarded by Grinwald (135) in 1982 and still holds good. Subsequent work by different investigators has shown that $[Na^+]_i$ rises rapidly during ischemia and remains so for about 8-10 minutes after reperfusion whereas the acidosis takes about 30 seconds to resolve. Grinwald

(136) in a subsequent work showed that the functioning of sodium-potassium pump failed in IR and this could possibly explain the Ca^{2+} overload seen on reperfusion, possibly due to the effect of ROS on the sarcolemmal Na^{+}-K^{+} ATPase (137). Some studies have shown that cytosolic Ca^{2+} concentration rises even during ischemia but none of the effects of calcium overload are seen during ischemia alone. Impaired myofilaments responsiveness to Ca^{2+} (138) and the proteolytic degradation of the various proteins (139) occur during reperfusion. Thus reperfusion would seem essential for the occurrence of Ca^{2+} overload and the different cardiac abnormalities associated with IR injury. Some of the mechanisms responsible for Ca^{2+} overload are discussed below.

(i) Sodium-hydrogen exchanger (NHX)

The NHX or antiporter is one of the membrane transporters that regulate the cardiomyocyte pH, the others being the Na^{+}-HCO_3^{-} symport, Cl^{-}-HCO_3^{-} and the Cl^{-}-OH^{-} exchangers. The first two are activated under conditions of acidosis while the latter two respond to alkaline conditions (140). The NHX is the most important of these all and has five different isoforms of which NHX1 is the primary isoform present in cardiomyocytes (141). NHX1 maintains the intracellular pH and cell volume by exchanging H^{+} for Na_{+} in a stoichiometric ratio of 1:1 (142). The glycosylated NHX1 protein has a molecular weight of 110 kDa and its activity is regulated by the hydrophilic carboxy C-terminus cytoplasmic domain that via phosphorylation dependent reactions (143). Acidosis is the main stimulus for the activation of the NHX1 (144) and most of the cell H^{+} is extruded through it. The

antiporter has an H^+ sensor that is receptive to changes in the pH where proton binding to a NHX1 oligomer causes conformational changes causing its activation. The NHX can be activated by other means too apart from acidosis like ischemic metabolites such as H_2O_2 (145) and lysophosphatidylcholine (146). Most of these act by stimulation of the phosphoinositide hydrolysis and activation of PKC resulting in phosphorylation and activation of NHX1 (141) and MAPKs (145). Under normal conditions the rate of Na^+ entry via NHX and efflux by Na^+-K^+ ATPase would balance each other (147). During ischemia ATP depletion causes the inhibition of Na^+-K^+ ATPase (148,149) or Na^+-K^+ pump failure (136) resulting in accumulation of intracellular Na^+ and eventually Ca^{2+} overload by exchanging Na^+ for Ca^{2+} via the NCX (reverse mode)(150). Na^+-K^+ ATPase has been suggested to be able to remove the acidosis induced Na^+ load all by itself in the absence of Na^+ entry (151). NHX inhibitors like amiloride attenuate the ouabain induced elevation in [Na+] and the entry of Ca2+through the NCX (147) suggesting Na^+ overload to be the primary defect. Amiloride has been shown to benefit when given during ischemia and reperfusion both and not during reperfusion alone (18). But for the concomitant Na^+ and eventual Ca^{2+} overload, NHX activation on reperfusion would have been beneficial, as it would restore the cellular pH to normal. Some recent studies have strongly implicated the NCX in the genesis of Ca^{2+} overload by demonstrating its overexpression in animals that showed enhanced IR injury possibly by increasing the Ca^{2+} entry (152).

(ii) Sodium-calcium exchanger (NCX)

The NCX extrudes calcium for Na^+ under normal conditions but has a reverse mode that gets activated during IR causing inflow of Ca^{2+}. The NCX is normally driven by an electrochemical gradient and the Na^+ to Ca^{2+}ratio is 3:1 (153). A reversal in the ionic concentrations or membrane potential can put the NCX in the reverse mode. In IR, the reverse mode of the NCX plays a substantial role in the development of Ca^{2+} overload and IR injury (154). Sarcolemmal depolarization and Increased Na^+ concentration are instrumental in this role of the reverse mode. Failure of the Na^+-K^+ ATPase due to ATP depletion during ischemia (149) causes depolarization of the sarcolemma and Na+ overload, which is further accentuated by the intracellular acidosis. Hearts reperfused with low Na^+ concentration buffers have shown a depression in the development of Ca^{2+} overload implicating Na^+overload and the NCX in the genesis of Ca^{2+} overload and IR injury (155). Similar results have been obtained with NCX (156) and NHX inhibitors (157). This is consistent with the observation that over expression of NCX is associated with an increased susceptibility to IR injury (152).

From the above discussion, it would emerge that genesis of Ca^{2+} overload during IR depends upon the status of the NHX and NCX. An important corollary to this observation is that for Na^+ overload to develop, the failure of the Na^+-K^+ pump is needed (136,148,149). Na^+-K^+ ATPase depression would also result in a reversal of the ionic concentration gradient that normally exists between Na^+ and Ca^{2+}causing an extrusion of Na^+ and not Ca^{2+} by the reverse mode of the NCX.

(2) Decreased responsiveness to Ca^{2+}

Though the exact underlying pathology responsible for decreased responsiveness of the myofilaments to Ca^{2+} is not known, evidence available indicates some structural abnormalities at the level of myofilaments that would alter their response to Ca^{2+} during excitation contraction. Previous studies from our laboratory have shown that ROS cause oxidation of the critical thiol groups in the myofilaments (158). Such structural modifications of the myofilaments could explain the decreased responsiveness to Ca^{2+} as well as the contractile abnormalities due to IR injury. ROS cause a reduction in the content of reduced glutathione and increase in oxidized glutathione (159). Taken together, changes in the content of these have been shown to decrease the Ca^{2+} sensitivity of the myofilaments (160). It would be interesting to point out that this effect of ROS on the myofilaments may possibly provide for the integration of the calcium and oxyradical hypotheses. Another important factor that could alter not only the contractility of myofilaments but also their response to Ca^{2+} during excitation contraction coupling is their proteolytic degradation by the Ca^{2+} activated neutral protease calpain. the role of calpain in cardiac pathology is discussed below.

Calpain

AIDS (acquired immunodeficiency syndrome) and cancer research in the 1980's and 1970's stimulated lot of interest in proteases. It was found that retroviruses involved in AIDS bud to form new particles that infect new cells.

These particles are activated upon cleavage by a viral protease (reverse transcriptase). In 1989 Manuel Navia and colleagues solved the structure of this viral protease (161) stimulating research to find an inhibitor for it. Saquinavir (1995) became the first protease inhibitor to be used for the treatment of AIDS (162) in combination with the antiviral drugs already being used.

Proteases are broadly classified into endo and exopeptidases. The endopeptidases or the proteinases cleave amino acids from the N-terminus while the exopeptidases cleave at the C-terminus. Proteolysis can be limited to the cleavage of a few amino acids resulting in the activation of the protein or it could lead to complete degradation into constituent amino acids. Endopeptidases have, for long, been shown to play an important role in different pathophysiological cellular functions that include cell growth, normal inflammatory response, blood clotting, infections, fertilization, tissue remodeling, cancer and apoptosis. Most proteins undergo ubiquitination, a process where the proteins are identified by ubiquitin (a 76 amino acid highly conserved protein) before being degraded by the 26S proteasome (163). Ubiquitin is thought to remove unwanted proteins and actively regulate the cell cycle. About 80-90% of proteolysis in mammalian cells is by the ubiquitin-proteasome pathway. Some of the important ubiquitin regulated processes include signal transduction, regulation of kinases and phosphatases, transcription, cell cycle progression, protein degradation, biogenesis and spermatogenesis, cell death and apoptosis. Dysfunction in this pathway can lead to Alzheimer's, Pick's and Parkinson's disease (164).

Endopeptidases are classified into the serine (trypsin, chymotrypsin), cysteine (calpains, papains, lysosomal cathepsins), aspartate (pepsin, rennin, HIV protease) and the metalloproteases (involve a Zn atom and regulate tissue remodeling; include MMP1, 3, 9 etc). Calpain is a ubiquitously present cytosolic cysteine protease present in many tissues, including the myocardium (165,166.) that causes limited proteolysis (167). The 2 isoforms, I (μ) and II (m), are present ubiquitously in various tissues alongside the tissue specific calpains. Studies so far have described calpains in animals and not in the plant kingdom.

The name 'calpain' was decided upon at the International conference on protein catabolism in 1991 (168). A Ca^{2+} dependent neutral proteinase was first identified as being similar to calpain in 1968 (169). It was found to be involved in the removal of the z-line in rabbit skeletal muscles in 1972 (170) and as an activator of PKC in rat liver in 1977 (171). Calpain has a large 80kDa subunit and a small 30kDa subunit. The latter is similar in all the different homologues thus forming a large family of proteinases due to the similar 30kDa cysteine protease domain. But these homologues are distinct from I and II isoforms due to difference in their other domain structures. These dissimilar domains are specific to the tissue / organ functions in which the homologues are expressed. Calpain (EC 3.4.22.17) has a Ca^{2+} binding domain similar to calmodulin and its activity is regulated by Ca^{2+} concentration. Calpain I and II are called μ and m respectively due to their micro and milli molar Ca^{2+} requirements for activation (181). The 30k domain is further made of two domains, N and C-terminal domains. The C-terminal domain

is similar to domain IV of the large 80kDa subunit and is believed to regulate the calpain activity through Cys^{105}, His^{262} and Asn^{286} residues (172).

Ischemia and IR have been shown to activate the different isoforms of calpain (173). The Ca^{2+} concentration needed for calpain activation (>10μm) (shown by in vitro studies) is unattainable under normal physiological conditions and cannot be attained under pathological conditions as well without the cell bursting (174). Matsumura et al found calpain was activated much before the cell achieved Ca^{2+} concentrations of 1μm, while others have shown that Ca^{2+} concentrations around 10μm are needed for activating μ-calpain (175). Thus mechanisms other than Ca^{2+} concentration would be needed to activate calpains, which are discussed below. Rat liver cell nuclear proteins have been shown to be proteolyzed by m-calpain (176). DNA addition to the medium lowered m-calpain requirement for Ca^{2+} indicating some complex formation between m- calpain and DNA (177). The 30k subunit has been suggested to regulate Ca^{2+} sensitivity of the large subunit by associating and dissociating from it (178). But subsequent studies have shown that the two subunits remain associated during catalysis as well (179). Similarly, limited proteolysis of the calpain large subunit into a 76 kDa subunit, and the 30k subunit into a smaller 18k unit has been suggested to lower the Ca^{2+} requirement (180). Subsequent studies have shown that this autolysis does occur but is not essential for proteolytic activity (181). Calpain is not bound to the membranes under normal conditions but increase in the cell [Ca^{2+}] translocates it to

the membranes. Molinari et al showed that membrane bound calpain is in a non-autolyzed form (182). This study documents two important observations: (i) it showed that autolysis of calpain was not needed for activation and (ii) during activation the enzyme gets translocated to the membrane. Membrane translocation could be one of the ways by which the enzyme escapes the endogenous inhibitor calpastatin. It is also proposed that calpain interacts with membrane proteins, and not with membrane phospholipids and that this association does not affect its Ca^{2+} requirement (183).

Role of Calpain in IR

Calpain has been shown to degrade the membrane cytoskeleton proteins like fodrin (calspectin or non-erythroid spectrin) and ankyrins that maintain cell membrane integrity (21, 184). Yoshida et al showed that ischemia activated m-calpain while μ-calpain was activated during reperfusion. But reperfusion decreased the m-calpain activity. They found degrdation of α-fodrin and calpastatin during ischemia and IR. They also showed that creatine kinase (CK), a diagnostic enzyme for AMI, levels were reduced in hearts treated with calpain inhibitors (21). Matsumura et al showed that in addition to fodrin other cytoskeletal proteins such as desmin and α-actinin were also degraded by calpain. The reduction in degradation of these proteins seen with leupeptin and calpain inhibitor I correlated well with improved cardiac contractility suggesting an association between calpain degradation of the cytoskeletal proteins and cardiac contractility (22). Recently Tsuji et al have shown that fodrin is probably the only cytoskeletal

protein to be degraded in IR induced proteolysis and did not find evidence for the proteolysis of other earlier reported proteins (185). Similarly, studies have shown the Ca^{2+} release channel of the SR (25) and Ca^{2+} pump ATPase (26) to be possible targets for calpain though the identity of the proteins mentioned is not clear. L-type Ca^{2+} channel (24) and RyR from the skeletal muscle have also been shown to be degraded by calpain (186).

Ca^{2+} overload occurs in patients of AF and calpain mediated proteolytic degradation of atrial proteins and alterations in the structural integrity of the sarcomere has been implicated in the pathogenesis of AF (27). Various studies have shown that the Ca^{2+} sensitivity of myofilaments is reduced in IR (28,29.). Goa et al have shown that the Ca^{2+} responsiveness of skinned muscle fibres decreases when exposed to Calpain I by studying their steady state force Ca^{2+} relationship (30) thus holding calpain activation during IR to be responsible for altering the myofilament structure and hence response to Ca^{2+}. Persistence of immunoreactivity of the contractile protein troponin T (TnT) in the serum of patients recovering from myocardial infarction (MI) is one of the diagnostic tools for MI (187). Calpain has been suggested to partially degrade TnT to facilitate cross-linking of its subunits with other proteins (188). Cross-linked proteins have been suggested to be responsible for the persistent immunoreactivity seen in MI patients.

Protease inhibitors

Protease inhibitors (PI) have been used in different conditions where the heart is exposed to ischemia or IR injury. One of the important uses of PIs is in cardioplegic solutions used for cardioplegic arrest during different surgical procedures like cardiopulmonary bypass, or cardiac transplants. These solutions include the St. Thomas cardioplegic solution (189) and the University of Wisconsin solution (190) that use aprotinin and other PIs (191) to prevent proteolytic damage to the myocardial tissue during preservation. Yamamoto et al showed that gabexate mesilate, a serine protease inhibitor enhanced the cardioprotective effect of the cardioplegic solutions suggesting a possible causal role for proteases in stunning seen during cardiac transplants and bypass surgeries (9). Shibata et al showed similar results confirming the protective effects of serine protease inhibitors nafamostat and gabexate mesilate when added to the St. Thomas cardioplegic solution during ischemia (191).

The different calpain inhibitors used in various studies include Leupeptin, E64d, calpain inhibitor I (N-acetyl-leu-leu-norlucinal) and II (N-acetyl-leu-leu-methioninal), ZLLLaL (benzyloxycarbonyl-Leu-Leu-cinal) and ZLLaL (benzyloxycarbonyl-Leu-Leucinal). Calpastatin (CS) is the endogenous calpain inhibitor that has been shown by different studies to be degraded by ischemia and IR (173). But CS does not inhibit the activity of p94, the muscle specific calpain. E64 was first obtained from the cultures of Aspergillus japonicus and has two derivatives E64c and E64d (192). E64d is the membrane permeable derivative of

E64c. Yoshida et al have shown that E64d [and dimethylsulfoxide (DMSO)] attenuates calpain activity while preventing the α-fodrin and calpastatin proteolysis by calpain (21). Leupeptin is an exogenous reversible cysteine protease inhibitor like E64 and is produced by fermentation in actinomycetes (192). It has been shown to inhibit calpain and decrease proteolysis in many tissues including the myocardium (21, 193-196) in various pathological conditions. It is a cell-penetrating aldehyde that has been used for many years in calpain studies (192, 203). Leupeptin is not toxic to many tissues, including the myocardium (195, 196). AK 295 [benzyoxycarbonyl-leucylaminobutyric acid-$CONH(CH_2)_3$-morpholine] and AK275 [benzyoxycarbonyl-leucylaminobutyric acid-$CONH(CH_2CH_3]$ are some of the newer and more powerful protease inhibitors that have been developed (197).

III. Statement of the problem and hypothesis to be tested

Restoration of blood flow is critical for resuscitation of the ischemic myocardium. But reperfusion is known to depress the cardiac contractile function. More than two decades of research on IR has identified Ca^{2+} overload and free radical production as the two mutually nonexclusive mechanisms responsible for post ischemic cardiac dysfunction. The downstream mediators of Ca^{2+} overload that induce cardiac dysfunction are speculative. Therefore it becomes imperative to investigate the contractile machinery of cardiomyocytes and the different mechanisms involved in maintaining Ca^{2+} homeostasis. Earlier studies have shown alterations in contractile proteins like troponin I and T and cytoskeletal proteins such as fodrin, desmin and actinin to be responsible for cardiac dysfunction. Activation of Ca^{2+} dependent neutral proteases such as calpain by Ca^{2+} overload during IR has been associated with the degradation of cytoskeletal and contractile proteins mentioned above.

Previous studies from our laboratory have shown that IR causes SR dysfunction by depressing the SR Ca^{2+} uptake and release. This could be due to a reduction in the protein content or depression in the phosphorylation of SR Ca^{2+} cycling proteins. We investigated the mechanisms that may be responsible for this reduction in protein content of SR Ca^{2+} cycling proteins or depression in the kinase activity resulting in SR dysfunction. We worked on the hypothesis that Ca^{2+}activated protease, calpain, may induce proteolytic degradation of SR proteins involved in Ca^{2+}homeostasis and results in cardiac dysfunction in IR hearts. This

aspect has not been investigated before. This work is expected to shed new light on the mechanisms by which Ca^{2+} overload may be mediating cardiac injury during IR.

IV. MATERIALS AND METHODS

Heart perfusion and experimental protocol. Male Sprague-Dawley rats weighing 225-275 g were anaesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). The hearts were rapidly excised, cannulated to the Langendorff apparatus, perfused with Krebs-Henseleit-solution (37^0C) and gassed with a mixture of 95% O_2 and 5% CO_2 at a pH of 7.4, containing (in mM): 120 NaCl, 25 $NaHCO_3$, 11 glucose, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$ and 1.25 $CaCl_2$. The hearts were electrically stimulated at a rate of 300 beats/min (Harvard 6002 stimulator from Harvard Apparatus, Holliston, MA) and perfusion rate was maintained at 10 ml/min. A water-filled latex balloon was inserted in the left ventricle and connected to a pressure transducer (Model 1050BP; BIOPAC SYSTEM INC., Goleta, CA) to record left ventricular systolic and diastolic pressure measurements. Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and diastolic pressures. The left ventricular end diastolic pressure (LVEDP) was adjusted at 10 mm Hg at the beginning of the experiment and left ventricular pressures were differentiated to estimate the rate of ventricular pressure development (+dP/dt) and the rate of ventricular pressure decline / decay (-dP/dt) using the Acknowledge 3.5.3 software for Windows (BIOPAC SYSTEM INC., Goleta, CA). All hearts were stabilized for a period of 20 min before being assigned to different groups in this study and were maintained at a constant temperature (37^0C) throughout the experiment. The control hearts were perfused for a period of 90 min after the 20 min stabilization.

Experimental Protocol:

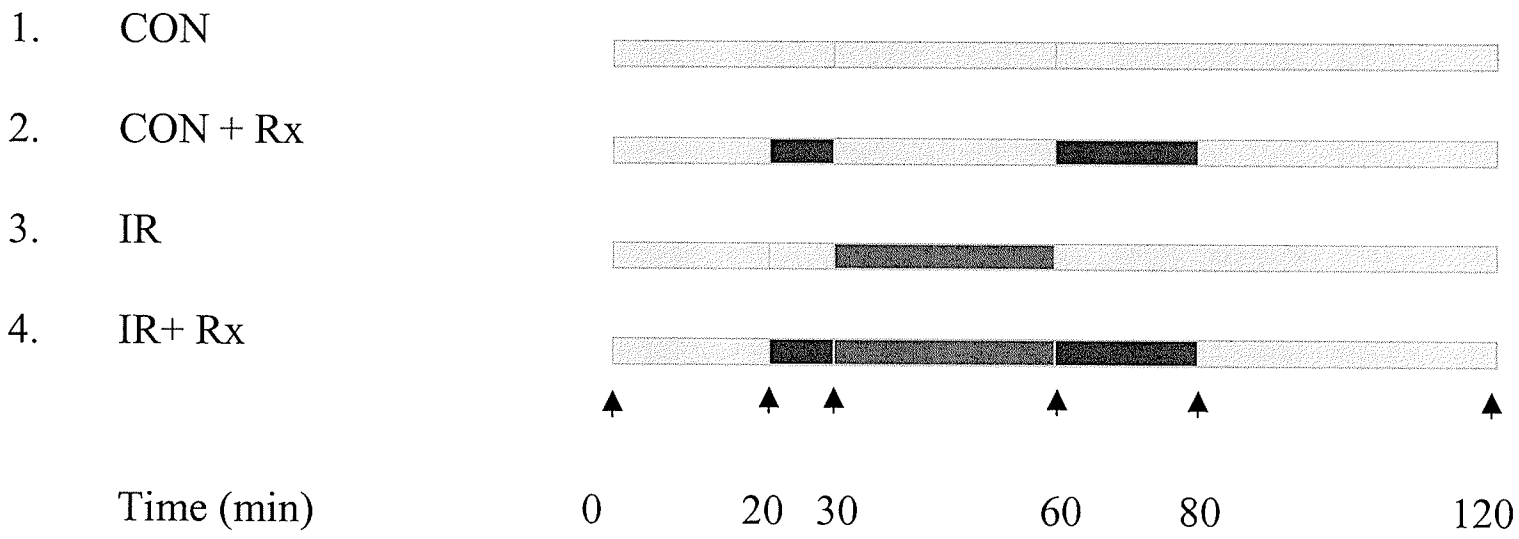


Figure. 2. Experimental protocol for perfusing isolated rat hearts under different conditions. Panels 1 and 2 show control hearts treated with and without leupeptin. Panels 3 and 4 show I/R hearts treated with and without leupeptin. Global ischemia (red bars) was induced by stopping coronary flow for 30 min and I/R by reperfusing the globally ischemic hearts for 60 min. Hearts were treated with leupeptin (deep blue bars) for 10min before and 20 min after ischemia in the I/R study. CON=Control, Rx=Treatment, IR=Ischemia Reperfusion.

Hearts were made globally ischemic by stopping the coronary flow for 30 min. Reperfusion of the 30 min globally ischemic hearts for a period of 60 min induced I/R. In another group of experiments, the I/R hearts were treated with a calpain inhibitor leupeptin (25μM). Leupeptin was infused for 10 min before inducing ischemia and for 20 min after ischemia beginning at the onset of reperfusion. To observe for any possible effects of the drug on control hearts, a group was added where control hearts were perfused with leupeptin for the same duration as the treatment group. Another series of experiments were designed to see the effect of leupeptin pre-treatment on hearts made globally ischemic for 30 min without being reperfused. The control hearts in this series were perfused for 60 min whereas global ischemia was induced for 30 min in the ischemic group. Preischemic leupeptin infusion was given for 10 min in the treated group. The scheme for perfusion is shown in Figure.2. The final concentration of Leupeptin was 25μM and this concentration of leupeptin was decided upon after a dose response was done with different concentrations of leupeptin (Table 1.). At the end of the experiments the hearts were stored at -70°C for 2 to 3 days before use.

SR preparation. SR vesicles were obtained by a method described previously (198) with slight modifications. Briefly, the ventricular tissue was pulverized and homogenized in a mixture of (in mM): 10 $NaHCO_3$, 5 NaN_3, 15 Tris-HCl at pH 6.8 (10 ml/g tissue) with a polytron homogenizer (Brinkmann, Westbury, NY). The homogenate was then centrifuged for 20 min at 9,500 rpm to remove cellular debris. The supernatant was further centrifuged for 45 min at

Table 1. Hemodynamic parameters of the isolated perfused rat hearts with different concentrations of leupeptin as compared to the control and ischemic-reperfused hearts.

Concentration (in μM)	LVDP (mm Hg)	LVEDP (mm Hg)	+dP / dt (mm Hg/sec)	-dP / dt (mm Hg/sec)
CON	112 ± 2.3	4.3 ± 0.9	2454.3 ± 111.2	1674.3 ± 227.7
IR	36.3 ± 9.2	56.3 ± 2.7	576.7 ± 62.2	509.0 ± 137.2
15	40.9 ± 9.7	51.7±5.6	613.0 ± 70.1	648.2 ± 39.6
20	71.0 ± 5.0	47.1 ± 9.1	1071.0 ± 54.6	1151.0 ± 52.8
25	84.3 ± 4.8	32.7 ± 4.7	1630.0 ± 81.7	1288.7 ± 87.7
40	29.9 ± 8.2	57.5 ± 6.2	538.0 ± 63.9	415.0 ± 86.2
50	27.7 ± 6.3	47.0 ± 9.0	505.0 ± 54.4	375.0 ± 54.1

n=3 for all groups. Left ventricular developed pressure (LVDP), Left ventricular end diastolic pressure (LVEDP), Left ventricular pressure development (+dP/dt) and left ventricular pressure decay (-dP/dt).CON=control, IR=Ischemia reperfusion. Data expressed as Mean ±SEM.

19,000 rpm (Beckman, JA 20). The supernatant, containing the cytosolic fraction, was aliquoted and the pellet was suspended in 8 ml of a buffer containing 0.6 M KCl and 20 mM Tris-HCl (pH 6.8) and centrifuged for 45 min at 19,000 rpm. The final pellet containing the SR fraction was suspended in a buffer containing 250 mM sucrose and 10 mM histidine, pH 7.0 and aliquoted. All solutions contained a cocktail of protease inhibitors consisting of aprotinin, leupeptin, AEBMSF and 0.1% phenylmethylsulphonyl fluoride (PMSF). Techniques have previously been established in our lab whereby activities of marker enzymes are used to determine the purity of membrane preparations using ouabain-sensitive Na^+-K^+ ATPase (as a SL marker) and rotenone-insensitive NADPH cytochrome c reductase and glucose-6-phosphatase (as SR marker). These have shown negligible cross contamination with other sub-cellular organelles (2-4%).

Protein Estimation. Protein concentration in each sample of SR was determined using Lowry's method. Varying concentrations of bovine serum albumin (BSA) in deionised distilled water (DDW) were used to generate a standard curve. All standards and samples were run in duplicate. 2 ml of working solution containing 2% potassium sodium tartarate, 1% $CuSO_4$ and 2% Na_2CO_3 (in 0.1N NaOH) in a ratio of 1:1:100 was added to blanks, standard and samples and vortexed. After 10 min 0.2 ml of 1N phenol reagent (Folin and Ciocaltues reagent) was added to each tube. After 20 min the absorbance of the samples at 623 nm was determined using an Ultrospec 2100 pro spectrophotometer (from Biochrom). The

protein concentration of samples was determined using a standard curve obtained with BSA on a custom made computer software in microsoft excel.

Calpain activity measurements. Calpain activity was measured in the cytosolic fraction of the heart homogenates. A calpain assay kit from Calbiochem was used to detect the calpain activity. The assay is based on the fluorometric detection of the cleavage of calpain substrate Ac-LLY-AFC. Calpain cleaves AFC, which can be read in a fluorometer. The cytosol was collected as discussed above after the second centrifugation. Protein estimation was done by the Lowry's method and protein concentration in each sample was normalized to 200μg by adding the extraction buffer provided in the kit to obtain a final volume of 85μl. All samples were run in duplicates. 1-2 μl of active calpain (1μg/μl) was added to 83 μl of the extraction buffer and used as a positive standard. For the negative standard, 1-2 μl of a calpain inhibitor (Z-LLY-FMK) was added to the cytosol from control samples and the final volume was made up to 85μl using the extraction buffer. 10μl of a 10X reaction buffer and 5μl of the substrate was added to all the samples including the standards. The reaction was carried out in a 96 well plate. The plate was protected from light and incubated for 1 hour at 37^0C with shaking. The samples were read in a Gemini fluorescence microplate reader from Molecular devices. The excitation filter was set at 400 nm and the emission filter was set at 505 nm. The results were expressed as relative fluorescent units (RFU).

Measurement of Ca^{2+}-uptake. Calcium-uptake activity of SR vesicles was measured by a procedure described previously (198). A total volume of 250 μl

contained (in mM): 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 μM ruthenium red. Ruthenium red was added as an inhibitor of the Ca^{2+}-release channel under the assay conditions mentioned above. The reaction was initiated by adding SR vesicles (10 μl of 2mg/ml protein) at 37^0C and terminated after 1 min by filtering 200 μL aliquot of the incubation mixture through 0.45 μm Millipore filters. The filters were washed with 5 ml washing buffer and dried at 60^0C for 1 hour. 10 ml of scintillation fluid was added to each of the scintillation vials containing the filters and were counted in a liquid scintillation counter. The Ca^{2+}-uptake reaction was linear during 2 min of the incubation period.

Measurement of Ca^{2+}- induced Ca^{2+}- release. The Ca^{2+}-release activity of SR vesicles was measured by a procedure adapted from a previously described method (198, 199). The SR fraction (62.5 μl of 0.5 mg/ml protein) was suspended in a total volume of 625 μl of loading buffer containing (in mM): 100 KCl, 5 $MgCl_2$, 5 potassium oxalate, 5 NaN_3, and 20 Tris-HCl (pH 6.8). The SR fraction was incubated with 10 μM $^{45}CaCl_2$ (20 mCi/L) and 5 mM ATP for 45 min at room temperature and Ca^{2+}-induced Ca^{2+}-release was carried out by adding 1 mM EGTA plus 1 mM $CaCl_2$ to the reaction mixture. The reaction was terminated at 10 seconds by Millipore filtration technique. Radioactivity in the filter was counted in 10 ml of scintillation fluid. The Ca^{2+}-induced Ca^{2+}-release was completely prevented (95 to 97%) by the treatment of SR preparations with 20μM ryanodine.

Measurement of CaMK and PKA activities. The SR preparations used in phosphorylation experiments were isolated in the presence of a phosphatase inhibitor to prevent any dephosphorylation from occurring during the isolation procedure. 1 mM sodium pyrophosphate was added to both the homogenization buffer and the phosphorylation assay medium. The CaMK and PKA activities of the cytosolic and SR preparations were measured by using Upstate Biotechnology (Lake Placid, NY) assay kits. The assay kit measures the phosphotransferase activity of PKA or CaM Kinase in immunoprecipitate and column fractions. The assay kit for CaMK activity is based on the phosphorylation of a specific substrate peptide (KKALR-RQETVDAL) by the transfer of the g-phosphate of [g-32 P] ATP by CaMK II. Because the SR and cytosolic CaMK also phosphorylated the exogenous substrate, the activity was calculated as the difference between the values obtained in the presence and absence of the exogenous substrate. The assay dilution buffer (ADB) I (for PKA) and II (CaMK), the substrate and inhibitor cocktail were taken from the kit in a concentration of 10µl in eppendorf tubes along with sample and DDW. The radioactive mixture is made by mixing P_{32} and MgATP from the kit in a concentration of 1:9. The reaction was started by adding 10µl of the radioactive mixture at 1 min interval to all the tubes and incubated for 10 min at 30^0C. Spotting the reaction mixture (25µl) on numbered phophocellulose filter papers stopped the reaction. Subsequently, 3 washings were done with phosphoric acid and one with acetone to remove any excess radioactivity. The

assay kit for PKA activity measurement is based on the phosphorylation of a specific substrate (kemptide) by using the transfer of the g-phosphate of [g-32 P] ATP by PKA. The phosphorylated substrates in both assays were then separated from the residual [g-32 P] ATP with P81 phosphocellulose paper. This was quantified by using a scintillation counter after adding 10 ml of scintillation fluid to each vial containing the phosphocellulose paper.

Western blot analysis. The protein content of Ca^{2+}-pump ATPase (SERCA2a; 105kDa), ryanodine receptor (RyR), phospholamban (PLB), calsequestrin (CQS) and CaMK and PKA were determined as described by some other investigators (200). Protein samples (20 µl of 2 mg/ml total protein/lane) were separated by electrophoresis through a Mini SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) in 5% (for RyR), 10% (for SERCA2a), 12% (for CQS, CaMK and PKA), and 15% (for PLB) gels. Samples for SERCA2a, PLB, CQS, CaMK and PKA were transferred to polyvinylidene difluoride membranes (PVDF) while that for RyR was transferred to nitrocellulose membrane. The membranes were probed with monoclonal anti-SERCA2a (1:1,400), monoclonal anti-ryanodine receptor (1:1,400 both from Affinity Bioreagents Inc., Golden, CO), monoclonal anti-phospholamban (1:2,000), or polyclonal anti-calsequestrin (1:2,000) antibodies. The antibodies for PLB, CQS and PKA were purchased from Upstate Biotechnology, Lake Placid, NY while the CaMK antibody was purchased from Santa Cruz Biotechnology, Inc. Antibody-antigen complexes in all membranes were detected by the chemiluminescence's ECL kit (Amersham Life

Science, Oakville, ON, Canada). Protein bands were visualized on Hyperfilm-ECL. An Imaging Densitometer model GS-800 (Bio-Rad Ltd., Hercles, CA) was used to scan the protein bands; these were quantified using the Quantity one 4.4.0 software from Bio Rad. It is pointed out that a linear relationship between the density of blots and protein load was observed when 5, 10, 20 and 30 μg membrane protein was used per lane.

Statistical analysis. Results are expressed as mean ± S.E.M. and statistically evaluated by one-way Analysis of Variance (ANOVA) test and the student t-test. A level of $P<0.05$ was considered the threshold for statistical significance between the control and various experimental groups and the groups themselves.

V. RESULTS

Cardiac function in myocardial ischemia/reperfusion. Measuring LVDP, LVEDP, +dP/dt and -dP/dt assessed cardiac function in the isolated perfused hearts. Hearts subjected to global ischemia for 30 min failed to generate LVDP, +dP/dt and -dP/dt but showed a marked increase in LVEDP (Table. 2). Reperfusion of the ischemic hearts for 60 min recovered the contractile function, as represented by 30 % improvement in LVDP, +dP/dt and -dP/dt of the respective pre-ischemic values but there was a marked increase in the LVEDP (Figure. 3, Panels A to D). The recovery of contractile activity in IR hearts was markedly improved by leupeptin (25μM) treatment; this was reflected by an 80-85% recovery of LVDP and between 66 to 90 % recovery in +dP/dt and -dP/dt, respectively, in comparison to pre-ischemic values (Figure.5, Panels A, C and D). A marked reduction in LVEDP was observed with leupeptin treatment in comparison to the IR group (Figure. 3, Panel B). Leupeptin treatment did not affect the cardiac function in the control group.

SR function. As SR plays a central role in determining the cardiac function the SR function was assessed by studying Ca^{2+}-uptake and Ca^{2+}-release activity in the isolated SR preparations in the control, ischemic and IR hearts. SR Ca^{2+}-uptake (nmol/mg protein/min) was significantly reduced to about 42 % of the control values in the ischemic and IR groups (Figure. 4, Panel A and Table 2). Leupeptin treatment improved Ca^{2+}-uptake activity in IR hearts to 97 % of the control values. Ca^{2+}-uptake activity in the ischemic group did not show any significant recovery

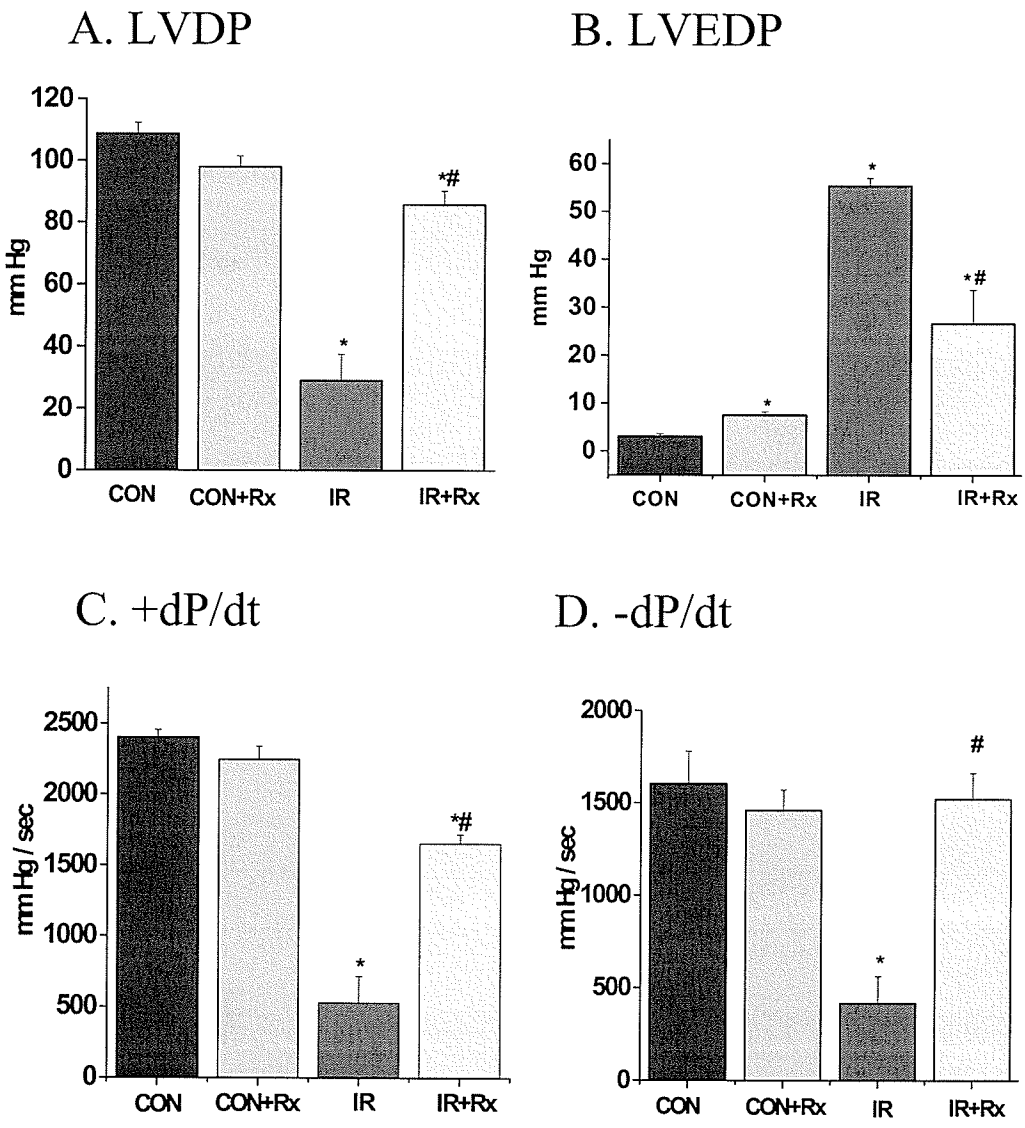


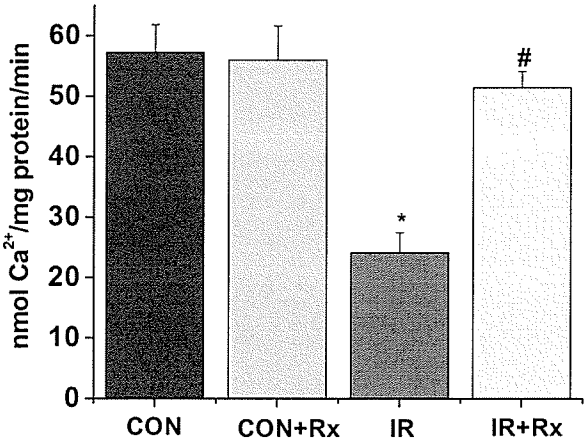
Figure.3. Cardiac function of the isolated perfused rat hearts subjected to I/R treated with and without leupeptin (25μM) compared to the control hearts. Panel A: Left ventricular developed pressure (LVDP), B: left ventricular end diastolic pressure (LVEDP). Panel C: Left ventricular pressure development (+dP/dt) and D: left ventricular pressure decay (-dP/dt). CON=Control (2hr), CON+Rx=control with leupeptin treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+Rx=IR hearts treated with leupeptin. [(n=6 for all groups). Data expressed as Mean ±SEM. *P<0.05 in comparison to control, # P<0.05 in comparison to the IR group].

Table 2. Cardiac function, Ca^{2+}uptake and cytosolic calpain activity of isolated perfused rat hearts pretreated with Leupeptin (10 min before onset of ischemia) and exposed to 30 min of global ischemia.

GROUP	LVDP (mm Hg)	LVEDP (mm Hg)	+dP/dt (mm Hg/sec)	-dP/dt (mm Hg/sec)	Ca^{2+} UPTAKE (nmol Ca^{2+} /mg protein)	CYTOSOLIC CALPAIN ACTIVITY
CON	98.0 ± 4.7	6.5 ± 0.5	2196.7 ± 116.3	1408.4 ± 81.0	46.1 ± 2.9	60.9 ± 10
CON +Rx	98.3 ± 5.9	9.8 ± 4.0	2219.0 ± 154	1428.0 ± 69.5	43.2 ± 3.0	62.5 ± 11
ISCH	1.4 ± 0.3*	43.1 ± 5.1*	17.1 ± 0.7*	17.1 ± 0.9*	18.9 ± 1.9*	106.2 ± 9.5*
ISCH +Rx	1.0 ± 0.1*	39.5 ± 6.5*	12.4 ± 1.1*	12.4 ± 1.0*	23.7 ± 2.9*	96.6 ± 11*

Cytosolic calpain activity is expressed as relative fluorescent units. n=5-6 hearts for all groups. Left ventricular developed pressure(LVDP), Left ventricular end diastolic pressure (LVEDP), Left ventricular pressure development and decay (±dP/dt). CON=Control (1hr), CON+Rx=control with leupeptin pretreatment, ISCH=30 min ischemia and ISCH+Rx=ischemic hearts with pretreatment. *P<0.05 in comparison to the control. Data expressed as Mean ±SEM.

A. SR Ca^{2+} uptake



B. SR Ca^{2+} release

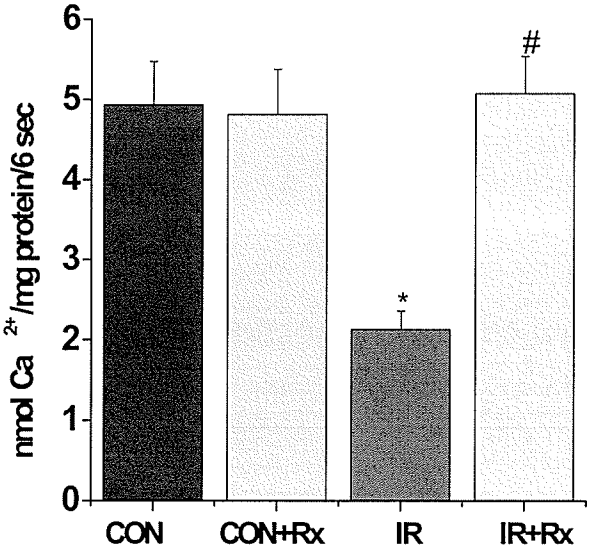


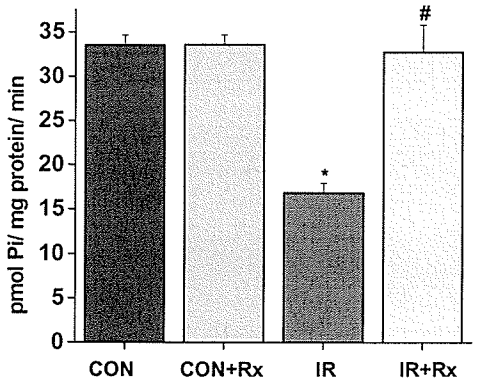
Figure.4. Effect of Leupeptin (25μM) on SR function in I/R hearts treated with and without leupeptin. (n=6). Panel A: Ca^{2+} uptake. Panel B: Ca^{2+} release. CON=Control (2hr), CON+Rx=control with leupeptin treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+Rx=IR hearts treated with leupeptin. [(n=6). Data expressed as Mean ±SEM. *$p<0.05$ in comparison to the control & # $p<0.05$ in comparison to the IR group)]

with leupeptin treatment for 10 min in the preischemic phase when compared to the ischemic group (Table 2). Control hearts treated with leupeptin showed no significant difference in SR Ca^{2+}-uptake activity in comparison to the control hearts.

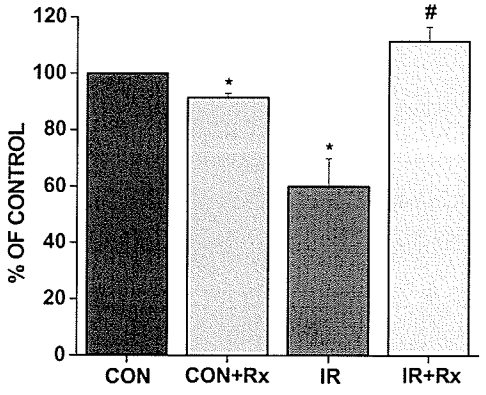
Ca^{2+}-induced Ca^{2+}-release (CICR) was significantly decreased in IR hearts to 43 % of the control values (Figure.4, Panel B). Leupeptin treatment markedly improved the SR Ca^{2+}-release from the I/R hearts to near normal values.

CaMK and PKA Activity. The SR associated CaMK and PKA phosphorylate SR calcium cycling proteins (SERCA2a, RyR and PLB). Thus changes in SR function may partly be linked to abnormalities in SR protein phosphorylation. Therefore we studied SR associated CaMK and PKA activity. SR CaMK and PKA activity was significantly reduced by 54 % and 51 % respectively in the IR group in comparison to the control group. This was consistent with the reduction in the protein content of these (Figure.5 and Figure.6). Treatment of the I/R hearts with leupeptin markedly improved the SR associated CaMK and PKA activities as well as their protein content. Leupeptin had no effect on the SR associated CaMK and PKA activities of control hearts. Hearts exposed to I/R showed a decreased protein content for CaMK II (δ isoform by 44 %) and PKA (α isoform by 40 %) in comparison to the controls. Treatment of the I/R hearts with leupeptin markedly improved the CaMK and PKA protein content (Figure 5 and 6, panel B &C).

A. SR CaM kinase II activity



B. Immunoreactive CaMK II (55kDa)



C. Comassie stained membrane

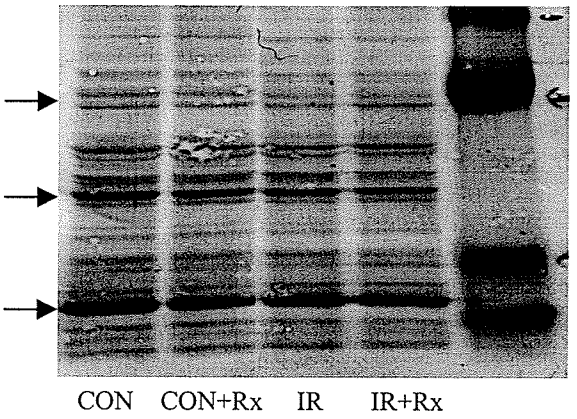


Figure.5. SR Calcium calmodulin dependent protein kinase-II (CaM kinase II) activity of the control and I/R hearts treated with and without Leupeptin (25μM). Panel A: SR CaMK II activity. Panel B: Western blot autoradiogram with analysis for CaMK II protein content. Panel C: Comassie stained membrane. Non specific bands (arrows) indicating loading across lanes. CON=Control (2hr), CON+Rx=control with leupeptin treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+Rx=IR hearts treated with leupeptin. [(n=5-7). Data expressed as Mean ±SEM.. (*$p<0.05$ in comparison to the control & # $p<0.05$ in comparison to the IR group)].

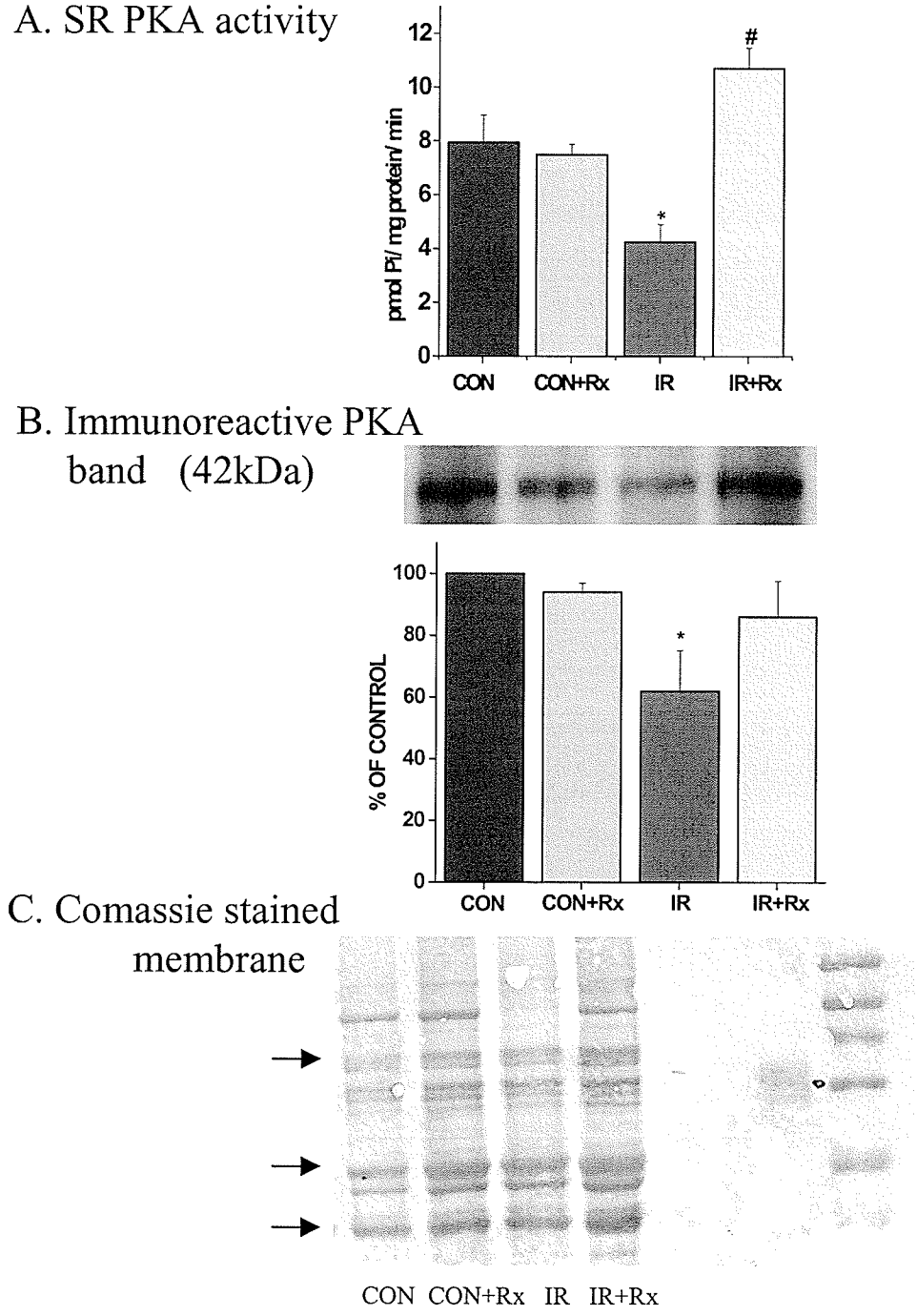


Figure.6. SR cAMP dependent protein kinase (PKA) activity of the control and I/R hearts treated with and without Leupeptin (25μM). Panel A: PKA activity. Panel B: Western blot autoradiogram and analysis for PKA protein content. Panel C: Comassie stained membrane. Non specific bands (arrows) indicating loading across lanes. CON=Control (2hr), CON+Rx=control with leupeptin treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+Rx=IR hearts treated with leupeptin. [(n=5-6). Data expressed as Mean ±SEM. (*$p<0.05$ in comparison to the control)].

To examine whether the changes in CaMK and PKA activities were restricted to the SR copartment only, we studied the activities of these enzymes in the cytosolic compartment also. But the cytosolic CaMK and PKA activity was unaffected by I/R in comparison to the control hearts.

SR protein content. Alterations in the SR function may be directly linked to changes in the expression of the SR - Ca^{2+} cycling proteins. Therefore we examined the content of SR proteins, namely: SERCA2a, RyR, PLB, and CQS. Reperfusion of the ischemic hearts decreased the protein content of SERCA2a (by 55%), RyR (by 70%), and PLB (by 40%) (Figures. 7, 8 and 10) in comparison to the controls. Leupeptin treatment recovered the protein content SERCA2a, RyR and PLB to normal values. The CQS protein content was higher in the IR group in comparison with control and leupeptin treated IR hearts (Figure. 9).

Calpain activity. We observed calpain activation during ischemia and IR, which is consistent with some of the other (21, 173, 185, 201) ex vivo studies that have shown similar results. The calpain activity from the cytosolic fraction of IR hearts was significantly higher than that seen in the ischemic hearts. IR hearts showed cytosolic calpain activity that was nine times of that observed in the respective perfused controls (Figure. 11) while the ischemic hearts showed twice as much calpain activity as the controls (Table 2). The calpain activity in the control hearts was insignificant. We did not do any differential studies to identify which isoform of calpain is being activated during ischemia and or IR and our results

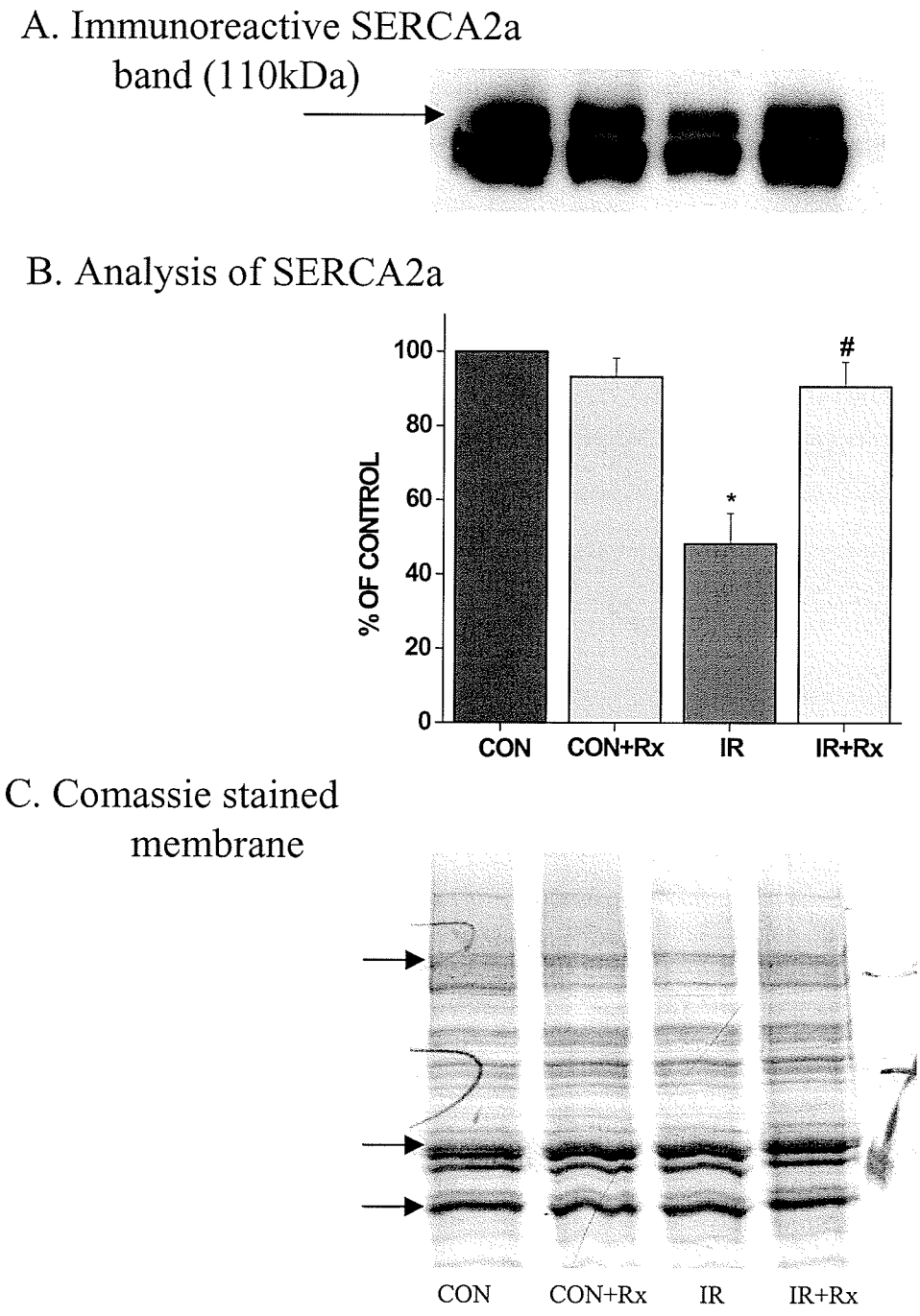
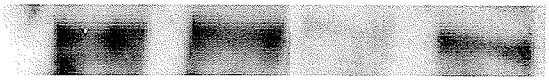
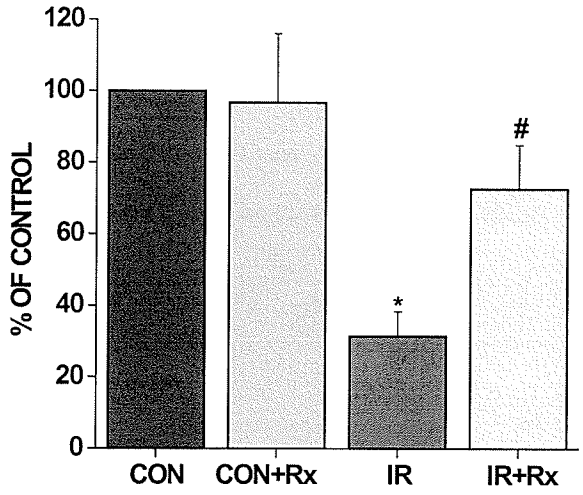


Figure. 7. Western blot analysis of sarco (endo) oplasmic reticulum Ca2+ATPase (SERCA2a) from SR preprations of control and IR hearts treated with and without leupeptin. Panel A: Autoradiogram of RyR. Panel B: Analysis of protein content. Panel C: Comassie stained membrane. Non specific bands (arrows) indicating loading across lanes. [(n=5-6 for all groups). Data expressed as Mean ± SEM. CON+Rx=control with leupeptin treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+Rx=IR hearts treated with leupeptin. *P<0.05 in comparison to the control and #P<0.05 in comparison to the IR group].

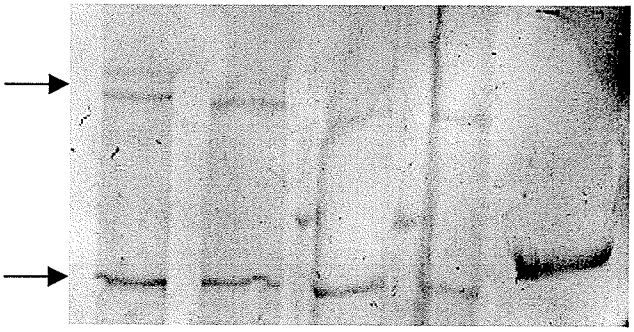
A. Immunoreactive RyR band (450kDa)



B. Analysis of RyR



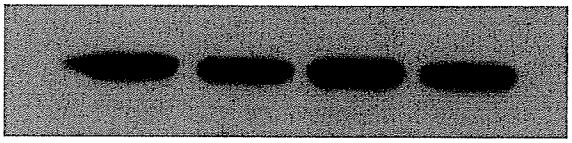
C. Comassie stained membrane



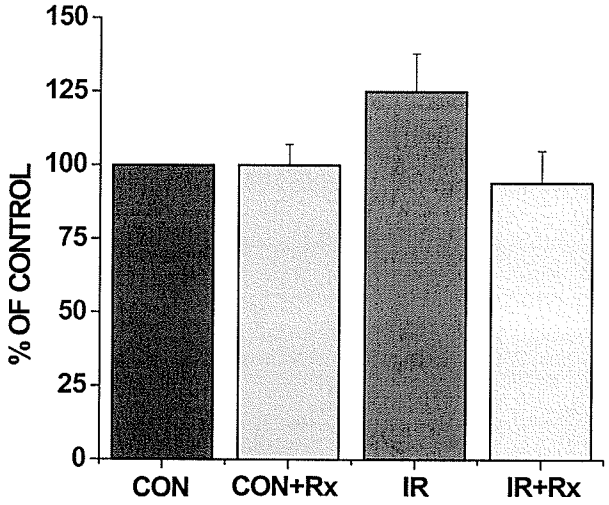
CON CON+Rx IR IR+Rx

Figure. 8. Western blot analysis of Ryanodine (RyR) receptor protein form SR preprations of control and IR hearts treated with and without leupeptin. Panel A: Autoradiogram of RyR. Panel B: analysis of protein content. Panel C: Comassie stained membrane of RyR. Non specific bands (arrows) indicating loading across lanes. [(n=5-6 for all groups). Data expressed as Mean ±SEM. CON+Rx=control with leupeptin treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+Rx=IR hearts treated with leupeptin. *P<0.05 in comparison to the control and #P<0.05 in comparison to the IR group].

A. Immunoreactive CQS band (60kDa)



B. Analysis of CQS



C. Comassie stained membrane

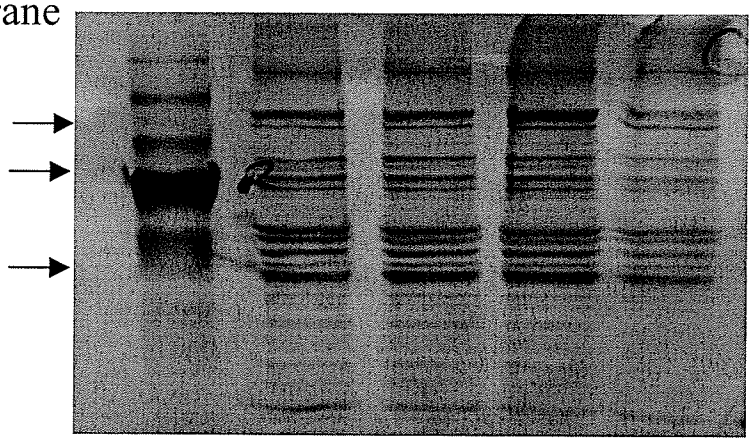
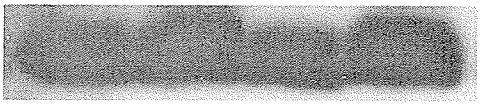
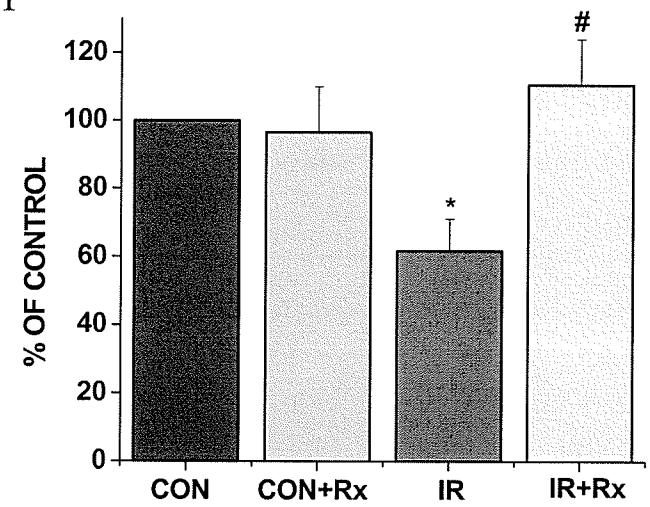


Figure. 9. Western blot analysis of Calsequestrin (CQS) from SR preprations of control and IR hearts treated with and without leupeptin. Panel A: Autoradiogram of CQS. Panel B: Analysis of protein content. Panel C: Comassie stained membrane. Non specific bands (arrows) indicating loading across lanes. [(n=5-6 for all groups). Data expressed as Mean ±SEM. CON+Rx=control with leupeptin treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+Rx=IR hearts treated with leupeptin.]

A. Immunoreactive PLB band (25kDa)



B. Analysis of PLB



C. Comassie stained membrane

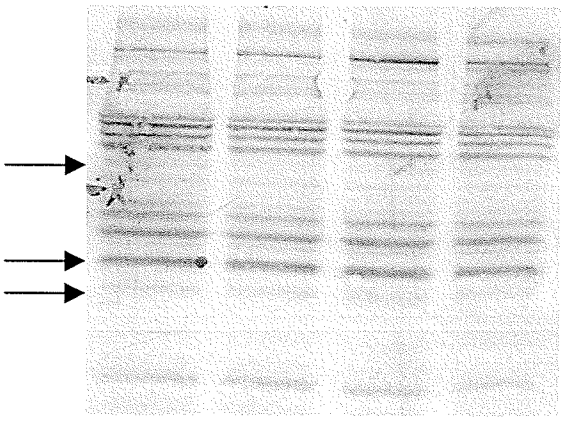


Figure. 10. Western blot analysis of Phospholamban (PLB) from SR preprations of control and IR hearts treated with and without leupeptin. Panel A: Autoradiogram of PLB. Panel B: Analysis of protein content. Panel C: Comassie stained membrane. Non specific bands (arrows) indicating loading across lanes. [(n=5-6 for all groups). Data expressed as Mean ±SEM. CON+Rx=control with leupeptin treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+Rx=IR hearts treated with leupeptin. *P<0.05 in comparison to the control and #P<0.05 in comparison to the IR group].

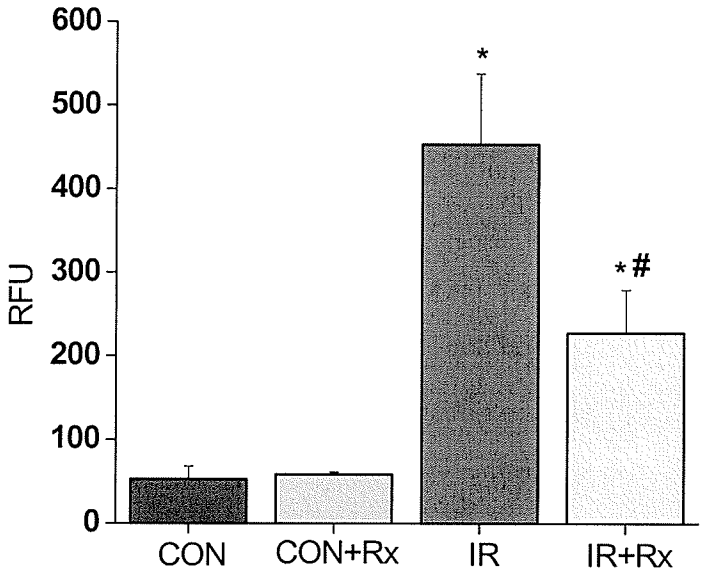


Figure. 11. Calpain activity of the cytosolic fraction from ischemia reperfused hearts treated with and without leupeptin in comparison to the control hearts. The results are expressed as relative fluorescent units (RFU). (n=5-6 for all groups. Data expressed as Mean ±SEM. P*<0.05 in comparison to the control. P #<0.05 in comparison to the IR group).

reflect the total cytosolic calpain activity. Our experiments for calpain activity in the SR fractions did not detect any cleavage of the fluroscent substrate indicating no calpain activity in the SR.

VI. Discussion

Our results show a depression in cardiac function as evident from a decrease in the LVDP, +dP/dt and –dP/dt and an increase in LVEDP during ischemia and IR (Figure.3, Table. 2). The SR plays a central role in cardiac contractility and depressed cardiac function could be due to a decrease in SR Ca^{2+} uptake and release activities as observed in our study. This decrease in SR function was observed during both ischemia and IR (Figure.4, Table 2). The decrease in the SR Ca^{2+} uptake and release may be due to a reduction in the protein content of the SR Ca^{2+} cycling proteins (SERCA2a, RyR and PLB) or due to a depression in the SR regulatory mechanism such as protein phosphorylation. Western blotting analysis showed a decrease in the protein content of the SR Ca^{2+} cycling proteins SERCA2a, RyR and PLB but not CQS during IR (Figure.7, 8, 9 and 10). The lack of change in the protein content of CQS suggests that reduction in content of SR proteins is not a generalized phenomenon in IR. This decrease in protein content could explain the SR dysfunction observed during IR. These results are consistent with previously published work from our laboratory (62, 63, and 202). The regulation of SR function was investigated by examining the activities of CaMK II and PKA. Our study shows that both CaMK II and PKA activities are depressed during IR (Figure. 5, 6). This was consistent with a reduction in the protein content of both CaMK II and PKA (Figure. 5, 6) and could explain the depressed activity of CaMK II and PKA. This is the first study to show a decrease in protein content of CaMK II and PKA in IR. Hence a decrease in the protein content of the SR Ca^{2+}

cycling and regulatory proteins could contribute for depression in SR Ca^{2+} uptake and release which in turn would lead to cardiac contractile dysfunction during IR. The *ex vivo* Langendorff perfused heart model used in this study is a well established model in our laboratory and has been used to study the different aspects of IR injury (62, 63).

Calpain activation observed in our study was consistent with some of the earlier studies that have reported calpain activation during IR (21, 26, and 173). Calpain activity was significantly higher during IR as compared to ischemia (Figure.11, Table.2). But calpain activity during ischemia was twice as compared to the respective perfused control hearts (Table 2). This would indicate that some amount of calpain activity is present even during ischemia contrary to an earlier report that had suggested calpain activation during IR and not ischemia (26). Calpain activity observed during IR was almost 9 times that of the respective perfused controls (Figure. 11). Calpain activity was minimal in control hearts perfused for 1 or 2 hours. In order to establish a link between the depressed cardiac function and calpain activation during ischemia and IR, we used a calpain inhibitor leupeptin to examine any beneficial effects. Experiments with different concentrations of leupeptin suggested that 25μM was the best dose (Table. 1). IR hearts treated with leupeptin during reperfusion showed an improvement in cardiac contractility and SR function (Figure. 5 and 6), and these experiments were consistent with a corresponding increase in the protein content of SERCA2a, RyR and PLB as compared to the IR Values (Figure. 7, 8 and 10). Leupeptin treatment

also improved the CaMK II and PKA activity and prevented a decrease in their respective protein contents during IR (Figure 5, 6). The cytosolic calpain activity in the IR hearts was significantly reduced by leupeptin treatment as compared to the untreated hearts (Figure. 11). Ischemic hearts treated with leupeptin before the onset of ischemia did not show any significant improvement in the contractile parameters or the SR function (Table. 2). Neither did treatment decrease the cytosolic calpain activity in the ischemic hearts (Table. 2). This would suggest that pretreatment with leupeptin does not afford protection against IR injury and that only treatment given at the onset of reperfusion was effective.

Activation of calpain may be responsible for the cardiac dysfunction observed in IR. Our results strongly suggest IR induced proteolytic degradation of SR Ca^{2+} cycling and regulatory proteins by calpain. This may be responsible for depressed SR function and its regulation because treatment with calpain inhibitor leupeptin partly or completely reversed these changes. The beneficial results of leupeptin (dissolved in water) that we have observed in our study compliment some of the other earlier works that have shown beneficial effects with leupeptin against calpain without using DMSO as a solvent (203, 204). Since we did not use dimethylsulfoxide (DMSO) as a solvent for leupeptin (highly water soluble) in our study, these beneficial effects cannot be attributed to DMSO as some previous studies have shown (21, 205). DMSO by itself has been shown to reduce the calpain activity in IR hearts (21, 173), and thus the results of some earlier studies seem questionable.

The proteolytic degradation observed cannot be attributed to lysosomal proteases like cathepsins as earlier studies have shown that they are released when irreversible myocellular damage appears (206, 207). Moreover cathepsins need an acidic pH for their activity, which can be attained during ischemia but acidosis is reversed within the first few minutes of reperfusion. This would make their role in IR injury seem doubtful. Kitakazae et al have shown that acidosis seen in early reperfusion actually protects against myocardial stunning (208). Therefore it is tempting to suggest that the decrease in protein content of SR Ca^{2+} cycling and regulatory proteins that we have observed in this study is due to the proteolytic action of calpain alone and leupeptin inhibition of calpain activity improves the cardiac dysfunction during IR. This may be a downstream mechanism of Ca^{2+}overload mediated cardiac injury in addition to the damaging effects of calpain on contractile proteins like troponin T and I and cytoskeletal proteins such as fodrin, desmin and α-actinin (21-25,27) and some of the SL channel proteins such as the L-type Ca^{2+} channel (24) observed by some other studies. Similarly, our work does not show any calpain activity in the SR samples from hearts exposed to IR (data not shown), an aspect that has not been looked into by earlier studies. We did not do differential studies to see which isoform of calpain is being activated during ischemia or IR. We intend to study the activation of specific calpain isoforms in IR during future studies. Yoshida et al (173) have shown that m-calpain activity is increased during ischemia but reperfusion decreases this activity and activates the μ-calpain isoforms instead. It is probable that the calpain activity we

observed during ischemia reflects an increase in activity of the m-calpain isoform. Calpastatin, the endogenous inhibitor of calpain has been shown to be degraded by calpain during ischemia and IR (21). It is possible that leupeptin may be acting by inhibiting the degradation of calpastatin during ischemia and IR. Employing *in vitro* techniques two earlier studies have suggested cardiac ryanodine receptor Ca^{2+} release channel and the Ca^{2+} pump ATPase as possible targets for calpain (25,26), and though specific antibodies had not been used to identify these respective proteins, the results are similar to our *ex vivo* findings. In our study we have used specific monoclonal antibodies for identifying the different SR proteins involved in Ca^{2+} homeostasis. This is therefore the first study to show that SR Ca^{2+} cycling and regulatory proteins may be target for calpain mediated proteolytic degradation/modulation resulting in SR dysfunction and eventually leading to cardiac contractile abnormalities.

VII. Conclusions

We studied the activation of calpain during IR and its effects on cardiac performance, SR function and the different SR proteins involved in Ca^{2+} homeostasis in an isolated perfused rat heart. These parameters were examined by perfusing the hearts with calpain inhibitor, leupeptin. Our results suggest the following:

1. Cardiac contractility was depressed in ischemia and IR and this depression was closely associated with SR dysfunction
2. The reduction in SR function correlated with a decrease in the SR protein content of RyR, SERCA2a and PLB during IR.
3. SR dysfunction was also associated with a depression in the SR CaMK II and PKA activities. This decrease was consistent with a reduction in protein content of CaMK II and PKA.
4. Perfused control hearts showed negligible calpain activity. Calpain activity was 9 times higher in hearts exposed to IR in comparison to controls.
5. Calpain activity during ischemia was significantly less than that observed during IR but twice that observed in comparison to controls.
6. Treatment with leupeptin attenuated calpain activity during IR and improved the cardiac contractility and SR function. This correlated with an increase in the SR protein content and kinase activity.

VIII. References

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