L-ARGININE ATTENUATES ISCHEMIA REPERFUSION INDUCED CHANGES IN CARDIAC PERFORMANCE AND SARCOPLASMIC RETICULUM FUNCTION

By

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A Thesis submitted to the University of Manitoba

In partial fulfillment of the requirements

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

MASTER OF SCIENCE

DEDICATED TO

MY PARENTS, FOR THEIR INSPIRATION AND

ENCOURAGEMENT.

MY HUSBAND AND BEST FRIEND, Dr. RAJA BALRAJ SINGH

FOR ALL HIS SUPPORT.

MY SON YUVRAJ SINGH, WHOSE AFFECTION AND LOVE

MADE ME A PROUD MOTHER AND WHOSE AMAZING CO-

OPERATION AND UNDERSTANDING HELPED ME ACHIEVE

WHAT I HAD SET OUT TO.

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ANOVA……analysis of variance

ATP……adenosine triphosphate

BSA……bovine serum albumin

Ca^{2+}……calcium

NO……nitric oxide

LA ……L-arginine

CAMK……Ca^{2+}/ Calmoculin dependent protein kinase

cAMP……cyclic adenine mono phosphate

PKA……cAMP dependent protein kinase

cGMP……cyclic guanosine mono phosphate

GTP……Guanosine tri -phosphate

PKG……cGMP dependent protein kinase

L-NAME……N^G-nitro-L-Arginine methyl ester

CON……control hearts

+dP/dt……rate of pressure development

-dP/dt ……rate of pressure decay

IR……Ischemia Reperfusion

H^+……hydrogen ion / proton

O_2^-……superoxide

$ONOO^-$……peroxynitrite

IR+LA ……ischemia-reperfusion hearts treated with L-arginine

K^+ .....potassium ion

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

LVDP.....left ventricular developed pressure

LVEDP .....left ventricular end diastolic pressure

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

Na^+.....sodium

P_i.....inorganic phosphate

PLB.....phospholamban

ROS.....reactive oxygen species

RNS.....reactive nitrogen species

RyR.....ryanodine receptor

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

NADH.....Nicotinamide adenine dinucleotide

NADPH.....Nicotinamide adenine dinucleotide phosphate

TNFα.....Tumor necrosis factor α

FAD.....Flavin adenine dinuleotide

FMN.....Flavin mono nucleotide

AEBMSF.....4-(2-Aminoethyl) benzenemethylsulfonyl fluoride

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ABSTRACT

Ischemia reperfusion (IR) injury is associated with a spectrum of events such as myocardial stunning, ventricular arrhythmias and cell death. It is well documented in the clinical settings including coronary angioplasty, cardiac bypass surgery and heart transplantation. Although various mechanisms have been suggested to explain the pathogenesis of myocardial IR injury, oxygen free radicals and cytosolic calcium (Ca^{2+}) overload have emerged as the prominent and mutually non-exclusive pathways mediating this phenomenon. Since nitric oxide (NO) is an important modulator of cardiac contractility with profound effects on both systolic and diastolic phases, extensive research exploring the role of NO in myocardial IR injury has been done. In view of the fact that the sarcoplasmic reticulum (SR) has a central role in maintaining Ca^{2+} homeostasis in the cardiac myocytes and is an important determinant of cardiac contractility; we studied the effects of exogenous NO upon the administration of its precursor (L-arginine) on the IR induced SR dysfunction as a possible mechanism for contractile abnormalities. Isolated perfused rat heart subjected to 30 min global ischemia followed by 60 min reperfusion was used as a model for IR injury. IR caused a depression in cardiac function and reduction in SR Ca^{2+} uptake and release. L-arginine treatment caused significant recovery in cardiac contractile function in IR hearts and prevented the decrease in SR Ca^{2+} uptake and release. IR induced depression in SR Ca^{2+} cycling proteins as well as Ca^{2+} regulating proteins were also attenuated by L-arginine. Treatment of IR hearts with NO synthase (NOS) inhibitor, L-NAME, ameliorated

the functional recovery seen with L-arginine treatment. Thus our results show that administration of L-arginine attenuated the IR induced cardiac contractile abnormalities and SR dysfunction.

INTRODUCTION

Significant work has been done in the last three decades in myocardial ischemia and subsequent effects induced by reperfusion of the ischemic myocardium (1,2). Many laboratories have shown functional, biochemical, ultrastructural and microvascular damage after brief periods of myocardial ischemia. From these, the concept of "myocardial stunning" has emerged that refers to prolonged, reversible changes in myocardial contractile function following a single or multiple periods of regional ischemia as well as global ischemia (3). The severity and duration of these post-ischemic changes depend on the length and intensity of the ischemia, as well as on the condition of the myocardium at the onset of the ischemic episode. Stunning also occurs in a several other situations including in myocardium located adjacent to infarcted tissue, transient increase in myocardial O_2 demands in the presence of incomplete coronary obstruction during both systole and diastole, in isolated perfused hearts rendered ischemic or anoxic, and in clinical situations, such as following ischemic arrest in open heart surgery, thrombolytic reperfusion, and in patients with coronary artery disease, such as unstable angina, exercise induced ischemia and angioplasty (4,5). Stunning is considered to be the fundamental component of hibernating myocardium, in which there is persistent contractile dysfunction in a viable myocardial tissue with reduced coronary blood flow (6).

Various experimental approaches (from brief episode to prolonged partial ischemia) and animal models have been developed to investigate the pathogenesis

of myocardial stunning. The most commonly used laboratory model to study myocardial stunning is the isolated Langendorff method of retrograde perfused heart. This model has proven to be useful for understanding the pathogenetic mechanisms of myocardial stunning at the level of excitation contraction coupling (7,8). Due to various experimental approaches using various species, different theories to explain the development of myocardial stunning primarily diverge into two hypotheses, oxidative stress caused by reactive oxygen species (ROS) and cytosolic Ca^{2+} overload (1,2,9,10). The oxidative stress results due to the excessive formation of oxidants and depletion of natural antioxidant mechanisms in the heart. The major oxidants involved in causing myocardial injury include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). These oxygen free radicals and oxidants are reported to cause lipid peroxidation altering membrane permeability and cause defects in the sarcolemmal proteins such as Ca^{2+} ATPase and Na^+-K^+ ATPase and intracellular proteins such as SR Ca^{2+} ATPase affecting normal Ca^{2+} homeostasis. The Ca^{2+} hypothesis postulates that altered intracellular Ca^{2+} homoeostasis may result in myocardial stunning. (These mechanisms are discussed in detail elsewhere). The former hypothesis was tested primarily in *in vivo* experimental settings using large mammalian models such as dogs and pigs, where as the latter was tested in *in vitro* experimental settings using rodent models. Consequently, it is conceivable that the divergent results can be explained by species difference, as well as differences in the experimental settings. There have been some other mechanisms put forth to

explain the contractile dysfunction in stunned myocardium. Insufficient energy production, resulting from decrease in ATP levels, was blamed in some earlier studies. However, numerous subsequent studies found no correlation between myocardial ATP levels and contractile dysfunction during post-ischemic phase. Other proposed mechanisms include impaired energy use by myofibrils, impairment in sympathetic neural responsiveness, impairment in myocardial perfusion or damage to extracellular collagen matrix. The studies on alterations in gene expression in large mammalian models of IR demonstrate that stunning elicits broad changes in gene and protein regulation not observed in the heart previously (11,12).

The involvement of NO in myocardial IR has attracted a great deal of interest in many research laboratories. In 1999, Xu and colleagues localized neuronal type nitric oxide synthase (nNOS) in the murine and human cardiac SR. In addition, other NOS isoforms have also been localized in the cardiac SL and mitochondria. It can therefore be inferred that NO may have important impact on the intracellular Ca^{2+} levels and thus the cardiac contractility. The mechanisms underlying are not very clear. The release of NO is reported to be markedly impaired and early event during the reperfusion period. Different modalities of NO replacement such as, NO gas, inorganic or organic NO donating compounds or the NO precursor substrates such as L-arginine are widely used to treat the deleterious consequences of the reperfusion injury. Majority of research so far has demonstrated that NO replacement therapy is capable of producing

cardioprotection in the settings of myocardial IR. Recent development of transgenic mice deficient in various NOS isoforms has helped tremendously to elucidate the pathophysiological roles of NO in IR injury.

REVIEW OF LITERATURE

Calcium (Ca^{2+}) is a ubiquitous second messenger molecule essential for electrical activity and contractility of heart. Its critical physiological role in muscle contraction was first demonstrated about 120 years ago in 1883 when Ringer showed that the presence of extracellular Ca^{2+} is essential for the maintenance of contraction in frog hearts (13). It is now well established that Ca^{2+} plays an important role in cellular integrity, regulation of metabolism, cell growth and differentiation (14). In human heart cells Ca^{2+} also regulate pacemaking and atrioventicular conduction (15). A steep electrochemical for Ca^{2+} (about 100,000 fold) exists across the sarcolemma (SL) membrane with extracellular Ca^{2+} concentration of 10^{-3} M and intracellular free Ca^{2+} concentration of 10^{-6} M. Such a concentration gradient is attributed to intricate but coordinated functioning of different cellular organelles such as SL, SR and the mitochondria. Since SR plays a major role in the regulation of intracellular Ca^{2+} handling in cardiomyocytes, this review of literature is focused on discussing the SR function in normal heart and in disease pathologies such as IR injury.

Ca^{2+} Homeostasis in the normal heart

SL and SR are the most important cellular organelles that regulate intracellular Ca^{2+}concentrations in the cardiomyocyte and thus Ca^{2+}homeostasis during the systolic and diastolic phases of cardiac contraction. Excitation- contraction - relaxation (E-C-R) coupling in normal cardiac muscle is initiated with the wave of electrical excitation that depolarizes the SL membrane. This permits the influx of

small amount of Ca^{2+} from the extracellular space via the voltage gated L-type Ca^{2+} channel (also called dihydropyridine receptors). This slight rise in intracellular Ca^{2+} triggers the release of relatively large amount of Ca^{2+} from the SR stores through the Ca^{2+} release channel. This phenomenon, known as calcium induced calcium release (CICR) (16,17) is fundamental to cardiac contractility and links surface membrane depolarization to Ca^{2+} activation of the contractile apparatus (18). The amount of Ca^{2+} released depends on the SR Ca^{2+} content as well as the intensity of SL Ca^{2+} current (19). This permits Ca^{2+} transient amplitude to be graded, a unique feature of cardiac tissue where all cells contract simultaneously (20). However, it was also shown that Ca^{2+} release does not occur from all portions of the SR and it is not regenerative, so it was hypothesized that CICR is under local control (21). The transient increase in free Ca^{2+} activates the myofilaments by binding to troponin C, which then switches off the inhibitory complex and allows the actomyosin interaction and cross bridge formation. As a result there is cell contraction with the generation of force.

For the occurrence of diastolic relaxation multiple cellular processes operate to extrude Ca^{2+} back to extracellular space and thus restore intracellular Ca^{2+} to the resting level. a) **SR Ca^{2+} pump ATPase**: actively pumps major portion of Ca^{2+} from the cytosol to the SR (22,23). b) **Sodium calcium exchanger** (NCX): extrudes some of the Ca^{2+} out of the cytosol (24,25). **SL Ca^{2+} pump ATPase**: pumps put negligible amount of Ca^{2+} out of the cell. d) **Mitochondrial uniport**: located within the inner mitochondrial membrane. Although this is inconsequential

with regards to cardiac contraction since a small amount of Ca^{2+} is accumulated, this Ca^{2+}can stimulate several Krebs' cycle enzymes and ATP synthesis that can match energy demand and energy supply (26,27). It has been shown that relative concentration of these mechanisms is species dependent (28,29). In rat ventricular myocardium, SR Ca^{2+} ATPase pump is higher than rabbit due to greater concentration of the pump molecules and is responsible for 92% of Ca^{2+} uptake while 71% of Ca^{2+} is removed by NCX. In humans, rabbit, cat, ferret and guinea pigs, the SR Ca^{2+} ATPase pump removes 70% of Ca^{2+}, whereas 25-30% is removed by NCX.

Structural aspect of SR system

Using suitable ultra centrifugation techniques SR membranes can be isolated. The isolated SR appears granular or vesicular on gross examination under a light microscope. Freeze fracture electron micrograph revealed that these vesicles are composed of a fine network system enclosed by a lipid bilayer and consists of three components (30,31); a) *Longitudinal or network SR*: It is a major region spreading throughout the myocyte and surrounds the myofibrils. This area is concerned with the uptake of Ca^{2+} from the cytosol and initiate relaxation (32). b) *Junctional SR*: This expanded part lies in close proximity to the T-tubular systems of sarcolemmal membrane, also known as the sub-sarcolemmal cisternae. These functional units release Ca^{2+} to initiate the contractile cycle. The cisternae and T tubules are connected by electron dense feet at the coupling site (33). c) *Corbular SR:* This region is confined to the I-band of the sarcomere. Both the junctional SR

and corbular SR are extensions of the network SR. The SR membrane is composed of several intrinsic proteins that are key regulators of cardiac ECR coupling and of functional significance. These include the Ca^{2+}–release channel or ryanodine receptor (RyR), Ca^{2+} pump ATPase (SERCA2a), phospholamban (PLB) and calsequestrin (CQS). The detail of each of these proteins is given below.

SR Ca^{2+} –release channel (RyR)

Cardiac contraction is triggered by the release of Ca^{2+} from the SR Ca^{2+} release channels, also referred to as the ryanodine receptors (RyR). Majority of the channels are located in the SR cisternae that lie in proximity to the T tubule invaginations of the sarcolemmal membrane. RyR lie in close association with L-type Ca^{2+} channels and this spatial association of the two channels allows the CICR phenomenon and is critical for the signal amplification process of the cardiac ECR coupling. The channel acquired this nomenclature from its capability to bind the plant alkaloid ryanodine. At lower concentrations (<10 μM) ryanodine keep the channel in the open state and low conductance configuration (34); at higher concentrations it can completely close the channel (35). This channel is also opened by methylxanthines such as caffeine (36). Apart from RyR, the other release channel on cardiac SR is 1, 4, 5 triphosphate sensitive channels (37,38). It contributes negligibly to Ca^{2+} release required for contraction but might be important for intercellular coupling. Using molecular cloning and cDNA sequencing analysis, RyR was identified in three different isoforms, RyR1, RyR2, RyR3 encoded by three different genes (39). RyR2 is the only isoform expressed

in cardiac tissue and is considered the largest protein identified in the SR consisting of 4969 amino acids with a molecular weight of 565 kDa (40). The unusual high molecular mass of RyR may be due to numerous endogenous modulatory ligands such as Ca^{2+}, calmodulin, nitric oxide, L-type Ca^{2+} channels and an associated protein known as FK 506 binding protein (FKBP). RyR forms a functional complex due to its association with several other proteins such as CQS, junctin, triadin and FKBP (41). This large protein forms the foot structure seen in electron micrograph of cardiac diads (42). RyR is known to be phosphorylated by Ca^{2+}/calmodulin dependent protein kinase (CaMK) and by cyclic adenine monophosphate (cAMP) –dependent protein kinase (PKA) at Ser -2809.

The sarco-endoplasmic reticulum Ca^{2+} –pump ATPase (SERCA)

This protein is responsible for the uptake of Ca^{2+} from the cytosol to SR lumen. SERCA2a activity determines the amount of sequestered Ca^{2+} to be available for release in the next wave of excitation and is responsible for the restoration of Ca^{2+} gradient between intracellular compartment and SR lumen side. Molecular cloning analysis has identified three SERCA genes, SERCA 1, 2, and 3 (43) which is spliced alternatively in several isoforms. SERCA1a is mainly expressed in fast twitch skeletal muscle while SERCA1b is abundant in fetal and neonatal stages (44). SERCA2a is the cardiac and slow twitch skeletal isoform (45) whereas SERCA2b is expressed in non-muscle and neuronal cells (46). SERCA3 is mainly expressed in epithelial and endothelial cell types (47). It is now well established that SERCA2a is the only isoform that is expressed in normal or stressed

myocardium (48). The SERCA2a pump (105 kDa), a Ca^{2+} stimulated and Mg^{2+} - dependent ATPase protein (49) constitutes 35-40% of the SR proteins (50) and is concentrated in the longitudinal component of SR (51). SERCA actively transport two Ca^{2+}-ions per ATP molecule hydrolysis against a high ionic gradient ranging between 100 nM -10 μM in the cytosol and 1 nM in the SR (52). The function and Ca^{2+} activation of SERCA can be modulated by several factors. The most predominant, indirect mechanism being the phosphoprotein phospholamban. It will be discussed later in the section. As a direct modulation, SERCA2a is under the control of Ca^{2+}/calmodulin dependant protein kinase (CAMK), which phosphorylates SERCA on serine (Ser) -38 and enhances the V_{max} for Ca^{2+} transport activity (by increasing ATP hydrolysis) to facilitate Ca^{2+} transport into the SR (53,54) Other direct factors that modulate SERCA activity and expression are thyroid hormones, insulin, and nitrosylation.

Phospholamban (PLB)

PLB is key SR membrane protein that critically regulates the SERCA2a function and thus cardiac contractility (55,56,57). It has been shown to decrease the apparent Ca^{2+} affinity of SERCA2a in its dephosphorylated form, making available more Ca^{2+} for the cardiac contraction (inotropic effect) (58,59). This dephosphorylation occurs via an SR associated type-1 phosphatase (60). During β-adrenergic stimulation, increased levels of cAMP cause phosphorylation of PLB that removes its inhibition on SERCA and facilitates Ca^{2+} transport into the SR, thereby causing cardiac relaxation (lusitropic effect) (61,62). PLB can be

phosphorylated at different sites by various protein kinases, at threonine (Thr)-17 by Ca^{2+}/calmodulin dependent protein kinase (63), at Ser-16 by cAMP dependent protein kinase (PKA) (64,65) and at Ser-10 by protein kinase C (66). It has been shown that Ser-16 phosphorylation precedes Thr-17 or Ser-10 phosphorylation and is also responsible for the β-adrenergic increase in cardiac relaxation (61,67).

Two possible mechanisms have been proposed to explain the stimulation of Ca^{2+} uptake by PKA dependent phosphorylation. The enhanced SERCA2a turnover rate and increased efficiency of the SERCA2a for Ca^{2+}or an increased coupling ratio (68,79). It is a remarkably conserved protein since it is coded by one gene in all species and is expressed predominantly in cardiac muscle, small amounts in slow twitch skeletal muscle (56). Cardiac PLB protein is comprised of two molecular weight forms (70), the monomeric active form (6 kDa) and the pentameric form (27 kDa), which is suggested to be the inactive reservoir for the monomeric form (71). The oligomeric structure has been confirmed by the fact that pentameric form, which is composed of 5 identical subunits, upon boiling dissociates into identical monomers having 52 amino acids (72). The PLB monomer contains two major domains. 1) Highly hydrophobic C-terminal domain or transmembrane domain, inserted into the SR membrane and functions as inhibitory domain that regulate affinity of SERCA for $Ca^{2+}$and 2) N-terminal amphiphilic cytosolic domain containing cytosolic IA domain that modulates transmembrane inhibitory activity and cytosolic IB domain which is involved in regulatory interaction between PLB and SERCA2a (73). It has been proposed that

PLB exists in its pentameric structure so as to form a hydrophobic Ca^{2+} selective ion channel pore in the lipid bilayer (74). Understanding the dynamics of PLB interaction with SERCA2a may prove an effective tool whereby in vivo inhibition of PLB activity can be used to enhance the cardiac contractility and maintain Ca^{2+} homeostasis in various cardiac pathologies.

Calsequestrin (CQS)

CQS is a moderate affinity and high capacity Ca^{2+} binding (≈50mol Ca^{2+} per mol protein) glycoprotein, localized in the lumen of the junctional SR (75,76). The primary physiological function of CQS is sequestration of large amount of Ca^{2+} in the lumen of SR, thereby reducing luminal levels of free Ca^{2+} and facilitating further uptake by the SERCA pump. Among the two isoforms, cardiac and fast skeletal muscle, only one is expressed in the developing adult and aging cardiac tissue (77,78,79). CQS (55 kDa) is composed of 396 amino acid residues based on SDS-PAGE analysis (80). It forms a network in the center of the terminal cisternae and is anchored to the junctional portion of SR membrane in close proximity with the RyR (81). Traidin has been proposed to link CQS with the junctional SR membrane and therefore to the RyR. The direct intermolecular interaction between the two proteins may be involved in regulating coordinated Ca^{2+} release (82). In addition, CQS has been proposed to function as Ca^{2+} buffer that reduces the lumen Ca^{2+} gradient and avoid intraluminal Ca^{2+} overload. CQS protein contains highly acidic C terminal tail (361-391) containing three closely spaced Ser residues, which may act as a substrate for the casein kinase II (83). Later Cala and Jones

localized endogenous P_i in the cluster of Ser residues and demonstrated that Ser-378 was rapidly phosphorylated in vivo by casein kinase II (84). However the functional significance of such phosphorylation event is presently unknown.

Factors modulating SR function and Regulation

The phosphorylation and dephosphorylation of SR Ca^{2+} cycling proteins are the key regulatory processes that modulate the movement of Ca^{2+} during cardiac contractile cycle. These events are accomplished by an SR associated CaMK (85) and PKA phosphorylation of these proteins (86). CaMK phosphorylates RyR, SERCA2a, and PLB whereas PKA phosphorylates RyR and PLB (87,88). The dephosphorylation mechanisms that exist serve to reverse the effects of phosphorylation and this is fulfilled by protein phosphatases, which hydrolyze the phospho-ester bond formed by protein kinases. These phosphatases are therefore involved intricately in regulating signal transduction pathways and the cellular proteins (89) and dephosphorylate both CaMK and PKA phosphorylated substrates (90). SERCA2a affinity for Ca^{2+} is inhibited by the protein-protein interaction on its cytoplasmic and transmembrane domains by the dephosphorylated form of PLB during diastole (91). 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. PLB phosphorylation by PKA and CaMK relieves this inhibition resulting in increased SERCA2a affinity for Ca^{2+}, enhanced SR Ca^{2+}-uptake and improved cardiac relaxation. SR releases its load of Ca^{2+} with the next wave of depolarization. There

is also a specific RyR site (Ser-2809), which is phosphorylated by CaMK (92). Such a phosphorylation may either increase or decrease RyR channel opening (93).

Thus it is evident from the preceding discussion that Ca^{2+} homeostasis in the myocyte is strictly maintained by the interplay of membrane proteins like RyR, SERCA2a, PLB and CQS and their phosphorylated - dephosphorylated state controlled by various protein kinases and protein phosphatases. IR injury affects some of these Ca^{2+} regulating mechanisms resulting in calcium overload and contractile dysfunction.

PATHOGENESIS OF MYOCARDIAL IR INJURY

OXIDATIVE STRESS

Oxidative stress is a detrimental state induced by excessive formation of reactive oxygen species (ROS). ROS are highly reactive species due to the presence of one or more unpaired electrons in their outer orbits. The most commonly encountered ROS radicals in the biological systems include superoxide (O_2^-), hydroxyl (OH^-) and hydrogen peroxide (H_2O_2). The initial proposition for the harmful effects of the ROS was provided by Geschman et al (94). In 1980's the role of ROS in relation to stunned myocardium was studied by many investigators in anesthetized open chest dog model (95,96,97). These studies demonstrated that administration of free radical scavengers improved the functional recovery of stunned myocardium. These type of results with oxygen radical scavengers provided indirect evidence for a causative role of ROS in the pathogenesis of myocardial stunning. The first definitive evidence that demonstrated the production of ROS after myocardial stunning was provided by Bolli and colleagues by the use of alpha –phenyl N –tert –butyl nitrone (PBN) and electron paramagnetic resonance spectroscopy (EPR) (98). They also found that the administration of hydroxyl radical scavenger mercaptopropionylglycine (MPG) one minute before reperfusion resulted in a marked improvement in regional function, which suggested that myocardial stunning is primarily the result of reperfusion injury consequent to a burst to ROS released during the first few minutes of reflow (99).

Sources of free radicals

Free radicals are produced under normal physiological circumstances by the electron transport chain in the mitochondrial matrix during oxidative phosphorylation (100). Although 98% of the high-energy electrons are involved in the ATP generation, about 1-2% results in the O_2^- formation. This was reported to happen at the level of complex I, II (101) or III in the mitochondria (102,103,104). These free radicals are neutralized by the cellular antioxidant defense mechanisms that include various enzymes such as mitochondrial manganese, cytosolic copper and zinc superoxide dismutase (Mn-SOD and Cu/Zn-SOD), and glutathione peroxidase (GSH-PO) (105). Various SOD enzymes scavenge O_2^- to form H_2O_2 that forms water and molecular oxygen under normal conditions, but under the stressful states may form highly reactive hydroxyl ion (OH^-) via the Fenton (106) and Haber-Weiss reactions (107). NAD(P)H oxidases mediate the flow of electrons from the substrate NADH (108,109) or NADPH (110) to the molecular oxygen forming superoxide. They are produced by the neutrophils (phagocytic) and also by the cells in the vasculature and the cardiac myocytes (non phagocytic). These oxidases are the major sources of superoxide production in response to stimuli like cytokines or certain types of growth factors.

Role in IR

The oxidative stress is considered to be a fundamental event in causing series of events observed during I/R injury. The ROS are reported to modulate gene expression as shown by upregulation of growth factor gene (111), decrease in the

mRNA levels for myofibrillar proteins (112), decrease in the mRNA levels for the SR proteins (113), induction of proto-oncogenes such as c-fos and c-jun mRNA which represent a rapid mechanism of transcriptional adaptation to stress (114). The O_2^- metabolites also induce lipid peroxidation of polyunsaturated fatty acids of the cellular membranes (115). This results in their disruption and increased permeability to several ions. In addition, lipid peroxidation also produces cytotoxic and mutagenic metabolites, which are capable of causing dysfunction of various subcellular proteins. ROS can also mediate oxidation of thiol groups contained within structural proteins such as ryanodine receptors (116), protein unfolding (117,118) as well as protein fragmentation and polymerization (119). Oxidative stress also results in depression of SL Ca^{2+} ATPase and Na^+ K^+ ATPase activities, which cause decreased Ca^{2+} efflux and increased Ca^{2+} influx (120,121). There is depression of SR Ca^{2+}ATPase activity and thus cause inhibition of Ca^{2+} sequestration from the cytosol resulting in Ca^{2+} overload (122,123,124).

ALTERED CALCIUM HOMEOSTASIS

A) INTRACELLULAR Ca^{2+} OVERLOAD

Kusuoka and associates (125) were the first to suggest that a transient period of Ca^{2+} overload during early reperfusion may be a critical factor in causing myocardial stunning in isovolumic ferret hearts perfused in the Langendorff mode. Later, Marban and associates (126) directly measured intracellular free Ca^{2+} and found that intracellular Ca^{2+} significantly increased during the first 15 minutes of ischemia and decreased rapidly during reperfusion. Different mechanisms operate

to induce Ca^{2+} overload. During the ischemic episode there is depression of mitochondrial oxidative phosphorylation due to lack of or decreased supply of O_2. This will lead to rapid depletion of high-energy phosphate compounds and an accumulation of H^+ and inorganic phosphates (P_i), causing acidosis. All these factors cause a shift in myocardial metabolism from aerobic to anaerobic. This cytoplasmic acidification causes activation of Na^+-H^+ exchanger (NHX) (127) resulting in an increase in the intracellular concentration of Na^+. Under normal conditions, the rate of Na^+ entry (via NHX) and efflux (via Na^+-K^+ ATPase) would balance each other (128). Lack of ATP also inhibits the SL Na^+-K^+ ATPase activity (129, 130) further increasing the intracellular concentration of Na^+. This was confirmed by NMR measurements showing that the intracellular concentration of Na^+ is increased during ischemia (131). Until reperfusion begins, Na^+ accumulates and the acidosis prevents the activity of Na^+-Ca^{2+} exchanger (NCX). Reperfusion of the myocardium causes reversal of the acidosis and subsequently activates NCX, which works in a reverse mode (132) to expel Na^+ out of the cell in exchange for extracellular Ca^{2+} resulting in Ca^{2+} overload. This rise in intracellular Ca^{2+} occurs during 9-15 min of ischemia (133). Many other factors are also implicated in the causation of intracellular Ca^{2+} overload. Abnormal cardiac membrane permeability due to incorporation of long chain fatty acids resulting from impaired mitochondrial function during ischemia also contributes to Ca^{2+} overload (134). Due to the lack of ATP, impairment of mechanisms responsible for the removal of Ca^{2+} from the cytosol either to the

extracellular space or to the Ca^{2+} storage sites, such as SL Ca^{2+} ATPase and SR Ca^{2+} ATP ase respectively, would cause Ca^{2+} overload. SR Ca^{2+} uptake and release activities due to defects in regulation of SR function by phosphorylation and dephosphoryation mechanisms have been reported to be impaired in ischemia. This may also lead to Ca^{2+} overload. Increase in the Ca^{2+} influx through the L-type Ca^{2+} channels is also held responsible for the transient Ca^{2+} overload. Support for this has emerged from several studies in which it has been shown that L-type Ca^{2+} channel blockers such as verapamil or nifedipine attenuate stunning (135,136)

B) DECREASED MYOFILAMENT SENSITIVITY TO CALCIUM

It has been shown by number of investigators that transient Ca^{2+} overload in stunned myocardium results in a decreased responsiveness of the myofilaments to intracellular Ca^{2+} as manifested by decrease in the maximal force generation by the cardiac myocyte and relative insensitivity to extracellular Ca^{2+}. The underlying mechanisms are not clearly known. It has been that Ca^{2+} overload activates Ca^{2+} dependent protease Calpain-I (137,138) that causes proteolytic degardation, dephosphorylation and covalent modification of myofilament proteins, which can result in decreased responsiveness. Oxidative stress is also an important mechanism. Previous studies from our laboratory (139) and also from Marban's group (140) have shown that ROS causes oxidation of the critical thiol groups in the myofilments that may underlie the decreased responsiveness to Ca^{2+}.

NITRIC OXIDE (NO)

NO has one of the most prestigious and unique historical backgrounds. Initially considered to be just a simple atmospheric pollutant, it has traveled a long way today to become the most sought after molecule in the research arena. *Science,* honored NO as the molecule of the year in the year 1992 (141). In the year 1998 Robert Furchgott, Louis Ignarro and Ferid Murad were jointly awarded Nobel Prize for their remarkable work in the field of NO. In the present scenario it is considered to be the universal regulator of metabolism. This review would be incomplete without mentioning some of the studies that laid the foundation stone to the huge success of NO molecule. Organic nitrates are in clinical use for more than 100 years to relieve pectanginal pain and other conditions such as congestive heart failure, pulmonary hypertension, fibrinolysis, and complications after cardiac catheterization (142). The NO donating properties of this compound were not known at that time. In 1990's its mechanism of action was defined (143). The earliest nutritional studies relating NO to the biological system came in 1916 (144). Subsequently, it was confirmed that NO is produced endogenously in the human intestine (145). In vitro study using mice model specifically demonstrated that activated macrophages are the primary producers of intermediary nitrogen compounds in the body (146). In 1977, Murad and colleagues (147) and later others found that NO activates soluble guanylate cyclase (sGC) that forms cyclic GMP from GTP that leads to vascular relaxation. In 1980, Furchgott and Zawadski proposed the concept of NO as a biological messenger in vasculature in a

landmark study (148). They proposed that on stimulation of muscarinic receptors by acetylcholine and other agonists like bradykinin or histamine, a labile substance is released from the endothelial lining that caused the relaxation of the underlying vascular smooth muscle. The substance was termed as endothelium derived relaxing factor (EDRF). Comparison of the physiological and biochemical properties of EDRF and NO led Furchgott (149) to suggest that EDRF was identical with NO. Ignarro and coworkers (150) and also Salvador Moncada and colleagues (151) independently provided experimental evidence that EDRF released from the vasculature was indistinguishable from NO in terms of chemical properties, stability, biological activity susceptibility to an inhibitor and to a potentiator, suggesting that EDRF and NO are identical. However Meyer et al showed that vasorelaxant properties of EDRF more closely resemble S-nitrosocysteine than NO (152).

BIOCHEMISTRY OF NO

In order to gain insight into the pathophysiological and sometimes paradoxical effects of NO it is very essential to have clear understanding of the biological chemistry of NO. In vivo NO is generated in a biosynthetic pathway involving family of enzymes called NO synthase (NOS), which share a common basic structural organization and requirement for substrate cofactors for enzymatic activity (153,154,155). These enzymes catalyse the formation of NO and L-citrulline from basic amino acid L-arginine (156). This reaction requires NADPH and molecular oxygen (O_2) as cosubstrates (157) as well as tetrahydrobioptin

(H4B), which is a cofactor for NOS. It maintains the structural and functional stability of the enzyme and is also essential for the efficient generation of NO (158). Insufficient H4B is reported to cause reduced NO formation and enhanced free radical generation (159). Using isotopic labeling it was established that the nitrogen atom of NO is derived from L-arginine and oxygen atom originated from molecular oxygen. Biochemical studies and sequence analysis revealed that NOS isoforms are closely related family of proteins encoded by three different genes sharing 50-60% amino acid homology (160). They are subdivided into two groups. First group of enzymes are termed constitutive and includes neuronal NOS or NOS1 (165 kDa) purified from rat and porcine cerebellum (161) and endothelial NOS or NOS3 (135kDa) purified from bovine vascular endothelium (162). They are expressed in all cells and their activation does not require new protein synthesis. Their activities are regulated by intracellular Ca^{2+} concentration although they can also be activated by shear stress and produce NO. Second group includes inducible (iNOS or NOS2) isoform purified from murine macrophages (159). iNOS is not expressed in the resting cells. Factors such as tumor necrosis factor α (TNFα), endotoxins lipopolysaccharides (LPS) may induce its expression. NOS2 is bound tightly to calmodulin even under resting intracellular Ca^{2+} concentrations, thus intracellular Ca^{2+} does not affect the function of NOS2.

NOS isoforms have a dimerical structure with two identical subunits with three domains in each subunit. Carboxy C terminal reductase domain; amino terminal oxygenase domain, and calmodulin binding domain, which links

reductase and oxygenase domains. Carboxy domain contains FAD and FMN moieties and facilitates electron transfer from NADPH to oxygenase domain of the opposite subunit of the dimer. Amino terminal domain catalyses the conversion of arginine to citrulline and NO and contain H4B prosthetic group. Calmodulin binding domain joins the reductase and oxygenase domain. The binding of calmodulin is required for the electron flow from flavin prosthetic group in the reductase domain to heme and converts oxygen and l-arginine to NO and L-citrulline (163,164).

NO produced from constitutive NOS enzymes mediate normal physiologic response such as vascular dilation and the neurotranmission. In contrast NO generated from NOS2 is usually larger in amount and mediate immunological roles. Under normal physiological circumstances when NO is present at low concentrations (<1μM), it directly reacts with the biological substrate and mediate regulatory and functional aspect in different organ systems. During pathological states, such as in active inflammation or infection, large amounts of NO is produced (>1μM) leading to the formation of reactive nitrogen species (RNS) that mediates the indirect actions of NO (165,166).

PHYSIOLOGICAL AND PATHOLOGICAL ASPECTS

NO is considered to be fundamental signaling molecule in diverse range of pathophysiological processes in the body. Once NO is generated, multiple pathways exists for it to modulate signal transduction pathways. Interestingly its behavior is paradoxical, beneficial in one setting acting as a messenger and a

modulator and potentially cytotoxic in another leading to pathological outcome. This dichotomy can be explained by several factors such as relative concentration produced, species and surrounding molecular redox milieu in which NO is produced. NO exerts its biological effects by two separate downstream pathways, cGMP dependent and cGMP independent. These are explained in brief with respect to their relationship in cardiovascular system.

CARDIOVASCULAR SYSTEM

cGMP dependent pathway

NO is predominantly produced in the coronary endothelial cells in response to pulsatile blood flow and shear stress (167). Once NO is produced, it diffuses within the cell and binds to the heme moiety contained within the soluble guanyl cyclase (sGC) causing the removal of the distal histidine and resulting in a five coordinated nitrosyl complex that activates the enzyme (168,169). Reaction between NO and guanylate cyclase produces an iron – nitrosylc complex that becomes activated to form cGMP, a key intracellular messenger that mediates numerous biological effects of NO (170,171). NO also inhibits platelet aggregation by a mechanism dependent on cGMP and in concert with prostacyclin. NO is also reported to interact with leukocytes and inhibit their activation. Furthermore it inhibits the proliferation of smooth muscle cells and modulates microvascular permeability.

cGMP independent pathway

In the cGMP independent pathway, redox regulated covalent modification of proteins at thiol residues contained within a specific sequence of amino acids occur (172). This protein nitrosylation activates various proteins involved in the E-C coupling including the L-type Ca^{2+} channel (173) and RyR (174).

Effects of NO on cardiac myocyte

Coronary endothelial cells are the primary source of NO produced by eNOS, which acts in an autocrine and paracrine manner and control cardiac contractile function. It is now well established that all the three NOS isoforms are expressed in the cardiac myocyte. nNOS is expressed in the pre and post ganglionic fibers innervating the sinoatrial and atrioventricular nodes, in subepicardial neuron cells and in intrinsic cardiac neurons (175). Recent investigations by Xu and colleagues (176) showed that there is nNOS like is present on cardiac SR. In addition Hare et al (177) showed that nNOS co-immunoprecipitated with the RyR. Very recently genetic studies have aided to the better understanding of the role of NO and have revealed that different NO isoforms are critically involved in regulating basal contractile function and also modulates fundamental events of myocardial E-C coupling and thus may have profound effect on calcium handling in the heart (178,179). iNOS can be expressed in infiltrating inflammatory cells, coronary microvascular and endocardial endothelial cells, coronary vascular smooth muscle, and cardiac myocyte depending upon the stimulus (180,181). eNOS is expressed predominantly coronary vessels and endocardial endothelail cells. eNOS also

localizes to the SL and t-tubular system where it gets inactivated by caveolin 3 until it gets displaced by Ca^{2+}/Calmodulin. This isoform is also reported to be present on the mitochondria (182). This suggests that NO may modulate mitochondrial oxidative phosphorylation and energy production and thus myocardial contractility.

Inducible NOS is considered the major part of host defense system and almost every cell type is capable of expressing this isoform once they are exposed to bacterial endotoxin and or inflammatory cytokines (183). NO is affective against various invading pathogens and tumor cells.

In males NO enhances smooth muscle relaxation of corpus cavernosum by increasing the tissue concentration of cGMP and thus mediates penile erection (184). The blockbuster drug Viagra (sildenafil citrate) has been in clinical use to treat impotence, which specifically inhibits cGMP phosphodiesterase, and increase cGMP. In females NO inhibits uterine contraction during pregnancy but facilitates the same during labour (185).

NO is reported to serve number of important functions in CNS. It establishes ordered synaptic connections between neurons, mediates synaptic plasticity, induces long term memory (189) and plays important role in cerebral blood flow and ischemia, neuroendocrine secretion, visual transduction and olfaction.

ROLE OF NO IN IR INJURY

Studies investigating the role of NO in myocardial damage and dysfunction during IR have drawn very inconsistent findings. It is controversial whether NO plays detrimental or beneficial role in the settings of IR in the heart. Some studies have demonstrated that myocardial NO synthesis is increased due to enhanced NOS synthesis during IR (187,188) and NOS inhibitors decrease the functional impairment observed during myocardial IR (189,190). Other reports have suggested that EDRF declined resulting in myocardial dysfunction and that the administration of NO donors or NOS substrates like L-arginine improves the functional recovery of reperfused myocardium (191,192). So far majority of studies have shown that NO is cardioprotective during IR and only a few have come up with the opposite results. The prevailing confusion has been tried to explain on the basis of unique NO biochemistry and different parameters such as dose, timing species etc. Recently studies were performed by gene deletion or overexpression of different NOS isoforms in mice under controlled conditions to elucidate their role in disease pathologies such as IR (193,194,195). The direct measurements of constitutive NO in the coronary effluent of the isolated working hearts subjected to IR have shown that constitutive NO release is impaired during myocardial IR (196, 197). This led to the augmented myocardial damage and the administration of NO or its biological substrates such as L-arginine attenuated the myocardial damage.

NO REPLACEMENT THERAPY

Since IR is characterized by deficit in NO levels, a logical therapeutic approach is to administer NO so as to restore physiological levels of NO. An appropriate dose response or careful titration needs to be performed to determine the optimal NO concentration. NO is usually given in three forms, NO gas, NO donating compounds that release NO in the biological solutions, and enzymatic precursor substrate such as L-arginine. Use of NO in the form of NO gas first attempted by Johnson et al in feline model of myocardial ischemia and reperfusion, where NO was reported to prevent myocardial necrosis (198). Since then various forms of NO replacement strategies have been used in the settings of myocardial IR. It includes inorganic NO donors such as sodium nitrite ($NaNO_2$)(199), organic NO donors such as SPM-5185, SIN-1 (200, 201) or L-arginine (202, 203).

MATERIALS AND METHODS

The experimental protocol was approved by the Animal care committee of the University of Manitoba and conforms to the Canadian council on animal care and use of experimental animals.

Perfusion and Experimental Protocol

Male Sprague-Dawley rats weighing 250-300g were injected intraperitoneally with the anesthetic mixture containing ketamine (60 mg/kg) and xylazine (10 mg/kg). After thoracotomy, the heart was rapidly excised from the root of aorta, and arrested in an ice-cold perfusion solution. It was then cannulated to the Langendorff apparatus through the ascending aorta and perfused in a retrograde manner with Krebs-Henseleit (K-H) solution (37°C 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 a constant flow of 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 the left ventricular systolic and diastolic pressures. The left ventricular developed pressure (LVDP) was calculated as difference between the systolic and diastolic pressures. The left ventricular end diastolic pressure (LVEDP) was adjusted between 5-10 mm Hg at the beginning of the experiment and the 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). Data were recorded online through analogue digital interface (MP100, BIOPAC SYS INC), stored and processed using the ACKNOWLEGDE 3.5.3 software for Windows. All hearts were stabilized with K-H medium for a period of 10 min at a constant temperature of 37^0C. The hearts were randomly distributed among six experimental groups consisting of; (a) Control hearts (C) were perfused for a period of 100 min with K-H medium. (b) In the second group (IR) hearts were exposed to global no-flow ischemia for 30 min followed by reperfusion for 60 min. (c) In the third group (LA) IR hearts were treated with 1.5 mM of L-arginine (SIGMA), a biological precursor of nitric oxide (NO), for 10 min before inducing ischemia and for 20 min after ischemia beginning at the onset of reperfusion. A dose response was done with 0.75, 1.5, 3, 6 and 12 mmol to determine the best cardioprotective dose of L-arginine (d) A fourth group (C+LA) was added to observe for any possible effects of the drug on control hearts; control hearts were perfused with L-arginine for the same duration as the treatment group. (e) The fifth group (LN) consisted of IR hearts treated with L-NAME, which is a selective inhibitor of nitric oxide synthase (NOS), (f) while in the sixth group (LA+LN) IR hearts were treated with a combination of L-arginine and L-NAME. The protocol for perfusion in different groups is shown in Figure1.

Experimental Protocol:

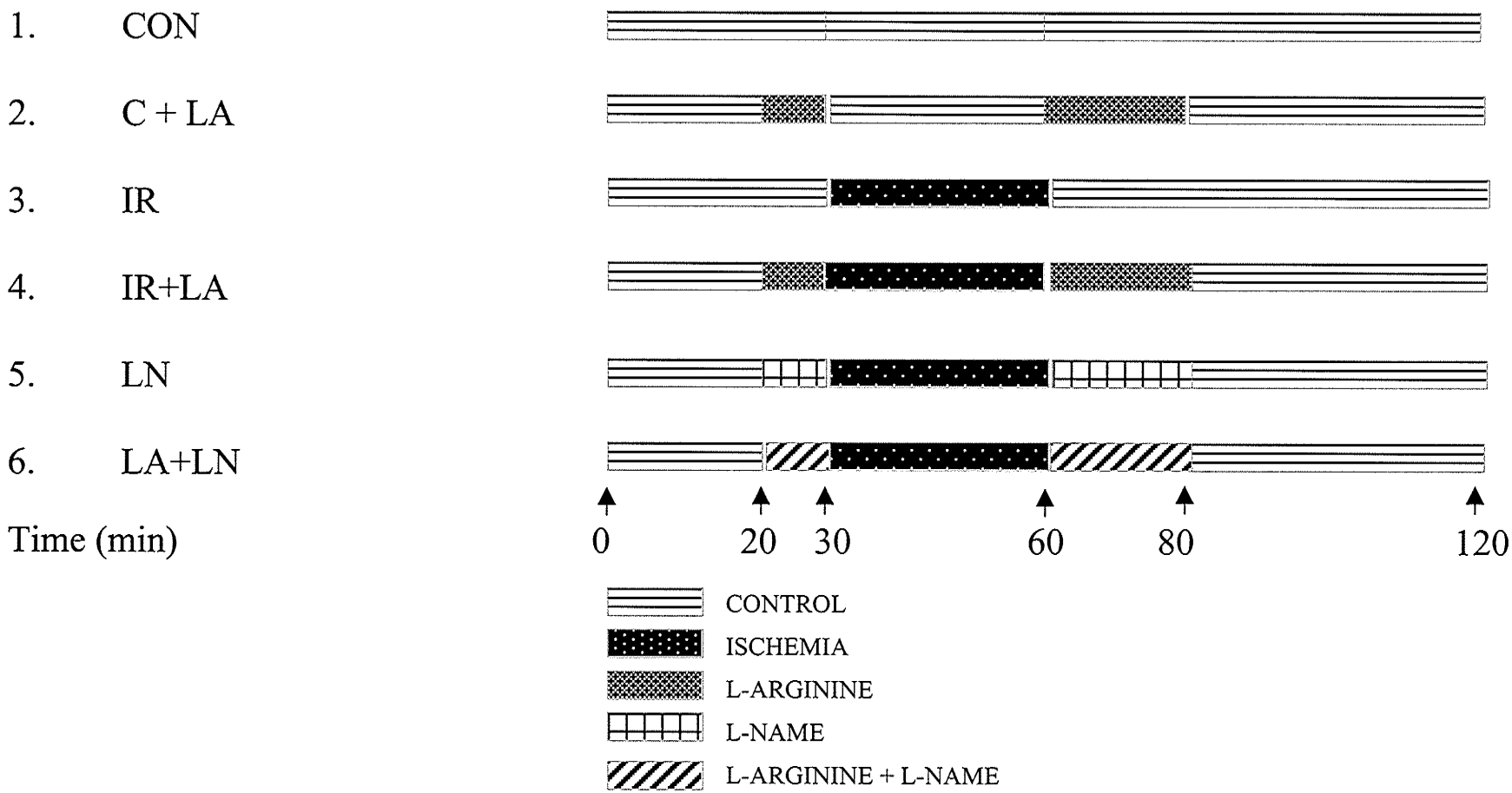

Figure 1. Experimental protocol for perfusing isolated rat hearts under different conditions. Panels 1 and 2 show control hearts treated with and without L-arginine. Panels 3 and 4 show IR hearts treated with and without L-Arginine (LA), while panel 5 and 6 show IR hearts treated with L-NAME (LN) or a combination of L-arginine and LN. Global ischemia was induced by stopping coronary flow for 30 min and IR by reperfusing the globally ischemic hearts for 60 min. CON=Control, IR=Ischemia reperfusion, LA=IR+L-arginine and LN=L-NAME.

At the end of the experiments the hearts were freeze clamped and stored at -70°C for 2 to 3 days before use.

SR Isolation and Membrane Preparation

SR vesicles were isolated according to a method described previously (204) with slight modifications. The left ventricular tissue was pulverized and homogenized twice for 20 sec each at half maximum setting 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 (Beckman JA 20) to remove cellular debris. The supernatant was centrifuged for 45 min at 19,000 rpm (Beckman, JA 20). The supernatant thus obtained (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) aliquoted and stored at -70^0C for further studies. All solutions contained a cocktail of protease inhibitors consisting of aprotinin, leupeptin, AEBMSF and 0.1% phenylmethylsulphonyl fluoride (PMSF).

Protein Estimation

Protein concentration in each sample of SR and cytosol was determined using Lowry's method for protein estimation. Varying concentrations of bovine serum albumin (BSA / 0-40μg) in de-ionized double 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 Ciocalteau reagent) was added to each tube. After 20 min, the absorbance of the samples at 623 nm was measured using an Ultrospec 2100 pro spectrophotometer (Molecular Devices). The protein concentration of samples was determined using a standard curve obtained with BSA on a custom made computer software in Microsoft excel.

Nitric oxide determination

Amount of nitric oxide was determined in the coronary effluent and the cytosol by performing NO colorimetric assay (Roche). Coronary effluent was collected at 5, 15 and 30 min after reperfusion and immediately frozen in liquid nitrogen. NO was detected in these samples on the basis of the formation of nitrite from nitrates. NADPH converts the nitrate in the sample to nitrite in the presence of nitrate reductase. The nitrite formed reacts with sulfanilamide and N- (1-napthyl)-ethylenediamine dihydrochloride to give a red violet diazo dye, which was measured on the basis of its absorbance in the visible range at 550 nm. NO content was measured in the IR, C, LA and LN+LA groups.

Measurement of Ca^{2+}-Uptake

Calcium-uptake activity of SR vesicles was measured by a procedure described previously (204,209). A total volume of 250 µl of standard reaction mixture 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 0.25 ruthenium red. Ruthenium red was added to inhibit 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°C 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 (Beckman, USA). The Ca^{2+}-uptake reaction was linear during 2 min of the incubation period.

Measurement of Ca^{2+}- Induced Ca^{2+}- Release

Ca^{2+}-release activity of SR vesicles was measured by a procedure adapted from a previously described method (204,205,209). 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 20-µM ryanodine.

Measurement of CaMK and PKA activities

The SR and cytosolic 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 II 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 activities of protein kinases. The assay kit for CaMK activity is based on the phosphorylation of a specific substrate peptide (KKALR-RQETVDAL) by the transfer of the γ-phosphate of [γ-32 P] ATP by CaMK II. The SR and cytosolic CaMK also phosphorylated the exogenous substrate; the activities were 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 (for CaMK), the substrate and inhibitor cocktail were taken from the kit in a concentration of 10 µl in eppendorf tubes along with sample and DDW. The radioactive mixture is made by mixing ^{32}P and Mg ATP from the kit in a concentration of 1: 9. The reaction was started by adding 10 µl of the radioactive mixture at 1 min interval to all the tubes and incubated for 10 min at 30^0C. Spotting the reaction mixture (25 µl) 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 γ-phosphate of [γ - ^{32} P] ATP by PKA. The phosphorylated substrates in both assays were then separated from the residual [γ-^{32} P] ATP with P81 phosphocellulose paper. This was quantified by using a scintillation counter (Beckman, USA) after adding 10 ml of scintillation fluid to each vial containing the phosphocellulose paper.

Western blot analysis

The protein content of Ca^{2+}-cycling and regulatory proteins, Ca^{2+}-pump ATPase (SERCA2a), ryanodine receptor (RyR), phospholamban (PLB), calsequestrin (CQS), and CaMK and PKA were determined as described by some other investigators (209). PLB phosphorylation at Ser-16 and Thr-17 were also investigated. Protein content of cardiac specific NOS isoform, bNOS, was ascertained. Protein samples (20μg/ml) were suspended in equal volume with the laemmlli buffer containing 0.1M Tris–HCl (pH -6.8), 15%(W/V) sodium dodecyl sulphate (SDS), 15% glycerol, 8% β-mercaptoethanol and 0.002% bromophenol blue and then denatured by boiling for 10 min (except PLB). Protein samples (20μg per lane) were then separated by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) in 5% (for RyR), 8% (for SERCA2a & bNOS), 12% (for CQS, CaMK and PKA), and 15% (for PLB) gels. Samples for SERCA2a, PLB, bNOS, CQS, CaMK and PKA were transferred electrophoretically to polyvinylidene difluoride membranes (PVDF, Millipore Corporation Bedford, MA, USA.) while

that for RyR were transferred to nitrocellulose membrane at 100 volts. The transfer buffer contained 25 mM Tris HCl, 192 mM glycerine and 4% Methanol (v/v). The membranes were shaken overnight in blocking buffer (TBS, 10mM tris, 150 mM NaCl and 5% skimmed milk powder) at 4^0C. The membranes were probed with monoclonal anti-SERCA2a (1:1,400), monoclonal anti-ryanodine receptor (1:1000 both from Affinity Bioreagents Inc., Golden, CO), monoclonal anti-phospholamban (1:2,000), or polyclonal anti-calsequestrin (1:2,000) antibodies. bNOS antibody (1:100) was obtained from ABR while CaMK II (1:500), PKA (0.5-2μg/ml) and PLB at Ser-16 (1:500) were obtained from Santa Cruz Biotechnology, Inc. The antibodies for PLB, CQS and PKA were purchased from Upstate Biotechnology and PLB Thr-17 antibody from Badrilla, UK. The membranes were incubated for 90 min with a secondary antibody. An anti mouse–peroxidase linked antibody was used as a secondary antibody for SERCA2a and PLB and RyR while anti-goat antibodies were used for CaMK II and PLB at Ser-16. bNOS, PKA and PLB at Thr-17 were probed with anti-rabbit secondary antibodies. All secondary antibodies were linked with a horseradish peroxidase. The blots were rinsed in a washing buffer 3 times (15 min each). Antibody-antigen complexes in all membranes were detected by a chemiluminescence detection kit (ECL kit Amersham Life Science, Oakville, ON, Canada). Protein bands were visualized on Hyperfilm-ECL. An Imaging densitometer, model GS-800 (Bio-Rad Ltd., Hercules, CA) was used to scan the protein bands and quantified using the Quantity one 4.4.0 software from Bio-Rad. Equal protein loading was checked in

every experiment by staining the membrane with Ponceau red before immunoblotting and with Coomassie Brilliant blue at the end of the experiment.

Statistical Analysis

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

RESULTS

Cardiac Function

Cardiac function was assessed by measuring LVDP, LVEDP, +dP/dt and -dP/dt in isolated rat hearts perfused on the Langendorff apparatus. In hearts exposed to 30 min global no-flow ischemia followed by 60 min reperfusion, recovery in contractile function was observed as represented by 30-35 % improvement in LVDP, +dP/dt and -dP/dt (Figure 2A, 2C & 2D) of the respective pre-ischemic values but there was a marked increase in the LVEDP (Figure 2B). Contractile activity in IR hearts was markedly improved by L-arginine (1.5 mM) treatment as observed by an 80-85% recovery of LVDP and about 65 % recovery in +dP/dt and -dP/dt, in comparison to pre-ischemic values (Figure 2A, 2C & 2D). A marked reduction in LVEDP was also observed with L-arginine treatment in comparison to the IR group (Figure 2B). Improvement in hemodynamic parameters in IR hearts by L-arginine was seen upon treatment with different concentrations of L-arginine (Table 1).

SR Function

SR function is integral to cardiac Ca^{2+} homeostasis and alterations in SR can adversely affect cardiac contractility. SR Ca^{2+}-uptake and release in the IR hearts were assessed upon treatment with 1.5mM L-arginine. A marked depression in both SR Ca^{2+}-uptake and Ca^{2+}-release activities in the IR hearts was attenuated by L-arginine treatment (Figure 3). The improvement in SR function in IR hearts was assessed with different concentrations of L-arginine to compliment

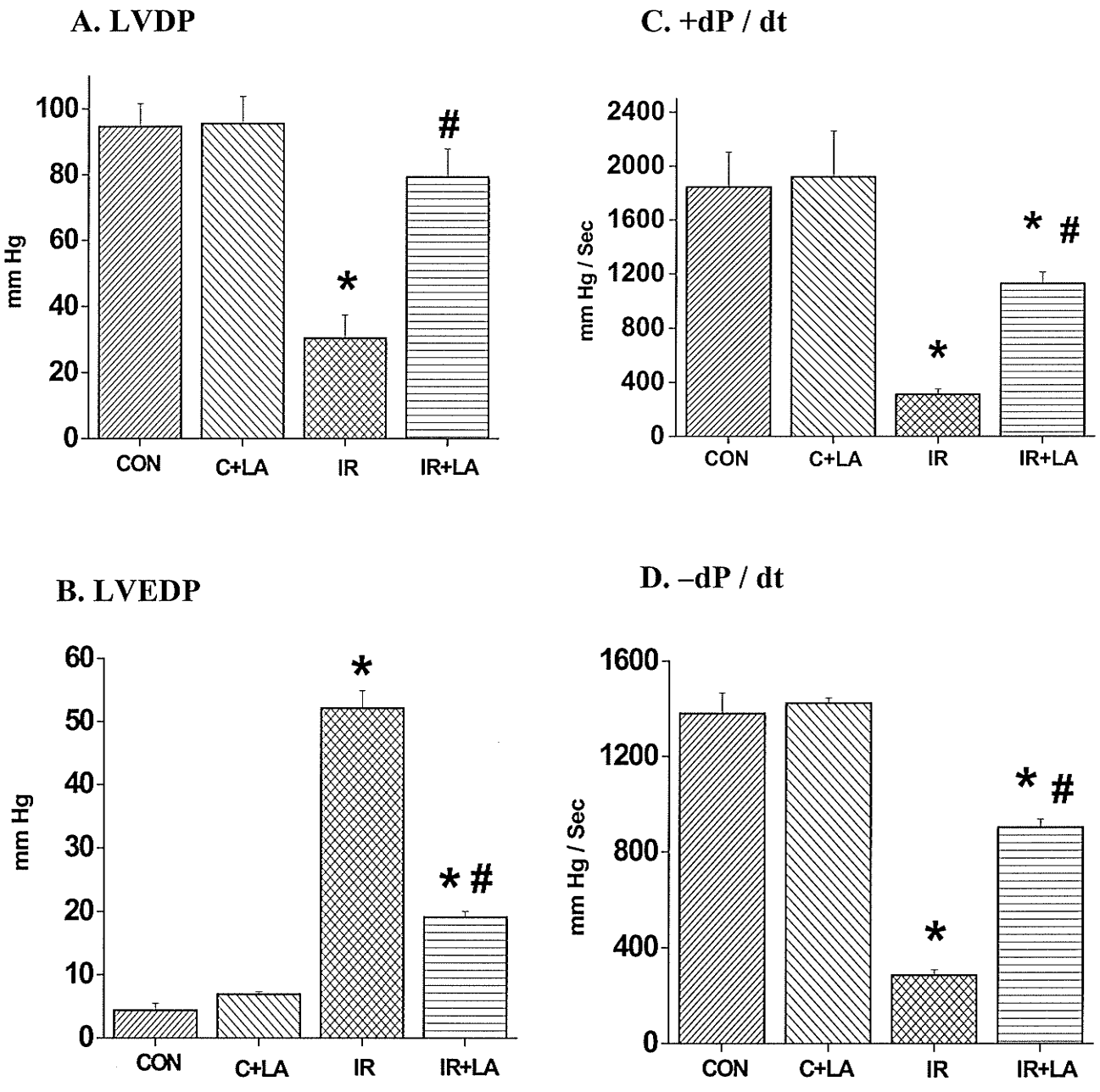

Figure 2. Cardiac function of the isolated perfused rat hearts subjected to IR treated with and without L-arginine (1.5mM) compared to the control hearts. Panel A: Left ventricular developed pressure (LVDP), B: left ventricular end diastolic pressure (LVEDP), Panel C: Left ventricular rate of pressure development (+dP/dt), Panel D: left ventricular rate of pressure decay (-dP/dt). CON=Control (2hr), C+LA=control with L-arginine treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+LA=IR hearts treated with L-arginine. n=5-6 for each group. Data expressed as Mean ± SE. *P<0.05 in comparison to control, # P<0.05 in comparison to the IR group.

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

Concentrations		LVDP (mm Hg)	LVEDP (mm Hg)	+dP / dt (mm Hg/sec)	-dP / dt (mm Hg/sec)
A.	CON	94 ± 6	3.4 ± 0.7	1844 ± 2.9	1380 ± 85
B.	IR	30 ± 7.2*	52 ± 2.7*	310 ± 39*	285 ± 22*
C.	IR + L-arginine (mM)				
i)	0.75	69 ± 6.7#	44 ± 3.6	1080 ± 70#	903 ± 39#
ii)	1.5	80 ± 9#	21 ± 2.1#	1128 ± 85#	901 ± 34#
iii)	3.0	75 ± 4.8#	31 ± 4.7#	1215 ± 82#	976 ± 88#
iv)	6.0	85 ± 8.2#	15 ± 6.2#	1639 ± 63#	1107 ± 86#
v)	12.0	91 ± 6.3#	13.0 ± 9#	1720 ± 54#	1502 ± 54#

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. n=5 for each group. Data expressed as Mean ± SE. *P<0.05 in comparison to the control and #P<0.05 in comparison to IR.

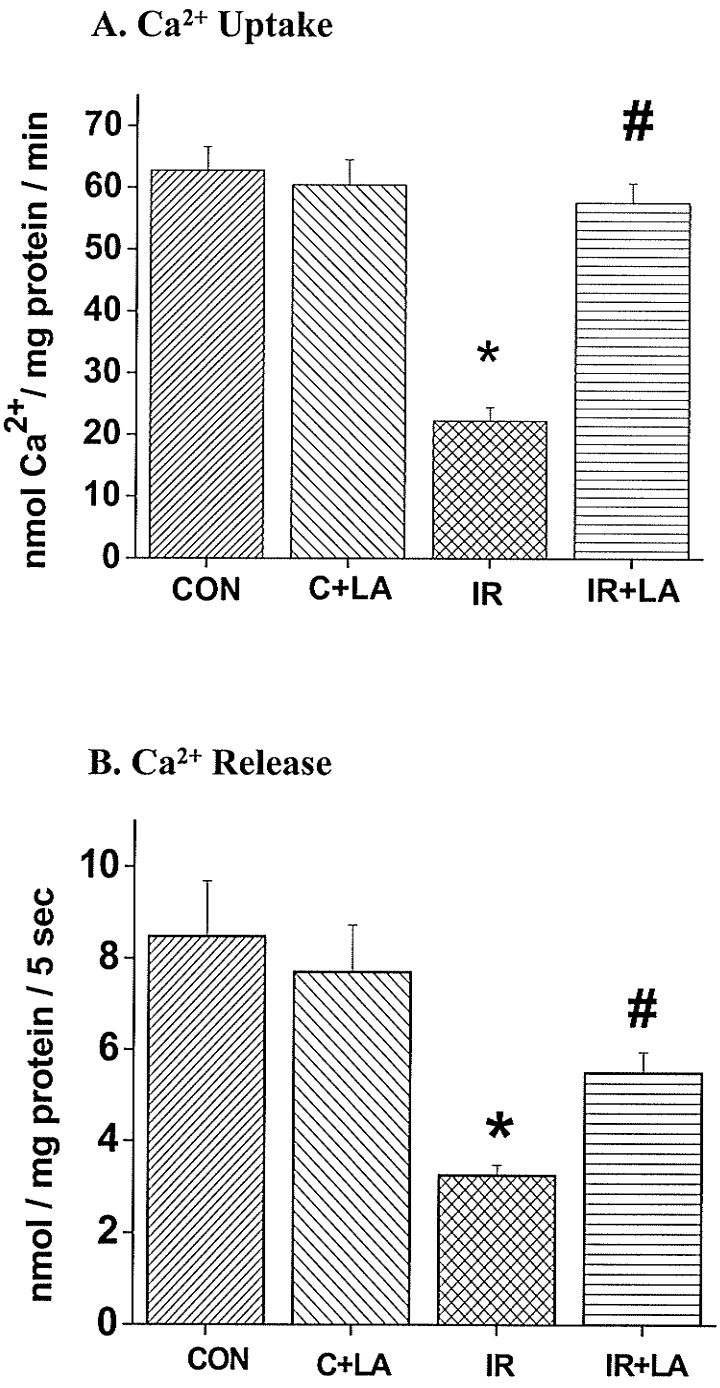

Figure 3. SR Ca^{2+} uptake (Panel A) and release (Panel B) in I/R hearts treated with and without L-arginine (1.5mM) in comparison to the control. CON=Control (2hr), C+LA=control with L-arginine treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+LA=IR hearts treated with L-arginine. n=5-6 for each group. Data expressed as Mean ± SE. *$p<0.05$ in comparison to the control & # $p<0.05$ in comparison to the IR group.

the hemodynamic data (Figure 4). SR Ca^{2+}-uptake and release were assessed in the isolated SR preparations from control and IR hearts treated with and without L-arginine. SR Ca^{2+}-uptake (nmol/mg protein/min) was significantly reduced to about 35-40 % of the control values in IR hearts (Figure 3A), which was improved by treatment with L-arginine to about 75 % of the control values (Figure 3A).

Ca^{2+}-induced Ca^{2+}-release (CICR) was significantly decreased in IR hearts to 35 % of the control values. L-arginine treatment markedly improved the SR Ca^{2+}-release from IR hearts to about 65 % of the control values (Figure 3B).

SR Protein Content

The content of SR Ca^{2+} cycling proteins; SERCA2a, PLB, CQS, were examined as alterations in levels of these proteins can influence SR function. Reperfusion of the ischemic hearts decreased the protein content of SERCA2a (to 60 %), and PLB (to 70%) (Figure 5A, 5B) of the control values. L-arginine treatment attenuated the decrease in protein content of SERCA2a, and PLB. There was no significant change in the protein content of CQS in the IR group in comparison to the control and L-arginine treated IR hearts (Figure 6A).

NO content in SR and cytosol

Recent studies have localized an isoform of NOS to the SR and thus we investigated changes in the bNOS protein content in SR samples obtained from IR hearts. Our results show a significant decrease in the bNOS protein content in IR (to about 20 % of control), which was attenuated by treatment with L-arginine

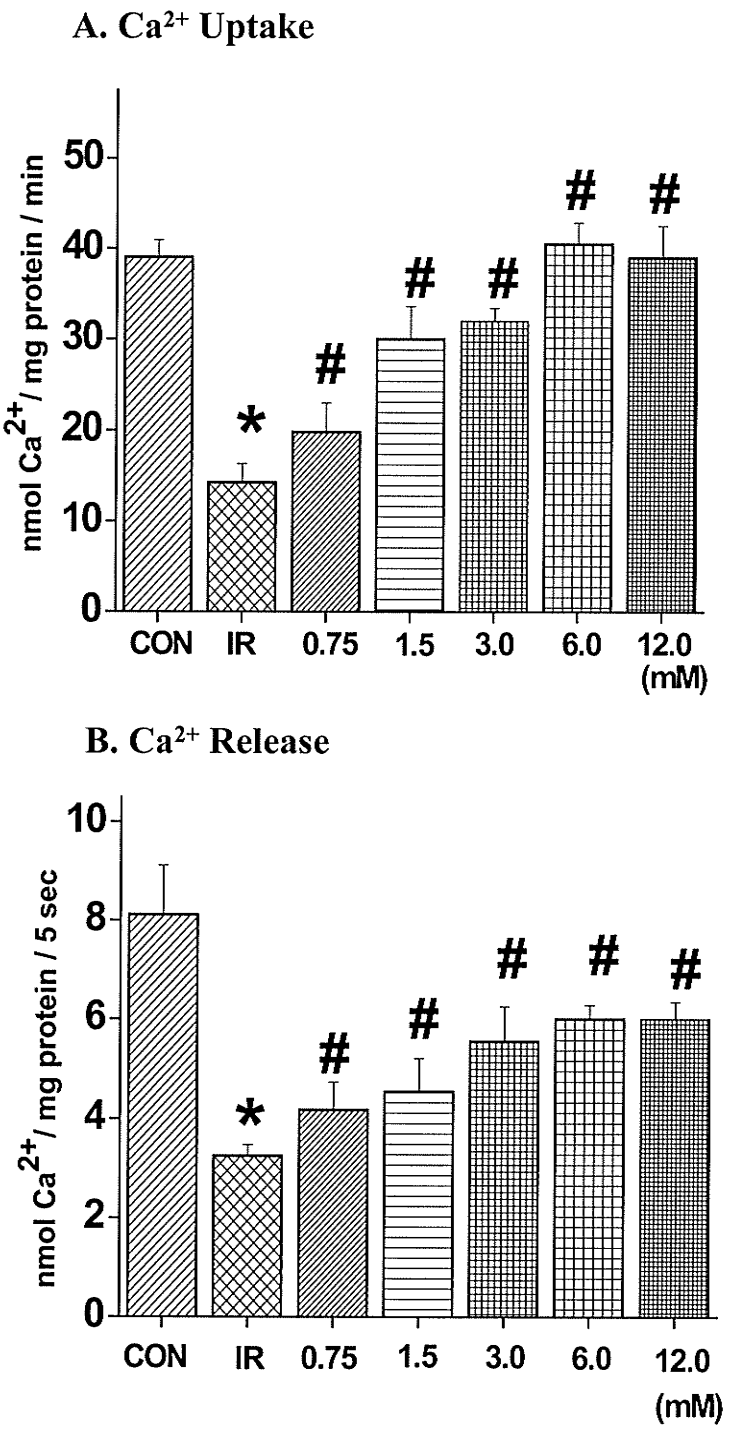

Figure 4. Effect of different concentrations of L-arginine (mM) on SR Ca^{2+} uptake (Panel A) and release (Panel B) in I/R hearts treated with and without L-arginine in comparison to the control. CON=Control (2hr), IR=60 min reperfusion of hearts exposed to 30 min ischemia. n=5-6 for each group. Data expressed as Mean ± SE. *$p<0.05$ in comparison to the control and #$p<0.05$ in comparison to IR.

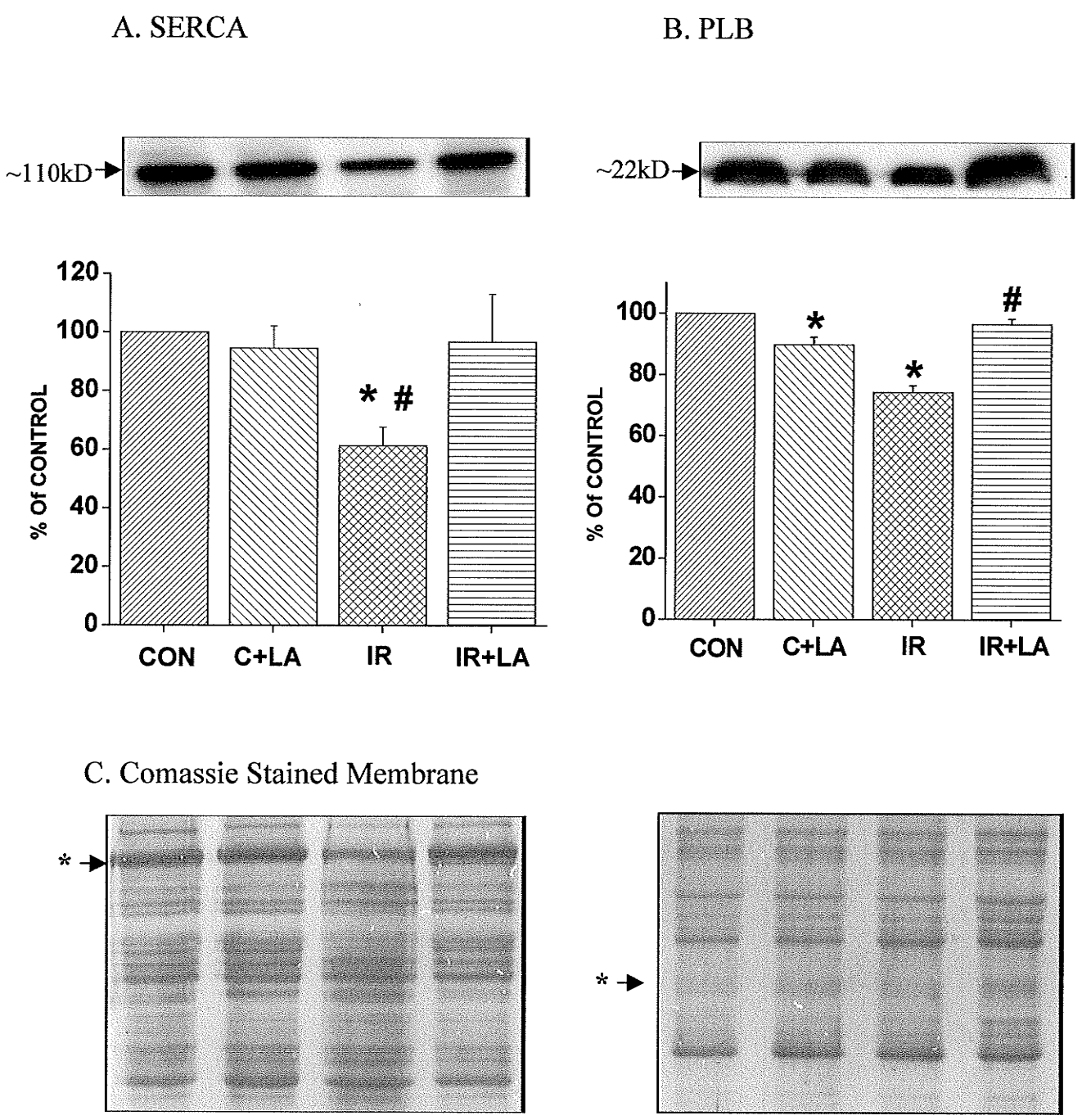

Figure 5. Immunoreactive band and western blot analysis for protein content of SR samples treated with and without L-Arginine (LA) as compared to the controls. Panel A: SERCA2a, Panel B: PLB. Commassie stained membranes showing equal protein loading (20μg protein per lane was loaded), * = band of interest. CON=Control, IR=Ischemia Reperfusion, LA=L-Arginine. [(n=5-6 for each group). Data expressed as Mean ± SEM. *P<0.05 in comparison to control, # P<0.05 in comparison to the IR+LA group].

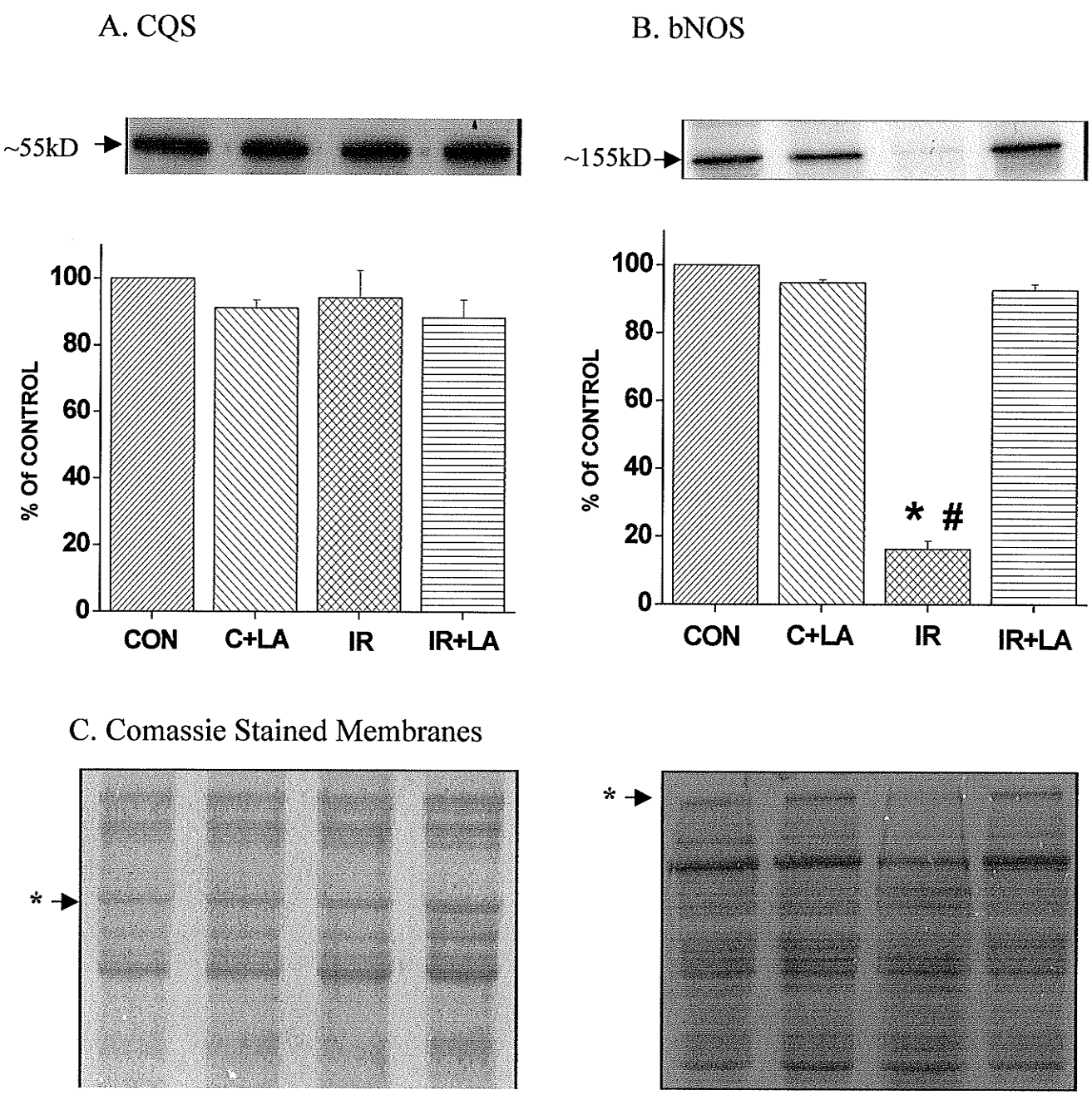

Figure 6. Immunoreactive band and western blot analysis for protein content of SR samples treated with and without L-Arginine (LA) as compared to the controls. Panel A: SERCA2a, Panel B: bNOS. Commassie stained membranes showing equal protein loading (20μg protein per lane was loaded), * = band of interet. CON=Control, IR=Ischemia Reperfusion, LA=L-Arginine. [(n=5-6 for each group). Data expressed as Mean ± SE. *P<0.05 in comparison to control, # P<0.05 in comparison to the IR+LA group].

treatment (Figure 6B). We also observed that NO content in the cytosol was reduced by 50% in IR groups as compared to controls (Figure 7). Treatment with 1.5mM L-arginine restored NO to control levels.

SR protein phosphorylation

SR Ca^{2+}-uptake is regulated by phosphorylation mediated by SR associated CaMK and PKA and therefore changes in SR function can be partly attributed to abnormalities in PLB. PLB phosphorylation at both Ser-16 and Thr-17 was reduced in IR hearts (Fig 8A, 8B). Treatment of IR hearts with L-arginine significantly improved PLB phosphorylation at both sites. To examine whether attenuation in PLB phosphorylation was due to changes in SR associated PKA and CaMK activities, these enzymatic activities were studied. Our results show a significant reduction in PKA and CaMK activity in the IR group to about 35 % of the control values (Figure 9A & 10A). Treatment of the IR hearts with L-arginine markedly improved the SR associated PKA and CaMK activities. L-arginine had no effect on the SR associated PKA and CaMK activities of control hearts (Figure 9A & 10A). In order to determine if the changes in CaMK and PKA activities in IR hearts were compartmentalized to the SR alone, we studied the activities of these enzymes in the cytosolic compartment also. Both cytosolic CaMK and PKA activities were unaffected by IR in comparison to the control hearts (Figures 9B & 10B). 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 (Figure 11).

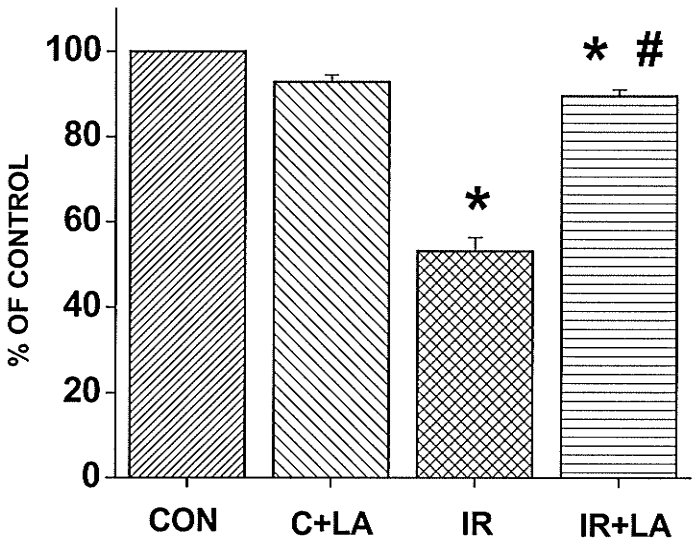

Figure 7. Cytosolic nitric oxide content in control and IR hearts treated with and without L-arginine (1.5mM). CON=Control (2hr), C+LA=control with L-arginine treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+LA=IR hearts treated with L-arginine. n=4-5 for each group. Data expressed as Mean ± SE. *$p<0.05$ in comparison to the control and #$p<0.05$ in comparison to the IR hearts.

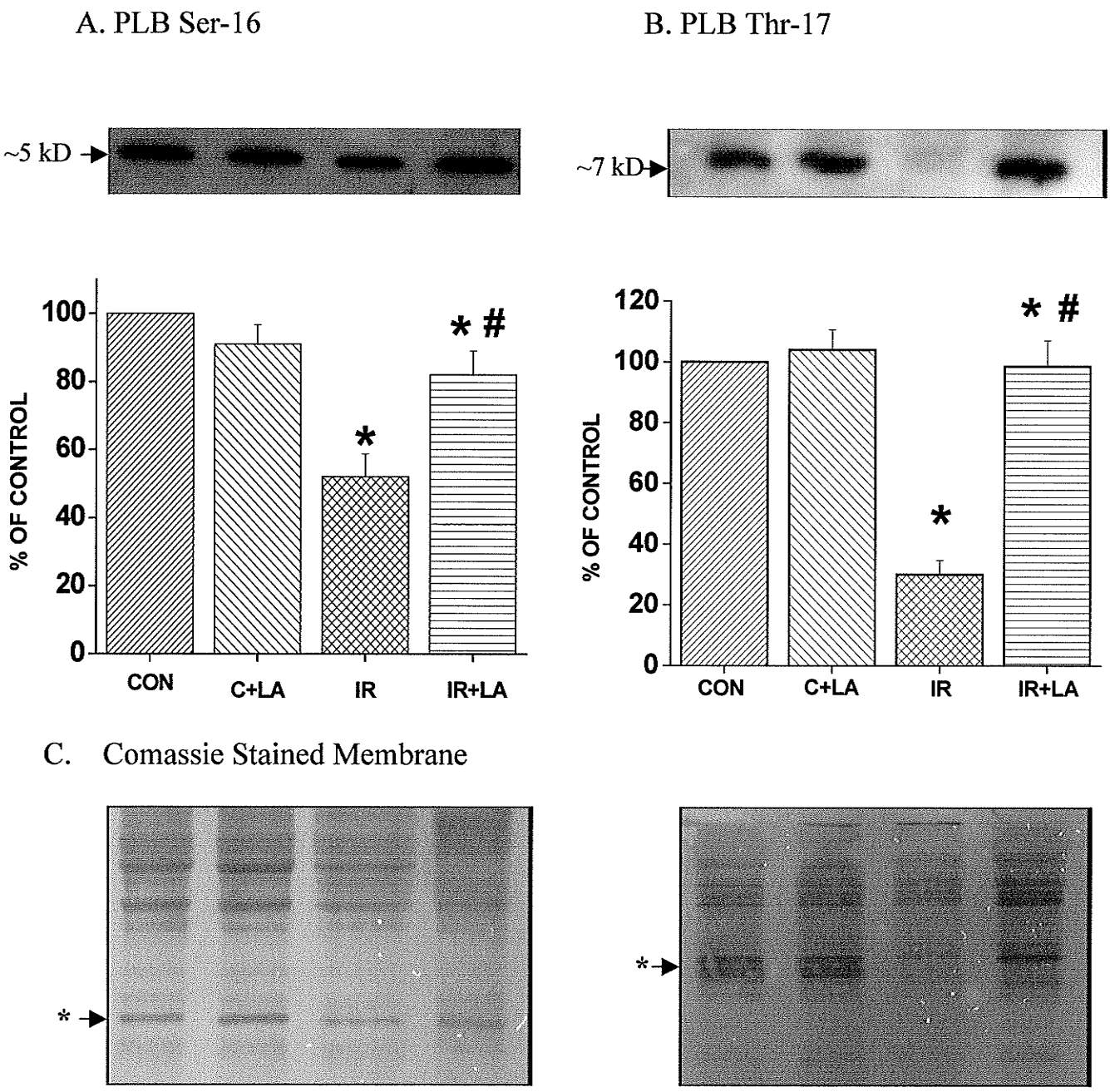

Figure 8. Western blot analysis of SR PLB Ser-16 and Thr-17 in hearts treated with and without L-Arginine (LA) as compared to the controls. Panel A: PLB-Ser 16 western blot and analysis for protein content. Panel B: PLB-Thr-17 western blot and analysis for protein content. Panel C: Commassie stained membranes showing equal protein loading (20μg protein per lane was loaded). * = Band of interest. CON=Control, IR=Ischemia Reperfusion, LA=IR+L-Arginine. n=5-6 for each group. Data expressed as Mean ± SE. *P<0.05 in comparison to control, # P<0.05 in comparison to IR.

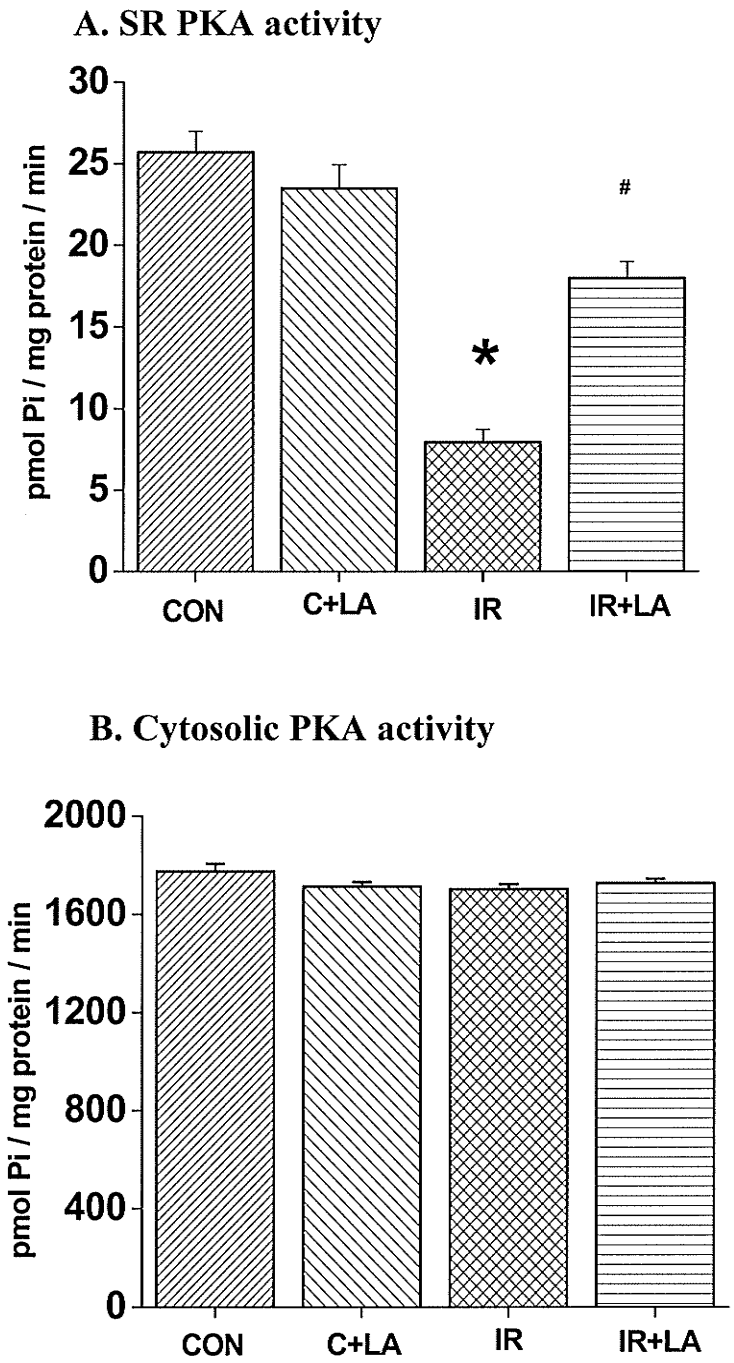

Figure 9. SR and cytosolic cAMP dependent protein kinase (PKA) activity of the control and IR hearts treated with and without L-arginine (1.5 mM). Panel A: SR PKA activity. Panel B: Cytosolic PKA activity. CON=Control (2hr), C+LA=control with L-arginine treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+LA=IR hearts treated with L-arginine. n=5-6 for each group. Data expressed as Mean ± SE. *$p<0.05$ in comparison to the control and #$p<0.05$ in comparison to the IR hearts.

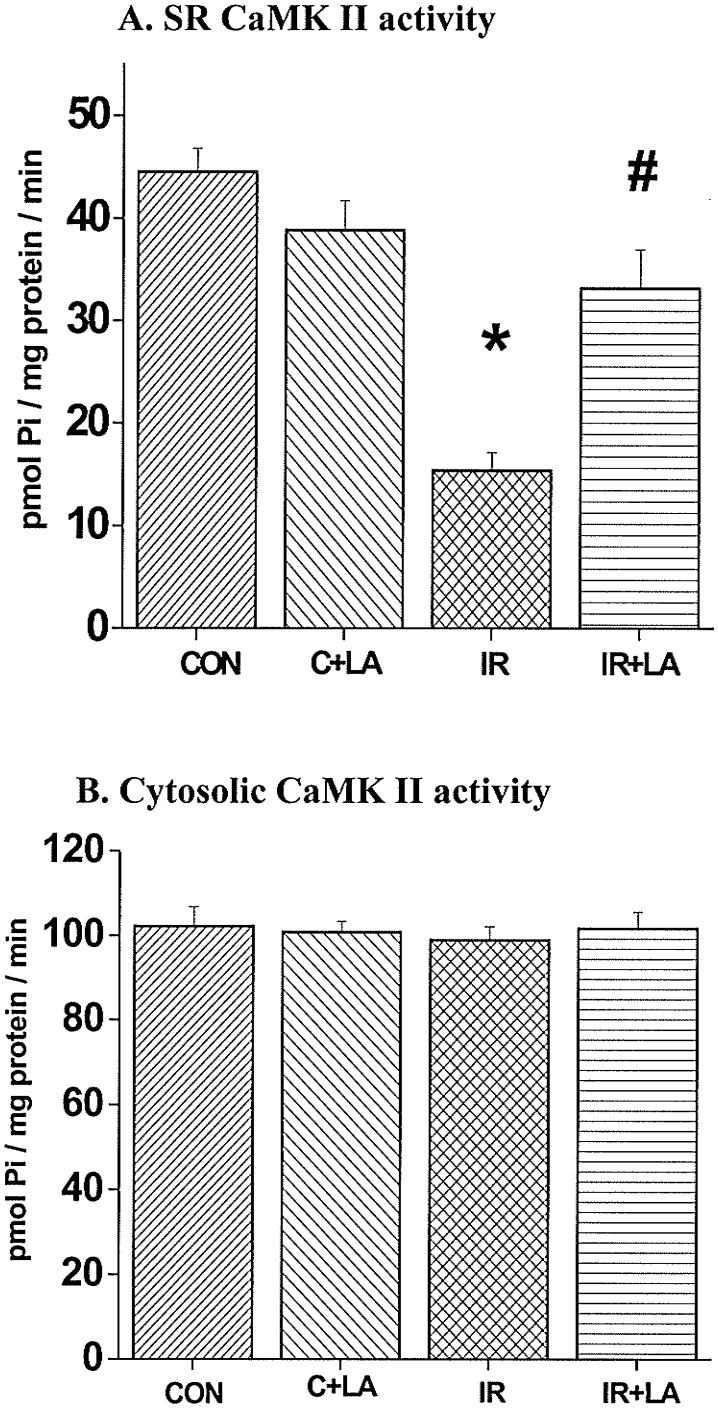

Figure 10. SR and cytosolic calcium calmodulin dependent protein kinase-II (CaM kinase II) activity of the control and IR hearts treated with and without L-arginine (1.5 mM). Panel A: SR CaMK II activity. Panel B: Cytosolic CaMK II activity. CON=Control (2hr), C+LA=control with L-arginine treatment, IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+LA=IR hearts treated with L-arginine. n=4-5 for each group. Data expressed as Mean ± SE. *$p<0.05$ in comparison to the control and #$p<0.05$ in comparison to the IR hearts.

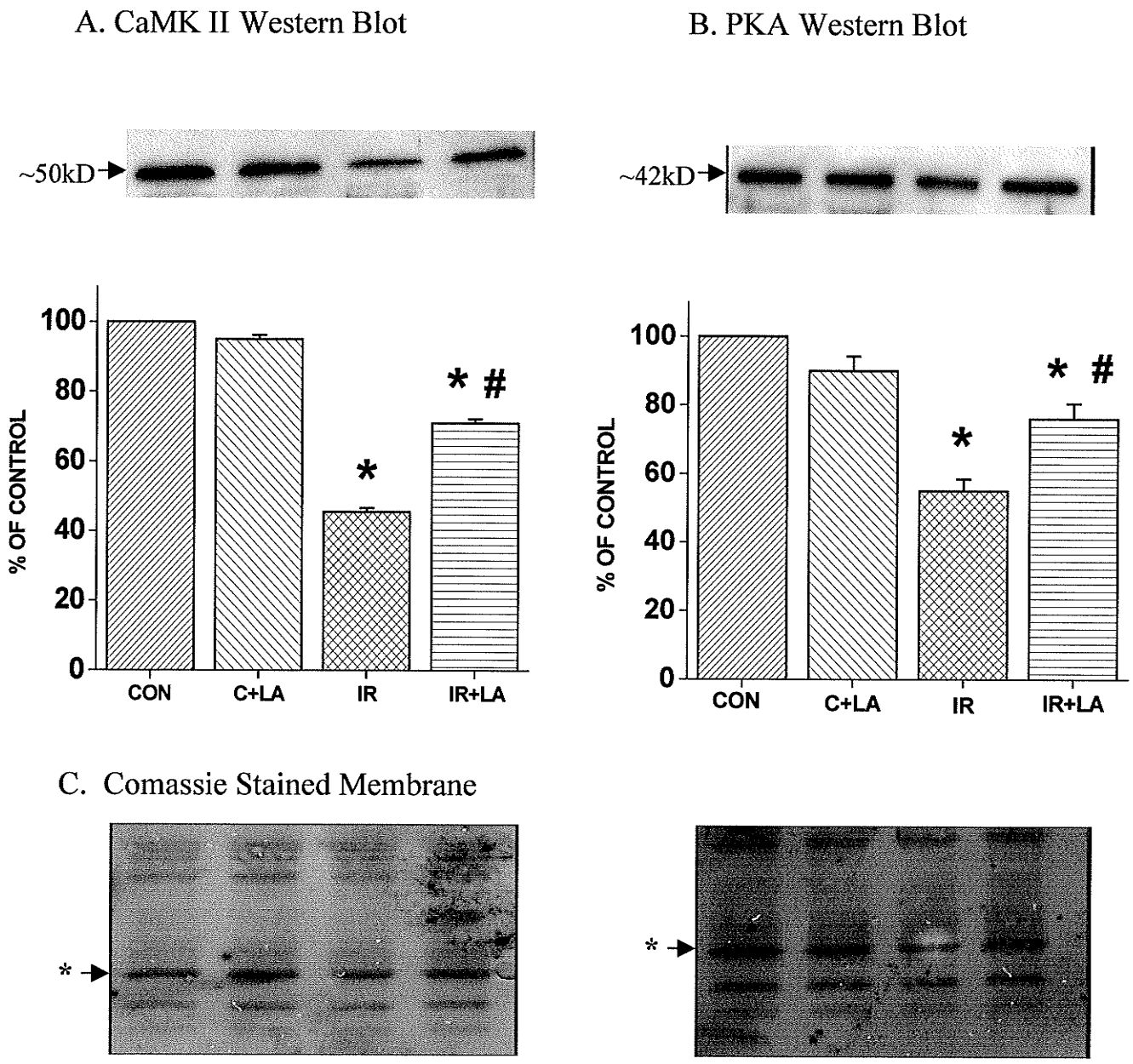

Figure 11. Western blot analysis of SR CaMK and PKA in IR hearts treated with and without L-Arginine (LA) as compared to the controls. Panel A: CaMK II (δ) immunoreactive band and analysis for protein content. Panel B: PKA immunoreactive band and analysis for protein content. Panel C: Commassie stained membranes showing equal protein loading (20μg protein per lane was loaded). *=band of interest. CON=Control, IR=Ischemia Reperfusion, LA=IR+L-Arginine n=4-5 for each group. Data expressed as Mean ± SE. *P<0.05 in comparison to control, # P<0.05 in comparison to IR.

There was a significant recovery of these protein levels in IR hearts upon L-arginine treatment (Figure 11).

Effect of L-NAME in the absence and presence of L-arginine in IR hearts

In order to examine mechanisms of the beneficial effects of L-arginine in the IR hearts, IR hearts were treated with L-NAME, an inhibitor of NO synthase, in the absence and presence of L-arginine. Treatment of IR hearts with L-NAME had no significant effects on the IR induced depression in LVDP, +dP/dt and -dP/dt. However LVEDP was increased further (Figure12). On the other hand, the beneficial effects of L-arginine on contractile function were attenuated by L-NAME (Figure 12). Similarly, L-NAME attenuated improvement in SR function of IR hearts by L-arginine treatment (Figure 13). There was no significant improvement in SR function of IR hearts with L-NAME alone (Figure 13). The results suggest that the beneficial effects of L-arginine on IR hearts may be due to the formation of NO.

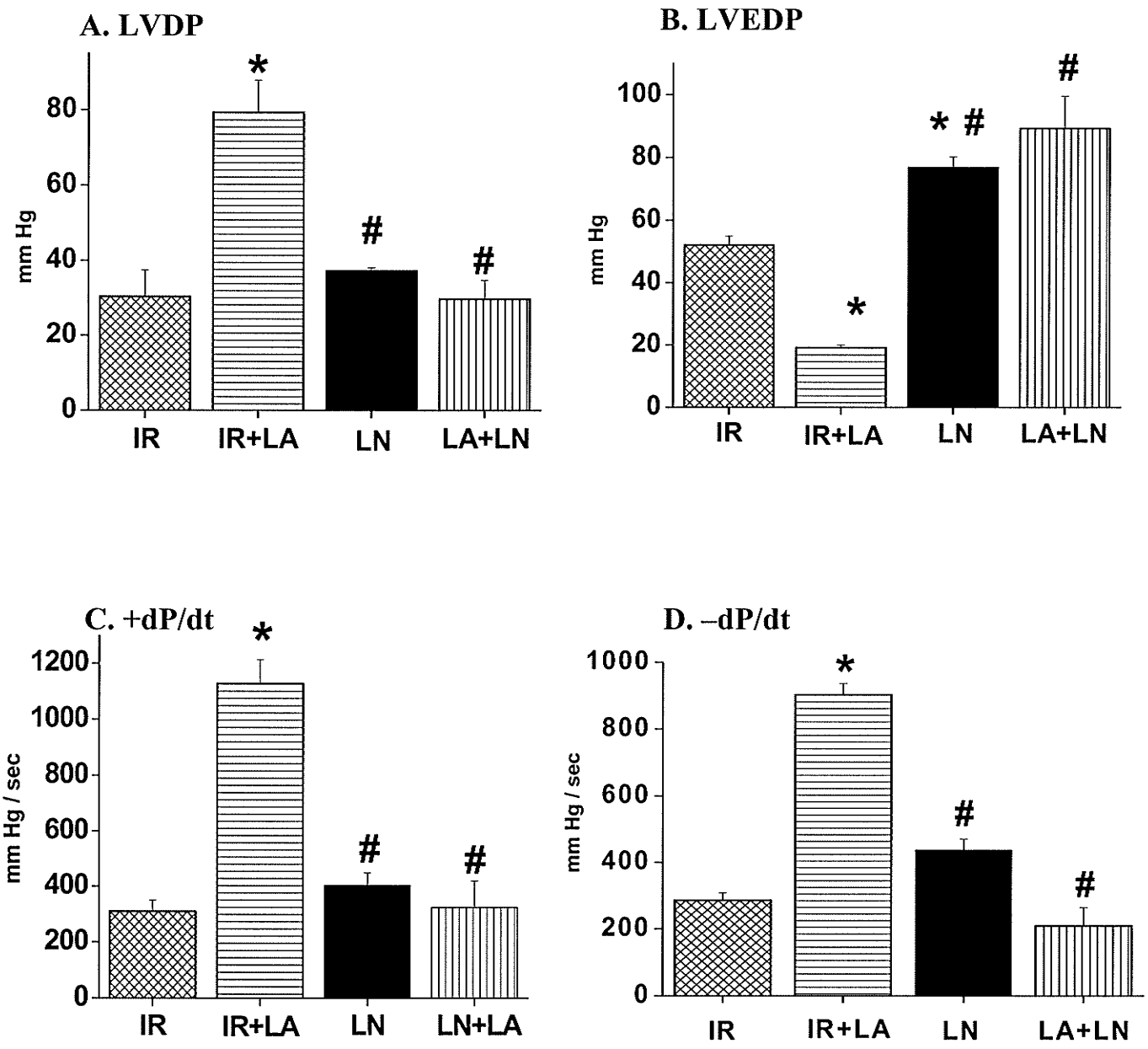

Figure 12. Cardiac function of the isolated perfused rat hearts subjected to I/R treated with and without L-Arginine (LA) (1.5mM), L-NAME (LN) (100μM) and a combination of the two. Panel A: Left ventricular developed pressure (LVDP), B: Left ventricular end diastolic pressure (LVEDP), Panel C= Rate of pressure development (+dP/dt) and Panel D=Rate of pressure decay (-dP/dt). IR=60 min reperfusion of hearts exposed to 30 min ischemia. n=5-6 for each group. Data expressed as Mean ± SE. *P<0.05 in comparison to IR, # P<0.05 in comparison to the IR+LA group.

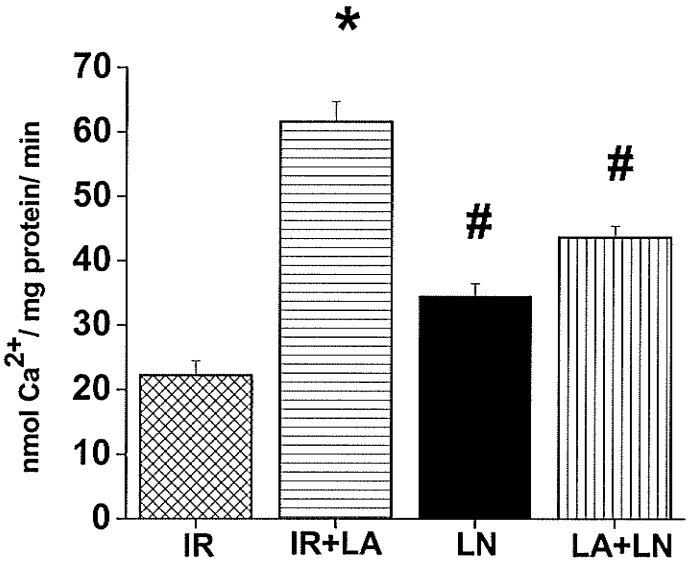

Figure 13. SR Ca^{2+} uptake in IR hearts treated with L-arginine (1.5mM), L-NAME and a combination of the two in comparison to IR. IR=60 min reperfusion of hearts exposed to 30 min ischemia and IR+LA=IR hearts treated with L-arginine (LA), LN=L-NAME. n=5-6 for each group. Data expressed as Mean ± SE. *$p<0.05$ in comparison to IR & # $p<0.05$ in comparison to the IR+LA group.

DISCUSSION

This study was designed to investigate cardiac contractile abnormalities and alterations in SR mediated Ca^{2+} uptake and release during IR. Since the *ex-vivo* Langendorff perfused rat heart is a well-established model to study different aspects of IR injury, this system was employed in this investigation. Induction of IR caused a depression in cardiac function as evident from a decrease in the LVDP, +dP/dt and –dP/dt and an increase in LVEDP during IR (Figure 2). Because SR plays a central role in cardiac contractility by maintaining Ca^{2+} homeostasis, its proper functioning is critical for contraction and relaxation, depressed cardiac contractility could be due to a decrease in SR function as observed in the present study. The decrease in SR Ca^{2+} uptake and release may be due to a reduction in protein content of the SR Ca^{2+} cycling proteins (SERCA2a and RyR) or due to a depression in the SR regulatory mechanisms such as protein phosphorylation by PLB. These results are consistent with the previous studies (113,123,124,207). Western blot analysis showed a decrease in the protein content of SERCA2a and PLB. A change in SR protein contents was not a generalized phenomenon since we did not see any changes in CQS protein content. This decrease in protein content could explain the alterations in SR Ca^{2+} uptake and release observed during IR. The regulation of SR function was investigated by examining the activities of CaMK II and cAMP associated PKA. It was observed that both CaMK II and PKA activities are depressed during IR, which is consistent with a decrease in their protein content. These results thus demonstrates that the

contractile abnormalities in IR hearts are not only associated with changes in SR function but may also be related to alterations in SR Ca^{2+}-cycling proteins and defects in the SR regulating mechanisms.

In order to show if IR induced changes in cardiac performance and SR function are associated with alterations in NO production, we measured cytosolic levels of NO and SR bNOS content. The results demonstrate that cytosolic NO levels are depressed in IR hearts. Reduced levels of NO in the hearts during IR are consistent with some of the earlier studies (196,197). Western blotting analysis showed a decrease in the protein content of NO synthase (bNOS) in the SR during IR. This form of NOS, recently localized to the SR (176) may be in part, responsible for the endogenous production of NO in cardiac cells, independent of NO production by the coronary endothelium (by eNOS). The decrease in protein content of bNOS could explain the decreased NO content in the cytosol. To investigate whether cardiac contractile impairment and SR dysfunction in IR hearts was associated with depletion of NO inside the cardiac myocyte and whether it could be reversed upon administration of an exogenous substrate of NO, IR hearts were treated with NO precursor, L-Arginine. A combination of pre-ischemic and post- ischemic L-arginine improved the NO content and also prevented a decrease in the protein content of bNOS during IR (Figure 7 & 6D). L-arginine treatment attenuated SR dysfunction and recovered SR protein content resulting in an improvement in cardiac contractile abnormalities. L-arginine treatment also improved the SR regulation by recovering PLB protein

phosphorylation by CaMK and PKA in the IR hearts at Thr-17 and Ser-16. Phosphorylation of PLB by CaMK and PKA are key events that relieve inhibition on SERCA2a and stimulate SR Ca^{2+}-uptake. PLB phosphorylation was downregulated in IR hearts suggesting increased inhibition of SERCA2a and reduced Ca^{2+}-uptake. Treatment with L-arginine could have in part relieved the inhibition of SERCA2a by PLB leading to the improvement in SR Ca^{2+}-uptake in the IR hearts. The reduction in SR associated CaMK and PKA activities were consistent with a reduction of their respective protein contents in IR hearts. These observations support the view that the beneficial effects of L-arginine on the IR induced contractile abnormalities may be due to improvement of SR Ca^{2+}- uptake and release activities as well as attenuation of changes in regulatory mechanisms for SR function in IR hearts.

For studying the mechanisms of the beneficial effects of L-arginine on the IR hearts, we perfused isolated hearts with L-NAME, an inhibitor of NOS, alone and in combination with L-arginine. L-NAME given in combination with L-arginine attenuated the improvement in cardiac contractility and SR function due to L-arginine in IR hearts. This would suggest a critical role for NO in maintaining cardiac contractility and SR function. The attenuation of the improvement in cardiac contractility and SR function observed with L-arginine treatment (of IR hearts) by L-NAME may in part be due to reduced production of NO by bNOS, which is inhibited by L-NAME. L-NAME given alone did not affect cardiac contractility during IR. While it is difficult to explain the increase in LVEDP in IR

hearts several mechanisms could possibly explain the cardioprotective effects of NO seen during IR. Exogenously given L-arginine has been shown to stimulate NOS activity (208). The decrease in NO production observed in our study, which is consistent with alterations in bNOS protein content may in part be responsible for the changes in SR function seen in our study during IR. Generation of NO by exogenously administrated L-arginine could have prevented a decrease in SR Ca^{2+} cycling and regulating proteins and protein phosphorylation thereby improving SR function and cardiac contractility. Depression in NO production due to alterations in bNOS may provide a new mechanism responsible for causing SR dysfunction resulting in cardiac contractile abnormalities during IR. Exogenous administration of L-arginine increases the NO content directly or by stimulating the endogenous production through bNOS and may function either by improving SR function by preventing downregulation of critical SR Ca^{2+} handling proteins directly or by preserving SR protein phosphorylation in IR hearts. A recent study from our laboratory has shown that calpain is activated during IR and this is responsible for causing abnormalities in SR Ca^{2+} handling and its regulation resulting in cardiac contractile dysfunction (209). It is shown that calpain and ubiquitin –proteasome are also the major proteolytic mechanisms for the degradation of NOS isoenzymes (210) and nitric oxide is reported to inhibit calpain-mediated proteolysis in skeletal muscle (211,212). It may thus be speculated that NO may inhibit proteolysis of SR proteins in cardiac myocytes by proteases (such as calpain) that are activated during IR.

CONCLUSIONS

1. Depressed cardiac performance due to IR was associated with a reduction in the SR Ca^{2+}- uptake and Ca^{2+}- release activities.
2. Impaired SR function in IR hearts was associated with changes in SR Ca^{2+} –cycling proteins as well as regulating mechanisms for SR Ca^{2+}- transport.
3. L-arginine, an NO precursor produced an improvement in both cardiac performance and SR function and SR regulation in IR hearts.
4. The beneficial effects of L-arginine on cardiac performance and SR function in IR hearts were attenuated by, L-NAME, an inhibitor on NO synthesis.
5. IR depressed the cytosolic levels of NO and decreased the bNOS content in SR; these effects of IR were prevented by L-arginine.
6. From the results in this study, it can be concluded that IR reduces the production of NO and this may result in SR dysfunction and abnormal contractile function.

REFERENCES

1. Dhalla NS, Golfman L, Takeda S, Takeda N, Nagano M. Evidence for the role of oxidative stress in acute ischemic heart disease: a brief review. Can J Cardiol 1999; 15:587-593.
2. Dhalla NS, Elmoselhi AB, Hata T, Makino N. Status of myocardial antioxidants in ischemia-reperfusion injury. Cardiovasc Res 2000; 47:446-456.
3. Bolli R. Myocardial stunning in man. Circulation 1982; 86:1671-1691.
4. Kloner RA, Allen J, Cox TA, Zheng Y, Ruiz CE. Stunned left ventricular myocardium after exercise treadmill testing in coronary artery disease. Am J Cardiol 1991; 68:329-334.
5. Kloner RA, Przyklenk K, Kay GL. Clinical evidence for stunned myocardium after coronary artery bypass surgery. J Card Surg 1994; 9:397-402.
6. Rahimtoola SH. The hibernating myocardium. Am Heart J 1989; 117:211-221.
7. Van Eyk JE, Powers F, Law W, Larue C, Hodges RS, Solaro RJ. Breakdown and release of myofilament proteins during ischemia and ischemia/reperfusion in rat hearts: identification of degradation products and effects on the pCa-force relation. Circ Res 1998; 82:261-271.

8. Gao WD, Perez NG, Seidman CE, Seidman JG, Marban E. Altered cardiac excitation-contraction coupling in mutant mice with familial hypertrophic cardiomyopathy. J Clin Invest 1999; 103:661-666.

9. Kloner RA, Bolli R, Marban E, Reinlib L, Braunwald E. Medical and cellular implications of stunning, hibernation, and preconditioning: an NHLBI workshop. Circulation 1998; 97:1848-1867.

10. Bolli R. Mechanism of myocardial "stunning". Circulation 1990; 82:723-738.

11. Frass O, Sharma H, Knoll R, Duncker D, McFalls E, Verdouw P, Schaper W. Enhanced gene expression of calcium regulatory proteins in stunned porcine myocardium. Cardiovasc Res 1993; 27:2037-2043.

12. Brand T, Sharma H, Fleischmann K, Duncker D, Mcfalls E, Verdouw P, Schaper W. Proto-oncogenes expression in porcine myocardium subjected to ischemia reperfusion. Circ Res 1992; 71:1351-1360.

13. Ringer SA. A further contribution regarding the influence of different constituents of the blood on the contraction of heart. J Physiol (Lond.)1883; 4:29-42.

14. Dhalla NS, Pierce GN, Panagia V, Singal PK, Beamish RE. Calcium movements in relation to heart function. Basic Res Cardiol 1982; 77:117-139.

15. Katz AM. Molecular biology of calcium channels in the cardiovascular system. Am J Cardiol 1997; 80:17I-22I.

16. Fabiato A, Fabiato F. Calcium release from the sarcoplasmic reticulum. Circ Res 1977; 40:119-129.

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

18. Fleischer S, Inui M. Biochemistry and biophysics of excitation –contraction coupling. Annu Rev Biophys Chem 1989; 18:333-364.

19. Bassani JW, Yuan W, Bers DM. Fractional SR Ca^{2+} release is regulated by trigger Ca^{2+} and SR Ca^{2+} content in cardiac myocytes. Am J Physiol 1995; 268:C1313-1319.

20. Stern MD, Lakatta EG. Excitation contraction coupling in the heart. The state of question. FASEB J 1992; 6:3092-3100.

21. Niggli E, Lederer WJ. Voltage –independent calcium release in heart muscle. Science 1990; 250:565-568.

22. Lewartowski B, Wolska B. The role of sarcoplasmic reticulum and Na^{+}-Ca^{2+} exchange in the Ca^{2+} extrusion from the resting myocytes of guinea pig heart: comparison with rat. J Mol Cell Cardiol 1993; 2591:75-91.

23. Lipp P, Pott L, Callewaert G, Carmeliet E. Calcium transients caused by calcium entry are influenced by the sarcoplasmic reticulum in guinea-pig atrial myocytes. J Physiol 1992; 454:321-338.

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

25. Negretti N, O'Neill SC, Eisner DA. The relative contributions of different intracellular and sarcolemmal systems to relaxation in rat ventricular myocytes. Cardiovasc Res 1993; 27:1826-1830.

26. Brandes R, Bers DM. Intracellular Ca^{2+} increases the mitochondrial NADH concentration during elevated work in intact cardiac muscle. Circ Res 1997; 80:82-87.

27. Hansford RG. Physiological role of mitochondrial Ca^{2+} transport. J Bioenerg Biomembr 1994; 26:495-508.

28. Bassani JW, Bassani RA, Bers DM. Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms. J Physiol 1994; 476:279-293.

29. Bers DM. Cardiac excitation-contraction coupling. Nature 2002; 415:198-205.

30. Scales DJ. Aspects of the mammalian cardiac sarcotubular system revealed by freeze fracture electron microscopy. J Mol Cell Cardiol 1981; 13:373-380.

31. Jewett PH, Leonard SD, Sommer JR. Chicken cardiac muscle: its elusive extended junctional sarcoplasmic reticulum and sarcoplasmic reticulum fenestrations. J Cell Biol 1973; 56:595-600.

32. Forbes MS. The membrane systems and cytoskeletal elements of mammalian myocardial cells. Cell Muscle Motil 1983; 3:89-155.

33. Sommer JR and Waugh RA. The ultrastructure of the mammalian cardiac muscle with special emphasis on the tubular membrane systems. A review. Am J Pathol 1976; 82:192-232.

34. Rousseau E, Smith JS, Meissner G. Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channel. Am J Physiol 1987; 253:C364-368.

35. Meissner G. Ryanodine activation and inhibition of the Ca2+ release channel of sarcoplasmic reticulum. J Biol Chem 1986; 261:6300-6306.

36. Meissner G, Henderson JS. Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide, and calmodulin. J Biol Chem 1987; 262:3065-3073.

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

38. Zucchi R, Ronca F, Ronca-Testoni S. Modulation of sarcoplasmic reticulum function: a new strategy in cardioprotection. Pharmacol Ther 2001; 89:47-65.

39. Marks AR, Tempst P, Hwang KS, Taubman MB, Inui M, Chadwick C, Fleisher S, Nadal –Ginard B. Molecular cloning and characterization of the ryanodine receptor/junctional complex cDNA from skeletal muscle. Proc Natl Acad Sci USA 1987; 86:8683-8687.

40. Ohtsu K, Willard HF, Khanna VK, Zorzato F, Green NM, MacLennan DH. Molecular cloning of cDNA encoding the Ca^{2+}release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J Biol Chem 1990; 265:13472-13483.

41. Zhang L, Kelley J, Schmeisser G, Kobayashi YM, Jones LR. Complex formation between junctin, traidin, calsequestrin, and the ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane. J Biol Chem 1997; 272:23389-23397.

42. Franzini –Armstrong C. Structure of sarcoplasmic reticulum. Fed Proc 1980; 39:2403-2409.

43. Arai M, Matsui H, Periasamy M. Sarcoplasmic reticulum gene expression in cardiac hypertrophy and heart failure. Circ Res 1994; 74:555-564.

44. Brandl CJ, Green NM, Korczak B, MacLennan DH. Two Ca^{2+} ATPase genes: homologies and mechanistic implications of deduced amino acid sequences. Cell 1986; 44:597-607.

45. Zarain-Herzberg A, MacLennan DH, Periasamy M. Characterization of rabbit cardiac sarco(endo)plasmic reticulum Ca^{2+}-ATPase gene. J Biol Chem 1990; 265:4670-4677.

46. Lytton J, Zarain-Herzberg A, Periasamy M, MacLennan DH. Molecular cloning of the mammalian smooth muscle sarco(endo)plasmic reticulum Ca^{2+}-ATPase. J Biol Chem 1989; 264:7059-7065.

47. Anger M, Samuel JL, Marotte F, Wuytack F, Rappaport L, Lompre AM. The sarco(endo)plasmic reticulum Ca^{2+}-ATPase mRNA isoform, SERCA 3, is expressed in endothelial and epithelial cells in various organs. FEBS Lett 1993; 334:45-48.

48. Anger M, Samuel JL, Marotte F, Wuytack F, Rappaport L, Lompre AM. In situ mRNA distribution of sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoforms during ontogeny in the rat. J Mol Cell Cardiol 1994; 26:539-550.

49. Komuro I, Kurabayashi M, Shibazaki Y, Takaku F, Yazaki Y. Molecular cloning and characterization of a Ca^{2+} Mg^{2+}-dependent adenosine triphosphatase from rat cardiac sarcoplasmic reticulum. Regulation of its expression by pressure overload and developmental stage. J Clin Invest 1989; 83:1102-1108.

50. Fabiato A, Fabiato F. Calcium and cardiac excitation-contraction coupling. Annu Rev Physiol 1979; 41:473-484.

51. Jorgensen AO, Shen AC, MacLennan DH, Tokuyasu KT. Ultrastructural localization of the Ca^{2+} Mg^{2+}-dependent ATPase of sarcoplasmic reticulum in rat skeletal muscle by immunoferritin labeling of ultrathin frozen sections. J Cell Biol 1982; 92:409-416.

52. Bers DM. Ca2+ transport during contraction and relaxation in mammalian ventricular muscle. Basic Res Cardiol 1997; 92:1-10.

53. Toyofuku T, Curotto Kurzydlowski K, Narayanan N, MacLennan DH. Identification of Ser38 as the site in cardiac sarcoplasmic reticulum Ca^{2+}-ATPase that is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase. J Biol Chem 1994; 269:26492-26496.

54. Lompre AM, Anger M, Levitsjy D. Sarco(endo)plasmic reticulum calcium pumps in the cardiovascular system: function and gene expression. J Mol Cell Cardiol 1994; 26:1109-1121.

55. Kadambi VJ, Kranias EG. Phospholamban: a protein coming of age. Biochem Biophys Res Commun 1997; 239:1-5.

56. Simmerman HK, Jones LR. Phospholamban: protein structure, mechanism of action, and role in cardiac function. Physiol Rev 1998; 78:921-947.

57. Koss KL, Kranias EG. Phospholamban: a prominent regulator of myocardial contractility. Circ Res 1996; 79:1059-1063.

58. James P, Inui M, Tada M, Chiesi M, Carafoli E. Nature and site of phospholamban regulation of the Ca^{2+} pump of sarcoplasmic reticulum. Nature 1989; 342:90-92.

59. Kim HW, Steenaart NA, Ferguson DG, Kranias EG. Functional reconstitution of the cardiac sarcoplasmic reticulum Ca^{2+}-ATPase with phospholamban in phospholipid vesicles. J Biol Chem 1990; 265:1702-1709.

60. Steenaart NA, Ganim JR, Di Salvo J, Kranias EG. The phospholamban phosphatase associated with cardiac sarcoplasmic reticulum is a type 1 enzyme. Arch Biochem Biophys 1992; 293:17-24.

61. Lindemann JP, Jones LR, Hathaway DR, Henry BG, Watanabe AM. beta-Adrenergic stimulation of phospholamban phosphorylation and Ca^{2+}-ATPase activity in guinea pig ventricles. J Biol Chem 1983; 258:464-471.

62. Kranias EG, Garvey JL, Srivastava RD, Solaro RJ. Phosphorylation and functional modifications of sarcoplasmic reticulum and myofibrils in isolated rabbit hearts stimulated with isoprenaline. Biochem J 1985; 226: 113-121.

63. Wegener AD, Simmerman HK, Lindemann JP, Jones LR. Phospholamban phosphorylation in intact ventricles. Phosphorylation of serine 16 and threonine 17 in response to beta-adrenergic stimulation. J Biol Chem 1989; 264:11468-11474.

64. Tada M, Kirchberger MA, Katz AM. Phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J Biol Chem 1975; 250:2640-2647.

65. Toyofuku T, Kurzydlowski K, Tada M, MacLennan DH.Amino acids Glu2 to Ile18 in the cytoplasmic domain of phospholamban are essential for functional association with the Ca^{2+}-ATPase of sarcoplasmic reticulum. J Biol Chem 1994; 269:3088-3094.

66. Simmerman HK, Collins JH, Theibert JL, Wegener AD, Jones LR. Sequence analysis of phospholamban. Identification of phosphorylation sites and two major structural domains. J Biol Chem 1986; 261:13333-13341.

67. Vittone L, Mundina C, Chiappe de Cingolani G and Mattiazzi A. cAMP and calcium-dependent mechanisms of phospholamban phosphorylation in intact hearts. Am J Physiol 1990; 258:H318–H325.

68. Kranias EG, Mandel F, Wang T, Schwartz A. Mechanism of the stimulation of calcium ion dependent adenosine triphosphatase of cardiac sarcoplasmic reticulum by adenosine 3',5'-monophosphate dependent protein kinase. Biochemistry 1980; 19:5434-5439.

69. Tada M, Yamada M, Ohmori F, Kuzuya T, Inui M, Abe H. Transient state kinetic studies of Ca^{2+}-dependent ATPase and calcium transport by cardiac sarcoplasmic reticulum. Effect of cyclic AMP-dependent protein kinase-catalyzed phosphorylation of phospholamban. J Biol Chem 1980; 255:1985-1992.

70. Bidlack JM, Shamoo AE. Adenosine 3',5'-monophosphate-dependent phosphorylation of a 6000 and a 22,000 dalton protein from cardiac sarcoplasmic reticulum. Biochim Biophys Acta 1980; 632:310-325.

71. Kimura Y, Kurzydlowski K, Tada M, MacLennan DH. Phospholamban inhibitory function is activated by depolymerization. J Biol Chem 1997; 272:15061-15064.

72. Wegener AD, Jones LR. Phosphorylation-induced mobility shift in phospholamban in sodium dodecyl sulfate-polyacrylamide gels. Evidence for a protein structure consisting of multiple identical phosphorylatable subunits. J Biol Chem 1984; 259:1834-1841.

73. Simmerman HK, Collins JH, Theibert JL, Wegener AD, Jones LR. Sequence analysis of phospholamban. Identification of phosphorylation sites and two major structural domains. J Biol Chem 1986; 261:13333-13341.

74. Kovacs RJ, Nelson MT, Simmerman HK, Jones LR.Phospholamban forms Ca selective channel in lipid bilayers. J Biol Chem 1998; 263:18364-18368.

75. Jorgensen AO, Campbell KP. Evidence for the presence of calsequestrin in two structurally different regions of myocardial sarcoplasmic reticulum. J Cell Biol 1984; 98:1597-1602.

76. Jorgensen AO, Shen AC, Campbell KP. Ultrastructural localization of calsequestrin in adult rat atrial and ventricular muscle cells. J Cell Biol 1985; 101:257-268.

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

78. Arai M, Aipert NR, Periasamy M. Cloning and characterization of the gene encoding rabbit cardiac calsequestrin. Gene 1991; 109:275-279.

79. Fliegel L, Ohnishi M, Carpenter MR, Khanna VK, Reithmeier RA, MacLennan DH. Amino acid sequence of rabbit fast-twitch skeletal muscle calsequestrin Proc Natl Acad Sci U S A 1987; 84:1167-1171.

80. Campbell KP, MacLennan DH. Purification and characterization of the 53000-dalton glycoprotein from the sarcoplasmic reticulum. J Biol Chem 1981; 256:4626-4632.

81. MacLennan DH, Wong PT. Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. Proc Natl Acad Sci U S A. 1971; 68:1231-1235.

82. Zhang L, Kelley J, Schmeisser G, Kobayashi YM, Jones LR. Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane. J Biol Chem 1997; 272:23389-23397.

83. Scott BT, Simmerman HKB, Collins JH, Nadal-Ginard B, Jones LR. Complete amino acid sequence of canine cardiac calsequestrin deduced by cDNA cloning. J Biol Chem 1998; 263:8958-8964.

84. Cala SE, Jones LR. Phosphorylation of cardiac and skeletal muscle calsequestrin isoforms by casein kinase II. Demonstration of a cluster of unique rapidly phosphorylated sites in cardiac calsequestrin. J Biol Chem 1991; 266:391-398.

85. Baltas LG, Karczewski P, Krause EG. The cardiac sarcoplasmic reticulum phospholamban kinase is a distinct delta-CaM kinase isozyme. FEBS Lett 1995; 373:71-75.

86. Kranias EG. Regulation of calcium transport by protein phosphatase activity associated with cardiac sarcoplasmic reticulum. J Biol Chem 1985; 260:11006-11010.

87. Le Peuch CJ, Haiech J, Demaille JG. Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cyclic adenosine monophosphate dependent and calcium--calmodulin-dependent phosphorylations. Biochemistry 1979; 18:5150-5157.

88. Xu A, Hawkins C, Narayanan N. Phosphorylation and activation of the Ca^{2+}-pumping ATPase of cardiac sarcoplasmic reticulum by Ca^{2+}/calmodulin-dependent protein kinase. J Biol Chem 1993; 268:8394-8397.

89. Cohen P. The structure and function of protein phosphatases. Annu Rev Biochem 1989; 58:453-508.

90. Kranias EG, Di salvo J. A phospholamban protein phosphatase activity associated with cardiac sarcoplasmic reticulum. J Biol Chem 1986; 261: 10029-10032.

91. Suzuki T, Wang JH. Stimulation of bovine cardiac sarcoplasmic reticulum Ca^{2+} pump and blocking phospholamban phosphorylation and

dephosphorylation by a phospholamban monoclonal antibody. J Bio Chem 1986; 261:7018-7023.

92. Witcher DR, Kovacs RJ, Schulman H, Cefale DC, Jones LR. Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. J Biol Chem 1991; 266:11144-11152.

93. Hain J, Onoue H, Mayrleitner M, Fleischer S, Schindler H. Phosphorylation modulates the function of the calcium release channel of sarcolasmic reticulum from cardiac muscle. J Biol Chem 1995; 270:2074-2081.

94. Gerschman R, Gilbert DL, Nye SW, Sweyer P, Ferrari R: oxygen poisoning and X-irradiation: a mechanism in common. Science 1954; 119:623-626.

95. Myers ML, Bolli R, Lekich RF, Hartley CJ, Roberts R. Enhancement of recovery of myocardial function by oxygen free radical scavenger after reversible regional ischemia. Circulation 1985; 72:915-921.

96. Gross GJ, Farber NE, Hardman HF, Warltier DC. Beneficial actions of superoxide dismutase and catalase in stunned myocardium in dogs Am J Physiol 1986; 250:H372-377.

97. Przyklenk K, Kloner RA. Superoxide dismutase plus catalase improve contractile function in the canine model of the "stunned myocardium". Circ Res 1986; 58:148-156.

98. Bolli R, Patel BS, Jeroudi MO, Lai EK, McCay PB. Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of

the spin trap alpha-phenyl N-tert-butyl nitrone. J Clin Invest 1988; 82:476-485.

99. Bolli R, Jeroudi MO, Patel BS, Aruoma OI, Halliwell B, Lai EK, McCay PB. Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury. Circ Res 1989; 65:607-622.

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

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

102. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med 2000; 29:222-230.

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

104. Sugioka K, Nakano M, Totsune-Nakano H, Minakami H, Tero-Kubota S, Ikegami Y. Mechanism of O_2^- generation in reduction and oxidation cycle of ubiquinones in a model of mitochondrial electron transport systems. Biochim Biophys Acta 1988; 936:377-385.

105. Augustin W, Wiswedel I, Noack H, Reinheckel T, Reichelt O. Role of endogenous and exogenous antioxidants in the defence against functional

damage and lipid peroxidation in rat liver mitochondria. Mol Cell Biochem 1997; 174:199-205.

106. Fenton H. Oxidation of tartaric acid in the presence of iron. J Chem Soc 1894; 10:157-158.

107. Haber F, Weiss J. The catalytic decomposition of hydrogen peroxide by iron salts. Proc Roy Soc 1934; 147:332-351.

108. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res 1994; 74:1141-1148.

109. Mohazzab KM, Kaminski PM, Wolin MS. NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. Am J Physiol 1994; 266:H2568-2572.

110. Pagano PJ, Ito Y, Tornheim K, Gallop PM, Tauber AI, Cohen RA. An NADPH oxidase superoxide-generating system in the rabbit aorta. Am J Physiol 1995; 268:H2274-2280.

111. Chua CC, Hamdy RC, Chua BH. Upregulation of vascular endothelial growth factor by H2O2 in rat heart endothelial cells. Free Radic Biol Med 1998; 25:891-897.

112. Torti SV, Akimoto H, Lin K, Billingham ME, Torti FM. Selective inhibition of muscle gene expression by oxidative stress in cardiac cells. J Mol Cell Cardiol 1998; 30:1173-80.

113. Temsah RM, Netticadan T, Chapman D, Takeda S, Mochizuki S, Dhalla NS. Alterations in sarcoplasmic reticulum function and gene expression in ischemic-reperfused rat heart. Am J Physiol 1999; 277:H584-594.

114. Peng M, Huang L, Xie ZJ, Huang WH, Askari A. Oxidant-induced activations of nuclear factor-kappa B and activator protein-1 in cardiac myocytes. Cell Mol Biol Res 1995; 41:189-197.

115. Girotti AW. Mechanisms of lipid peroxidation. J Free Radic Biol Med 1985; 1:87-95.

116. Anzai K, Ogawa K, Ozawa T, Yamamoto H. Oxidative modification of ion channel activity of ryanodine receptor. Antioxid Redox Signal 2000; 2:35-40.

117. Wolff SP, Dean RT. Fragmentation of proteins by free radicals and its effect on their susceptibility to enzymic hydrolysis. Biochem J 1986; 234:399-403.

118. Davies KJ. Protein damage and degradation by oxygen radicals. I. general aspects. J Biol Chem 1987; 262:9895-9901.

119. Dean RT, Roberts CR, Jessup W. Fragmentation of extracellular and intracellular polypeptides by free radicals. Prog Clin Biol Res 1985; 180:341-350.

120. Dixon IM, Kaneko M, Hata T, Panagia V, Dhalla NS. Alterations in cardiac membrane Ca^{2+} transport during oxidative stress. Mol Cell Biochem 1990; 99:125-133.

121. Dixon IM, Hata T, Dhalla NS. Sarcolemmal Na^+-K^+-ATPase activity in congestive heart failure due to myocardial infarction. Am J Physiol 1992; 262:C664-671.

122. Smart SC, Sagar KB, Schultz J, Warltier DC, Jones LR. Injury to the Ca^{2+} ATPase of the sarcoplasmic reticulum in anesthetized dogs contributes to myocardial reperfusion injury. Cardiovasc Res 1997; 36:174-184.

123. Osada M, Netticadan T, Tamura K, Dhalla NS. Modification of ischemia-reperfusion-induced changes in cardiac sarcoplasmic reticulum by preconditioning. Am J Physiol 1998; 274:H2025-2034.

124. Netticadan T, Temsah R, Osada M, Dhalla NS. Status of Ca^{2+}/calmodulin protein kinase phosphorylation of cardiac SR proteins in ischemia-reperfusion. Am J Physiol 1999; 277:C384-391.

125. Kusuoka H, Porterfield JK, Weisman HF, Weisfeldt ML, Marban E. Pathophysiology and pathogenesis of stunned myocardium. Depressed Ca^{2+} activation of contraction as a consequence of reperfusion-induced cellular calcium overload in ferret hearts. J Clin Invest 1987; 79:950-961.

126. Marban E, Koretsune Y, Corretti M, Chacko VP, Kusuoka H. Calcium and its role in myocardial cell injury during ischemia and reperfusion. Circulation 1989; 80:IV17-22.

127. Wu ML, Vaughan-Jones RD. Interaction between Na^+ and H^+ ions on Na-H exchange in sheep cardiac Purkinje fibers. J Mol Cell Cardiol 1997; 29: 1131-1140.

128. Frelin C, Vigne P, Lazdunski M. The role of the Na+/H+ exchange system in cardiac cells in relation to the control of the internal Na+ concentration. A molecular basis for the antagonistic effect of ouabain and amiloride on the heart. J Biol Chem 1984; 259:8880-8885.

129. Karmazyn M, Gan XT, Humphreys RA, Yoshida H, Kusumoto K. The myocardial Na^+-H^+ exchange: structure, regulation, and its role in heart disease. Circ Res 1999; 85:777-786.

130. Griese M, Perlitz V, Jungling E, Kammermeier H. Myocardial performance and free energy of ATP-hydrolysis in isolated rat hearts during graded hypoxia, reoxygenation and high Ke^+-perfusion. J Mol Cell Cardiol 1988; 20:1189-1201.

131. Grinwald PM. Calcium uptake during post-ischemic reperfusion in the isolated rat heart: influence of extracellular sodium. J Mol Cell Cardiol 1982; 14:359-365.

132. Kim D, Cragoe EJ Jr, Smith TW. Relations among sodium pump inhibition, Na-Ca and Na-H exchange activities, and Ca-H interaction in cultured chick heart cells. Circ Res 1987; 60:185-193.

133. Steenbergen C, Murphy E, Levy L, London RE. Elevation of cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart Circ Res 1987; 60:700-707.

134. Philipson KD, Ward R. Effects of fatty acids on Na^{+}-Ca^{2+} exchange and Ca^{2+} permeability of cardiac sarcolemmal vesicles. J Biol Chem 1985; 260:9666-9671.

135. Przyklenk K, Kloner RA. Effect of verapamil on postischemic stunned myocardium: importance of timing of treatment. J Am Coll Cardiol 1988; 11:614-623.

136. Przyklenk K, Ghafari GB, Eitzman DT, Kloner RA. Nifedipine administered after reperfusion ablates systolic contractile dysfunction of postishemic stunned myocardium. J Am Coll Cardiol 1989; 13:1176-1183.

137. Gao WD, Liu Y, Mellgren R, Marban E. Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium. A consequence of Ca^{2+}-dependent proteolysis. Circ Res 1996; 78:455-465.

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

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

140. Gao WD, Liu Y, Marban E. Selective effects of oxygen free radicals on excitation-contraction coupling in ventricular muscle. Implications for the mechanism of stunned myocardium. Circulation 1996; 94:2597-2604.

141. Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. Science 1992; 258:1898-1902.

142. Murrel V. Nito-glycerine as a remedy for angina pectoris. Lancet 1879; 1: 80-81.

143. Ahler J, Andersson RGG, Torfgard K, Axelsson KL. Organic nitrate esters: Clinical use and mechanisms of action. Pharmacol Rev 1991; 43:351-423.

144. Mitchell HH, Schonle HA, Grindy HS: The origin of nitrates in the urine. Journal of Biological Chemistry 1916; 24:461-490.

145. Tannenbaum SR, Fett D, Young VR, Land PD, Bruce WR. Nitrite and nitrate are formed by endogenous synthesis in the human intestine. Science 1978; 200:1487-1489.

146. Stuehr DJ, Marletta MA. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc Natl Acad Sci U S A 1985; 82:7738-7742.

147. Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proc Natl Acad Sci USA 1977; 74: 3203-3207.

148. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373-376.

149. Furchgott RF. Endothelium derived relaxing factor: discovery, early studies and identification as nitric oxide. Bio Sci Rep 1999; 19:235-251.

150. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. Nature 1987; 327:24-26.

151. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci USA 1987; 84:9265-9269.

152. Myers PR, Minor RL Jr, Guerra R Jr, Bates JN, Harrison DG. Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. Nature 1990; 345:161-163.

153. Moncada S, Palmer RM, Higgs EA. Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. Biochem Pharmacol 1989; 38:1709-1715.

154. Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol Toxicol 1990; 30:535-560.

155. Marletta MA. Nitric oxide: biosynthesis and biological significance. Trends Biochem Sci 1989; 14:488-492.

156. Palmer RM, Ashton DS, Moncada S. Vsacular endothelial cells synthesize nitric oxide from L-arginine. Nature 1988; 333:664-666.

157. Marletta MA. Nitric oxide synthase structure and mechanism. J Biol Chem 1993; 268:12231-12234.

158. Gross SS, Levi R. Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. J Biol Chem 1992; 267:25722-25729.

159. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci USA 1998; 95:9220-9225.

160. Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. J Biol Chem 1994; 269:13725-13728.

161. Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J 1992; 6:3051-3064.

162. Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, Murad F. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc Natl Acad Sci USA 1991; 88:10480-10484.

163. Su Z, Blazing MA, Fan D, George SE. The calmodulin-nitric oxide synthase interaction. Critical role of the calmodulin latch domain in enzyme activation. J Biol Chem 1995; 270:29117-29122.

164. Siddhanta U, Wu C, Abu-Soud HM, Zhang J, Ghosh DK, Stuehr DJ. Heme iron reduction and catalysis by a nitric oxide synthase heterodimer containing one reductase and two oxygenase domains. J Biol Chem 1996; 271:7309-7312.

165. Wink DA, Hanbauer I, Grisham MB, Laval F, Nims RW, Laval J, Cook J, Pacelli R, Liebmann J, Krishna M, Ford PC, Mitchell JB. Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. Curr Top Cell Regul 1996; 34:159-187.

166. Wink DA, Grisham MB, Mitchell JB, Ford PC. Direct and indirect effects of nitric oxide in chemical reactions relevant to biology. Methods Enzymol 1996; 268:12-31.

167. Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. Am J Physiol 1986; 250: H1145-1149.

168. Ignarro LJ. Heme-dependent activation of guanylate cyclase by nitric oxide:a novel signal transduction mechanism. Blood Vessels 1991; 28:67-73.

169. Stone JR, Marletta MA. Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. Biochemistry 1994; 33:5636-5640.

170. Chinkers M, Garbers DL. Signal transduction by guanylyl cyclases. Annu Rev Biochem 1991; 60:553-575.

171. Murad F. The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication. Recent Prog Horm Res 1994; 49:239-248.

172. Stamler JS, Lamas S, Fang FC. Nitrosylation. The prototypic redox-based signalling mechanism. Cell 2001; 106:675-683.

173. Campbell DL, Stamler JS, Strauss HC. Redox modulation of L-type calcium channels in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and S-nitrosothiols. J Gen Physiol 1996; 108: 277-293.

174. Xu L, Eu JP, Meissner G, Stamler JS. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. Science 1998; 279:234-237.

175. Klimaschewski L, Kummer W, Mayer B, Couraud JY, Preissler U, Philippin B, Heym C. Nitric oxide synthase in cardiac nerve fibers and neurons of rat and guinea pig heart. Circ Res 1992; 71:1533-1537.

176. Xu KY, Huso DL, Dawson TM, Bredt DS, Becker LC. Nitric oxide synthase in cardiac sarcoplasmic reticulum. Proc Natl Acad Sci USA 1999; 96:657-662.

177. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobai IA, Lemmon CA, Burnett AL, O'Rourke B, Rodriguez ER, Huang PL, Lima JA, Berkowitz DE, Hare JM. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature 2002; 416:337-339.

178. Ashley EA, Sears CE, Bryant SM, Watkins HC, Casadei B. Cardiac nitric oxide synthase 1 regulates basal and beta-adrenergic contractility in murine ventricular myocytes. Circulation 2002; 105:3011-3016.

179. Khan SA, Skaf MW, Harrison RW, Lee K, Minhas KM, Kumar A, Fradley M, Shoukas AA, Berkowitz DE, Hare JM. Nitric oxide regulation of myocardial contractility and calcium cycling: independent impact of neuronal and endothelial nitric oxide synthases. Circ Res 2003; 92:1322-1329.

180. Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium. Br J Pharmacol 1992; 105:575-580.

181. Smith JA, Radomski MW, Schulz R, Moncada S, Lewis MJ. Porcine ventricular endocardial cells in culture express the inducible form of nitric oxide synthase. Br J Pharmacol 1993; 108:1107-1110.

182. Kanai AJ, Pearce LL, Clemens PR, Birder LA, VanBibber MM, Choi SY, de Groat WC, Peterson J. Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. Proc Natl Acad Sci USA 2001; 98:14126-14131.

183. Nathan C, Hibbs J. Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr Opin Immunol 1991; 3:65-70.

184. Ignarro LJ, Bush PA, Buga GM, Wood KS, Fukuto JM, Rajfer J. Nitric oxide and cyclic GMP formation upon electrical stimulation cause

relaxation of corpus cavarnosum smooth muscle. Biochem Biophy Res Commun 1990; 170:843-850.

185. Yallampalli C, Garfield RE, Byam-Smith M. Nitric oxide inhuibits uterine contractility during pregnancy but not during delivery. Endocinology 1993; 133:1899-1902.

186. Bohme GA, Bon C, Lemaire M, Reibaud M, Piot O, Stutzmann JM, Doble A, Blanchard JC. Altered synaptic plasticity and memory formation in nitric oxide synthase inhibitor-treated rats. Proc Natl Acad Sci USA 1993; 90:9191-9194.

187. Zweier JL, Wang P, Kuppusamy P. Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. J Biol Chem 1995; 270:304-307.

188. Depre C, Fierain L, Hue L. Activation of nitric oxide synthase by ischemia in the perfused heart. Cardiovasc Res 1997; 33:82-87.

189. Depre C, Vanoverschelde JL, Goudemant JF, Mottet I, Hue L. Protection against ischemic injury by nonvasoactive concentrations of nitric oxide synthase inhibitors in the perfused rabbit heart. Circulation 1995; 92:1911-1918.

190. Naseem SA, Kontos MC, Rao PS, Jesse RL, Hess ML, Kukreja RC. Sustained inhibition of nitric oxide by NG-nitro-L-arginine improves myocardial function following ischemia/reperfusion in isolated perfused rat heart. J Mol Cell Cardiol 1995; 27:419-426.

191. Wang QD, Morcos E, Wiklund P, Pernow J. L-arginine enhances functional recovery and Ca^{2+}-dependent nitric oxide synthase activity after ischemia and reperfusion in the rat heart. J Cardiovasc Pharmacol 1997; 29:291-296.

192. Draper NJ, Shah AM. Beneficial effects of a nitric oxide donor on recovery of contractile function following brief hypoxia in isolated rat heart. J Mol Cell Cardiol 1997; 29:1195-1205.

193. Jones SP, Girod WG, Palazzo AJ, Granger DN, Grisham MB, Jourd'Heuil D, Huang PL, Lefer DJ. Myocardial ischemia-reperfusion injury is exacerbated in absence of endothelial cell nitric oxide synthase. Am J Physiol 1999; 276:H1567-1573.

194. Kanno S, Lee PC, Zhang Y, Ho C, Griffith BP, Shears LL 2nd, Billiar TR. Attenuation of myocardial ischemia/reperfusion injury by superinduction of inducible nitric oxide synthase. Circulation 2000; 101:2742-2748.

195. Brunner F, Maier R, Andrew P, Wolkart G, Zechner R, Mayer B. Attenuation of myocardial ischemia/reperfusion injury in mice with myocyte-specific overexpression of endothelial nitric oxide synthase. Cardiovasc Res 2003; 57:55-62.

196. Maulik N, Engelman DT, Watanabe M, Engelman RM, Maulik G, Cordis GA, Das DK. Nitric oxide signaling in ischemic heart. Cardiovasc Res 1995; 30:593-601.

197. Engelman DT, Watanabe M, Engelman RM, Rousou JA, Flack JE 3rd, Deaton DW, Das DK. Constitutive nitric oxide release is impaired after ischemia and reperfusion. J Thorac Cardiovasc Surg 1995; 110:1047-1053.

198. Johnson G 3rd, Tsao PS, Lefer AM. Cardioprotective effects of authentic nitric oxide in myocardial ischemia with reperfusion. Crit Care Med 1991; 19:244-252.

199. Johnson G 3rd, Tsao PS, Mulloy D, Lefer AM. Cardioprotective effects of acidified sodium nitrite in myocardial ischemia with reperfusion. J Pharmacol Exp Ther 1990; 252:35-41.

200. Siegfried MR, Carey C, Ma XL, Lefer AM. Beneficial effects of SPM-5185, a cysteine containing NO donor in myocardial ischemia-reperfusion. Am J Physiol 1992; 263:H771-777.

201. Siegfried MR, Erhardt J, Rider T, Ma XL, Lefer AM. Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia-reperfusion. J Pharmacol Exp Ther 1992; 260:668-675.

202. Nakanishi K, Vinten-Johansen J, Lefer DJ, Zhao Z, Fowler WC 3rd, McGee DS, Johnston WE. Intracoronary L-arginine during reperfusion improves endothelial function and reduces infarct size. Am J Physiol 1992; 263:H1650-1658.

203. Weyrich AS, Ma XL, Lefer AM. The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. Circulation 1992; 86:279-288.

204. Netticadan T, Temsah RM, Kawabata K, Dhalla NS. Sarcoplasmic reticulum Ca $^{2+}$/calmodulin –dependent protein kinase is altered in heart failure. Circ Res 2000; 86:596-605.

205. Netticadan T, Temsah RM, Kent A, Elimban V, Dhalla NS. Depressed levels of Ca^{2+} -cycling proteins may underlie sarcoplasmic reticulum dysfunction in the diabetic heart. Diabetes 2001; 50:2133-2138.

206. Meyers M, Schillinger W, Pieske B, Holubarsch C, Heilmann C, Posival H, Kuwajima G, Mikoshiba K, Just H, Hasenfuss G. Alteration of sarcoplasmic reticulum proteins in failing human dialated cardiomyopathy. Circulation 1995; 92: 778-784.

207. Temsah RM, Dyck C, Netticadan T, Chapman D, Elimban V, Dhalla NS. Effect of beta-adrenergic blockers on sarcoplasmic reticular function and gene expression in the ischemic-reperfused heart. J Pharmacol Exp Ther 2000; 293:15-23.

208. Wang QD, Morcos E, Wiklund P, Pernow J. L-arginine enhances functional recovery and Ca^{2+}-dependent nitric oxide synthase activity after ischemia and reperfusion in the rat heart. J Cardiovasc Pharmacol 1997; 29:291-296.

209. Singh RB, Chohan PK, Dhalla NS, Netticadan T. The sarcoplasmic reticulum proteins are targets for calpain action in the ischemic-reperfused heart. J Mol Cell Cardiol 2004; 37:101-110.

210. Kone BC, Kuncewicz T, Zhang W, Yu ZY. Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. Am J Physiol Renal Physiol 2003; 285:F178-190.

211. Michetti M, Salamino F, Melloni E, Pontremoli S. Reversible inactivation of calpain isoforms by nitric oxide. Biochem Biophys Res Commun 1995; 207:1009-1014.

212. Koh TJ, Tidball JG. Nitric oxide inhibits calpain-mediated proteolysis of talin in skeletal muscle cells. Am J Physiol Cell Physiol 2000; 279:C806-812.